Mycobacterium tuberculosis

INTERACTIONS WITH THE IMMUNE SYSTEM

INFECTIOUS AGENTS AND PATHOGENESIS

Series Editors: Mauro Bendinelli, *University of Pisa*
Herman Friedman, *University of South Florida*

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MYCOBACTERIUM TUBERCULOSIS
Interactions with the Immune System
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INTERACTIONS WITH THE IMMUNE SYSTEM

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Preface to the Series

The mechanisms of disease production by infectious agents are presently the focus of an unprecedented flowering of studies. The field has undoubtedly received impetus from the considerable advances recently made in the understanding of the structure, biochemistry, and biology of viruses, bacteria, fungi, and other parasites. Another contributing factor is our improved knowledge of immune responses and other adaptive or constitutive mechanisms by which hosts react to infection. Furthermore, recombinant DNA technology, monoclonal antibodies, and other newer methodologies have provided the technical tools for examining questions previously considered too complex to be successfully tackled. The most important incentive of all is probably the regenerated idea that infection might be the initiating event in many clinical entities presently classified as idiopathic or of uncertain origin.

Infectious pathogenesis research holds great promise. As more information is uncovered, it is becoming increasingly apparent that our present knowledge of the pathogenic potential of infectious agents is often limited to the most noticeable effects, which sometimes represent only the tip of the iceberg. For example, it is now well appreciated that pathologic processes caused by infectious agents may emerge clinically after an incubation of decades and may result from genetic, immunologic, and other indirect routes more than from the infecting agent in itself. Thus, there is a general expectation that continued investigation will lead to the isolation of new agents of infection, the identification of hitherto unsuspected etiologic correlations, and, eventually, more effective approaches to prevention and therapy.

Studies on the mechanisms of disease caused by infectious agents demand a breadth of understanding across many specialized areas, as well as much co-operation between clinicians and experimentalists. The

series *Infectious Agents and Pathogenesis* is intended not only to document the state of the art in this fascinating and challenging field but also to help lay bridges among diverse areas and people.

M. Bendinelli
H. Friedman

Preface

Tuberculosis once again occupies a special position in the areas of infectious diseases and microbiology. This disease has been important to mankind since even before biblical times. Tuberculosis has been a major cause of morbidity and mortality in humans, especially in highly urbanized Europe, until a few decades ago. Indeed, this disease became a center of many novels, plays, and operas, since it appeared to be quite popular to have the heroine dying of "consumption." Most importantly, tuberculosis also became the focus of attention for many investigations during the 19th and even the 20th centuries. Major advances were made in the areas of isolation and identification of *M. tuberculosis* and related microorganisms. The discovery, by Robert Koch, that tuberculosis was caused by an infectious agent revolutionized our thinking about diseases. Koch's postulates were developed with tuberculosis in mind and became a focal point for many advances in microbiology and medicine.

Studies with mycobacteria as a central focus have also led to revolutionary new concepts about immunology in general. Koch himself showed that those exposed to *M. tuberculosis* develop a skin hypersensitivity or allergy to the microorganism's antigens, an observation which was the starting point for many important developments. Indeed, immediate-type hypersensitivity and atopic or IgE-mediated allergy were defined in relation to the delayed-type cutaneous hypersensitivity evidenced with the tubercle bacillus. In the mid 1940s Merrill Chase showed conclusively that tuberculin hypersensitivity could be transferred from skin test-positive to negative guinea pigs not by serum but only by means of cells, thus setting the stage for the explosive interest in cellular immunology. Jules Freund's technique of inducing enhanced immune responses to "weak antigens" with an oil adjuvant containing mycobacteria was also popularized about this time. It was soon shown

that mycobacteria and their products increased resistance to a wide variety of other microbial agents, thus inspiring interest in "natural" resistance mechanisms to infectious agents as well as to tumors.

The advent of streptomycin and other effective chemotherapeutic agents seemed to signal the end of continued research in this area. Nevertheless, basic immunologists, perhaps unaware of the importance of their studies of infectious diseases, became interested in mechanisms of cellular immunity using mycobacterial antigens as a "tool." In the late 1960s, one of Merrill Chase's graduate students, Barry Bloom, using such antigens, developed reproducible methods for examining soluble mediators of cellular immunity, such as the migration inhibitory factor, the first of several lymphokines shown subsequently to be involved in immune reactions. In the 1970s, investigators in France and Japan showed that the minimal subunit of mycobacterial cell wall, muramyl dipeptide, could serve as an adjuvant and an immunological stimulant. Thus, beginning in the 19th century and up until the present day, there have been many advances made by studying the tubercle bacillus, both as a pathogen and as an important tool to dissect the immune response system.

Medical scientists, as well as the population at large, often forget that tuberculosis is still the cause of much suffering in less developed regions of the world. According to World Health Organization data, there are at least 30 million individuals who are affected by tuberculosis in the world today and as many as one million individuals die as a result of it every year. Although this disease is a major public health problem in developing countries, there has been recent concern that the disease might once again manifest itself with renewed virulence in countries where sanitation and improved medical care have lowered the rate of infection. The aging of populations, the increased numbers of individuals with chronic diseases such as cancer and AIDS, and even immunodeficiencies due to therapy or other causes associated with the reduced frequency of natural vaccination may be factors that expose developed countries to such risk. The medical and scientific community has also been made aware of the importance of mycobacteria as pathogens by the observation that *M. avium intracellulare* is a major cause of morbidity and indeed mortality in patients with AIDS.

For these reasons, further investigation of *M. turberculosis* is imperative. There are many aspects of considerable practical importance that remain to be elucidated. That the host's response determines the histological and clinical manifestations, as well as the eventual outcome of infection, has been known since the pioneering work of Koch. Nevertheless, there are still many gaps in our present knowledge of the ways in which the microorganism interacts with the host's immune system to

cause disease or immunity. For example, we still don't know how tubercle bacilli may remain dormant within the tissues for many years or the reasons for the breakdown of resistance which may lead to reactivation and progression of infection. Nor do we understand the mechanisms which, under certain conditions, lead to anergy, a most interesting expression of immunoregulation. Neither do we know why the BCG vaccine, which has been used routinely and apparently successfully in many developed countries, has failed to confer significant protection in regions of high endemia. In fact, it has been urged that new vaccines must be developed, and efforts are already under way in this direction using genetic engineering technologies.

The editors and authors of this volume believe it very timely, then, to attempt to bring together all the burgeoning information on *M. tuberculosis* and its interactions with the immune system. We anticipate that this volume will help to provide an environment for interdisciplinary studies of this important area of investigation. Few infections, if any, illustrate the complexity and the double-edged nature of immune responses as effectively as tuberculosis.

H. Friedman
M. Bendinelli

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1

Mycobacterial Antigens

Reagents for Tuberculin Skin Testing and Serodiagnosis of Tuberculosis

L. F. AFFRONTI

1. INTRODUCTION

Despite the fact that knowledge of the cause and cure of tuberculosis has been known for years, it still is one of the most communicable diseases in the world and remains as one of the largest single causes of disease and death today, particularly in developing nations. At least 15 million people suffer from tuberculosis currently, which claims more than 3 million lives each year. This is particularly disturbing, since tuberculosis is a disease for which not only chemotherapeutic treatment is known and available but also because a standard regimen of antituberculosis drugs can be administered rather inexpensively and has been shown to be highly effective.

Probably no other bacterium has been so extensively studied than the organism that causes tuberculosis, *Mycobacterium tuberculosis*. Since its isolation by Koch in 1882, it has been boiled, autoclaved, ground up,

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subjected to high pressure, and sonicated, autolyzed, and extracted with a variety of reagents and by a host of different physicochemical procedures—all for the purpose of obtaining basic knowledge about its biology and chemistry for furthering our understanding of its pathogenesis and for possible uses in diagnosis.

Although mycobacteria were the first pathogenic organisms to be described more than a century ago,[1] the information obtained by application of an array of procedures and approaches noted above have yielded a limited body of knowledge when comparison is made to the other microorganisms of medical importance, particularly with respect to time and effort expended. This appears to be so, despite the application by many investigators of the more modern physicochemical methods in isolating, purifying, and characterizing these mycobacterial antigens. That the antigens of the tubercle bacillus have proved important in the pathogenesis of tuberculosis is unmistakingly clear. I have been of the opinion that if we know their precise chemical makeup as well as the possible biologic effects of each on their cellular environments when they are released, we would better understand the mechanisms of tuberculous disease and consequently how to combat it. Thus, it is important to isolate the antigens in as pure form as possible and to determine the chemical and biologic properties of each. It is possible that these different antigens may have different uses—in skin testing, serologic testing, and perhaps prevention.

Historically, the first intracutaneous immunodiagnostic test used in humans was the tuberculin skin test introduced by von Pirquet[2] in 1907. This test used a heated and essentially autolytic crude broth containing a mixture of proteins and other antigens of varying chemical composition, called Koch's Old tuberculin (OT), which was scratched into the skin. Although of practical value as an aid in the diagnosis of tuberculosis, Koch's OT and subsequent tuberculin preparations known as purified protein derivatives (PPD) suffered from their lack of specificity and reproducibility and by their inability to distinguish between active disease and prior sensitization due to infection with *M. tuberculosis* or other closely related mycobacteria. A tuberculin antigen that would be monospecific for detecting infections only with *M. tuberculosis* would be of great diagnostic and epidemiologic benefit, but such a product is not yet available. Thus, a need continues to be recognized for obtaining a more sensitive and specific skin-test antigen.

Because of my own special interest in mycobacterial antigens relates to their application and use both in skin testing and in the serodiagnosis of tuberculosis, this discussion emphasizes within that context on past and current knowledge of mycobacterial antigens derived from culture filtrate and bacterial extracts, with special consideration given to immu-

noreactive proteins and polysaccharides and their isolation, separation, and biologic characterization. Newer directions for obtaining immunodiagnostic reagents, including molecular biologic approaches for examining antigens from *M. tuberculosis,* are also considered and the benefits associated with this line of research assessed.

2. CULTURE FILTRATE AND BACILLARY EXTRACTS AS SOURCE MATERIALS FOR TUBERCULIN PREPARATION

The study of mycobacterial antigens for use in skin testing and in serodiagnosis has involved products that have originated both from culture filtrate material and from extracts prepared from whole bacilli, reflecting a lack of agreement and controversy among workers in the field as to the most appropriate source material to be used for the isolation and purification of the many antigens from tubercle bacilli. Today, there is general agreement in that there appear to be no significant qualitative antigenic differences that can be detected in both culture filtrate and whole cell extracts prepared from the same organism under constant conditions, although obvious quantitative differences can be demonstrated.[3]

2.1. From Heated Culture Filtrate

Koch's Old tuberculin for skin testing was originally prepared from the heated and concentrated glycerine peptone broth on which *M. tuberculosis* had grown for 6–8 weeks. This obviously contained many autolytic products derived from both the bacillus and medium on which it was grown and resulted in a crude product that varied in potency and specificity each time it was prepared. It was clear from the early work of Seibert *et al.*[4,6] on electrophoretic studies of different concentrated tuberculins that they varied significantly in their proportions of the different protein components and in their content of polysaccharides and nucleic acid, depending on such factors as length of growth and strain of bacilli studied. Realizing that variation in potency and specificity of OT exist, the need for a pure tuberculin became a desirable and necessary goal.

These recognized discrepancies led to the systematic investigation of standard strains of tubercle bacilli and their culture filtrates precipitated by trichloroacetic acid (TCA) and alternatively, in subsequent studies, from the culture filtrate of tubercle bacilli grown on a chemically defined synthetic medium precipitated by ammonium sulfate. These products were given the name PPD by Seibert[4] in the United States and

purified tuberculin (PT) by Lind[5] in Denmark. These preparations were much reduced in polysaccharides, nucleic acid, and lipid levels and thus represented much purer materials for use in skin testing and serodiagnosis than had originally been available. It is also important to realize that these products were never considered as single antigens but rather as complex mixtures of antigens. Although it is recognized by biochemists that an attempt to isolate and purify protein antigens from heated materials is not an optimal approach for separating and purifying individual constituents because of the obvious denaturation and degradation associated with the original heated source material, this approach would have been abandoned were it not for the hazards involved in working with highly infectious bacilli in large volumes and for the concern over both greater antigenicity and increased sensitization in experimental animals injected with unheated undenatured proteins.

2.2. From Unheated Culture Filtrate

It became increasingly clear, however, that a new strategy for separating the proteins of the culture filtrate should be undertaken, since it was difficult to separate both denatured and undenatured proteins from heated starting materials. A more logical approach to the study of these proteins was made by fractionating raw unheated tuberculin; these antigens were obtained by an alcohol and acetic acid fractionation procedure proposed by Seibert,[6] that resulted in three different proteins and two distinct polysaccharides. Her pioneering work to isolate and purify these fractions; the careful physiochemical characterization studies that followed have withstood the test of time and served as a useful reference point against which subsequent fractionation procedures by many investigators could be compared.

A number of investigators[7–9] showed that the yield of antigens and the composition of the culture filtrate can be greatly influenced by such factors as age of culture, type of medium used, temperature, and final pH at the time of harvest. That such variations exist emphasizes the importance in carefully defining a reference standard obtained from preparation of a single large batch of tuberculin. Such a product, designated PDD-S (standard), was prepared from *M. tuberculosis* by Seibert and Glenn[10] in 1941; this serves as the reference product in the United States. Similar large-batch PPD preparations were made by Affronti[11] from other species of mycobacteria for the U.S. Public Health Service tuberculosis skin-testing program in 1958.

2.3. From Bacillary Extracts

Some investigators have chosen to use bacillary extracts as the source material for the study of mycobacterial antigens. These prepara-

tions have been obtained from bacilli in a variety of ways, including sonication, freezing and drying, and grinding, and subjecting the organisms to extreme pressures.

Little antigenic differences were noted by Janicki *et al.*[12] when they compared *M. tuberculosis* extracts prepared by sonication and high-pressure methods. Other investigators using electrophoretic and immunodiffusion techniques demonstrated no major antigenic differences between those antigens present in the culture filtrates and those in the bacillary extracts.[13] As similarly seen with cultures filtrates, the composition of antigens in the bacillary extracts can vary, depending on the age of the culture at harvest and other factors.[14] Of special note, however, are the studies by Wayne and Sramek,[15] who showed that extracts prepared from resting mycobacterial cells in synchrony contained antigens unidentifiable from those in extracts obtained from cells that were actively dividing, underscoring the important role played by different conditions and state of growth in antigen production.

These occurrences of antigenic variations argue convincingly for the need to provide workers with a reliable reference and classification system that may be used to identify and possibly relate the antigens present in the many preparations produced by various laboratories for study. Such a system was advocated and developed by Janicki and a group of interested investigators,[16] who used immunoelectrophoresis methodology and reagents produced under the U.S.–Japan Cooperative Medical Sciences Program. Both reference antisera and antigens were produced commercially on a large scale and made available to interested workers. This system readily identified 11 major mycobacterial antigens and served as a common reference system for those investigators whose studies were primarily supported by the U.S.–Japan Cooperative Medical Science Program and who had a need to relate their products to a common point of reference. Although this system had limited application, identifying only 11 of the plethora of antigens that can be demonstrated by other immunoelectrophoretic techniques with hyperimmune antisera,[17] it facilitated the study and identification of common mycobacterial antigens in both culture filtrates and cell sonicate extracts.

Polyacrylamide gel electrophoresis (PAGE) was first introduced in 1965 by Affronti *et al.*[18] to separate and identify mycobacterial antigens present in protein and polysaccharide fractions. Among the advantages offered by this powerful analytic system for defining both culture filtrates and cell extracts are its reproducibility under standardized conditions, requirement for extremely small sample size and volume, and adaptability for the identification and nomenclature of individual antigens characterized by their respective mobilities and resultant R_f values. Identification of separated components by specific differential stains is

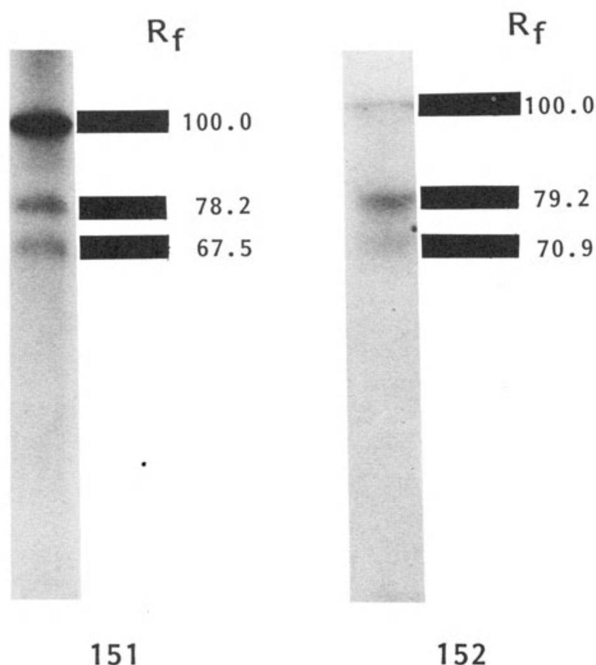

FIGURE 1. Polyacrylamide gel electrophoretic (PAGE) pattern and R_f values of the protein components present in the polysaccharide I fractions from two different H37Ra culture filtrates run at identical nitrogen levels.

an additional benefit of this procedure. Figure 1 depicts the electrophoretic pattern and R_f values of the stained protein components present in the polysaccharide I fractions of two different H37Ra culture filtrates run at the same nitrogen levels, illustrating how individual components in a mixture of constituents can be identified on the basis of their R_f values.

Modified PAGE and immunoelectrophoresis procedures that served to increase the resolution and sensitivity of the specific techniques employed were also subsequently introduced and applied. For example, two-dimensional immunoelectrophoresis was used to study the antigenic relationship among different mycobacteria.[19] A large number of shared antigens were stained, observed, and identified among the various mycobacterial species examined. A similar observation was reported by Chaparas *et al.*,[20] who sought improved resolution by applying a variation of the immunoelectrophoretic method termed the fused rocket technique to the antigens of *M. tuberculosis* and other mycobacteria he had studied. Of special note was the superior resolving power reported by the

Scandinavian group, who proposed a reference nomenclature for the antigens of bacillus Calmette–Guérin (bCG) strain of *M. bovis* studied by a two-dimensional crossed immunoelectrophoresis technique.[21] Although it is clear that several modifications employing both immunoelectrophoresis and PAGE methods have been applied to the study of the identification and nomenclature of mycobacterial antigens, except for the specific laboratory at which the identification system most likely originated, wide acceptability of a system for identification and nomenclature of fractionated components has not found favor.

Stanford and Beck[22] addressed the problem of analysis and nomenclature of the mycobacterial antigens by simply numbering the antigens detected by immunodiffusion systems. This approach was abandoned and replaced by a classification by antigen distribution wherein the antigens that appear as distinct lines on immunodiffusion analysis are placed into four groups. Group I includes those antigens present in all mycobacterial species; group II includes antigens that are present in the slowly growing species; group III includes those antigens restricted to the rapidly growing species; and group IV includes those antigens restricted to individual species.

This classification scheme appears to be more applicable to the needs of the mycobacterial taxonomist in attempting to classify and define separate species and related taxons within the genus.

3. EARLIER STUDIES ON THE PREPARATION OF TUBERCULINS FOR SKIN TESTING

Attempts to improve the specificity and sensitivity of the tuberculin skin test as well as to minimize the variations observed in the potency of OT preparations available for use resulted in the interest and stimulation by a number of investigators to isolate and purify mycobacterial antigens for possible diagnostic and epidemiologic use. It became apparent as more experience was gained in using OT in both chemical and epidemiologic studies that these variations and lack of uniformity impaired the value of the skin test.

3.1. Old Tuberculin

The first serious attempt to obtain diagnostically purified skin-test fractions was made by Seibert from OT, a concentrated culture filtrate prepared according to the classic method used by Koch.

In these early studies, OT was prepared by concentrating glycerol peptone broth filtrate on which tubercle bacilli had grown for 6–8 weeks

to one tenth its original volume.[23] Koch believed that the active component in this material, which he originally advocated for the treatment of tuberculosis, was protein or at least closely associated to it; Koch tried to isolate the active principle from the culture filtrate by the addition of various precipitants. Among the various protein precipitants he used was alcohol; when he made comparative skin tests using the alcohol-precipitated crude fraction and OT in tuberculosis patients, the alcohol-precipitated crude fraction had a much more potent tuberculin effect than did his OT. Koch believed this observation to be of only theoretical interest, and he did not pursue these purification procedures any further.[24] It was an obvious disappointment that Koch's tuberculin, which had been at first received with so much enthusiasm as a cure for tuberculosis, did not materialize. However, its use as an aid in the diagnosis of tuberculosis was soon recognized worldwide and ironically fulfilled Koch's earlier prediction that it would have good diagnostic value.

3.2. Purified Tuberculins

It soon became apparent that it was difficult to separate the active protein principle in tuberculin from the many other components of the glycerin broth medium from which it was prepared. In order to avoid these difficulties, a chemically defined synthetic medium was employed by Seibert[25] in which tubercle bacilli were grown for 6–8 weeks. The live bacteria were filtered off and the unheated culture filtrate concentrated by ultrafiltration through gun-cotton membranes and subsequently precipitated by ammonium sulfate. Although a considerable amount of polysaccharide was found associated with the protein precipitate, this impurity could be removed by repeated solution and precipitation. Such an essentially polysaccharide-free tuberculin, designated MA-100, was later prepared by Masucci and McAlpine,[26] who extended the previous work of Seibert. In comparisons made with OT in humans, it was found to be significantly more potent. However, as with other tuberculins derived from unheated culture filtrates, MA-100 had a sensitizing effect when injected repeatedly into the skin.

3.3. Purified Protein Derivatives

With the encouragement and support of the National Tuberculosis Association and following years of investigation on improving a tuberculin for use in skin testing, Seibert[27] in 1934 prepared a highly specific protein of low molecular weight and nonsensitizing character that had the stability and other requirements for a standardized tuberculin. Tubercle bacilli were grown on a synthetic medium of Dorset for 6–8

weeks. The culture was heated to kill the bacilli, the organisms filtered off, and the culture filtrate concentrated by ultrafiltration on collodion membranes. This was followed by precipitation of the concentrated material with TCA. In order to remove the acetic acid, the precipitate was washed repeatedly with ether, resulting in a light tan-colored dry powder. This product was referred originally by Seibert as SOTT (synthetic medium old tuberculin precipitated by trichloracetic acid); later, the initials PPD (purified protein derivative, because it represented a form of the protein that had now possibly been degraded by the heat) were used to designate the manufactured product. Molecular-weight determination and protein analysis of this fraction have proved it to be approximately 2000–4000, characterizing it as a polypeptide. Seibert regarded this low-molecular-weight polypeptide as being not only the smallest unit possessing specific tuberculin activity but also as representing the basic unit from which larger tuberculin-active aggregates were formed. Although the antigenicity of the protein molecule is gradually lost as it is reduced in size, its specific tuberculin potency remained unchanged. The concept of an active protein molecule proposed by Seibert during this early period derived its support from her investigations with this particular PPD tuberculin. Although it was reported that skin responses were elicited in sensitive patients given very small doses of this PPD intracutaneously, PPD was unable to induce sensitization in experimental animals even when repeatedly injected with large amounts of this product. Specifically, it could neither elicit Arthus responses in guinea pig and rabbits nor produce precipitins or precipitate highly immune sera. Thus, this nonsensitizing character is an essential attribute that a standard tuberculin must meet if it is to be used in skin testing.

3.4. PPD-S

Continuing efforts to produce an even purer PPD than was prepared commercially and available during the 1930s resulted in a more improved tuberculin that represented a large single batch of PPD.[10] This was subsequently adopted both as the U.S. government's standard and as the International Standard for tuberculin in 1952.[28] Studies with this product (PPD-S) showed it to be purer and more potent than any of the previous preparations. Seibert's electrophoresis studies demonstrated that the previous method of precipitation with TCA did not separate the nucleic acid from protein but that it would in fact precipitate them together. However, it was found that by precipitating with half-saturated ammonium sulfate at neutrality and at low temperature, significant separation can be made, and a product resulted that was a very potent protein for use in skin testing.[10]

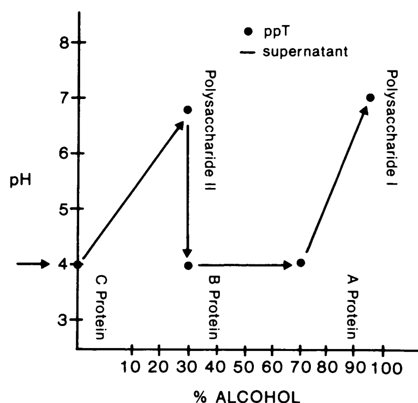

FIGURE 2. Fractionation scheme for isolating three different proteins and two distinct polysaccharides. (From Seibert.[6])

This tuberculin standard was prepared from the DT strain of *M. tuberculosis* grown on Long's medium for 8–10 weeks. Repeated precipitation of the heated concentrated ultrafiltrate by half-saturated ammonium sulfate in the cold and repeated washings on gun-cotton membranes resulted in a protein that had an estimated molecular weight of 10,500. The protein content was approximately 92.1%, with low amounts of nucleic acid and polysaccharides, of 1.2% and 5.9% respectively. Some heterogeneity was noted upon ultracentrifugation, and moving boundary electrophoresis showed the presence of at least three components of different mobilities. As with the PPD prepared by the TCA acid method, frequent skin tests with PPD-S were nonsensitizing.[29]

The classic and probably most studied of the fractionation procedures for preparing mycobacterial antigens were those developed by Seibert and associates[6] for fractionating unheated culture filtrates. Her protocol, patterned after the Cohn serum protein fractionation scheme, used alcohol at low temperature with careful control of pH. With this scheme of differential solubility, as noted in Fig. 2, three protein fractions, designated A, B, and C in order of increasing electrophoretic mobility, and two serologically reactive polysaccharides, termed polysaccharides I and II, were obtained. These fractions, which could be reproducibly isolated, had different physicochemical and biologic properties. Protein A had a molecular weight of 35,000–42,000 and appeared to be more potent as a skin-test material than PPD-S when given in equivalent protein concentrations. The B protein was estimated to have a molecular weight of 20,000 and of a tuberculin potency intermediate between that of the most potent A and the least potent C proteins. These differences in potency appeared to be more consistent when compared in sensitized animals but yielded variable results when compared in humans, most likely because of the variation in individual sensitivity. Ami-

no acid analyses indicated no significant differences in amino acid composition among the A-, B-, and C-protein fractions.[30]

Polysaccharide I is obtained from the concentrated culture filtrate as a residual component after the removal by acid and alcohol precipitation of the A, B, and C proteins and polysaccharide II. It is almost immobile and could be obtained in relatively pure form in a moving boundary preparative electrophoresis apparatus by permitting other components of the concentrated culture filtrate to migrate away from this electrophoretically homogeneous substance. It has a molecular weight of 9000 and is a heteropolysaccharide composed of mannose arabinose and galactose.[31] Sometimes polysaccharide I is connected to a nitrogenous residue, which has been identified as glucosamine or as those amines and amino acids that make up the mucopeptide complex.[32]

Biologic analysis of polysaccharide I indicates that it is active as a haptene in serologic tests and is incapable of eliciting delayed skin-test reactions both in infected animals and in humans. It is nonantigenic in its pure form but, when contaminated with minute amounts of protein, can elicit both *in vivo* and *in vitro* cell-mediated hypersensitivity responses, very likely attributable to its contaminating protein.[33–34]

The polysaccharide II fraction appears to be more restricted in its distribution among the different strains of tubercle bacilli than polysaccharide I. Its presence is inconstant, since it may not be produced by a particular culture that had previously produced it in copious amounts under identical conditions. When present, it gives a characteristic opalescence to the culture filtrate. Polysaccharide II has a slow mobility in moving boundary electrophoresis, and ultracentrifugation studies indicate it has a molecular weight of 100,000. This polyglucosan is antigenic when injected into animals inducing antibodies that react in both precipitin and complement-fixation tests. It fails to elicit a delayed skin response in infected animals or humans.[35]

An attempt to relate these fractions obtained according to Seibert's classic method to the numbered antigens of the U.S.–Japan immunoelectrophoresis nomenclature was reported by Daniel and Affronti.[36] These workers demonstrated that the A protein contained antigens 1, 2, 4, 5, and 6, with antigen 6 being the most dominant.

The B protein contained antigens 1, 2, 5, 6, and 7. It differed from the A protein by having much less antigen 6 but significant amounts of another antigen, antigen 7. By contrast, the C protein was composed of antigens 2, 6, and 7 but in significantly lower amounts than observed for the other two proteins. It also contained two nonidentified and minor anodal components. Polysaccharide I, the heteropolysaccharide, showed two precipitin arcs upon immunoelectrophoretic analysis that consisted mainly of antigen 2, with the other arc representing small amounts of

TABLE I
Antigenic Relationships of Seibert's Tuberculin Fractions with the U.S.–Japan Immunoelectrophoresis Identification System

U.S.–Japan antigen identification number	Protein			Polysaccharide	
	A	B	C	I	II
1	P	P	—	P	—
2	P	P	PS	P	—
3	—	—	—	—	P
4	P	—	—	—	—
5	P	P	—	—	—
6	PL	PS	PS	—	—
7	—	PL	PS	—	—

P, present; PL, present in large amount; PS, present in small amount.

antigen 1. Polysaccharide II, the high-molecular weight polyglucosan, has been identified as antigen 3. Table I summarizes these relationships with the U.S.–Japan Immunoelectrophoresis reference nomenclature.

4. SURVEY OF OTHER FRACTIONATION MODALITIES FOR SEPARATING AND PURIFYING MYCOBACTERIAL ANTIGENS

It should be clear at this point that both the culture filtrates and mycobacterial extracts are highly complex mixtures of proteins, lipids, sugars, and nucleic acids. Successful separation and isolation of the many antigenic components in the mixtures require the application of different fractionation modalities other than those that have already been considered.

Since the introduction by Seibert of the ethanol–acetic acid solubility methods for purification of tuberculins and their subsequent characterization and preparation by moving boundary electrophoresis,[6] additional physicochemical methods have become available in the laboratory and are now part of the technologic armamentarium used by investigators seeking to isolate and purify mycobacterial antigens for study. No attempt is made here to provide a detailed review of these methods. The reader is directed to the two reviews by Daniel and Janicki[3] and Daniel[37] for a more comprehensive treatment of such techniques applicable to fractionation. Rather, it is the intent of this section to review only selected physicochemical procedures that reflect various representative approaches chosen by different investigators for isolating

and purifying mycobacterial antigens and that, as part of the published description of the fraction, also provided information about its biologic characterization by demonstrating either tuberculin activity, species specificity, or both.

Kniker and LaBorde[38] applied ion-exchange chromatography to separate 12 fractions from the culture filtrates of four strains of *M. tuberculosis* comprising antigens whose relationships were subsequently analyzed by serologic methods. None was pure or contained single antigenic components. These investigators concluded that a single physicochemical method probably would not be able to separate pure antigens from complex source materials such as culture filtrates. Subsequently, upon applying this method to Seibert's A, B, and C proteins, Kniker[39] reported that each protein was associated with a separate peak but that each, when analyzed by serologic methods, contained multiple antigens. Moreover, the eluted peaks, when analyzed, had greater absorbency at 280 nm than at 260 nm, implying that the scheme proposed for fractionation by Seibert, involving prior alcohol and acetic acid treatment, results in purer products, with accompanying decreases in both polysaccharide and nucleic acid contaminants. Antigenic analysis of these fractions by immunodiffusion revealed some species specificity, but nonspecific antigens were broadly distributed throughout the fractions, limiting the value of this method in recovering fractions containing single or specific antigens.

The investigations of Yamamura *et al.*[40–43] applied ion-exchange chromatography with DEAE-cellulose in order to purify further a tuberculin fraction, which they termed tuberculin active peptide (TAP). The original material they reported on was a less pure product obtained by extracting heat-killed mycobacterial cell walls. The molecular weight was estimated at 5000–10,000 and, although it was able to elicit delayed skin-test responses, it was not immunogenic.

Working with enzymatically degraded cell walls of *M. tuberculosis,* Kotani and associates[44] also employed ion-exchange chromatography after gel filtration of the enzymatic digest to effect purification of a tetrapeptide and a tripeptide fraction of the peptidoglycan cell wall. When these peptides are complexed with mannose, arabinose, and galactose, reaction with rabbit antisera prepared against tubercle bacilli occurs. It is unclear as to what particular moiety is responsible for this reaction.

Lind[45] applied carboxymethylcellulose ion-exchange chromatography to the culture filtrate of *M. tuberculosis* but was unsuccessful in obtaining fractions that did not have multiple antigens that overlapped with others.

As reported by Daniel and Janicki,[3] moderate separation was ob-

tained by Baer and Chaparas, who employed molecular exclusion chromatography using Sephadex G25 and G50 to fractionate acid-soluble and acid-insoluble constituents from the culture filtrate of *M. bovis,* BCG strain. Although the acid-insoluble portion contained many antigens, as determined by serologic methods, and displayed tuberculin activity in both homologously and heterologously sensitized animals, the acid-soluble portion was of special interest. These investigators succeeded in separating it into three fractions. The first fraction was believed to contain mainly polysaccharides. Interestingly, it exhibited tuberculin activity, since it elicited typical delayed skin-test responses when injected into sensitized animals. Moreover, when assessed by cell-mediated *in vitro* assays, this fraction inhibited the migrating macrophages obtained from sensitized animals but failed to stimulate mitogenesis in guinea pig lymphocytes. Upon skin testing human tuberculin reactors at significantly lower doses than had been used to elicit positive reactions in guinea pigs, this diluted fraction failed to produce any skin reactions. Protease and trypsin treatment failed to eliminate skin-test responses; it remained unclear as to the chemical component responsible, since it is well established that only a small amount of contaminating protein is required to elicit a skin response in highly sensitized animals and humans. The other two fractions, each of which contained nearly as much protein as polysaccharide, was capable of eliciting a delayed skin response in sensitized animals and inhibited macrophage migration but, unlike the first fraction, had the ability to stimulate mitogenesis.

Janicki *et al.*[46] attempted to fractionate *M. tuberculosis* culture filtrates by continuous-flow paper electrophoresis. These workers obtained a pool of polysaccharide fractions and a pool of protein-enriched fractions, essentially all of which contained multiple antigens when subsequently analyzed by immunodiffusion.[47]

Although this zonal electrophoretic technique can be of moderate effectiveness in separating polysaccharide (because of the cathodal nature of the molecule), it has only limited value in providing individual antigens. Biologic characterization of the polysaccharide fraction showed that it neither stimulated mitogenesis in cultured blood lymphocytes from tuberculosis patients nor induced precipitins when injected into guinea pigs,[48] although it could react with antisera from highly sensitized animals.[49] In this regard, immunodiffusion studies[50] supported and confirmed the presence of polysaccharide antigens in the fraction when it was reacted with sera from patients with tuberculosis.

Daniel and associates have been successful in employing affinity chromatography techniques as a tool for isolating and purifying antigens from complex mixtures. Early work by Daniel and Wisnieskski[51] resulted in the observation that polysaccharides from mycobacterial

culture filtrates react reversibly with the lectin concanavalin A (Con A). Passing crude mixtures through a column containing Con A complexed to agarose in the form of agarose beads permits the isolation of polysaccharides and glycoproteins that bind to the lectin through their sugar moieties. Such a separation is not caused by the physicochemical properties of the molecules but depends on the degree of specificity of the interacting sites between molecules. Consequently, various molecules in a mixture that have the same or very similar reactive sites can be recovered together in spite of very different physicochemical properties. It must be remembered that although this technique can separate antigens irrespective of molecular size, it will not isolate monospecific molecules, because different antigenic determinants other than those specifically interacting with the reactive sites would also be present in the isolated antigens.

Using this technique, Daniel[52] isolated three polysaccharides from the culture filtrate of *M. tuberculosis;* one was characterized as a Con A-nonreactive polysaccharide and the other two were Con A-reactive polysaccharides. Of these latter two, one was of high concanavalin affinity and the other of low affinity. Immunologic characterization showed that these polysaccharides react with animal antisera in immunodiffusion and hemagglutination systems and have the capability to elicit immediate and delayed hypersensitivity skin responses in laboratory animals. Protein was present in these fractions, but treatment with proteolytic enzymes did not appear to eliminate its activity.

Further analysis of these polysaccharides by Daniel and Misaki as described by Daniel and Janicki[3] indicated that the Con A-nonreactive polysaccharide was arabinogalactan and was identified with antigen 2 of the U.S.–Japan reference system. Analyses of both the high Con A-reactive and low Con A-reactive polysaccharide were identified as arabinomannan, correlating with antigen 1 of the reference system. Contaminating each of the polysaccharides was a glycan, reported as nonantigenic.

These careful comparative studies provide conclusive evidence that arabinomannan and arabinogalactan identify as antigen 1 and antigen 2, respectively, of the U.S.–Japan reference system and confirms by more modern sophisticated laboratory techniques the earlier pioneering work of Seibert, who similarly showed that her polysaccharide I fraction had the same sugar composition.

The versatility of immunoabsorbent affinity chromatography was employed by both Laguerre and Turcotte[53] in preparing immunoabsorbents with antisera against specific mycobacteria antigens for furthering their purification. After establishing optimal conditions for disassociating bound antigen from immunoabsorbent columns, Daniel and Ander-

son[54] were able to apply this technique to the purification of an antigen from *M. tuberculosis* H37Ra culture filtrate and that could be identified as antigen 5 by the U.S.–Japan reference system. This antigen had tuberculin skin-test activity nearly equal to that of PPD when injected in sensitized animals and when first described appeared to have greater species specificity than PPD.[55] Subsequent studies[56] in humans showed antigen 5 to be no more specific than PPD, however. Characterization of this cytoplasmic protein indicates that it has a molecular weight ranging from 28,500 to 35,000 and is believed to consist of a single peptide chain containing no disulfide linkages. It appears to be free of measurable carbohydrate, as determined by analysis but has been reported to bind with low affinity to Con A, implying it must be associated with a carbohydrate moiety. Cell-mediated *in vitro* studies showed that antigen 5 stimulated mitogenesis and migration inhibitory factor (MIF) production by lymphocytes from sensitized guinea pigs. Human lymphocytes from tuberculin-positive donors also responded to antigen 5 with MIF production but, interestingly, were not stimulated to undergo mitogenesis.

Although an array of physicochemical methods, including the many variations and modification of ion exchange, molecular exclusion, and affinity chromatography, and both moving boundary and PAGE have been variously applied by many investigators in attempts to isolate and purify mycobacterial antigens, no single method led successfully to the satisfactory recovery of improved fractions containing single or specific antigens for skin testing or serodiagnosis of tuberculosis over those available today. An obvious outcome from these many studies was the realization that mycobacterial antigens are heterogeneous and that the use of a single method or procedure to produce a fraction of the level of purity and specificity desired would be unlikely. Rather, it would most probably require a sequence of fractionation and purification procedures to fractionate mycobacterial antigens successfully from complex mixtures; this was the next logical modality considered.

Using a combination of ammonium sulfate precipitation, preparative electrophoresis, and ion-exchange chromatography, Yoneda and Fukui[57,58] were successful in ultimately obtaining fractions from culture filtrates of *M. tuberculosis* containing single antigens. Unfortunately, this approach gave very small yields of materials for study. They isolated two antigens designated α and β, which were considered major extracellular proteins. Molecular-weight calculations based on ultracentrifugation estimates were 30,000 for the α-fraction and 100,000–150,000 for β. Ultracentrifugation analysis of the β-fraction showed that it had two components and therefore was not as pure as the α-antigen.

The α-antigen was found in the A, B, and C proteins prepared according to Seibert's fractionation method and appeared to be wide-

spread among mycobacterial species, although it was not found in the rapidly growing mycobacteria, i.e., *M. phlei, M. fortuitum,* and *M. smegmatis.* Therefore, α would belong to the group II classification of Stanford, i.e., antigens present in slow-growing mycobacteria.[59] In sensitized animals, it elicited large skin reactions but diminished or weak reaction when subjected to heat, thus acknowledging its known physicochemical property of heat sensitivity. It was found to precipitate antiserum and induced antibodies when injected into animals. Although this approach of Yoneda and Fukui employing a combination of methods was one of the first to give productive results, recovery of yields was small, and research protocols were labor-intensive and complex, limiting its value and application on a larger scale. By employing the agar-gel diffusion technique and absorbed anti-α-antigen sera as a taxonomic tool to differentiate related mycobacterial strains, subsequent studies by Tasaka *et al.*[60] recently showed that each purified protein antigen possesses determinants that are apparently specific for the species from which it was isolated.

Daniel and Ferguson[61] isolated two protein fractions from the culture filtrate of *M. tuberculosis* by combining a series of physicochemical methods that included prior precipitation with ammonium sulfate, followed by gel filtration with P-300 acrylamide, ion-exchange chromatography, and zonal electrophoresis. The two proteins identified as α_1, and α_2 had an estimated molecular weight that ranged from 45,000 to 48,000. These two proteins were biologically characterized as displaying tuberculin activity in sensitized guinea pigs and were capable of stimulating mitogenesis with cells from PPD-positive humans donors.[62] It was later shown that α_2 had identity with antigen 6 of the U.S.–Japan reference system and demonstrated increased specificity when skin tested in sensitized guinea pigs.[63] It is a major protein of *M. tuberculosis* and other closely related mycobacterial species. It was also identified as a major antigen of Seibert's A protein by Daniel and Affronti[36]

A multiphysicochemical separation approach was undertaken by Nagai *et al.*,[64] who attempted to fractionate a PPD preparation made from the heated culture filtrate of *M. tuberculosis.* These workers employed precipitation by ammonium sulfate, followed by gel filtration, ion-exchange chromatography, and electrophoresis in acrylamide gel. They obtained five protein fractions with molecular weights of approximately 10,000 each. Subsequent studies by Nagai *et al.*[65] on the unheated culture filtrate of *M. bovis* BCG, in which they used fractionation procedures similar to those they employed when working with a heated PPD-like material some years earlier, yielded an interesting protein designated MPB 70. Nagai and co-workers described this protein to be 15,100–18,000 M_r and to make up the major protein component of the

cellular filtrate, considered highly specific. It was characterized as having an R_f value of 0.70 on PAGE. It appears to have a high level of specificity, since it was reported that very small skin-test reactions were given by guinea pigs sensitized with mycobacterial strains other than *M. bovis*. Subsequent studies by Miura *et al.*[66] demonstrated that detectable amounts of this protein are produced by some, but not all, BCG substrains examined and that it elicited skin reactions in guinea pigs sensitized with live BCG but not when sensitized with heat-killed organisms.

Studies by Kuwabara and Tsumita[67–69] were of special interest, since these workers isolated a protein from heat-killed acetone-extracted *M. tuberculosis* and *M. bovis* cells, which was a potent tuberculin in both sensitized animals and infected humans. By using a combination of enzymatic digestion, ammonium sulfate precipitation, and both ion-exchange chromatography and gel filtration, the crude protein was finally crystallized with 85% ammonium sulfate and 50% acetone. This labor-intensive protocol suffered from low yields but produced an extremely pure and potent antigen. It had a molecular weight of 9700, not unlike that reported for PPD-S and prepared from the concentrated culture filtrate of *M. tuberculosis* by Seibert and Glenn.[10] Kuwabara[69] succeeded in determining the amino acid sequence of this protein. Upon generating many peptide fragments of various size from this protein by enzymatic degradation and subsequently studying their tuberculin activities, it was deduced that the maximum tuberculin activity of one of the prime candidate fragments that was isolated resided in the 6-amino acid sequence, ASN-GLY-SER-GLN-MET-ARG. When skin-tested in sensitized animals, it possessed tuberculin activity but at a level less than that elicited by the parent protein. Subsequent studies by Savrda,[70] who synthesized this 6-amino acid peptide, failed to demonstrate any tuberculin activity in sensitized animals either when the peptide was administered alone or when conjugated to bovine serum albumin (BSA). It did produce weak cross-reactions with PPD, however, in radioimmunoassay (RIA).

Moreover, Savrda[71] later synthesized an octapeptide and a hexadecapeptide that did elicit skin reactions in sensitized guinea pigs, but at a dose twice that used in his previous study. By contrast, Toida *et al.*[72] failed to detect any skin reactivity when they used either a hexadecapeptide or an octadecapeptide synthesized in accordance with sequence originally described by Kuwabara.[69]

Following an approach similar to the above studies, but using an antigen that had been isolated after affinity chromatography by elution from an immunoadsorbent to which a monoclonal antibody had been coupled and designated BCG-a,[73] Minden *et al.*[74] determined the se-

quence of the 20-terminal amino acid residues of this antigen and chemically synthesized several peptides corresponding to different portions of it. Of the peptides synthesized, one designated as BCG-a P showed significant immunologic similarity between it and antigens found in sonicated extracts by BCG and H37Rv but not in those from the nontuberculous mycobacteria examined. Western blot analysis suggested that this component has a molecular weight of 10,000 and is capable of inducing and eliciting both humoral and cellular immune responses. Minden *et al.* point out the potential benefits of combined monoclonal antibody methodology and affinity chromatography to prepare, standardize, and identify mycobacterial antigens for a possible broad range of immunodiagnostic and immunogenic uses.

Polyacrylamide gel electrophoresis is a fractionation technique that not only offers a high degree of resolution, simplicity, and reproducibility for analysis and study of biologic materials but is also an excellent tool for following homogeneity and purity of the isolated fraction. This procedure separates components in a complex mixture on the basis of molecular size and charge. An advantage of this procedure is that the pore size of the polyacrylamide gel can be tailor-made to meet the requirements of separating different classes of molecules by modifying the concentration of the polyacrylamide employed. Affronti *et al.*[18] were the first to apply this technique as an analytic tool for following tuberculin fractionation. Preparations obtained by precipitations by various concentrations of ammonium sulfate of unheated culture filtrate of *M. tuberculosis* and fractions obtained by the method of Seibert[6] were analyzed. Since reproducible Rf values can be determined and specific histochemical staining procedures can be employed to identify the nature of the separated components, this procedure has much to offer as a potential reference system. Improvements in this powerful tool were subsequently introduced by Affronti *et al.*,[75] who characterized and compared mycobacteria antigens by discontinuous pore-gradient gel electrophoresis and by Wright *et al.*,[76] by applying a two-dimensional PAGE procedure in further characterizing these mycobacterial antigens. Both modifications underscored the tremendous heterogeneity and complexity of antigens associated with *M. tuberculosis* and related mycobacterial species.

Among other workers who employed PAGE were Roszman *et al.*,[77] who were able to recover apparently specific antigens from culture filtrates of mycobacterial species, including *M. bovis, M. avium,* and two strains of the *M. intracellular-avium* complex. Eluates from these four culture filtrates each contained anodic components when electrophoresed and stained. These investigators were able to isolate single antigens

from some eluates, and these were analyzed and identified by immunodiffusion. Of particular interest was an antigen that was specific only for *M. bovis.*

In an attempt to obtain large yields of antigens, a preparative PAGE method was developed and described by Minden and Farr,[78] who isolated fractions from sonicated cells of *M. tuberculosis, M. bovis* BCG, and *M. fortuitum.* Among the many isolated antigens obtained by this procedure was one of special interest—a rapidly moving mucopolysaccharide with a molecular weight in the range of 9000–12,000 and was soluble in 50% ammonium sulfate. Its antigenicity was abolished by pronase, and there was a suggestion of species specificity because of the positive results obtained from antiserum binding studies.

In an extensive cooperative study[12,13] involving participation by Daniel, Chaparas, Goldstein, Good, Janicki, and Wright, preparative PAGE was also applied to the fractionation of unheated culture filtrate and cell extracts of *M. tuberculosis.* It was the aim of this collaborative effort to elute the electrophoresed antigens from individual gel slices and determine its identity with the U.S.–Japan reference system. The specificity and potency of each fraction were assessed by skin testing in groups of sensitized guinea pigs. The antigens were also surveyed for their ability to induce blast transformation in cultured lymphocytes. Maximum antigenic activity and species specificity were reported to reside in those fractions containing antigens 5 and 6 of the U.S.–Japan nomenclature. The polysaccharide-associated antigens 1, 2, and 3 did not induce blast transformation and elicited nonspecific skin-test reactions in sensitized guinea pigs. Finally, an extremely anodal antigen that had also been isolated and that had been speculated to be the same as the highly anodal antigen described by both Roszman and Minden and Farr in their studies noted above neither displayed skin-test activity nor induced blast transformation in tissue-culture assays.

Affronti *et al.*[79] undertook to fractionate PPD-S, the international standard, by a preparative PAGE apparatus that they designed and developed in the laboratory of Ouchterlony. Electrophoretic patterns showed multiple components when stained by specific protein stains. Skin testing in sensitized animals with eluates from 7% and 15% gel segments, which represent moieties of low molecular weight, elicited the reactions of maximal activity, confirming the early work of Seibert and Glenn,[10] who showed that tuberculin activity also resided in low-molecular weight components.

Encouraged by the promising results obtained by applying the preparative gradient gel electrophoresis method first described by Affronti[18] for fractionation of products of mycobacterial origin and its subsequent application for separating more specific components from PPD-S,

a cooperative interlaboratory study was initiated with the support of the World Health Organization.[80,81] Its aim was to study the above-mentioned PAGE method for fractionation of *M. tuberculosis* on a large scale. The study enlisted the cooperation of four laboratories—that of Affronti in Washington, D.C., Ouchterlony, Lind, and Ridell in Goteborg, Sweden, Wisingerova in Prague, Czechoslovakia, and Magnusson in Copenhagen, Denmark. The project involved the growth and harvesting of 1500 cultures of *M. tuberculosis* H37Rv and the ultrafiltration of 150 liters of the concentrated culture filtrate resulting in two fractions: one containing molecules of 1000 and 10,000 M_r and the other of greater than 10,000 M_r. A PPD designated PPD-V3, made according to the method of Seibert[10] was prepared from the greater than 10,000 M_r fraction, since that fraction was the most active, as revealed by a lymphocyte stimulation assay. The preparative PAGE method employed a multistage gel for protein separation. The PPD migrates through a four-stage gel column consisting of a gel concentration of 3.5%, 4.75%, 7%, and 15%. Eluates prepared from each of the four segments were evaluated by comparative skin tests in groups of sensitized guinea pigs, using PPD-RT23 as a reference standard. Although the largest number of precipitinogens was found in the 15% fraction, when analyzed by immunodiffusion, the 7% fraction was the most potent skin-test antigen in homologously and heterogously sensitized animals.

Significant levels of cross-reactivity could be demonstrated with all four fractions examined, however, reflecting the presence of shared epitopes that exist in antigens of the other mycobacterial species. Of particular interest was the observation that an increased species specificity was associated with the eluate from the 7% fraction as compared with the reference tuberculin PPD-RT23 in sensitized guinea pigs. Whether this observed difference in guinea pigs would have application in human skin testing remains to be determined. It is clear that no single-step fractionation procedure, including this preparative PAGE system, is optimal for isolation and purification of mycobacterial antigens. The production of semipure fractions resulting from this method, however, can serve as an improved starting material for further purification.

5. SEROLOGIC METHODS FOR DIAGNOSIS OF TUBERCULOSIS

A variety of serologic methods have been employed to demonstrate the increase of antibodies against *M. tuberculosis* infections. These include complement fixation, agglutination, and hemagglutination techniques. None of these tests has been completely successful, however, and all have limitations in terms of sensitivity, specificity, and reproducibility.

Using a modification of the precipitation in gel method described by Oakley and Fulthorpe,[82] Parlett *et al.*[83] applied this one-dimensional gel double-diffusion procedure (the tube GDD test) to demonstrate antibodies in the sera from 48 tuberculosis patients. Only 2 of 38 healthy individuals gave positive reactions. These encouraging results stimulated further studies using this technique and in 1959, Parlett and Youmans[84] reported on a double-blind study evaluating the specificity and sensitivity of this test. This extensive study of 1097 tuberculosis sera from patients was classified according to the extent of disease. It showed that 84.2% of patients with advanced pulmoanary disease gave positive results; 73.5% of patients with moderately advanced disease were positive, as were 57.8% of patients with minimal disease. It was of interest that 73.3% of patients infected with atypical mycobacteria gave positive results, while 43% of samples of sera from patients with nonpulmonary tuberculosis did so. Only 2.1% of patients with disease other than tuberculosis gave positive reactions. Finally, they reported no correlation between the presence of tuberculin hypersensitivity and the presence of humoral antibody.

In a subsequent comparative study, Lind[85] reported that the gel tube test was 30% more sensitive than the Ouchterlony double-diffusion plate technique. Although these immunodiffusion studies showed promise for developing a specific and sensitive serodiagnostic test, experience by other investigators gave conflicting and varying results. For example, Grange and Kardjito[86] obtained positive results with only 28% of the sera from tuberculosis patients they tested, underscoring the problem of low specificity they associated with this test. Thurston[87] pointed out that attempts to employ immunodiffusion as a clinical diagnostic test suffered in ways similarly applicable to agglutination reactions as a serodiagnostic test for tuberculosis, attributing the shortcomings of this technique not only to the presence of cross-reacting antigens in the reagents employed but also to the inherent variability of the individual's immune responses over time, emphasizing a widely acceptable view that antibody production follows a dynamic course in development of disease.

The soluble antigen fluorescent antibody (SAFA) procedure was first applied to the serodiagnosis of tuberculosis by a group of investigators who used mycobacterial antigens prepared by Affronti and Seibert.[88] The test is an indirect fluorescent antibody procedure that employs tuberculin proteins absorbed to cellulose acetate disks. These are reacted with patient's sera and labeled antiglobulins. This test is technically simple to perform and objective, since the reactions result in fluorescence measured in a fluorometer. In using a crude antigenic preparation to evaluate 50 normal human sera, serum antibody was detected in more than 60% of the samples, reflecting the lack of sufficient specificity and sensitivity

associated with the crude tuberculin reagent first employed by Toussaint *et al.*[89]

Fractionation of the crude antigen preparation according to the methods of Seibert in an attempt to improve the value of the test was undertaken by Affronti *et al.*[90] High antibody levels were generally detected in patients with advanced tuberculosis than in patients diagnosed as having minimal or moderately advanced disease. In evaluating these sera, it was reported that the antibody titer rose following the initiation of chemotherapy. These investigators emphasized, however, in comparing measurements of central tendency derived from fluorometer readings of 208 serial serum samples from tuberculosis patients in various stages of disease, that all sera were reactive at some time during the course of testing with different more purified antigen preparations, thus making correlation of antibody level with stage of disease of limited value. Employing the SAFA test to evaluate 191 sera, of which 126 were from cases of tuberculosis and 65 controls, Kiran and Shriniwas[91] viewed the test as useful in the early diagnosis of tuberculosis.

In comparing tuberculosis patients treated for 3 months and after 1 year of therapy, Kiran and Shriniwas found that significant levels of antibody occurred in sera from patients treated for 3 months, a finding not unlike that reported by Affronti *et al.*[90] After 1 year of treatment, the number of cases with high antibody levels decreased significantly, but in all cases a demonstrable yet low level of antibody could be detected. They speculated that this low antibody level could be attributable to the immunosuppressive properties described by Ellner and Daniel[92] for arabinomannose, the polysaccharide that would be released upon the death of the tubercle bacillus following chemotherapy.

A highly sensitive RIA technique based on the binding of radiolabeled antigen to antibody, followed by precipitation of the complex by an anti-immunoglobulin, was employed by Minden and Farr.[78] Using goat anti-IgG, they were able to measure and compare the precipitated mycobacterial [^{131}I]antigen–antibody complex from the sera of tuberculosis patients and healthy controls. The tuberculous sera were found to bind the greatest amount of labeled antigen while sera from the control group bound the least.

Other primary binding studies by Minden *et al.*[93] demonstrated the universal presence of broadly cross-reactive antibodies in the sera of humans and laboratory animals that cross-react with antigenic determinants of both mycobacteria and other naturally occurring microorganisms of the environment, thereby adding to the extreme difficulty of developing any new specific serologic test for the diagnosis of tuberculosis.

Although the experience noted above describes RIA procedures for

detecting antibody in patient serum, an alternative approach would be to employ such a test for detecting antigen in the patient's specimen (e.g., sputum, spinal fluid). Straus *et al.*[94] reported on such a test, which would be particularly useful in cases of extrapulmonary tuberculosis, for which bacterial isolation of the tubercle bacillus is usually unrewarding.

In the RIA, routine clinical specimens (e.g., sputum, spinal fluid) can be prepared for culture according to standard methods and grown on Middlebrook 7H9 medium for 4 days. Aliquots are removed, autoclaved, and used for the assay. The RIA procedure has the sensitivity of detecting about 1 μg antigen or equivalent to detecting 1×10^3 microorganism/ml. This method has the benefit of providing a measure of safety that is unattainable when processing specimens for bacteriologic culture, because heat-killed specimens are used. Along with being readily adaptable for assaying large numbers of samples in a clinical laboratory, this procedure has great potential in being safe, rapid, and sensitive.

During the early 1970s, Engvall and Perlmann[95] introduced the enzyme-linked immunosorbent assay (ELISA) as a rapid, sensitive procedure for the detection and measurement of antibodies in sera of patients infected with different pathogenic microorganisms. This assay was soon recognized for its importance in the serodiagnosis of a number of infectious diseases. Application of this procedure for detecting antibodies to *M. tuberculosis* was reported by Nassau *et al.*[96] using a crude mycobacterial antigen. In spite of a significant overlap between values of the tuberculosis and control sera, they believed that this technique had potential as a useful serodiagnostic test. Subsequent studies by Tandon *et al.*,[97] using PPD as antigen, showed better discrimination between tuberculous and control sera and resulted in a false-positive incidence of 4%. In a study employing ELISA to evaluate antibodies to *M. tuberculosis* in IgG, IgA, and IgM classes in tuberculosis and other diseases, Grange *et al.*[98] reported that high levels of IgG were present in tuberculous sera when tested with a crude sonicate of *M. bovis* (BCG). However, in their subsequent ELISA studies,[99] these workers reported little discrimination among patients with tuberculosis and tuberculin-positive controls. Using a more purified glycolipid antigen from *M. bovis* (BCG), Reggiardo *et al.*[100] reported a better separation between sera from active tuberculosis patients and tuberculin-positive individuals, indicating the importance of the role that appropriate antigen selection obviously plays in this procedure. This importance is further emphasized by the work of Daniel *et al.*,[101] who similarly demonstrated a significant increase in IgG antibody level of tuberculous sera when assayed with a highly purified protein fraction from *M. tuberculosis*, named antigen 5.

Correlation of antibody titer with extent of disease was noted in studies by Viljanen *et al.*,[102] who reported that increased antibody levels in the sera of tuberculous patients were observed for all immu-

noglobulin classes examined, except for antibodies of the IgA class in patients diagnosed with minimal disease.

The ability of the ELISA to differentiate patients with active tuberculosis from other pulmonary diseases and BCG-vaccinated individuals was reported by Kalish *et al.*,[103] using PPD as antigen. In an attempt to improve the specificity and sensitivity of this assay, five serologically active antigens, including PPD-S, the international standard, prepared from *M. tuberculosis* were evaluated against a reference tuberculous serum by Affronti.[104] Of the antigens assessed, the A protein of Seibert was the most sensitive, detecting the highest antibody levels in the serum assayed. Although reactive, PPD-S was the least potent. Antibody levels using these five antigens against a reference serum are compared in Fig. 3.

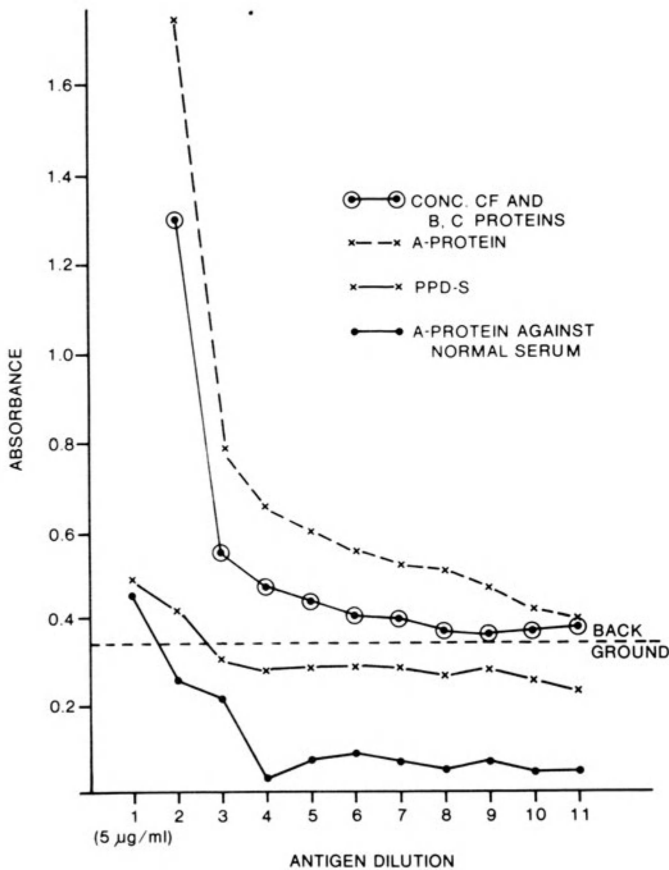

FIGURE 3. Comparison of antibody levels using different mycobacterial antigen fractions against a tuberculous reference system. (From Affronti[104].)

Encouraged by these results, a comparative study was undertaken by Affronti *et al.*,[105] using tuberculoproteins A and C to measure the amount of reactive IgG in the sera from advanced and moderately advanced tuberculosis patients, leprosy patients, and normal individuals. The A protein detected significant levels of IgG antibodies in patients with both advanced and moderately advanced disease and in patients with leprosy. Although patient sera from all stages of tuberculous disease and leprosy were also reactive when assayed with the C protein, it was concluded that the A protein was consistently more potent than the C protein in detecting mycobacterial antibodies, demonstrating that the A protein is an antigen of potentially serodiagnostic value. It is of additional interest that comparative skin-testing studies conducted by Vandiviere *et al.*[106] more than two decades ago reported that the A protein possessed greater potency as a skin-test antigen than the C protein or PPD-S, when administrated in equivalent protein concentrations. Because the A protein has a molecular weight ranging from 35,000 to 42,000 and contains antigens 1, 2, 5, and 6 and probably 4 when compared with the U.S.–Japan reference standard, Affronti attributes the greater potency manifested by the A protein to its large molecular weight and large number of antigens.

Another approach that has recently gained attention is the alternative use of the ELISA test to detect the presence of mycobacterial antigens, rather than antibody, in body fluids of patients. This has been investigated with promising results by several workers. The application of ELISA by Sada *et al.*[107] to detect mycobacterial antigens in the cerebrospinal fluid (CSF) of patients with tuberculous meningitis suggests that it may be a useful tool in clinical practice. By studying CSF samples from 16 patients with either bacteriologically confirmed or presumptive diagnosis of tuberculous meningitis employing an assay with commercially obtained antiBCG hyperimmune sera, significantly high absorbance readings were consistently observed. One false-positive finding out of 22 negative samples tested resulted in a specificity of 95%. Of 16 CSF samples tested, three were false negative, resulting in a sensitivity of 81%.

A similar approach was followed recently by Yanez *et al.*,[108] who examined the ELISA in detecting mycobacterial antigens in patient sputum. Using a double-antibody sandwich technique and employing commercially available rabbit anti-BCG sonic extract immunoglobulin, a specificity of only 80% and a sensitivity of 59% were obtained, with a positive predictive value of 59% and a negative predictive value of 80%. Possible explanations for the low sensitivity observed in the detection of acid-fast bacilli in sputum included destruction of bacilli by routine procedures used to process sputum that can result in protein denaturation and antigen being coated with body fluids or secretions, preventing

them from reacting with antibody. Procedures to minimize or reduce these adverse conditions should readily improve the practical value and benefits of such a test.

It should be clear at this point that one of the major difficulties in evaluating the antibody response in tuberculosis is the presence of epitopes that *M. tuberculosis* share with other closely related mycobacteria in the environment, resulting in antigenic cross-reactions that complicate any attempt to develop a specific and reactive serodiagnostic test for tuberculosis. A practical approach for resolving this problem is the application of immunoblotting techniques using partially purified antigens as probes that react specifically with serum antibodies in patients with tuberculosis. Immunoblot analysis involves electrophoresing selected mycobacterial antigens on SDS–PAGE slabs and electrophoretically transferring the separated components to nitrocellulose paper, where they are reacted with the patient's serum. Bound antibody is detected with avidin–horseradish peroxidase complexed to biotinylated anti-human IgG and the color developed by a suitable chromogen. Applying this method and employing the Seibert, A, B, and C proteins and polysaccharide I and II fractions from *M. tuberculosis*, Coates *et al.*[109] identified antigens in these fractions that reacted with antibody from patients with active tuberculosis. Most sera from the normal control group in their study also reacted with these same antigens, indicating that such antigens may share determinants with other closely related environmental bacteria, including other less pathogenic mycobacteria (i.e., the atypical mycobacteria) to which we are continuously exposed and become infected during the course of normal living. Of particular interest, however, was that antibody activity was noted consistently with antigens comprising two of the Seibert fractions: antigens in the high-molecular weight polyglucosan fraction, polysaccharide II, and the A-protein fraction.

Immunoblotting sera from tuberculous patients with antigens comprising the A-protein fractions revealed that all tuberculous and 25% of the control sera contained antibody that reacted with a 32,000-M_r antigen and a heterogeneous high-molecular-weight antigen. Three antigens, a 10,000-M_r antigen, a 30,000–44,000-M_r antigen, and a high-molecular weight antigen were found in the polysaccharide II fraction that reacted with sera from 70% or more of patients with tuberculosis and from 20–70% of the control sera. A fourth antigen that comprised the polysaccharide II fraction, with a molecular weight ranging from 17,000 to 28,000, reacted with antibody in 64% of the sera from patients with tuberculosis but with only 1 of 15 normal sera, suggesting that this antigen may represent a serodiagnostically useful polypeptide. Immunoblot profiles of the A protein and polysaccharide II fractions are noted in Fig. 4. It was also noteworthy that when a comparison of the

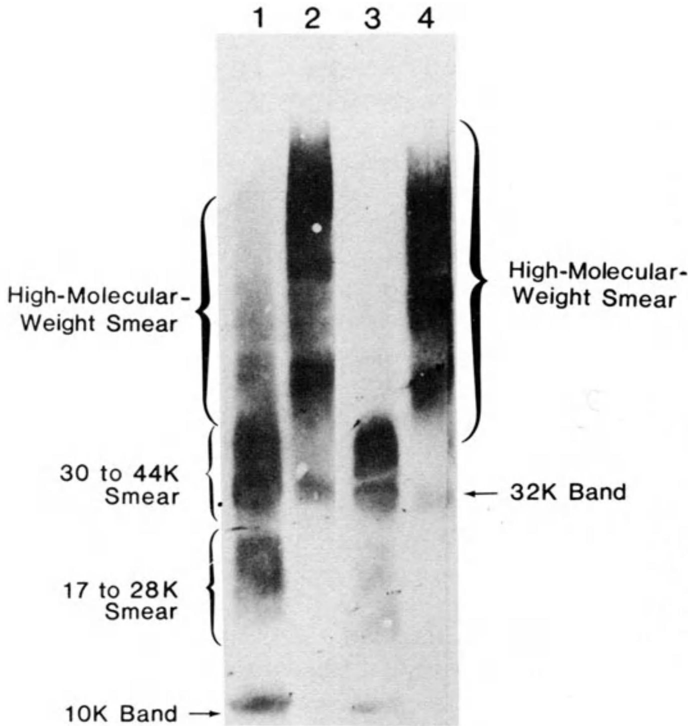

FIGURE 4. Immunoblots of A protein and polysaccharide II fractions. Sodium dodecyl sulfate–polyacrylamide gradient gels of Seibert fractions were electrophoretically transferred to nitrocellulose paper and reacted with a 1 : 200 dilution of a serum pool from patients with tuberculosis. Lanes 1 and 3, Seibert polysaccharide II fraction; lanes 2 and 4, Seibert A protein fraction. Lanes 1 and 2, after transfer, were reacted with a single serum sample from a patient with tuberculosis. Lanes 3 and 4 were reacted with different individual serum samples from a patient with tuberculosis. Identified are the HMW-smear antigen, 30–44K-smear antigen, 17–28K-smear antigen, and 10K-band antigen observed in the Seibert polysaccharide II fraction. In the A protein fraction, a HMW-smear antigen and a 32K-band antigen are identified. (From Coates *et al.*[109])

polysaccharide II fraction and PPD was undertaken by the immunoblot procedure and probed with tuberculous sera, antibody reacted with antigens in PPD of molecular weight similar to the 10,000-, 17,000- to 28,000-M_r band, and the 30,000–44,000-M_r band of the polysaccharide II fraction.

6. MOLECULAR BIOLOGIC APPROACHES FOR EXAMINING ANTIGENS FROM *M. TUBERCULOSIS*

With the introduction of molecular biologic and genetic engineering techniques for potentially defining the full complement of protein

antigens possessed by an infectious agent a powerful new strategy has now become available that can potentially result in the development of effective, specific, and well-characterized reagents for use in the sero-diagnosis of tuberculosis and skin testing. DNA and antibody-cloning techniques developed only during recent years are resulting in significant advances and improvements for the diagnosis and treatment of a number of infectious diseases.

Surveying the genome of *M. tuberculosis* for DNA sequences that encode for specific immunologically reactive proteins and cloning them in a suitable host-vector system is an attractive approach, being undertaken by a number of workers using tools of molecular biology noted above. Clark-Curtiss *et al.*,[110] for example, developed DNA libraries of three mycobacterial species in *Escherichia coli* using a cosmid vector, i.e., a vector that combines properties of both plasmids and λ phage and observed no expression of mycobacterial DNA in *Escherichia coli* unless the mycobacterial DNA was linked to a strong promotor. By contrast, Young *et al.*[111] reported on the cloning and expression of *M. tuberculosis* DNA in *E. coli* using the bacteriophage vector λ gt11. This is a 43.7-kb linear double-stranded bacteriophage cloning vector designed expressly for cloning small *Eco*RI fragments with *Eco*RI linkers. An important feature of this vector is that it expresses the foreign DNA insert as a fusion protein linked to the galactosidase polypeptide of *E. coli,* ensuring that the genomic insert will be efficiently transcribed and translated in *E. coli.* Recombinant libraries generated from λ gt11 and subsequently expressing their corresponding proteins can be screened with antibody probes. Three different *M. tuberculosis* antigens having molecular weights of 16,000, 18,000, and 33,000, as detected by three different monoclonal antibodies were produced using this host vector system. Such methodology offers a novel approach for providing large amounts of well characterized highly specific antigens of interest for subsequent use.

A similar approach was recently undertaken by van Embden *et al.*,[112] who reported on the cloning and expression of *M. bovis* BCG DNA in *E. coli* with the bacteriophage λ vector EMBL3. This cloning vector can accommodate up to 23-kb inserts, making it a useful vector for genomic library constructions. Among many clones tested for expression of mycobacterial antigens was one that produced a 64,000-M_r antigen. Immunoelectrophoresis studies showed that antigens cross-reacting with this protein are distributed in a variety of mycobacteria; this common antigen would be classified as belonging to group I of Stanford's classification scheme.

Embden *et al.*[112] are of the opinion that it will be difficult to clone genes expressing antigens specific for either *M. tuberculosis* or *M. bovis* BCG, since it has been convincingly shown by the immunoelectrophoretic studies of Closs *et al.*[21] that these two species contain essentially the

same antigens. In spite of this, the pioneering monoclonal antibody study by Coates *et al.*[113] and the subsequent work of Kolk *et al.*[114] on characterizing mycobacterial antigens give strong evidence that species-specific determinants on antigens may permit the identification and differentiation between closely related mycobacterial species. This finding is in contrast to recent studies on monoclonal antibody reactivity to *M. tuberculosis* by Andersen *et al.*,[115] which support the view that no monoclonal antibodies capable of identifying epitopes only on *M. tuberculosis* have yet been produced.

On a theoretical basis, it would not be unreasonable to expect, however, that by selecting clones that produce specific antigenic determinants using appropriate monoclonal antibodies as probes, specific and sensitive antigens could be obtained that would have possible diagnostic value. Giving validity to this approach are the results of a recently reported study by Chandramuki *et al.*,[116] who made practical use of a reverse passive hemagglutination immunodiagnostic test to detect the presence of a unique mycobacterial antigen, MY4,[117] in the CSF of tuberculous meningitis patients by employing a monoclonal antibody probe that had been developed specifically against it. This monoclonal antibody, designated ML34, was coupled to sheep red blood cells and used to detect the mycobacterial antigen and immune complexes in CSF. The monoclonal antibody was of the IgM isotype capable of reacting with mycobacterial species, including *M. tuberculosis, M. leprae* and *M. bovis.* Antigen was demonstrated in 88% of culture-positive and in 73% of culture-negative tuberculous meningitis patients. The test was of limited diagnostic value, however, since it also detected antigen in 21% of patients with pyogenic meningitis and 8% of control subjects. It is important to recognize that although the results of this study are disappointing from a diagnostic point of view, the novel approach used by these workers employing hybridoma techniques for the production of monoclonal antibodies having the potential of identifying single antigenic determinants has promise and merits further investigative efforts in the search for reagents of diagnostic usefulness. Unlimited amounts of homogeneous antibody can be produced by this technique, and standardization of the antigen of interest against the desired monoclonal antibodies becomes a practical procedure.

By applying a combination of recombinant DNA molecular biologic and genetic engineering methodologies to the complex problems recognized for decades as being singularly associated with antigens of the mycobacteria, a new approach is brought into the investigative arena, which optimistically promises to provide the necessary tools to achieve the long sought-after goal of more sensitive, specific, and effective antigens for use in skin testing, serodiagnosis, and prevention of tuberculosis.

REFERENCES

1. Koch, R., 1882, Aetiologie der tuberculose, *Berl. Klin. Wochenschr.* **19:**221–230.
2. von Pirquet, C., 1907, Demonstration zur tuberkulindiagnose durch hautimpfung, *Berl. Klin. Wochenschr.* **48:**699.
3. Daniel, T. M., and Janicki, B. W., 1978, Mycobacterial antigens: Review of their isolation, chemistry and immunological properties, *Microbiol. Rev.* **42:**84–113.
4. Seibert, F. B., 1934, The isolation and properties of the purified protein derivative of tuberculin, *Am. Rev. Tuberc.* **30:**713–720.
5. Lind, P., 1947, Purified tuberculin, its preparation and properties, *Acta Tuberc. Scand.* **31:**112–159.
6. Seibert, F. B., 1949, The isolation of three different proteins and two polysaccharides from tuberculin by alcohol fractionation. Their chemical and biological properties, *Am. Rev. Tuberc.* **59:**86–101.
7. Castelnuovo, G. M., Duncan, M. E., and Bellezza, G., 1964, Mycobacterial antigens: A study of antigens in bacterial extracts and in culture filtrates of different ages, *Tubercle* **45:**246–254.
8. Lind, A., Norlin, M., and Ouchterlony, O., 1970, Studies on the precipitinogenic pattern of mycobacteria cultivated at various temperatures, *Pneumologie* **142:**283–289.
9. Magnusson, M., Kim, H. K., and Bentzon, M. W., 1963, Tuberculin production. 1. Yield of tuberculoprotein from various media, *Acta Pathol. Microbiol. Scand.* **58:**363–375.
10. Seibert, F. B., and Glenn, J. T., 1941, Tuberculin purified protein derivative. Preparation and analysis of a large quantity for standard, *Am. Rev. Tuberc.* **44:**9–25.
11. Affronti, L. F., 1959, Purified protein derivatives (PPD) and other antigens prepared from atypical acid-fast bacilli and *Nocardia asteroids, Am. Rev. Tuberc.* **79:**284–295.
12. Janicki, B. W., Wright, G. L., Jr., Good, R. C., and Chaparas, S. D., 1976, Comparison of antigens in sonic and pressure cell extracts of *Mycobacterium tuberculosis, Infect. Immun.* **14:**425–437.
13. Daniel, T. M., Good, R. C., and Janicki, B. W., 1975, Immunoelectrophoresis of *Mycobacterium tuberculosis* antigens. Comparative analysis of cell extract and culture filtrate antigens, *Am. Rev. Respir. Dis.* **112:**639–644.
14. Turcotte, R., and Des Ormeaux, Y., 1972, Influence of the age of mycobacterial cultures on the protein and carbohydrate composition of tuberculins, *Can. J. Microbiol.* **18:**637–645.
15. Wayne, L. G., and Sramek, H. A., 1979, Antigenic differences between extracts of actively replicating and synchronized resting cells of *Mycobacterium tuberculosis, Infect. Immun.* **24:**363–370.
16. Janicki, B. W., Chaparas, S. D., Daniel, T. M., Kubica, G. P., Wright, G. L., Jr., and Yu, G. S., 1971, A reference system for antigens of *Mycobacterium tuberculosis, Am. Rev. Respir. Dis.* **104:**602–604.
17. Axelsen, J. H., Harboe, M., Closs, O., and Godal, T., 1974, BCG antibody profiles in tuberculoid and lepromatous leprosy, *Infect. Immun.* **9:**952–958.
18. Affronti, L. F., Parlett, R. C., and Cornesky, R. A., 1965, Electrophoresis on polyacrylamide gels of protein and polysaccharide fractions from *Mycobacterium tuberculosis, Am. Rev. Respir. Dis.* **91:**1–5.
19. Roberts, D. B., Wright, G. L., Jr., Affronti, L. F., and Reich, M., 1972, Characterization and comparison of mycobacterial antigens by two-dimensional immunoelectrophoresis, *Infect. Immun.* **6:**564–573.
20. Chaparas, S. D., Brown, T. M., and Hyman, I. S., 1978, Antigenic relationships among species of *Mycobacterium* studied by fused rocket immunoelectrophoresis, *Int. J. Syst. Bacteriol.* **28:**547–560.

21. Closs, O., Harboe, M., Axelsen, N. H., Bunch-Christensen, K., and Magnusson, M., 1980, The antigens of *Mycobacterium bovis* strain BCG, studied by crossed immunoelectrophoresis: A reference system., *Scand. J. Immunol.* **12**:249–263.
22. Stanford, J. L., and Beck, A., 1968, An antigenic analysis of the mycobacteria. *Mycobacterium fortuitum Myco. kansasii, Myco phlei, Myco. smegmatis* and *Myco. tuberculosis, J. Pathol.* **95**:131–139.
23. Koch, R., 1891, Fortsetzung der Mitteilunger uber ein Heilmittel gegen tuberculose, *Dtsch. Med. Woschenschr.* **17**:101–102.
24. Chaparas, S. D., 1984, Immunologically based diagnostic tests with tuberculin and other mycobacterial antigens, in: *The Mycobacteria A Sourcebook* (G. P. Kubica and L. G. Wayne, eds.), pp. 195–220, Dekker, New York.
25. Seibert, F. B., 1928, The chemical composition of the active principle of tuberculin. XL. An improved and simplified method for making a standard undenatured tuberculin of any desired strength and a method of chemical assay, *J. Biol. Chem.* **78**:345–362.
26. Masucci, P., and McAlpine, K. L., 1930, Biochemical studies of bacterial derivatives. X. Preparation of human tubercle bacillus protein MA-100, *Proc. Soc. Exp. Biol. Med.* **27**:661–663.
27. Seibert, F. B., 1934, The isolation and properties of the purified protein derivative of tuberculin, *Am. Rev. Tuberc. (Suppl.)* **30**:713–720.
28. WHO, 1952, *World Health Organization Technical Report,* Ser. No. 56, *Expert Committee on Biological Standardization, Fifth Report, July 1952,* p. 6.
29. Tukey, J. W., DuFour, E. H., and Seibert, F. B., 1950, Lack of sensitization following repeated skin tests with standard tuberculin (PPD-S), *Am. Rev. Tuberc.* **62**:77–86.
30. Seibert, F. B., Soto-Figueroa, E., and DuFour, E. H., 1955, Isolation, identification, and classification of proteins of tuberculin and the tubercle bacillus, *Am. Rev. Tuberc.* **71**:704–721.
31. Birnbaum, S. E., and Affronti, L. F., 1968, Chemical and serological relationships between the heteropolysaccharides of *Mycobacterium tuberculosis* and *Mycobacterium kansasii, J. Bacteriol.* **95**:559–564.
32. Birnbaum, S. E., 1968, A comparison of the immunochemistry of polysaccharides from *M. tuberculosis* and the atypical mycobacteria, dissertation, George Washington University, Washington, D.C.
33. Birnbaum, S. E., and Affronti, L. F., 1970, Mycobacterial polysaccharides. I. Serological and skin testing studies on mycobacterial polysaccharides, *Int. Arch. Allergy Immunol.* **37**:88–97.
34. Heilman, D. H., 1967, *In vitro* studies on polysaccharides of *Mycobacterium tuberculosis* and delayed hypersensitivity, *Am. Rev. Respir. Dis.* **96**:198–203.
35. Seibert, F. B., Stacey, M., and Kent, P. W., 1949, An antigenic polysaccharide, "Polysaccharide II," isolated from tuberculin, *Biochim. Biophys. Acta* **3**:632–640.
36. Daniel, T. M., and Affronti, L. F., 1973, Immunoelectrophoretic analysis of antigenic constituents of Seibert fractions of mycobacterial culture filtrates. Identification with the proposed United States–Japan reference nomenclature, *Am. Rev. Respir. Dis.* **108**:1244–1248.
37. Daniel, T. M., 1984, Soluble mycobacterial antigens, in: *The Mycobacteria. A Sourcebook* (G. P. Kubica. and L. G. Wayne, eds.), pp. 417–465, Dekker, New York.
38. Kniker, W. T., and LaBorde, J. B., 1964, The separation of mycobacterial antigens by ion-exchange chromatography. I. Details of chromatographic and immunologic procedures and results with four strains of *M. tuberculosis, Am. Rev. Respir. Dis.* **89**:29–40.
39. Kniker, W. T., 1965, The use of ion-exchange chromatography for mycobacterial antigen isolation, *Am. Rev. Respir. Dis. (Suppl.)* **92**:19–28.

40. Someya, S., Hayashi, O., and Yamamura, Y., 1962, Studies on the antigenicity of tuberculin active peptide, *Am. Rev. Respir. Dis.* **86**:542–546.
41. Someya, S., Koyama, K., Asami, N., Kataoka, T., and Yamamura, Y., 1966, Studies on the tuberculin reactivity of tuberculin peptide, *Jpn. J. Microbiol.* **10**:221–227.
42. Yamamura, Y., Onoue, K., and Azuma, I., 1968, Chemical and immunological studies on peptides and polysaccharides from tubercle bacilli, *Ann. NY Acad. Sci.* **154**:88–97.
43. Yamamura, Y., Onoue, K., and Tahara, T., 1969, Purification and the properties of tuberculin active peptides, *Z. Immunitaetsforch. Allergie Klin. Immunol.* **137**:171–176.
44. Kotani, S., Yanagida, I., Kato, K., and Matsuda, T., 1970, Studies on peptides, glycopeptides and antigenic polysaccharide–glycopeptide complexes isolated from an L-11 enzyme lysate of cell walls of *Mycobacterium tuberculosis* strain H37Rv, *Biken J.* **13**:249–275.
45. Lind, A., 1965, Immunologic analysis of mycobacterial fractions, *Am. Rev. Respir. Dis. (Suppl.)* **92**:54–62.
46. Janicki, B. W., Aron, S. A., and Raychaudhuri, A., 1972, Separation and isolation of mycobacterial antigens by continuous-flow electrophoresis, *Am. Rev. Respir. Dis.* **106**:779–783.
47. Janicki, B. W., Goldstein, R. A., and Aron, S. A., 1971, Immunoelectrophoretic studies of the precipitating antibody response in tuberculosis, *Am. Rev. Respir. Dis.* **103**:894.
48. Janicki, B. W., Aron, S. A., Schechter, G. P., and McFarland, W., 1972, Tuberculin antigens active in human lymphocyte blastogenesis, *Proc. Soc. Exp. Biol. Med.* **141**:809–813.
49. Janicki, B. W., and Aron, S. A., 1973, Failure of isolated tuberculopolysaccharides to induce precipitins in the guinea pig, *Annu. Mtg. Am. Soc. Microbiol.* 123 (abst.).
50. Janicki, B. W., Goldstein, R. A., and Aron, S. A., 1971, Immunoelectrophoretic studies of the precipitating antibody response in tuberculosis, *Am. Rev. Respir. Dis.* **103**:894 (abst.).
51. Daniel, T. M., and Wisnieski, J. J., 1970, The reaction of concanavalin A with mycobacterial culture filtrates, *Am. Rev. Respir. Dis.* **101**:762–764.
52. Daniel, T. M., 1974, The purification of mycobacterial polysaccharides with concanavalin A, *Am. Rev. Respir. Dis.* **110**:634–640.
53. Laguerre, M., and Turcotte, R., 1977, Use of sepharose-conjugated BCG antibodies for the purification of tuberculin-active components. *Can. J. Microbiol.* **23**:695–700.
54. Daniel, T. M., and Anderson, P. A., 1978, The isolation by immunoabsorbent affinity chromatography and physicochemical characterization of *Mycobacterium tuberculosis* antigen 5, *Am. Rev. Respir. Dis.* **117**:533–539.
55. Daniel, T. M., Ellner, J. J., Todd, L. S., McCoy, D. W., Payne, V. D. H., Anderson, P. A., and Bhe, F. T., 1979, Immunobiology and species distribution of *Mycobacterium tuberculosis* antigen 5, *Infect. Immun.* **24**:77–82.
56. Daniel, T. M., Balestrino, E. A., Balestrino, O. C., Davidson, P. T., Debanne, S. M., Kataria, S., Kataria, Y. P., and Scocozza, J. B., 1982, The tuberculin specificity in humans of *Mycobacterium tuberculosis* antigen 5, *Am. Rev. Respir. Dis.* **126**:600–606.
57. Fukui, Y., and Yoneda, M., 1961, Extracellular proteins of tubercle bacilli. III. Column chromatographical purification of the proteins of a virulent strain (H37Rv) of *Mycobacterium tuberculosis*, *Biken J.* **4**:187–196.
58. Fukui, Y., Hirai, T., Uchida, T., and Yoneda, M., 1965, Extracellular proteins of tubercle bacilli. IV. α and β antigens as major extracellular protein products and as cellular components of a strain (H37Rv) of *Mycobacterium tuberculosis*, *Biken J.* **8**:189–199.

59. Grange, J. M., 1984, The humoral immune response in tuberculosis: Its nature, biological role, and diagnostic usefulness, *Adv. Tuberc. Res.* **21**:1–78.
60. Tasaka, H., Nomura, T., and Matsuo, Y., 1985, Specificity and distribution of alpha antigens of *Mycobacterium avium-intracellulare, Mycobacterium scrofulaceum* and related species of mycobacteria, *Am. Rev. Respir. Dis.* **132**:173–174.
61. Daniel, T. M., and Ferguson, L. E., 1970, Purification and characterization of two proteins from culture filtrates of *Mycobacterium tuberculosis* H37Ra strain, *Infect. Immun.* **1**:164–168.
62. Daniel, T. M., and Hinz, C. F., Jr., 1974, Reactivity of purified proteins and polysaccharides from *Mycobacterium tuberculosis* in delayed skin test and cultured lymphocytes mitogenesis assays, *Infect. Immun.* **9**:44–47.
63. Daniel, T. M., and DeMuth, R. W., 1977, Immunochemical analysis of a major antigen of *Mycobacterium szulgai, J. Infect. Dis.* **135**:778–786.
64. Nagai, S., Nagasuga, T., Matsumoto, J., and Kohda, K., 1974, Isolation of tuberculin skin test reactive proteins from heated culture filtrate of *Mycobacterium tuberculosis* H37Rv, *Am. Rev. Respir. Dis.* **108**:17–28.
65. Nagai, S., Matsumoto, J., and Nagasuga, T., 1981, Specific skin reactive protein from culture filtrate of *Mycobacterium bovis* BCG, *Infect. Immun.* **31**:1152–1160.
66. Miura, K., Nagai, S., Kinomoto, M., Haga, S., Tokunaga, T., 1983, Comparative studies with various substrains of *Mycobacterium bovis* BCG on the production of an antigenic protein, MPB 70, *Infect. Immun.* **39**:540–545.
67. Kuwabara, S., and Tsumita, T., 1974, Primary structure of tuberculin active protein from tubercle bacilli, *Jpn. J. Exp. Med.* **44**:129–132.
68. Kuwabara, S., 1975, Purification and properties of tuberculin-active protein from *Mycobacterium tuberculosis, J. Biol. Chem.* **250**:2556–2562.
69. Kuwabara, S., 1975, Amino acid sequence of tuberculin-active protein from *Mycobacterium tuberculosis, J. Biol. Chem.* **250**:2563–2568.
70. Savrda, J., 1980, Synthesis and biological assays of a peptide from a tuberculin-active protein, *Infect. Immun.* **30**:686–693.
71. Savrda, J., 1983, Synthesis and biological assays of peptides from a tuberculin active protein, *Infect. Immun.* **40**:1163–1169.
72. Toida, I., Yamamoto, S., Takuma, S., Suzuki, T., and Hirato, M., 1985, Lack of tuberculin activity of synthetic peptides, *Infect. Immun.* **50**:614–619.
73. Minden, P., Kelleher, P. J., Freed, J. H., Nielsen, L. D., Brennan, P. J., McPheron, L., and McClatchy, J. K., 1984, Immunological evaluation of a component isolated from *Mycobacterium bovis* BCG with a monoclonal antibody to *M. bovis* BCG, *Infect. Immun.* **46**:519–525.
74. Minden, P., Houghton, R. A., Spear, J. R., and Shinnick, T. M., 1986, A chemically synthesized peptide which elicits humoral and cellular immune response to mycobacterial antigens, *Infect. Immun.* **53**:560–564.
75. Affronti, L. F., Wright, G. L., Jr., and Reich, M., 1972, Characterization and comparison of mycobacterial antigens by discontinuous pore gradient gel electrophoresis, *Infect. Immun.* **5**:474–481.
76. Wright, G. L., Jr., Affronti, L. F., and Reich, M., 1972, Characterization and comparison of mycobacterial antigens by two-dimensional polyacrylamide gel electrophoresis, *Infect. Immun.* **5**:482–490.
77. Roszman, T. L., Mallmann, V. H., Mallmann, W. L., and O'Reilly, D., 1968, Immunologic responses evoked by mycobacterial components. Disc electrophoretic separation of specific mycobacterial antigens, *Am. Rev. Respir. Dis.* **97**:103–112.
78. Minden, P., and Farr, R. S., 1969, Binding between components of the tubercle bacillus and humoral antibodies, *J. Exp. Med.* **130**:931–954.

79. Affronti, L. F., Grow, L., Ouchterlony, O., Lind., A., and Norlin, M., 1971, Fractionation of the international standard tuberculin, PPD-S, *Am. Rev. Respir. Dis.* **103:**893 (abst.).
80. Affronti, L. F., Lind, A., Ouchterlony, O., Ridell, M., and Wisingerova, E., 1986, An evaluation of the polyacrylamide gel electrophoresis fractionation method for the production of *Mycobacterium tuberculosis* skin test preparation. I. Production, physicochemical characterization, and serological analysis, *J. Biol. Std.* **14:**177–185.
81. Affronti, L. F., Bentzon, M. W., Lind, A., Magnusson, M., Ouchterlony, O., Ridell, M., and Wisingerova, E., 1986, An evaluation of the polyacrylamide gel electrophoresis fractionation method for the production of *Mycobacterium tuberculosis* skin test preparations. II. Comparative skin testing on guinea pigs, *J. Biol. Std.* **14:**187–200.
82. Oakley, C. L., and Fulthorpe, A. J., 1953, Antigenic analysis by diffusion, *J. Pathol.* **65:**49–60.
83. Parlett, R. C., Youmans, G. P., Rehr, C., and Lester, W., 1958, The detection of antibodies in the serum of tuberculous patients by an agar double diffusion precipitatin technique. *Am. Rev. Tuberc.* **77:**462–472.
84. Parlett, R. C., and Youmans, G. P., 1959, An evaluation of the specificity and sensitivity of a gel double diffusion test for tuberculosis, *Am. Rev. Respir. Dis.* **80:**153–166.
85. Lind, A., 1961, A comparison between the plate and tube technique for the demonstration of precipitins in sera from tuberculous patients, *Int. Arch. Allergy. Appl. Immunol.* **18:**305–317.
86. Grange, J. M., and Kardjito, T., 1982, Serological tests for tuberculosis: Can the problem of how specificity be overcome? *Indian J. Chest. Dis.* **24:**108–117.
87. Thurston, J. R., 1964, The present status of serologic test for the diagnosis of tuberculosis, *Proc. U.S. Anim. Health Assoc.* **68:**314–319.
88. Affronti, L. F., and Seibert, F. B., 1965, Some early investigations of *Mycobacterium tuberculosis, Am. Rev. Respir. Dis.* **92:**1–8.
89. Toussaint, A. J., Fife, E. H., Parlett, R. C., Affronti, L. F., Wright, G. L., Jr., Reich, M., and Morse, W. C., 1969, A soluble antigen fluorescent antibody test for the serodiagnosis of *Mycobacterium tuberculosis* infections, *Am. J. Clin. Pathol.* **52:**708–713.
90. Affronti, L. F., Fife, E. H., and Grow, L., 1973, Serodiagnostic test for tuberculosis, *Am. Rev. Respir. Dis.* **108:**822–825.
91. Kiran, U., and Shriniwas, 1985, Soluble antigen fluorescent antibody test in serodiagnosis of pulmonary tuberculosis, *Eur. J. Respir. Dis.* **67:**204–208.
92. Ellner, J. J., and Daniel, T. M., 1979, Immunosuppression by mycobacterial arabinomannan, *Clin. Exp. Immunol.* **35:**250–257.
93. Minden, P., McClatchy, J. K., and Farr, R. S., 1972, Shared antigens between heterologous bacterial species, *Infect. Immun.* **6:**574–582.
94. Straus, E., Wu, N., Quraishi, M. A. H., and Levine, S., 1981, Clinical applications of the radioimmunoassay of secretory tuberculin protein, *Proc. Natl. Acad. Sci. USA* **78:**3214–3217.
95. Engvall, E., and Perlmann, P., 1972, Enzyme-linked immunosorbent assay (ELISA). III. Quantitation of specific antibodies by enzyme labeled anti-immunoglobulin in antigen coated tubes, *J. Immunol.* **109:**129–135.
96. Nassau, E., Parson, E. R., and Johnson, G. D., 1976, The detection of antibodies to *Mycobacterium tuberculosis* by enzyme-linked immunosorbent assay (ELISA), *Tubercle* **57:**67–70.
97. Tandon, A., Saxena, R. P., and Saxena, K. C., 1980, Diagnostic potentialities of enzyme-linked immunosorbent assay in tuberculosis using purified tuberculin antigen, *Tubercle* **61:**87–89.

98. Grange, J. M., Gibson, J., Nassau, E., and Kardjito, T., 1980, Enzyme-linked immunosorbent assay (ELISA): A study of antibodies of *Mycobacterium tuberculosis* in the IgG, IgA, and IgM classes in tuberculosis, sarcoidosis, and Crohn's disease, *Tubercle* **61:**145–152.
99. Kardjito, T., Handayo, I., and Grange, J. M., 1982, Diagnosis of active tuberculosis by immunological methods. I. The effect of tuberculin reactivity and previous BCG vaccination on the antibody levels determined by ELISA, *Tubercle* **63:**269–274.
100. Reggiardo, I., Vasquez, E., and Schnoper, L., 1980, ELISA tests for antibodies against mycobacteria glycolipids, *J. Immunol. Methods* **34:**55–60.
101. Daniel, T. M., Oxtoby, M. J., Pinto, E. M., and Moreno, E. S., 1981, The immune spectrum in patients with pulmonary tuberculosis, *Am. Rev. Respir. Dis.* **123:**556–559.
102. Viljanen, M. K., Eskola, J., and Tala, E., 1982, Enzyme-linked immunosorbent assay (ELISA) for antibodies to purified protein derivative of tuberculin PPD, *Eur. J. Respir. Dis.* **63:**257–262.
103. Kalish, S. B., Radin, R. C., Levitz, D., Zeiss, C. R., and Phair, J. P., 1983, The enzyme-linked immunosorbent assay method for IgG antibody to purified protein derivative in cerebrospinal fluid of patients with tuberculous meningitis, *Ann. Intern. Med.* **99:**630–633.
104. Affronti, L. F., 1985, Useful immunologic tests for tuberculosis, *Clin. Immunol. Newsl.* **6:**55–57.
105. Affronti, L. F., Taylor, R., and Hassan, E. M., 1986, Comparison between tuberculoprotein A and C in the serodiagnosis of tuberculosis by enzyme-linked immunosorbent assay, *Int. Arch. Allergy Appl. Immunol.* **80:**81–84.
106. Vandiviere, H. M., Vandiviere, H. R., and Seibert, F. B., 1961, Comparison between purified tuberculoproteins and the international standard tuberculin, *J. Infect. Dis.* **108:**45–52.
107. Sada, E., Ruiz-Palacios, G. M., L'opez-Vidal Y., and Ponce de Le'on, S., 1983, Detection of mycobacterial antigens in cerebrospinal fluid of patients with tuberculous meningitis by enzyme-linked immunosorbent assay, *Lancet* **2:**651–652.
108. Yanez, M. A., Coppola, M. P., Russo, D. A., Delaha, E., Chaparas, S. D., and Yeager, H., Jr., 1986, Determination of mycobacterial antigens in sputum by enzyme immunoassay, *J. Clin. Microbiol.* **23:**822–825.
109. Coates, S. R., Hansen, D., Schecter, G., Slutkin, G., Hopewell, P., Affronti, L. F., and Echenberg, D. F., 1986, Identification of *Mycobacterium tuberculosis* antigens in Seibert fractions by immunoblotting, *J. Clin. Microbiol.* **24:**126–130.
110. Clark-Curtiss, J. E., Jacobs, W. R., Docherty, M. A., Ritchis, L. R., and Curtiss, R. III, 1985, Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae*, *J. Bacteriol.* **161:**1093–1102.
111. Young, R. A., Bloom, B. R., Grosskinsky, C. M., Ivanyi, J., Thomas, D., and Davis, R., 1985, Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA, *Proc. Natl. Acad. Sci. USA* **82:**2583–2587.
112. Thole, J. E. R., Dauwerse, H. G., Das, R. K., Groothuis, D. G., Schouls, L. M., and van Embden, J. D. A., 1985, Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli*, *Infect. Immun.* **50:**800–806.
113. Coates, A. R. M., Allen, B. W., Hewitt, J., Ivanyi, J., and Mitchison, D. A., 1981, Antigenic diversity of *Mycobacterium tuberculosis* and *Mycobacterium bovis* detected by means of monoclonal antibodies, *Lancet* **2:**167–169.
114. Kolk, A. H. J., Ho, M. L., Klatser, P. R., Eggelte, T. A., Kuijper, S., deJonge, S., and van Leeuwen, J., 1984, Production and characterization of monoclonal antibodies to *Mycobacterium tuberculosis*, *M. bovis* (BCG) and *M. leprae*, *Clin. Exp. Immunol.* **58:**511–521.

115. Andersen, A. B., Yuan, Z., Haslov, K., Vergmann, B., and Bennedsen, J., 1986, Interspecies reactivity of five monoclonal antibodies to *Mycobacterium tuberculosis* as examined by immunoblotting and enzyme-linked immunosorbent assay, *J. Clin. Microbiol.* **23**:446–451.
116. Chandramuki, A., Allen, P. R., Keen, M., and Ivanyi, J., 1985, Detection of mycobacterial antigen and antibodies in the cerebrospinal fluid of patients with tuberculous meningitis, *J. Med. Microbiol.* **20**:239–247.
117. Praputpittaya, K., and Ivanyi, J., 1985, Detection of an antigen (MY4) common to *M. tuberculosis* and *M. leprae* by "tandem" immunoassay, *J. Immunol. Methods* **79**:149–157.

2

Role of Adjuvant and Immunogenic Moieties of *M. tuberculosis* in Pathogenicity

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1. INTRODUCTION

Immune responses can be nonspecifically enhanced or modified by administrating certain substances together with the antigens. These substances, called adjuvants, are obtained from bacteria, viruses, parasites, and plants and are highly heterogeneous with respect to their nature and origin. Probably the strongest and most common adjuvants are substances derived from bacteria, especially *Mycobacterium tuberculosis*. Since the historical experiments by Freund *et al.*[1,2] established that administration of antigens incorporated in water-in-oil emulsion together with killed mycobacteria induced delayed-type hypersensitivity (DTH) and enhanced antibody production, mycobacteria have received a great deal of attention in immunology. Their biologic activities are versatile. For example, the administration of mycobacteria in water-in-oil emulsion induces not only enhancement of DTH and antibody production but also adjuvant polyarthritis,[3,4] granuloma formation,[5] autoimmune disease,[6,7] suppression of tumor growth,[8] and increased susceptibility to endotoxin.[9] Therefore, many investigators have made efforts to define chemically the adjuvant moieties of mycobacteria. In recent years, Lederer *et al.*[10] identified a minimal adjuvant active structure, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), which was then synthesized by

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Merser *et al.*[11] This molecule has been confirmed to exhibit versatile biologic activities similar to those of mycobacteria and to affect immunocompetent cell functions.

By contrast, the immunogenic moieties of *M. tuberculosis,* purified protein derivatives (PPD), tuberculin active peptide (TAP), and Old tuberculin (OT) are also important substances in immune responses. One major manifestation of host response to tuberculous infection is the development of DTH to protein or polypeptide antigens of tubercle bacilli. Besides the induction of DTH response, these immunogenic moieties have been shown to play an important role in immune regulation. This chapter discusses the role in pathogenesis of moieties of *M. tuberculosis* by reviewing observations done in our own and other laboratories.

2. ROLE OF ADJUVANT MOIETIES OF *M. TUBERCULOSIS*

2.1. Adjuvant Activity of Bacterial Cell Wall Fraction, Wax D, Cord Factor and Water-Soluble Adjuvant

In 1949 Raffel *et al.*[12] demonstrated for the first time that the adjuvant of intact mycobacterial cells could be reproduced by the wax extracted from *M. tuberculosis.* Following this, the adjuvant activity of wax D, which was further fractionated from such wax fraction, was demonstrated; the injection of wax D with an antigen in a water-in-oil on induced increased antibody production, DTH[13,14] and adjuvant polyarthritis.[15] In addition to wax D, cord factor extracted from bacterial cells was also shown to have adjuvant activities. When cord factor with antigen in Freund's incomplete adjuvant was injected into animals, increased antibody levels were produced.[16] Moreover, Bekierkunst *et al.*[17] found that intravenous injection of cord factor into mice produced extensive lung gramuloma.

In contrast with these water-insoluble adjuvants, Adam *et al.*[18,19] prepared a water-soluble adjuvant (WSA) by lysozyme digestion of purified *M. smegmatis* cell walls. In *in vivo* experiments, the WSA replaced mycobacterial cells in Freund's complete adjuvant and stimulated antibody production and induction of DTH.[20] However, the WSA produced neither arthrogenic activity, as observed in wax D, nor the side effects elicited by mycobacterial cells.[20] In *in vitro* experiments, the adjuvant effect of WSA on antibody formation by mouse lymphoid cells was examined by Modolell *et al.*[21] WSA significantly increased the numbers of antibody-forming cells against both T-dependent and -independent

antigens. The adjuvant effect of WSA on antibody formation against T-independent antigen was also observed following elimination of T cells by anti-θ serum plus complement and using lymphoid cells from C3H/nu/nu mice, suggesting that, at least *in vitro,* it is not mediated by T cells. The WSA did not have a direct B-cell-stimulating activity and showed adjuvant activity when it was preincubated with macrophages, but not when it was preincubated with macrophage-depleted cells. Therefore, WSA enhanced the immune response by stimulating macrophages directly. In addition, Juy and Chedid[22] reported that WSA-activated macrophages inhibit tumor growth both *in vitro* and *in vivo.* Oppenheim *et al.*[23] reported that WSA stimulates human peripheral and mouse peritoneal cells to produce a factor that is mitogenic for mouse thymocytes. Since the factor was produced by adherent cells (i.e., macrophages), but not by nonadherent cells, the mediator induced by WSA is probably interleukin 1 (IL-1). These investigators also showed that $P388D_1$, a macrophage cell line, could be stimulated by WSA to produce IL-1. Collectively, these findings suggested that the target cell for WSA action is the macrophage.

2.2. Adjuvant Activities of MDP

Following the discovery of WSA and peptidoglycan, efforts were made in several laboratories to find adjuvants of lower molecular weight that could replace WSA or peptidoglycan. Thus, the synthetic glycopeptide *N*-acetylmuramyl-L-alanyl-D-isoglutamine, or muramyl dipeptide (MDP) (Fig. 1), a minimal adjuvant active structure, was found by Ellouz *et al.*[10] and synthesized by Merser *et al.*[11] Shortly afterward, Kotani *et al.*[24] reported similar findings. Following their reports, MDP has been found to possess various biologic activities other than adjuvanticity (Table I).

2.2.1. Adjuvant Activities of MDP in Immune Response

Muramyl dipeptide suspended with an antigen in water-in-oil emulsion has been shown to have a strong adjuvant effect on the production of humoral antibody and on the induction of DTH.[25] Moreover, MDP also displays adjuvant activity for IgE production.[26] In addition to these *in vivo* experiments, the adjuvant activity of MDP *in vitro* was demonstrated by Specter *et al.,*[27] who reported that cultures of normal spleen cells stimulated with graded concentrations of MDP produced markedly enhanced antibody plaque-forming cell (PFC) responses to sheep red blood cells (SRBC).

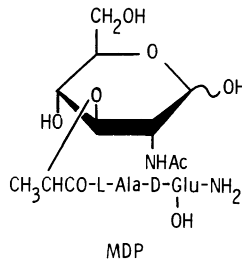

FIGURE 1. Muramyl dipeptide (MDP).

2.2.2. *Target Cells for the Adjuvant Activity of MDP*

Löwy *et al.*[28] demonstrated the importance of T cells as mediators of the adjuvant activities of MDP. In their experiments, CBA mice were reconstituted with various splenic cell types harvested from donors that had received SRBC and MDP 24 hr earlier. When the donor splenic cells were treated with anti-θ serum plus complement and reconstituted with normal thymocytes, the MDP adjuvant activity was completely abolished in the recipients. By contrast, when the same spleen cells were depleted of their adherent cells (i.e., macrophages) and reconstituted with normal macrophages, no effect on the adjuvant activity of MDP was detected. Sugimoto *et al.*[29] also investigated the mechanism of MDP action by studying antihapten PFC and hemagglutinin responses to hapten–carrier conjugates in mice. These investigators showed that MDP could augment the generation of carrier-specific helper cell function. However, in these *in vivo* experiments, the involvement of macrophages in the adjuvant activity of MDP could not be excluded. It was therefore possible

TABLE I
Biologic Activities of MDP

Adjuvant activity
Nonadjuvant activities
Granuloma formation in animals
Necrotic inflammatory reaction in guinea pigs
Enhanced proliferation of bone marrow granulocyte–macrophage progenitor cells
Enhanced host resistance against infection
Induction of suppressor T cells
Pyrogenicity and somnogenicity through central nervous system

that macrophages were the initial target cells of MDP and that an adjuvant-activated macrophage–T-cell interaction was involved in the generation of adjuvant-activated helper T cells. Sugimura *et al.*[30] examined the initial target cells of MDP adjuvant activity *in vitro,* using cell-separation procedures. The results showed that MDP enhanced the immune responses following direct interaction with antigen-stimulated T and B cells shortly after being triggered by antigen and that there was no macrophage requirement for MDP to elicit the adjuvant action in primary anti SRBC PFC response *in vitro.* These reports showed that the mechanism by which MDP promotes immune responsiveness seemed to be mediated by T cells, but not by macrophages. In contrast with these reports, the importance of macrophages as mediators of MDP adjuvant activities was shown by Fevrier *et al.*[31] studying the *in vitro* PFC response to SRBC. In their experiments, when the supernatant from MDP-stimulated spleen cells was added to cultured spleen cells, anti-SRBC PFC numbers were increased. The factor(s) was produced by spleen cells treated with anti-θ serum plus complement but not by cells treated with antimacrophage serum plus complement. Moreover, the factor(s) was showed to be released by adherent spleen cells following MDP stimulation. They concluded that the target cells for MDP are the macrophages, which release factor(s) ultimately acting on B cells through T cells. Moreover, MDP was shown to be able to activate macrophages and to produce lymphocyte activating factor (LAF), which is probably IL-1. Thus, LAF from MDP-stimulated macrophages may play an important role in MDP adjuvant activity.

2.2.3. Genetic and Gender Control of Adjuvanticity

The adjuvanticity of MDP was shown in mice to be under genetic control. Staruch and Wood[32] showed that MDP in BALB/c mice markedly enhanced the secondary antibody response against bovine serum albumin (BSA) *in vitro* but had little enhancing effect in C57BL/10Sn mice under the same conditions. These investigators also showed that MDP was influenced by at least two genes, present inside and outside the major histocompatibility complex. In addition, Damais *et al.*[33] reported that the mitogenic activity of MDP, measured as uptake of [^{3}H]thymidine by spleen cells, was strain dependent; DBA/2, BALB/c, AKR, CBA, and Swiss mice were high responders, while C3H/He and C57BL/6 mice were low responders. This strain difference in susceptibility to the adjuvant and mitogenic activities of MDP was different from that observed in bacillus Calmette–Guerin (BCG)- or BCG cell wall (CW)-induced lung granuloma response.[34,35] Gender-associated differences were reported

in adjuvant and reticuloendothelial system (RES)-stimulated activity[36]; female mice showed much higher adjuvant activity, as measured by antibody production and RES-stimulated activity, as measured by clearance of carbon particles, than did male mice. These differences might be related to the levels of estrogens shown to be stimulatory to RES-macrophage phagocytic function.

2.3. Biologic Activities of MDP Other Than Adjuvanticity

Muramyl dipeptide has various biologic activities other than the adjuvant activities cited in Table I. This section discusses and correlates some of the various biologic activities of MDP other than adjuvanticity that have been reported by several laboratories.

2.3.1. Granuloma Formation

Mycobacteria are well known to induce granuloma formation. Therefore, the chemical component of mycobacteria responsible for this action has been studied. Emori and Tanaka[37] found MDP to induce massive granulomas in guinea pigs and rats. The footpads of these animals were injected with a water-in-oil emulsion containing 100 μg MDP without protein antigen. Two weeks after MDP injection, the draining lymph nodes showed the formation of massive granulomas, which were indistinguishable from those evoked by killed mycobacteria. By contrast, the BCG CW-induced lung granuloma response in mice has been reported to be controlled genetically and to involve cell-mediated immunity; for example, C57BL/6 mice were high responders and C3H/He mice low responders.[35] We showed that lung granuloma was produced in both C57BL/6 and C3H/He mice when 300 μg MDP coupled with mycolic acid (mycoloyl-MDP) in water-in-oil-in-water (w/o/w) emulsion was injected intravenously (IV)[38] (Fig. 2). Therefore, lung granuloma induced with mycoloyl-MDP in mice might be nonspecific in contrast with the involvement of cell-mediated immunity in BCG CW-induced lung granuloma response.

2.3.2. Necrotic Inflammatory Reaction

Recently, Nagao and Tanaka[39] noted an interesting phenomenon. Severe and extensive necrotic inflammation with exudation and hemorrhage was observed in guinea pig footpads that had been injected with tubercule bacilli in water-in-oil emulsion 4 weeks earlier, 24 hr after an intracutaneous injection of MDP. This reaction was observed in guinea pigs but not in mice, rats, and rabbits and was different from the usual

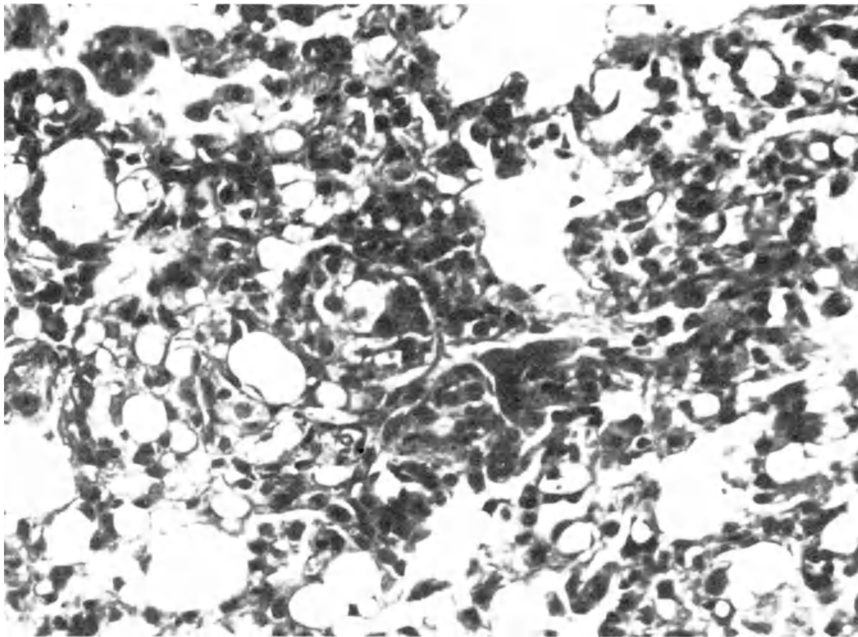

FIGURE 2. Mycoloyl-MDP-induced lung granuloma formation. Lung from a C3H/He mouse 1 week after the injection of emulsion containing 300 μg mycoloyl-MDP and 300 μg PPD. (From Yamamoto *et al.*[38])

Schwartzman reaction, worsened by heparin, a Schwartzman reaction inhibitor. The mechanism for this necrotic reaction remains obscure.

2.3.3. Effect of MDP on Proliferation of Bone Marrow Granulocyte–Macrophage Progenitor Cells

Adjuvants such as bacterial lipopolysaccharide, lipid A, and bacterial CW components were reported to affect myelopoiesis. Wuest and Wachsmuth[40] showed that MDP induced a dose-dependent increase in the number of bone marrow granulocyte–macrophage progenitor cells, the maximum effect being reached at a dose of about 10 mg/kg and the 50% effect at a dose of about 0.1 mg/kg. Moreover, they showed that low doses of MDP (0.1–1 mg/kg) provoked lymphocytosis and larger doses of MDP (10 mg/kg upwards) produced lymphocytopenia and an increase in the number of young staff neutrophils and monocytes. Galelli and Chedid[41] reported that MDP elicited *in vivo* a response characterized by a rise in the level of monocyte–macrophage colony-stimulating activity in

serum, a proliferation of multipotential stem cells in the bone marrow and an expansion of granulocyte–macrophage progenitors in the spleen.

2.3.4. Stimulation by MDP of Host Resistance against Infection

The natural resistance of host to infection can be increased by prophylactic administration of various bacterial products. Therefore, MDP, an important adjuvant moiety of bacterial CW, was examined for its ability to enhance host resistance against extracellular and intracellular pathogens. Chedid *et al.*[42] showed that MDP and various analogues administered to mice by various routes enhanced their nonspecific resistance against *Klebsiella pneumoniae* and were effective even when administered after challenge infection. Matsumoto *et al.*[43] showed that when mice treated with MDP were challenged with *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Escherichia coli.,* the degree of protection ranged from minor, slightly higher and the highest, respectively. By contrast, MDP was also reported to be effective in protecting against *Streptococcus pneumoniae,*[44] *Candida albicans,*[45] and *Trypanosoma cruzi.*[46] We reported that in mice, mycoloyl-MDP was capable of enhancing lung granuloma formation and host resistance against *M. bovis* infection, whereas MDP was not.[38] The importance of phagocytic cells, such as polymorphonuclear leukocytes and macrophages, for microbicidal activity has been emphasized. Recently, MDP was shown to activate the microbicidal activity of these cells.[47] That is, MDP stimulated the chemotactic mobility,[48] phagocytic activity,[45] and superoxide anion production[45,49,50] in polymorphonuclear leukocytes and macrophages. MDP-induced cellular stimulated states are closely associated with the enhancement of host-defense mechanisms to intracellular and extracellular organisms. However, other studies showed that MDP was ineffective in protecting against infections with *Listeria monocytogenes,*[51] *Mycobacterium leprae,*[52] and *Schistosoma mansoni.*[53] Thus, the effectiveness of MDP as a stimulant of host resistance is not clearly established in the literature, but this probably depends on differences in experimental conditions, such as administration form and route.

2.3.5. Immune Suppression by MDP

Contrary to its adjuvant activity, MDP was shown to induce a depression of immune responses. Leclerc *et al.*[54] showed that the anti-SRBC PFC response was suppressed in mice that had been injected intraperitoneally with 500 μg MDP, 1, 2, and 3 days before immunization with SRBC. These workers also indicated that *in vitro* generation of cytotoxic T cells from mice that had been treated with MDP was de-

creased. In their analysis of MDP-induced immunosuppression, they found T-cell-enriched populations of spleen cells from mice injected with MDP to exhibit a suppressive activity against anti-SRBC PFC response that could be removed by treatment with anti-Thy 1 antiserum plus complement. Moreover, Kishimoto *et al.*[55] showed that antigen-conjugated MDP or 6-0-mycoloyl-MDP had a selective suppressive effect on the induction of IgE antibody response. Preadministration of dinitrophenyl (DNP)-mycoloyl-MDP completely inhibited the induction of anti-DNP IgE antibody response; this effect was attributed to the induction of radiosensitive DNP-specific suppressor T (Ts) cells by DNP-mycoloyl-MDP.

By contrast, we reported that BCG CW-induced or live *Listeria*-induced DTH responses were suppressed in mice that had been injected IV with 500 μg MDP in water-in-oil-in-water emulsion 2 weeks before immunization[56] (Figs. 2 and 3). However, when injected intraperitoneally or subcutaneously with 500 μg MDP in w/o/w emulsion or in

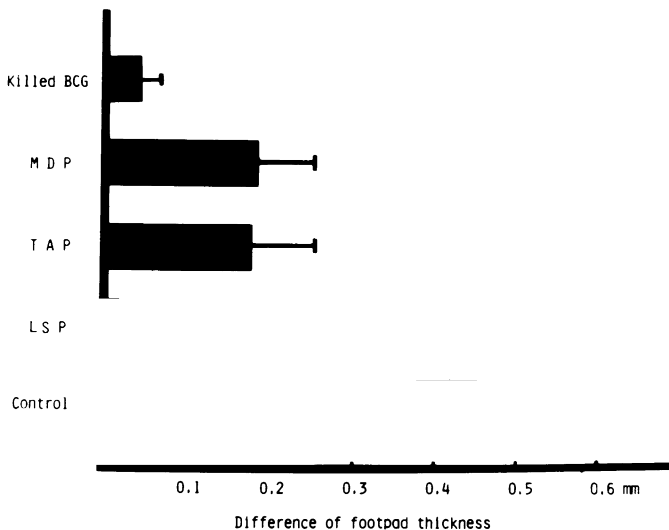

FIGURE 3. DTH to PPD in C3H mice previously injected intravenously (IV) with muramyl dipeptide (MDP), tuberculin-active peptide (TAP) or *Listeria*-soluble protein (LSP). Each group of six C3H mice was injected IV with a water-in-oil-in-water (w/o/w) emulsion of MDP, TAP, or LSP at a dose of 500 μg. The other two groups were injected with the w/o/w vehicle emulsion above or with 1 mg heat-killed BCG and were used as controls. All mice were immunized subcutaneously with oil-treated BCG CW 2 weeks after IV injection. Four weeks later, footpad testing was performed with 10 μg purified protein derivatives (PPD). Mean footpad swelling ±SD are shown. Control versus heat-killed BCG, $p < 0.001$; control versus MDP, $p < 0.02$; control versus TAP, $p < 0.01$. (From Kato *et al.*[56])

saline 2 weeks earlier, the BCG CW-immunized mice showed no DTH suppression. Therefore, IV injection of MDP in w/o/w emulsion seems to be more effective in inducing DTH suppression than that in saline, since Parant *et al.*[57] reported that 75–95% of ^{14}C-labeled MDP in saline injected IV into mice was eliminated in the urine within 2 hr. The MDP-induced DTH suppression was analyzed by use of a macrophage migration inhibition (MI) assay.[56] MI-positive peritoneal exudate cells (PEC) from BCG CW-immunized mice were mixed with spleen cells from mice that had been injected with 500 μg MDP in water-in-oil emulsion and the mixture was examined for MI activity. No MI activity was observed, suggesting that the MI activity of PEC was suppressed by the spleen cells from MDP-treated mice (Table II). The suppressive activity was eliminated by treatment of spleen cells with anti-θ serum plus complement but not with anti-mouse Ig plus complement, indicating that MDP induced Ts cells resident in the spleen (Table III). Thus, the MDP-induced Ts cells were supposed to suppress the elaboration or release of MI factor from BCG CW-sensitized T cells. Such suppressor cells also suppressed the MI activity of PEC from live *Listeria*-immunized mice and

TABLE II
Inhibitory Effect of Splenic Nonadherent Cells from MDP-, TAP-, and LSP-Injected C3H mice on MI Activity of BCG CW-Effector PEC[a,b,c]

Treatment group [b]	Presence of PPD	Area of migration (cm^2)	MI activity[d] (%)
Vehicle	–	3.0 ± 0.4	57 ($p < 0.01$)
(positive control)	+	1.7 ± 0.3	
MDP	–	3.5 ± 0.7	86 ($p > 0.05$)
	+	3.0 ± 0.1	
TAP	–	2.8 ± 0.3	93
	+	2.6 ± 0.6	
LSP	–	3.0 ± 0.3	57 ($p < 0.01$)
	+	1.7 ± 0.1	

[a]From Kato *et al.*[56]
[b]BCG, bacillus Calmette–Guérin; CW, cell wall; LSP, *Listeria*-soluble protein; MDP, muramyl dipeptide; MI, migration inhibition; PEC, peritoneal exudate cells; PPD, purified protein derivative; TAP, tuberculin-active peptide.
[c]Nonadherent cells were obtained from five mice injected IV with 500 μg MDP, tuberculin-active peptide TAP, or LSP in water-in-oil-in-water emulsion 2 weeks earlier. Nonadherent cells from five mice injected IV with vehicle alone served as a control. All these nonadherent cells were mixed in a ratio of 1 : 9 with BCG CW-effector PEC harvested from 20 mice immunized with BCG CW and were assayed for inhibition of the MI activity of BCG CW-effector PEC.
[d]MI activity (percentage migration inhibition) = (migration area of the mixed cells with 50 μg/ml PPD/migration area of the mixed cells without PPD) × 100. The significance of differences between the migration area of the mixed cells with PPD and that without PPD was determined by Student's *t*-test.

TABLE III
Inhibitory Effects of Anti-θ and Antimouse Ig-Treated MDP-Nonadherent Cells on MI Activity of BCG CW-effector PEC[a,b]

BCG CW-effector PEC mixture	Presence of PPD	Area of migration (cm^2)	MI activity (%)
Control nonadherent cells	−	3.1 ± 0.1	45 ($p < 0.001$)
	+	1.4 ± 0.2	
MDP nonadherent cells	−	3.1 ± 0.5	94
	+	2.9 ± 0.4	
Anti-θ and C-treated MDP nonadherent cells	−	2.9 ± 0.3	52 ($p < 0.001$)
	+	1.5 ± 0.1	
Antimouse Ig and C-treated MDP nonadherent cells	−	2.5 ± 0.1	108
	+	2.7 ± 0.6	

[a]From Kato *et al.*[56]
[b]After treatment with anti-θ serum plus complement (C) or with antimouse Ig serum plus C, MDP nonadherent cells were assayed for inhibition of the MI activity of BCG CW-effector PEC, as indicated in the footnotes to Table II.

were active across the H-2 barrier. Furthermore, the MDP-induced Ts cells were shown to suppress both DTH induction and expression phases in BCG CW-sensitized mice.

2.3.6. *Effect of MDP on the Central Nervous System*

Some constituents of gram-positive bacteria, especially group A *Streptococcus pyogenes,* are well known to cause fever when injected intravenously in rabbits. Roberson and Schwach[58] and Rotta and Bednár[59] reported that purified preparation of CW or peptidoglycans isolated from *Streptococcus pyogenes* and other streptococci elicited a reproducible febrile response in rabbits. Following these findings, Kotani *et al.*[60] and Dinarello *et al.*[61] showed that MDP has a definite pyrogenic effect and elicits leukocyte pyrogen in rabbits. Riveau *et al.*[62] showed that MDP given by the intracerebroventricular route has a tremendously (~200,000-fold) higher pyrogenicity, eliciting a strong and lasting febrile response, although no circulating endogenous pyrogen could be detected in plasma and cerebrospinal fluid. By contrast, Krueger *et al.*[63] reported the sleep-promoting effect of MDP. Intraventricular infusion of MDP increased hourly slow-wave sleep as well as raised body temperature in rabbits and cats. These results showed that MDP administered intraventricularly induced a somnogenic effect in animals, suggesting that MDP may induce pyrogenicity and somnogenicity by a direct effect on brain structures that control body temperature and sleep. However, whether these effects of

MDP on the CNS are associated with pathogenesis in tuberculosis is unknown.

3. ROLE OF THE IMMUNOGENIC MOIETIES OF *M. TUBERCULOSIS*

It is generally accepted that bacterial proteins or polypeptides, immunogenic moieties, as well as adjuvant substances play an important role in the DTH response induced by tubercle bacilli and that they also function actively in eliciting the tuberculin reaction. Since the original tuberculin preparation described by Koch in 1891 and named Old tuberculin (OT), efforts were pursued in several laboratories to make tuberculin-active proteins. Their biologic activity was studied mainly as capacity to elicit tuberculin reactions in tuberculous subjects or animals. This section reviews the other biologic activities associated with such proteins.

3.1. Lymphokine Production

Mycobacterium-sensitized T lymphocytes release a series of factors, i.e., lymphokines, affecting the immune state induced by specific antigens of mycobacteria. Originally, Bloom and Bennett[64] showed that Mycobacterium-sensitized lymphocytes cultured with a specific antigen, PPD, could release the macrophage MIF, which inhibited the migration of normal guinea pig peritoneal macrophages from capillary tubes. Detection of MIF from sensitized T lymphocytes exposed to antigens is regarded today as a valid *in vitro* correlate of DTH. Other lymphokine produced include the skin reactive factor, mitogenic factor, lymphotoxin, macrophage activation factor, and IL-2. Recently, Takatsu *et al.*[65] reported that *Mycobacterium*-sensitized T cells released T-cell-replacing factor, which acted on allogeneic as well as syngeneic B cells.

3.2. Desensitization in Cellular Immunity

An injection of large doses of antigen into animals with DTH can render the animals specifically unreactive to subsequent intradermal challenge with that antigen. The unresponsiveness is short-lived, lasting several days, and is related to the dose of antigen given. Yamamoto and Takahashi[66] showed that the IV injection of BCG protoplasm into animals sensitized by *Mycobacterium* reduced the dermal reaction to BCG protoplasm and simultaneously induced a high level of MIF in serum. This dermal unresponsiveness is probably due to the high level of serum MIF elaborated in response to IV challenge with a large dose of BCG

protoplasm; MIF may inhibit the accumulation of monocytes inflowing from blood circulation in the tuberulin reaction site, and this result lead to the expression of dermal desensitization. Yamamura *et al.*[67] showed that repeated injections of TAP were effective in desensitizing cutaneous hypersensitivity in rabbits sensitized with heat-killed tubercle bacilli and prevented cavity formation in which cellular immunity might play an important role. Although they did not refer to the release of MIF by desensitization, released MIF might be associated with the prevention of cavity formation.

3.3. Mycobacterial Antigen-Induced DTH Suppression

We showed that the induction of antigen nonspecific Ts cells was achieved by injection of heat-killed BCG.[68] Nonspecific Ts cells, which suppress DTH elicited by both BCG CW and *Listeria monocytogenes*, were also shown to be induced by IV injection of MDP.[56] We also showed that in contrast with MDP-induced DTH suppression, Ts cells induced by IV injection of TAP, suppressed BCG CW-induced DTH but not *Listeria*-induced DTH[56] (Figs. 3 and 4). In addition, the TAP-induced Ts cells

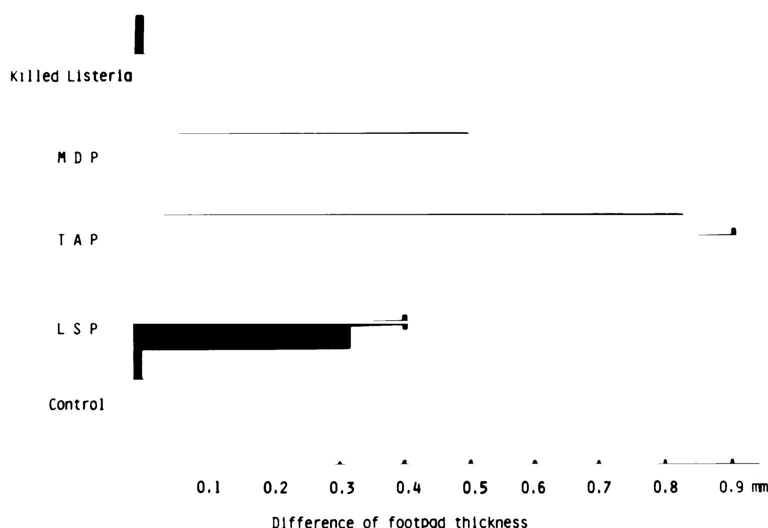

FIGURE 4. DTH to *Listeria*-soluble proteins (LSP) in mice previously injected intravenously (IV) with MDP, TAP, or LSP. C3H mice that had been treated by IV injection with MDP, TAP, LSP, or heat-killed *Listeria* 2 weeks earlier were immunized with 10^3 live *Listeria*. Seven days later, the footpad test was performed with 10 μg LSP. Mean footpad swellings ±SD are shown. Control versus heat-killed LSP, $p < 0.001$. (From Kato *et al.*[56])

TABLE IV
Inhibitory Effects of Anti-θ and Antimouse Ig-Treated TAP Nonadherent Cells on MI Activity of BCG CW-Effector PEC[a,b]

BCG CW-effector PEC mixture	Presence of PPD	Area of migration (cm^2)	MI activity (%)
Control nonadherent cells	−	2.8 ± 0.3	71 ($p < 0.01$)
	+	2.0 ± 0.2	
TAP nonadherent cells	−	2.9 ± 0.1	90
	+	2.6 ± 0.4	
Anti-θ and C-treated TAP nonadherent cells	−	2.5 ± 0.3	68 ($p < 0.05$)
	+	1.7 ± 0.5	
Antimouse Ig and C-treated TAP nonadherent cells	−	2.6 ± 0.3	100
	+	2.6 ± 0.2	

[a]From Kato *et al.*[56]
[b]TAP nonadherent cells were treated and tested in the MI assay as the MDP nonadherent cells in Table III.

suppressed the MI activity of PEC from BCG CW-immunized mice but not that from *Listeria*-immunized mice (Tables II, IV, and V). Therefore, two different Ts cell subsets, induced by both adjuvant and immunogenic moieties of BCG, might be produced in BCG-induced DTH suppression. They might act *in vivo* by interfering with the lymphokine-dependent mechanism by which DTH effector T cells elicit DTH, since they evidently suppressed the MI activity of PEC from BCG CW-immu-

TABLE V
Inhibitory Effects of Splenic Nonadherent Cells from MDP-, TAP- and LSP-injected C3H mice on MI activity of live *Listeria*-Effector PEC[a,b]

Treatment group	Presence of LSP	Area of migration (cm^2)	MI activity (%)
Vehicle (positive controls)	−	3.3 ± 0.1	58 ($p < 0.001$)
	+	1.9 ± 0.3	
MDP	−	3.0 ± 0.2	90 ($p > 0.05$)
	+	2.7 ± 0.2	
TAP	−	3.2 ± 0.2	50 ($p < 0.001$)
	+	1.6 ± 0.2	
LSP	−	3.5 ± 0.1	97
	+	3.4 ± 0.4	

[a]From Kato *et al.*[56]
[b]The inhibitory effects of MDP nonadherent cells, TAP nonadherent cells, and LSP nonadherent cells on the MI activity of live *Listeria*-effector PEC were assayed as described in the footnotes to Table II.

nized mice. The antigen specificity of TAP-induced Ts cells suggests that this subset acts via antigen receptors or anti-idiotypic receptors to inhibit the ability of antigen-sensitive DTH effector T cells to elaborate lymphokines. The adjuvant-induced Ts cells may, instead, act on these lymphokine-releasing effector T cells by nonspecific mechanisms or factors derived from the suppressor cells. Recently, Colizzi *et al.*[69] reported that spleen cells from mice injected IV with BCG, released factors in culture that suppressed DNA synthesis induced by concanavalin A (Con A) *in vitro.* They also showed that both macrophages and T cells produced inhibiting factors and that the macrophage factor had a molecular weight of 10,000–30,000, while the T-cell factor had a molecular weight of 50,000–70,000. Little information is available on supressor factors possibly produced by MDP- and TAP-induced Ts cells. However, it can be speculated that the suppressor factors from MDP- and TAP-induced Ts cells may be involved in suppression of DTH *in vivo,* since these cells reside in the spleen and do not circulate.

4. SUMMARY

Since Freund's original observation on the adjuvant effect of mycobacteria on immune response,[1,2] mycobacteria have been accepted as the most potent enhancing agents for immune response. This finding prompted a lengthy search to determine the chemical structure of the *Mycobacterium* fraction exhibiting adjuvant activity; recently, the important relevant structure, MDP, has been identified.[10] MDP is not immunogenic and is devoid of the several side effects elicited by administration of whole mycobacteria. MDP with an antigen in water-in-oil emulsion was capable of replacing whole mycobacteria in increasing antibody production and inducing DTH.[25] The biologic activities of MDP other than its adjuvant activity have been outlined. Interestingly, MDP was shown to produce antigen-nonspecific Ts cells active in humoral and cell-mediated immunity in spite of its nonimmunogenic activity.[54,56] Therefore, MDP seems to be an immunomodulator as well as an immunostimulator. However, the mechanisms involved in the induction of antigen nonspecific Ts cells by MDP are still unknown. The above-mentioned findings obtained in experimental animals suggest that the administration route and dose of MDP, should it be used in the therapy of human infectious diseases or tumors, should be examined first to ensure that the MDP has a beneficial effect without inducing Ts cells or other side effects.

We have also described the biologic activities of the immunogenic moieties of *M. tuberculosis.* One of the main biologic activities is the in-

duction from T cells sensitized with mycobacteria of lymphokines, that play an important role in immune responses. Moreover, we made a few remarks on the regulatory effects of the immunogenic moieties of *M. tuberculosis* on immunity, i.e., desensitization and antigen-specific Ts-cell induction involved in immunity against tuberculosis.

The study of the various biologic activities of adjuvant and immunogenic moieties of *M. tuberculosis* may lead to a better understanding of the host–parasite relationship in experimental and human tuberculosis. It may also stimulate research on various topics that are not apparently linked to the adjuvant activity of *M. tuberculosis*, such as myelopoiesis, pyrogenicity, or somnogenicity.

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REFERENCES

1. Freund, J., Casals, J., and Hosmer, E. P., 1937, Sensitization and antibody formation after injection of tubercle baccili and paraffin oil, *Proc. Soc. Exp. Biol. Med.* **37:**509–513.
2. Freund, J., Stern, E. R., and Pisani, T. M., 1947, Isoallergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and mycobacteria in water-in-oil emulsion, *J. Immunol.* **57:**179–194.
3. Pearson, C. M., 1956, Development of arthritis, periarthritis and periostitis in rats given adjuvants, *Proc. Soc. Exp. Biol. Med.* **91:**95–101.
4. Waksman, B., Pearson, C. M., and Sharp, J. T., 1960, Studies of arthritis and other lesions induced in rats by injection of mycobacterial adjuvant. II. Evidence that the disease is a disseminated immunologic response to exogenous antigen, *J. Immunol.* **85:**403–411.
5. Spector, W. G., and Lykke, A. W. J., 1966, The cellular evolution of inflammatory granulomata, *J. Pathol.* **92:**163–177.
6. Kabat, E. A., Wolf, A., and Benzer, A. E., 1946, Rapid production of acute disseminated encephalomyelitis in rhesus monkey by injection of brain tissue with adjuvants, *Science* **104:**362–363.
7. Morgan, I. A., 1947, Allergic encephalomyelitis in monkeys in response to injection of normal monkey nervous tissue, *J. Exp. Med.* **85:**131–140.
8. Zbar, B., and Tanaka, T., 1971, Immunotherapy of cancer: Regression of tumors after intralesional injection of living *Mycobacterium bovis, Science* **172:**271–273.
9. Suter, E., Velman, G. A., and Hoffman, R. G., 1958, Sensitivity of mice to endotoxin after vaccination with BCG (Bacillus Calmette-Guérin), *Proc. Soc. Exp. Biol. Med.* **99:**167–173.
10. Ellouz, F., Adam, A., Ciorbaru, R., and Lederer, E., 1974, Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives, *Biochem. Biophys. Res. Commun.* **59:**1317–1325.
11. Merser, C., Sinay, P., and Adam, A., 1975, Total synthesis and adjuvant activity of bacterial peptidoglycan derivatives. *Biochem. Biophys. Res. Commun.* **66:**1315–1322.
12. Raffel, S., Arnaud, L. E., Dukes, C. D., and Huang, J. S., 1949, The role of the wax of tubercle bacillus in establishing delayed hypersensitivity, *J. Exp. Med.* **90:**53–71.

13. Tanaka, A., Tanaka, L., Tsubone, T., Kuroda, Y., and Sugiyama. K., 1965, Fractionation and characterization of wax D, peptidoglycolipid of mycobacteria tuberculosis. II. Adjuvanticity of the subfractions of wax D of H37Ra and wax D of other strains of *M. tuberculosis, Int. Arch. Allergy Appl. Immunol.* **28:**340–352.
14. White, R. G., Jollès, P., Samour, D., and Lederer, E., 1964, Correlation of adjuvant activity and chemical structure of wax D fraction of mycobacteria, *Immunology* **7:**158–171.
15. Pearson, C. M., and Wood, F. D., 1959, Studies of polyarthritis and other lesions induced in rats by injection of mycobacterial adjuvant. I. General clinical and pathological characteristics and some modifying factors, *Arthritis Rheum.* **2:**440–449.
16. Kato, M., 1972, Antibody formation to trehalose-6,6′-dimycolate (cord factor) of *Mycobacterium tuberculosis, Infect. Immun.* **5:**203–211.
17. Bekierkunst, A., Levij, I. S., Yarkoni, E., Vilkas, E., Adam, A., and Lederer, E., 1969, Granuloma formation induced in mice by chemically defined mycobacterial fractions, *J. Bacteriol.* **100:**95–102.
18. Adam, A., Ciorbaru, R., Petit, J. F., and Lederer, E., 1972, Isolation and properties of a macromolecular, water-soluble, immunoadjuvant fraction from the cell wall of *Mycobacterium smegmatis, Proc. Natl. Acad. Sci. USA* **69:**851–854.
19. Adam, A., Ciorbaru, R., Petit, J. F., Lederer, E., Chedid, L., Lamensans, A., Parant, M., Rosselet, J. P., and Berger, F. M., 1973, Preparation and biological properties of water-soluble adjuvant fractions from delipidated cells of *Mycobacterium smegmatis* and *Nocardia opaca, Infect. Immun.* **7:**855–861.
20. Chedid, L., Parant, M., Parant, F., Gustafson, R. H., and Berger, F. M., 1972, Biological study of a non-toxic, water-soluble immunoadjuvant from mycobacterial cell wall, *Proc. Natl. Acad. Sci. USA* **69:**855–858.
21. Modolell, M., Luckenbach, G. A., Parant, M., and Munder, P. G., 1974, The adjuvant activity of a mycobacterial water-soluble adjuvant (WSA) *in vitro.* I. The requirement of macrophages, *J. Immunol.* **113:**395–403.
22. Juy, D., and Chedid, L., 1975, Comparison between macrophage activation and enhancement of nonspecific resistance to tumors by mycobacterial immunoadjuvants, *Proc. Natl. Acad. Sci. USA* **72:**4105–4109.
23. Oppenheim, J. J., Togawa, A., Chedid, L., and Mizel, S., 1980, Components of mycobacteria and muramyl dipeptide with adjuvant activity induce lymphocyte activating factor, *Cell. Immunol.* **50:**71–81.
24. Kotani. S., Watanabe, Y., Kinoshita, F., Shimono, T., Morisaki, I., Shiba, T., Kusumoto, S., Tarumi, T., and Ikenaka, K., 1975, Immunoadjuvant activities of synthetic *N*-acetylmuramyl-peptides or -amino acids, *Biken J.* 18:105–111.
25. Azuma, I., Sugimura, K., Taniyama. T., Yamawaki, M., Yamamura, Y., Kusumoto, S., Okada, S., and Shiba, T., 1976, Adjuvant activity of mycobacterial fractions: Adjuvant activity of synthetic *N*-acetylmuramyl-dipeptide and the related compounds, *Infect. Immun.* **14:**18–27.
26. Ohkuni, H., Norose, Y., Ohta, M., Hayama, M., Kimura, Y., Tsujimoto, M., Kotani, S., Shiba, T., Kusumoto, S., Yokogawa, K., and Kawata, S., 1979, Adjuvant activities in production of reaginic antibody by bacterial cell wall peptidoglycan or synthetic *N*-acetylmuramyl dipeptide in mice, *Infect. Immun.* **24:**313–318.
27. Specter, S., Cimprich, R., Friedman, H., and Chedid, L., 1978, Stimulation of an enhanced *in vitro* immune response by a synthetic adjuvant, muramyl dipeptide, *J. Immunol.* **120:**487–491.
28. Löwy, I.. Bona, C., and Chedid, L., 1977, Target cells for the activity of a synthetic adjuvant: Muramyl dipeptide, *Cell. Immunol.* **29:**195–199.
29. Sugimoto, M., Germain, R. N., Chedid, L., and Benacerraf, B., 1978, Enhancement of

carrier-synthetic helper T cell function by the synthetic adjuvant, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), *J. Immunol.* **120:**980–982.
30. Sugimura, K., Uemiya, M., Saiki, I., Azuma, I., and Yamamura, Y., 1979, The adjuvant activity of synthetic *N*-acetylmuramyl-dipeptide: Evidence of initial target cells for the adjuvant activity, *Cell. Immunol.* **43:**137–149.
31. Fevrier, M., Birrien, J. L., Leclerc, C., Chedid, L., and Liacopoulos, P., 1978, The macrophage, target cell of the synthetic adjuvant muramyl-dipeptide (MDP), *Eur. J. Immunol.* **8:**558–562.
32. Staruch, M. J., and Wood, D. D., 1982, Genetic influences on the adjuvanticity of muramyl dipeptide *in vivo, J. Immunol.* **128:**155–160.
33. Damais, C., Parant, M., Chedid, L., Lefrancier, P., and Choay, J., 1978, *In vitro* spleen cell responsiveness to various analogs of MDP (*N*-acetylmuramyl-L-alanyl-D-isoglutamine), a synthetic immunoadjuvant, in MDP high-responder mice, *Cell. Immunol.* **35:**173–179.
34. Allen, E. M., Moore, V. L., and Stevens, J. O., 1977, Strain variation in BCG-induced chronic pulmonary inflammation in mice. I. Basic model and possible genetic control by non-H-2 genes, *J. Immunol.* **119:**343–347.
35. Yamamoto, K., and Kakinuma, M., 1978, Genetic control of granuloma response to oil-associated BCG cell wall vaccine in mice, *Microbiol. Immunol.* **22:**335–348.
36. Tanaka, A., Nagao, S., Nagao, R., Kotani, S., Shiba, T., and Kusumoto, S., 1979, Stimulation of reticuloendothelial system of mice by muramyl dipeptide, *Infect. Immun.* **24:**302–307.
37. Emori, K., and Tanaka, A., 1978, Granuloma formation by synthetic bacterial cell wall fragment: muramyl dipeptide, *Infect. Immun.* **19:**613–620.
38. Yamamoto, K., Kakinuma, M.. Kato, K., Okuyama, H., and Azuma, I., 1980, Relationship of anti-tuberculous protection to lung granuloma produced by intravenous injection of synthetic 6-0-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine with or without specific antigens, *Immunology* **40:**557–564.
39. Nagao, S., and Tanaka, A., 1985, Necrotic inflammatory reaction induced by muramyl dipeptide in guinea pigs sensitized by tubercle bacilli, *J. Exp. Med.* **162:**401–412.
40. Wuest, B., and Wachsmuth, E. D., 1982, Stimulating effect of N-acetyl muramyl dipeptide in vivo: Proliferation of bone marrow progenitor cells in mice, *Infect. Immun.* **37:**452–462.
41. Galelli, A., and Chedid, L., 1983, Modulation of myelopoiesis in vivo by synthetic adjuvant-active muramyl peptides. Induction of colony-stimulating activity and stimulation of stem cell proliferation, *Infect. Immun.* **42:**1081–1085.
42. Chedid, L., Parant, M., Parant, F., Lefrancier, P., Choay, J., and Lederer, E., 1977, Enhancement of nonspecific immunity to *Klebsiella pneumoniae* infection by a synthetic immunoadjuvant (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) and several analogs, *Proc. Natl. Acad. Sci. USA* **74:**2089–2093.
43. Matsumoto, K., Ogawa, H., Nagase, O., Kusama, T., and Azuma, I., 1981, Stimulation of nonspecific host resistance to infection induced by muramyldipeptide, *Microbiol. Immunol.* **25:**1047–1058.
44. Humphers, R. C., Henika, P. R., Ferraresi, R. W., and Krahenbuhl, J. L., 1980, Effects of treatment with muramyl dipeptide and certain of its analogs on resistance to *Listeria monocytogenes* in mice, *Infect. Immun.* **30:**462–466.
45. Cummings, N. P., Pabst, M. J., and Johnston, R. B., 1980, Activation of macrophages for enhanced release of superoxide anion and greater killing of *Candida albicans* by injection of muramyl dipeptide, *J. Exp. Med.* **152:**1659–1669.
46. Kierszenbaum, F., and Ferraresi, R. W., 1979, Enhancement of host resistance against

Trypanosoma cruzi infection by the immunoregulatory agent muramyl dipeptide, *Infect. Immun.* **25**:273–278.

47. Hadden, J. W., Englard, A., Sadlik, J. R., and Hadden, E. M., 1979, The comparative effects of isoprinosine, levamisole, muramyl dipeptide and SM 1213 on lymphocyte and macrophage proliferation and activation *in vitro*, *Int. J. Immunopharm.* **1**:17–27.
48. Ogawa, T., Kotani, S., Fukuda, K., Tsukamoto, Y., Mori, M., Kusumoto, S., and Shiba, T., 1982, Stimulation of migration of human monocytes by bacterial cell walls and muramyl dipeptides, *Infect. Immun.* **38**:817–824.
49. Pabst, M. J., and Johnston, R. B., 1980, Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or lipopolysaccharide, *J. Exp. Med.* **151**:101–114.
50. Kaku, M., Yagawa, K., Nagao, S., and Tanaka, A., 1983, Enhanced superoxide anion release from phagocytes by muramyl dipeptide or lipopolysaccharide, *Infect. Immun.* **39**:559–564.
51. Finger, H., and Wirsing von König, C-H., 1980, Failure of synthetic muramyldipeptide to increase antibacterial resistance, *Infect. Immun.* **27**:288–291.
52. Krahenbuhl, J. L., Humphres, R. C., and Ferraresi, R. W., 1980, Effect of adjuvant immunotherapy of infection with *M. leprae* and *M. marinum*, *Int. J. Leprosy* **48**:504–505.
53. Olds, G. R., Chedid, L., Lederer, E., Adel, A., and Mahmoud, F., 1980, Induction of resistance to *Schistosoma mansoni* by natural cord factor and synthetic lower homologues, *J. Infect. Dis.* **141**:473–478.
54. Leclerc, C., Bourgeois, E., and Chedid, L., 1982, Demonstration of muramyl dipeptide (MDP)-induced T suppressor cells responsible for MDP immunosuppressive activity, *Eur. J. Immunol.* **12**:249–252.
55. Kishimoto, T., Hirai, Y., Nakanishi, K., Azuma, I., Nagamatsu, A., and Yamamura, Y., 1979, Regulation of antibody response in different immunoglobulin classes. VI. Selective suppression of IgE response by administration of antigen-conjugated muramylpeptides, *J. Immunol.* **123**:2709–2715.
56. Kato, K., Yamamoto, K., Kimura, T., Azuma, I., and Askenase, P. W., 1984, Suppression of BCG cell wall-induced delayed-type hypersensitivity by pretreatment with killed BCG: Induction of nonspecific suppressor T cells by adjuvant portion (MDP) and of specific suppressor T cells by the antigen portion (TAP), *J. Immunol.* **132**:2790–2795.
57. Parant, M., Parant, F., Chedid, L., Yapo, A., Petit, J. F., and Lederer, E., 1979, Fate of the synthetic immunoadjuvant, muramyl dipeptide (^{14}C-labeled) in the mice, *Int. J. Immunopharmacol.* **1**:35–41.
58. Roberson, B. S., and Schwab, J. H., 1961, Endotoxic properties associated with cell walls of group A streptococci. *J. Infect. Dis.* **108**:25–34.
59. Rotta, J., and Bednar, B., 1969, Biological properties of cell wall mucopeptide of hemolytic streptococci, *J. Exp. Med.* **130**:31–47.
60. Kotani, S., Watanabe, Y., Shimono, T., Harada, K., Shiba, T., Kusumoto, S., Yokogawa, K., and Taniguchi, M., 1976, Correlation between the immunoadjuvant activities and pyrogenicities of synthetic *N*-acetylmuramyl-peptides or -amino acids, *Biken J.* **19**:9–13.
61. Dinarello, C. A., Elin, R. J., Chedid, L., and Wolff, S. M., 1978, The pyrogenicity of the synthetic adjuvant muramyl dipeptide and two structural analogues, *J. Infect. Dis.* **138**:760–767.
62. Riveau, G., Masek, K., Parant, M., and Chedid, L., 1980, Central pyrogenic activity of muramyl dipeptide, *J. Exp. Med.* **152**:869–877.
63. Krueger, J. M., Pappenheimer, J. R., and Karnovsky, M. L., 1982, Sleep-promoting effects of muramyl peptides, *Proc. Natl. Acad. Sci. USA* **79**:6102–6106.

64. Bloom, B. B., and Bennett, B., 1966, Mechanism of a reaction in vitro associated with delayed-type hypersensitivity, *Science* **153:**80–82.
65. Takatsu, K., Tominaga, A., and Hamaoka, T., 1980, Antigen-induced T cell-replacing factor (TRF). I. Functional characterization of a TRF-producing helper T cell subset and genetic studies on TRF production, *J. Immunol.* **124:**2414–2422.
66. Yamamoto, K., and Takahashi, Y., 1971, Macrophage migration inhibition by serum from desensitized animals previously sensitized with tubercle bacilli, *Nature (New Biol.)* **233:**261–262.
67. Yamamura, Y., Ogawa, Y., Maeda, H., and Yamamura, Y., 1974, Prevention of tuberculous cavity formation by desensitization with tuberculin-active peptide, *Am. Rev. Respir. Dis.* **109:**594–601.
68. Kato, K., and Yamamoto, K., 1982, Suppression of BCG cell wall-induced delayed-type hypersensitivity by BCG pre-treatment. II. Induction of suppressor T cells by heat-killed BCG injection, *Immunology* **45:**655–661.
69. Colizzi, V., Ferluga, J., Garreau, F., Malkovsky, M., and Asherson, G. L., 1984, Suppressor cells induced by BCG release non-specific factors *in vitro* which inhibit DNA synthesis and interleukin-2 production, *Immunology* **51:**65–71.

3

Genetic Background of the Host and Expression of Natural Resistance and Acquired Immunity to *M. tuberculosis*

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1. INTRODUCTION

Recently, the World Health Organization (WHO) outlined a strategic approach for the design of an effective vaccine against tuberculosis.[1] A fundamental part of the vaccine development depends on a clearer understanding of the role of genetic factors in host responses to *Mycobacterium tuberculosis*. The aim of this chapter is to review studies that have dealt with the genetic control of susceptibility and resistance to tuberculosis in both humans and animal models.

By the 1960s, the concept of genetic susceptibility and resistance to tuberculosis had been firmly established by the studies of Lurie and Dannenberg[2] and Gray,[3] which demonstrated that inbred animal strains differed in their response to the same dose of *M. tuberculosis*. Furthermore, Mackaness[4] showed that the granuloma formation that occurs during tuberculosis infection is the result of cell-mediated immune reac-

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tions. The important concept demonstrated by these early studies was that the host response to *M. tuberculosis* consists of two phases: the preimmune or innate phase of resistance to the establishment of infection, and the subsequent emergence of immunity that leads either to elimination of mycobacteria or to progression of the disease.

2. INHERITED RESISTANCE TO TUBERCULOSIS IN RABBITS

The earliest formal genetic analysis of the trait of resistance and susceptibility to tuberculosis was begun during the 1930s.[2] The experimental system comprised a colony of rabbits derived from four families selectively inbred for resistance or susceptibility. The typing criterion used for selective inbreeding was the quantitation of primary pulmonary tubercles that developed 5 weeks after inhalation of virulent human type *M. tuberculosis* (H37Rv). Alveolar macrophages (AM) from resistant (R) rabbits were capable of trapping twice as many bacteria in a given period of time as the susceptible (S) rabbits and, by 5 weeks, the R rabbits had developed only 5% the number of primary tubercles as the S animals. Most importantly, the lesions in S rabbits contained more viable bacilli.[5] Resistance to the progress of tuberculosis, characterized by increased survival after virulent infection, was associated with a more rapid maturation of monocytes to epitheloid cells, which resulted in enhanced restriction of bacillary multiplication. A similar pattern was observed when intradermal inoculation of avirulent *M. bovis,* or bacillus Calmette–Guérin (BCG), organisms was studied.[6] Again, the BCG nodules in R rabbits ulcerated more quickly but healed faster, whereas the opposite kinetics were observed in susceptible rabbits. Furthermore, BCG vaccination caused a five-fold increase in the resistance of R-type rabbits against a subsequent virulent challenge with H37Rv. The susceptible animals were rendered only 1.2-fold more resistant by the same treatment. Moreover, the R rabbits produced antibodies and developed cellular hypersensitivity to tuberculin more rapidly than S rabbits. The trait of resistance to tuberculosis was shown to be dominant, multiple and additive over susceptibility by mendelian analysis experiments.[7] The genetic studies conducted by Lurie *et al.*[7] revealed that certain inbred rabbits are innately endowed with a superior capacity to sequester *M. tuberculosis* within AM and to generate an immune response. Although the gene(s) regulating resistance to tuberculosis in rabbits was not identified, the experimental results have withstood the test of time and are now being confirmed, extended, and explained in experimental models of tuberculosis in inbred strains of mice.

3. GENETIC CONTROL OF TUBERCULOSIS AND BCG INFECTION IN THE MOUSE

3.1. Earlier Studies on Intraspecies Variation in Resistance/Susceptibility to *M. tuberculosis*

Strain variation in resistance to mycobacterial infections among mice has been noted since the mid-1940s. Early reports by Donovick *et al.*[8] showed that the survival time of various mouse strains after intravenous (IV) infection with *M. tuberculosis* varied considerably. The C57BL, DBA/1, and CF1 strains were the most susceptible, while the Swiss Albino, C3H, CFN, and Strong A (A/St) strains were the most resistant.

The unusual susceptibility of the C57BL/6 (B6) strain was also reported by Gray.[3] Mice of this strain, when infected IV, could not restrict the proliferation of the bacilli in their reticuloendothelial organs and succumbed early in the course of infection. Sever and Youmans[9] observed a similar growth pattern of the avirulent *M. tuberculosis* H37Ra strain, the attenuated R1Rv strain, and the *M. bovis* (BCG) strain in the spleen, liver, and lungs of A/St and C57BL/6 mice. Mice of the C57BL/6 strain were, however, more susceptible to infection with the virulent *M. tuberculosis* H37Rv than the A/St and the CF1 strain, as measured by median survival time after IV inoculation.[10] Using *M. tuberculosis* (Vallée) as an infectious agent, Lynch *et al.*[11] performed a mendelian segregation analysis of the trait of resistance to tuberculosis in (C57BL/6J × Swiss)F_1 hybrids and backcross populations. Continuous backcrossing showed that the trait of innate resistance was controlled by a single dominant gene that, with all probability, was identical to the chromosome 1 *Bcg* gene (see Section 3.3).

3.2. Genetic Resistance to *M. tuberculosis* and BCG in Biozzi Mice

The resistance to *M. tuberculosis* and *M. bovis* (BCG) in Swiss mice selectively bred for high (HL) or low (LL) antibody responsiveness to sheep red blood cells has also been examined.[12] The quantitative differences in antibody production in the two lines of mice have been shown to be related to the segregation of polygenic characters determined by about 10–12 independent loci.[13] One hypothesis offered to explain the HL and LL phenotypes derives from the observation that LL-type macrophages exhibit superior metabolic activity.[14] Macrophages from LL mice also express stronger bacteriostatic activity than do HL macrophages.[15] Consequently, the LL mice are more resistant to *in*

vivo infection with *Salmonella typhimurium*[15] and BCG.[12] By contrast, LL mice did not exhibit the same innate resistance when infected with virulent H37Rv by the IV route.[12] The mortality reached 100% in LL animals by 4 weeks after infection, whereas HL mice, as well as F_1 hybrids derived from LL × HL crosses, survived the infection. The results of the HL/LL murine studies differed considerably from those of Lurie and Dannenberg's rabbits, which were also selectively inbred for macrophage function.[2] The R rabbits exhibited superior resistance against both BCG and H37Rv. It should be noted, however, that the H37Rv bacteria were administered to the rabbits by the aerogenic route as opposed to the IV route used in the murine experiments. Since natural infection by *M. tuberculosis* is most often contracted by the respiratory route,[16] it is possible that alveolar macrophages express genetic factors not found in splenic macrophages; thus, the resistance of AM may be more relevant to the study of infection with *M. tuberculosis*.

3.3. The Bcg Gene: Genetics and Phenotypic Expression

Another murine genetic system shown to control the course of mycobacterial infection is the *Bcg* gene located on chromosome 1.[17] All inbred strains of mice examined thus far fall into either of two categories when infected with a low dose [10^4 colony-forming units (CFU)] of BCG Montreal: Bcg^s (susceptible) or Bcg^r (resistant). According to these experimental conditions, the IV-injected BCG multiplies rapidly in the spleens and livers of Bcg^s mice during the first 3–4 weeks of infection. No multiplication occurs in that time period in the reticuloendothelial organs of Bcg^r mice. Moreover, the phenotype of resistance to BCG can be demonstrated within 24–48 hr as a significantly lower number of viable BCG bacilli in Bcg^r spleens as compared with Bcg^s spleens. This difference becomes more significant as the infection progresses to its peak 3 weeks later, when Bcg^s mice manifest 100–1000-fold more BCG bacteria in their spleens than do Bcg^r mice. The trait of resistance to BCG infection was found to be under the control of a single autosomal-dominant gene.[18] Mapping of the *Bcg* gene was achieved by typing recombinant inbred (RI) strains for the trait of resistance/susceptibility to BCG.[17] The B×H and B×D RI strains used for this study were derived from the pairs of progenitors expressing the allelic forms of the *Bcg* gene, i.e., the C57BL/6 (B, Bcg^s) and C3H/HeJ (H, Bcg^r) or DBA/2J (D, Bcg^r). The strain distribution pattern of Bcg^s and Bcg^r alleles in 36 RI strains was found to be similar to the strain distribution pattern of two isoenzyme loci, *Idh-1* (isocitrate dehydrogenase) and *Pep-3* (peptidase 3), located on the centromeric part of chromosome 1. The gene order was established by recombination

frequencies to be *Idh-1–Bcg–Pep-3*. An interesting outcome of the mapping studies was the discovery of an identical strain distribution pattern among *Bcg*$^{r/s}$, *Ity*$^{r/s}$, and *Lsh*$^{r/s}$ phenotypes that control resistance/susceptibility to BCG, *Salmonella typhimurium*, and *Leishmania donovani*, respectively. These results suggest that the *Bcg–Ity-Lsh* genes are either the same gene or in a tight linkage, as no proven recombination events have occurred among the three phenotypes in all cases tested so far.[19] Finally, infection with *M. lepraemurium* has also been shown to follow *Bcg* gene strain distribution.[20]

These experiments constitute a clear example that host resistance to the growth of mycobacteria and other intracellular pathogens *in vivo* is under the control of a single genetic locus. The *Bcg* gene is expressed independently of H-2 genes and is not dependent on natural killer (NK) or thymus-derived lymphocytes.[21] It has been confirmed both *in vivo* and *in vitro* that the cell that expresses the *Bcg* gene is the resident macrophage.[21,22]

The molecular mechanism of the *Bcg* gene action is the subject of current studies. At a descriptive level, the *Bcg* gene appears to have pleiotropic effects[23]: the macrophages of *Bcg*^{r} phenotype, when compared with *Bcg*^{s} macrophages, exhibited several characteristics of macrophage activation, such as enhanced spreading, a higher oxidative respiratory burst to a variety of activating stimuli, enhanced Ia antigen expression, superior support of nonspecific as well as antigen-induced mitogenesis, and quantitatively superior bactericidal activity. The observed functional differences between *Bcg*^{r} and *Bcg*^{s} macrophages macrophages may thus be explained by their state of activation: the *Bcg*^{r} macrophages are already primed for activation.[23] The *Bcg*^{r} macrophage, when exposed to the membrane perturbation caused by the phagocytosis of mycobacteria, becomes promptly activated for antimycobacterial activity. By contrast, the *Bcg*^{s} macrophage requires a sequence of priming and activating stimuli (including mycobacterial cell wall products and lymphokines) before the full extent of its antimycobacterial activity is expressed. Whether the *Bcg* gene product controls the enhanced responsiveness of the macrophage or interferes with the action of regulating stimuli (i.e., prostaglandins) is unclear. The action of the *Bcg* gene, defined as the level of macrophage priming for activation *in vitro*, can be observed *in vivo* as an interference with mycobacterial proliferation in the sites of infection. Interestingly enough, only those mycobacterial strains that show an intermediate level of virulence are clearly restricted by the *Bcg* gene. Its role can be completely masked when avirulent strains of mycobacteria are used in experimentation, but its effect (i.e., antimycobacterial activity) can be overcome by the presence of rapidly multiplying mycobacteria of high virulence.[24]

3.4. Segregation Analysis of the Mode of Inheritance of the Trait of Resistance to *M. tuberculosis*

In studies employing the highly virulent H37Rv strain of *M. tuberculosis,* the presence of additional host genetic elements, acting in accord with the *Bcg* gene, were identified.[25] In these experiments, various inbred mouse strains were typed according to their median survival time (MST) after IV inoculation of H37Rv bacteria. A clear difference in MST was noted in only two strains: I/St, MST = 21 days and A/Sn, MST = 62.7 days. The F_1 hybrids derived from I × A crosses were uniformly more resistant than the A parents, suggesting that susceptibility to H37Rv in the I strain is a recessive trait. Further backcross analysis of F_1 × I/St showed that susceptibility to tuberculosis was determined by a single autosomal gene provisionally designated *Ts-1*.

Interestingly, when 24 other strains were typed, a spectrum of MST was observed. The pattern emerged as follows (in descending order of MST): A/Sn, AKR, CBA, BRSUNT, BALB/c, B10.WB, B6, and I/St. It was therefore evident that A/Sn and I/St represented extreme resistant and susceptible *Ts-1* alleles, respectively, whereas the remainder expressed varying degrees of resistance that, incidentally, closely followed *Bcg* gene strain segregation. Another observation brought out by these studies was the beneficial effect of heterozygosity or F_1 hybrid resistance to tuberculosis. For example, the MST in C57BL/6 (B6) and BALB/c mice was 25 and 31 days, respectively, whereas the MST in (B6 × BALB/c) F_1 hybrids was 84 days. However, analysis of seven RI strains derived from BALB/c × B6 (C×B) mice showed that five of the strains were as sensitive as the progenitors, whereas two RI were more resistant. Data from the RI analysis suggest that the trait of resistance to *M. tuberculosis* in intermediate-type strains is probably controlled by more than one gene, *Bcg* possibly being among them. Nickonenko *et al.*[25] also pointed out a potential role for H-2 genes in regard to the observation of heterozygosis in protection against the disease. In $H\text{-}2^{j/j}$ homozygous F_1 mice, the usual observed increase in MST among F_1 progeny was absent, whereas in F_1 $H\text{-}2^{a/j}$ heterozygous mice, the MST was greatly increased.

It should be pointed out that the number of references concerning the innate resistance of mice to BCG infection far outnumbers those on tuberculosis. However, many investigators have dealt with the immunogenetic control of tuberculosis infection; these studies are discussed in the following section.

3.5. Genetic Control of Immunologic Responses to *M. tuberculosis*

In experimental murine models of tuberculosis, the phase of immunity becomes demonstrable, approximately 7–14 days after infection

with *M. tuberculosis.*[26] Since major histocompatibility complex H-2-linked genes are known to regulate the immune response,[27] it is conceivable that the H-2 haplotype of the host may determine the quality of the immune response that develops.

There is ample evidence to indicate that animals immunized with BCG are more resistant to a secondary challenge of virulent H37Rv *M. tuberculosis* than are nonimmune animals.[4,6,12,28] Several immune parameters have been associated with antituberculosis resistance, including delayed-type hypersensitivity (DTH) to tuberculin or purified protein derivative (PPD),[29] macrophage activation by thymus-derived (T) lymphocyte soluble factors,[30,31] and the ability of immune T cells to transfer resistance against tuberculosis.[28] To date, most of the evidence suggests that the activated macrophage is the effector cell, which destroys tubercule bacilli in the infected animal.[16] Yet it is clear that if the host lacks T lymphocytes, the ability of the animal to resist infection with tuberculosis is greatly reduced.[28] The activation of specific T cells by mycobacterial antigens induces the secretion of lymphokines (LK), i.e., γ-interferon (IFN_{γ}) and interleukin-2 (IL-2).[32] IFN_{γ} is a potent activator of macrophages *in vitro.*[33] The characteristics of LK-activated macrophages include enhancement of cytotoxicity toward several intracellular parasites, including *M. tuberculosis.*[30] In the tuberculosis-infected host, it has been proposed that a high concentration of T-cell-derived LK within the granuloma may lead to the rapid acquisition of properties of activated macrophages by incoming blood monocytes.[16] In the presence of sufficient LK, a greater proportion of activated macrophages would favor the intracellular destruction of bacteria and subsequent elimination of infection. The contribution of *M. tuberculosis*-specific antibodies in the elimination of bacteria is unlikely, since serum from immune animals cannot transfer immunity[34] and may actually enhance the growth of mycobacteria.[35]

3.5.1. H-2 Restriction of Immunoregulatory Events in M. tuberculosis Infection

Patterson and Youmans[31] demonstrated that splenic lymphocytes isolated from H37Ra-immunized mice were able to augment the bacteriostatic activity of normal peritoneal exudate cells (PEC) *in vitro.* The addition of immune lymphocytes caused a 40% reduction in the number of virulent H37Rv bacteria inside PEC as compared with the addition of normal cells. Similar inhibition of bacterial growth inside PEC was also observed when cell-free supernatants derived from cultures of immune spleen cells were added to the test system. The results of the study implied that H37Rv bacteria induced the elaboration of factors from

H37Rv-immune, but not normal, splenic lymphocytes. The factor alone was capable of increasing the bacteriostatic activity of PEC.

Evidence for major histocompatibility complex (MHC) restriction of lymphocyte activation in tuberculosis was later presented by Rosenthal and Shevach[36] in guinea pig strains 2 and 13. The study in question examined the ability of allogeneic and syngeneic macrophages to induce T-lymphocyte proliferation to PPD *in vitro.* It was shown that only syngeneic macrophages were capable of presenting PPD antigen to *M. tuberculosis*-immune responder T lymphocytes. In addition, the requirement for histocompatibility antigen recognition between T cells and macrophages was further proved by experiments employing alloantisera directed at strain 2 and 13 MHC determinants.

Recently, T-cell lines derived from *M. tuberculosis*-immune mice have been employed to examine H-2 restriction of macrophage activation.[30] It was established that immune T cells were able to inhibit intracellular multiplication of H37Rv bacteria inside macrophages only when those macrophages shared H-2 IA region determinants with the T-cell line. Furthermore, it was shown that supernatants collected from cultures of immune T cells could activate macrophage bacteriostatic activity against *M. tuberculosis* in an H-2 independent manner. Thus, all the above studies strongly suggest that although host elimination of *M. tuberculosis* due to macrophage activation by T-cell LK is under H-2 I region control, the LK alone can exert its function in a genetically unrestricted manner.

3.5.2. Genetic Control of Lymphokine Production in Mycobacterial Infections

As it has been clearly shown that macrophages activated by T-cell-derived LK inhibit the growth of *M. tuberculosis,* it is conceivable that differential production of LK among mouse strains may account for the differences in susceptibility to *M. tuberculosis* infection.

In a study by Neta and Salvin,[37] 12 strains of mice were tested for the ability to produce migration inhibition factor (MIF) and IFN_{γ} in response to stimulation with tubercle antigens. The inbred strains that were high producers of MIF *in vivo* were associated with the $H\text{-}2^{d}$ haplotype (BALB/cByJ, DBA/2, C57BL/KJ), whereas low responders were of the $H\text{-}2^{k}$ type (AKR, CBA,CaJ, C3H/Hc). The release of lFN into the circulation of the mice paralleled the production of MIF.

The production of IFN_{γ} and IL-2 in various strains of mice infected with BCG[38] or *M. lepraemurium*[39] has been investigated. The amounts of IFN_{γ} produced in the serum of mouse strains after BCG immunization and PPD challenge were found to vary widely. Specifically, B6 and

BALB/c strains were high and low responders, respectively, a result opposite to that reported by Neta and Salvin.[37] Further studies on F_1, F_2, and backcross mice of BALB/c × B6 hybrids led to the conclusion that high or low BCG–PPD provoked IFN_γ production is determined by a major autosomal locus.[38] However, it is obvious that the ability of the B6 strain to secrete high levels of IFN_γ, a potent activator of macrophage bacteriostatic activity does not correlate with the high susceptibility of B6 to tuberculosis.[25]

The susceptibility or resistance of inbred strains of mice to infection with *M. lepraemurium* (MLM) is determined by the *Bcg* genotype. Hoffenbach *et al.*[39] explored the possibility that strain susceptibility to MLM may be associated with low IL-2 activity or serum levels of specific anti-MLM antibodies. The ability of spleen cells to produce IL-2 *in vitro* after MLM infection was found to be decreased in C57BL/6, DBA/1, and DBA/2 strains only. As these strains show either phenotypic expression of the *Bcg* gene, no correlation between low IL-2 production and MLM-susceptibility was evident. Similarly, there was no influence of the *Bcg* gene on serum antibody titers.

3.5.3. Immunologic Consequences of the Bcg Gene

The influence of the host *Bcg* genotype on the subsequent development of immunity has not yet been firmly established. Clearly, in some instances, it has been shown that *Bcg*^{s} animals, which allowed significant multiplication of BCG in the liver and spleen, developed immune responses such as granulomas and protective immunity to heterologous pathogens that were absent in *Bcg*^{r} mice.[40,41] Conversely, it has also been reported that in spite of the inability of BCG organisms to establish an obvious infection in *Bcg*^{r} mice, these animals nevertheless developed evidence of protective immunity.[42,43] The dilemma may be resolved by comparing not the absolute presence or absence of immunity, but the type and quality of responses that develop after BCG infection in *Bcg*^{r} and *Bcg*^{s} animals.

3.5.4. H-2 Control of Antibody Production to M. tuberculosis Antigens

The role of the murine H-2 complex in controlling anti-*M. tuberculosis* antibody production has also been investigated.[44] In this study, the ability of H37Rv-immune C57BL/6, CBA, and H-2 recombinant BALB/c and B10 mice to produce antibodies against several distinct protein antigens of *M. tuberculosis* was determined. C57BL/6 and CBA/Ca were found to be high and low antibody responders, respectively. Further

analysis of H-2 recombinants revealed that the trait of high or low responsiveness was consistent with H-2^b and H-2^k haplotypes, respectively. However, background genes could still be observed to exert significant control over antibody production to at least two of the *M. tuberculosis* proteins. The results suggest that H-2 immune response genes do control antibody responses to certain proteins of *M. tuberculosis.* Although it has previously been shown that antibodies are not protective in tuberculosis infection, the observed H-2 control of antibody secretion may actually reflect differences in T-cell activation, since the protein epitopes of *M. tuberculosis* are T-dependent antigens (J. Ivanyi and K. Sharp, personal communication).

3.5.5. Immunogenetics of Granuloma Formation

The formation of granulomas in response to BCG, *M. tuberculosis,* or *M. lepraemurium* infection is believed to be a T-cell-dependent reaction.[44,45] In an investigation of the granulomatous response to BCG in T-cell-deficient nude mice, it was shown that granulomas were only formed if the nude host received H-2 I-region-compatible T lymphocytes.[44] The results of such studies demonstrate that the granulomatous response to mycobacteria is under H-2 genetic control. In another study,[46] various inbred strains of mice were injected with BCG-cell wall (CW) vaccine to determine whether the extent of the granulomatous reaction was genetically controlled. It was found that inbred strains could be classified into three categories according to lung weights 1 month after BCG vaccination: C57BL/6, SJL/J, and AKR/Hc were high responders, whereas C3H/Hc, C3H/HeB, DBA/1J, and P/J were low responders. The strains C57BL/10SnJ, DBA/2, and BALB/c were characterized as intermediate responders. The segregation analysis demonstrated that the trait of lung granuloma formation in the BCG-CW model was under polygenic control, with a minor involvement of the H-2 locus. In fact, the Igh locus has been shown to control the granulomatous reaction that occurs after IV injection of BCG in an oil-in-saline emulsion.[47] In *M. lepraemurium* infection, the extent of granuloma formation in the footpad is controlled by H-2 and non-H-2 genes.[45,48]

The data are suggestive of a polygenic control of host responses to mycobacteria. Several years ago, an hypothesis to explain the genetic control of tuberculosis was put forth by Lurie and Dannenberg,[2] who proposed that certain genes, for example, *Bcg,* control the innate resistance to infection. In chronic bacterial diseases such as tuberculosis, however, the immune response plays a crucial role in resistance; thus, H-2-linked genetic control of infection becomes superimposed on innate resistance.

4. GENETIC CONTROL OF TUBERCULOSIS IN HUMANS

4.1. Immunoregulation in Tuberculosis and Leprosy

In recent years, much attention has been centered on immunoregulatory defects in both leprosy and tuberculosis. The clinical manifestations of leprosy are defined according to a scale based on the histopathologic and immunologic status of the patient.[49] It has been clearly shown that persons suffering from tuberculoid leprosy exhibit immunologic responsiveness against the causative organism, *M. leprae,* whereas persons with lepromatous leprosy are anergic or unresponsive to the antigens of *M. leprae.*[50] Numerous reports have described the existence of both suppressor adherent cells[51,52] and suppressor T lymphocytes[53] in the peripheral blood of LL patients. Other immunologic defects associated with LL are defective IL-2[54] and IFN_{γ}.[55]

A clinical spectrum of human tuberculosis has also been described.[56] Patients were classified according to the extent of tubercular lesions, response to chemotherapy, skin-test reaction, amount of anti-PPD antibodies in serum, and leukocyte migration to PPD. Such comparisons distinguished the patients into four groups with two polar forms: reactive (RR) and unreactive (UU). The RR type was characterized by localized lesions, a prompt response to chemotherapy, skin-test positivity, and a low titer of anti-PPD antibodies. The UU patients exhibited diffuse lesions, a poor response to antitubercular drugs, high levels of antibody, and a negative skin test. However, the use of such a classification system for human tuberculosis is not as widely accepted as the Ridley-Jopling diagnostic scale in leprosy. Nevertheless, the extent of clinical tuberculosis has been shown to correlate with the immunologic responsiveness of the patient and the similarities to the leprosy spectrum are striking. The studies described in the following section illustrate the forms of immunologic anergy characterized in tuberculosis.

An analysis of T-helper/T-suppressor cell ratios in tuberculosis patients has been performed by employing monoclonal (OKT) antibodies specific for T-cell phenotypes.[57] It was shown that lymphocytes isolated from patients with newly diagnosed tuberculosis expressed an average $T4^+$ helper /$T8^+$ suppressor cell ratio of 2 after stimulation with PPD *in vitro.* By contrast, the ratio was decreased to 1.4 in patients with tuberculosis pleurisy and advanced pulmonary disease and was due to an increase in the percentage of $T8^+$ suppressor cells. However, the functional role of the $T8^+$ subset in suppression of PPD responses in advanced pulmonary tuberculosis patients was not examined.

It has been demonstrated that lymphocytes isolated from tuberculosis patients who were negative skin-test reactors as well as patients

with advanced disease had poor lymphoproliferative responses to PPD *in vitro*.[58–60] The weak response of lymphocytes from pleurisy patients could be reversed by the removal/depletion of adherent monocytes prior to culture.[59] Another study showed that the PPD response of T cells could be abrogated by the addition of adherent mononuclear cells (AMC) derived from tuberculosis patients.[61] A more recent report[62] showed that suppression of tuberculosis lymphocyte responses to PPD *in vitro* occurred when autologous AMC were added in a 1 AMC : 2 T-cell ratio. The suppressive activity in AMC was abrogated by specific depletion of monocytes with OKM1 antisera plus complement. The AMC cell population did not require the presence of T lymphocytes, and the suppressor mechanism appeared to be prostaglandin independent.

A defect in the ability to produce IFN has also been described in tuberculosis.[60] The study comprised 25 tuberculosis patients and 25 age-matched controls of identical ethnic origin. A group of nine tuberculosis patients were unable to produce IFN_{α} after stimulation of their peripheral blood lymphocytes with PPD *in vitro,* were unresponsive to PPD in the skin test, and also had weak lymphoproliferative activity to PPD *in vitro*. The remainder of the patients exhibited no signs of depressed immunity compared to control individuals. The inability of the nine patients to produce IFN to PPD was apparently an antigen-specific defect, since levels of IFN equivalent to normal controls were obtained if staphylococcal enterotoxin-A was employed as a stimulant. These results support the findings of an earlier study[58] that reported PPD unresponsiveness in 49 of 200 (25%) tuberculosis patients. In both studies, no obvious correlation was made between immunologic anergy and the extent of clinical disease in unresponsive patients. The report by Lenzini *et al.,*[56] however, observed a definite correlation between patients with severe tuberculosis, a negative skin test, and the type and extent of lesions.

4.2. HLA Restriction of Immune Responses

Although the investigators who carried out the studies discussed in the preceding section did not perform genetic analyses of the anergic tuberculosis patients, the evidence of disordered immune responses in tuberculosis lends support to the theory that the human leukocyte-antigen (HLA) complex might be associated with such defects.

The HLA system is composed of four closely linked loci on chromosome number 6: HLA-A, -B, -C, and -D/DR.[63] Each of the loci is polymorphic, and approximately 8–39 codominant alleles have been described for each locus. The products of the HLA-A, -B, and -C loci are analogous to murine H-2-K and -D antigens, in that both are expressed

on almost all cells of the body. Class I HLA-A, -B, and H-2-K,-D gene products are known to function as target recognition structures for cytotoxic T lymphocytes (CTL).[64] The target cell, when infected by a virus, and the CTL must share at least one class I antigen for lysis to occur. Class II gene products, or HLA-D/DR antigens, restrict the immune response at the level of antigen presentation to lymphocytes.[65] Genetic HLA-D/DR or H-2-I region restriction is evidenced by the abrogation of antigen presentation by macrophages to lymphocytes when there is histoincompatibility at the class II locus or when antibodies directed at class II antigens are added to the test system. Such experiments illustrate the central role of the HLA complex in controlling immunoregulatory events.

The lymphoproliferative response of normal human cloned T cells to PPD antigen has been shown to be restricted by class II HLA-D/DR and -MB determinants.[66] A total of 15 PPD-specific T-cell clones were derived from a single donor and examined for the ability to proliferate to PPD in the presence of HLA-D/DR or HLA MB-matched or mismatched antigen presenting cells (APC) obtained from a panel of donors. Only those APC that shared HLA-D/DR or -MB determinants with the T cells were found to be able to present the antigen. Furthermore, the addition of monoclonal antibodies directed at HLA-D/DR antigens inhibited lymphocyte proliferation. The inhibition occurred by the selective action of monoclonal antibodies on APC but not responding lymphocytes, implying that HLA class II restriction occurred at the level of the APC. The results suggest that human T-cell responses to PPD are class II restricted and that clonally distributed PPD reactive T cells may be restricted by different class II molecules. These experiments extend the findings of Hirschberg *et al.*,[67] where class II antigens were shown to restrict PPD antigen presentation to peripheral blood lymphocytes.

4.3. Studies on HLA and Tuberculosis

The search for associations between HLA markers and susceptibility to tuberculosis infection is based on the following rationale: since tubercle bacteria are known to induce a wide range of immune responses in the host, certain HLA alleles may predispose, while others protect against tuberculosis. It is not presumed that HLA genes control innate susceptibility to tuberculosis, but rather modulate the extent or severity of clinical manifestations.

The existence of genetic predisposition to tuberculosis has been suggested by numerous twin studies.[68,69] Twin studies are designed to establish whether a trait is genetically determined by the presence of a greater concordance rate in monozygotic (MZ) as opposed to dizygotic

(DZ) twins. In the best analyzed study,[68] 205 twin pairs were deemed suitable for examination after a survey of 20,000 tuberculosis patients in sanitoriums. It was found that the tuberculosis rate was two times greater among MZ pairs than among DZ co-twins. Similarly, other studies have reported that the tuberculosis concordance rate was consistently greater in MZ pairs.[69] The twin data thus provide substantial evidence for a role of genetic factors in host susceptibility to tuberculosis.

Singh *et al.*[70] examined HLA-haplotype segregation in 25 multiple tuberculosis-case families in North India. Family studies are intended to establish whether the transmission of HLA phenotypes from parents to children show a preferential segregation among affected siblings as compared with healthy siblings. According to this analysis, the inheritance pattern of HLA-DR2 was present more frequently than expected among affected siblings. Specifically, the transmission of DR2 from either diseased or healthy parents to diseased offspring occurred in 21 of 27 and in 15 of 17 cases, respectively, in contrast to the lower transmission of this phenotype to healthy offspring. In addition, the percentage frequency of HLA-DRw6 was decreased among affected siblings. These data argue in favor of a susceptibility gene to tuberculosis being HLA-DR2 associated.

The data from the North India study were also analyzed according to the lod-score method.[71] Lod scores are used to determine whether inheritance data are compatible with either dominant or recessive modes of inheritance. It was found that the data favored a dominant mode of inheritance. Singh *et al.* pointed out that such a result was surprising, in view of the apparent recessive mode of inheritance in tuberculoid leprosy.[72] It should also be mentioned that the HLA-DR2 allele has been shown to have a significant association with the tuberculoid form of leprosy, whereas HLA-DRw6 is significantly reduced.[72] The data therefore strongly implicate HLA-DR2 as a susceptibility gene in both tuberculosis and leprosy.

An investigation conducted on 124 North Indian pulmonary tuberculosis patients reported no associations between HLA-A, -B, and -C antigens and tuberculosis.[73] However, a slight increase in HLA-DR2 and a clear decrease in HLA-DRw6 was noted in patients compared with controls. The association between HLA DR2 and tuberculosis is therefore stronger within families than it is in the general North Indian population. This was taken as evidence that genetic factors modulating tuberculosis infection probably include non-HLA genes.

Several studies have investigated the association between HLA markers and tuberculosis in different populations. Hwang *et al.*[74] analyzed HLA phenotypes in a black American urban population. The data were computed to estimate the relative risk of disease. The results indicated a significant increase of HLA-DR5 in patients compared with con-

trols, resulting in a risk factor of 3.84. A decrease was found in HLA-DR6y in tuberculosis patients compared with controls. Hwang *et al.* believe that the HLA-DR6y phenotype is probably identical to HLA-DRw6 and thus supports the findings of Singh and co-workers. No significant differences were noted in the distribution of HLA-A antigens but HLA-B5 was significantly decreased in patients.

In another study of a black urban population, a 20% increase in the frequency of HLA-Bw15 was found in tuberculosis patients compared with controls.[75] Interestingly, the increase in antigen frequency also was correlated with the extent of the disease. Although two of the investigations have compared HLA associations in tuberculosis among black Americans, it is not known why HLA-Bw15 was not found in both studies. It was suggested that genetic inhomogeneity and geographic variation may have contributed to the discrepancy.[74]

In a Mexican-American population, 100 patients with active disease and 100 healthy controls were HLA-A, -B, and -C phenotyped.[76] The results confirmed previous observations that the HLA-A2 and -A9 phenotypes are increased among Mexican-Americans. Although the study did detect a greater frequency of HLA-B15, -B7, and -B17 among tuberculosis patients, the differences were not statistically significant compared with controls. However, the frequency of HLA-B15 distribution in 19% of patients versus 4% of controls approximated the 20% HLA-B15 increase found among blacks.[75]

In a Newfoundland community,[77] 589 individuals were typed for HLA-A and -B alleles. Among the 46 persons who had a history of tuberculosis, 26 (56%) were HLA-B8 positive. The difference was significant upon comparison with controls, where the B8 antigen was found in 110 persons, or 20% of the community. The interesting observation was made by Selby *et al.* that HLA-B8 is also associated with increased susceptibility to myasthenia gravis, Addison's disease, and a number of other autoimmune disorders.[77] The possible link of HLA-B8 with both tuberculosis and autoimmune diseases may not be a chance association as evidence exists for autoimmune reactions in both tuberculosis and leprosy.[51,78]

5. CONCLUSIONS

The genetic control of host resistance to mycobacterial infections is complex and, as intuitively expected, polygenic. Several firm conclusions can be made by the analysis of the presently available data:

1. At least in animal models, innate resistance/susceptibility to *M. tuberculosis,* BCG, and *M. lepraemurium* is under genetic control.

2. H-2 restriction of macrophage–lymphocyte interaction in tuberculosis has been demonstrated. Furthermore, certain H-2 haplotypes have been shown to influence the quality of immune responses which develop to *M. tuberculosis* as well as BCG and *M. lepraemurium.*
3. HLA restriction of immune responses to *M. tuberculosis* has been confirmed. Several immunologic disorders have been described in tuberculosis, but genetic components have not been investigated.
4. Currently, the HLA-DR2 allele has been associated with susceptibility to tuberculosis. HLA-DRw6 may protect against the disease. A dominant mode of inheritance has been suggested in family studies.
5. Different ethnic populations express different HLA alleles associated with tuberculosis.

What might be a practical approach to the future research on the genetics of resistance in tuberculosis and in other mycobacterial diseases? Clearly, it must begin with the appreciation that the apparent complexity of the genetic regulation is mainly attributable to the numerous steps in the host response to these agents. One can at least separate the stages of resistance to infection, resistance to mycobacterial proliferation, and the clearance of mycobacteria from the infected foci. Thus, one should use, at least in experimental models, well-defined criteria of resistance in order to segregate the polygenic response into a sequence of events under separate, and possibly monogeneic control. Furthermore, one must take into account that such factors as virulence of the microorganisms and the route of inoculation significantly influence the outcome of infection; therefore, the conditions of infection must be standardized in the search for the host's regulatory elements.

The other point we would like to make is that there is certainly an influence of events regulated by a particular gene on the course of host response regulated by the next gene in the sequence. For example, the failure to establish a clear function of MHC-linked immune-response genes in an infection in which immunity plays such a dominant protective role may be the result of the effect of natural resistance genes on the course of the immune response has largely been ignored. In the murine model of IV BCG infection with the relatively low dose of BCG inoculum (10^4 CFU), the need for a specific immune response to control the bacterial growth in the organs of innately resistant *Bcg*^{r} hosts is practically nonexistent. Therefore, whatever MHC (H-2) haplotype is carried by various *Bcg*^{r} hosts, they all will appear as resistant to infection. This, clearly, is not due to the presence of effective acquired immunity,

but rather is an expression of efficient macrophage function in those animals. The influence of the H-2 locus on the development of specific immune responses to BCG can thus only be studied, under the same experimental conditions, in the mouse strains that are innately susceptible and that permit the progression of the infection to the point that an immunogenic load is achieved.

Finally, it is important to define what particular parameters of the specific immune response one wishes to study and to correlate this parameter with the pertinent *in vivo* phenomenon, i.e., the clearance of infection or the resistance to reinfection. Failure to do that may result in the search for the genetic variation in epiphenomena that may be associated with the particular course of infection but are only casually related to the actual host defense mechanisms or to the outcome of infection.

Such painstaking processes will probably result in the discovery of several genes, their chromosomal location, and the more precise definition of their phenotypic expression. These efforts may possibly lead to the isolation of those genes as biochemical entities.

The search for human genes of resistance to mycobacterial infection obviously cannot follow the same strategy. The failure to find a clear association between HLA haplotypes and the phenotype of susceptibility to tuberculosis again rests with the HLA-directed processes being superimposed on those that are coded for by yet unknown genes, i.e., *Bcg* gene homologues. One may hope for the availability of mouse genomic probes that could be used to search for homologous sequences in the susceptible individuals in endemic areas.

Eventually, one may attempt the use of random restriction fragment length polymorphism probes to distinguish chromosomal segments that co-segregate with the trait of susceptibility or resistance in families. Such an approach should result in much better correlations with pertinent chromosomal markers than those obtained until now using the HLA probes.

REFERENCES

1. WHO, 1983, Plan of action for research in the immunology of tuberculosis: Memorandum from a WHO meeting, 1983, *Bull. WHO* **61**:779–785.
2. Lurie, M. B., and Dannenberg, A. M., Jr., 1965, Macrophage function in infectious disease with inbred rabbits, *Bacteriol. Rev.* **29**:466–476.
3. Gray, D. F., 1960, Variations in natural resistance to tuberculosis, *J. Hyg.* **58**:215–227.
4. Mackaness, G. B., 1968, The immunology of antituberculous immunity, *Am. Rev. Respir. Dis.* **97**:337–344.
5. Lurie, M. B., Zappasodi, P., and Tickner, C., 1955, On the nature of genetic resistance to tuberculosis in the light of the host–parasitic relationships in natively resistant and susceptible rabbits, *Am. Rev. Tuberc. Pulm. Dis.* **72**:297–323.

6. Lurie, M. B., Zappasodi, P., Cardona-Lynch, E., and Dannenberg, A. M., Jr., 1952, The response to the intracutaneous inoculation of BCG as an index of native resistance to tuberculosis, *J. Immunol.* **68:**369–387.
7. Lurie, M. B., Zappasodi, P., Dannenberg, A. M., Jr., and Weiss, G. H., 1952, On the mechanism of genetic resistance to tuberculosis and its mode of inheritance, *Am. J. Hum. Genet.* **4:**302–314.
8. Donovick, R., McKee, C. M., Jambar, W. P., and Rake, G., 1949, Use of the mouse in standardized test for anti-tuberculous activity of compounds of natural or synthetic origin: Choice of mouse strains, *Am. Rev. Tuberc.* **60:**109–120.
9. Sever, J. L., and Youmans, G. P., 1957, The enumeration of non-pathogenic viable tubercule bacilli from the organs of mice, *Am. Rev. Tuberc.* **75:**280–294.
10. Youmans, G. P., and Youmans, A. S., 1972, Response of vaccinated and non-vaccinated syngeneic C57BL/6 mice to infection with *M. tuberculosis, Infect. Immun.* **6:**748–754.
11. Lynch, C. J., Pierce-Chase, C. H., and Dubos, R., 1965, A genetic study of susceptibility to experimental tuberculosis in mice infected with mammalian tubercle bacilli, *J. Exp. Med.* **121:**1051–1070.
12. Gheorghiu, M., Mouton, D., Lecoeur, H., Lagranderie, M., Meuel, J. C., and Biozzi, G., 1985, Resistance of high and low antibody responder lines of mice to the growth of avirulent (BCG) and virulent (H37Rv) strains of mycobacteria, *Clin. Exp. Immunol.* **59:**177–184.
13. Biozzi, G., Mouton, D., Stiffel, C., and Bouthillier, Y., 1984, Major role of macrophage in quantitative genetic regulation of immune responsiveness and anti-infections immunity, *Adv. Immunol.* **36:**189–234.
14. Wiener, E., and Dandieri, A., 1974, Differences in antigen handling by peritoneal macrophages from the Biozzi high and low responder mice, *Eur. J. Immunol.* **4:**457–463.
15. Plant, J., and Glynn, A. A. 1982, Genetic control of resistance to *Salmonella typhimurium* infection in high and low antibody responder mice, *Clin. Exp. Immunol.* **50:**283–290.
16. Dannenberg, A. M., Jr., 1983, Pathogenesis of tuberculosis: Native and acquired resistance in animals and humans, in: *Immunology of Tuberculosis and Leprosy: A Symposium.* U.S.–Japan Cooperative Medical Sciences Program, pp. 344–354.
17. Skamene, E., Gros, P., Forget, A., Kongshavn, P. A. L., St. Charles, C., and Taylor, B. A., 1982, Genetic regulation of resistance to intracellular pathogens, *Nature (Lond.)* **297:**506–510.
18. Gros, P., Skamene, E., and Forget, A., 1981, Genetic control of natural resistance to *Mycobacterium bovis* (BCG) in mice, *J. Immunol.* **127:**2417–2421.
19. Potter, M., O'Brien, A. D., Skamene, E., Gros, P., Forget, A., Kongshavn, P. A. L., and Wax, J., 1983, A Balb/c congenic strain of mice that carries a genetic locus (**Ity**^{r}) controlling resistance to intracellular parasites, *Infect. Immun.* **40:**1234–1235.
20. Brown, I. N., Glynn, A. A., and Plant, J., 1982, Inbred mouse strain resistance to *Mycobacterium lepraemurium* follows the *Ity/Lsh* pattern, *Immunology,* **47:**149–156.
21. Gros, P., Skamene, E., and Forget, A., 1983, Cellular mechanisms of genetically controlled host resistance to *Mycobacterium bovis* (BCG), *J. Immunol.* **131:**1966–1972.
22. Stach, J-L., Gros, P., Forget, A., and Skamene, E., 1984, Phenotypic expression of genetically-controlled natural resistance to *Mycobacterium bovis, J. Immunol.* **132:**888–892.
23. Denis, M., Buschman, E., Forget, A., Pelletier, M., and Skamene, E., 1986, Pleiotropic effects of the *Bcg* gene: Regulation of Ia dependent macrophage function, *6th International Congress of Immunology,* abst. No. 5.12.11, 1986.
24. Denis, M., Forget, A., Pelletier, M., Turcotte, R., and Skamene, E., 1986, Control by

the *Bcg* gene of early resistance in mice to infections with BCG substrains and atypical mycobacteria. *Clin. Exp. Immunol.* **63:**517–525.
25. Nickonenko, B. V., Apt, A. S., Moroz, A. M., and Averbakh, M. M., 1985, Genetic analysis of susceptibility of mice to H37Rv tuberculosis infection: Sensitivity versus relative resistance, in: *Genetic Control of Host Resistance and Malignancy,* Vol. 3 (E. Skamene, ed.), pp. 291–298, Liss, New York.
26. Blanden, R. V., Lefford, M. J., and Mackaness, G. B., 1969, The host response to Calmette–Guérin bacterius infection in mice, *J. Exp. Med.* **129:**1079–1101.
27. Snell, G. D., 1981, Studies in histocompatibility, *Science* **213:**172–178.
28. Orme, I. M., and Collins, F. M., 1983, Protection against *Mycobacterium tuberculosis* infection by adoptive immunotherapy: Requirement for T-cell deficient recipients, *J. Exp. Med.* **158:**74–83.
29. Nakamura, R. M., and Tokunaga, T., 1978, Strain difference of delayed-type hypersensitivity to BCG and its genetic control in mice, *Infect. Immun.* **22:**657–664.
30. Rook, G. A. W., Champion, B. R., Steele, J., Varey, A. M., and Stanford, J. L., 1985, I-A restricted activation by T cell lines of anti-tuberculosis activity in murine macrophages, *Clin. Exp. Immunol.* **59:**414–420.
31. Patterson, R. J., and Youmans, G. P., 1970, Demonstration in tissue culture of lymphocyte-mediated immunity to tuberculosis, *Infect. Immun.* **1:**600–603.
32. Huygen, K., Zhen-Xi, Z., and DeClerc, Q. E., 1983, Failure of athymic nude mice sensitized with bacillus Calmette-Guérin to produce interferon in response to purified protein derivative, *Proc. Soc. Exp. Biol. Med.* **172:**260–264.
33. Nathan, C. F., Murray, H. W., Wiebe, M. E., and Tubin, B. Y., 1983, Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity, *J. Exp. Med.* **158:**670–689.
34. Reggiardo, Z., and Middlebrook, G., 1974, Failure of passive serum transfer of immunity against aerogenic tuberculosis in guinea pigs, *Proc. Soc. Exp. Biol. Med.* **145:**173–175.
35. Forget, A., Benoit, J. C., Turcotte, R., and Gusew-Chartrand, N., 1976, Enhancement activity of anti-mycobacterial sera in experimental *Mycobacterium bovis* (BCG) infection in mice, *Infect. Immun.* **13:**1301–1306.
36. Rosenthal, A. S., and Shevach, E. M., 1974, Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes, *J. Exp. Med.* **138:**1194–1212.
37. Neta, R., and Salvin, S. B., 1980, *In vivo* release of lymphokines in different strains of mice, *Cell. Immunol.* **51:**173–178.
38. Huygen, K., and Palfliet, K., 1984, Strain variation in IFN-γ production of BCG-sensitized mice challenged with PPD, *Cell. Immunol.* **85:**75–81.
39. Hoffenbach, A., Lagrange, P. H., and Bach, M. A., 1985, Strain variation of lymphokine production and specific antibody secretion in mice infected with *Mycobacterium lepraemurium, Cell. Immunol.* **91:**1–11.
40. Pelletier, M., Forget, A., Bourassa, D., Gros, P., and Skamene, E., 1982, Immunopathology of BCG infection in genetically resistant and susceptible mouse trains, *J. Immunol.* **129:**2179–2185.
41. Buschman, E., Denis, M., Forget, A., and Skamene, E., 1986, Immunological consequences of innate resistance or susceptibility in congenic mouse strains, in the *Sixth International Congress of Immunology,* abst. 5.12.10.
42. Bourassa, D., Forget, A., Pelletier, M., Skamene, E., and Turcotte, R., 1985, Cellular immune response to *Mycobacterium bovis* (BCG) in genetically susceptible and resistant congenic mouse strains, *Clin. Exp. Immunol.* **62:**31–38.
43. Orme, I. M., and Collins, F. M., 1984, Demonstration of acquired resistance in *Bcg*^{r}

inbred mouse strains infected with a low dose of BCG Montréal, *Clin. Exp. Immunol.* **56:**81–88.
44. Veda, K., Yamazaki, S., and Yanamoto, S., 1983, H-2 I region restriction phenomenon in T cell dependent granuloma formation to *Mycobacterium bovis* BCG, *J. Reticuloendothel. Soc.* **34:**475–484.
45. Closs, O., Lovik, M., Wigzell, H., and Taylor, B. A., 1983, H-2 linked gene(s) influence the granulomatous reaction to viable *Mycobacterium lepraemurium* in the mouse, *Scand. J. Immunol.* **18:**59–63.
46. Yanamoto, K., and Kakinuma, M., 1978, Genetic control of granuloma response to oil-associated BCG cell wall vaccine in mice, *Microbiol. Immunol.* **22:**335–348.
47. Schrier, D. J., Sternick, J. L., Allen, E. M., and Moore, V. L., 1982, Immunogenetics of BCG-induced anergy in mice. Control by genes linked to the IgH complex, *J. Immunol.* **128:**1466–1470.
48. Adu, H. O., Curtis, J., and Turk, J. L., 1983, Role of the major histocompatibility complex in resistance and granuloma formation in response to *Mycobacterium lepraemurium* infection, *Infect. Immun.* **40:**720–725.
49. Ridley, D. S., and Jopling, W. H., 1966, Classification of leprosy according to immunity: A five-group system, *Int. J. Leprosy* **34:**255–273.
50. Myrvang, B., Godal, T., Ridley, D. S., Froland, S. S., and Song, Y. K., 1973, Immune responsiveness to *Mycobacterium leprae* and other mycobacterial antigens throughout the clinical and histopathological spectrum of leprosy, *Clin. Exp. Immunol.* **14:**541–553.
51. Bullock, W. E., and Fasal, P., 1971, Studies of immune mechanisms in leprosy. III. The role of cellular and humoral factors in impairment of the *in vitro* immune response, *J. Immunol.* **106:**888–899.
52. Nath, I., van Rood, J. J., Mehra, N. K., and Vaidya, M. C., 1980, Natural suppressor cells in human leprosy: The role of HLA-D-identical peripheral lymphocytes and macrophages in the *in vitro* modulation of lymphoproliferative responses, *Clin. Exp. Immunol.* **42:**203–210.
53. Mehra, V., Mason, L. H., Rothman, W., Reinherz, E., Schlossman, S. F., and Bloom, B. R., 1980, Delineation of a human T cell subset responsible for lepromin-induced suppression in leprosy patients, *J. Immunol.* **125:**1183–1188.
54. Haregwoin, A., Godal, T., Mustafa, A. S., Belehu, A., and Yemaneberhan, T., 1983, T-cell conditioned media reverse T-cell unresponsiveness in lepromatous leprosy, *Nature (Lond.)* **303:**342–344.
55. Nogueria, N., Kaplan, G., Levy, E., Sarno, E. N., Kusher, P., Piperno, A. G., Vieza, L., Gould, V. C., Levis, W., Steinman, R., Yip, Y., and Cohn, Z. A., 1983, Defective γ-interferon production in leprosy: Reversal with antigen and interleukin-2, *J. Exp. Med.* **158:**2165–2170.
56. Lenzini, L., Rottoli, P., and Rottoli, L., 1977, The spectrum of human tuberculosis, *Clin. Exp. Immunol.* **27:**230–237.
57. Shiratsuchi, H., and Tsuyuguchi, I., 1984, Analysis of T cell subsets by monoclonal antibodies in patients with tuberculosis after *in vivo* stimulation with purified protein derivative of tuberculin, *Clin. Exp. Immunol.* **57:**271–278.
58. Nash, D. R., and Douglass, J. E., 1980, Anergy in active pulmonary tuberculosis: A comparison between positive and negative reactors and an evaluation of 5 TU and 250 TU skin test doses, *Chest* **77:**32–37.
59. Fujiwara, H., Okuda, Y., Fukukawa, T., and Tsuyuguchi, I., 1982, In vitro tuberculin reactivity of lymphocytes from patients with tuberculous pleurisy, *Infect. Immun.* **35:**402–409.
60. Onwubalili, J. K., Scott, G. M., and Robinson, J. A., 1985, Deficient immune interferon production in tuberculosis, *Clin. Exp. Immunol.* **59:**405–413.

61. Kleinhenz, M. E., Ellner, J. J., and Daniel, T. M., 1981, Immunosuppressive cellular interactions in pulmonary tuberculosis, *Clin. Res.* **29:**448A.
62. Kleinhenz, M. E., and Ellner, J. J., 1985, Immunoregulatory adherent cells in human tuberculosis: Radiation-sensitive antigen-specific suppression by monocytes, *J. Infect. Dis.* **152:**171–176.
63. Dausset, J., 1981, The major histocompatibility complex in man: Past, present and future concepts, *Science* **213:**1469–1474.
64. Zinkernagel, R. M., and Doherty, P. C., 1979, MHC-restricted cytotoxic T-cells: Studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction specificity, function and responsiveness, *Adv. Immunol.* **27:**52–177.
65. Erb, P., and Feldmann, M., 1975, The role of macrophages in the generation of T-helper cells. I. The requirement for macrophages in helper cell induction and characteristics of the macrophage–T-cell interaction, *Cell. Immunol.* **19:**356–367.
66. Pawelec, G., Schneider, E. M., Muller, C., and Wernet, P., 1985, HLA-DR, MB- and novel DC-related determinants restrict purified protein derivative of tuberculin (PPD)-stimulated human T cell proliferation, *Eur. J. Immunol.* **15:**12–17.
67. Hirschberg, H., Bergh, O. J., and Thorsby, E., 1979, Clonal distribution of HLA restricted antigen-reactive T cells in man, *J. Exp. Med.* **150:**1271–1276.
68. Comstock, G. W., 1978, Tuberculosis in twins: A reanalysis of the Prophit survey, *Am. Rev. Respir. Dis.* **117:**621–624.
69. Fine, P. E. M., 1981, Immunogenetics of susceptibility to leprosy, tuberculosis and leishmaniasis. An epidemiological perspective, *Int. J. Leprosy* **49:**437–454.
70. Singh, S. P. N., Mehra, N. K., Dingley, H. B., Pande, J. N., and Vaidya, M. C., 1983, Human leukocyte antigen (HLA)-linked control of susceptibility to pulmonary tuberculosis and association with HLA-DR types, *J. Infect. Dis.* **148:**676–681.
71. Singh, S. P. N., Mehra, N. K., Dingley, H. B., Pande, J. N., and Vaidya, M. C., 1984, HLA haplotype segregation study in multiple case families of pulmonary tuberculosis, *Tissue Antigens* **23:**84–86.
72. DeVries, R. R. P., Mehra, N. K., Vaidya, M. C., Gupta, M. D., Meera Khan, P., and van Rood, J. J., 1980, HLA-linked control of susceptibility to tuberculoid leprosy, *Tissue Antigens* **16:**294–304.
73. Singh, S. P. N., Mehra, N. K., Dingbey, H. B., Pande, J. N., and Vaidya, M. C., 1983, HLA-A, -B, -C, and -DR antigen profile in pulmonary tuberculosis in North India, *Tissue Antigens* **21:**380–384.
74. Hwang, C. H., Khan, S., Ende, N., Mangura, B. T., Reichman, L. B., and Chou, J. 1985, The HLA-A, -B, and -DR phenotypes and tuberculosis, *Am. Rev. Respir. Dis.* **132:**382–385.
75. Al-Arif, L. I., Goldstein, R. A., Affronti, L. F., and Janicki, B. W., 1979, HLA-Bw15 and tuberculosis in a North American black population, *Am. Rev. Respir. Dis.* **120:**1275–1278.
76. Cox, R. A., Arnold, D. R., Cook, D., and Lundberg, D. I., 1982, HLA phenotypes in Mexican Americans with tuberculosis, *Am. Rev. Respir. Dis.* **126:**653–655.
77. Selby, R., Barnard, J. M., Buchler, S. K., Crumley, J., Larsen, B., and Marshall, W. H., 1978, Tuberculosis associated with HLA-B8 Bfs in a Newfoundland community study, *Tissue Antigens* **11:**403–408.
78. Van Eden, W., Holoshitz, J., Nero, Z., Frenkel, A., Klajman, A., and Cohen, I. R., 1985, Arthritis induced by a T-lymphocyte clone that responds to *Mycobacterium tuberculosis* and to cartilage proteoglycans, *Proc. Natl. Acad. Sci. USA* **82:**5117–5120.

4

Immune Response to Mycobacteria

Characterization of Immunocompetent Cells in Tuberculous Lesions of Humans

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1. THE FIRST CONTAGION: THE ALVEOLAR MACROPHAGES REPRESENT THE FIRST-LINE DEFENSE IN DEEP LUNG

Tuberculosis is an extremely severe infectious disease that commits the defense mechanisms of the host profoundly and for a long time. Antibiotic therapy is undoubtedly the most important treatment in such disease for blocking the growth of tubercle bacilli, but complete recovery as well as immunity against reinfections depend on the perfect functioning of the immunocompetent system.

When *Mycobacterium tuberculosis* penetrates the respiratory apparatus (or another organ of the body), immunologic changes occur in order to defend the host both from the development of the intercurrent infection and from possible future infections by the same agent. Nevertheless, it must be pointed out that the first penetration of *M. tuberculosis* in the

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airways does not necessarily cause the disease. If inhaled tubercle bacteria stop at the trachea or bronchi level without reaching the deep lung, they can be easily removed from the tracheobronchial tree by the mucociliary clearance. If *M. tuberculosis* reaches the alveolar spaces, the possibility of developing the disease is related to two basic aspects: (1) the characters of the mycobacterial agents (bacterial charge and virulence); and (2) the genetic and acquired factors of host resistance or susceptibility to the infection.

The role of genetic factors in the immune response to *M. tuberculosis* has been extensively investigated, especially in animals.[1–3] The factors that may play an important role in controlling resistance to tuberculosis are multiple, including race, and major histocompatibility complex, but the most important is surely the efficiency of alveolar phagocytic cells in blocking the growth of tubercle bacteria. In fact, even when the characteristics of the infecting agent (virulence, charge) are the same, there is considerable individual variation in the response of macrophages to mycobacteria. In some cases, the mycobacteria are destroyed by macrophages; in others, the macrophages can ingest but not kill the mycobacterium, which can survive without growing within the phagocytic cells; in still other cases, the macrophages are unable either to phagocyte or destroy *M. tuberculosis,* which can grow and cause the disease. Undoubtedly, what determines whether the disease progresses or regresses is the power of the macrophage to inhibit the growth of tubercle bacilli within its cytoplasm.[1]

The killing of tubercle bacilli by macrophages is strictly dependent, at first, on the ingestion of mycobacteria and, afterward, on the fusion of primary lysosomes with the mycobacteria containing phagocytic vacuoles. It follows that one of the most important genetic factors connected to tuberculosis resistance (or susceptibility) can be identified in the congenital power of macrophages. On the other hand, the congenital ability of macrophages to kill tubercle bacilli may also be influenced by the virulence characteristics of the infecting *M. tuberculosis* strain. The virulence might be identified in secretory substances or in cell-wall constituents that can interfere with various phases of the phagocytic process, for example, by preventing the fusion of lysosomes with phagocytic vacuoles or by rendering the mycobacterial cell-wall resistant to lysosomial hydrolytic enzymes. For instance, some mycobacterial strains are resistant to the action of oxygen radicals, because they are protected by a thick lipidic wall and are able to produce a catalase that destroys hydrogen peroxide.

Soon after the first contagion, the cellular defenses are exiguous, scarcely effective, and limited, at least in the early phase, to the resident alveolar macrophages (and some recruited circulating macrophages).

These cells must be activated in order to enhance their ability to kill the tubercle bacilli. At any rate, the first contagion is a crucial moment in the tuberculous infection because the message to other immunocompetent cells (especially lymphocytes) comes from this phase, and the subsequent course of the infection is strictly dependent on the ensuing interactions of the immunocompetent cells.

In this regard, we should underline that the macrophage is a prototypical accessory or antigen-presenting cell. In other terms, this cell exerts a crucial function in antigen processing and presentation and in promoting specific immune responses through T cells.

2. IMMUNITY IN TUBERCULOSIS IS CELL MEDIATED

Interactions among the various populations of immunocompetent cells, particularly between macrophages and T lymphocytes, characterize the immunologic response to tuberculosis as acquired cellular immunity or cell-mediated immunity, classified also as a type IV immunologic reaction by Gell and Coombs.

The first observations regarding the immunology of tuberculosis were reported by Koch[4] at the end of the nineteenth century. Koch observed a higher resistance to tuberculous infection in guinea pigs reinfected with the homologous *M. tuberculosis* than in guinea pigs infected for the first time—the Koch phenomenon. A localized slow granulomatous response appeared at the site of the primary infection, while an early indurative response (maximal at 72 hr) appeared at the site of secondary infection, followed by rapid healing. Moreover, Koch observed that a similar response could be induced in the skin of tuberculosis patients by the intradermal injection of heat-killed tubercle bacilli or tuberculin extracted from them. In subsequent years, many other workers[1,5,6] confirmed the essentially cellular nature of the antituberculous immune response. There is no doubt that immunity against tuberculosis is not antibody dependent but is carried out by alveolar macrophages as effector cells and by lymphocytes as mediator cells. For example, it has been shown[7] that antituberculosis immunity can be transferred to normal recipients by infusions of lymphoid cells harvested from tuberculin-hypersensitive donors, but not by hyperimmune serum.

As originally described by Koch, the injection of tubercle bacilli or tuberculin in the skin of tuberculosis patients is followed by an intradermal granulomatous reaction at the site of injection, i.e., delayed-type hypersensitivity (DTH). In naturally infected individuals, immunity and DTH usually develop concomitantly, suggesting that they may be the

expression of immunologic events in which the same immunocompetent cells play a critical role.[8]

Recent studies have shown that T lymphocytes have an active role in the generation of a positive tuberculin response to the intradermal injection of purified protein derivative (PPD). Scheynius *et al.*[9] demonstrated that at the site of a PPD injection, the lymphocytes expressing the surface antigen recognized by the monoclonal antibody (MAb) Leu 1 increased with time. Moreover, at 48 and 96 hr postinjection, most of these T lymphocytes expressed the helper/inducer phenotype, whereas few expressed the suppressor/cytotoxic phenotype. These data are of interest because these T cells are the best lymphokine producers. These studies have also demonstrated the presence of a high proportion of Ia-positive mononuclear cells (HLA-DR expressing cells). In the work by Konttinen *et al.*,[10] the mononuclear cells infiltrating a positive PPD response was analyzed *in situ* more in detail by immunohistochemical methods; the results confirmed that macrophages and T lymphocytes are numerically the most important cellular populations involved in the DTH skin reaction. However, while such cells appeared to be the dominant effector cells at 72 hr, the study also showed that in the early phases of the reaction (6, 24, and 48 hr), there is a mobilization of epidermal Langerhans cells (known to be efficient antigen-presenting cells) to the perivascular spaces in the dermis, suggesting that these cells may play a role in the initial phases of DTH. Relatively little is known about the sites and kinetics of dendritic cell production and of their migration patterns in the organism. It has been proposed that circulating dendritic cells represent the principal cell type required for the start of several immune responses.[11] It seems possible that the entry of such powerful accessory cells into a lesion may intensify the immune response at the local level. In fact, in the autologous mixed lymphocyte reaction, the dendritic cells were demonstrated to stimulate autologous T cells, and their activity was up to 10 times higher than that of macrophages.[12]

By comparing the immunologic responses in the human lung infected by tubercle bacilli and in the skin after PPD injection, we can see some important differences, however. In DTH, it is the epidermal Langerhans cell that appears in the very early phases and, probably, represents the principal cell type responsible for starting and intensifying the reaction. In pulmonary tuberculosis, the cell that appears to start the immunologic response is the alveolar macrophage, which represents the first-line defense against air polluters in the deep lung. The fundamental role of alveolar macrophages is to phagocyte and inhibit or kill the tubercle bacilli (see Section 1). Macrophages can efficiently kill *M. tuberculosis*, but to do so they must be activated.

3. T LYMPHOCYTES ACTIVATE ALVEOLAR MACROPHAGES FOR KILLING TUBERCLE BACILLI

There is no doubt that in tuberculosis infections the primary function of alveolar macrophages is to ingest and kill the bacilli. However, soon after contagion, macrophages must also sensitize the T lymphocytes by the presentation of processed antigen(s) or its products. In order for T lymphocytes to receive an efficient antigenic signal, they must also receive two additional signals from macrophages: (1) the antigen must be presented complexed with an HLA-class II molecule, and (2) the macrophage-secreted soluble factor interleukin-1 (IL-1) must interact with specific receptors present on the plasma membrane of T lymphocytes.

The contact with processed mycobacterium antigens and the action of IL-1 activate the T cell to release a number of lymphokines. Among these, the most important are interleukin-2 (IL-2), which induces the proliferation of T and B lymphocytes and activates natural killer (NK) cells, γ-interferon (IFN_{γ}) which acts on several cell populations and also activates the accessory cells; macrophage activation factor, which activates the macrophages; and migration inhibitory factor, which inhibits the migration of macrophages and causes them to accumulate at the site of infection.

When activated by lymphokines, macrophages change their morphology (e.g., they become larger) and exhibit increased enzymatic and metabolic activities. In this phase, they have an increased ability to ingest the tubercle bacilli, to inhibit their growth and kill them (Fig. 1).

T cells and macrophages accumulate at the site of infection, thus forming the tuberculous granuloma. In granuloma formation, not only T lymphocytes and their soluble factors but also macrophages play a critical role. In particular, some investigations[3] have shown the possible different functions exerted by macrophages in the same granuloma: some may produce soluble factors active on T cells, some act as antigen-presenting cells, and some display high enzymatic activity (e.g., increased production of proteases).

The tuberculous granuloma is the typical immunologic granuloma; it is quite similar to those of sarcoidosis, of berylliosis, and of mycotic infections. This granuloma is characterized by a high cellular turnover, since activated macrophages enter it continuously; the new arrived macrophages phagocytize and replace the destroyed ones and also ingest the tubercle bacilli released by them.

Therefore, the critical role of T cells and macrophages in the tuberculosis immune response, where the latter represent the effectors or the

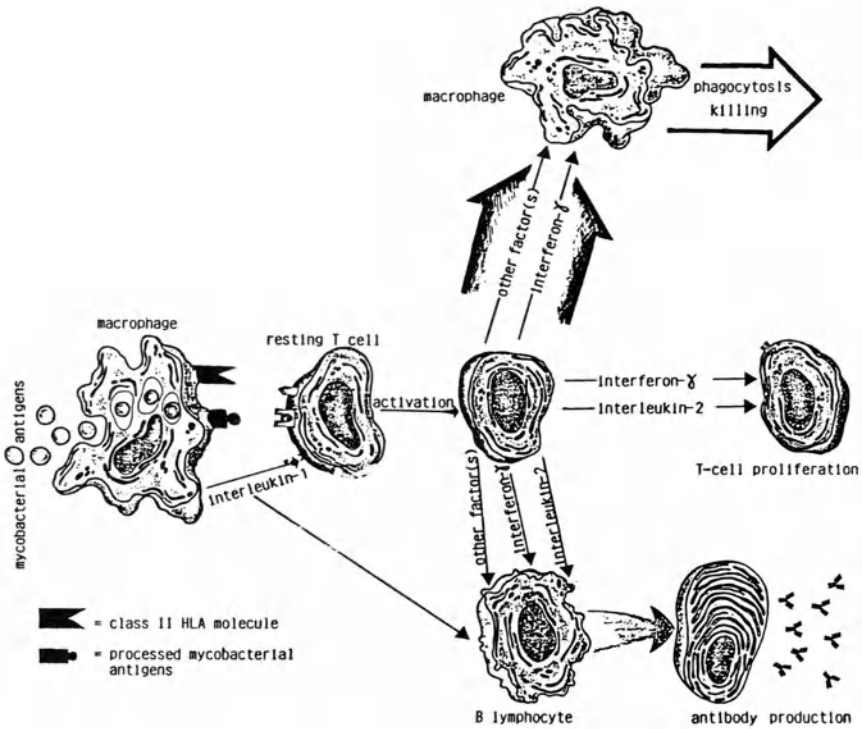

FIGURE 1. Interactions between the immune system and mycobacterial antigens.

armed arm and the former the mediator or memory of control, appears evident. Clearly, immunocompetent T lymphocytes are the most important cells involved in acquired antituberculosis immunity, as T-cell-depleted hosts (e.g., congenitally athymic or affected by other immunodeficiency syndromes) are unable to show tuberculin DTH or acquired antituberculosis resistance after a tuberculosis challenge.[13]

Whether specific antibodies are involved in antituberculosis protection has long been discussed. After several demonstrations that hyperimmune serum gives little, if any protection, it is known that specific antibodies exhibit poor direct antituberculosis activity. We believe that they may play an active role, by helping macrophages in the phagocytosis of the tubercle bacilli without influencing their killing.

4. *IN VITRO* MODEL OF HUMAN TUBERCULOIMMUNITY

Although the local DTH skin reaction to PPD offers a simple method for studying the immunologic events that follow the entry of my-

cobacterial products in a host and the cellular subsets involved, a more direct approach would undoubtedly be the study of the immunocompetent cellular populations infiltrating the sites of infection. With this in mind, it appears obvious that a great deal of information can be obtained by studying specimens of lung infected by *M. tuberculosis;* such studies have been extensively performed in animals but not very often in man.

Some characteristics of the local cellular immunity were recently analyzed in a group of patients affected by tuberculous pleurisy.[14] In showing that T lymphocytes were more dominant in tuberculous pleural effusions than in the peripheral blood, this investigation confirms the compartmentalization of specifically tuberculin-reactive lymphocytes and emphasizes the importance of studying tuberculosis immunity at the site of infection.

In the past, several attempts have been made to establish *in vitro* models of human tuberculosis immunity, which might be used to analyze the immunologic response to mycobacterial antigens. We recently designed a series of experiments for studying in detail the various events that follow the stimulation of lymphocytes with PPD *in vitro.* Since it was unconceivable that all the T lymphocytes were responsive to PPD, we employed the 5/9 MAb to fractionate the T-cell population. The 5/9+ cell fraction of normal individuals has been found to contain all the T cells responsive to various soluble antigens, such as tetanus toxoid[15] and thyroglobulin.[16]

In addition to proliferative responsiveness, which is only one of the aspects of the immune response, we investigated the production of the lymphokines IL-2 and IFN_{γ}. Attention was also focused on the macrophage activation inducible by soluble factor(s) derived from PPD-stimulated T-cell cultures.

We first analyzed the proliferative response to PPD of peripheral blood mononuclear cells (PMNC) isolated from skin-test-positive subjects in dose–response and time–response assays. Peripheral mononuclear cells were isolated from defibrinated blood by density-gradient centrifugation on Ficoll–Urovison and then washed three times with RPMI 1640. Each subject's PMNC was cultured with PPD, respectively, both in dose–response (PPD: 0.05, 0.5, and 1 μg/ml) and in time–response experiments (days 4, 7, and 10). Purified protein derivative (PPD) for *in vitro* research (activity: about 20 IU/1 μg) was provided by Sclavo (Siena, Italy) and diluted in RPMI 1640. Cultures were set up in triplicate in U-bottomed microtiter trays (Sterilin, England) at a concentration of 200,000 PMNC/well; 18 hr before the end of the culture, cells were pulsed with [^{3}H]thymidine (1 μCi/well), and then cultured for another 18 hr and harvested on glass-wool filters using an automated cell harvester (Titertek D-001, Flow Laboratory, Scotland). Thymidine

incorporation was measured in a β-counter (Beckman, Fullerton, California). The proliferation peaked at 7 days of culture and decreased afterward (Table I). The range of PPD dose levels in cultures was 0.05–1 μg/ml; the optimal concentration of PPD for lymphocyte proliferation was 1 μg/ml. In order to study which cell subset was responsible for the proliferative response to PPD, PMNCs from four healthy tuberculin-reactive subjects were first fractionated into T and non-T cells by E-rosette formation and density gradient centrifugation.[16] The T-enriched (90–95% purity) population was further fractionated into 5/9+ and 5/9− subsets by a further density gradient using ox red blood cells coated by the 5/9 monoclonal antibody as described by Ling *et al.*,[17] obtaining 5/9+ and 5/9− fractions of more than 95% and 99% purity, respectively. Mixtures of the various T-cell subsets (total, 5/9+, 5/9−) and autologous non-T cells (as a source of antigen-presenting cells) were cultured with PPD (1 μg/ml). Cultures were set up in triplicate in U-bottomed microtiter trays (100,000 T cells plus 50,000 non-T cells/well) for 7 days. Results showed that purified 5/9+ cells responded maximally to PPD (24,167 ± 5877 cpm) whereas no proliferation was detectable in the 5/9− cell population (6658 ± 1782 cpm) ($p < 0.01$; Student's *t*-test). Since the subset proliferating to PPD expresses the 5/9 antigen, we investigated whether cell proliferation was affected by the addition of the relevant MAb. An anti-Ia, i.e., D1.12,[18] was added as control, since anti-Ia MAb was previously shown to block PPD-induced responses.[19] Thus, 10 μg/well of the two MAbs was added to the onset of cultures containing PMNC and PPD. Figure 2 shows that, whereas complete abrogation of cell proliferation was observed in the cultures containing the anti-Ia MAb, the 5–9 MAb caused a slight inhibition only, even when added at 50 μg/well (data not shown). These results would suggest that the mole-

TABLE I
Proliferative Response of Peripheral Mononuclear Cells to Purified Protein Derivative[a]

PPD dose (μg)	Day 4	Day 7	Day 10
1	8307 ± 1704	22,239 ± 6740	8495 ± 4305
0.5	4481 ± 969	16,460 ± 4005	8021 ± 2566
0.05	4475 ± 1415	12,908 ± 2341	9227 ± 2063

[a] Data are expressed as the means ±SD of cpm (four healthy tuberculin-reactive subjects).

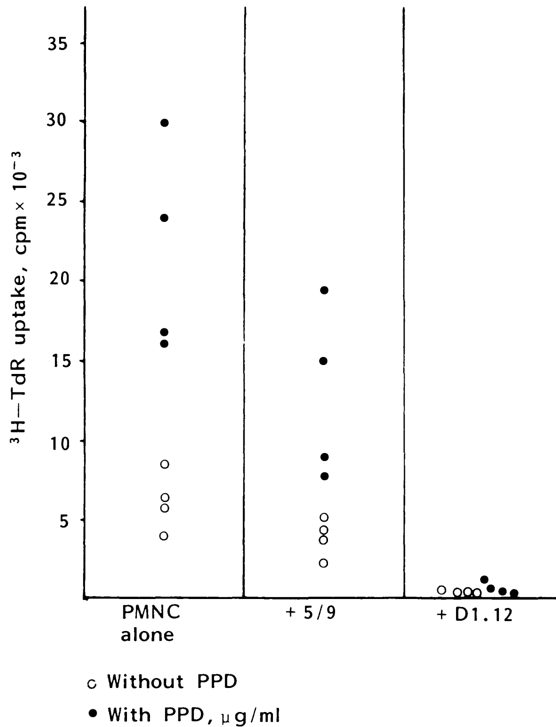

FIGURE 2. Effects of addition of D1.12 (anti-Ia) and 5/9 monoclonal antibodies on PPD-induced proliferation of peripheral mononuclear cells.

cule recognized by the 5/9 MAb is not directly involved in PPD-induced responsiveness.

In a previous study in autoimmune thyroid disease, we had found a thyroglobulin-induced proliferation of T8+ cells (i.e., 5/9−), which was mediated by the 5/9+ subset.[20] Experiments were designed to analyze this phenomenon in PPD-stimulated cultures. We had previously shown that the proliferative response to PPD was confined to the T-cell subset expressing the 5/9 surface antigen and that the 5/9+ subpopulation contained the cell required for the induction of alloreactive cytotoxic T lymphocyte precursor (CTL-P) in mixed lymphocyte culture. Since all T8+ cells have been shown to be CTL-P,[21] it was possible that 5/9+ cells were required as regulatory cells for the PPD-induced proliferative response of T8+ cells. Freshly isolated 5/9+ and 5/9− populations were analyzed for expression of the T8 antigen; 92% of T8+ cells were confined to the 5/9− cell fraction.[20] In these experiments, all the culture combinations contained 50,000 irradiated (5000 rad) autologous non-T

cells as the source of antigen-presenting cells. The proportion of T8+ cells was evaluated in those culture combinations in which cell proliferation in response to PPD was observed, i.e., in those containing 5/9+ cells alone or a mixture of 5/9+ and 5/9− cells (reconstituted population). After 7 days in culture, virtually no T8+ cells were found in the 5/9+ population (data not shown), indicating that the T8 antigen cannot be expressed *de novo* under these experimental conditions. The expression of T8 antigen was evaluated with the B9.4 monoclonal antibody in basal conditions (day 0) and in 7 day cultures with or without PPD (1 μg/ml) in four healthy tuberculin-reactive subjects. At day 0, the percentage of T8+ cells detected was 22.2 ± 2.9 (mean ± SEM); after day 7, the percentage of T8+ was 12.08 ± 0.8 with PPD and 17.5 ± 1.7 without PPD. In addition, only 12.8% of T8+ cells were detected in the reconstituted populations. These data provide evidence that PPD does not induce a recruitment of T8+ cells derived from precursors present in the 5/9− subset. This concept was further supported by experiments in which 5/9− cells were cultured with irradiated 5/9+ cells, since no proliferation was observed (data not shown).

We then investigated lymphokine production. Freshly isolated 5/9+ and 5/9− populations were cultured with PPD in the presence of autologous antigen-presenting cells. After 96 hr, the supernatants were collected and tested for IL-2 following the method of Gillis *et al.*[22] As shown in Table II, IL-2 was detected only in the supernatants fluids of 5/9+ T cell. Also, IFN_{γ} was produced only by PPD-stimulated 5/9+ T cells. The supernatants of these cells contained 50–144 units/ml, whereas no IFN_{γ}

TABLE II
Interleukin-2 Production by 5/9 − and 5/9+ T Cells after PPD Stimulation[a,b]

Subject	5/9− T cells	Total T cells	5/9+ T cells
1	ND[c]	ND	4.5
2	ND	ND	11
3	ND	19.2	11.8
4	ND	ND	12.5

[a] Interleukin-2 was assayed in 96-hr supernatants of total, 5/9+, and 5/9− T lymphocytes from four healthy tuberculin-reactive subjects. The cells were cultured in the presence of 1 μg/ml PPD and 50,000 irradiated (5000 rad) autologous non-T cells as a source of antigen presenting cells.

[b] Data expressed as percentage of the maximal proliferation of CTL-L cells induced by the supernatant of IL-2 producing Jurkat cell line stimulated with mitogen.

[c] ND, not dosable.

was detected in the supernatants of unstimulated 5/9+ and PPD-stimulated or -unstimulated 5/9− cells.

To define a further event presumably associated with the PPD-induced response, we tested supernatants of PPD-stimulated PMNC and 5/9+ and 5/9− cell cultures on the recently described monocytic line ZC-1.6.[23] In preliminary experiments, the supernatants of PMNC and 5/9+ cells appeared capable of inducing hydrogen release by this cell line, whereas 5/9− cell supernatants did not.

Collectively, these data demonstrate that both proliferative response and lymphokine production induced by PPD are strictly confined to a subset of T lymphocytes identified by the 5/9 MAb. PPD is unable to stimulate 5/9− T cells both to proliferate and to produce IL-2 and IFN_γ. In addition, no direct or indirect (through the 5/9+ subset) recruitment of T8+ cells was demonstrated. As expected, these results tend to exclude an involvement of the cytotoxic branch in the response to PPD stimulation.

Since the final event in DTH is the activation of macrophages, we investigated the effect of supernatants of 5/9+ and 5/9− T cells stimulated with PPD on the human monocytic cell line ZC-1.6, which can be activated immunologically to release hydrogen peroxide.

The supernatants of 5/9+ T cells, which contained remarkable amounts of IFN_γ, elicited a sharp release of hydrogen peroxide; this might suggest that in our PPD-stimulated system it is the IFN_γ that is, at least in part, responsible for macrophage activation. The macrophage is known to be one of the preferential targets of IFN_γ activity. Nevertheless, it is conceivable that additional macrophage-activating factor(s), distinct from IFN, may act on macrophages, as recently proposed by Andrew *et al.*[24] on the basis of experiments with PPD-stimulated human T-cell clones.

The production of antibody directed to PPD is generally considered a phenomenon of negligible biologic relevance. However, we recently provided interesting experimental evidence of a closed relationship between the *in vivo* presence of anti-PPD antibodies and activity of tuberculosis infections.[25] The presence of high levels of 5/9+ T cells in the bronchoalveolar lavage (BAL) of patients with active miliaric tuberculosis[26] suggests the possibility that IL-2 produced by 5/9+ T cells upon PPD stimulation acts on B cells to stimulate the production of specific antibodies. IL-2 was recently demonstrated to promote the proliferation of B cells.[27] Furthermore, IFN_γ has been found to exert a direct helper effect on B lymphocytes[28]; therefore, the possibility exists that IFN_γ acts synergistically with other helper factor(s) in the stimulation antibody responses to PPD. Studies are currently in progress at our

laboratory to investigate this hypothesis. It is also possible, however, that IL-2, produced as a consequence of PPD stimulation, enhances the secretion of other soluble factor(s) by the activated T cells. Finally, since it was reported that IL-1 production by accessory cells of patients with pulmonary tuberculosis is increased,[29] it is likely that IL-1 acts on B cells as a cofactor in their clonal expansion,[30] thereby contributing to an explanation of the presence of anti-PPD antibodies in patients with active tuberculosis.

The experimental data reported here provide detailed evidence that PPD can stimulate the immune system of humans by acting on T cells confined within a subset identified by 5/9 MAb. This early event is followed by the release of soluble factor(s) that lead to the activation of several other immunocompetent cell populations, including macrophages. It is conceivable that these data will find useful clinical applications.

5. LYMPHOKINES AND PPD RESPONSIVENESS

The immune response to PPD *in vitro* results in IL-2 and IFN_{γ} production by a specialized subset of T cells. However, these data were obtained by studying normal subjects. It is known that in patients with active tuberculosis, an immunologic hyporesponsiveness to PPD can occur.

Fujiwara *et al.*[29] proposed that defective cellular response to PPD is a consequence of the hyperproduction of IL-1 they detected in tuberculous patients. The finding of IL-1 hyperproduction is extremely interesting per se, but the proposed suppressive effect of IL-1 on PPD-induced T-cell proliferation is in disagreement with current views. In fact, IL-1(s) was never found to be directly suppressive for T cells.[30] In Fujiwara's investigation, most likely some other factor(s) affected the suppression. Presumably, the inhibition of PPD responsiveness is not due to prostaglandins, since Toossy *et al.*[31] failed to restore the response by adding indomethacin. In the same study, a reduction of IL-2 production and of IL-2 receptor expression on T cells upon PPD stimulation were detected in the patients, and the effects appeared to be related to the defective proliferative response of T cells to PPD.

These data are of great clinical interest, since they provide a better understanding of the mechanisms leading to cell-mediated hyporesponsiveness in tuberculosis. The defective IL-2 production and IL-2 receptor expression observed by Toossy *et al.*[31] are important, since similar alterations have been demonstrated or suggested in many immunologic

disorders.[32] Although these observations do not seem to fit with the increased IL-1 production found by Fujiwara *et al.*,[29] we believe that many uncertainties may be solved by the use of recombinant IL-1 in similar experiments.

Unfortunately, these failed to provide information on IFN_γ. We have previously demonstrated that normal IFN_γ production can coexist with defective IL-2 production in the same culture.[33] Since IFN_γ and other macrophage-activating factor(s) seem to play the crucial role of promoting macrophage phagocytic activities, assay of these factors in single patients might yield interesting prognostic indications.

6. THE TUBERCULOUS LESION IN HUMANS: CHARACTERIZATION OF THE IMMUNOCOMPETENT CELLS

Pulmonary tuberculosis, as is true of many other granulomatous diseases affecting the lung (e.g., sarcoidosis,[34] extrinsic allergic alveolitis) is characterized by a compartmentalization of the immune response at the site of infection. Therefore, in the active stage of the disease, we can expect an increase of specifically tuberculin-reactive lymphocytes in the tuberculous lesions with depletion of the same cells in the peripheral blood. This expectation was easily verified in tuberculous pleurisy, where analysis of the pleural exudate obtained by thoracentesis permitted a detailed investigation of the immunologic cells present in the pleural fluid.

Shimokata *et al.*[14] compared the characteristics and functions of lymphocytes in the pleural exudate and peripheral blood and found that the lymphocytes in pleural fluid contained higher proportion of T cells and produced more IFN_γ following PPD stimulation, especially in patients with more intense skin reactions. The compartmentalization of immunocompetent lymphocytes was subsequently confirmed by other workers,[35,36] who found an increase of both T4 lymphocytes and the T4/T8 ratio in the pleural fluids and a reduction of these parameters in the peripheral blood. These *in vivo* findings are in perfect agreement with the experimental evidence obtained by our group on the T-lymphocyte response to mycobacterial antigens.

The study of the immunocompetent cells in tuberculous pulmonary infiltrates might appear more difficult, since the cells must be recovered from the deep lung. However, using the new techniques of bronchologic investigations developed in recent years, we were able to examine specimens from the alveolar districts without surgical procedures. The cells

infiltrating the site of infection were obtained by the relatively simple method of BAL selectively performed in the infected lobes with the help of the fiberoptic bronchoscope.

In the BAL of a group of patients with active pulmonary tuberculosis, Bariffi *et al.*[37] found a significant increase in the total number of T cells and in the proportion of the helper–inducer subset. This study did not show evidence of any significant reduction of the T8 lymphocytes or changes in the T4/T8 ratio.

Teles de Araujo *et al.*[38] also found significantly increased numbers of lymphocytes in the BAL from tuberculous patients and normal T4/T8 ratios. Nevertheless, some patients in this group who were affected by nonreactive forms of tuberculosis showed a significant decrease in total T lymphocytes and in the helper–inducer subpopulation with reduction of the T4/T8 ratio. This report suggested that different forms and/or stages of active tuberculosis may reflect different immunologic host responses and may therefore present differences in the distribution of lymphocyte subsets and in the soluble factor(s) produced, as well as in the cooperation between the immunocompetent cells.

In examining this hypothesis, Ainslie *et al.*[36] investigated the variations in T-lymphocyte numbers and subsets of both the BAL fluid and peripheral blood from patients with different forms and stages of pulmonary tuberculosis. Patients were classified as being affected by localized or disseminated pulmonary tuberculosis; the latter group was also arbitrarily subdivided into high-lymphocyte responders (BAL lymphocytes $>$ 50,000/ml) and low-lymphocyte responders (BAL lymphocytes $<$ 50,000/ml). In both groups with disseminated disease, the T8 cells were increased and the T4 cells decreased in the BAL as well as in the peripheral blood. In addition, in some patients with disseminated pulmonary tuberculosis studied after 8 weeks of treatment, bronchoalveolar lymphocytes and T4/T8 ratios were found to be remarkably increased. In patients with localized pulmonary tuberculosis, the T4/T8 ratio was normal in the peripheral blood but was found to be lower in BAL from the affected lobes than in BAL from the unaffected lobes. These results are apparently in disagreement with those reported by Teles de Araujo *et al.*[38] and Bariffi *et al.*[37] Ainslie *et al.*[36] suggested that the increase of suppressor T lymphocytes in BAL might involve a suppressor cell activating factor released by alveolar macrophage stimulated by some component of mycobacteria. This factor might act only locally at the site of infection.

Interleukin-1 is a complex of different molecules secreted mainly by monocytes and macrophages. This monokine exerts a crucial role in initiating and perpetuating the granulomatous process. Alveolar macrophages of BAL from tuberculosis patients have been investigated for

their capability to release IL-1 under specific antigenic stimulation. Baughman *et al.*[39] demonstrated that alveolar macrophages from skin-positive subjects with no clinical evidence of tuberculosis infection released significantly enhanced amounts of IL-1 under PPD stimulation compared with alveolar macrophages from skin negative healthy patients. In the same study, alveolar macrophages from tuberculin skin-negative and skin-positive patients with active tuberculosis were also investigated. Only the alveolar macrophages of the skin-positive subjects released significant amounts of IL-1 following PPD stimulation. This finding was in agreement with the data obtained in skin-positive healthy patients.

This evidence concerning the immunologic characterization of the tuberculous cellular infiltrate in humans suggests the following conclusions:

1. In tuberculous pleurisy, there is no doubt that the immunocompetent cells are compartmentalized in the pleural fluid, and the immune response is characterized by an increase in T lymphocytes primarily of the helper–inducer subset.
2. In active pulmonary tuberculosis, differences exist in the immune cells infiltrating the site of infection among the various forms and stages of the disease. These differences may reflect variations in the different antigenic stimulation driven by different infecting mycobacteria or congenitally different immunologic responses as well as the form and/or stage of tuberculosis. Undoubtedly, in reactive (tuberculin skin-positive) patients with the active form of pulmonary tuberculosis, or after therapy, alveolar macrophages show an increased ability to release IL-1 under specific antigenic stimulation: moreover, alveolar lymphocytosis, increased T helper–inducer subset, and T4/T8 ratio can characterize the reactive forms more frequently than the others.

7. PERSPECTIVES AND CONCLUSION

In 1983 the World Health Organization (WHO) published a Memorandum,[40] a plan of action for research in the immunology of tubercolosis. Some of the main points to be pursued were T-cell cloning, production of MAb to define the heterogeneity of mycobacterial antigens and their epitopes, the role of immunoregulatory molecules such as lymphokines and monokines, and relationships with the clinical status.

This chapter has touched on all these points, except the antigenic diversity of *M. tuberculosis*. This aspect is considered in the present sec-

tion, as it is probably the only point in which the concept of immunologically guided prevention of tuberculosis should be implicated.

Recently a large number of MAb to different epitopes of mycobacteria were developed.[41] The data indicate that different antigenic determinants could elicit different immune responses, thereby opening new exciting perpectives for tuberculosis prevention. In fact, Ottenhoff *et al.*[42] recently demonstrated that HLA-DR4 is associated with high responsiveness to the species-specific antigens of *M. tuberculosis,* but not to the antigens shared with other mycobacteria. This phenomenon strongly suggests an association between DR4 and immune response to *M. tuberculosis* and possibly that different Ir genes control the response to different epitopes of the same antigenic complex. If genetic factors do indeed regulate susceptibility to tuberculosis and the intensity of immune response to specific *M. tuberculosis* antigens, it might become possible to predict the susceptibility and resistance to infection of each individual.

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REFERENCES

1. Lurie, M. B., 1964, *Resistance to Tuberculosis: Experimental Studies in Native and Acquired Defense Mechanisms,* Harvard University Press, Cambridge, Massachusetts.
2. Lurie, M. B., and Dannenberg, A. M., Jr., 1965, Macrophage function in infectious disease with inbred rabbits, *Bacteriol. Rev.* **29:**466–476.
3. Dannenberg, A. M., Jr., 1982, Pathogenesis of pulmonary tuberculosis, *Am. Rev. Respir. Dis. (Suppl)* **125:**25–30.
4. Koch, R., 1890, Weitere mittheilung uber ein heilmittel gegen tuberkulose, *Dtsch. Med. Wochenschr.* **16:**1029–1032.
5. Mackaness, G. B., 1954, The growth of tubercle bacilli in monocytes from normal and vaccinated rabbits, *Am. Rev. Tuberc.* **69:**495–504.
6. Suter, E., 1953, Multiplication of tubercle bacilli within mononuclear phagocytes in tissue cultures derived from normal animals vaccinated with BCG, *J. Exp. Med.* **97:**235–247.
7. Chase, M. W., 1945, The cellular transfer of cutaneous hypersensitivity to tuberculin, *Proc. Soc. Exp. Biol. Med.* **59:**134–135.
8. Mackaness, G. B., 1971, The induction and expression of cell-mediated hypersensitivity in the lung, *Am. Rev. Respir. Dis.* **104:**813–828.
9. Scheynius, A., Klareskog, L., and Forsum, U., 1982, *In situ* identification of T lymphocyte subsets and HLA-DR expressing cells in the human skin tuberculin reaction, *Clin. Exp. Immunol.* **49:**325–330.
10. Konttinen, Y. T., Bergroth, V., Visa-Tolvanen, K., Reitamo, S., and Forstrom, L., 1983, Cellular infiltrate in situ and response kinetics of human intradermal and epicutaneous tuberculin reactions, *Clin. Immunol. Immunopathol.* **28:**441–449.
11. Van Voorhis, W. C., Hair, L. S., Steinman, R. M., and Kaplan, G., 1982, Human

dendritic cells. Enrichment and characterization from peripheral blood, *J. Exp. Med.* **155:**1172–1187.

12. Kuntz Crow, M., and Kunkel, H. G., 1982, Human dendritic cells: Major stimulators of the autologous and allogeneic mixed leucocyte reactions, *Clin. Exp. Immunol.* **49:**338–346.
13. Collins, F. M., 1979, Cellular antimicrobial immunity, *CRC Crit. Rev. Microbiol.* **7:**27–91.
14. Shimokata, K., Kawachi, H., Kishimoto, H., Maeda, F., and Ito, Y., 1982, Local cellular immunity in tuberculous pleurisy, *Am. Rev. Respir. Dis.* **126:**822–824.
15. Corte, G., Mingari, M. C., Moretta, A., Damiani, G., Moretta, L., and Bargellesi, A., 1982, Human T cell subpopulations defined by a monoclonal antibody. I. A small subset is responsible for proliferation to allogenic cells or to soluble antigens and for helper activity for B cell differentiation, *J. Immunol.* **128:**16–19.
16. Canonica, G. W., Cosulich, M. E., Croci, R., Ferrini, S., Bagnasco, M., Dirienzo, W., Ferrini, O., Bargellesi, A., and Giordano, G., 1984, Thyroglobulin-induced T-cell in vitro proliferation in Hashimoto's thyroiditis: Identification of the responsive subset and effect of monoclonal antibodies directed to Ia antigens, *Clin. Immunol. Immunopathol.* **32:**132–141.
17. Ling, N. R., Bishop, J., and Jefferies, R., 1977, Use of antibody coated red cells bearing surface immunoglobulins, *J. Immunol. Methods* **15:**273–289.
18. Carrel, S., Tosi, R., Gross, N., Tanigaki, N., Carcagnola, A. N., and Accolla, R. S., 1981, Subset of human Ia-like molecules defined by monoclonal antibodies, *Mol. Immunol.* **18:**403–411.
19. Hirshberg, H., Braathen, L. R., and Thorsby, E., Antigen presentation by vascular endothelial cells and epidermal Langerhans cells: The role of HLA-Dr, *Immunol. Rev.* **66:**57–61.
20. Canonica, G. W., Caria, M., Bagnasco, M., Cosulich, M. E., Giordano, G., and Moretta, L., 1985, Proliferative response of T8+ cytolytic T lymphocytes to thyroglobulin in human autoimmune thyroiditis. Analysis of cell interactions and culture requirements, *Clin. Immunol. Immunopathol.* **36:**40–48.
21. Moretta, A., Pantaleo, G., Moretta, L., Mingari, M. C., and Cerottini, J. C., 1983, Quantitative assessment of the pool sizes and subset of cytolytic T lymphocytes within human resting or alloactivated peripheral blood T cell populations, *J. Exp. Med.* **158:**571–585.
22. Gillis, S., Ferm, M. M., Ou, W., and Smith, K. A., 1978, T cell growth factor: Parameters of production and a quantitative microassay for activity, *J. Immunol.* **120:**2027–2032.
23. Damiani, G., Zocchi, E., Martinoli, C., Querzola, F., and Zaccheo, D., 1985, Establishment and characterization of a new human cloned myelomonocytic cell line (ZC-1.6), *Exp. Hematol.* **13:**861–868.
24. Andrew, P. W., Rees, A. D. M., Scoging, A., Dobson, N., Matthews, R., Whittall, J. T., Coates, A. R. M., and Lowrie, D. B., 1984, Secretion of a macrophage-activating factor distinct from interferon-γ by human T cell clones, *Eur. J. Immunol.* **14:**962–964.
25. Melioli, G., Machi, A. M., and Scordamaglia, A., 1985, L'immunità nella tubercolosi. I. Aspetti umorali, *Lotta contro Tuberc. Mal. Polm. Soc.* **55:**275–278.
26. Barbolini, S., Bisetti, A., Magnani, C., Rossi, G., Squadrini, F., Velluti, G., and Zucchi, L., 1985, L'immunità nella tubercolosi. III. Basi morfo-funzionali, *Lotta contro la Tuberc. Mal. Polm. Soc.* **55:**287–291.
27. Mingari, M. C., Gerosa, F., Carra, G., Accolla, R. S., Moretta, A., Zubler, R. H., Waldman, T. A., and Moretta, L., 1984, Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells, *Nature (Lond.)* **312:**641–643.

28. Romagnani, S., Giudizi, M. G., Biagiotti, R., Almerigogna, F., Mingari, C., Maggi, E., Liang, C., and Moretta, L., 1986, B cell growth factor activity of interferon-γ. Recombinant human interferon-γ promotes proliferation of anti-μ-activated human B lymphocytes, *J. Immunol.* **136:**3513–3516.
29. Fujiwara, H., Kleinhenz, M. E., Wallis, R. S., and Ellner, J. J., 1986, Increased interleukin-1 production and monocyte suppressor cell activity associated with human tuberculosis, *Am. Rev. Respir. Dis.* **133:**73–77.
30. Oppenheim, J. J., Kovacs, E. J., Matsushima, K., and Durum, S. K., 1986, There is more than one interleukin 1, *Immunol. Today* **7:**45–56.
31. Toossi, Z., Kleinhenz, M. E., and Ellner, J. J., 1986, Defective interleukin-2 production and responsiveness in human pulmonary tuberculosis, *J. Exp. Med.* **163:**1162–1172.
32. Kromer, G., Schauenstein, K., and Wick, G., 1986, Is autoimmunity a side effect of interleukin 2 production? *Immunol. Today* **7:**199–200.
33. Canonica, G. W., Ciprandi, G., Caria, M., Dirienzo, W., Shums, A., Norton-Koger, B., and Fudenberg, H. H., 1985, Defect of autologous mixed lymphocyte reaction and interleukin-2 in aged individuals, *Mech. Ageing Dev.* **32:**205–212.
34. Rossi, G. A., Sacco, O., Cosulich, E., Risso, A., Balbi, B., and Ravazzoni, C., 1986, Helper T-lymphocytes in pulmonary sarcoidosis, *Am. Rev. Respir. Dis.* **133:**1086–1090.
35. Rossi, G. A., Balbi, B., Manca, F., Celada, F., and Ravazzoni, C., 1985, Tuberculous pleurisy: Proliferative response to PPD of circulating and pleural T lymphocytes, abst. 142. in: *Bronchitis and Emphysema* (L. Allegra and G. Rizzato, eds.), GIMT.
36. Ainslie, G. M., Bateman, E. D., and Solomon, J. A., 1986, Variation of T lymphocyte numbers and subsets in different forms and stages of pulmonary tuberculosis, *Am. Rev. Respir. Dis.* **133:**A39.
37. Bariffi, F., Pesci, A., Bertorelli, G., Parpanesi, M., and Maffei, M., 1985, Studio dell'immunità cellulo-mediata in soggetti affetti da tubercolosi polmonare, *Lotta contro Tuberc. Mal. Polm. Soc.* **55:**298–305.
38. Teles de Araujo, A., Raymundo, E., and Freitas e Costa, M., 1986, Bronchoalveolar lavage in pulmonary tuberculosis, *Bull. Eur. Physiopathol. Respir.* **22:**94s.
39. Baughman, R. P., Hendricks, D. E., Corser, B. C., and Bullock, W. E., 1986, Antigen specific interleukin 1 production by alveolar macrophages in patients with tuberculous infections, *Am. Rev. Respir. Dis.* **133:**A39.
40. World Health Organization, 1983, Plan of action for research in the immunology of tuberculosis: Memorandum from WHO Meeting, *Bull. WHO* **61:**779–785.
41. Engers, H. D., Bloom, B. R., and Godal, T., 1985, Monoclonal antibodies against mycobacterial antigens, *Immunol. Today* **6:**347–348.
42. Ottenhoff, T. H. M., Torres, P., Terencio De Las Aguas, J., Fernandez, R., Van Eden, W., De Vries, R. R. P., and Stanford, J. L., 1986, Evidence for an HLA-DR4-associated immune-response gene for Mycobacterium tuberculosis, *Lancet* **2:**310–312.

5

The Tubercle Bacillus–Human Macrophage Relationship Studied *In Vitro*

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1. THE CELLULAR FOCUS OF TUBERCULOSIS

Tuberculosis is an infection of the monocytic phagocyte (MP) system.[1–8] The disease-causing bacilli multiply in cells of this system as determined by bodily environment. For example, they reproduce freely in alveolar macrophages (MP) at the beginning of human infection because these cells lack resistance, are located in oxygen-rich areas that support the oxygen needs of these aerobic bacteria, and are not yet receiving signals for immune activation from the body's T-cell system.[4,9–11] But later, the bacteria are inhibited by MP, for instance, in lymph nodes, because these cells are in oxygen-poor areas and are being defensively activated by immune lymphokines that T cells responding to bacillary antigens have begun to produce.[4,7–9,12] The MP may also respond detrimentally to infection-exacerbating molecular signals, like γ-globulin produced by tuberculoprotein-hypersensitive lymphocytes.[5,7–9,13] Then, they actively promote bacillary growth.

Tubercle bacillus–human MP interactions such as these can now be studied in fully controllable cellular and molecular detail with an *in vitro* model of human tuberculosis using cultures of blood MP and lymphocytes.[8] No longer is it necessary to depend on animal, or animal cell

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research, which, though informative, has never been directly relevant to the human disease.[3,5,9,10,13–15] The following is a description of the tubercle bacillus–human MP relationship derived from 5 years of research with this model.

2. THE MODEL AND THE DATA IT PRODUCES

The model was developed from rabbit,[16–19] guinea pig,[18,20,21] and mouse[22–24] cell precedents. Tubercle bacillus infections of cultured human cells had been described before,[6,25–30] but not with purified native human MP until technical advances in methods of cell culturing made this possible.[31] Detailed descriptions of the model and the kinds of data it produces have been published.[8,31–34] The description summarizes the model for interpretation of the information to be reviewed.

The human monocytes used are adherent cells from peripheral blood cultured in spot monolayers of approximately 5×10^5 cells per spot, in disposable plastic petri dishes, three spots per 35-mm diameter dish. Each dish contains 1.5 ml (large excess relative to number of cultured cells) of RPMI-1640 medium, supplemented with 1% unheated autologous normal serum (NS), and is kept at pH 7.2 in 7.5% CO_2. The monocytes are cultured for 7 days, and the resulting macrophages are then infected with log-phase highly virulent Erdman tubercle bacilli, stored for this purpose in one-use aliquots at −90°C.[10]

After infection, the MP are cultured 7 days more so that their responses to the bacilli can be observed. These responses are characterized and measured either by microscope, i.e., for percentage MP infected and number of acid-fast bacilli (AFB) per MP, or by culture, i.e., culture counts of bacterial colony-forming units (CFU) from lysates of the MP cultures, using replicate samples of the cultures taken at 0 time (immediately after infection) and at 4 and 7 days. Nearly all bacillary multiplication is intracellular; the bacilli do not multiply significantly in NS-supplemented RPMI-1640, even though no antibiotic except penicillin is present.[32,34]

3. MACROPHAGE UPTAKE OF TUBERCLE BACILLI

This uptake has been studied by infecting the MP with standardized concentrations of ultrasonically dispersed,[10] mostly single (human infections usually begin with an infectious unit of two to three bacilli[11]) Erdman tubercle bacilli for 30 min at 37°C. After infection, the cells were washed, and some samples were fixed and stained for AFB counts,

TABLE I

Quantitative Characteristics of the Infection of Cultured Human Macrophages with Virulent Tubercle Bacilli[a,b]

Infecting suspension (CFU/ml)			Ratio	Percentage macrophages infected			AFB/infected macrophage				Lysate 10^5 MP (CFU/ml)			Ratio[d]
N	Mean	SEM	(bacilli : MP)	*N*	Mean	SEM	*N*	Mean	SEM	Bac./10^5 macrophages[c]	*N*	Mean	SEM	(AFB : CFU)
14	2.8×10^6	0.2	1.0	16	42.9	3.5	16	3.62	0.28	1.6×10^5	16	6.8×10^4	0.7	2.4
15	1.4×10^6	0.1	0.5	19	35.9	2.	20	3.02	0.15	1.1×10^5	19	2.2×10^4	0.2	5.0
10	7.2×10^5	0.04	0.25	15	20.9	2.0	15	2.12	0.22	4.4×10^4	15	1.3×10^4	0.1	3.4

[a] AFB, acid-fast bacilli; CFU, colony-forming units; *N*, number of samples (experiments); SEM, standard error of the mean; MP, macrophage.

[b] Data from 52 experiments with macrophages from 15 donors using three different concentrations of Erdman bacilli for infection. Observations made after 30 min of infection at 37°C.

[c] Product of percentage of macrophages infected × mean AFB/MP × 10^5.

[d] Bacilli per 10^5 macrophages, calculated by AFB counts divided by CFU/ml of 10^5 lysed macrophages.

while others were lysed for dilution, plating, and CFU counting. Data from 52 experiments using 15 different donors are summarized in Tables I and II. Table I presents data from all subjects, and Table II presents data from two subjects with different responses.

Data were obtained from experiments using three different concentrations of infecting suspension (first column of tables) expressed as culturable bacilli (CFU) per ml. The second column of Table I shows the calculated ratio of the bacilli per MP. AFB counts produced data on the percentage of MP infected and the mean AFB per infected MP (columns 3 and 4). A calculation of AFB/10^5 MP made from these data is shown in column 5 to permit direct comparison with CFU/10^5 MP (column 6) and the ratio of AFB : CFU (last column). The numbers of MP per ml MP lysate (i.e., 10^5) in the CFU counts were determined by both direct counting of enlarged photomicrographs of the cultures and by counts of MP nuclei.[32,34] The data in Tables I and II provide the following information about tubercle bacillus uptake by normal human MP.

Only a portion of the MP are infectable, less than one half from the average donor, regardless of the ratio of bacilli to MP used, confirming a previous prediction.[35] Increases in the bacillus-to-MP ratio produced diminishing increases in proportions of MP infected. The average maximum percentage of infected MP was 42.9 at the highest (1 : 1) ratio used. Interpolation of the data indicates that a theoretical infection of 8 : 1 ratio would produce only 3% more infected MP, 46%.

Increasing the bacillus-to-MP ratio increased the proportions of MP infected more than the number of bacilli per MP (compare columns 4, 5, and and 6, Table I), most probably by increasing the probability of MP–bacillus contact.

The average MP susceptible to infection was likely to be infected more than once during the 30-min period of infection used in these experiments (see column 4, Table I). Infected MP thus were not refractory to reinfection.

Two of three infecting bacilli were either dead (nonculturable), killed by MP that they had infected, or both (see column 7, Table I). Counts of total bacilli compared with culturable bacilli indicated that about one half the bacilli in infecting suspensions (prepared from frozen-thawed stock aliquots) were culturable. Consequently, the added proportion of nonculturable bacilli among phagocytized bacteria probably consisted of debilitated microorganisms killed by the ingesting MP. Thus, in certain circumstances, here probably a combination of MP activation caused by bacillary ingestion,[2] natural microbicidal activity,[36] and freeze-thaw injury of the bacilli.[10,37] human MP can kill,[2,4,9,12] rather than just inhibit,[15] tubercle bacilli.

Significant differences among subjects in respect to the way in which MP becomes infected can be seen in Tables I and II. Table II

TABLE II
Quantitative Characteristics of the Infection of Cultured Human Macrophages with Virulent Tubercle Bacilli: Mean Data from Two Different Subjects, RM and GB[a,b]

Infecting suspension (CFU/ml)	Percentage of macrophages infected	AFB/infected macrophage	AFB/10^5 macrophages	Lysate of 10^5 MP (CFU/ml)	Ratio (AFB : CFU)
		Subject RM (means of nine experiments)			
2.8×10^6	64.4 (4.9)	3.9 (0.7)	2.5×10^5	9.7×10^4 (1.4)	2.6
1.4×10^6	31.1 (1.4)	2.5 (0.06)	7.7×10^4	2.0×10^4 (0.3)	3.9
7.2×10^5	15.3 (2.4)	1.95 (0.3)	3.0×10^4	1.65×10^4 (0.25)	1.8
		Subject GB (means of six experiments)			
2.8×10^6	29.4 (5.6)	3.6 (1.2)	1.1×10^5	6.2×10^4 (2.4)	1.8
1.4×16^6	39.5 (6.9)	3.3 (0.4)	1.3×10^5	1.6×10^4 (0.4)	8.1
7.2×10^5	10	1.9	1.9×10^4	9.7×10^3	2.0

[a]See footnotes to Table I for explanations.
[b]Figures in parentheses are SEM.

summarizes the results from nine experiments with subject RM, and six with subject GB of the 15 subjects from whom data were obtained for Table I. The most important difference shown by data in Table II is that while the numbers of bacilli accumulated by any one phagocytic MP are similar for different subjects (column 3, Table II), all the MP of exceptional subjects are infectable (subject RM, column 2). This is evident from a direct proportionality between the bacillus-to-MP ratio and the proportion of MP becoming infected and the corresponding total AFB and CFU counts (columns 4 and 5). It is a direct demonstration of an inherent cellular difference of response to tuberculosis among human subjects.[2,9]

An average of two contacts between tubercle bacilli and MP occurred for each event of phagocytosis. As suggested by the data in Table I, this is especially evident from data for subject RM in Table II. Thus, at a 1 : 1 ratio of bacilli to MP, of the 100% of MP infectable, only 64.4% (column 2) became infected. Tubercle bacilli that make contact with human phagocytic cells may not be ingested; when they are, ingestion is rapid—15 sec.[38]

Table II shows another exceptional activity of some subjects' MP—that they make many freshly ingested tubercle bacilli non-acid-fast (compare columns 4 and 5 for subject RM). This observation is further illustrated and discussed below.

3.1. Influence of Culture Medium on Uptake of Tubercle Bacilli by Human Macrophages

Table III summarizes data from six experiments with two donors. MP were used immediately after isolation or after 3 or 7 days of culture in RPMI-1640 or Newman-Tytell[39] media and were infected with *M. tuberculosis* (Erdman) or *M. bovis* (BCG).[40] Bacillary uptake was improved by RPMI for one donor's MP (MM) but not the other's (AC). The uptake was not affected by which bacillus or age of MP was used.

The pH value is a medium property that is partly regulated by the concentration of CO_2 in the incubator. Higher concentrations (lower pH) seemed to inhibit bacillary uptake. Some data in Table IV show, however, that the higher concentration was suppressing bacillary acid-fastness rather than bacillary uptake, because differences by AFB counts between high and low CO_2 concentrations were absent from CFU counts. The numbers of AFB and CFU per infected MP were similar in the compared concentrations of CO_2. This means that the observed difference in total bacillary uptake is due to about one fourth the infected MP being able to make ingested bacilli non-acid-fast without necessarily killing them. Further evidence for this is given below.

TABLE III
Differences between Uptake of Tubercle Bacilli in Human Monocytic Phagocytes (MP) Cultured in Newman-Tytell or RPMI-1640 Medium

		AFB/MP, RPMI[b] / AFB/MP, N-T		CFU, RPMI[c] / CFU, N-T	
MP donor	Age of MP[a]	Erdman	BCG	Erdman	BCG
AC	7	ND	1.28	ND	ND
MM	7	1.08	0.98	1.32	3.20
AC	3	1.15	0.35	0.95	0.84
MM	3	2.12	3.17	1.50	3.20
AC	0	0.97	0.57	0.56	1.00
MM	0	1.52	1.52	1.61	1.52
Means		1.37 (0.2)[d]	1.31 (0.4)	1.19 (0.19)	1.63 (0.42)
Means, donor AC		1.06 (0.09)	0.73 (0.28)	0.76 (0.12)	0.92 (0.08)
Means, donor MM		1.57 (0.30)	1.89 (0.66)	1.47 (0.08)	2.1 (0.55)

[a]Day of culture after isolation at time of infection.
[b]Ratios of mean AFB taken up by 10^5 MP cultured in RPMI-1640 (RPMI) versus Newman-Tytell (N-T). Ratios were obtained separately for Erdman and BCG bacilli.
[c]Ratios of mean CFU taken up by 10^5 MP cultured in RPMI versus N-T.
[d]Figures in parentheses are SEM.

3.2. Influence of Immune Lymphokines on Uptake

Table V summarizes data relating to this factor from 13 experiments with nine different subjects. Lymphokine was produced by incubating the lymphocytes of subjects who exhibited immunity, due to immunization with BCG or an experimental vaccine, TESA, with a soluble immunizing antigen.[31] Placebo supernates were obtained from incubating lymphocytes from nonimmune donors similarly. The MP were incubated with 1.25% test or placebo lymphokine for 24 hr before infection. AFB and CFU counts both detected fewer bacilli in lymphokine-exposed than in placebo-exposed MP. While this could mean that lymphokine suppressed phagocytosis, we think it more likely that the lymphokine-activated MP killed one third to one half the bacilli they ingested. A proportion of "missing" bacilli, as measured by CFU, could be seen visualized microscopically in the MP as non-acid-fast objects that resemble bacteria.

3.3. Influence of Human Serum on Uptake

Human MP that have been cultured for 7 days in RPMI-1640 medium with 1% unheated NS will phagocytize tubercle bacilli equally well during the 30-min period of infection described above in RPMI-1640

TABLE IV

Effects of CO_2 Concentration on Uptake of Virulent Tubercle Bacilli as Measured by AFB and CFU Counts

Experiment	Subject	CO_2 concentration[a]	Mean % MP infected	Mean AFB per MP	Mean AFB[b] ($\times 10^4$)	Mean CFU[c] ($\times 10^4$)
1	MM	6.5	45.2	2.2	9.9	1.5
	MM	8.5	35.0	2.2	7.7	2.2
2	AC	6.5	31.8	2.0	6.4	1.0
	AC	8.5	24.0	1.8	4.3	1.0
3	AC	6.5	32.0	2.0	6.4	1.5
	AC	8.5	22.2	1.8	4.0	1.1
Mean ratios[d]			0.74 (0.02)	0.93 (0.03)	0.69 (0.05)	1.2 (0.25)

[a] Percentage of CO_2 in incubator air.
[b] By AFB counts.
[c] Per 10^5 MP.
[d] Ratios of 8.5% CO_2 values / 6.5% CO_2 values. SEM in parentheses.

TABLE V
Effects of Antigen-Generated Lymphokines on Uptake of Virulent Tubercle Bacilli as Measured by AFB and CFU Counts

Experiment	Subject	Source of immune response[a]	Ratio of control : lymphokine[b]	
			AFB	CFU
D-64	DS	BCG	1.8	1.1
D-66	PH	BCG	1.1	0.94
D-67	MS	TESA[c]	2.0	0.76
D-72	CC	BCG	1.5	1.6
D-56	KG	BCG	1.5	1.6
D-75	MS	TESA	2.4	1.3
D-55	RJ	BCG	0.92	1.22
D-60	PH	BCG	1.3	2.0
Means (SEM)			1.57 (0.17)	1.34 (0.15)
D-83	JD	None	0.9	0.8
D-70	RM	None	1.0	0.6
D-57	MS	None	2.1	0.74
D-59	MS	None	1.0	1.0
D-76	MM	None	0.63	1.6
Means (SEM)			1.1 (0.25)	0.95 (0.18)

[a]Subjects were exhibiting immune responses at time of experiment, as separately tested[31] due to type of immunization shown.
[b]Ratio of mean AFB or CFU per MP for cells incubated in medium only or medium with lymphokine supernate (see text).
[c]TESA is an experimental immunizing antigen.[31]

alone, supplemented with 1% or 5% unheated NS, or a 10% serum substitute (A. J. Crowle and M. H. May, unpublished observations). This agrees with the idea that the bacilli promote their own uptake to obtain a suitable microenvironment for replication.[38] Cultured J-111 human leukemic monocytes also readily phagocytize tubercle bacilli.[38] The bacilli do not so readily infect nonphagocytic human cells. For example, they have been reported to require high concentrations of horse serum to infect HeLa cells.[25]

3.4. Individual Variations in Macrophage Uptake and Killing of Tubercle Bacilli

Donors are reported to differ in the phagocytosis of tubercle bacilli by their MP.[40] This is only conditionally confirmed by the data in Table VI. These data are from 62 experiments using MP from 11 different subjects infected in the same way with Erdman bacilli, with a bacillus-to-

TABLE VI
Uptake of Tubercle Bacilli by Macrophages from Different Donors[a]

Donor[b]	Percentage MP infected	Mean AFB per infected MP	Mean AFB/10^5 MP[c] ($\times 10^5$)	Mean CFU/10^5 MP ($\times 10^5$)
AC	40 (7)	3.1 (0.4)	1.21 (0.4)	0.27 (0.12)
DS	38 (5)	2.5 (0.3)	0.93 (0.07)	0.27 (0.10)
GB	37 (4)	3.5 (0.4)	1.25 (0.2)	0.20 (0.06)
PH	34 (7)	3.1 (0.4)	1.10 (0.2)	0.22 (0.06)
MS	34 (4)	2.7 (0.6)	0.97 (0.03)	0.21 (0.04)
JB	34 (8)	3.5 (1.2)	1.50 (0.5)	0.27 (0.10)
KG	33 (3)	3.3 (0.4)	1.12 (0.2)	0.29 (0.05)
RJ	32 (9)	3.0 (0.4)	1.00 (0.3)	0.29 (0.08)
RM	31 (3)	3.0 (0.2)	0.92 (0.10)	0.39 (0.17)
RD	31 (3)	3.3 (0.3)	1.02 (0.2)	0.15 (0.03)
TB	28 (1)	2.2 (0.2)	0.72 (0.03)	0.19 (0.05)
Means		3.0 (0.12)	1.07 (0.065)	0.248 (0.019)

[a]Numbers in parentheses are SEM.
[b]Donors arranged by rank from high to low for percentage of MP infected, second column.
[c]Expressed in numbers per 10^5 MP to make AFB data in this column directly comparable with CFU data, last column, and taking into account all (infected and uninfected) MP.

MP ratio of 0.5, equal to that of the middle infecting concentration (Table I).

The donors in Table VI are ranked by mean percentage of MP infected by AFB counts. While it is obvious that donors at opposite ends of the spectrum are significantly different, only a large study such as this, or experiments with subjects who happen to be at the opposite ends,[40] would show such differences. That is because there are also large differences between bleedings from individual subjects that overlap the generally small differences between means of MP infected for most of the subjects. Rankings for the percentage of cells infected and mean AFB per cell (columns 2 and 3) do not coincide. Therefore, there should be little actual difference between total numbers of bacilli phagocytized by different whole cultures of MP from different subjects, which is true according to total counts by AFB or CFU (columns 4 and 5).

Table VII shows the mean ratios of CFU/AFB from data in Table VI. These, representing the ratios of living to dead bacilli, are ranked from highest numbers of AFB uptake downward. They show that, on average, only one in four AFB counted microscopically was culturable and confirm the conclusions from Table I that about three fourths of the bacilli in the infecting suspension are not culturable after being phagocytized by human MP. While some probably are not culturable because they have been injured or killed by the processes of freezing/

TABLE VII
Ratios of CFU : AFB per 10^5 MP from Data in Table VI Ranked from High to Low in AFB/MP

Rank[a]	Subject	CFU : AFB[b]	Ratio CFU/AFB[b]
Highest	JB	$2.5 \times 10^4 : 1.5 \times 10^5$	0.17
	GB	$2.0 \times 10^4 : 1.35 \times 10^5$	0.15
	AC	$2.7 \times 10^4 : 1.2 \times 10^5$	0.23
	KG	$2.9 \times 10^4 : 1.1 \times 10^5$	0.26
	PH	$2.2 \times 10^4 : 1.1 \times 10^5$	0.20
	RD	$1.5 \times 10^4 : 1.02 \times 10^5$	0.15
	RJ	$2.9 \times 10^4 : 1.0 \times 10^5$	0.29
	MS	$2.1 \times 10^4 : 9.6 \times 10^4$	0.22
	DS	$2.7 \times 10^4 : 9.3 \times 10^4$	0.29
	RM	$3.9 \times 10^4 : 9.2 \times 10^4$	0.42[c]
Lowest	TB	$1.9 \times 10^4 : 7.2 \times 10^4$	0.26
		$N = 11$ Mean = 0.24 SD = 0.078 SEM = 0.024	

[a]Rank, highest to lowest by counts of AFB per 10^5 MP.
[b]Per 10^5 MP.
[c]More than 2 SD of the mean (SD) different from the mean of all values.

storage/thawing associated with their use in infecting suspensions,[10,37] the single ratio of 0.42 for subject RM in Table VII, different from all others by greater than 2 SD, suggests an added reason: while approximately one half the infecting bacilli are inherently not culturable (as has already been suggested from Table I), the remainder that cannot be cultured (about another one fourth) must be killed by most donors' MP during phagocytosis. Thus, either the MP of most subjects can kill one half the viable, virulent Erdman bacilli they ingest or one half these subjects' MP can kill all the bacilli they ingest. Either way, these data are strong evidence that some normal (i.e., not immune-activated) human MP are able to kill virulent tubercle bacilli during phagocytosis.

This is an unexpected finding because (1) MP are not supposed to have any defense against tubercle bacilli until activated by immune lymphokine[4,5,7,8,12,17]; and (2) even when activated, they are supposed to be only bacteriostatic.[15] It can best be explained by assuming that some MP in a population are able to kill newly ingested bacilli but that others[2,13] are not, and it is the susceptible ones that support the development of tuberculosis at the cellular level. Apparently, all the MP of subject RM are susceptible, which probably explains why (Table II) they are all

infectable. This subject may be a human counterpart of natively BCG-susceptible mice.[41]

4. NATURE OF BACILLARY GROWTH IN CULTURED HUMAN MONOCYTIC PHAGOCYTES

Bacilli harvested from log-phase 7H9 broth cultures continue to grow exponentially in human MP. Figure 1 illustrates this with plots of

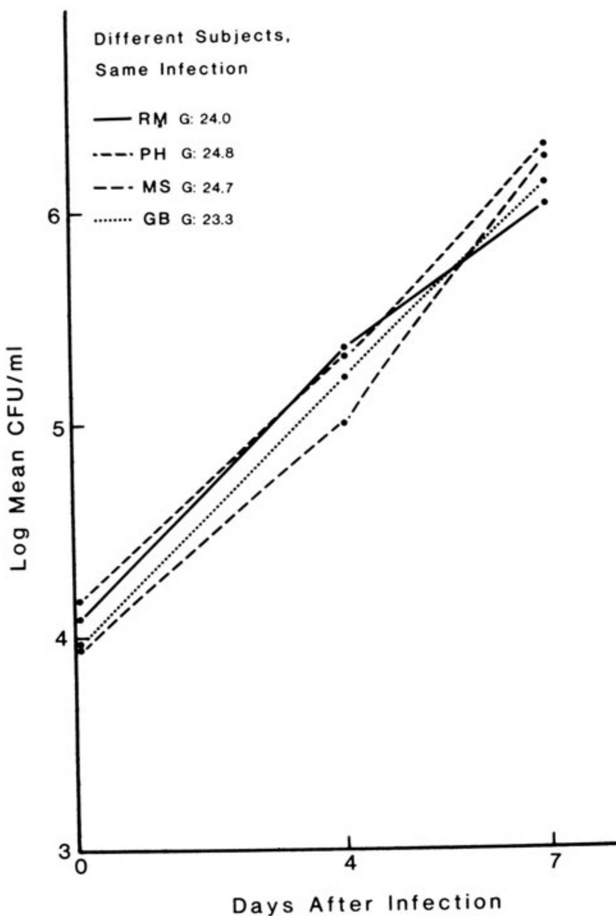

FIGURE 1. Rates of Erdman tubercle bacillus growth in cultured MP from four different normal subjects, as indicated by CFU per ml of MP lysate taken at 0 time and 4 and 7 days postinfection. Each data point is the mean of five values, with SEM usually being less than 10% of the mean. One ml of lysate was the product of an average of 1×10^5 cultured MP. *G* is bacillary generation time (hr) calculated from the mean slope of bacillary replication.

intracellular bacillary replication in four experiments using MP from different donors, all infected with the same batch of bacilli in a ratio to MP of 0.25 (lowest concentration in Table I). Mean generation times (*G*) calculated from these plots indicated intramacrophage bacillary dividing times of 23–25 hr, in agreement with results from large numbers of other experiments.

This rate is readily influenced, however, by MP microenvironment, experimental conditions, and methods of counting bacilli. The somewhat longer *G* of virulent H37Rv of 26 and 34 hr, respectively, in (human) HeLa and amniotic cells[30] might be the result of counts being made microscopically (AFB) rather than by the more sensitive counting of cultured CFU. BALB/c mouse MP in our model are more resistant than human MP, if tested in comparable conditions using medium supplemented with 1% normal mouse serum ($G = 65$ hr), but they falsely appear to be more permissive ($G = 20$ hr) in medium supplemented with 10% fetal calf serum (FCS), because this serum promotes bacillary replication. *G* values for tubercle bacilli in cultured peritoneal MP from mice estimated from published reports are 51 hr[23] or 24 hr in FCS,[7] from rabbits 31 hr[16] or 24 hr[42] and from guinea pigs approximately 24 hr.[21,42] When exponentially growing bacilli have been used for infection, intracellular growth in animal cells has been exponential,[16] just as it is in human MP. Bacillary growth is said to be exponential with $G = 24$ hr in the lungs of living mice.[43] *G* for Erdman bacilli in aerated 7H9 bacteriologic culture medium is about 16 hr (A. J. Crowle, unpublished observations).

4.1. Variations in Intracellular Bacterial Generation Times Measured by CFU and AFB Counts

Figure 2 summarizes data from a series of 16 experiments testing bleedings from one normal donor over a period of nearly 70 weeks, where *G* was determined from both AFB and CFU counts. These data are shown to illustrate the consistency of results possible by CFU counts in our model, to show that there may be significant abnormalities in G for a normal donor, and to demonstrate that AFB counts of tubercle bacilli may sometimes be quite erroneous.

Usually, *G* by CFU stayed close to its mean of 23.0 hr (±2.1 hr, SEM). However, there were some significant deviations from normal, e.g., at 61 and 67 weeks. Interestingly, the major abnormalities correlated with donor stress (graduate student oral examinations), suggesting that donor serum (1% NS supplement in the medium) can influence the resistance of human MP to tubercle bacilli. This suggestion is confirmed by other data.

In most cases, in this model and for MP from most donors, AFB-

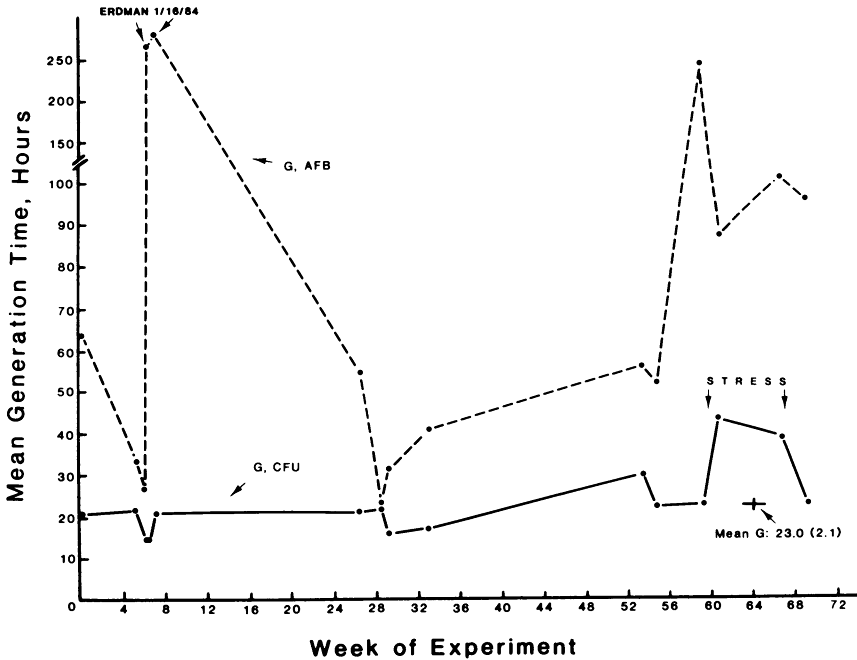

FIGURE 2. Generation times simultaneously determined in a series of experiments with MP from a normal subject (GB) by AFB and CFU counts. Three different batches of Erdman bacilli were used to infect the MP in different experiments. Batch 1/16/84, used only twice as indicated, gave large discrepancies between AFB and CFU count generation time because large proportions of the bacilli replicating within the MP were not acid-fast. "Stress" indicates a period of difficult student examinations for the subject. The mean *G* of 23.0 hr and its SEM (parentheses) were calculated from CFU values excluding those of the period of stress.

and CFU-determined *G* values correlate.[33] Figure 2 demonstrates, however, that they may disagree unequivocally by fault of the AFB counts. This may be due partly to characteristics of the infecting bacilli (e.g., batch 01/16/84 of Erdman used in the two experiments at 6 and 7 weeks only, as indicated), but more frequently is due to properties of a donor's MP that make replicating bacilli non-acid-fast. Discrepancies such as these definitively confirm previous evidence[44] that important proportions of tubercle bacilli living and multiplying in cultured human MP can be non-acid-fast and indicate that AFB counts alone are undependable enumerators of tubercle bacilli. However, such counts do still need to be made in *in vitro* experiments on human tuberculosis, because they indicate the purity of infection and the numbers and various characteristics of MP.[32] These results directly confirm various previous indirect obser-

vations[9,12,45–48] that the conventional acid-fast stain is an undependable indicator of human tuberculosis.

4.2. Effect of Original Intensity of Infection on Intramacrophage Generation Time (*G*) of Erdman Tubercle Bacilli

Early experiments with Erdman-infected cultured human MP showed that several-day periods of the infection were desirable to detect such phenomena as an expression of tuberculoimmunity[31] but also should not be extended beyond 7 days, when MP begin to be killed by accumulating loads of bacilli and adjacent MP become secondarily infected.

Figure 3 shows that this time limitation results from exponential growth of the bacilli regardless of concentration of infecting bacilli at concentrations below those causing multiple infection of single MP (bacillus to MP $\leq$ 0.5, in this model). In 7 days, one bacillus in a MP will have produced over 60 progeny, which usually are sufficient to kill the MP. Consequently, after 7 days the exponential bacterial growth curve breaks and turns downward as MP that are needed for the bacilli to grow in are killed and lost from the culture. When MP cultures are infected so as to respond as in Fig. 3, deviations from the normal (G = 24 hr) slope or from linearity are biologically meaningful indicating, for instance, expression of immunity, anti-immunity, or various responses to drugs, hormones, or lymphokines. Note that the mean G for infections of bacillus-to-MP ratios of 0.125, 0.25, and 0.5 are about the same, but shorter for the ratio of 1 (Fig. 3). The shorter G for this heavy infection probably is an artifact of either secondary infection from prematurely overloaded MP being killed and releasing bacilli to infect adjacent MP or MP–MP interstimulation, resulting from overinfection, which enhances intracellular bacillary replication.[8,34]

The normal cultured human MP can tolerate loads of 30 to 50 bacilli, 10-fold less than it will tolerate of *M. avium*[14,49] That is because tubercle bacilli are toxic for these MP, as they are for animal MP[16,19] and human myeloid monocytes,[27] and as shown morphologically. Microenvironment affects this tolerance. For instance, in 500 U/ml γ-interferon (IFN_γ) most human MP will be killed at the beginning of an experiment by just the few infecting bacilli.[34] The tolerance also is lowered by miscellaneous factors, such as the pH of the medium rising to 7.4 or higher even briefly or traces of Tween 80 surfactant present in the infection medium as carryover from culturing of the bacilli.

4.3. Effect of Strains of Bacillus

Tubercle bacillus virulence correlates partly with survival and rate of multiplication in human MP. These virulence properties, necessary

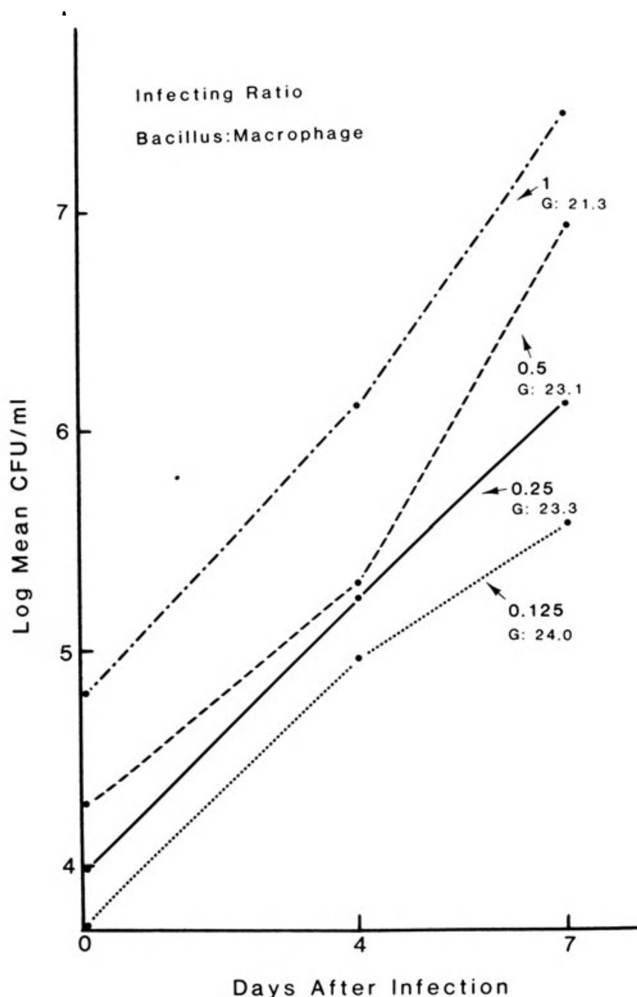

FIGURE 3. Generation times (*G*, hr) of Erdman bacilli in cultured MP from subject AC infected at the four different bacillus-to-MP ratios of 1, 0.5, 0.25, and 0.125. See Fig. 1 for further explanation of data presentation.

for tuberculosis to be established, have been reported for human cell (HeLa, amniotic cell) cultures,[28–30] animal cell cultures,[17,20] and whole-animal experiments.[4,7,41,43] They ranked six strains of BCG in agreement with their methods of virulence measurement.[40] Bacillary virulence can be increased by passaging through MP cultures.[24] A strain of BCG was produced in this way that could replicate in human MP as fast as virulent Erdman bacilli, but it remained unable to cause progressive infection of CF-1 mice.[40] This is important because it indicates that the rate of tubercle bacillus replication in normal MP is only a partial mea-

sure of bacillary virulence, which misses the additional ability to survive and possibly multiply in immune lymphokine-activated MP.[1,3–5,7,8,12]

In addition to attenuated BCG, some virulent tubercle bacilli have been compared in the human MP model. Table VIII summarizes the essential results for three strains of tubercle bacilli isolated from patients in the World Health Organization (WHO) BCG trial at Chingleput India. These were provided by Dr. Donald W. Smith and were of high, moderate, or low virulence in guinea pigs.[50] Erdman bacilli and the high virulence strain #36 behaved consistently (low SEM) and similarly (mean *G* values of 23.0 and 24.5 hr, respectively). However, the other two strains behaved inconsistently (high SEM) and were not clearly different in virulence as indicated by guinea pig testing. Their variable rates of intra-MP replication suggest low virulence, with replication rates depending on small variations of responses to them of the MP. The partial disagreement between cultured human MP and *in vivo* guinea pig evaluations of these two strains probably has at least two causes. One is the absence of mechanisms of acquired immunity in the MP analysis, and the other could be the large difference between people and guinea pigs in susceptibility to tuberculosis.

4.4. Differences in Intracellular Generation Time as Determined by AFB or CFU Methods of Counting

Figure 2 demonstrated some particular but occasional discrepancies between AFB and CFU counts. Tables IX and X describe consistent discrepancies between them, mainly indicating that significant propor-

TABLE VIII
Comparison of Generation Times (*G*, hr) in Normal Human Macrophages of Tubercle Bacilli of Different Virulence[a]

	Strain of bacillus							
	Erdman		36 (high)		50 (moderate)		44 (low)	
Experiment	CFU[b]	G	CFU	G	CFU	G	CFU	G
H 261 I[c]	8.1	22.9	9.5	24.2	17.0	27.6	5.6	23.3
H 252 I[d]	15.0	22.0	18.0	25.5	65.0	37.5	2.8	17.3
H 250 I[d]	8.5	24.0	8.7	25.3	38.0	37.5	7.3	40.9
H 249 I[d]	13.0	23.1	20.0	23.1	24.0	23.1	22.0	19.9
Mean (SEM)	23.0 (0.4)		24.5 (0.6)		31.4 (3.6)		25.4 (5.3)	

[a]Erdman compared with similar infections with strains of 36, 50, and 44 graded by experimental infection in guinea pigs[50] as high, moderate, or low virulence, respectively.
[b]CFU $\times 10^3$ per 10^5 MP at time 0, immediately postinfection.
[c]Klett 5-infection suspension (0.25 : 1 = bacillus to MP).
[d]Klett 10-infection suspension (0.5 : 1 = bacillus to MP).

tions of healthy replicating tubercle bacilli are regularly non-acid-fast in cultured human MP.

Table IX summarizes data from 81 experiments using MP from 10 different donors over a period of 3 years, showing *G* by CFU counts to be regularly less than G by AFB (means of 22.5 versus 54.6 hr), with an average ratio of 0.41, indicating that more than one half the culturable bacilli regularly were not being counted as AFB.

This difference cannot be explained by extracellularity of the uncounted AFB or by the inherent inefficiency of AFB counting, because tubercle bacilli do not multiply extracellularly in this model[32]; also, phagocytosis data show that AFB counts are able to detect considerable proportions of bacilli undetectable by CFU. It is most likely due to the non-acid-fastness of discrepant numbers of bacilli.[44,46,48] This explanation agrees with disparities between AFB and CFU counts indicating consistently higher proportions of CFU that are not acid-fast in the MP from some donors, e.g., GB and MS (Table IX) than most others. Practically, AFB counts in the MP of such donors are highly variable (note high SEM for donors GB and MS), unreliable relative to true numbers of tubercle bacilli, and potentially misleading (Fig. 2).

Since AFB/CFU discrepancies depend partly on infecting bacillus

TABLE IX
Differences in Intramacrophage Generation Time (*G*) of Erdman Bacilli Dependent on Method of Bacillary Counting: AFB versus CFU

MP donor	Number of experiments	Mean *G*[a] CFU	Mean *G*[a] AFB	Mean ratio CFU/AFB
KG	7	25.7 (1.4)	58.2 (7.9)	0.44
GB	16	23.0 (2.0)	93.9 (28.6)	0.25
JB	4	31.3 (6.8)	62.9 (24.1)	0.50
MM	12	24.8 (1.4)	34.9 (4.2)	0.71
PH	6	20.1 (1.4)	45.5 (6.2)	0.44
RM	20	19.5 (1.1)	34.1 (2.8)	0.57
DS	3	18.0 (0.6)	51.3 (5.5)	0.35
AC	6	18.2 (1.8)	38.4 (4.2)	0.47
MS	4	19.8 (1.8)	79.8 (21.8)	0.25
TB	3	24.4 (1.4)	47.2 (2.5)	0.52
Total means	81	22.5 (1.3)	54.6 (6.2)	0.41

[a]Data from experiments of all kinds, including those in which MP were being incubated in immune[8,31] or anti-immune[8,34,56] lymphokines. Only MP from donor GB were normal in all experiments performed with a single donor. These data are assembled to contrast results to the two different modes of counting rather than represent normal *G* values. Mean *G* expressed as hr (+SEM).

TABLE X
Comparison of Intramacrophage Generation (*G*) Times of Erdman Bacilli by AFB and CFU Methods of Counting in Experiments Using One Batch and Concentration of Bacilli

Experiment	MP donor	Generation times[a]		Ratio CFU/AFB
		CFU	AFB	
D-81	JB	22.5	68.4	0.33
D-82	PH	22.5	46.9	0.48
D-83	JD	16.9	86.3	0.20
D-64	DS	16.9	56.3	0.30
D-66	PH	24.8	71.3	0.34
D-67	MS	24.6	168.0	0.14
H 263 I	GB	23.3	60.0	0.39
H 264 I	RM	24.0	72.2	0.33
D-78	RM	16.5	84.4	0.20
D-72	CC	25.7	53.4	0.48
Mean, normal MP[b] (±SEM)		23.9 (0.5)	77.2 (15.6)	0.36 (0.04)
Mean, permissive MP (±SEM)		16.8 (0.1)	75.7 (7.5)	0.23 (0.03)

[a]Mean *G* calculated from CFU and AFB counts (in hours).
[b]Based on *G* from CFU counts, there are two populations of MP. Means and SEM values were calculated separately, as shown.

characteristics (see Fig. 2), Table X provides results from 10 experiments with MP from nine donors, in which this variable was eliminated using the same batch of Erdman bacilli for infection at the same ratio of 0.25 for bacilli to MP. This more consistent infection identifies two different responses of human MP to infection: one normal (mean $G = 23.9$ hr by CFU counts) and one permissive (mean $G = 16.8$ hr). This difference is due to certain effects of subjects' serum on the MP (see Section 4.5). Note that AFB counts are not sensitive enough to detect these differences.

The two mean ratios of *G* for CFU versus AFB counts in Tables IX and X agree in indicating that more than one half the culturable bacilli are not acid-fast. Differences between means for the normal and permissive MP further indicate, by the lower ratio for the permissive MP, that the fastest-growing bacilli are likely to include the greatest proportion that are not acid-fast.

In vitro, tubercle bacilli are known to be non-acid-fast for two opposite reasons[45,46,48]: (1) are injured, dying, or dead; and (2) they are in a stage of culture, usually early, lacking acid-fastness. We have previously given examples in cultured human MP of the first reason (tuber-

culoimmunity,[8,31] antimycobacterial drugs[44,51,52]). The data in Tables IX and X and other data (Fig. 2) clearly provide several examples probably related to the second reason. However, our data do not indicate whether they are not acid-fast because they are in a particular growth stage or because of the peculiarities of their intramacrophage microenvironment.

The practical value of CFU and AFB counts in this MP model is that they demonstrate MP–tubercle bacillus interactions. Another method of bacterial counting for this purpose is to measure [^{3}H]uracil incorporation by the bacilli.[53] From several experiments (unpublished), we can confirm that this method is rapid, convenient, and objective, but we have been unable to get results with it that are internally consistent or that will agree consistently with CFU counts.

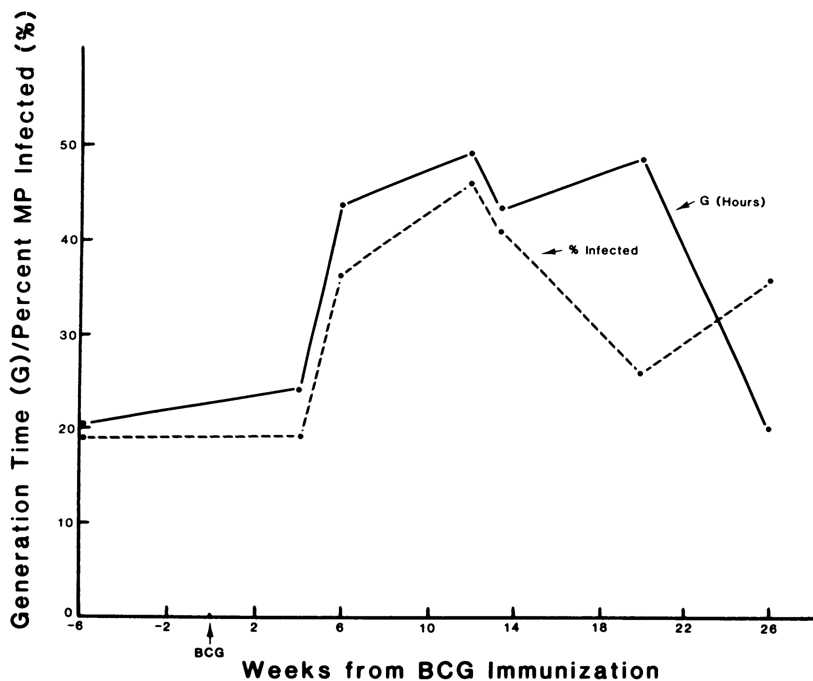

FIGURE 4. Generation times and percentages of MP initially infected for a series of experiments using MP from BCG-immunized subject KG, in which the MP were incubated in medium supplemented with 1% unheated KG serum. The response of KG's MP had been studied under the same conditions for 9 months before the beginning of these experiments and had remained stable at the value indicated for the experiment done 6 weeks before immunization.

TABLE XI
Effects of Donor Serum on Intramacrophage Growth of Virulent Tubercle Bacilli

Experiment	Donor	Generation times[a] by CFU counts		Ratio NS/SS
		NS[b]	SS	
H 257 I	RM	18.8	25.5	0.74
D-62	RJ	26.3	31.3	0.84
D-65	MS	24.0	27.8	0.86
H 259 I	RM	19.8	18.4	1.08
H 260 I[c]	RD	45.0	33.2	1.36
T-3	KG	21.4	19.7	0.92
D-66	PH	24.8	25.7	0.97
D-67	MS	24.6	39.9	0.62
D-69	MM	22.5	22.5	1.00
H 264 I	RM	24.0	26.6	0.90
D-70	RM	16.5	28.5	0.58
D-72	CC	25.7	24.6	1.04
D-73	ER	28.3	30.0	0.94
D-76	MM	27.2	28.7	0.95
D-77	DS	30.0	20.1	1.50
D-78	RJ	25.1	25.9	0.97
D-79	RM	24.0	24.6	0.98
LR-2	PH	45.6[d]	48.0	0.95
D-80	MS	24.6	21.9	1.12
H 271 I	ME	19.3	19.5	0.99
H 273 I	RM	24.8	26.8	0.93
D-81	JB	22.5	23.3	0.97
D-82	PH	22.5	27.6	0.85
D-83	JD	16.9	33.4	0.51
LR-6	NE	26.3	30.0	0.88
LR-7	AC	24.8	30.0	0.83
LR-8	DS	21.3	25.5	0.84
Mean (±SD)		23.8 (2.5)	26.2 (4.7)	0.95 (0.13)

[a]Mean G (in hr).
[b]NS = 1% normal (autologous donor) serum; SS = 10% serum substitute, to provide the same albumin concentration as 1% NS (see text).
[c]Underscored data indicate experiments with G values by CFU that are significantly different (>2 SD) from the population mean.
[d]Infection with BCG; all other experiments infected with Erdman.

4.5. Effects of Donor Serum

For technical reasons, MP in our model have usually been cultured in medium supplemented with 1% unheated autologous normal serum (NS).[32] NS is not as neutral as originally thought.

It may be protective. This was discovered in a long-term series of

experiments with BCG-immunized subjects in which the negative control group was MP incubated in medium with the 1% donor serum, i.e., NS, and as shown in Fig. 4. In Fig. 4, the mean *G* for this group is plotted for a subject (KG) relative to time of immunization. A statistically significant (comparison with mean *G* for this subject before immunization) antituberculosis resistance appeared at 6–20 weeks after immunization. This finding correlated with the development of opsonins, as shown, and with a new ability of the subject's lymphocytes to make immune lymphokine[31] when incubated with immunizing antigen (not shown). Thus, a subject's serum may affect results in this model, in this case, specifically because of containing immune-response lymphokines.

To investigate this type of finding, a serum substitute[54] was compared with autologous serum in a large number of experiments. Table XI summarizes results from 27 consecutive experiments with 15 different donors, showing that autologous serum can be protective or antiprotective even for nonimmunized subjects.

Instances of these autologous NS-mediated abnormalities are underscored. Exclusion of these from statistical analysis results in a mean *G* in NS of 23.8 hr, essentially the same as in the various other experiments

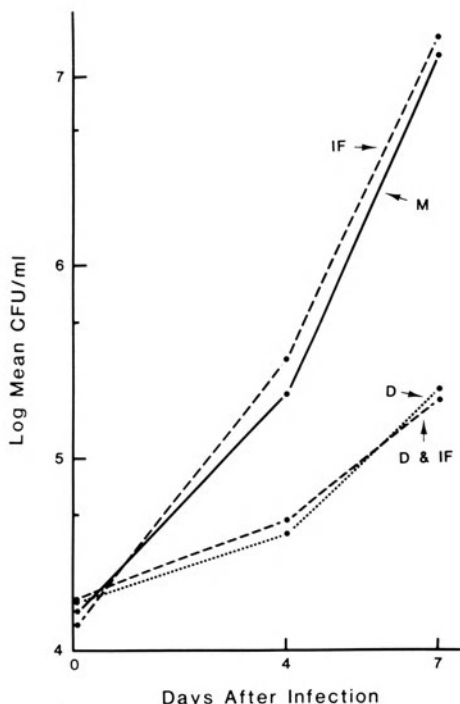

FIGURE 5. Protectiveness of 4 μg/ml 1, 25(OH)$_2$ vitamin D$_3$ against tubercle bacillus infection in MP from normal donor ME, whether used with 50 U/ml of recombinant human IFN$_\gamma$ (D and IF) or alone (D). In the two groups showing no protection, MP were incubated in medium only (M) or in IFN only (IF).

(see Fig. 2 and Table X). These abnormalities are more than 2 SD different from the normal mean of 23.8 hr.

Three of the abnormalities indicate increased resistance of the MP and two show decreased resistance. One of the former is an artifact obviously explained by the use of attenuated BCG for infection. Since it is not a serum-caused abnormality, it shows up in both NS and SS.

The specific reasons for these NS-related abnormalities are unknown, but they can be reproduced in physiologically reasonable ways. NS-mediated increased resistance can be reproduced by adding 1,25$(OH)_2$ vitamin D_3[55] to the serum and decreased resistance by adding IFN_γ.[34,56]

The recently discovered[55,57] protectiveness of 1,25$(OH)_2$vitamin D_3, the hormonally active form of vitamin D, is illustrated in Fig. 5. Table XII shows that the hormone protects in NS but not in SS. The effective concentration of 4 μg/ml (Fig. 5 and Table XII) at 10^{-5}M might seem unphysiologically high.[58] That may be due to imperfect use of the hormone in these early experiments. Additional experiments are indicating that it is effective in much smaller quantities as a cofactor for priming human MP to respond to antituberculosis immune lymphokine. The necessity of NS for 1,25$(OH)_2$ vitamin D_3 protectiveness is not yet understood but may relate to delivery of the hormone to the responding MP by plasma carriers.[55]

An antiprotective effect of IFN_γ for tubercle bacillus-infected human MP[34] can account for the accelerated replication of the intracellular bacilli in experiments D-70 and D-83 (see Table XI). The effect

TABLE XII
Antituberculosis Protectiveness of 1,25$(OH)_2$ Vitamin D_3 for Human Macrophages in Medium Supplemented with Normal Serum (NS) but Not a Serum Substitute (SS)

	Generation times (in hr), by CFU counts in					
Subject	NS	NS + D_3	Ratio $\frac{NS + D_3}{NS}$	SS	SS + D_3	Ratio $\frac{SS + D_3}{SS}$
ME	21.8 (3.2)[a]	73.2 (16.9)	3.4	20.1 (1.2)	22.7 (2.3)	1.1
LR	24.6 (1.0)	97.4	3.9	28.6 (0.8)	—	—
RJ	23.6 (2.1)	40.9	1.7	28.6 (2.7)	24.8	0.9
AC	20.9 (1.0)	31.3	1.5	29.2 (5.2)	27.8	1.0
Means	23.4 (0.9)	55.1 (13.0)	2.4 (0.5)	26.6 (2.2)	25.1 (1.5)	1.0 (0.06)

[a]Figures in parentheses are SEM for the *G* shown, where these were available from multiple experiments. Generation times without SEM are from single experiments.

is apparently due to stimulation by the lymphokine of the MP to make mycobacterial growth-enhancing factors.[8,56]

These observations and explanations of serum-factor regulation of tubercle bacillus replication in cultured human MP are more than laboratory curiosities. They directly substantiate long-suspected[59,60] connections among sunlight, diet, vitamin D, and resistance to tuberculosis. IFN_γ circulates in both pathologic[61] and physiologic[62] conditions and in high concentrations in tuberculin-positive animals following antigenic stimulation.[4,5] Thus, it could easily account for NS-caused increased MP susceptibility to tubercle bacilli, in a manner relevant to the tuberculosis-exacerbating mycobacterial growth-enhancing factors postulated by Canetti[9] to explain explosive bacterial growth in some forms of human tuberculosis, as well as some aspects of reactivation tuberculosis.[8,56]

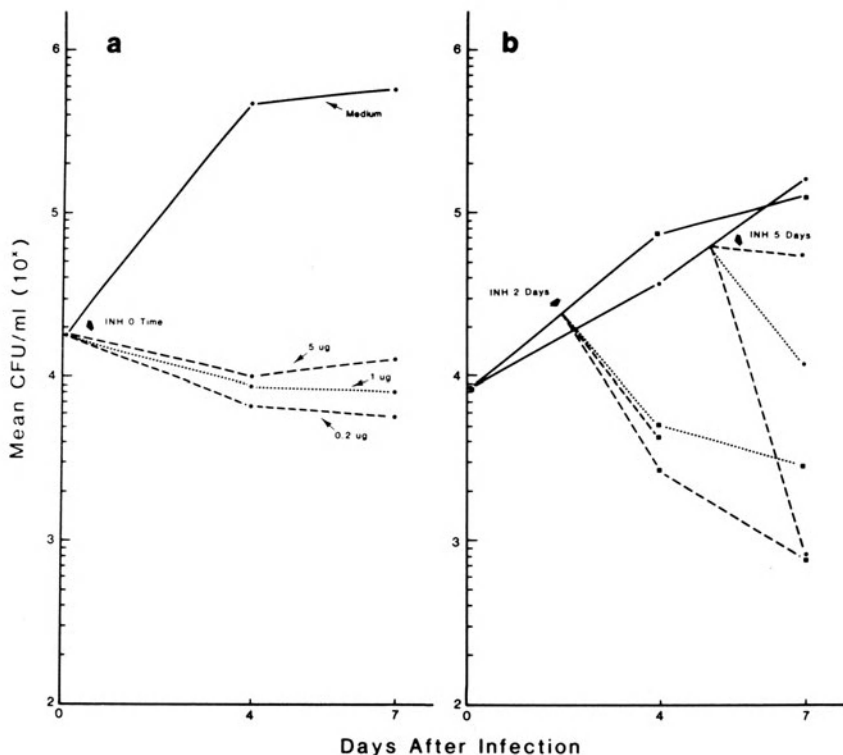

FIGURE 6. Isoniazid (INH) added to cultured human MP immediately after infection with Erdman bacilli is only mycobacteriostatic (a), even at high concentration (MIC = 0.05 μg/ml), but added at 2 or 5 days after infection is mycobactericidal (B).

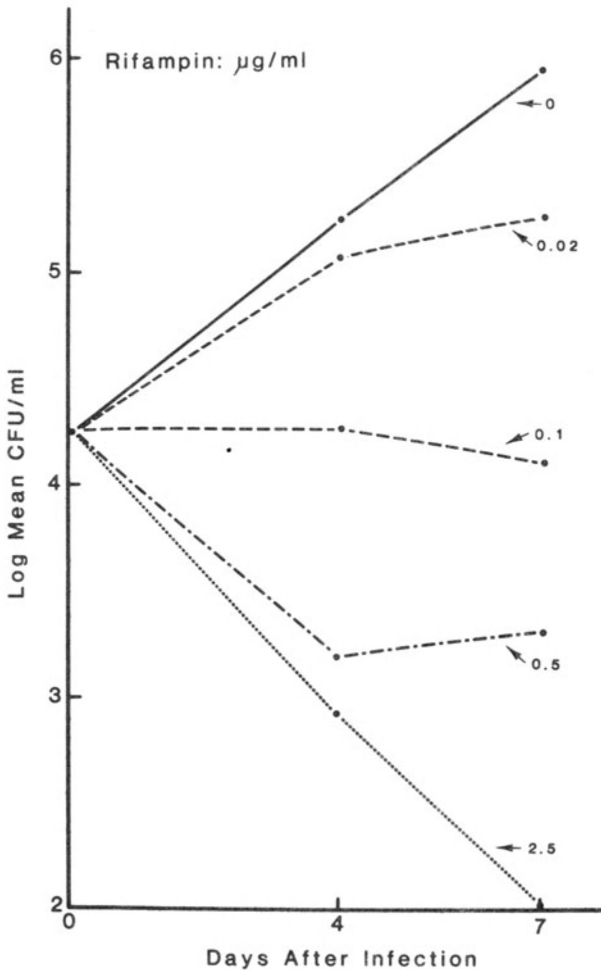

FIGURE 7. Rifampin added to cultured human MP immediately after infection is mycobacteriostatic at its minimal inhibitory concentration of 0.1 μg/ml and mycobactericidal at higher concentrations.

4.6. Effects of Antituberculosis Drugs

The effects of antituberculosis drugs are reviewed relative to the knowledge they provide about the tubercle bacillus–human host relationship. Streptomycin (SM), ethambutol (EMB), pyrazinamide (PZA), isoniazid (INH), rifampin (RIF), ceforanide, and ofloxacin have been tested against Erdman[33,44,51,52] or other[63,64] tubercle bacilli in cultured

human MP. Each drug has exhibited activity in the model remarkably similar to its clinical activity.[65]

SM suffers a 50–100-fold loss of activity.[33] This corresponds with its poorer effects *in vivo* than in bacteriologic culture and can be explained by the low pH of mycobacterium-containing phagolysosomes,[66] which greatly depresses its effectiveness.[67]

EMB seems more effective clinically than would be expected from purely bacteriologic studies, and the model reveals why. Freely entering the MP,[68] it there becomes unexpectedly effective against intracellular bacilli in exponential growth.[52] This seems due to cooperation between

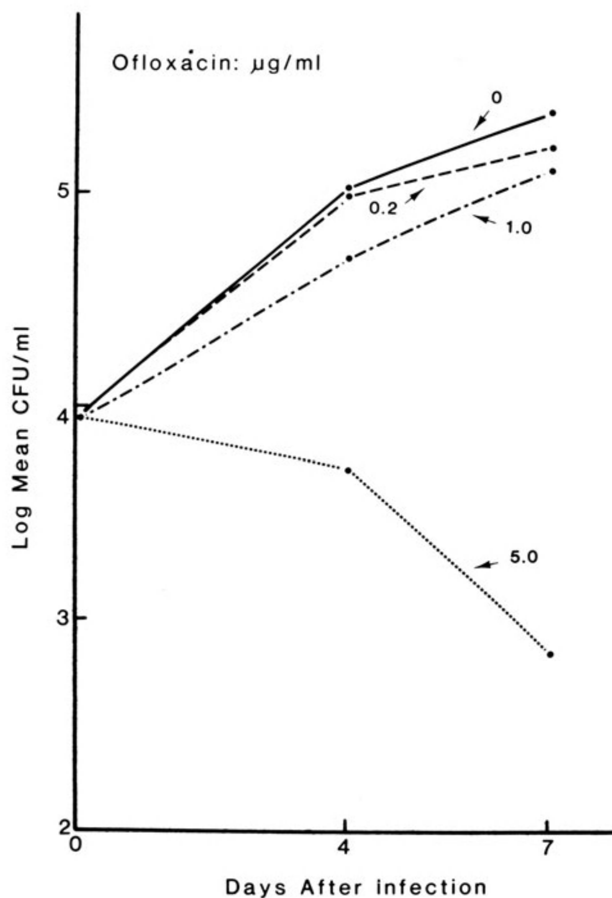

FIGURE 8. Ofloxacin, like rifampin, is mycobactericidal when added to cultured human MP immediately postinfection.

MP and EMB, in which the EMB impairs bacterial cell wall formation[46,48,69]; the wall-defective bacilli then are killed by the MP.

PZA is ineffective against tubercle bacilli in normal bacteriologic culture medium,[70] but its minimal inhibitory concentration in MP in the model is equal to the lowest effective plasma concentration in patients.[71] This confirms that tubercle bacilli are contained by MP in acidic phagolysosomes (see also Section 6) because PZA requires the acidity to be effective.[70,71]

INH is bactericidal in the test tube and freely crosses cell membranes[73] but nevertheless is unable alone to produce relapse-free cures of tuberculosis.[73] The model provided an explanation for this paradox by showing INH to inhibit its own cidal effects against intracellular bacilli that are not multiplying[75] (Fig. 6). RIF alone is able to produce relapse-free cures[76] and in the model did not interfere with its own actions, as did INH (Fig. 7).

Ofloxacin is supposed to be most effective against multiplying tubercle bacilli,[77] like INH. However, its antibacterial activity is probably not directed against primary microbial functions of replication, for it behaves more like RIF in the model than INH (Fig. 8) and does not interfere with its own mycobactericidal effectiveness.

Unlike PZA, ceforanide was known to be effective against tubercle bacilli in bacteriologic culture but not in tuberculous patients[78] (M. D. Iseman, unpublished observations). By also proving ineffective in the model, it confirmed the close correlation of the model with clinical tuberculosis.[65]

5. MORPHOLOGY OF THE TUBERCLE BACILLUS–HUMAN MONOCYTIC PHAGOCYTE RELATIONSHIP BY OPTICAL MICROSCOPY

The MP in our model are developed by 7-day incubation of plastic-adherent blood monocytes and progress through stages of differentiation that are well characterized for human monocytes: monocytes → young macrophages → macrophages → epithelioid cells → giant cells.[2,79–81] These changes and stages parallel the progression of monocyte form and function in the human body in tuberculosis.[4,9] This is important, because some stages of this progression are absent from whole animal models,[5,83] and may account for some well-known disparities between human and animal tuberculosis.[3,5]

At the beginning of an experiment, after the first 30 min of incubation in 35-mm petri dishes, the adherent monocytes form a spot, averaging 7 mm in diameter, of loosely distributed, comma-shaped cells. After

3 days' incubation, the cells begin to transform into macrophages; by 7 days, when they are infected, they are nearly all macrophages. The cells average 5×10^4 per spot (direct photomicrographic counts and indirect counts by nuclei of lysed cells), and are >98% viable by dye exclusion, 99% positive for nonspecific esterase, and 100% positive for neutral red uptake.[32,34,55] During differentiation, the cells change in ability to pro-

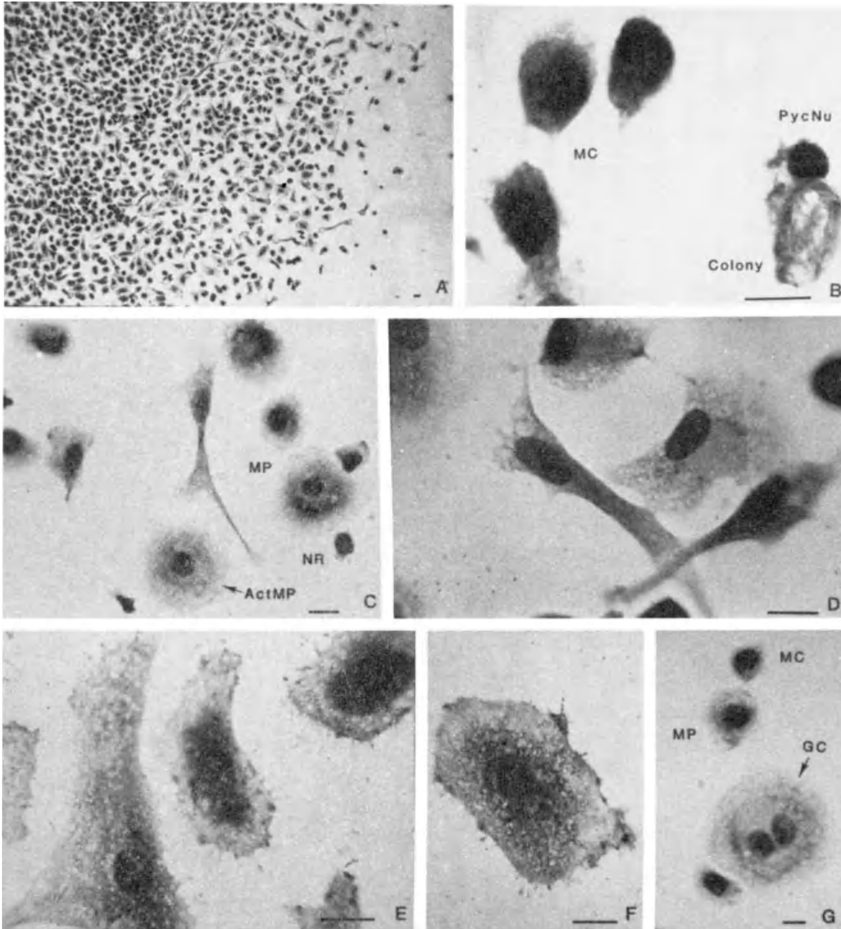

FIGURE 9. Typical microscopic appearances of cultured MP in the *in vitro* model of tuberculosis infection after 7 days of culture, immediately before infection. (A–G) Bar = 10 μm. Edge of a typical spot of adherent cells (A). MC, monocyte; PycNu, pyknotic cell nucleus (shown in B with a microcolony of tubercle bacilli); MP, macrophage; NR, nuclear remnant; ActMP, activated microphage, GC, giant cell.

duce oxidative metabolites but do not show much change in susceptibility to tubercle bacilli.[36]

Figure 9 illustrates the appearance of the cells, just before infection. They remain in their original 7-mm-diameter spot of deposition (Fig. 9A), as they do during the subsequent 7 days of infection. This finding agrees with time-lapse photomicrographic observations that they undulate and change shape, giving an appearance of migrating, but do not move much from place to place.[82]

These cells are morphologically heterogeneous (Table XIII; see also Fig. 9), corresponding closely with previous descriptions of similar cells in culture[81,82] or *in vivo*.[4,9]

A change of medium on the sixth day of culture and infection 24 hr later, as routinely done, stimulates their appearance of being activated.[2,3,12,80,82] The cells become enlarged and vacuolized, show increased numbers of cytoplasmic granules and ruffled surfaces, and develop pale-staining nuclei that contain two to three prominent nucleoli (Fig. 10A).

When the culture is infected with 0.25 bacilli per MP, about 10% of the MP become infected with one or two bacilli (Fig. 10A) that are brightly acid-fast. The progeny of these bacilli accumulate in clusters, loosely packed and not corded (Fig. 10B–D). Intracellular tubercle bacilli have generally been reported by others as noncorded (rabbit MP,[16,17] guinea pig MP[20,42]), but occasionally in corded accumulations (human monocytoid J-111 cells,[38] HeLa cells[28,29]). Bacilli that accumu-

TABLE XIII
Morphologic Types, Characteristics, and Proportion in Culture of Human MP at 7 Days and just before Infection[a]

Morphologic type	Total area[b]	Nuclear area[b]	Ratio (cytoplasm: nucleus)	Percentage of population
Nuclear remnant	52	52	0	5.2
Monocyte	154	55	0.8	5.9
Young macrophage	302	24	6.3	9.8
Mature macrophage	625	118	1.6	56.9
Stretched macrophage	525	61	7.2	7.8
Epitheloid cell	1200	74	8.3	14.4
Multinuclear giant cell	1626	64[c]	5.7	0.002

[a]Incubation as described in text in RPMI-1640 medium supplemented with 1% unheated autologous serum.
[b]Means from direct measurements (in mm^2) of calibrated photomicrographs, from the proportions of each cell type in a total of 100 cells counted.
[c]Area of one nucleus.

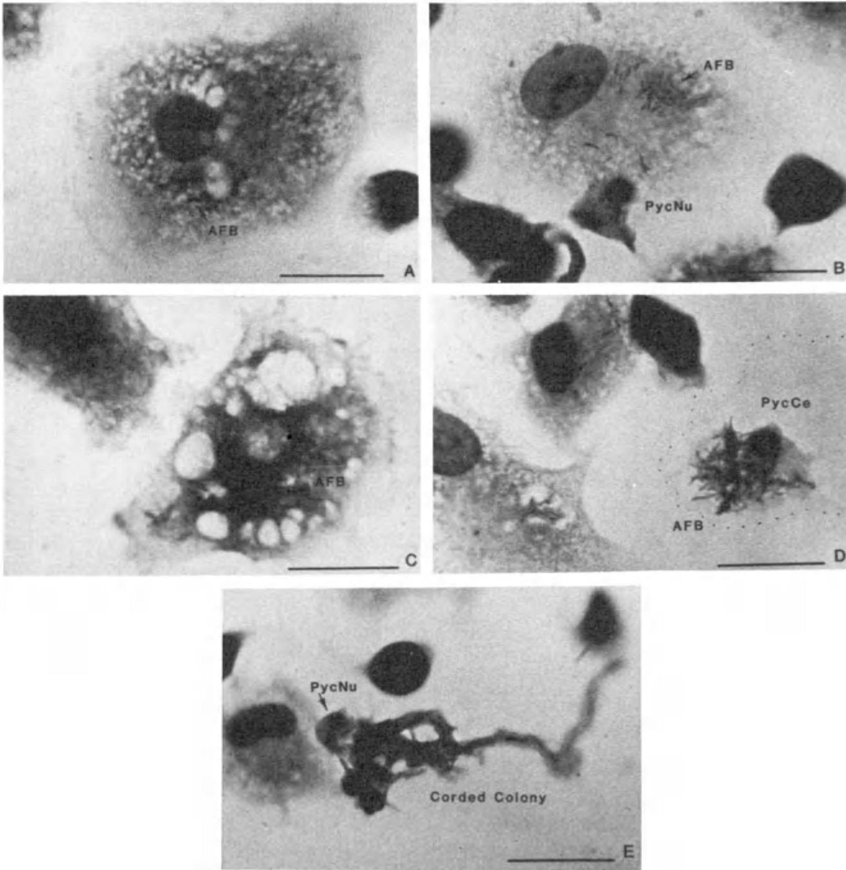

FIGURE 10. Typical microscopic appearances of cultured human MP infected with Erdman tubercle bacilli, as explained in text. (A–E) Bar = 10 μm. (D) PycCe, pyknotic cell shrunk from its original size, indicated by the dotted outline.

late in healthy large macrophages, and especially epithelioid cells,[2] tend to stain lightly acid-fast and sometimes non-acid-fast, but bacilli in small macrophages or monocytes, or in larger cells that are damaged by the infection tend to stain strongly acid-fast (Fig. 10B,D). Because the bacilli are toxic for human MP,[16,20,27,42] pyknotic cells with shrunken, darkly stained nuclei appear late in the infection[42] (Fig. 10B, D, E). Bacilli growing out from killed MP form corded microcolonies (Fig. 10E). By relative dispersion characteristics, these extracellular bacilli appear to be hydrophobic, while the noncorded intracellular bacteria appear to be hydrophilic, like *in vivo*-grown tubercle bacilli.[43]

6. MORPHOLOGY OF THE TUBERCLE BACILLUS–HUMAN MONOCYTIC PHAGOCYTE RELATIONSHIP BY ELECTRON MICROSCOPY

Figure 11 shows electron photomicrographs of this relationship corresponding to the optical photomicrographs in Fig. 10. The bacilli are

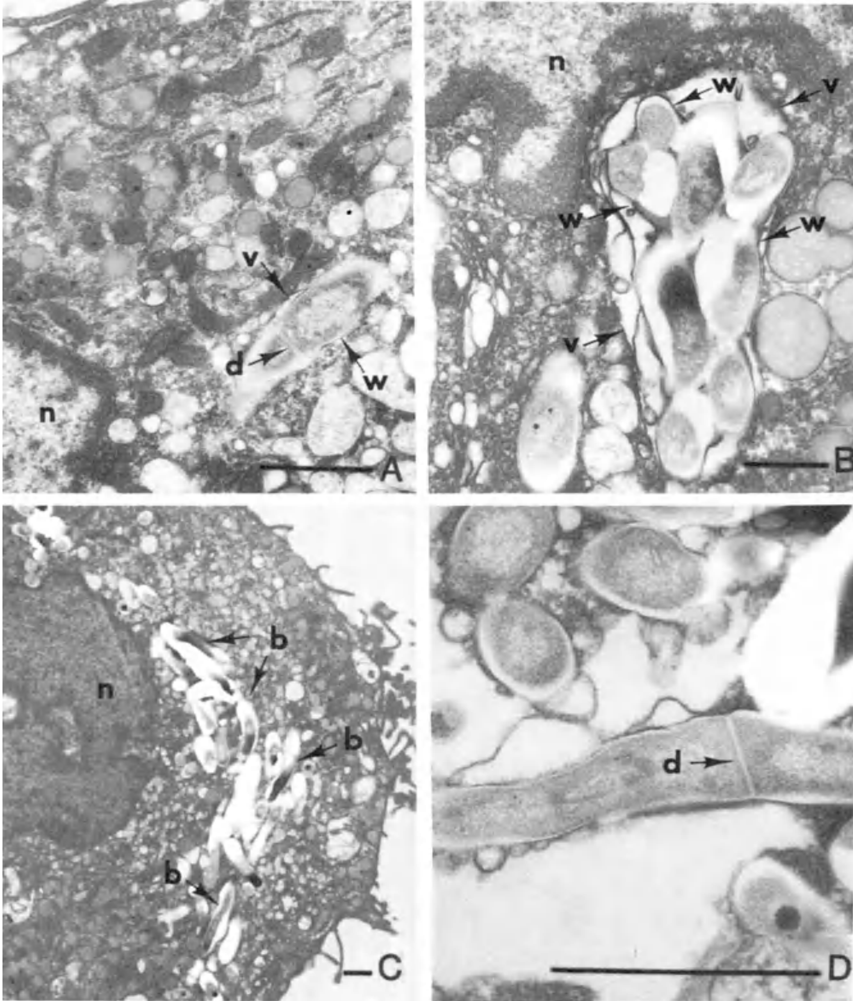

FIGURE 11. Transmission electron microscopic appearances of tubercle bacillus-infected cultured human MP. (A–D) Bar = 3 μm. v, vacuolar wall; w, bacterial cell wall; n, MP nucleus; b, tubercle bacillus; d, division of replicating bacteria. (Electron micrographs prepared by Dr. Albert E. Vatter.)

not free in MP cytoplasm; rather, they always multiply in tightly enclosing vacuoles (Fig. 11A, corresponding to Fig. 10A). They accumulate randomly but in compact masses in vacuoles that enlarge to accommodate their growth. A healthy infected cell develops two to three prominent nucleoli in its nucleus (Fig. 11B, C, corresponding to Fig. 10D). The bacilli have a tough envelope, as they are difficult to section cleanly. Their toxicity causes the breakdown of MP cytoplasm (Fig. 11D), and formation of the pyknotic cell (Fig. 10D).

These tubercle bacillus–human MP relationships may not be seen with other mycobacteria. Thus, in the same model and under the same conditions, the relationship with *M. avium* is quite different.[49] *M. avium* also replicates exponentially, but it is not toxic for the human MP, and can accumulate to as many as 10^3 coccobacilli per healthy MP. It loses its acid-fastness in these MP and replicates in vacuoles that divide with the dividing bacteria; each bacterium is surrounded in its individual vacuole by a large electron-lucent zone. Whether grown in the MP or extracellularly, *M. avium* is hydrophilic and does not form well-organized cords.

7. SYNOPSIS OF THE TUBERCLE BACILLUS–HUMAN MONOCYTIC MACROPHAGE RELATIONSHIP AS REVEALED BY THE *IN VITRO* MODEL

Human peripheral blood monocytes differentiate through young macrophages to adult macrophages in 6 days of culture. The 3-day cells are slightly less susceptible to virulent Erdman bacilli than monocytes or 7-day MP, but as populations in cultures, none of these kinds of cells can do more than slow the growth of these bacilli. The bacilli grow exponentially in the MP, doubling every 24 hr. Tubercle bacilli are readily and rapidly ingested by human MP, even in the absence of serum, requiring on average two contacts between a bacillus and a MP per ingestion. One half the healthy virulent bacilli are killed as they are ingested, but those that are not multiply in the cells they have infected until they destroy them. Varying proportions of the bacilli can be non-acid fast, as they exist and multiply in human MP.

The intracellular bacilli stay within vacuoles and accumulate in loose noncorded groups. They are toxic for MP and cause cells with an average of 30 or more to become pyknotic. This toxicity is much increased by high concentrations (500 U/ml) of IFN_γ so that just the few bacilli used for infection suffice to lyse the MP.

The MP sustain and nourish the bacilli; some lymphokines, notably IFN_γ in low concentrations (5–50 U/ml), generally enhance this nour-

ishment. But the MP can inhibit the bacilli when stimulated by other molecules such as immune lymphokine or 1,25$(OH)_2$vitamin D_3.

The effects of antimycobacterial drugs on tubercle bacilli in cultured human MP are similar both qualitatively and quantitatively to their known effects in patients.

The *in vitro* model of human tuberculosis as described here has revealed molecular and cellular details of human tuberculosis that were not previously accessible. It is hoped that this new information will materially improve the understanding of human tuberculosis and its control.

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REFERENCES

1. Briger, E. M., 1949, The host parasite relationship in tuberculous infection. II, *Tubercle* **30**:242–253.
2. Pearsall, N. N., and Weiser, R. S., 1970, *The Macrophage*, Lea & Febiger, Philadelphia.
3. Nelson, D. S., 1979, Macrophages as effectors of cell-mediated immunity, in: *Phagocytes and Cellular Immunity* (H. H. Gadebusch, ed.), pp. 57–100, CRC Press, Boca Raton, Florida.
4. Dannenberg, A. M., Jr., 1984, Chemical and enzymatic host factors in resistance to tuberculosis, in: *The Mycobacteria*, Part B (G. P. Kubica and L. G. Wayne, eds.), pp. 721–760, Dekker, New York.
5. Lagrange, P. H., 1984, Cell-mediated immunity and delayed hypersensitivity, in: *The Mycobacteria*, Part B (G. P. Kubica and L. G. Wayne, eds.), pp. 681–720, Dekker, New York.
6. Thacore, H., and Willett, H. P., 1966, The formation of spheroplasts of *Mycobacterium tuberculosis* in tissue culture cells, *Am. Rev. Respir. Dis.* **93**:786–796.
7. Youmans, G. P., 1979, *Tuberculosis*, W. B. Saunders, Philadelphia.
8. Crowle, A. J., Douvas, G. S., and May, M. H., 1983, The cellular and molecular nature of human tuberculoimmunity, *Bull. Int. Union Tuberc.* **58**:72–80.
9. Canetti, G., 1955, *The Tubercle Bacillus in the Pulmonary Lesion of Man*, Springer, New York.
10. Brown, I. N., 1983, Animal models and immune mechanisms in mycobacterial infection, in: *The Biology of the Mycobacteria*, Vol. 2 (C. Ratledge and J. Stanford, eds.), pp. 173–234, Academic, New York.
11. Bates, J. H., 1984, Transmission, pathogenesis, pathology, and clinical manifestations of tuberculosis, in: *The Mycobacteria*, Part B (G. P. Kubica and L. G. Wayne, eds.), pp. 991–1005, Dekker, New York.
12. Lowrie, D. B., 1983, Mononuclear phagocyte–mycobacterium interaction, in: *The Biol-*

ogy of the Mycobacteria, Vol. 2 (C. Ratledge and J. Stanford, eds.), pp. 235–279, Academic, New York.
13. Rook, G. A. W., 1983, An integrated view of the immunology of the mycobacterioses in guinea pigs, mice and men, in: *The Biology of the Mycobacteria*, Vol. 2 (C. Ratledge and J. Stanford, eds.), pp. 279–319, Academic, New York.
14. Brieger, E. M., 1949, The host–parasite relationship in tuberculous infection. I, *Tubercle* **30:**277–236.
15. Lefford, M. H., 1981, Immunology of *Mycobacterium tuberculosis*, in: *Immunology of Human Infection.* Part 1: *Bacteria, Mycoplasma, Chlamydiae, and Fungi* (A. J. Nahmias and R. J. O'Reilly, eds.), pp. 345–368, Plenum, New York.
16. Hsu, H. S., 1965, In vitro studies on the interactions between macrophages of rabbits and tubercle bacilli. I. Cellular basis of native resistance, *Am. Rev. Respir. Dis.* **91:**488–498.
17. Mackaness, G. B., Smith, N., and Wells, A. Q., 1954, The growth of intracellular tubercle bacilli in relation to their virulence, *Am. Rev. Tuberc.* **69:**479–494.
18. Suter, E., 1952, Multiplication of tubercle bacilli within phagocytes cultivated in vitro, and effect of streptomycin and isonicotinic acid hydrazide, *Am. Rev. Tuberc.* **65:**775–776.
19. Fong, J., Schneider, P., and Elberg, S. S., 1957, Studies on tubercle bacillus–monocyte relationship. II, *J. Exp. Med.* **105:**25–38.
20. Berthrong, M., and Hamilton, M. A., 1958, Tissue culture studies on resistance in tuberculosis. I. Normal guinea pig monocytes with tubercle bacilli of different virulence, *Am. Rev. Tuberc.* **77:**436–449.
21. Berthrong, M., and Hamilton, M. A., 1959, Tissue culture studies on resistance in tuberculosis. II. Monocytes from normal and immunized guinea pigs infected with virulent human tubercle bacilli, *Am. Rev. Tuberc.* **79:**221–231.
22. Cahall, D. L., and Youmans, G. P., 1975, Macrophage migration inhibitory activity of mycobacterial growth inhibitory factor and the effect of a number of factors on mycobacterial growth inhibitory factor activity, *Infect. Immun.* **12:**851–857.
23. Hart, P. D., and Armstrong, J. A. 1974, Strain virulence and the lysosomal response in macrophages infected with *Mycobacterium tuberculosis, Infect. Immun.* **10:**742–746.
24. Zlotnik, A., and Crowle, A. J., 1982, Lymphokine-induced mycobacteriostatic activity in mouse pleural macrophages, *Infect. Immun.* **37:**786–793.
25. Shepard, C. C., 1955, Phagocytosis by HeLa cells and their susceptibility to infection by human tubercle bacilli, *Proc. Soc. Exp. Biol. Med.* **90:**392–396.
26. Lewis, M. R., 1925, The formation of macrophages, epithelioid cells and giant cells from leukocytes in incubated blood, *Am. J. Pathol.* **1:**91–100.
27. Brosbe, E. A., Sugihara, P. Y., and Smith, C. R., 1967, Cinematographic observations of mycobacterium-infected J-111 cells, *Tex. Rep. Biol. Med.* **25:**48–64.
28. Shepard, C. C., 1957, Growth characteristics of tubercle bacilli and certain other mycobacteria in HeLa cells, *J. Exp. Med.* **105:**39–49.
29. Shepard, C. C., 1957, Use of HeLa cells infected with tubercle bacilli for the study of antituberculosis drugs, *J. Bacteriol.* **73:**494–498.
30. Shepard, C. C., 1958, A comparison of the growth of selected mycobacteria in HeLa, monkey kidney, and human amnion cells in tissue culture, *J. Exp. Med.* **107:**237–246.
31. Crowle, A. J., and May, M., 1981, Preliminary demonstration of human tuberculoimmunity in vitro, *Infect. Immun.* **31:**453–464.
32. Douvas, G. S., and Crowle, A. J., 1985, Macrophages I. Model for studying antimicrobial immunity in vitro, *Pract. Methods Clin. Immunol.* **9:**81–90.
33. Crowle, A. J., Sbarbaro, J. A., Judson, F. N., Douvas, G. S., and May, M. H., 1984, Inhibition by streptomycin of tubercle bacilli within cultured human macrophages, *Am. Rev. Respir. Dis.* **130:**839–844.

34. Douvas, G. S., Looker, D. L., Vatter, A. E., and Crowle, A. J., 1985, Gamma interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria, *Infect. Immun.* **50:**1–8.
35. Soderberg, L. S. F. and Solotorovsky, M., 1979, Host–parasite interactions with macrophages in cultured, in: *Phagocytes and Cellular Immunity* (H. H. Gadebusch, ed.), pp. 101–137, CRC Press, Boca Raton, Florida.
36. Douvas, G. S., Berger, E. M., Repine, J. E., and Crowle, A. J., 1986, Natural mycobacteriostatic activity in human monocyte-derived adherent cells, *Am. Rev. Respir. Dis.* **134:**44–48.
37. Rosenthal, S. R., 1980, *BCG Vaccine: Tuberculosis—Cancer*, PSG, Littleton, Massachusetts.
38. Brosbe, E. A., Sugihara, P. Y., and Smith, C. R., 1967, Cinematographic observations of mycobacterium-infected J-111 cells, *Tex. Rep. Biol. Med.* **25:**48–64.
39. Johnson, W. D., Jr., Mei, B., and Cohn, Z. A., 1977, The separation, long-term cultivation, and maturation of the human monocyte, *J. Exp. Med.* **146:**1613–1626.
40. Crowle, A. J., and May, M. H., 1983, Replication of lyophilized and cultured BCG in human macrophages, *Am. Rev. Respir. Dis.* **128:**673–679.
41. Gros, P., Skamene, E., and Forget, A., 1983, Cellular mechanisms of genetically controlled host resistance to *Mycobacterium bovis* (BCG), *J. Immunol.* **131:**1966–1972.
42. Suter, E., 1952, The multiplication of tubercle bacilli within normal phagocytes in tissue culture, *J. Exp. Med.* **96:**137–150.
43. Segal, W., 1984, Growth dynamics of in vivo and in vitro grown mycobacterial pathogens, in: *The Mycobacteria,* Part A (G. P. Kubica and L. G. Wayne, eds.), pp. 547–574, Dekker, New York.
44. Crowle, A. J., Sbarbaro, J. A., and May, M. H., 1986, Inhibition by pyrazinamide of tubercle bacilli within cultured human macrophages, *Am. Rev. Respir. Dis.* **134:**1052–1055.
45. Nyka, W., 1974, Studies on the effect of starvation on mycobacteria, *Infect. Immun.* **9:**843–850.
46. Imaeda, T., 1984, Cell wall deficient forms, in: *The Mycobacteria,* Part A (G. P. Kubica and L. G. Wayne, eds.), pp. 671–680, Dekker, New York.
47. Breiger, E. M., 1955, Tubercle bacilli in infected tissues grown on tissue culture, *CIBA Found. Symp.* **9:**224–233.
48. Barksdale, L., and Kim, K., 1977, Mycobacterium, *Bacteriol. Rev.* **41:**219–372.
49. Crowle, A. J., Tsang, A. Y., Vatter, A. E., and May, M. E., 1986, Comparison of 15 laboratory and patient-derived strains of *Mycobacterium avium* for ability to infect and multiply in cultured human macrophages, *J. Clin. Microbiol.* **24:**812–821.
50. Smith, D. W., Reeser, R., and Musa, S., 1985, In vitro correlates of virulence and bacillemic tendency for tubercle bacilli isolated from persons in South India, *Abst. Annu. Mtg. Am. Soc. Microbiol.* **85:**25.
51. Crowle, A. J., Sbarbaro, J. A., Iseman, M. D., and May, M. H., 1988, Effects of isoniazid and of ceforanide against virulent tubercle bacilli in cultured human macrophages, *Tubercle* (in press).
52. Crowle, A. J., Sbarbaro, J. A., Judson, F. N., and May, M. H., 1985, The effect of ethambutol on tubercle bacilli within cultured human macrophages, *Am. Rev. Respir. Dis.* **132:**742–745.
53. Rook, G. A., and Rainbow, S., 1981, An isotope incorporation assay for the antimycobacterial effects of human monocytes, *Ann. Immunol.* **132D:**281–289.
54. Stevenson, H. C., Schlick, E., Griffith, R., Chirigos, M. A., Brown, R., Conlon, J., Kanapa, D. J., Oldham, R. K., and Miller, P., 1984, Characterization of biological response modifier release by human monocytes cultures in suspension in serum-free medium, *J. Immunol. Methods* **70:**245–256.

55. Crowle, A. J., Ross, E. J., and May, M. H., Inhibition by 1,25 $(OH)_2$-vitamin D_3 of the multiplication of virulent tubercle bacilli in cultured human macrophages, *Infect. Immun.* **55:**2945–2950.
56. Crowle, A. J., 1987, Certain lymphokines stimulate human macrophages to support the growth of tubercle bacilli, in: *Proceedings of the International Symposium on Advances in Immunization* (H. Hanssen, ed.) (in press).
57. Rook, G. A. W., Steele, J., Fraher, L., Barker, S., Karmali, R., O'Riordan, J., and Stanford, J., 1986, Vitamin D_3, gamma interferon, and control of proliferation of *Mycobacterium tuberculosis* by human monocytes, *Immunology* **57:**159–163.
58. Gray, T. K., and Cohen, M. S., 1985, Vitamin D, phagocyte differentiation and immune function, *Surv. Immunol. Res.* **4:**200–212.
59. Holick, M. F., 1986, Sunlight and skin: Their role in vitamin D nutrition for humans, in: *Nutrition and the Skin* (D. A. Roe, ed.), pp. 15–44, Liss, New York.
60. Davies, P. D. O., 1985, A possible link between vitamin D deficiency and impaired host defence to *Mycobacterium tuberculosis, Tubercle* **66:**301–306.
61. O'Kelly, P., Thomsen, L., Tilles, J. G., and Cesario, T., 1985, Inactivation of interferon by serum and synovial fluids, *Proc. Soc. Exp. Biol. Med.* **178:**407–411.
62. Baba, T., Ochiya, T., Yamaguchi, K., Baba, A., Takase, T., and Uyeno, K., 1986, Monocyte activating factor in sarcoidosis. I. Existence of the factor in sarcoidosis sera, *Clin. Immun. Immunopathol.* **39:**24–36.
63. Carlone, N. A., Acocella, G., Cuffini, A. M., and Forno-Pizzoglio, M., 1985, Killing of macrophage-ingested mycobacteria by rifampicin, pyrazinamide, and pyrazynoic acid alone and in combination, *Am. Rev. Respir. Dis.* **132:**1274–1277.
64. Liss, R. H., 1982, Bactericidal activity of ethambutol against extracellular *Mycobacterium tuberculosis* and bacilli phagocytized by human alveolar macrophages, *S. Afr. Med. J. (Spec. Issue)* **62:**15–19.
65. Crowle, A. J., 1986, Studies of antituberculosis chemotherapy with an in vitro model of human tuberculosis, *Semin. Resp. Infect.* **1:**262–264.
66. Sprick, M. G., 1956, Phagocytosis of *M. tuberculosis* and *M. smegmatis* stained with indicator dyes, *Am. Rev. Tuberc.* **74:**552–565.
67. Eagle, H., Levy, M., and Fleischman, R., 1952, The effect of the pH of the medium on the antibacterial action of penicillin, streptomycin, chloramphenicol, terramycin, and bacitracin. *Antibiot. Chemother.* **2:**63–75.
68. Johnson, J. D., Hand, W. L., Francis, J. B., King-Thompson, N., and Corwin, R. W., 1980, Antibiotic uptake by alveolar macrophages, *J. Lab. Clin. Med.* **95:**429–439.
69. Winder, F. G., 1982, Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of the mycobacteria, in: *The Biology of the Mycobacteria,* Vol. 1 (C. Ratledge and J. Stanford, eds.), pp. 354–441, Academic, New York.
70. Butler, W. R., and Kilburn, J. O., 1982, Improved method for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide, *J. Clin. Microbiol.* **16:**1106–1109.
71. Fox, W., And Mitchison, D. A., 1975, Short-course chemotherapy for pulmonary tuberculosis, *Am. Rev. Respir. Dis.* **111:**325–353.
72. Bass, J. B., Jr., Farer, L. S., Hopewell, P. C., and Jacobs, R. F., 1986, Treatment of tuberculosis and tuberculosis infection in adults and children, *Am. Rev. Respir. Dis.* **134:**355–363.
73. Dickinson, J. M., Aber, V. R., and Mitchison, D. A., 1977, Bactericidal activity of streptomycin, isoniazid, rifampin, ethambutol, and pyrazinamide alone and in combination against *Mycobacterium tuberculosis, Am. Rev. Tuberc.* **116:**627–635.
74. Fox, W., 1981, Whither short-course chemotherapy, *Br. J. Dis. Chest* **75:**331–357.
75. Schaefer, W. B., 1954, The effect of isoniazid on resting tubercle bacilli, *Am. Rev. Tuberc.* **69:**125–127.

76. Snider, D. E., Graczyk, J., Bek, E., and Rogowski, J., 1984, Supervised six-months treatment of newly diagnosed pulmonary tuberculosis using isoniazid, rifampin and pyrazinamide with and without streptomycin, *Am. Rev. Respir. Dis.* **130:**1091–1094.
77. Tsukamura, M., Nakamura, E., Yoshi, S., and Amano, H., 1985, Therapeutic effect of a new antibacterial substance ofloxacin (DL 8280) on pulmonary tuberculosis, *Am. Rev. Respir. Dis.* **131:**352–356.
78. Heifets, L., Iseman, M. D., Cook, J. L., Lindholm-Levy, P. J., and Drupa, I., 1985, Determination of in vitro susceptibility of *Mycobacterium tuberculosis* to cephalosporins by radiometric and conventional methods, *Antimicrob. Agents Chemother.* **27:**11–15.
79. Schrek, R., 1958, Slide-chamber method to measure sensitivity of cells to toxic agents, *Arch. Pathol. Lab. Med.* **66:**569–576.
80. Sutton, J. S., and Weiss, L., 1966, Transformation of monocytes in tissue culture into macrophages, epithelioid cells, and multinucleated giant cells, *J. Cell. Biol.* **28:**303–332.
81. Vernon-Roberts, B., 1972, *The Macrophage*, Cambridge University Press, London.
82. Rabinowitz, Y., and Schrek, R., 1962, "Monocytic" cells of normal blood, Shilling and Naegeli leukemia, and leukemic reticuloendothelieosis in slide chambers, *Blood* **20:**453–470.
83. Ridley, D. S., 1983, The histopathological spectrum of the mycobacterioses, in: *The Biology of the Mycobacteria*, Vol. 2 (C. Ratledge and J. Standord, eds.), pp. 129–172, Academic, New York.

6

Regulation and Pharmacology of Granulomatous Inflammation

VERNON L. MOORE

1. INTRODUCTION

Granulomatous inflammation consists of collections of macrophages, epithelioid macrophages, and sometimes lymphocytes around a nidus such as the tubercle bacillus, *Schistosoma mansoni* eggs, or other foreign material (usually particulate). This type of inflammation can be classified according to cell type, organization, whether it exhibits low turnover or high turnover, or whether it is nonimmunologic (foreign body) or immune based. All these aspects have been described in recent reviews.[1,2] An important example of granulomatous inflammation is tuberculosis, for which the pathology has also been extensively described. In tuberculosis, the development of granulomatous inflammation is a host response to the tubercle bacillus that is probably beneficial in augmenting the killing of *Mycobacterium tuberculosis* and in sequestrating the organisms to prevent dissemination. However, in tuberculosis as well as other granulomatous diseases, granulomatous inflammation is clearly a two-edged sword, since it also results in extensive inflammation, necrosis, calcification, and fibrosis. The maximal development of granulomatous

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inflammation in tuberculosis is probably dependent on inflammation induced by the development of delayed hypersensitivity to antigens of the tubercle bacillus. Indeed, it is this immune response that is primarily responsible for producing activated macrophages responsible for controlling the growth and pathogenesis of *M. tuberculosis.*

In addition to being important as a mechanism of host resistance in tuberculosis, granulomatous inflammation has other important pathologic roles. In schistosomiasis (*mansoni*), granulomatous inflammation plays an important and morbid role in the development of hepatic fibrosis and portal hypertension.[3,4] In fact, granulomatous inflammation in general has a tendency to develop into fibrosis, and it is this fibrosis that constitutes the important component of several important diseases, e.g., idiopathic pulmonary fibrosis, sarcoidosis, hypersensitivity pneumonitis, and the sclerosis of glomerulonephritis.

From another point of view, granulomatous inflammation has been the focus of the interrelationship between the immune response and inflammation. In particular, many years of investigation on models of granulomatous inflammation induced in animal models by *S. mansoni* or the bacillus Calmette–Guérin (BCG) strain of *M. bovis* have demonstrated that the immune response is essential for the maximal development of this type of inflammation. These studies in animal models have also shown that granulomatous inflammation is under genetic control and is regulated by suppressor T cells in ways similar to the immune response to more simple antigens.

Since several excellent reviews have already been prepared on the morphologic and functional aspects of granulomatous inflammation,[1–3] the present chapter reviews the mechanisms that regulate this type of inflammation. In addition, the major aspects of what is known about the pharmacologic control of granulomatous inflammation are summarized, focusing on the information obtained from animal models of granulomatous inflammation. The principal animal models that have been used are as follows: (1) murine schistosomiasis (mansoni); (2) BCG-induced granulomatous inflammation in rabbits and mice, using either killed BCG or BCG cell walls; and (3) models of host resistance to BCG and related intracellular pathogens in mice.[3–7] The last topic is covered much more thoroughly in Chapter 9, this volume.

2. MURINE SCHISTOSOMIASIS AS A MODEL OF GRANULOMATOUS INFLAMMATION

Schistosomiasis is an infection afflicting approximately 200 million people and is thus one of the most important infectious diseases, particularly in third-world countries. The pathogenesis of schistosomiasis (*man-*

soni) involves the development of granulomatous inflammation primarily in the liver and lungs. Inflammation is due to the development of delayed hypersensitivity to antigens in eggs deposited by the worms.[4] This leads to granulomatous inflammation that results in fibrosis and portal hypertension, the major cause of morbidity in the disease. Several years ago, Warren[4] used a model of murine schistosomiasis (*mansoni*) to show that the granulomatous inflammation that developed was dependent on the genesis of delayed hypersensitivity to antigens released from the eggs. The evidence for this was obtained by cell and serum transfer studies, neonatal thymectomy, and pharmacology. Thus, the development of an accelerated granulomatous response is immunospecific, e.g., whereas mice sensitized with either *S. mansoni* or *Ascaris suis* eggs developed accelerated granulomatous inflammation when challenged with specific antigen, they failed to do so when challenged with the irrelevant antigen. Also, the capacity to develop accelerated granulomatous inflammation was transferable with spleen cells from specifically sensitized mice but could not be transferred with immune serum. Other studies showed that T lymphocyte deficient mice (neonatal thymectomy or *nu/nu* nude) could not develop maximal granulomatous inflammation. Collectively, these data indicate that T cells and not serum antibodies are involved in the production of *S. mansoni*-induced granulomatous inflammation in mice. Later studies have shown that the lymphokine, migration inhibitory factor (MIF), which may be involved in delayed hypersensitivity, is produced by sensitized T cells from animals with schistosomiasis.[9]

Another interesting and important phenomenon in murine schistosomiasis (*mansoni*) is termed modulation.[4] Even in the presence of large worm burdens, granulomatous inflammation subsides. The phenomenon has been shown to be due to the development of a subset of T suppressor cells that are I-J^{+}.[10] First, modulation can be transferred with spleen cells, but not with serum, from animals with subsiding lesions to animals with progressive lesions.[11] The cells that mediate modulation are Lyt 2^{+} I-J^{+}, sensitive to low doses of cyclophosphamide and to adult thymectomy.[10,12,13] Also, transfer of modulation requires compatibility at the *H-2 I-J* locus.[14] Cells of the same phenotype can suppress MIF production *in vitro* by spleen cells from *S. mansoni*-infected mice.[9] This is an important system of immunomodulation that has been demonstrated both *in vitro* and *in vivo*. It may limit the progressive development of granulomatous inflammation into fibrosis or the extent of fibrosis, when it occurs. Undoubtedly, other types of immunoregulation occur but have not been studied extensively. It is known, for example, that anti-idiotypic antibodies appear 2–3 weeks after the onset of disease in mice; they may play a role in immunoregulation.[15] Also, soluble immune-response suppressor (SIRS) is present in the serum of mice 20

weeks postinfection and is released from isolated granulomas *in vitro*.[16] This molecule could also play a role in immunoregulation in this model of granulomatous inflammation.

3. GRANULOMATOUS INFLAMMATION INDUCED BY KILLED BCG OR BY BCG CELL WALLS

During the 1960s, Myrvik *et al.*[17,18] showed that the IV injection of killed BCG (suspended in light mineral oil) caused intense granulomatous inflammation in the lungs of rabbits. Subsequent studies showed that sensitized rabbits developed accelerated granulomatous inflammation, that the development of accelerated granulomas was immunospecific,[19,20] and that cells from the lungs of rabbits with granulomas were inhibited from migrating onto glass surfaces in the presence of (PPD).[21] The migration inhibition reaction is generally considered an *ex vivo* test for delayed hypersensitivity. Other studies from that laboratory showed that cells from inflamed lungs produced a migration inhibitory factor for normal rabbit macrophages similar physically to classic migration inhibitory factor (MIF).[22] Thus, killed BCG can produce granulomatous inflammation in rabbit lungs that appears to be dependent on delayed hypersensitivity for its maximal expression.

During the late 1970s, granulomatous pulmonary inflammation induced by BCG was developed in inbred mice in order to study further the role of immunity as well as the genetics and immunoregulation of this response.[5] Investigators demonstrated that whereas certain strains of inbred mice (C57BL/10, C57BL/6) developed intense granulomatous pulmonary inflammation, other strains (CBA, C3H) were low responders.[5,23] In the high responders, marked splenomegaly occurred, reaching values as high as 10 times normal. Investigations using T-lymphocyte-depleted animals showed that responsiveness in C57BL/6 mice was dependent on T cells.[24] The generation of responsiveness turned out to be complex and multigenic. However, genes linked to both the *H-2* and *Igh* complex seemed to influence the development of granulomatous inflammation.[25]

Another study showed that low-responder CBA/J mice could be converted to high responders by pretreatment with cyclophosphamide.[26] Cyclophosphamide at certain doses and given prior to antigen seems to eliminate suppressor T cells preferentially. In these studies, the cyclophosphamide effect was reversible with splenic T cells from BCG-sensitized mice. Thus, in this system, at least one type of low responsiveness occurs because of the development of a population of suppressor T cells that limit the development of granulomatous inflamma-

tion. This is similar to the modulation of granulomatous inflammation in murine schistosomiasis (*mansoni*) by suppressor T cells (see Section 2). Also, granulomatous pulmonary inflammation induced in mice by BCG cell walls can be upmodulated by treatment with cyclophosphamide.[27] (Studies on granulomatous inflammation induced by BCG cell walls are discussed below.) Therefore, in three experimental systems, the development of T-suppressor cells plays a major role in controlling the extent of granulomatous inflammation. Although speculative, these immunoregulatory cells may also play a key role in controlling the development of fibrosis as a sequelae to granulomatous inflammation.

Although anergy is discussed in Chapters 8 and 9, this volume, high-responder mice injected with killed BCG develop peripheral anergy as assessed by several parameters: (1) they do not develop delayed hypersensitivity to PPD when challenged in the footpad,[28] (2) spleen cells from these mice do not respond well to PPD or mitogen,[28] and (3) high-responder mice immunized with sheep erythrocytes are suppressed in their ability to synthesize antibodies.[28] This situation is similar to that in human diseases such as tuberculosis and sarcoidosis, in which there is peripheral anergy when localized or systemic granulomatous inflammation is present. One possible role for anergy in the control of granulomatous inflammation is by active suppressor mechanisms. Support for this idea stems from studies showing that spleen cells from high responder, anergic C57BL/6 mice produce factors *ex vivo* that suppress both BCG-induced granulomatous inflammation and delayed hypersensitivity to sheep erythrocytes[29] and by another study which showed that anergy was mediated by adherent cells, presumably macrophages.[30] More studies are obviously needed in this area.

The genetics of BCG-induced anergy is also of interest. Anergy is influenced by both *H-2* and *Igh* genes in the mouse and, at least in the strains studied, these appear to be the only genes involved.[31,33] Although not known, it is possible that the development of anergy involves intermediate suppressor T cells that are involved in the genesis of suppressor macrophages. It would be interesting to study the development of granulomatous inflammation in mice that are high responders and nonanergic to determine whether they develop progressive and fibrotic inflammation. Interestingly, Yamamoto and Kakinuma[6] reported that SJL mice are high responders to BCG cell walls and respond to PPD peripherally.

Japanese investigators have also studied granulomatous pulmonary inflammation in mice induced by BCG cell walls.[6,27,33–36] Their results parallel those done using killed BCG, in several ways. The strain distribution patterns of responsiveness are similar, i.e., C57BL/6 mice are high responders; C3H/He and CBA mice are low responders.[6] Also,

C57BL/6 high-responder mice are anergic to PPD, but C3H low-responder mice are responsive to PPD peripherally.[6] These workers have also shown that T cells are required for maximal development of granulomatous inflammation[35] and that cyclophosphamide treatment augments the development of granulomatous inflammation.[27]

However, in addition to the above findings, this group has provided important new information on the pathogenesis of granulomatous inflammation. They demonstrated that treatment with cyclophosphamide augmented granulomatous inflammation not only in low-responder C3H/He mice but also in high-responder C57BL/6 mice as well.[27] Thus, it is likely that cyclophosphamide-sensitive cells, perhaps T-suppressor cells, modulate the granulomatous response even in high-responder animals. In addition, these investigators showed that the difference between high and low responsiveness is apparently unrelated to the distribution of BCG cell walls, since [^{125}I]-BCG cell walls did not accumulate preferentially in the lungs of C57BL/6 high-responder mice.[35]

Using radiation chimeras, Kakinuma *et al.*[35] showed that low responders could be converted into high responders with high-responder bone marrow. Also high responders become low responders when reconstituted with low-responder bone marrow. These studies demonstrate that bone marrow-derived cells are essential for the expression of the low- or high-responder genotype. This group of investigators have also related the high-responder/low-responder genotype to host defense.[34] Pulmonary macrophages from high-responder C57BL/6 mice were more efficient in killing both *Mycobacterium bovis* and *Listeria monocytogenes* compared with macrophages from low-responder C3H/He mice. Pulmonary macrophages from high-responder mice also had increased oxygen consumption and were inhibited from migrating onto glass surfaces in the presence of PPD. These results suggest that granulomatous inflammation in the lungs of high-responder mice is associated with the development of delayed hypersensitivity, which in turn activates macrophages for host defense.

4. GENETIC CONTROL OF HOST RESISTANCE TO BCG

Although this topic is fully discussed in Chapter 3, this volume, this group has done important work relating the genetic control of resistance to later development of granulomatous inflammation and immune-based host resistance.[7,37] The *BCG* locus controls the early phase of resistance to *M. bovis* (strain BCG Montreal). In mice that inherit the *Bcg*^{s} (BCG-sensitive) gene, there is rapid growth of BCG in the spleen

and liver for approximately 3 weeks. After 3 weeks, the growth of bacteria wanes due to the development of granulomatous inflammation. This later development of host resistance in association with granulomatous inflammation is correlated with the appearance of splenomegaly and delayed hypersensitivity to PPD. Most mouse strains that inherent the *Bcg*^{r} (BCG-resistant) allele do not develop delayed hypersensitivity, splenomegaly, or granulomatous inflammation. Apparently, expression of the *Bcg*^{r} gene is sufficient to control bacterial growth without the development of delayed hypersensitivity and granulomatous inflammation. The phenotypic expression of this gene is unknown, but it has been suggested that it is an intracellular bacteriostatic mechanism.[7] This gene may regulate resistance to a wide variety of intracellular parasites and is thus crucial in host resistance. Presumably, this mechanism also is operative in humans. It is possible that individuals who inherit this gene can control certain infections sufficiently without developing granulomatous inflammation with its possible deleterious effects.

5. PHARMACOLOGIC CONTROL OF GRANULOMATOUS INFLAMMATION

Control of granulomatous disease by drugs is important, since this is the primary means of modulating this type of inflammation. The classes of agents in use for chronic inflammatory disease that involve granulomatous inflammation are corticosteroids, cyclooxygenase inhibitors (aspirinlike drugs), putative disease modifiers (D-penicillamine and gold), cytotoxic agents, and immunoregulants. 5-Lipoxygenase inhibitors, histamine-2 (H_2)-receptor antagonists, and angiotensin-converting enzyme inhibitors are being evaluated in animal models. In general, the development of pharmacologic agents to control or cure this type of inflammation has been unsatisfactory. Corticosteroids are highly effective anti-inflammatory agents, but their toxic side effects prevent their widespread use. Aspirinlike drugs constitute the primary treatment for such diseases as rheumatoid arthritis and osteoarthritis. However, it is widely believed that their main effects are alleviation of pain and edema, without effects on the prevention of erosion of cartilage and subchondral bone. D-Penicillamine and gold salts are effective in a percentage of cases of rheumatoid arthritis but also have toxic side effects. The first authentic immunoregulants to be used in chronic inflammatory disease are niridazole and cyclosporin A. Niridazole is the drug of choice for schistosomiasis[38] (*mansoni*) and seems to inhibit delayed hypersensitivity preferentially by an unknown mechanism.[39–41] Cyclosporin A is being used to prevent transplantation rejection and is being tested

clinically in a wide variety of human chronic inflammatory diseases. Its toxicity may limit its widespread and chronic use. A great deal of time and effort is being spent by major pharmaceuticals manufacturers to develop novel, effective, and safe anti-inflammatory agents. These efforts hold much promise for the control and even cure of chronic inflammatory diseases. The remainder of this chapter briefly discusses each of the above categories of pharmacologic agents.

Cyclooxygenase inhibitors are the initial drugs used in rheumatoid and osteoarthritis. They are effective analgesics and reduce edema. However, rheumatoid arthritis is mediated and perpetuated by immunologic mechanisms. Scattered reports state that cyclooxygenase inhibitors suppress delayed hypersensitivity, but definitive studies are lacking. Also, no definitive studies have been done on the effect of cyclooxygenase inhibitors on delayed hypersensitivity in humans. In experimental animals, cyclooxygenase inhibitors have been shown to augment immune responses, presumably by preventing the synthesis of prostaglandin E_2 (PGE_2), which enhances the activity of suppressor T cells.[42] In practical application, this effect of cyclooxygenase inhibitors on augmentation of delayed hypersensitivity may be minimal, since these pharmacologic agents have been used chronically for years in humans without gross observation of enhancement of immune responses.[42]

Corticosteroids are the classic anti-inflammatory agents. They have multiple effects on the inflammatory response, but the contribution of each of these pathways on each individual inflammatory response is unknown.[43] Corticosteroids are effective immunoregulatory agents, particularly in delayed hypersensitivity, where they seem to work by inhibiting the synthesis of interleukin-2 (IL-2).[43] They also suppress the synthesis of IL-1, which may contribute to their anti-inflammatory effects. Corticosteroids induce a family of glycoproteins known as lipomodulin or lipocortin. These molecules prevent phospholipase A_2 from liberating arachidonic acid from membrane phospholipids. Since arachidonic acid is the substrate for both cyclooxygenase and lipoxygenases, corticosteroids act as dual inhibitors of the synthesis of prostaglandins (including thromboxanes) and leukotrienes. Numerous reports ascribe the anti-inflammatory effects of corticosteroids to this mechanism. However, it is by no means certain that this is the major mechanism of action of corticosteroids. Although corticosteroids have antiedema properties similar to those of cyclooxygenase inhibitors, they are not analgesic in models of pain in which cyclooxygenase inhibitors are highly effective, i.e., acetic acid-induced writhing in mice.[44] It is possible that the analgesic effects of corticosteroids in chronic inflammation are secondary to the prevention of cellular infiltration into sites of

inflammation.[43] Although the mechanism of action of corticosteroids as anti-inflammatory agents is important, their toxic side effects prevent their widespread chronic usage.

D-Penicillamine and gold salt are secondary disease-modifying agents used in rheumatoid arthritis. Studies on their mechanism of action suggest that D-penicillamine works by inhibiting macrophage function and that the mode of action of gold compounds as an inflammatory agent is by suppression of T lymphocytes.[44] Because of their inconsistent beneficial effects as well as their toxicity, novel effective and safe therapeutic agents are needed.

Although corticosteroids are immunoregulants, they also have other major anti-inflammatory effects. Niridazole was probably the first authentic immunoregulant used in chronic inflammatory disease. This is the drug of choice for schistosomiasis (*mansoni*).[38] In humans, this agent suppresses delayed hypersensitivity to PPD, mumps, and schistosomal antigens.[38,39] In experimental murine schistosomiasis (*mansoni*), niridazole suppresses delayed hypersensitivity to *Schistosoma mansoni* egg antigens, allograft rejection, and the generation of cytotoxic T cells.[40,41] It seems to have minimal effects on antibody synthesis. Niridazole is a prodrug and is not active as an immunoregulant in *in vitro* systems. One of its active forms is 1-thiocarbamoyl-2-imidazolidinone (TCI), which is found in mouse and human blood after niridazole is given systemically.[40,41] TCI is reported to be extremely potent as a systemic immunoregulant.[40,41] In order to achieve such potency, this compound must be highly specific (receptor antagonist or enzyme inhibitor?) and must have very favorable pharmacokinetics by the oral route. The parent compound, niridazole, is much less potent by the oral route (~100 mg/kg).[40,41] It is surprising that neither niridazole nor TCI has been tried in other major chronic inflammatory diseases in human subjects.

Cyclosporin A is another authentic immunoregulatory drug that is currently receiving much attention. Although developed as a drug to ameliorate transplantation rejection, it is being tried clinically in a wide spectrum of chronic inflammatory diseases. Its mode of action is complex, but a major pathway of inhibition seems to be suppression of IL-2 synthesis.[45] Its toxicity may prevent widespread chronic usage.

Experimentally, H_2 receptor antagonists, i.e., cimetidine and ranitidine, seem to augment immune responses.[46] Mechanistically, this probably occurs because suppressor T cells (or at least certain subpopulation of these cells) bear H_2-receptors. This raises the possibility that H_2-agonists would be effective immunoregulants. In general, this approach may not be feasible because of side effects such as excess acid secretion by cells in the gastrointestinal (GI) tract. This effect may not have impor-

tant clinical application, since patients have been on drugs such as cimetidine and ranitidine for long periods without general evidence of immunoenhancement.

Angiotensin-converting enzyme (ACE) inhibitors (captopril, enalapril) have been developed for the suppression of hypertension. Studies in man suggested that patients with sarcoidosis and certain other granulomatous diseases had elevated blood levels of ACE.[47] Experimental animal studies in BCG-induced granulomatous inflammation and schistosomiasis (*Schistosoma mansoni*) showed elevated ACE levels either in bronchoalveolar lavage fluid or in serum.[48,49] Treatment of animals with high doses of captopril not only reduced ACE levels but suppressed granulomatous inflammation as well.[48] The possible relationship of ACE to chronic inflammation is unclear. However, recent studies from our laboratory, using a bioavailable compound closely related to enalapril and exhibiting potent inhibitory activity toward ACE, did not show suppression of BCG-induced granulomatous inflammation in mice (J. Metzger and V. Moore, unpublished data). We are dubious about the use of ACE inhibitors in chronic inflammation.

There is a great deal of interest presently about the use of 5-lipoxygenase inhibitors as inflammatory agents. LTB_4 is a potent chemotactic factor for neutrophils, causes increased adherence of these cells to endothelium, and activates them for increased metabolic activity by interaction with specific cellular receptors. LTC_4 and LTD_4 have potent action on airways and blood vessels that results in bronchoconstriction and increased vascular permeability. Kunkel *et al.*[50] assessed the effects of 5-lipoxygenase inhibitors on *Schistosoma mansoni*-induced granulomatous inflammation. Using nordihydroguarietic acid (NDGA) and nafazatrom as 5-lipoxygenase inhibitors by intraperitoneal injection, these workers showed that granulomatous inflammation was suppressed. Treatment with these compounds also suppressed Ia expression and lowered the levels of LTC_4 and 5-HETE *in vivo*. NDGA was mostly selective for lowering of 5-lipoxygenase products. Although nafazatrom lowered the levels of both 5-lipoxygenase and cyclooxygenase products, indomethacin (which only lowered cyclooxygenase products) did not suppress granulomatous inflammation and augmented Ia expression. Although not known, it is possible that these drugs inhibited granulomatous inflammation by lowering Ia expression and affecting antigen presentation. It should also be stressed that both NDGA and nafazatrom are antioxidants that could be affecting the ability of reactive oxygen products to mediate inflammation. As more specific 5-lipoxygenase inhibitors are produced, answers to these questions could be forthcoming.

6. SUMMARY

Although the current status of drug therapy in chronic inflammation is not satisfactory, several strategies appear to be promising for the development of effective and safe drugs. By contrast, some strategies that are being pursued do not appear promising.

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REFERENCES

1. Adams, D. O., 1976, The granulomatous inflammatory response—A review, *Am. J. Pathol.* **84:**164–191.
2. Garrett, K. C., Richerson, H. B., Hunninghake, G. W., 1984, Pathogenesis of granulomatous lung diseases. II. Mechanisms of granuloma formation, *Am. Rev. Respir. Dis.* **130:**447–483.
3. Boros, D. L., 1978, Granulomatous inflammation, *Prog. Allergy* **24:**184–267.
4. Warren, K. S., 1980, The relevance of schistosomiasis, *N. Engl. J. Med.* **303:**203–206.
5. Moore, V. L., Schrier, D. J., Sternick, J. L., and Allen, E. M., 1980, Genetic control of granulomatous inflammation, in: *Basic and Clinical Aspects of Granulomatous Diseases* (D. Boros and T. Yoshida, eds.), pp. 201–214, Elsevier–North-Holland, Amsterdam.
6. Yamamoto, K., and Kakinuma, M., 1978, Genetic control of granuloma response to oil-associated BCG cell wall vaccine in mice, *Microbiol. Immunol.* **22:**335–348.
7. Skamene, E., Gros, P., Forget, A., Kongshavn, P. A. L., St. Charles, C., and Taylor, B. A., 1982, Genetic regulation of resistance to intracellular pathogens, *Nature (Lond.)* **297:**506–509.
8. Phillips, S. M., DiConza, J. J., Gold, J. A., and Reid, W. A., 1977, Schistosomiasis in the congenitally athymic (nude) mouse. I. Thymic dependency of eosinophilic, granuloma formation and host morbidity, *J. Immunol.* **118:**594–599.
9. Chensue, S. W., Boros, D. L., and David, C. S., 1980, Regulation of granulomatous inflammation in murine schistosomiasis. *In vitro* characterization of T lymphocyte

subsets involved in the production and suppression of migration inhibition factor, *J. Exp. Med.* **151:**1398–1412.

10. Chensue, S. W., Wellhausen, S. R., and Boros, D. L., 1981, Modulation of granulomatous hypersensitivity. II. Participation of Ly1$^+$ and Ly2$^+$ T lymphocytes in the suppression of granuloma formation and lymphokine production in *Schistosoma mansoni*-infected mice, *J. Immunol.* **127:**363–367.
11. Colley, D. G., 1976, Adoptive suppression of granuloma formation, *J. Exp. Med.* **143:**696–700.
12. Colley, D. G., Lewis, F. A., and Todd, C. W., 1979, Adoptive suppression of granuloma formation by T lymphocytes and by lymphoid cells sensitive to cyclophosphamide, *Cell. Immunol.* **46:**192–196.
13. Colley, D. G., 1981, T lymphocytes that contribute to the immunoregulation of granuloma formation in chronic murine schistosomiasis, *J. Immunol.* **126:**1465–1468.
14. Green, W. F., and Colley, D. G., 1981, Modulation of *Schistosoma mansoni* egg-induced granuloma formation: I–J restriction of T cell-mediated suppression in a chronic parasitic infection, *Proc. Natl. Acad. Sci. USA* **78:**1152–1156.
15. Powell, M. R., and Colley, D. G., 1985, Demonstration of splenic auto—Anti-idiotypic plaque-forming cells in mice infected with *Schistosoma mansoni*, *J. Immunol.* **134:**4140–4145.
16. Aune, T. M., Freeman, C. L., Jr., and Colley, D. G., 1985, Production of the lymphokine soluble immune response suppressor (SIRS) during chronic experimental schistosomiasis mansoni, *J. Immunol.* **135:**2768–2771.
17. Myrvik, Q. N., Leake, E. S., and Fariss, B., 1961, Studies on pulmonary alveolar macrophages from the normal rabbit: A technique to procure them in a high state of purity, *J. Immunol.* **86:**128–132.
18. Myrvik, Q. N., Leake, E. S., and Oshima, S., 1962, A study of macrophages and epithelioid-like cells from granulomatous (BCG-induced) lungs of rabbits, *J. Immunol.* **89:**745–751.
19. Kawata, H., Myrvik, Q. N., and Leake, E. S., 1964, Dissociation of tuberculin hypersensitivity as mediator for an accelerated pulmonary granulomatous response in rabbits, *J. Immunol.* **93:**433–438.
20. Moore, V. L., Myrvik, Q. N., and Leake, E. S., 1973, Specificity of a BCG-induced pulmonary granulomatous response in rabbits, *Infect. Immun.* **7:**743–746.
21. Galindo, B., and Myrvik, Q. N., 1970, Migratory response of granulomatous alveolar cells from BCG-sensitized rabbits, *J. Immunol.* **105:**227–237.
22. Moore, V. L., and Myrvik, Q. N., 1974, Inhibition of normal rabbit alveolar macrophages by factor(s) resembling migration inhibition factor, *J. Reticuloendothel. Soc.* **16:**21–26.
23. Allen, E. M., Moore, V. L., and Stevens, J. O., 1977, Strain variation in BCG-induced chronic pulmonary inflammation in mice. I. Basic model and possible genetic control by non-H-2 genes, *J. Immunol.* **119:**343–347.
24. Sternick, J. L., Allen, E. M., Ripani, L. M., Schrier, D. J., and Moore, V. L., 1981, BCG-induced granulomatous pulmonary inflammation and splenomegaly in mice: A T cell-dependent response, *Cell. Immunol.* **65:**211–220.
25. Sternick, J. L., Schrier, D. J., Moore, V. L., 1983, Genetic control of BCG-induced granulomatous inflammation in mice, *Exp. Lung Res.* **5:**217–228.
26. Moore, V. L., Mondloch, V. M., Pedersen, G. M., Schrier, D. J., and Allen, E. M., 1981, Strain variation in BCG-induced chronic pulmonary inflammation in mice: Control by a cyclophosphamide-sensitive thymus-derived suppressor cell, *J. Immunol.* **127:**339–342.
27. Yamamoto, K., Kato, K., Kakinuma, M., and Brehmer, W., 1982, Cellular regulation of lung granuloma formation and delayed-type hypersensitivity induced with BCG cell

walls. Further evidence of involvement of cellular immunity in lung granuloma formation, *Zentralbl. Bakteriol. Mikrobiol. Hyg. [A]* **251**:357–368.
28. Allen, E. M., and Moore, V. L., 1979, Suppression of phytohemagglutinin and lipopolysaccharide responses in mouse spleen cells by bacillus Calmette–Guérin, *J. Reticuloendothel. Soc.* **26**:349–356.
29. Allen, E. M., Sternick, J. L., Schrier, D. J., and Moore, V. L., 1981, BCG-induced chronic pulmonary inflammation and splenomegaly in mice: suppression of PHA-induced proliferation, delayed hypersensitivity to sheep erythrocytes, and chronic pulmonary inflammation by soluble factors from adherent spleen cells, *Cell. Immunol.* **58**:61–71.
30. Schrier, D. J., Allen, E. M., and Moore, V. L., 1980, BCG-induced macrophage suppression in mice: Suppression of specific and non-specific antibody-mediated and cellular immunologic responses, *Cell. Immunol.* **56**:347–356.
31. Schrier, D. J., Sternick, J. L., Allen, E. M., and Moore, V. L., 1982, Immunogenetics of BCG-induced anergy in mice: Control by genes linked to the *Igh* complex, *J. Immunol.* **128**:1466–1469.
32. Callis, A. H., Schrier, D. J., David, C. S., and Moore, V. L., 1983, Immunogenetics of BCG-induced anergy in mice. Control by *Igh*- and *H-2*-linked genes, *Immunology* **49**:509–516.
33. Kakinuma, M., Onoé, K., Yasumizu, R., and Yamamoto, K., 1983, Strain differences in lung granuloma formation in response to a BCG cell-wall vaccine in mice. Failure of antigen presentation by low-responder macrophages, *Immunology* **50**:423–431.
34. Kato, K., Yamamoto, K., Okuyama, H., and Kimura, T., 1984, Microbicidal activity and marphological characteristics of lung macrophages in *Mycobacterium bovis* BCG cell wall-induced lung granuloma in mice, *Infect. Immun.* **45**:325–331.
35. Kakinuma, M., Onoé, K., Okada, M., Kimura, T., Kato, K., Okuyama, H., Morikawa, K., and Yamamoto, K., 1981, Failure of C3H mice to develop lung granuloma after intravenous injection of BCG cell wall vaccine. Demonstration of a defect in lymphoid cells, *Immunology* **43**:1–9.
36. Kakinuma, M., and Yamamoto, K., 1985, Strain differences in lung granuloma formation in response to a BCG cell-wall vaccine in mice. Demonstration of two types of low responders, *Immunology* **55**:91–95.
37. Bourassa, D., Forget, A., Pelletier, M., Skamene, E., and Turcotte, R., 1985, Cellular immune response to *Mycobacterium bovis* (BCG) in genetically-susceptible and resistant congenic mouse strains, *Clin. Exp. Immunol.* **62**:31–38.
38. Webster, L. T., Jr., Butterworth, A. E., Mahmoud, A. A. F., Mngola, E. N., and Warren, K. S., 1975, Suppression of delayed hypersensitivity in schistosome-infected patients by niridazole, *N. Engl. J. Med.* **292**:1144–1147.
39. Daniels, J. C., Fajordo, I., and David, J. R., 1975, Two stages in lymphocyte mediator production by differential susceptibility to blockade using niridazole, *Proc. Natl. Acad. Sci. USA* **72**:4569–4597.
40. Tracy, J. W., Kazura, J. W., and Webster, L. T., Jr., 1982, Suppression of cell-mediated immune responses *in vivo* and *in vitro* by 1-thiocarbamoyl-2-imidazolidinone, Immunopharmacology **4**:187–200.
41. Gautman, S. C., Scissors, D. L., and Webster, L. T., Jr., 1982, Further observations on the effects of 1-thiocarbamoyl-2-imidazolidinone (TCI) on cell-mediated immunity, *Immunopharmacology* **4**:201–212.
42. Goodwin, J. S., and Ceuppens, J., 1983, Regulation of the immune response by prostaglandins, *J. Clin. Immunol.* **3**:295–314.
43. Claman, H. N., 1984, Anti-inflammatory effects of conticosteroids, *Clin. Immunol. Allergy* **4**:317–329.
44. Nakamura, H., Mizushima, Y., Seto, Y., Motoyoshi, S., and Kadokawa, T., 1985, Dex-

amethasone fails to produce antipyretic and analgesic actions in experimental animals, *Agents Actions* **16:**542–547.
44. Lipsky, P. E., 1983, Remission-inducing therapy in rheumatoid arthritis, *Am. J. Med.* **75**(suppl. 48)**:**40–49.
45. Shevach, E. M., 1985, The effects of cyclosporin A on the immune system, *Annu. Rev. Immunol.* **3:**397–423.
46. Rocklin, R. E., 1982, Modulation of inflammatory and immune responses by histamine, in: *Immunopharmacology* (P. Sirois and M. Rola-Pleszczynski eds.), pp. 49–74, Elsevier Biomedical Press, New York.
47. Silverstein, E., Friedland, J., Lyons, H. A., and Gourin, A., 1976, Markedly elevated angiotensin-converting enzyme in lymph nodes containing non-necrotizing granulomous in sarcoidosis, *Proc. Natl. Acad. Sci. USA* **73:**2137–2141.
48. Schrier, D. J., Ripani, L. M., Katzenstein, A. L., and Moore, V. L., 1982, Role of angiotensin-converting enzyme in Bacille Calmette–Geúrin-induced granulomatous inflammation. Increased angiotensin-converting enzyme levels in lung lavage and suppression of inflammation with captopril, *J. Clin. Invest.* **69:**651–657.
49. Weinstock, J. V., Ehrinpreis, M. N., Boros, D. L., and Gee, J. B., 1981, Effect of SQ 14225, an inhibitor of angiotensin 1-converting enzyme, on the granulomatous response to *Schistosoma mansoni* eggs in mice, *J. Clin. Invest.* **67:**931–936.
50. Kunkel, S. L., Chensue, S. W., Mouton, C., and Higashi, G. I., 1984, Role of lipoxygenase products in murine pulmonary granuloma formation, *J. Clin. Invest.* **74:**514–524.

7

Cytolytic Cells in *M. tuberculosis* Infections

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GENNARO DE LIBERO

1. INTRODUCTION

Tuberculosis is a bacterial infectious disease that, left untreated, often takes a chronic course.[1] To a great extent, this chronicity is due to the fact that the etiologic agents, *Mycobacterium tuberculosis, M. bovis,* and *M. africanum* have developed means to survive or even grow in one of the host's most potent effector cells, the mononuclear phagocytes. These cells are particularly specialized to engulf, kill, and degrade invading microorganisms, yet at first sight may not look like an attractive habitat for microbial living. Thus, the evasion mechanisms used by pathogenic mycobacteria must be highly effective. Although these mechanisms have hitherto not been fully elucidated, it is generally believed that they are manifold, including (1) resistance to reactive oxygen metabolites and lysosomal enzymes, (2) inhibition of phagosome–lysosome fusion, and (3) perhaps evasion into the cytoplasm.[2]

Because of their intracellular habitat, pathogenic mycobacteria are relatively well shielded from humoral defense mechanisms. However, during intracellular replication, some bacterial antigens become accessible to host cell processing and are subsequently expressed on the surface of the infected cells. These bacterial antigens can now be identified by

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mycobacteria-specific precursor T cells triggered to proliferate and differentiate into effector T cells. Effector T lymphocytes will then set into motion appropriate defense mechanisms. Although there is no doubt that T cells are the crucial mediators of antimycobacterial immunity and that the steps described lead to the acquisition of antimycobacterial resistance, little is known about the fine mechanisms and the T-cell sets involved.

1.1. Existence of Two Distinct T-Cell Types

It is now a generally accepted dogma of cellular immunology that T cells recognize foreign antigens within the context of self-molecules encoded by genes of the major histocompatibility complex (MHC) rather than by reacting with native foreign molecules directly. Furthermore, recent studies have provided compelling evidence that the T-cell lineage can be divided into two major sets on the basis of phenotype and antigen-recognition pattern: T cells that bear the CD4 phenotype recognize antigen plus class II MHC molecules, whereas T cells of the CD8 phenotype react with antigen plus class I MHC molecules.[3] Furthermore, current evidence suggests that the CD4 and CD8 molecules on T cells may be directly involved in antigen recognition and that they interact with a monomorphic structure of class II or class I molecules, respectively. The CD4 and CD8 molecules, as well as the restricting MHC elements, have been identified in the murine and human system. The CD4 molecule is seen by anti-T4 antibodies in human and anti-L3T4 antibodies in mice, while anti-T8 antibodies in humans and anti-Lyt2 antibodies in mice define the CD8 molecule. Class II molecules are encoded by the HLA-D/DR, DQ, DP loci of the human and by the H-2 I-A, I-E loci of the murine MHC. The HLA-A, -B, and -C loci of the human and the H-2 K, and L loci of the murine MHC encode the class I molecules.

1.2. Existence of Two Major Effector Functions of T Cells

Although exceptions exist, there seems to be a predilection of the two T-cell sets for different functions. Class II-restricted CD4 T lymphocytes preferentially induce or activate novel effector functions in their target cells, hence are often referred to as helper/inducer T cells. Class I-restricted CD8 T lymphocytes, by means of direct cell–cell contact, preferentially lyse their target cells, hence are often designated cytolytic T cells. It has been postulated that the division of the T-cell lineage into two separate sets has developed as a strategy for the efficient combat of intracellular infectious agents.

Cytolytic T lymphocytes should be adequate for limiting the spreading of obligate intracellular pathogens by destroying infected target cells. Viruses represent the typical proponents of this type of pathogens, since they require living host cells for their replication. Furthermore, class I molecules are expressed by virtually all nucleated cells. Because viruses can infect different types of host cells, antigen recognition in the context of class I molecules by CD8 T lymphocytes of cytolytic type should permit unrestricted scanning of all host cells for viral infections.

By contrast, the activation of effector mechanisms should be adequate for defense against facultative intracellular bacteria, which can survive and often even multiply in the extracellular compartment. Mycobacteria can be seen as the typical proponents of this type of pathogens. They are located within mononuclear phagocytes, which possess a high antibacterial potential (i.e., in which bacteriocidal functions can be induced by lymphokines) and which express class II antigens. Thus, infected macrophages can be recognized and activated by CD4 T cells of helper/inducer type.

1.3. T-Cell Sets and Functions Relevant to Acquired Resistance against Tuberculosis

While the scheme described above undoubtedly has great value, situations can be envisaged in which neither of these two functions alone is sufficient for effective elimination of the infectious agent. Such a scenario could occur, for example, if a facultative intracellular pathogen is resistant against novel effector functions induced in cells with high antibacterial potential or if the pathogen resides in cells with low antibacterial potential. Under these circumstances, activation of infected host cells by lymphokines from helper/inducer T cells would not be sufficient for the effective elimination of pathogens and would depend on additional mechanisms.

It appears that in tuberculosis, such cases can indeed occur. Certain types of tissue macrophages, e.g., Kupffer cells, have a low antibacterial potential and are relatively refractory to activation by T-cell-derived lymphokines. Early after systemic infection, however, most bacteria are trapped within these cells.[4] Furthermore, it appears that in granulomas, multinucleated giant cells contain mycobacteria that they fail to destruct.[5] *M. tuberculosis* has developed multiple evasion mechanisms, rendering this pathogen resistant even to activated macrophage functions, which may suffice for destruction of other intracellular pathogens possessing less complex evasion mechanisms. It is therefore easily conceivable that activated macrophages, while succeeding in destroying less resistant intracellular bacteria such as *Listeria monocytogenes* or *Legionella pneumophila,* will fail to do so with *M. tuberculosis.* Recently, we have even

found heterogeneous susceptibility toward activated macrophages within the species *M. tuberculosis*.[6] In this study, γ-interferon (IFN_{γ})-activated macrophages could inhibit growth of *M. bovis* and *M. tuberculosis* H37Rv but not of *M. tuberculosis* Middelburg.

We have therefore considered the possibility that in tuberculosis, besides helper/inducer T cells, cytolytic T cells are required for effective protection. This chapter describes the generation of class I-restricted CD8 T-cell lines and clones from *M. tuberculosis*-immune mice, which are capable of lysing mycobacteria-primed macrophages in an antigen-specific and class I-restricted manner. We assume that these data provide primary evidence that CD8 T cells with cytolytic activity participate in the immune response against *M. tuberculosis*. This does not exclude a role for helper/inducer T cells in the defense against tuberculosis. Rather, we would like to suggest that a synergistic interplay between these two T-cell sets and functions is needed for effective combat of tuberculosis and other intracellular bacterial infections.

2. RESULTS

2.1. Establishment of CD8 T-Cell Lines with Cytolytic Activity from *M. tuberculosis*-Immune Mice

C57BL/6 mice were immunized subcutaneously (SC) with killed *M. tuberculosis* H37Ra in Freund's complete adjuvant or, alternatively, were infected intravenously (IV) with 10^6 live *M. bovis* strain BCG Phipps organisms. After 8–14 days, draining lymph nodes or spleens, respectively, were collected and single cell suspensions prepared. Cells were cultured in the presence of killed *M. tuberculosis* or *M. bovis*, respectively, and accessory cells and interleukin-2 (IL-2)-containing supernatant. After 8 days, the vast majority of these cells were $Thyl^+$, $L3T4^-$, and Lyt2+, as judged by analysis with the fluorescence-activated cell sorter. A representative cell phenotype profile is shown in Fig. 1. Native lymph node cells from *M. bovis*-infected mice have a CD4:CD8 ratio of >1. Thus, this short-term culture resulted in a preferential expansion of CD8 T cells.

Because the CD8 phenotype is primarily associated with target cell lysis, we wanted to assess whether these T cells were capable of lysing macrophages expressing mycobacterial antigens. In the model of experimental infection of mice with *L. monocytogenes,* we have already shown that CD8 T cells are capable of lysing infected macrophages in an antigen-specific way.[7,8] In this system, we found bone marrow macrophages to be well suited as target cells. After 9–14 days of culture in hydro-

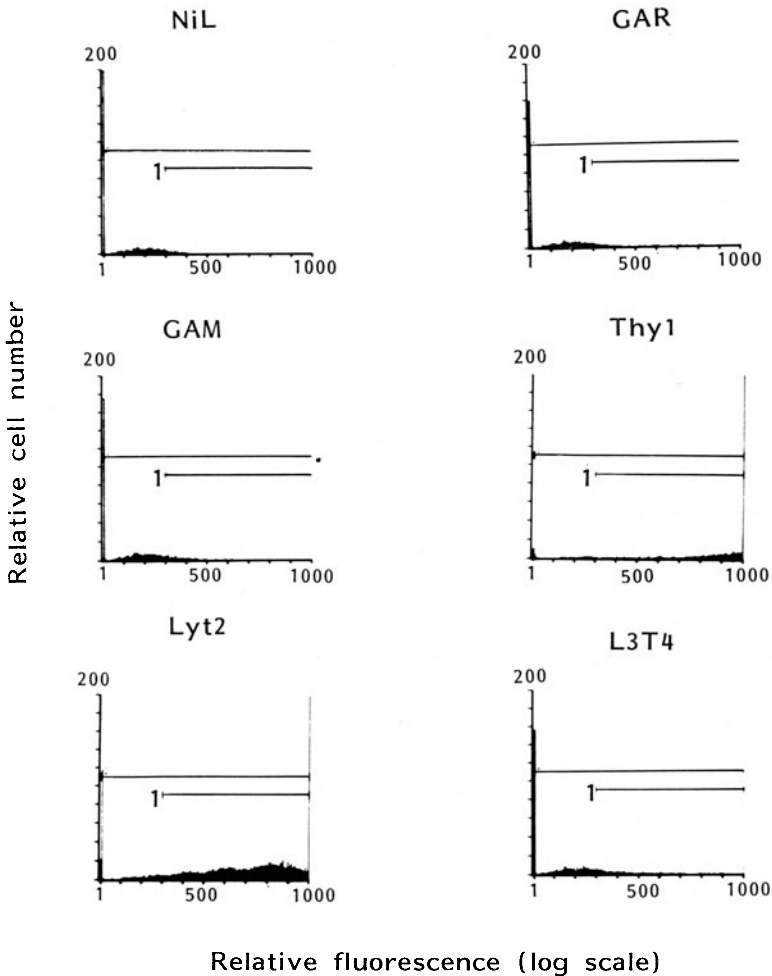

FIGURE 1. Phenotype analysis of a *M. tuberculosis*-reactive T-cell line. Cells were incubated with different monoclonal antibodies and in a second step with fluorescein isothiocyanate (FITC)-labeled goat anti-rat (GAR) or goat anti-mouse (GAM) antisera and analysed with a fluorescence activated cell sorter (FACS). 10,000 cells were counted per group. Cells alone, 2.2% fluorescence; GAR, 4.9%; GAM, 5.3%; Thy1, 89.5%; Lyt2, 87.1%; L3T4, 9%.

phobic Teflon bags containing medium supplemented with supernatants from L929 cells, the vast majority of the bone marrow cells were of macrophage lineage.[9] They expressed class I antigens and the MAC-1 marker but were totally devoid of class II molecules (Fig. 2). Furthermore, these cells were highly phagocytic and capable of processing and presenting bacterial antigens. Lysis of bone marrow macrophages ex-

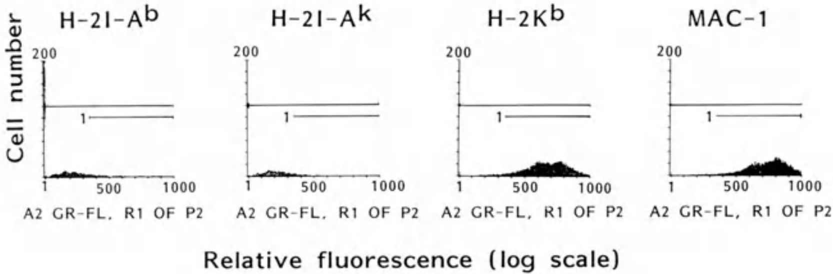

FIGURE 2. Phenotype analysis of bone marrow macrophages. Cells were labeled as described in Fig. 1. Cells alone, 2.1% fluorescence; GAR, 2.6%; GAM, 3.2%; I-A^b, 6.9%; I-A^k, 3.1%; H-$2K^b$, 94.4%; MAC-1, 96.2%.

pressing *L. monocytogenes* antigens was readily demonstrable using a 4-hr ^{51}Cr-release assay. Although lysis of bone marrow macrophages expressing mycobacterial antigens by mycobacteria-specific T cells was demonstrable in the 4-hr ^{51}Cr release assay, the system could be improved by extending the culture period to 12–18 hr and by employing a colorimetric method as a detection system.[8,10] In this assay, the reduction of neutral red uptake by macrophages is taken as parameter of target cell death.

Spleen cells from *M. bovis*-infected mice were restimulated *in vitro* as

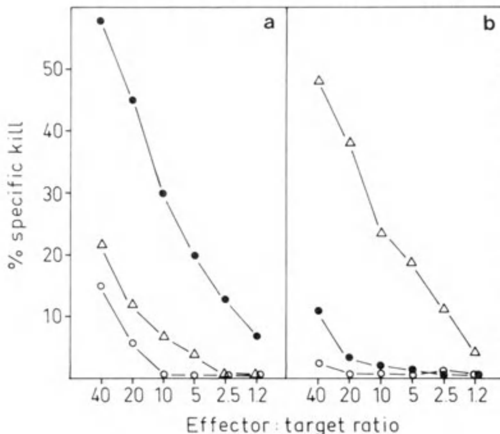

FIGURE 3. Antigen specificity of cytolytic T lymphocytes from *M. bovis* and *L. monocytogenes*-infected mice. *M. bovis* (a) and *L. monocytogenes* (b) immune cytolytic T cells were tested against *M. bovis*-infected (●), *L. monocytogenes*-infected (△), or uninfected bone marrow macrophages (○). C57Bl/6 mice were infected IV with *M. bovis* and spleen cells collected 14 days later. The *L. monocytogenes*-specific T-cell clone 1D7 has been described by Kaufmann *et al.*[7] The cells were grown in the presence of irradiated accessory cells, antigen (heat-killed bacteria), and IL-2-containing supernatant. Bone marrow macrophages were used as target cells.[8] Target cells (2×10^3/well) were labeled with ^{51}Cr and then incubated with effector cells for 4 hr. Specific killing was calculated according to the formula % specific lysis = $100 \times (A-B)/(C-B)$, where A is cpm from experimental wells, B is low control cpm, and C is high-control cpm. Spontaneous ^{51}Cr release <22%.

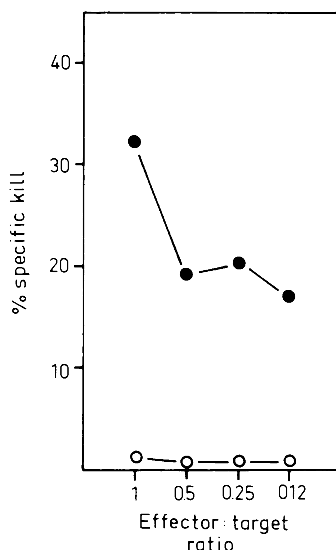

FIGURE 4. Cytolytic activity of T cells from *M. tuberculosis*-immunized mice. C57Bl/6 mice were immunized with killed *M. tuberculosis* H37Ra in incomplete Freund's adjuvant. After 14 days, cells from draining lymph nodes were grown in the presence of irradiated accessory cells, antigen (killed *M. tuberculosis* H37Ra), and exogenous IL-2-containing supernatant. After 10 days, the cells were tested for cytolytic activity with neutral red assay.[10] Cytolytic T cells were incubated with 10^5/well bone marrow macrophages in the presence (●) or absence (○) of 40 μg/well killed *M. tuberculosis* H37Ra for 18 hr. Afterward, cells were incubated with 0.036% neutral red, washed, and lysed. The optical absorbance was read at 540 nm. Killing was calculated according to the formula % lysis = 100 $(A-B)/A$, where A is absorbance of wells without T cells, and B is absorbance of experimental wells.

described above; afterward, T cells were added to cultures containing bone marrow macrophages and killed *M. bovis* BCG or *L. monocytogenes* organisms as antigen. As a control, a *L. monocytogenes*-specific CD8 T-cell clone was used. As shown in Fig. 3, T cells derived from *M. bovis*-infected mice were capable of lysing bone marrow macrophages in the presence of the homologous antigen, indicating that the T-cell response was antigen specific. By contrast, the *L. monocytogenes*-reactive T-cell clone only lysed macrophages presenting listerial antigen, leaving those macrophages that expressed mycobacterial antigen unaffected. Comparable cytolytic activities were also found with T cells from mice immunized with killed *M. tuberculosis* H37Ra (Fig. 4). Thus, both live and killed mycobacteria were capable of inducing cytolytic T lymphocytes.

Results from another experiment demonstrating antigen specificity are illustrated in Fig. 5. It can be seen that bone marrow macrophages primed with killed *M. bovis, M. tuberculosis,* and *M. scrofulaceum* were

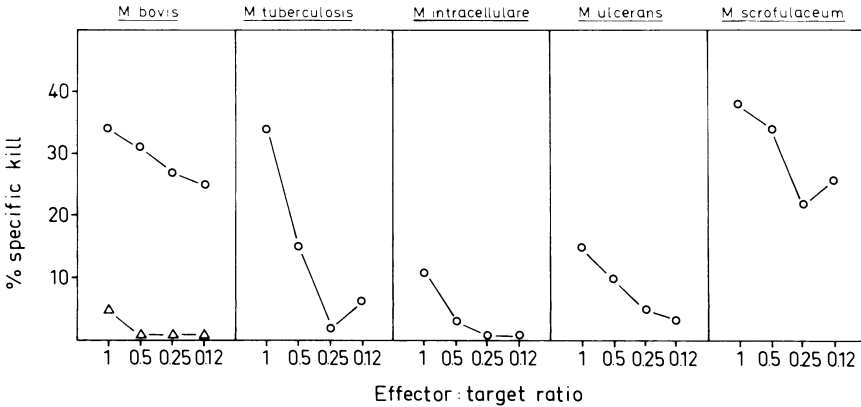

FIGURE 5. Antigen cross-reactivity of a *M. tuberculosis*-specific T-cell line. *M. tuberculosis*-specific T cells were incubated with bone marrow macrophages in the presence (○) or absence (△) of 10^6 heat-killed mycobacteria/well. Neutral red assay was performed as described in Fig. 4.

lysed by the T cells from *M. tuberculosis*-immune mice, whereas macrophages primed with killed *M. intracellulare* and *M. ulcerans* showed either no or only marginal lysis. Whether this selectivity was due to preferential recognition by cytolytic T cells of epitopes shared by *M. bovis, M. tuberculosis,* and *M. scrofulaceum,* but not by *M. intracellulare* and *M. ulcerans* or due to differential processing of a common antigen has to await further analysis. It may be interesting to note that soluble protein antigens from *M. tuberculosis* and *M. scrofulaceum* can induce necrotic skin-reactions in humans.[10]

2.2. Establishment of CD8 T-Cell Clones with Reactivity to *M. tuberculosis*

A T-cell line derived from *M. tuberculosis* inmune mice was cloned under limiting dilution conditions in the presence of antigen, accessory cells, and IL-2-containing supernatants, and afterwards, growing cells were expanded. Resulting T-cell clones had the CD8 phenotype; three of nine clones were also stained by the KJ16 antibody which reacts with the antigen receptor of approximately 20% of peripheral T cells from C57Bl/6 mice.[11]

2.3. Interferon-γ Secretion by *M. tuberculosis*-Reactive CD8 T-Cell Clones

Cloned T cells were tested for their capacity to proliferate and to secrete IL-2 and interferon-γ (IFN_γ). These clones were unable to pro-

TABLE I
IFNγ Secretion by *M. tuberculosis*-Reactive T-Cell Clones[a]

T-cell stimulation	IFN secretion by T-cell clone (U)					
	None	10A6	3A7	3C7	30E8	30H4
AC alone	<1	<1	<1	<1	<1	<1
AC + r−IL−2	<1	<1	<1	<1	<1	<1
AC + Ag	<1	<1	1	<1	3	3
r−IL−2 alone	ND	<1	<1	<1	1	1
AC + Ag+r−IL−2	<1	<1	81	27	9	9
AC + Ag+r−IL−2 + α-IFNγ	<1	<1	<1	<1	<1	<1

[a]Cloned T cells were co-cultured with accessory cells (AC), antigen (Ag, killed *M. tuberculosis* organisms), and/or r-IL-2. In the last lane, a specific rabbit anti-IFNγ antiserum (α-IFNγ) was added to the cultures. After 24 hr, supernatants were removed and IFN titers determined as described by Kaufmann *et al.*[7]

liferate or produce IL-2 and IFN_γ when they were cultured with accessory cells and mycobacterial antigens in the absence of IL-2. After stimulation by r-IL-2 alone, cloned T cells showed significant growth in the presence or absence of accessory cells and antigen. Importantly, in four out of five clones tested, IFN secretion could be induced by costimulation with r-IL-2, accessory cells, and antigen (Table I). The IFN activity was abrogated by treatment with a specific anti-IFN_γ antiserum (α-IFN_γ) indicating that it was of the IFN_γ class. These findings suggest that *M. tuberculosis*-reactive CD8 T-cell clones were able to secrete IFN_γ provided that IL-2, antigen, and accessory cells were present.

TABLE II
Cytolytic Activity of *M. tuberculosis*-Reactive CD8 T-Cell Clones[a]

Clone	Antigen	Percent specific lysis at effector : target ratio		
		0.5 : 1	0.25 : 1	0.12 : 1
10A6	+	32	16	10
	−	4	0	0
3A7	+	22	20	16
	−	6	17	16
3C7	+	97	41	36
	−	40	12	7
30E8	+	76	55	18
	−	3	1	1
30H4	+	75	71	49
	−	16	18	0

[a]The cytolytic activity of cloned T cells was determined in the neutral red assay in the presence or absence of killed *M. tuberculosis*, using bone marrow macrophages as target cells. For further details, see Fig. 4.

2.4. Cytolytic Activity of *M. tuberculosis*-Reactive CD8 T-Cell Clones

The cytolytic activity of the T-cell clones toward macrophages expressing mycobacterial antigens was assessed as described above. As summarized in Table II, *M. tuberculosis*-reactive CD8 T-cell clones were capable of lysing *M. tuberculosis*-primed bone marrow macrophages. Interestingly, the T-cell clone 10A6 that failed to secrete IFN_γ after stimulation with antigen and r-IL-2 showed *M. tuberculosis*-specific lysis, suggesting that *M. tuberculosis*-reactive cytolytic CD8 T-cell clones are heterogeneous with respect of IFN_γ production.

2.5. Class I Restriction of *M. tuberculosis*-Reactive CD8 T-Cell Clones

The genetic restriction of cytolysis by two of our clones could be identified. In this particular experiment, thioglycollate-induced peritoneal exudate cells were used as target cells, and cytolysis was assessed by ^{51}Cr release (Table III). Clones 30H4 and 10A6 specifically lysed *M. tuberculosis*-primed macrophages from syngeneic mice (C57 BL/6) and H-2D compatible H2K, H-2I disparate mice (B10.A(2R)). By contrast, macrophages from H-2K compatible H-2I, H-2D disparate mice (B10.MBR) were not killed in an antigen-specific way. These findings indicate that the two clones recognized antigen in association with H-2D-encoded molecules, hence were class I restricted. Whether the H-2D restriction is a common feature of mycobacteria-reactive CD8 T cells or mycobacterial antigens are also recognized by cytolytic T cells independent of H-2D molecules remains to be analyzed. In the *L. monocytogenes* model, until now we identified H-2K and not H-2D restricted cytolytic T-cell clones[7]; preliminary evidence points to a role of apparently H-2 nonrestricted CD8 T cells with cytolytic activity in antibacterial immunity[8] (S. H. E. Kaufmann, H. R. Rodewald, E. Hug, and G. De Libero, unpublished data).

2.6. Inhibition of Mycobacterial Growth by Cytolytic CD8 T Cells

We wanted to assess the fate of *M. bovis* inside macrophages after encounter with cytolytic T lymphocytes. Bone marrow macrophages were infected with live *M. bovis* BCG and, after 4 days, graded numbers of *M. tuberculosis*-reactive cytolytic T cells were added to these *M. bovis*-infected macrophages. After 18 hr, the cells were lysed and survival of *M. bovis* measured by [^{3}H]uracil uptake during a subsequent 6-hr period. Throughout the experiment, serum and antibiotics were omitted from the culture medium. The data depicted in Table IV demonstrate

TABLE III
Class I Restriction of Two *M. tuberculosis*-Reactive CD8 T-Cell Clones[a]

Target cells	MHC	Antigen	Percent specific lysis by clone 30 H4 at effector : target ratio				Percent specific lysis by clone 10A6 at effector : target ratio			
	KID		12 : 1	6 : 1	3 : 1	1.5 : 1	12 : 1	6 : 1	3 : 1	1.5 : 1
C57B1/6	bbb	+	52	28	22	4	49	30	46	4
		−	9	0	6	0	15	25	1	2
B10.A(2R)	kkb	+	71	44	30	22	71	65	53	45
		−	10	8	0	0	22	9	4	13
B10.MBR	bkq	+	22	17	16	19	13	23	9	8
		−	32	25	31	12	6	21	16	2

[a]The cytolytic activity of cloned T cells was determined in a 4 hr ^{51}Cr release assay. As a source of target cells, thioglycollate-induced peritoneal cells from *M. bovis* infected or uninfected mice were used.

TABLE IV
Growth Inhibition of *M. bovis* Inside Macrophages by Cytolytic T Cells

Cytolytic T cells	Cell number ($\times 10^4$)	[^{3}H]Uracil uptake[a] (cpm)	Percent inhibition[b]
—	—	18,576	—
M. tuberculosis-reactive T-cell line	45	2,317	87
	15	10,301	44
	5	18,511	1
M. tuberculosis-reactive T-cell clone	45	4,228	77
	15	18,578	1
	5	22,553	−20

[a]Bone marrow macrophages were infected with 2×10^6 live *M. bovis* BCG organisms. After 4 days, T cells were added; 18 hr later, cells were lysed and mycobacteria were pulsed for 6 hr with [^{3}H]uracil.
[b]% Inhibition was calculated according to the formula (1 − test/control) × 100.

that cytolytic T cells markedly inhibited [^{3}H]uracil uptake. Since this assay measures RNA synthesis by *M. bovis,* at the moment we cannot distinguish between bacteriostatic and bacteriocidal effects. Although formally not excluded, macrophage activation by lymphokines (e.g., IFN_γ) probably was not responsible for the antimycobacterial functions. In previous experiments, we found that bone marrow macrophages can be activated by IFN_γ to inhibit growth of *M. bovis* as measured by [^{3}H]uracil uptake.[6] However, in these experiments IFN_γ activation had to be initiated before or at the time of infection; at later times, IFN_γ had no or only marginal effects on mycobacterial growth. Furthermore, the T-cell clone 10A6 used in this experiment failed to secrete IFN_γ after stimulation with antigen and accessory cells (see Table I). Therefore, we would like to suggest that the inhibition of mycobacterial growth is a consequence of target cell lysis by cytolytic T cells. Although further studies aimed at the elucidation of the mechanisms involved in the anti-mycobacterial effects are required, this experiment already points to direct antimycobacterial effects of cytolytic T lymphocytes.

3. DISCUSSION

3.1. Possible Role of CD8 T Cells in Tuberculosis

This chapter describes class I-restricted CD8 T lymphocytes from *M. tuberculosis*-immune mice that specifically lyse mycobacteria-primed macrophages. The generation of these T cells depended on *in vivo* immunization with mycobacteria and expression of cytolytic activity re-

quired restimulation with antigen, accessory cells, and crude IL-2. Mycobacteria-specific cytolytic T cells did not develop from cells of normal or *L. monocytogenes*-immune mice. Both live and killed mycobacteria could be used for immunization, indicating that the cytolytic T cells were specific for structural antigens and that their generation did not depend on replicating organisms. However, a much higher bacterial load and Freund's incomplete adjuvant had to be used for immunization with killed *M. tuberculosis* compared with an infection with live *M. bovis.*

Hitherto, the participation of CD8 T lymphocytes with specific cytolytic activity in immunity to intracellular bacteria had not been shown. However, we have recently identified cells of similar type and function after immunization of mice with *L. monocytogenes*[7,8] and *M. leprae.*[13] Cytolytic T cells have also been found to develop during *Rickettsia tsutsugamushi* and *Theileria parva* infections.[14,15] In the human system, cytolytic T cells with specificity for mycobacterial antigens have been described.[16,17] These T lymphocytes, however, most probably belong to the class II-restricted CD4 T-cell set. Studies performed in the murine system strongly suggest that class II restricted CD4 T cells can lyse their target cells,[18] and we recently found that *L. monocytogenes*-specific class II restricted CD4 T cells can lyse *L. monocytogenes*-infected macrophages, provided that Ia expression had been induced.[19] It has been shown that during *M. bovis* infection, natural killer (NK) cells arise,[20] and NK cells have been implicated in protection against viral infection.[21] The antigen specificity of the cytolytic T cells described here distinguishes them from these NK cells as well as from lymphokine-activated NK cells.[22]

Although a role for cytolytic T-cell functions in resistance to, and pathology of, tuberculosis cannot be inferred directly from our experiments, data from the literature would support such a notion. In the model of experimental infection of mice with *L. monocytogenes,* evidence has accumulated that class I-restricted CD8 T cells are required for adoptive transfer of protection.[23–25] The dependence of adoptive protection on CD8 T cells has also been confirmed in infections of mice with the facultative intracellular pathogens, *M. tuberculosis, M. lepraemurium,* and *Brucella abortus.*[26–28]

During viral infection, class I-restricted CD8 T lymphocytes with cytolytic potential become readily demonstrable. In several viral systems, particularly the influenza system, the potential relevance of these T cells to acquired resistance has been studied. It has been shown that heterogeneous as well as cloned CD8 T cells that lyse influenza-infected target cells *in vitro* are also capable of conferring specific protection upon infected mice.[29,30] In a more detailed study, the involvement of IFN_{γ} in the *in vivo* protection against influenza by CD8 T cells was indirectly excluded, and evidence for direct cell–cell contact was obtained.[30] We

postulate that similar T-cell functions are involved in the host response against tuberculosis.

3.2. Possible Role of CD4 T Cells in Tuberculosis

In the infected host, tubercle bacilli are primarily located within the cells of the mononuclear phagocyte system. These cells express class II molecules, hence can present mycobacterial antigens to class II-restricted CD4 T lymphocytes of helper/inducer type. Once these T cells have been activated, they can recirculate through the body and scan for infected Ia^+ host cells. The secondary encounter between mycobacteria-specific T cells and infected macrophages results in the activation of the latter via T-cell derived lymphokines, including IFN_γ. Provided that the microorganisms residing inside lymphokine activated cells are susceptible to these newly acquired functions, this interaction will lead to growth inhibition or even destruction of the bacteria. This mechanism alone should be sufficient for the confinement of bacteria to discrete foci and often may suffice for their clearance from the host.

In many cases, the interaction of helper/inducer T cells with macrophages, however, may not suffice for protection primarily for two reasons. First, mononuclear phagocytes are highly heterogeneous with respect to their antibacterial capacities that can be induced by lymphokines. It appears that young blood monocytes possess the highest antibacterial potential, whereas many tissue macrophages have a much lower potential and to a great degree seem to be refractory to lymphokine activation. This deficiency has been clearly documented for Kupffer cells.[4] Second, tubercle bacilli have developed a wide range of mechanisms to evade from macrophage destruction. Thus, even mononuclear phagocytes in which antibacterial functions can be induced to a certain degree may fail to destroy virulent strains of *M. tuberculosis,* although they may be capable of doing so with many intracellular bacteria possessing less potent evasion mechanisms. Under these circumstances, helper/inducer T cells may greatly benefit from additional host mechanisms.

3.3. Possible Role of CD8 T Cells in Protection against Tuberculosis

Unlike CD4 T cells, CD8 T cells recognize antigen in association with class I molecules. It is generally assumed that viral but not bacterial or soluble protein antigens associate with class I molecules. Our experiments in the *M. tuberculosis* and the *L. monocytogenes*[7] system, however, suggest that antigens from intracellular bacteria can indeed be expressed in association with class I molecules. Thus, macrophages harbor-

ing mycobacteria should be able to stimulate not only helper/inducer but also cytolytic T-cell responses. Once cytolytic T cells have been generated, they are qualified to examine host cells for mycobacterial infection irrespective of their nature and origin because virtually all nucleated cells express class I antigens. In addition, we have obtained preliminary evidence that some cytolytic T cells are apparently H-2 nonrestricted. Although mycobacteria are predominantly located within macrophages, they can reside in some nonprofessional phagocytes. For example, *M. leprae* is known to preferentially inhabit Schwann cells and mycobacteria have been isolated from epithelial cells.[21,22] These nonprofessional phagocytes as well as tissue macrophages with low antibacterial potential are suspected to protect rather than attack intracellular organisms. Thus, they could provide an ideal habitat for persistent *M. tuberculosis* organisms, and their destruction may well be beneficial for the host.

The data summarized in Table IV provide preliminary evidence that target cell lysis has a direct inhibitory effect on the growth of intracellular mycobacteria. It can be assumed that through target cell lysis, lysosomal and/or cytoplasmic enzymes with potent antimicrobial activities are mobilized and brought into intimate contact with the microorganisms. In this way, inhibition of phagosome–lysosome fusion maintained in intact host cells could be overcome by cytolysis. The question as to how target cell lysis affects mycobacterial growth, however, needs to be further evaluated. Even if mycobacteria are not directly affected by host-cell destruction, they will become accessible to more powerful mononuclear cells by this mechanism. After being released, mycobacteria can be engulfed by immigrant blood monocytes with high antibacterial potential that can be called into action by T-cell-derived lymphokines. Although we assume that helper/inducer T cells are the major source of macrophage-activating lymphokines, our demonstration that CD8 T cells produce IFN_{γ} indicates that they can contribute to macrophage activation as well. IFN_{γ} secretion by CD8 T cells, however, depended on exogenous IL-2, indicating that in an *in vivo* situation they may also require help from CD4 T cells. We assume that in a tuberculous granuloma, transmission of persisting mycobacteria from aged macrophages to immigrant blood monocytes occurs and that this process is controlled by the synergistic interplay between helper/inducer and cytolytic T lymphocytes.

3.4. Possible Role of CD8 T Cells in Immunopathology

In recent studies, the distribution of CD4 and CD8 T cells in mycobacterial lesions has been studied histologically.[33] Tuberculoid leprosy lesions represent well-organized granulomas and contain only few sur-

viving mycobacteria. In these granulomas, CD4 T cells are primarily found in the necrotic center, with numerous CD8 T cells scattered over a surrounding mantle. Recently, CD8 T cells were identified as the major subset infiltrating the site of skin after challenge with tuberculin proteins in humans.[34] In addition, in murine listeriosis granuloma formation seems to be class I restricted.[35]

Highly activated cytolytic CD8 T cells inevitably will lead to tissue destruction. It has been suggested that the late-appearing necrotic response is protective when the challenge is superficial, probably because necrosis leads to sloughing of the infected focus.[11] By contrast, enhanced susceptibility was observed in animals with necrotizing skin-test reactivity to mycobacterial proteins after deep tissue infection.[36,37] Necrosis, the principal pathologic process in human secondary tuberculosis, therefore does not always correlate with protection. Until now, the mechanisms responsible for necrotic lesions have not been fully elucidated. Necrosis has been shown to occur during mycobacterial infections in animal models[38]; it also occurs after injection of soluble mycobacterial antigens.[39] However, it has not yet been demonstrated that necrotic responses to soluble proteins can be transferred with T cells. Cytolytic CD8 T lymphocytes, which we have shown to develop after *M. tuberculosis* immunization, could mediate such reactions in infectious diseases in a similar way, as they have been found to participate in transplant rejection.[40]

Cytolytic mechanisms therefore could have a dual effect. In well-

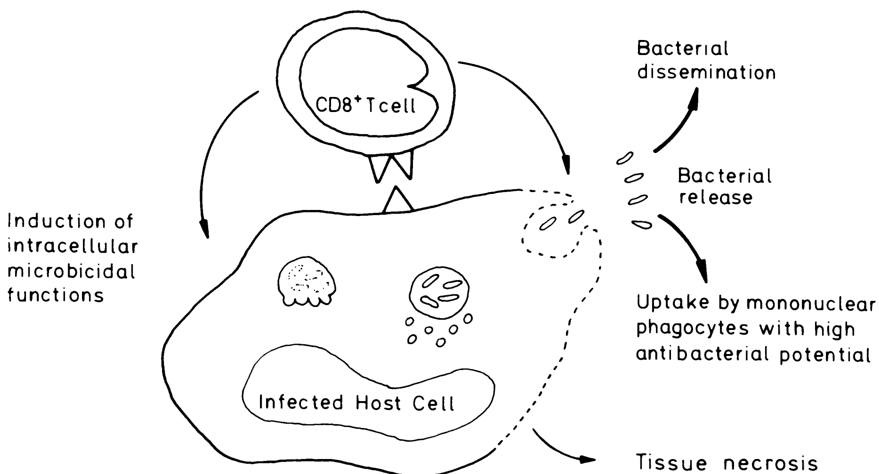

FIGURE 6. Possible consequences of the interaction between CD8 T lymphocytes and infected host cells in tuberculosis.

organized granulomas, in which cell destruction is confined to macrophages and to the site of bacterial growth, cytolytic mechanisms could be responsible for protection. When highly and diffusely activated, however, cytolytic T cells may cause severe tissue damage and facilitate bacterial dissemination. Granuloma liquification and caseation may represent such harmful alterations. In addition, detrimental consequences may also be preponderant in cases in which highly specialized host cells are the targets of cytolytic T cells. Such effects may occur in tuberculoid leprosy patients who express strong antibacterial T-cell immunity yet suffer from nerve lesions probably due to destruction of Schwann cells harboring *M. leprae*. Possible beneficial and harmful consequences of the interaction between CD8 T lymphocytes and infected target cells in tuberculosis are summarized in Fig. 6.

4. CONCLUDING REMARKS

At this point, it may be worth recalling Robert Koch's discovery of what is generally thought to be the first description of a delayed-type hypersensitivity reaction. In 1890, Koch observed that tuberculous guinea pigs strongly reacted to the local injection of *M. tuberculosis* material with induration, necrosis, and subsequent skin rejection. He observed that within the necrotic tissue, the conditions for the enclosed microorganisms became extremely hostile, causing bacteriostatic or even bacteriocidal effects. Koch concluded that necrotic reactions should be protective as long as tissue destruction remains confined to, and controlled in, circumscribed foci. Under uncontrolled conditions, however, destructive effects would be preponderant and, in the worst cases, cause death of the host. Although Koch assumed that the mycobacterial material directly induced these effects, we now know that they are mediated by specific T lymphocytes. The data discussed here suggest that not only helper/inducer but also cytolytic T-cell functions participate in the host response against *M. tuberculosis* and that a protective outcome will critically depend on an intricate balance between the different cell types involved. The availability of CD4[41] and CD8 T-cell clones with reactivity to *M. tuberculosis* may facilitate a better understanding of this and related questions.

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REFERENCES

1. Hahn, H., and Kaufmann, S. H. E., 1981, The role of cell-mediated immunity in bacterial infections, *Rev. Infect. Dis.* **3:**1221–1250.
2. Andrew, P. W., Jackett, P. S., and Lowrie, D. B., 1985, Killing and degradation of microorganisms by macrophages, in: *Mononuclear Phagocytes: Physiology and Pathology* (R. T. Dean and W. Jessup, eds.), pp. 311–335, Elsevier, Amsterdam.
3. Fitch, F. W., 1986, T-cell clones and T-cell receptors, *Microbiol. Rev.* **50:**50–69.
4. Lepay, D. A., Nathan. C. F., Murray, H. W., Steinman, R. M., and Cohn, Z. A., 1985, Murine Kupffer cells: Mononuclear phagocytes deficient in the generation of reactive oxygen intermediates, *J. Exp. Med.* **161:**1079–1096.
5. Lowrie, D. B., 1983, Mononuclear phagocyte–mycobacterium interaction, In: *Biology of Mycobacteria,* Vol. II (C. Ratledge and J. Stanford, eds.), pp. 235–278, Academic, London.
6. Flesch, I., and Kaufmann, S. H. E., 1987, Mycobacterial growth inhibition by interferon-γ activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis, J. Immunol.* **138:**4408–4413.
7. Kaufmann, S. H. E., Hug, E., and De Libero, G., 1986, *Listeria monocytogenes*-reactive T lymphocyte clones with cytolytic activity against infected target cells, *J. Exp. Med.* **164:**363–368.
8. De Libero, G., and Kaufmann, S. H. E., 1986, Antigen-specific $Lyt2^+$ cytolytic T lymphocytes from mice infected with the intracellular bacterium *Listeria monocytogenes, J. Immunol.* **137:**2688–2694.
9. Meerpohl, H. G., Lohmann Matthes, M. L., and Fischer, H., 1976, Studies on the activation of mouse bone marrow-derived macrophages by the macrophage cytotoxicity factor (MCF), *Eur. J. Immunol.* **6:**213–217.
10. Parish, C. R., and Müllbacher, A., 1983, Automated colorimetric assay for T cell cytotoxicity, *J. Immunol. Methods* **58:**225–237.
11. Rook, G. A. W., 1983, An integrated view of the immunology of the mycobacteriosis in guinea pigs, mice and men, in: *Biology of Mycobacteria,* Vol. II (C. Ratledge and J. Stanford, eds.), pp. 279–319, Academic, London.
12. Roehm, N., Herron, L., Cambier, J., Di Guisto, D., Haskins, K., Kappler, J., and Marrack, P., 1984, The major histocompatibility complex-restricted antigen receptor on T cells: Distribution on thymus and peripheral T cells, *Cell* **83:**577–584.
13. Chiplunkar, S., De Libero, G., and Kaufmann, S. H. E., 1986, *Mycobacterium leprae*-specific $Lyt2^+$ T lymphocytes with cytolytic activity, *Infect. Immun.* **54:**793–797.
14. Rollwagen, F. M., Dasch, G. A., and Jerrells, T. R., 1986. Mechanisms of immunity to rickettsial infection: Characterization of a cytotoxic effector cell, *J. Immunol.* **136:**1418–1421.
15. Eugui, E. M., and Emery, D. L., 1981, Genetically restricted cell-mediated cytotoxicity in cattle immune to *Theileria parva, Nature (Lond.)* **290:**251–254.
16. Hank, J. A., and Sondel, P. M., 1982, Soluble bacterial antigen induces specific helper and cytotoxic responses by human lymphocytes in vitro, *J. Immunol.* **128:**2734–2738.
17. Hansen, P. W., Madsen, M., Christiensen, S. E., Johnsen, H. E., and Kissmeyer-Nillsen,

F., 1984, Cell mediated PPD specific cytotoxicity against human monocyte targets: Evidence for restriction by class II HLA antigens, *Tissue Antigens* **23:**171–180.
18. Jones, B., Tite, J. P., and Janeway, C. A., 1986, Different phenotypic recipients of the mouse B cell tumor A20/25 are selected by antigen- and mitogen-triggered cytotoxicity of L3T4-positive, I-A-restricted T cell clones, *J. Immunol.* **136:**348–356.
19. Kaufmann, S. H. E., Hug, E., Väth, U., and De Libero, G., 1987, Specific lysis of *Listerial monocytogenes* infected macrophages by class II-restricted L3T4$^+$ T cells, *Eur. J. Immunol.* **17:**351–357.
20. Wolfe, S. A., Tracey, D. E., and Henney, C. S., 1977, BCG-induced murine effector cells. II. Characterization of natural killer cells in peritoneal exudates, *J. Immunol.* **119:**1152–1157.
21. Santoli, D., and Koprowsky, H., 1979, Mechanism of activation of human natural killer cells against tumor and virus-infected cells, *Immunol. Rev.* **44:**125–163.
22. Grimm, I. E. A., Mazumder, A., Zhang, H. Z., and Rosenberg, S. A., 1982, The lymphokine activated killer cell phenomenon: Lysis of NK-resistant fresh solid tumor cells by IL-2 activated autologous human peripheral blood lymphocytes, *J. Exp. Med.* **155:**1823–1841.
23. Kaufmann, S. H. E., Simon, M. M., and Hahn, H., 1979, Specific Lyt 123 T cells are involved in protection against *Listeria monocytogenes* and in delayed-type hypersensitivity to listerial antigens, *J. Exp. Med.* **150:**1033–1038.
24. Kaufmann, S. H. E., Hug, E., Väth, U., and Müller, I., 1985, Effective antibacterial protection against *Listeria monocytogenes* and delayed-type hypersensitivity to listerial antigens depend on cooperation between specific L3T4$^+$ and Lyt2$^+$ T cells, *Infect. Immun.* **48:**263–266.
25. Cheers, C., and Sandrin, M. S., 1983, Restriction in adoptive transfer of resistance to *Listeria monocytogenes.* II. Use of congenic and mutant mice show transfer to be H-2K restricted, *Cell Immunol.* **78:**199–205.
26. Orme, I. M., and Collins, F. M., 1984, Adoptive protection of the *Mycobacterium tuberculosis* infected lung: Dissociation between cells that passively transfer protective immunity and those that transfer delayed-type hypersensitivity to tuberculin, *Cell Immunol.* **84:**113–120.
27. Adu, H. O., Curtiss, J., and Turk, J. L., 1983, The resistance of C57BL/6 mice to subcutaneous infection with *Mycobacterium lepraemurium* is dependent on both T cells and other cells of bone marrow origin, *Cell. Immunol.* **78:**249–256.
28. Pavlov, H., Hogarth, M., McKenzie, I. F. C., and Cheers, C., 1982, *In vivo* and *in vitro* effects of monoclonal antibody to Ly antigens on immunity to infection, *Cell. Immunol.* **71:**127–138.
29. Lin, Y.-L., and Askonas, B. A., 1981, Biological properties of an influenza A virus-specific killer T cell clone. Inhibition of virus replication in vivo and induction of delayed-type-hypersensitivity reactions, *J. Exp. Med.* **154:**225–234.
30. Lukacher, A. E., Braciale, V. L., and Braciale, T. J., 1984, In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific, *J. Exp. Med.* **160:**814–826.
31. Saito H., Tomioka, H., Sato, K., and Watanabe, T., 1986, Abilities of human oligoneudroglial cells and mouse Schwann cells to phagocytose *Mycobacterium leprae* and other mycobacteria, *Infect. Immun.* **51:**157–162.
32. Mapother, M. E., and Songer, J. G., 1984, In vitro interaction of *Mycobacterium avium* with intestinal epithelial cells, *Infect. Immun.* **45:**67–73.
33. Modlin, R. L., Hofman, F. M., Meyer, P. R., Sharma, O. P., Taylor, C. R., and Rea, T. H., 1983, In situ demonstration of T lymphocyte subsets in granulomatous inflammation: Leprosy, rhinoscleroma and sarcoidosis, *Clin. Exp. Immunol.* **51:**430–438.

34. Beck, J. S., Morley, S. M., Gibbs, J. H., Potts, R. C., Ilias, M. I., Kardjito, T., Grange, J. M., Stanford, J., and Brown, R. A., 1986, The cellular responses of tuberculosis and leprosy patients and of healthy controls in skin tests to "new tuberculin" and leprosin A., *Clin. Exp. Immunol.* **64:**484–494.
35. Näher, H., Sperling, U., and Hahn, H., 1985, H-2K-restricted granuloma formation by Lyt-2$^+$ T cells in anti-bacterial protection to facultative intracellular bacteria, *J. Immunol.* **134:**569–572.
36. Wilson, G. S., Schwabacher, H., and Maier, I., 1940, The effect of the desensitization of tuberculous guinea-pigs, *J. Pathol.* **50:**89–109.
37. Seibert, F. B., 1933, Effect of sensitization with tuberculin protein upon development and course of experimental tuberculosis, *Proc. Soc. Exp. Biol. Med.* **30:**1274–1276.
38. Nyke, W., 1956, Enhancement of resistance to tuberculosis in mice experimentally infected with *Brucella abortus, Am. Rev. Tuber.* **73:**251–265.
39. Crowle, A. J.. 1959, Delayed hypersensitivity in several strains of mice studied with different tests, *J. Allergy* **30:**442–459.
40. Loveland, B. E., and McKenzie, I. F. C., 1982, Which T cells cause graft rejection?, *Transplantation* **33:**217–223.
41. Kaufman, S. H. E., and Flesch, I., 1986, Function and antigen-recognition pattern of L3T4$^+$ T-cell clones from *Mycobacterium tuberculosis* immune mice, *Infect. Immun.* **54:**291–296.

8

Anergy and Other Immunologic Perturbances in Mycobacterial Infections

Overview

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1. INTRODUCTION

The clinical and histologic features of any infectious disease are the result not only of the toxic and invasive properties of the pathogen but of the immune reactions of the host as well. In most cases, the host-immune response leads to a reaction that rapidly eliminates the pathogen, but in other cases the immune mechanisms fail and the outcome of the infection is a fatal one. In still other cases, the host and the pathogen engage in a long drawn-out conflict and the disease becomes chronic. Alternatively, the immune reaction may be abnormal or exaggerated and may damage the host more than the invader. In the case of mycobacteria, it is the cell-mediated immune (CMI) response rather than the production of antibody that appears to be of importance in overcoming the infections. Undoubtedly, antibodies are produced in response to naturally occurring and experimental mycobacterial infections, but there is no evidence that they play any role in host defense. Indeed, there is some evidence that serum factors (possibly antibody) may have harmful effects, through

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antibody-dependent immunopathology[1] or by enhancing the growth of the challenging mycobacteria.[2]

One of the first indications that an individual has been exposed to a mycobacterial infection is the development of a positive skin-test reaction to mycobacterial antigen. Equivalent *in vitro* tests have been developed evaluating the proliferative capacity of specific T cells or their ability to produce lymphokines, such as migration inhibitory factor (MIF) or γ-interferon (IFN_{γ}). The delayed-type hypersensitivity (DTH) reaction is thus an *in vivo* parameter of a normally functioning CMI. This immune reactivity can be temporarily reduced or ablated during viral infections or in patients having other underlying infections or ma-

TABLE I
Causes of False-Negative Tuberculin Reactions[a]

Acquired and constitutional factors known to alter cellular immune responses
Infections
Bacterial (typhoid fever, brucellosis, leprosy, syphilis, typhus, pertussis, streptococcal infection)
Viral (measles, mumps, chicken pox, influenza, mononucleosis, yellow fever, AIDS)
Fungal (South American blastomycosis, histoplasmosis, coccidioidomycosis)
Parasites (schistosomiasis, toxoplasmosis, malaria, filariasis)
Live attenuated viral vaccinations
Measles, mumps, rubella, polio
Diseases affecting the lymphoid organs
Hodgkin disease, lymphoma, chronic lymphocytic leukemia, sarcoidosis
Metabolic derangements
Myxedema, chronic renal failure, diabetes mellitus, prolonged zinc deficiency
Drugs
Corticosteroids, most immunosuppressive drugs (cyclosporin A)
Age
Newborn, elderly patients
Nutrition
Malnutrition with severe protein-calorie deficiency
Miscellaneous
Atopic dermatitis, burns, surgery, pregnancy, stress, graft-versus-host reaction, neuroendocrine disorders
Technical factors
Related to the tuberculin used
Improper storage or dilution (chemical denaturation, contamination)
Absorption (prevented by adding Tween 80)
Related to the timing or method of testing
Improper amount of tuberculin (Delayed administration after drawing into syringe, subcutaneous injection, preallergic state after infection)
Methodologic errors of reading
Nonexperienced reader, conscious or unconscious bias, recording errors

[a]Adapted from Snider.[3a]

lignant diseases.[1] Even in healthy persons, a temporary change can occur following vaccination with viral vaccine or during the development of an earlier applied positive tuberculin test.[3] In tuberculosis, the potential causes of a false-negative tuberculin reaction are numerous (Table I). They may be related to variation in potency of the tuberculin used or to errors in performing or reading the test. Nevertheless, it has long been recognized that occasional tuberculous patients can be tuberculin negative; recent studies suggest that in patients in whom active tuberculosis has recently developed, this may occur more frequently than reported by earlier authors.[1,4] This chapter focuses on such cases. Well-recognized causes of anergy, such as sarcoidosis, viral exanthems, severe malnutrition, advanced age, malignant lesions, and immunosuppressive and steroid drugs, are not considered, although the precise underlying mechanisms are frequently unknown. Most patients who do not react to tuberculin are often also unresponsive to recall antigen; evidence is accumulating that strongly suggests that tuberculosis, like leprosy and other mycobacterial diseases, are diseases in which an immune spectrum is present and that significant modulation of T- and B-lymphocyte-mediated responses occurs in many patients. The first part of this chapter deals with the immune spectrum observed in leprosy and tuberculosis and with the suspected immune mechanisms. The second part deals with experimental models aimed to reproduce an unresponsiveness comparable to that observed in human.

2. THE IMMUNE SPECTRUM IN HUMAN MYCOBACTERIAL DISEASES

2.1. Leprosy

2.1.1. Immune Defects and Clinical Status

Leprosy is caused by the obligate intracellular bacterium *M. leprae*. Contrary to widely held belief, *M. leprae* circulates extensively in endemic areas. However, most infected individuals never develop overt clinical manifestations of the disease, and others develop localized lesions which heal spontaneously. This implies that most individuals are able to cope immunologically with the microorganism and that those who develop the disease are in some way immunologically defective or hyperreactive with respect to it. Infection with *M. leprae* affects the peripheral nerves and the dermis, causing an accumulation of macrophages and other immune cells at the infected sites. The clinical pattern and ultimate outcome of the disease depend on the extent of the host response to the microorganism, which in turn controls bacillary multi-

plication. A systematic classification of the clinical manifestations of the disease, based on bacteriologic features, on the patient's immunity, and on the pathologic evaluation of the lesions has been proposed by Ridley and Jopling,[5] who defined an entire spectrum encompassing five main categories of disease. The extremes are known as lepromatous (LLp) and tuberculoid (TT) leprosy. In the latter, marked CMI is observed and bacterial growth or dissemination are controlled. In the former, where little if any CMI develops and bacterial multiplication seems uncontrolled, *M. leprae* disseminates throughout most of the dermis. Between the two polar groups there are borderline lepromatous (BL), mid-borderline (BB), and borderline tuberculoid (BT) patients. One should also note an intermediate group, between the BL and LLp, called lepromatous subpolar (LLs), which before therapy, is difficult to separate from the LLp group. Individuals who have upgrading reactions and positive conversion of the Mitsuda reaction after therapy are usually reclassified as LLs patient. New classification based on the development of *in vitro* tests and better knowledge of the cellular composition of the cutaneous lesions may soon be proposed.

The characteristics of the six primary groups are shown in Fig. 1. Immunologic unresponsiveness in LL patients is only CMI related, since a high percentage of these patient have high titers of antibody to the specific phenolic glycolipid 1 (gly-1) or proteins of *M. leprae*. Moreover,

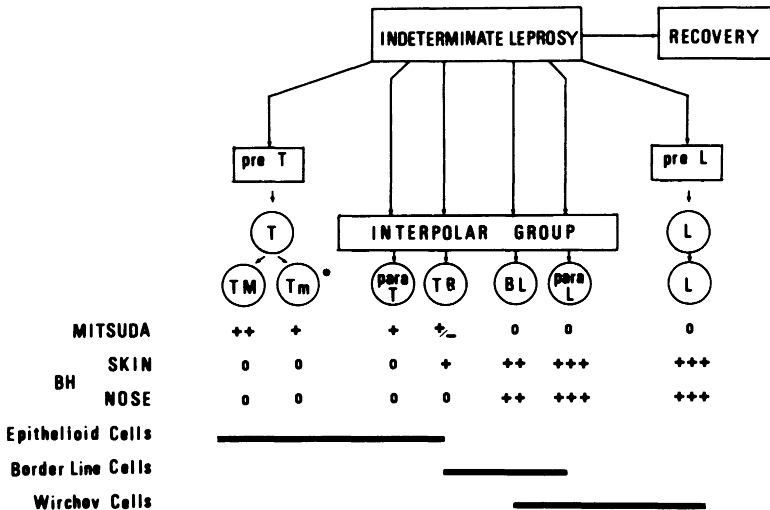

FIGURE 1. Bacterial, immunologic, and histopathologic classification of leprosy. TM, T_{major}; Tm, T_{minor}. (Adapted from Laguillon.)

some clinical manifestations in LL patients are related to high levels of circulating immune complexes (ICx) containing gly-1 antigen.[6]

Cölöglu[7] reviewed earlier reports of leprosy-associated impaired renal function, including renal amyloidosis, interstitial nephritis, pyelonephritis, and proliferative glomerulonephritis. In fact, kidney diseases are the most common cause of clinical deterioration and death in leprosy. Severity of kidney involvement was dependent on antibody levels and ICx concentration in serum. The immune injury of the kidney structures was of type II and III hypersensitivity reactions, involving ICx deposition on glomerular capillary walls and mesangium. Many of the patients had erythema nodosum leprosum. Not only is antibody to bacterial products present in serum or in tissues, but recent investigations have shown significant increases in autoantibodies in patients with leprosy.[1] The production, regulatory mechanisms, and role of such antibodies are as yet not fully understood. An acquired dysregulation of the autoreactive B-cell system, associated with defects in suppressor mechanisms, is a possible explanation.[8]

2.1.2. *Mechanisms of Immune Disorders in Leprosy*

The mechanisms leading to the reduced or absent CMI to *M. leprae* antigens in leprosy patients are yet not fully understood. The disease seems to develop in subjects who may already possess a defect in the ability to mount cellular hypersensitivity and granuloma reaction to mycobacterial antigen, as suggested by the negativity of the Mitsuda reaction and by a decreased *in vitro* responsiveness to mitogens in relatives of LL patients.[9] The extent of the immune deficit becomes more pronounced as the disease progresses toward the LL pole of the spectrum. The defect is specific according to many investigators, since the patient's inability to respond is directed only to *M. leprae* antigen, and it has been shown that a certain number of LL patients become positive to tuberculin after BCG vaccination. However, considerable recent evidence indicates that nonspecific depression may also occur, which seems to vary from patient to patient and from study to study.[10] After treatment with antibacterial drugs for months and years, LL patients regain a normal responsiveness to nonspecific antigens and mitogens, but rarely to lepromin, despite the elimination of detectable bacilli from tissue biopsy specimen. Thus, one is obliged to ask why some patients fail to limit their disease and why some progress toward one part of the spectrum and some to other parts.

Two main hypotheses have been suggested, as reported recently by Shepard.[11] The first postulates that in healthy contacts, or in those with self-limiting intermediate lesions, a brisk CMI reaction eliminates the

microorganisms. In the case of TT, a delay in the onset of CMI permits enough organisms to spread, so that bacterial eradication is not rapidly or easily achieved. In addition, low or poor regulatory T-cell mechanisms and persistence of antigen enable pathologic granulomatous reactions to be established, leading to dermal and neurologic involvement. The recent finding of HLA-DR2 linkage in TT patient is in favor of this hypothesis.[12] By contrast, in LL individuals, the onset of CMI is indefinitely delayed. However, this theory has been challenged on the basis of several clinical and immunologic observations showing that early leprosy might be associated with CMI, which then either increases or is apparently suppressed.[13]

The second hypothesis, involving acquired and active suppression, implies the existence of mechanisms for inhibiting CMI induction or expression. This might involve the production of suppressor cells or derived mediators, preventing the induction of immunologically committed competent cells or inhibiting them from reaching the macrophage engulfed bacteria, or the intervention of blocking antibodies or other humoral nonspecific factors. All these possibilities seem to occur. Production of soluble factors by macrophages loaded with *M. leprae* has been recently described.[14] Trapping of antigen-reactive lymphocytes in lymph nodes has been also demonstrated in numerous experimental and naturally occurring mycobacterial infections. Evidence of the presence in blood and lesions of specific suppressor OKT8 lymphocytes has been reported.[6]

However, even if the clinical, bacteriologic, immunologic, and histologic features of leprosy seem to result from a complex and multifactorial immune response, one should consider that affected individuals may not constitute a truly homogeneous population according to their immune responsiveness.[6,10] The first confirmation of such concept comes from analysis of the genetic factors controlling the course of *M. leprae* infection. As far as HLA-linked factors are concerned, most evidence exists regarding the development of TT leprosy (HLA-DR2) and to a lesser extent of LLp leprosy (HLA-DQ1). It seems that HLA-linked factors do not confer susceptibility or resistance to infection but rather modulate the type of the immune response to *M. leprae*.[15] For instance, individuals susceptible to the development of TT leprosy may be genetically different (HLA-DR2) from those who remain healthy after exposure to the bacillus.[12] Both are capable of eradicating the bacterium, but only the TT patients express an unregulated CMI response, indicating that the genetic factors involved might concern the regulatory mechanisms of T cells, and not the capacity to mount an effective protection. A second insight comes from studies on *in vivo* and *in vitro* restoration of

immune responsiveness. Immunotherapy using multiple injections of killed *M. leprae* and BCG vaccine in persistent nonresponders (LL or BL patients), after chemotherapy, show that only a percentage of patients (roughly 60%) are able to convert to immune responsiveness. Analogous variations in results were also observed in Mitsuda-negative contacts, although the percentage of nonresponders after immunotherapy was lower.[16] *In vitro* tests also suggest that LL and BL patients represent a heterogeneous group. This was clearly shown recently by Kaplan *et al.*[10] Using different parameters to evaluate the immune response to *M. leprae* and other cross-reactive antigens (BCG vaccine), such as T-cell proliferation or INF_{γ} release, and restoration of these responses with interleukin (IL-2), these investigators were able to characterize three types of subjects: the responder (usually in BT and TT patients), the low responder, and the nonresponder. Nonresponders and low responders are patients with LLp leprosy, but they cannot otherwise be distinguished on the basis of histopathologic classification, bacterial indices, or duration and type of treatment. When T cells from these patients were tested for bacillus Calmette–Guérin (BCG)-induced proliferation, about 50% of both low responders and nonresponders reacted, confirming earlier reports on the presence of BCG-reactive T cells in patients unable to respond to *M. leprae.* It will be interesting to determine whether patients who are restored by the Convit's immunotherapy are among the BCG responders. Peripheral blood T cells from all patients usually respond to a variable degree to mitogens, such as concanavalin A (Con A). Therefore, the T-cell defect in LL patients seems to be specific for *M. leprae* but is manifest as either nonresponsiveness or hyporesponsiveness. Attempts to overcome the unresponsiveness of T cells from LL patients by the addition of exogenous IL-2 have been reported with varying success.[17,20]

Results recently obtained by Kaplan and Cohn[21] suggest that the addition of exogenous purified IL-2 does not lead to *de novo* sensitization but instead appears to expand already sensitized cells. These facts might suggest that the absence of IL-2 production per se is not responsible for the anergic state. Furthermore, cell-depletion experiments showed that monocyte-mediated suppression was not limited to *M. leprae,* as it was also observed in response to BCG but only in the responsive population (responder and low responders). Also, OKT8 suppressor cells seemed to have no effect on IFN_{γ} release or on proliferation to *M. leprae* or BCG in nonresponders but did reduce the Con A response. It was concluded that in the T-cell anergy of the subpopulation of LL patients who are true nonresponders, neither monocytes (or their products) nor suppressor T cells are involved. Also, a simple lack of IL-2 production or

absence of IL-2 receptors was incompatible with the observed defect.[17] The last explanation is that there is a very low number or absence of *M. leprae*-responsive T cells in the circulation of these patients, as already described some years ago by Stoner *et al.*[22] However, a fundamental question remains: Is this lack of circulating specific T cells in the true nonresponder LL patients due to an absence of precursor specific T cell or to the blocking effect of *M. leprae*-induced suppressor factor(s)?[23] Careful prospective studies and rigorous *in vitro* tests in Mitsuda-negative healthy contacts, before and after vaccination with *M. leprae* and BCG, might throw some light on such difficult problem.

2.2. Tuberculosis

2.2.1. Anergy and Immunosuppression

Many clinical and histologic observations have suggested that the immune response that occurs in tuberculosis is not univocal and, as leprosy, a complete spectrum exists. One of the first indications of tuberculosis infection or successful contact with *M. tuberculosis* is the development of a positive tuberculin skin test. In a small percentage of individuals living in endemic areas, this early tuberculin DTH reaction will be lost as the infection progresses, and the state of anergy will persist throughout the remainder of the infection period.[24] However, efficient chemotherapy is usually associated with a reduction in the bacillary mass and restoration of skin reactivity. This situation seems different from the cases, described by various investigators,[4] of a disseminated rapidly fatal nonreactive tuberculosis, with many necrotic foci, full of microorganisms but with no cellular response. Patients with this clinical entity show essentially no manifestations of cell-mediated hypersensitivity and no epithelioid granuloma formation at sites of infection replaced by a massive infiltration of macrophages overloaded with enormous numbers of acid-fast bacteria (AFB). Hematologic abnormalities are usually present, including pancytopenia. The prognosis is poor even with adequate chemotherapy.[26]

Associated anergy is also observed in many patients with classic miliary tuberculosis, which might represent the other extreme of the spectrum, since they reportedly develop typical granulomas containing very few microorganisms.[27]

Several studies have examined the immune status of anergic tuberculous patients. *In vitro* tests of patients with tuberculin anergy have usually disclosed marked abnormalities. For instance, proliferative responses of circulating T cells to phytohemagglutinin (PHA), pokeweed

mitogen (PWM), or protein-purified derivative (PPD) are usually lower than in tuberculin-positive controls.[28–29]

A semiquantitative relationship between PPD skin reactivity and PPD-induced lymphocyte proliferation was observed, the leukocyte migration inhibition test was often negative, and sensitization to dinitrochlorobenzene (DNCB) frequently failed in anergic tuberculosis.[29] Additional detected abnormalities include increases in serum immunoglobulin G (IgG), IgM, IgA, α_1-globulin, and precipitins to PPD.[30] These findings suggest the existence in tuberculosis of a spectrum of immune responsiveness, with a reciprocal relationship between cellular and humoral reactivity (see Section 2.1.1.)[4] An analysis of the immunologic spectrum in tuberculosis was undertaken by Lenzini *et al.*,[31] who studied 66 patients and placed them into four groups: reactive (RR), reactive intermediate (RI), unreactive intermediate (UI), and unreactive (UU), using clinical and radiologic data, response to chemotherapy, skin testing, histology of resected lesions, leukocyte-migration test, and presence of antimycobacterial antibodies. The findings are summarized in Table II. This spectrum has some features in common with that observed in leprosy. The UU form or the rare syndrome disseminated nonreactive tuberculosis typifies one end of the spectrum and is somehow analogous to LLp. However, *M. tuberculosis* is far more virulent, permitting a chronic lepromatous form of tuberculosis to exist. Furthermore, this group must be small in endemic areas, possibly because the more susceptible persons dye in young age, and the population becomes relatively resistant over a period of several generations.[32]

At the other pole of the spectrum are most patients with chronic stable tuberculosis. These patients uniformly have demonstrable T lymphocyte mediated DTH to mycobacterial antigens, and low B lymphocyte mediated humoral responses. This spectrum is probably best demonstrated early during the course of the disease. At this time, a substantial number of patients most likely have some decreased specific and nonspecific T-cell function, but this might be related to specific trapping of reactive T cells into the foci of infection.[33] With stabilization and improvement of disease during therapy, T-lymphocyte responses appear to heighten and return to normal.[34] This was well documented by Roney *et al.*,[24] who found that 2 weeks of protein supplementation via high-calorie, high-protein hospital diet associated with effective antituberculosis chemotherapy restored PPD skin reactivity in 17 or 23 nonreactors. Zeitz *et al.*[30] showed that skin positivity was restored in all patients, 59–134 days after the original negative test. Mycobacterium specific humoral antibodies dropped and *in vitro* proliferation responses improved.

TABLE II
Spectrum of Human Tuberculosis as Defined on the Basis of Clinical, Bacteriologic, Histologic, and Immunologic Data[a]

Classification	Reactive (RR)			Reactive intermediate (RI)			Unreactive intermediate (UI)			Unreactive (UU)		
Clinical features	Micronodular localized tuberculosis			Nodular or micronodular localized tuberculosis with cavitation, unilateral or bilateral lymphadenopathy, tuberculous serositis			Nodular or micronodular chronic diffuse tuberculosis with cavitation and fibrosis, lymphadenopathy complication with fistula formation.			Acute miliary tuberculosis Disseminated tuberculosis		
Mycobacteria												
In sputum	–	–	–	–	–	–	+	+	–	+	+	+
In tissues	–	–	–	+	–	–	+	+	+	+	+	+
Immunologic changes in lymph nodes												
Germinal center plasma cells	–	–	+	–	–	+	+	+	+	–	–	+
Paracortical area	+	+	+	+	+	–	+	–	–	–	–	–
Skin test to PPD (%)												
Typical reaction		100			30			5			0	
Early reaction		0			13			15			—	
Mixed reaction		0			57			80			0	
Leukocyte migration inhibition	+	+	+	+	+	–	±	–	–	–	–	–
Humoral anti-PPD antibodies (% positive)		5			70			98			100	
Responsive to antimycobacterial treatment (%)		100			90			33			0	

[a]From Lenzini *et al.*[31]

2.2.2. *Mechanisms of Unresponsiveness*

The mechanisms of anergy in tuberculosis are unknown. Nevertheless, many theories have been put forward and several studies performed. In contrast with leprosy, because of the rarity of disseminated nonreactive tuberculosis, most studies have been done in patients who recovered after treatment, belonging to the intermediate group of the spectrum. Here again, various mechanisms may be interdependent, or different factors may operate in different subsets of unresponsive patients, being heterogeneous, as in LLp patients.

2.2.2.a. Compartmentalization. One possibility is that sensitized lymphocytes are sequestered at or near the site of tuberculous lesions and are thus unavailable to participate in the DTH skin reaction. El-Naggar and Higashi[35] found evidence of immunologic compartmentalization of T cells in the cerebrospinal fluid (CSF) of patients with tuberculous meningitis.

Thestrup-Pedersen[3] found evidence in 10 tuberculin-positive individuals that a significant suppression of the responses to stimulation with both PPD and PHA occurred after skin testing with PPD. The suppression was seen within 1 week from skin test. The results show that a transient suppression of CMI can be induced in normal individuals by the application of a small dose of antigen. Trapping of both specific and nonspecific T cells may be a factor in the nonspecific component of the anergy seen in some severe cases of tuberculosis. Perhaps trapping in lesions and in lymphoid tissues occurs only when active inflammatory processes are present.

The depressed PHA reactivity of such patients was also demonstrated to be dependent on macrophages,[39] suggesting another possibility, the presence of circulating suppressor cells.

Rook *et al.*[33] found that lymphocyte transformation in response to mycobacterial antigen in anergic patients was significantly higher when lymph node cells were used rather than peripheral blood lymphocytes. These workers postulated that there may be specific trapping of antigen-reactive cells in lymph nodes or in infected tissues, thereby depleting the peripheral circulation.

Pleurisy with effusion and other forms of tuberculous serositis may represent special cases of immunomodulation, with anergy resulting from circumstances that may be particular. Nevertheless, it may cast light on the general mechanisms of immune regulation, also because of the possibility of harvesting inflammatory cells from the serous cavities.

The propensity for tuberculin skin-test nonreactivity of patients with tuberculous effusions was remarked on by many early investigators and is well documented in about one third of patients.[36] For instance,

Catanzaro and Barker[37] showed that cultured pleural fluid lymphocytes from two patients with tuberculous pleurisy were more responsive to PPD than to peripheral blood lymphocytes. The possible sequestration of committed specific lymphocytes in the pleural fluid was subsequently explored by Ellner.[38] Two patients with tuberculin-negative skin tests had blood lymphocytes that responded poorly, but the pleural lymphocytes responded well to PPD. When depleted of the adherent cells, the peripheral blood lymphocytes recovered their responsiveness to the level displayed by pleural cells. These results indicate that the peripheral unresponsiveness was not due solely to sequestration of committed specific lymphocytes but also to the presence of suppressor adherent cells that did not enter the pleural cavity. That immunodepression in tuberculous serositis may be due to circulating suppressor cells is suggested also by the following studies.

2.2.2.b. Suppressor Mechanisms. The area has received much recent attention focusing particularly on subsets of immunocompetent cells which have suppressor function. IgG Fc receptor-bearing lymphocytes were demonstrated to be present in the peripheral blood of tuberculosis patients tested *in vitro* with PPD.[40]

Ellner[41] showed that patients with low lymphocyte proliferation to PPD were also anergic to tuberculin skin testing and had a greater proportion of circulating monocytes. Here again, depletion of adherent cells resulted in a 25-fold enhancement of responses to PPD in low responders, compared with a threefold increase in high responders. Adding backgraded number of adherent cells to purified T-cell populations resulted in a greater depression of PPD-induced proliferative response in the low responders than in the high responders.

Peripheral blood suppressor cells in human tuberculosis were also demonstrated by other authors.[42–44] Most of these studies tend to suggest that suppressor cells are antigen specific in their induction, being dependent on the presence of viable mycobacteria in the tissues but may act nonspecifically in producing suppressor factors. Suppressor cell activities have also been found to reside within B cells,[45] T cells,[40] or monocytes.[41] In a recent study it was found that monocytes may lack surface expression of DR antigen in association with diminished *in vivo* and *in vitro* responses to PPD.[46]

As a rule, tuberculin anergy and suppressor cell activity disappear after successful chemotherapy of the underlying disease both in humans[24,47] and in experimental infection.[34] However, in some cases of severe pulmonary tuberculosis, anergy and specific T-cell unresponsiveness remain.[29] Such unresponsiveness may be due to some form of antigenic overloading, brought about by the large mycobacterial population within the lungs or elsewhere.[48] Even many months after successful chemotherapy, the residual antigenic mass represents a persistent de-

posit not removed by macrophages, and circulating factors may sustain a state of high-dose tolerance.[34]

2.2.2.c. Serum Factors. A third possibility is that serum inhibitors or mycobacterial antigens may be involved in tuberculin anergy. Heilman and McFarland[49] demonstrated that serum or plasma from patients with active tuberculosis suppressed *in vitro* responses to mycobacterial or other antigens of lymphocytes cultured from normal donors. The serum from some patients with LLp has also been shown to contain suppressor activity.[50] Moreover, suppressive factors have been shown to be produced by macrophages from LLp patients.[14] The serum from cattle infected with *M. paratuberculosis,* with Johne disease, contains a suppressor substance that inhibits the response to mitogens and antigens.[51] The suppressive activity was shown to be associated with immunoglobulin, possibly complexed with antigen.[52]

Neta and Salvin[53] demonstrated that mycobacteria, in addition to immunopotentiating activities, contain suppressive substances as well. Suppressive factor(s) found in plasma could be mycobacterial in origin and exert their inhibitory action after release by adherent cells.[43,54] One candidate factor for nonspecific suppression was shown to be IFN_{α}, whose secretion by mononuclear cells of normal PPD-negative donors correlated well with the degree of nonspecific *in vitro* suppression.[6] This suppression is nonspecific, since it occurred with all mycobacterial species tested and with killed mycobacteria and is produced by both positive and negative tuberculin responders. A second condidate has been described by Ellner and Daniel[52] and by Kleinhenz *et al.*[55] Inhibition of blastogenesis was associated with the release of prostaglandin E_2 (PGE_2); when blood monocytes were cultivated with pooled plasma from tuberculous patients (TB plasma) containing the mycobacterial cell wall polysaccharide D-arabino-D-galactan (AG), the effect was inhibited by the presence of indomethacin or by passing the TB plasma over an immunoabsorbent column of Sepharose-linked antibody against mycobacterial AG. AG also increased monocyte attachment to plastic.

It is conceivable that numerous factors are involved in anergy: specific anergy might be mediated by antigen-specific suppressor T (Ts) cells, while generalized anergy might be due to the direct effect of plasma factors or cell wall antigen, and the two mechanisms might, respectively, be indomethacin independent or dependent.[56] Alternatively, mycobacterial polysaccharide induced suppression may ensure antigen specificity via an interaction with Ts cells.[6,55,57] Recently, it has been observed an increased sensitivity to the suppressive effects of PGE_2 on IFN_{γ} and proliferation to mitogen in the leukocytes of human neonates[58] and in the lymphocytes of adult mice with a variety of inflammatory diseases or in experimental diseases.[59] Finally, the situation might be further complicated by the likelihood that mycobacteria that

differ markedly in their interaction with macrophages, such as *M. leprae* and *M. tuberculosis,* end up in different types of macrophages.[9] Recent preliminary evidence from our laboratory suggests that *M. bovis* strain BCG is killed by those macrophages that ingest it first, but the remaining live BCG is able to grow later in other subpopulations of macrophages harvested from inflammatory foci (J. L. Stach, B. Hurtrel, and P. H. Lagrange, in preparation). Thus, certain strains of mycobacteria may have evolved strategies that promote uptake by macrophages subsets with limited ability to kill them or to process antigen, and are therefore unable to stimulate the right subset of T cells to induce protective immunity.

2.2.2.d. Genetic and Environmental Predispositions. Unrecognized protein or nutritional deficiencies have been shown to be associated with anergy in tuberculosis. In fact, restoration of skin reactivity was described by Rooney *et al.*[24] in anergic tuberculosis patients given a high-calorie, high-protein hospital diet with effective chemotherapy. Harrison *et al.*[60] reported that anergic rural Nigerians with active tuberculosis were frequently undernourished and that the size of the skin reaction correlated significantly with serum albumin and transferrin concentrations. However, other workers have found no significant difference in serum protein and albumin levels in patients with anergic tuberculosis as compared with those with normal skin reactivity.[32,61] Experimental murine infection have shown that, compared with controls, protein-calorie-restricted mice exhibited diminished early inflammatory nonspecific immune response, granuloma size, and DTH reaction to tuberculin. By contrast, bacterial multiplication was reduced in the organs of these animals (G. A. Conge, P. Gouache, P. H. Lagrange, and D. Lemonier, unpublished results).

Finally, it is conceivable that genetic factors predispose to high susceptibility to tuberculosis and tuberculin hyporesponsiveness.[32] Persson *et al.*[62] observed a high frequency of HLA B7 in patients with tuberculin-negative saracoidosis as opposed to tuberculin-positive persons. In a study of patients with malignant melanoma treated with BCG vaccine, Buckley *et al.*[63] reported that conversion of tuberculin sensitivity was significantly reduced in patients with B7 antigens, as opposed to B7-negative patients.

In a recent study, Cox *et al.*[64] showed that HLA B7 did not appear to be associated with a depressed tuberculin response in tuberculous patients or to influence the clinical course of disease. The failure to detect significant difference in the HLA class I antigens does not preclude the possibility that resistance or susceptibility to tuberculosis is encoded by the major histocompatibility complex (MHC).

The involvement of HLA-encoded genetic factors in leprosy has been evidenced most convincingly by family studies. In particular, HLA

class II (DR) antigens have been found to be associated with certain forms of disease. In a recent study[15] of the relationship between HLA and LLp, HLA haplotype segregation was analyzed in 28 families from Venezuela, and it was found that HLA DR3 was inherited preferentially by children with polar TT rather than LLp. This seemed to indicate that in this population a DR3-associated factor controls the type of disease that develops after infection with *M. leprae.* Furthermore, out of a group of 74 healthy Caucasian individuals living in a leprosy nonendemic area, HLA DR3 was absent in the 16 individuals who did not respond to any mycobacterial antigen tested.[65] These data show that an HLA-DR3-associated genetic factor controls, albeit indirectly, skin-test responsiveness to mycobacterial antigens. Similar findings were obtained in experimental infection of congenic mice having different H2 haplotypes but the same genetic background.[66] The expression of tuberculin sensitivity after BCG infection was shown to be dependent on at least two genes, one linked with H2 and the other, on chromosome 1, called *Bcg* gene.[67] Many different mechanisms seem to be involved in the unresponsiveness in tuberculosis and in leprosy. Some such factors are depicted in Fig. 2.

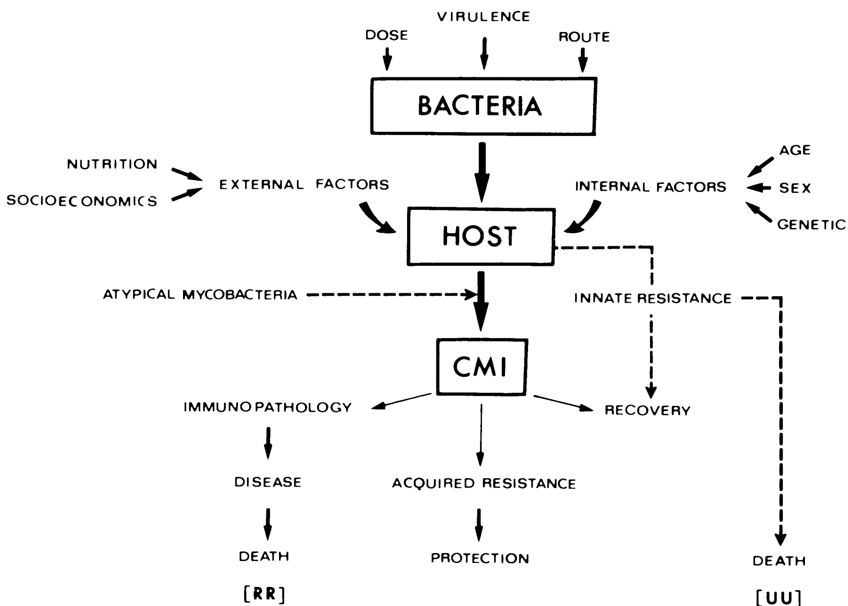

FIGURE 2. Schematic diagram of mycobacteria–host relationship and related factors influencing the outcome of infection or disease and the participation of cell-mediated immunity (CMI) in humans. (Adapted from Lagrange *et al.*[66])

Note, for instance, the particular pathway in unresponsive tuberculosis patient (called UU, by Lenzini *et al.*[31]), without any CMI and those initially RR who develop increased immunopathology leading to death. Similarly, there might be different pathways for recovery, including those in which CMI is involved and those bypassing the CMI thanks to good innate resistance or low infective inoculum. Without better tools than we have today for the study of the immunology and epidemiology of human mycobacterial infections, it will be extremely difficult to demonstrate clinically the existence of such pathways. It is hoped that experimental infections of laboratory animals may provide a better understanding of the spectrum of immune responses to mycobacteriosis.

3. THE IMMUNE SPECTRUM IN EXPERIMENTAL MYCOBACTERIAL INFECTIONS

3.1. Introduction

It is not the purpose of this chapter to analyze the advantages and limitations of animal models. Excellent reviews have recently appeared on this subject.[9,68] The main advantage of animal models is that dependent and independent variables, such as enumeration of AFB in target organs, dose, route, timing of infection, species, strain, and sex of the host, can be precisely controlled and selected. The pathogenic and immunogenic properties of different mycobacterial species and strains can be compared. The use of a single mycobacterial strain that is prepared, kept, and inoculated under rigidly identical conditions into selected isogenic strains of animals can also provide background knowledge of the nonspecific and specific mechanisms of resistance to infection and disease.

Variations in resistance to tuberculosis of different animal strains have been described since the beginning of the century in mice,[69–71] guinea pigs,[72] and rabbits.[73] Only recently has an analogous variability been described in the only animal in which *M. leprae* is able to multiply, the armadillo.[74]

Anergy in experimental mycobacterial infections has generally been studied after that an established DTH has vanished due to desensitization with a high dose of antigen or to infection progression. This state of skin-test anergy will usually persist throughout the remainder of the infection period.[34] By contrast, recent studies have examined the absence or low level of DTH to tuberculin that may occur after infection and the factors involved.[66]

3.2. Acquired Anergy

3.2.1. Desensitization

Injection of large doses of antigen into an animal with DTH can render the animal specifically unreactive to subsequent intradermal challenge with that antigen.[75] The unresponsiveness is usually short-lived and proportional to the dose of antigen given. *In vitro* (lymphocyte proliferation and lymphokine production) techniques have been shown to correlate with skin-test reactivity.[76] Desensitization has been used in order to analyze the relationship between DTH and acquired protection.[77] Desensitization of tuberculous guinea pigs with daily administration of increasing doses of tuberculin to the point at which intradermal responsiveness to tuberculin was lost was associated with persistence of immunity to reinfection. It was also shown that desensitized animals survived longer, with less caseation and fewer microorganisms in the lungs.[78] Moreover, acquired anergy in such animals is not always specific and is not accompanied by the loss of responsiveness in other sites. For instance, desensitized rats are still susceptible to tuberculin shock[79] and do not express DTH to heterogeneous recall antigens. Desensitization appears to work by means of an active environmental factor(s): when transferred into desensitized guinea pigs, specific immunocompetent lymphocytes lost their competence, while cells removed from desensitized animals regained their immunologic reactivity.[80] Two mechanisms responsible for anergy after desenstization have been proposed. One attributes it to a compartmentalization of antigen-reactive lymphocytes,[76] the second to an exaggeration of normal DTH regulatory mechanisms triggered by a regimen of antigen given intravenously(IV) that produces systemic activation of suppressor cells. Either mechanism has also been suggested for anergy induced by species and strains of mycobacteria that tend to disseminate after a subcutaneous (SC) injection.

3.2.2. Disseminated Mycobacterial Infections

When mice are infected SC with species of mycobacteria (*M. lepraemurium, M. ulcerans, M. avium*) that disseminate from the injection site, two distinct phases of immune reactivity can be distinguished. The first phase is characterized by the slow development of positive immune parameters: DTH to specific antigen, production of lymphokines (MIF), and enhanced responsiveness to unrelated antigens (i.e., SRBC). However, as the disease progresses, the CMI parameters are switched off and the microorganisms disseminate throughout the body.[81] Spleen cells harvested from such an anergic host exhibit reduced proliferative re-

sponsiveness to PHA, Con A, or allogeneic lymphocytes *in vitro*. The final phase of the disease resembles LLp. Similar findings were obtained when mice were inoculated IV with other disseminating mycobacterial species (e.g., *M. lepraemurium*)[82] or with nondisseminating mycobacteria (e.g., *M. bovis* st. BCG, *M. kansasii, M. habana*).[34]

Brown[68] recently published an excellent review on the immunologic abnormalities induced by *M. lepraemurium* or BCG infection in mice. Thymic involution and massive infiltration by infected macrophages, disturbance of lymphoid tissue architecture, alteration of lymphocyte traffic, impaired responses of both T-dependent and T-independent antigens, and depressed mitogenic activity and T-cell cytotoxicity are the most frequent abnormalities observed in experimental mycobacterial infections. After inoculation with invasive and disseminating yet highly immunogenic bacteria (*M. lepraemurium, M. avium*), all rodents infected systemically, and most of those infected SC develop a progressive lepromatous-type disease, associated with anergy and T-cell depletion. For this reason, the former infection has been used largely as an immunologic model to *M. leprae* infection in human subjects. However, the onset and rapidity of the gradual fall in antimycobacterial response that occurs as the infection progresses depend not only on the dose, route, and species of mycobacteria inoculated, but also on the genetic constitution of the animals.

Disseminated experimental infection with *M. leprae* given IV to Mangabey monkeys was shown to be associated with anergy and to an increasingly severe depression of blastogenic responses to PHA, Con A, and PWM. The polyclonal immunoglobulin plaque-forming cell response to PWM was also depressed.[83] These results indicate that the Mangabeys should provide a useful model for studies of the immunologic changes accompanying leprosy and their underlying mechanisms.

3.2.3. Mechanisms of Acquired Unresponsiveness

Although peripheral anergy develops after the IV infection of large doses of mycobacteria, the spleen of such animals does contain cells capable of transferring DTH and protection to normal naive recipients.[34] However, the same cells exhibit a reduced blastogenic responsiveness to mitogens or to allogeneic lymphocytes *in vitro*.[84] Decreased IL-2 activity was found to be an early indicator of T-cell immunodeficiency after *M. lepraemurium* infection[85] or after BCG inoculation.[86] Such results seem to indicate that two main mechanisms underlie the acquired unresponsiveness: trapping and suppressor cell activation.

3.2.3.a. Perturbation of Lymphocyte Recirculation. Several experimental approaches have shown evidence of an altered recirculation pattern of

specific committed lymphocytes. Chromium-51-labeled lymph node cells injected IV into mice with disseminated *M. ulcerans* infection were shown to be trapped in the lymph nodes at the expense of spleen and blood, as compared with normal mice.[87] Similar results were obtained by Zatz,[88] who showed that IV or SC injection of BCG resulted in increased localization of ^{51}Cr-labeled syngeneic lymph node cells in the spleen or draining nodes, respectively. This increase was biphasic in the spleen, occurring 1 or 2 days after BCG and reappearing 3 weeks later, but monophasic in the lymph nodes, lasting throughout the 24 days of the study.

Bullock[89] gave evidence that IV infusion of radiolabeled thoracic duct lymphocytes (TDL) from normal syngeneic donors failed to produce a significant increase of cell output and radioactivity in the thoracic duct lymph of *M. lepraemurium*-infected rats. Perturbation of lymphocyte recirculation took place 2–6 weeks after inoculation of viable *M. lepraemurium,* never occurred using heat-killed mycobacteria, and appeared to be secondary to granulomatous pathology in lymphoid organs. The spleen of the infected rats was shown to be the major trap for recirculating TDL and, to a lesser extent, the lymph nodes and liver.[90] If specific antigen-binding lymphocytes are chronically trapped in lymphoid organs that harbor mycobacterial antigen difficult to remove from the antigen-presenting cells, the conditions may prevail within the microenvironment of these organs, whereby their function in the amplification of an immune response is subverted to another, namely the induction of suppressor factors that may maintain a state of unresponsiveness.

3.2.3.b. Cell-Mediated Suppressor Mechanisms. Several investigators have analyzed the nature of the inhibitory cells evoked in animals infected with a high IV dose of BCG or *M. lepraemurium.* Most studies evaluated the suppression of proliferative responses to mitogens, others T dependent and independent antibody production *in vitro.*[91] Bullock *et al.*[92] demonstrated the presence of two cell populations in the spleen of *M. lepraemurium*-infected mice that suppress the primary antibody response of syngeneic spleen cells to SRBC. One suppressor population was sensitive to treatment with anti-Thy1 serum plus complement, and the other was adherent and appeared to be a macrophage. Demonstrability of suppressor cells correlates generally with number of mycobacteria present in the tissue and extent of their multiplication, although very high doses of killed microorganisms will produce a similar effect.[93] Also, lung granulomas associated with macrophages capable of potent suppression of both antibody and CMI reactivity were shown to be produced after IV injection of killed BCG in an oil-in-saline emulsion.[94] Later, the development of chronic granulomatous reactions and suppressor macrophages was shown to be under the control of a re-

cessive gene linked to the immunoglobulin heavy-chain allotype, but not to H_2.

When nondisseminating bacteria are used (i.e., BCG), the development of these suppressor cells does not prevent mice from recovering fully from massive BCG infection. In addition, suppressor cells develop after, rather than before, dissemination of ultimately fatal infections, such as *M. lepraemurium*[92] or *M. avium*.[95] Thus, induction of these suppressor cell populations seems to be a consequence of massive challenge by an unusual experimental route. Moreover, the treatment of heavily infected mice with an effective chemotherapy regimen before the development of specific anergy and cellular hyporesponsiveness results in complete absence of Ts-cell activity.[96] Usually such Ts-cell activity was found among the nonadherent spleen cell population.

Following SC infection with *M. lepraemurium,* a nylon–wool-adherent radiosensitive suppressor cell population was demonstrated in the spleen of BALB/c mice.[97] Such cells were able to decrease resistance when injected into newly infected syngeneic mice. No such cells were found in infected C57BL/6 mice, which are resistant to dissemination. This new phenomenon is of importance, since the suppressor cells involved, associated with dissemination, did not resemble those described after IV challenge. An analogous observation was made when BALB/c mice were infected IV with a low dose of BCG (Fig. 3). There was no difference in BCG multiplication during the first 3 weeks after challenge in normal or in prechallenge irradiated BALB/c mice, but later there was a better clearance of viable microorganisms in the spleen of irradiated mice as compared with normal mice. The low rate of clearance in normal mice was associated with a decline of the 42-hr DTH reaction to tuberculin, while protracted 42-hr DTH was observed in irradiated BALB/c mice. No such effect was observed in C57BL/6, A/J, C3H, or DBA/1 mice. These results indicate that the elimination of the regulatory mechanisms to manipulate various cell functions before or during infection, may induce restoration of immune responsiveness and acquired resistance. This may occur through modulation of the production of cellular mediators known to facilitate the proliferation and differentiation of immunocompetent lymphocytes.

3.2.3.c. Interleukin Production. In experimental murine leprosy, nonspecific T-cell suppression was marked, as measured by decreased proliferative response to Con A and in mixed lymphocyte reactions *in vitro*.[84] This was shown to be associated with a decreased production of IL-2 in the supernatant of Con A-stimulated spleen cells.[85]

Decreased IL-2 activity was found to be a sensitive indicator of T-cell immunodeficiency, since it occurred as early as 1 month after *M. lepraemurium* challenge. Whereas IL-1 production remained unaffected

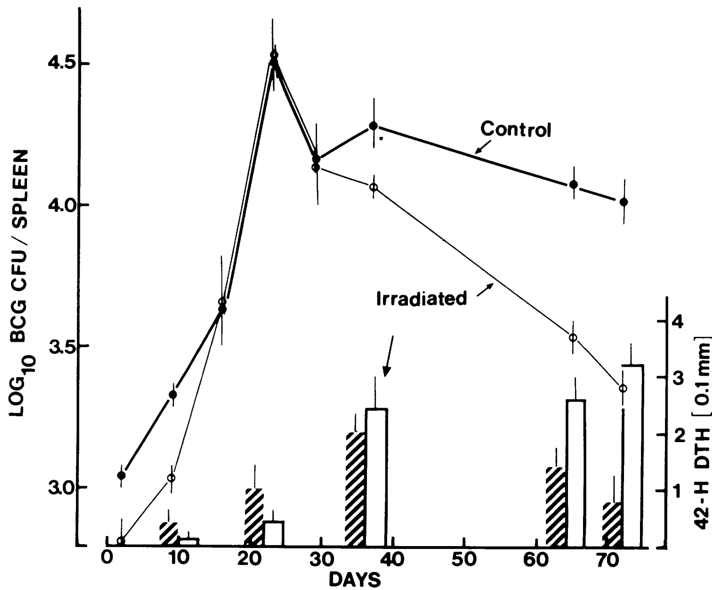

FIGURE 3. Bacterial growth curves in normal and irradiated (550 rad, 24 hr before challenge), BALB/c mice infected IV with 1×10^4 viable BCG Pasteur (1173 P2 strain). Hatched and white bars represent the 42-hr footpad swelling (DTH) reactions following injections of 4 μg tuberculin into the hind footpad of normal and irradiated respectively; five mice per group (mean ±SEM). CFU, colony-forming units; DTH, delayed-typed hypersensitivity.

over the entire observation period, IL-2 decreased as the infection progressed. This was observed also after SC injection of a high (1×10^8) or low (1×10^5) inoculum of *M. lepraemurium* but occurred when a threshold number of bacteria had reached the spleen.[98] When different strains of mice were infected IV with 1×10^7 *M. lepraemurium,* it was shown that after 3 months, the number of AFB recovered from the spleen correlated with the strain distribution of *Bcg* gene, controlling innate resistance as established for BCG infection.[67] However, decreased IL-2 production by spleen cells was only detected in C57BL/6 and DBA/1 mice (Table III). Specific antibody levels were found to be high in C57BL/6, A/J, and BALB/c mice. Thus, there was no evidence of a direct influence of the *Bcg* gene on lymphokine and specific antibody production.[99]

Preliminary evidence shows that syngeneic receipients of T-cell-enriched spleen cell suspensions from anergic C57BL/6 infected mice exhibited a decreased ability to control the local growth of *M. lepraemurium* inoculated SC prior to adoptive transfer.[100]

TABLE III
Number of Acid-Fast Bacteria Recovered from Spleen, Spleen Cell IL-2 Production, and Specific Antibody Levels in Various Inbred Strains of Mice Infected Intravenously with 1 × 10^7 *M. lepraemurium* for 3 Months[a]

Strains[b]	*Bcg* genotype	Log_{10} AFB/spleen[c]	IL-2[d] (%)	Antibody levels[f]
C57BL/6	ss	7.29 ± 0.14	26[e]	+++
BALB/C	ss	6.94 ± 0.23	94	++
DBA/1	ss	6.66 ± 0.21	46[e]	+/−
A/J	rr	6.35 ± 0.10	86	+++
CBA	rr	6.24 ± 0.09	91	+
DBA/2	rr	5.70 ± 0.34	63[e]	−/+
B6D2F1	sr	5.92 ± 0.33	70[e]	+/−

[a]Adapted from Hoffenbach *et al.*[99]
[b]Seven mice per group.
[c]Geometric mean ±SEM.
[d]IL-2 production is expressed as percentage of the value obtained for age matched normal mice.
[e]Significantly different from control values ($p < 0.05$, Mann–Withney test).
[f]Measured by solid-phase radioimmunoassay.

Although it was found that spleen cells from *M. lepraemurium*-infected mice did not lack IL-2 receptors, *in vivo* or *in vitro* restoration of T-cell responsiveness was not observed after administration of preparations containing IL-2 activity (A. Hoffenbach, unpublished results). This finding is in contrast with results reported in BCG infection.[101]

Orme *et al.*[102] found that spleen cells from heavily BCG-infected mice were able to transfer adoptive protection but were unresponsive to mitogens *in vitro*. Since the unresponsiveness was restored by supplementation of IL-2, it was suggested that the unresponsiveness may be due to reduction or consumption of available IL-2 in the culture medium.

More recently, a similar unresponsiveness was seen in mice chronically infected with *M. intracellulare*.[59] The effect was correlated with spleen and lung weight increase, number of viable bacteria in these organs, and reduced production of oxygen metabolites (i.e., superoxide anion) by spleen macrophages. A drastic reduction of IL-2 and IFN_γ production was also observed when unfractionated spleen cells from infected mice were stimulated with specific antigen, but not with Con A. In addition, these abnormalities were associated with increased production of PGE_2 by spleen macrophages and a higher susceptibility of T cells to PGE_2. *In vivo* daily treatment for 7 days of infected mice with IFN_γ, muramyl-dipeptide (MDP),or indomethacin significantly reduced spleen and lung weights as well as the viable counts of *M. intracellulare* in these organs. These data suggest that persistent infection with this my-

cobacterial species results primarily from failure of release of macrophage-activating lymphokines rather than from insufficient numbers of macrophages or abnormal macrophage–lymphokine receptor. No data are available on the radiosensitivity of macrophages producing high levels of PGE_2.

The acquired anergy and *in vitro* unresponsiveness that occur following a high IV dose of nondisseminating mycobacteria or after SC administration of disseminating mycobacteria have been studied primarily in mice. Lymphocyte trapping and induction of at least three types of regulatory suppressor cells may contribute to the nonspecific component of the defect. But in one case only (the radiosensitive suppressor cell, or the activated PGE_2-producing macrophage) have these cells been shown to have a detrimental effect on the course of infection. The properties of the regulatory cells depend chiefly on the species of mycobacteria (i.e., virulence factors), on the route of infection, and, to a lesser extent, on the genetic background of the mouse.[99]

3.3. Innate Specific Unresponsiveness

3.3.1. Immune Response to M. bovis BCG

The responsiveness to nonpathogenic (nonvirulent) mycobacterial (e.g., BCG) infection has been studied in different strains of mice by several workers.[71,103,104] High- (SWM/MS) and low-responder (C3H) inbred strains were used, and the development of DTH was measured by spleen index, footpad swelling, and disappearance of peritoneal macrophages.[103] The investigators concluded that high responsiveness was dominant. In a subsequent study, they suggested that the low responsiveness was due to Ts cells, since DTH was restored after cyclophosphamide treatment.[105] The interstrain difference was then explained on the basis of an inability of macrophages from low responders to present antigen to sensitized lymphocytes.[108]

Using a different model, it was shown in our laboratory[104] that mouse strains independently selected against mycobacteria presented high or low resistance to mycobacterial infections (*M. bovis* BCG, and *M. tuberculosis,* strain H37Rv) with differences in both the innate ability of nonimmune macrophages to inhibit the multiplication of the microorganisms in the spleen early in infection and in the ability to mount specific and nonspecific immune responses independently of the route and dose of the inoculum (Table IV). Later it was shown, using inbred strains of mice, that the difference in natural resistance can be attributed to gene(s) segregated in each strain[67] that control the innate microbicidal activity of macrophages.[107] This was best shown when mice were

TABLE IV

Natural and Acquired Resistance to *M. tuberculosis* H37Rv and Development of Immune Parameters after BCG Immunization in High-Antibody (H.ab) and Low-Antibody (L.ab) Producer Mice[a]

	H.ab		L.ab	
Mean survival time (days) after one IV injection of 1×10^6 H37Rv				
In normal mice	127.1 ± 5.8		59.2 ± 9.0	
In BCG vaccinated mice	186.6 ± 10.2		87.6 ± 4.6	
Antibodies to PPD (hemagglutination titer) 28 days after BCG vaccination	1/8192		1/512	
Delayed-type hypersensitivity to tuberculin (4 μg/mouse) 30 days after BCG vaccination with	24 hr	48 hr	24 hr	48 hr
2.2×10^5 viable BCG	7.6 ± 0.6	5.8 ± 0.4	1.8 ± 0.3	0.8 ± 0.3
2×10^6 viable BCG	10.0 ± 0.7	7.6 ± 0.8	2.2 ± 0.6	0.6 ± 0.4
2×10^7 viable BCG	14.8 ± 1.3	10.8 ± 1.4	4.2 ± 1.0	1.8 ± 0.5
Two injections of 2×10^6 BCG at 2-week interval	6.8 ± 1.1	6.4 ± 0.9	1.4 ± 0.3	1.2 ± 0.2
Stimulation index of spleen cells cultivated *in vitro* for 56 hr with 8×10^4 viable BCG in mice BCG vaccinated 42 days previously	Total spleen cells	Nonadherent cells	Total spleen cells	Nonadherent cells
	3.80 ± 0.20	4.60 ± 0.17	3.20 ± 0.32	2.75 ± 0.18
Log_{10} number of colony-forming units of BCG in spleen of mice injected with 3×10^6 viable BCG				
At 6 hr	5.48 ± 0.12		5.50 ± 0.21	
At 3 weeks	5.09 ± 0.16		<3.95	
At 5 weeks	4.18 ± 0.25		4.03 ± 0.28	
In draining lymph node after one injection on 1×10^7 viable BCG, 3 weeks previously	4.36 ± 0.32		3.16 ± 0.09	

[a] Adapted from Lagrange *et al.*[66,104]

inoculated IV with a low inoculum (1×10^4 viable bacteria per mouse) of BCG Montreal and only for the mycobacterial multiplication observed in the spleen after 3 weeks.[108] The absence of systemic granuloma formation and very low 48-hr DTH reactions to tuberculin was obtained after BCG infection in naturally resistant (*Bcg*^{r}) mouse strains.[109] Moreover, recent studies showed that mice that develop the 42-hr DTH reactivity to tuberculin belong to the natural susceptible (*Bcg*^{s}) strains (Fig. 4). They are then able to develop a dose-related acquired resistance to IV challenge with the homologous organisms.[110] Natural resistance or susceptiblity to *M. bovis* BCG is not linked to genes of the MHC, since it is located on chromosome 1,[67] but CMI responses, such as DTH reactions, involve cell cooperation between macrophages and T lymphocytes controlled by gene(s) encoded in the MHC. The influence of H2 haplotype on the level of DTH to tuberculin was analyzed in various inbred and recombinant lines of mice. These lines were of the same background, expressing the *Bcg*^{s} phenotype but had different H2 haplotypes. The results showed that the level of the DTH reaction is linked to the H2 haplotype: $H2^q$ lines gave the lowest level and $H2^k$ the highest, with $H2^b$ intermediate.[66] These results indicate that the expression level of a DTH reaction to tuberculin after BCG infection is influence at least by two genes. One located on chromosome 1 controls the type of the DTH reaction. All *Bcg*^{s} mice, except BALB/c, expressed a protracted 42-hr reaction, while *Bcg*^{r} mice never expressed such reaction independently of the time and route of infection. The second gene, located in the MHC, controls the intensity of the local inflammatory reaction, and possibly of immunopathologic phenomena. Thus, innate anergy or absence of inflammatory reaction may be observed in different strains of mice for different reasons.

3.3.2. Acquired Anergy, Immune Suppression, and Dissemination

Concerning the BALB/c mice, which represent an exception in that they are *Bcg*^{s} but express the 18-hr DTH reaction during the first month of infection[111] and a mixed reaction at later times (Fig. 4), it has been shown previously that whatever the route of BCG inoculation, they always exhibited dissemination and slow clearance rate (Fig. 3). This situation was modified when the mice were sublethally irradiated before challenge, since they become able to express the 42-hr DTH reaction. No such effect was observed in other *Bcg*^{s} or *Bcg*^{r} strains (Fig. 5 and 6). Thus, the induction of a particular subset of a T cell with regulatory functions may explain the unresponsiveness that occurs naturally in this strain of mice, even after a low inoculum of nondisseminating mycobacteria. Similar results were obtained in *Leishmania tropica* infections, in

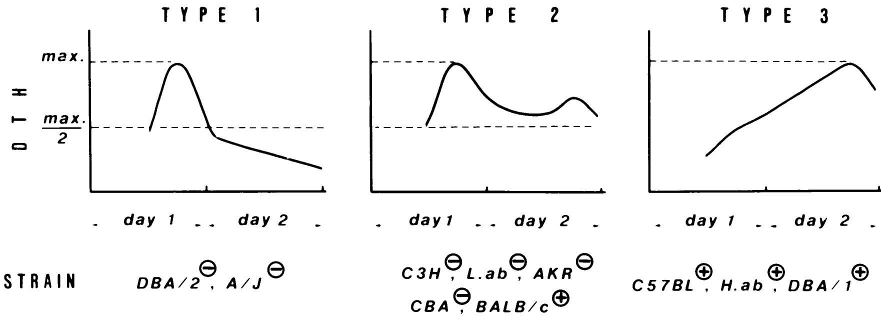

FIGURE 4. Time course of footpad swelling in various inbred mouse strains following the injection of 4 μg tuberculin 3 weeks after one SC injection of 1 × 10^6 BCG Pasteur (1173 P2 strain) into the contralateral footpad. The patterns observed can be classified into three types, according to maximum footpad swelling measured during the first and second day following elicitation. Comparison between DTH pattern and natural resistance to BCG infection showed a correlation between susceptibility and protracted type of DTH reaction in all strains tested, except BALB/c., (+, natural susceptibility; −, natural resistance; BCG, bacillus Calmette–Guérin; DTH, delayed-type hypersensitivity.

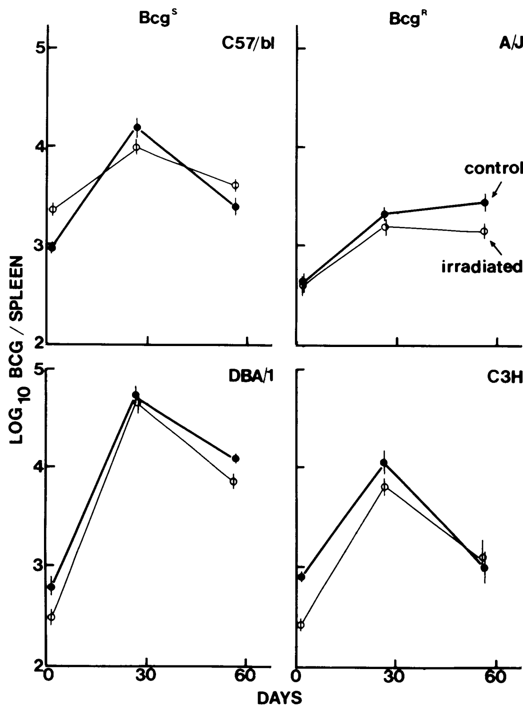

FIGURE 5. Growth curves of BCG strains 1173P2 in control and irradiated (550 rad, 1 day prior to challenge) mouse strains, expressing the two *Bcg* phenotypes, infected IV with 1 × 10^4 viable cells; 5 mice per group (mean ±SEM).

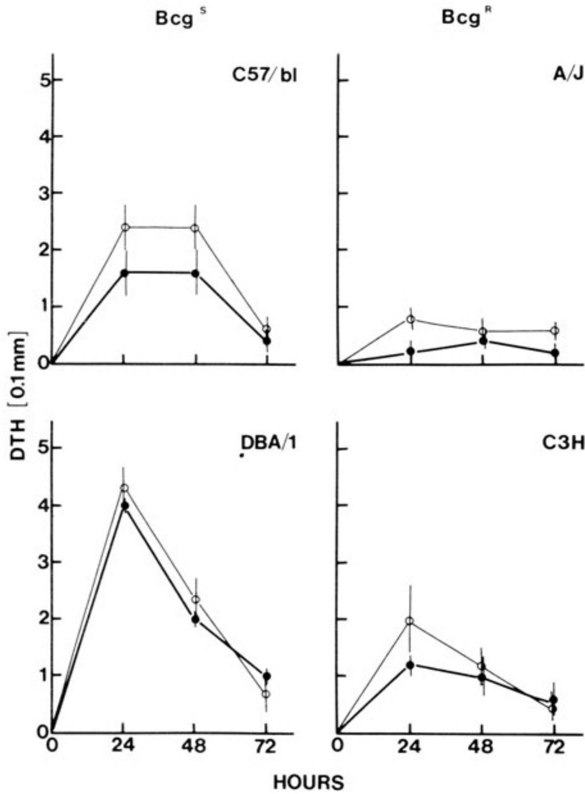

FIGURE 6. Time course of hind footpad swelling following injection of 4 μg tuberculin in control (●) and irradiated (○) mice of strains expressing the two *Bcg* phenotypes and infected SC with 1 × 10^6 viable BCG Pasteur (1173P2 strain) 58 days earlier; five mice per group (mean ±SEM).

which irradiation restored the strong antiparasite DTH response that develops in parallel with resistance to disease.[112] The suppressor cells involved were of thy-1$^+$, L3t4, and I-J$^-$ phenotype and were capable of inhibiting the induction and expression of DTH as well as reversing the healing of lesions.[113] Susceptibility to irradiation may suggest that suppressor cell generation during leishmania,[114] *M. lepraemurium,*[115] and BCG infections is secondary to a defect in macrophages or in a particular subpopulation of macrophages caused by these pathogens in BALB/c mice.

Thus, depending on time, multiplication, and persistence of the microorganisms, L3t4$^+$ lymphocytes will accumulate and function as suppressor cells, explaining why immune responses may vary from up-

grading reactions (if L3t4 are prevented to function) to downgrading reactions (if mycobacteria continue to multiply and disseminate). Such variations in immune responses may resemble those observed in BL or nonreactive intermediate tuberculosis.

3.3.3. Resistance of Mice after *M. lepraemurium* Infection

In these studies, resistance was measured as mean survival time (MST) after systemic challenge.[116] The MST showed a transition from the most susceptible (BALB/c) to the most resistant (DBA/2) strains. The pattern observed that resistance to infection is at least in part controlled by a gene, or group of genes, with the same strain distribution as *Bcg*, *Thy*, and *Lsh* genes.[117] However, when the same strains of mice were inoculated SC with a lower inoculum of *M. lepraemurium*, a different pattern of resistance was observed.[118] C57BL/6 but not C3H mice were able to limit bacterial multiplication, produced an immune granuloma, prevented the dissemination of the microorganism, and expressed a 48-hr DTH reaction. Since C57BL/6, but not BALB/c mice, are resistant to SC challenge and are very susceptible to IV infection with various pathogenic mycobacteria, it is possible that a same microorganism induces different effector mechanisms that are beneficial because they are self-limited during localized infection but toxic to the host (as demonstrated by fatal tuberculin shock[119]) and ineffective in protection after a high dose inoculated systemically. The mechanisms of such acquired unresponsiveness are different from those observed in BALB/c mice.

4. CONCLUSIONS

Numerous conclusions can be drawn from the above data. Individuals developing any of the mycobacterial diseases may temporarily lose or may never have the possibility to respond to mycobacterial antigens. When any of these infections become disseminated, specific and nonspecific local or systemic cell-mediated unresponsiveness occurs. Depending on the underlying mechanisms, in certain individuals this unresponsiveness is reversed by either chemotherapy or immunotherapy, or both, with clinical improvement, while others never recover from their specific unresponsiveness. It may be also important to consider that the regulatory mechanisms mounted by the mammalian immune system are basically the same in all mycobacterial infections but vary depending on genetic and environmental predispositions of the host and on the pathogenic capacity of the infecting species.

Several explanations for the *in vivo* and *in vitro* anergic states occurring in human mycobacterial infections have been put forward. They

are not mutually exclusive, but each may concern particular subsets of individuals belonging to the various groups of the immunologic spectrum. Infected individuals are more or less heterogeneous in immune response, showing geographic variation, and an even higher heterogeneity is observed in specific and nonspecific effectors and regulatory mechanisms. Thus, for an apparently similar anergic state, different regulatory mechanisms may be involved. Such newly acquired knowledge will be of great assistance for future strategy in controlling human mycobacterial infections.

The conclusions drawn from experimental models have been useful in the past, but some confusion exists in the literature concerning the definition of resistant and susceptible mouse strains.[9] This results from the use of different species and strains of mycobacteria (without any definition of their pathogenic capacity), inoculum doses, and route of administration, as well as from differences in the evaluation of resistance and pattern of CMI responsiveness or unresponsiveness.

There are several areas in urgent need of further *in vivo* and *in vitro* studies: fate and nature of interrelationship between living mycobacteria and several subpopulations of phagocytic cells; interactions between such subpopulations of infected cells and the recruitment, functions, and clonal expansion of specific T-cell subpopulations; relationships between regulatory cell pathways and natural or acquired resistance or susceptibility; and interrelationships between protection and immunotherapy. The amount and type of cell mediators also have a genetic basis, and further analysis of the responses of resistant and susceptible animals will yield information of value. The genes involved need to be identified and mapped, and techniques must be devised to distinguish at the molecular level between the phenotypic expression that influences innate and acquired resistance. It will then be possible to develop realistic *in vivo* and *in vitro* tests in human beings for evaluation of the innate potential to mount an effective acquired immunity before the first contact with a given mycobacterium species. Such work may also lead to the possibility of finding ways to increase natural resistance and of developing better adapted chemotherapy for individuals already suffering from mycobacterial diseases or methods other than classic vaccination for protection of genetically unresponsive individuals.[120]

REFERENCES

1. Chaparas, S. D., 1982, The immunology of mycobacterial infections, *C.R.C. Crit. Rev. Microbiol.* **9:**139–197.
2. Forget, A., Benoit, J. C., Turcotte, R., and Gusew-Chartrand, N., 1976, Enhancement activity of anti-mycobacterial sera in experimental *Mycobacterium bovis* (BCG) infection in mice, *Infect. Immun.* **13:**1301–1306.

3. Thestrup-Pedersen, K., 1974, Temporary suppression of lymphocyte transformation after tuberculin skin testing, *Immunology* **27**:965–971.
3a. Snider, D. E. Jr., 1982, The Tuberculin Skin Test, *Am. Rev. Respir. Dis.* **125**(Suppl. 3):108–118.
4. Daniel, T., 1980, The immunology of tuberculosis, *Clin. Chest Med.* **1**:189–201.
5. Ridley, D. S., and Jopling, W. H., 1966, Classification of leprosy according to immunity. A five group system, *Int. J. Lepr.* **69**:442–444.
6. Bloom, B. R., Learning from leprosy: A perspective on immunology and the third world, *J. Immunol.* **137**:1–10.
7. Cölogłu, A. S., 1979, Immune complex glomerulonephritis in leprosy, *Lepr. Rev.* **50**:213–222.
8. Avrameas, S., 1986, Natural autoreactive B cells and autoantibodies: The "know thyself" of the immune system, *Ann. Inst. Pasteur (Immunol.)* **137D**:150–156.
9. Rook, G. A. W., 1983, An integrated view of immunology of the mycobacterioses in guinea pigs, mice and men, in: *Biology of Mycobacteria*, Vol. 2 (C. Ratledge, and J. Stanford, eds.), pp. 279–319, Academic, London.
10. Kaplan, G., Weinstein, D. E., Steinman, R. M., Levis, W. R., Elvers, U., Patarroys, E., and Cohn, Z. A. 1985, An analysis of *in vitro* T cell responsiveness in lepromatous leprosy, *J. Exp. Med.* **162**:917–929.
11. Shepard, C. C., 1984, Immunity to leprosy and the Mitsuda reaction, *Int. J. Lepr.* **52**:74–77.
12. DeVries, R. R. P., Van Eden, W., and Van Rood, J. J., 1981, HLA-linked control of the course of *M. leprae* infections, *Lepr. Rev.* **52**:109–119.
13. Scott, G. C., Russell, D. A., Boughton, C. R., and Vincin, D. R., 1976, Untreated leprosy probability for shifts in Ridley-Jopling classification, *Int. J. Lepr.* **44**:110–121.
14. Salgame, P. R., Mahadevan, P. R., and Antia, N. H., 1983, Mechanism of immunodepression in leprosy: Presence of suppressor factor(s) from macrophages of lepromatous patients, *Infect. Immun.* **40**:1119–1126.
15. Van Eden, W., Gonzalez, N. M., De Vries, R. R. P., Convit, J., and Van Rood, J. J., 1985, HLA linked control of predisposition to lepromatous leprosy, *J. Infect. Dis.* **151**:9–14.
16. Convit, J., Aranzazu, N., Ulrich, M., Pinardi, M. E., Reyes, O. and Alvarado, J., 1982, Immunotherapy with a mixture of *Mycobacterium leprae* and BCG in different forms of leprosy and in Mitsuda negative contacts, *Int. J. Lepr.* **50**:415–424.
17. Haregewoin, A., Longley, J., Bjune, G., Mustafa, A. S., and Godal, T., 1985, The role of interleukin-2 (IL-2) in the specific unresponsiveness of lepromatous leprosy to *Mycobacterium leprae:* Studies *in vitro* and *in vivo*, *Immunol. Lett.* **11**:249–252.
18. Ottenhoff, T., Elferink, D., and De Vries, R. R. P., 1984, Unresponsiveness to *Mycobacterium leprae* in lepromatous leprosy *in vitro:* Reversable or not?, *Int. J. Lepr.* **52**:419–422.
19. Nath, I., Sathish, M., Jayaraman, T., Bhutani, L. K., and Sharma, A. K., 1984, Evidence for the presence of *M. leprae* reactive T lymphocytes in patients with lepromatous leprosy, *Clin. Exp. Immunol.* **58**:522–530.
20. Nogueira, N., Kaplan, G., Levy, E., Sarno, E. N., Kusher, P., Granelli-Piperno, A., Vieira, L., Colomber Gould, V., Levis, W. R., Steinman, R. M., Yip, Y. K., and Cohn, Z. A., 1983, Defective gamma interferon production in leprosy, reversal with antigen and interleukin 2, *J. Exp. Med.* **158**:2165–2170.
21. Kaplan, G., and Cohn, Z. A., 1985, Cellular immunity in lepromatous and tuberculoid leprosy, *Immunol. Lett.* **11**:205–209.
22. Stoner, G. L., Mshana, R., Town, R. N., and Belehu, A., 1982, Studies on the defect in cell-mediated immunity in lepromatous leprosy using HLA-D identical siblings. Ab-

sence of circulating suppressor cells and evidence that the defect is in the T-lymphocyte rather than the monocyte population, *Scand. J. Immunol.* **15:**33–48.

23. Nath, I., Jayaraman, J., Satish, M., Bhutani, L. K., and Sharma, A. K., 1984, Inhibition of interleukin 2 production by adherent cell factors from lepromatous leprosy patients, *Clin. Exp. Immunol.* **58:**531–538.
24. Rooney, J. J., Croceo, J. A., and Kramer, S., 1976, Further observations on tuberculin reactions in active tuberculosis, *Am. J. Med.* **60:**517–522.
25. Proudfoot, A. T., 1971, Cryptic disseminated tuberculosis, *Br. J. Hosp. Med.* **5:**773.
26. Grieco, M. H., and Chmel, H., 1974, Acute disseminated tuberculosis, as a diagnostic problem. A clinical study based on twenty-eight cases, *Am. Rev. Respir, Dis.* **109:**554–560.
27. Sahn, S. A., and Neff, T. A., 1974, Miliary tuberculosis, *Am. J. Med.* **56:**459–505.
28. Nash, D. R., and Douglas, J. E., 1980, Anergy in active tuberculosis: A comparison between positive and negative reactors and an evaluation of 5 TU and 250 TU test doses, *Chest* **77:**32–37.
29. McMurray, D. N., and Echeverri, A., 1978, Cell-mediated immunity in anergic patients with pulmonary tuberculosis, *Am. Rev. Respir. Dis.* **118:**827–834.
30. Zeitz, S., Ostow, J. H., and Van Arsdel, P. P., Jr., 1974, Humoral and cellular immunity in the anergic tuberculous patients, *J. Allergy Clin. Immunol.* **53:**20–26.
31. Lenzini, L., Rottoli, P., and Rottoli, L., 1977, The spectrum of human tuberculosis, *Clin. Exp. Immunol.* **27:**230–237.
32. Bates, J. H., 1982, Tuberculosis: susceptibility and resistance, *Am. Rev. Respir. Dis.* **125**(suppl. 3):20–24.
33. Rook, G. A. W., Carswell, J. W., and Stanford, J. L., 1976, Preliminary evidence for the trapping of antigen specific lymphocytes in the lymphoid tissues of "anergic tuberculosis," *Clin. Exp. Immunol.* **26:**129–132.
34. Collins, F. M., 1982, The immunology of tuberculosis, *Am. J. Rev. Respir. Dis.* **125** (suppl. 3):42–49.
35. El-Naggar, A., and Higashi, G. I., 1981, Tuberculosis meningitis E Rosette forming lymphocytes in cerebrospinal fluid, *Neurology (NY)* **31:**610–612.
36. Berger, H. W., and Mejia, E., 1973, Tuberculous pleurisy, *Chest* **63:**88–92.
37. Catanzaro, A., and Barker, A. F., 1976, Immunocompetence of pleural fluid lymphocytes, *Clin. Res.* **24:**326A.
38. Ellner, J. J., 1978, Pleural fluid and peripheral blood lymphocyte function in tuberculosis, *Ann. Intern. Med.* **89:**932–933.
39. Jensen, J. R., and Thestrup-Pedersen, K., 1982, *In vitro* changes in cell-mediated immunity following tuberculin skin testing in humans, *Acta. Pathol. Microbiol. Immunol. Scand.* [*C*] **90:**109–116.
40. Tsuyuguchi, I., Shiratsuchi, H., Teraoka, O., and Hirano, T., 1980, Increase in T cells bearing IgG Fc receptor in peripheral blood of patients with tuberculosis by *in vitro* stimulation with purified protein derivative, *Am. Rev. Respir. Dis.* **121:**951–957.
41. Ellner, J. J., 1978, Suppressor adherent cells in human tuberculosis, *J. Immunol.* **121:**2573–2579.
42. Katz, P., Goldstein, R. A., and Fauci, A. S., 1979, Immunoregulation in infection caused by *Mycobacterium tuberculosis.* The presence of suppressor monocytes and the alteration of a subpopulations of T lymphocytes, *J. Infect. Dis.* **140:**12–21.
43. Wadee, A. H., Sher, G. L., and Rabson, A. R., 1980, Production of a suppressor factor by human adherent cells treated with mycobacteria, *J. Immunol.* **125:**1380–1386.
44. Jaffe, M. I., and Rabson, A. R., 1981, Suppression of L.I.F. production *in vitro* but not blastogenesis in patients with tuberculous meningitis, *Clin. Immunol. Immunopathol.* **18:**245–253.

45. Bona, C., Audibert, F., Juy, D., and Chedid, L., 1976, Cell suppression in PPD induced blast specific response of human peripheral blood lymphocytes, *Clin. Exp. Immunol.* **26:**258–266.
46. Tweardy, D. J., Schacter, B. Z., and Ellner, J. J., 1984, Association of altered dynamics of monocytes surface expression of human leukocyte antigen DR with immunosuppression in tuberculosis, *J. Infect. Dis.* **149:**31–37.
47. Uberoi, S., Malaviya, A. N., Chattopadhyay, C., Kumar, R., and Shrinivas, X., 1975, Secondary immunodeficiency in miliary tuberculosis, *Clin. Exp. Immunol.* **22:**404–408.
48. Nyka, W., 1963, Studies on *M. tuberculosis* in lesions of human lung, *Am. Rev. Respir. Dis.* **88:**670–679.
49. Heilman, D. H., and McFarland, W., 1966, Inhibition of tuberculin-induced mitogenesis in culture of lymphocytes from tuberculous donors, *Int. Arch. Allergy* **30:**58–66.
50. Bullock, W. E., and Fansal, P., 1971, Studies of immune mechanisms in leprosy. III. The role of cellular and humoral factors in impairment of the *in vitro* immune response, *J. Immunol.* **106:**888–899.
51. Davies, D. H., Corbeil, L., Ward, D., and Ducan, J. R., A humoral suppressor of *in vitro* lymphocyte transformation responses in cattle with Johne's disease, *Proc. Soc. Exp. Biol. Med.* **145:**1372–1377.
52. Ellner, J. J., and Daniel, T. M., 1979, Immunosuppression by mycobacterial arabinomannan, *Clin. Exp. Immunol.* **35:**250–257.
53. Neta, R., and Salvin, S. B., 1979, Adjuvants in the induction of suppressor cells, *Infect, Immun.* **23:**360–365.
54. Bahr, G. M., Rook, G. A. W., and Stanford, J. L., 1981, Inhibition of the proliferative response of peripheral blood lymphocytes to mycobacterial or fungal antigens by co-stimulation with antigen from various mycobacterial species, *Immunology* **44:**593–598.
55. Kleinhenz, M. E., Ellner, J. J., Spagnuolo, P. J., and Daniel, T. M., 1981, Suppression of lymphocyte response by tuberculous plasma and mycobacterial arabinogalactan: Monocyte dependence and indomethacin reversibility, *J. Clin. Invest.* **68:**153–162.
56. Bahr, G. M., Rook, G. A. W., and Stanford, J. L., 1981, Prostaglandin dependent regulation of the *in vitro* proliferative response to mycobacterial antigens of peripheral blood lymphocytes from normal donors and from patients with tuberculosis or leprosy, *Clin. Exp. Immunol.* **45:**646–653.
57. Mustafa, A. S., and Godal, T., 1983, *In vitro* induction of human suppressor T cells by mycobacterial antigens. BCG activated $OKT4^+$ cells mediate suppression of antigen induced T cell proliferation, *Clin. Exp. Immunol.* **52:**29–37.
58. Wakasugi, N., Virelizier, J. L., Arenzana-Seidedos, F., Rothhut, B., Mencia-Huerta, J. M., Russo-Marie, F., and Fiers, W., 1985, Defective IFN gamma production in the human neonate. III. Role of increased sensitivity to the suppressive effects of prostaglandin E, *J. Immunol.* **134:**172–178.
59. Edwards, C. K., Hedegaard, H. B., Zlotnik, A., Gandadharam, P. R., Johnston, R. B., and Pabst, M. J., 1986, Chronic infection due to *Mycobacterium intracellulare* in mice: Association with macrophage release of prostaglandin E2 and reversal by injection of indomethacin, muramyl-dipeptide or interferon-gamma, *J. Immunol.* **136:**1820–1827.
60. Harrison, B. D. W., Tugwell, P., and Fawcett, I. W., 1975, Tuberculin reaction in adult Nigerian with sputum positive pulmonary tuberculosis, *Lancet* **1:**421–424.
61. Daniel, T. M., Oxtoby, M. J., Pinto, E. M., and Moreno, E. S., 1981, The immune spectrum in patients with pulmonary tuberculosis, *Am. Rev. Respir. Dis.* **123:**556–559.
62. Persson, I., Ryder, L. P., Nielson, L. S., and Svejgaard, A., 1975, The HLA B7 histocompatibility antigen in sarcoidosis in relation to hypersensitivity, *Tissue Antigens* **6:**50–53.

63. Buckley, C. E. III, White, D. H., and Siegler, H. F., 1976, HLA-B7 associated tuberculin hyporesponsiveness in BCG-treated patient, in: *HLA and Disease,* Vol. 58 (J. Dausset and L. Degos, eds.), pp. 175–179, INSERM, Paris.
64. Cox, R. A., Arnold, D. R., Cook, D., and Lundberg, D. I., 1982, HLA phenotypes in Mexican Americans with tuberculosis, *Am. Rev. Respir. Dis.* **126:**653–655.
65. Van Eden, W., De Vries, R. R. P., Stanford, J. L., and Rook, G. A. W., 1983, HLA-DR3 associated genetic control of response to multiple skin tests with tuberculins, *Clin. Exp. Immunol.* **52:**287–292.
66. Lagrange, P. H., Hurtrel, B., Brandely, M., and Thickstun, P. M., 1983, Immunological mechanisms controlling mycobacterial infections, *Bull, Eur. Physiopathol. Respir.* **19:**163–172.
67. Gros, P., Skamene, E., and Forget, A., 1981, Genetic control of natural resistance to *Mycobacterium bovis* (BCG) in mice. *J. Immunol.* **127:**2417–2421.
68. Brown, I. N., 1983, Animals models and immune mechanisms in mycobacterial infection, in: *Biology of Mycobacteria,* Vol. 2 (C. Ratledge and J. Stanford, eds.), pp. 173–234, Academic, London.
69. Gray, D. F., 1961, The relative resistance of rats and mice to experimental pulmonary tuberculosis, *J. Hyg. (Lond.)* **59:**471–477.
70. Lynch, C. J., Pierce-Chase, C. H., and Dubos, R. J., 1965, A genetic study of susceptibility to experimental tuberculosis in mice infected with mammalian bacilli, *J. Exp. Med.* **121:**1051–1070.
71. Forget, A., Skamene, E., Gros, P., Miailhe, A. C., and Turcotte, R., 1981, Differences in response among inbred mouse strains to infection with small doses of *Mycobacterium bovis* BCG, *Infect. Immun.* **32:**42–47.
72. Lewis, P. A., and Loomis, P., 1928, The capacity of guinea pigs to produce antibodies as affected by the inherence and as related to familial resistance to tuberculosis, *J. Exp. Med.* **47:**437–448.
73. Lurie, M. B., Abramson, S., and Heppleston, A. G., 1952, On the response of genetically resistant and susceptible rabbits to the quantitative inhalation of human type tubercle bacilli and the nature of resistance to tuberculosis, *J. Exp. Med.* **95:**119–137.
74. Kirchheimer, W. F., and Sanchez, R. M., 1981, Intraspecies differences of resistance against leprosy in nine-banded armadillos, *Lepr. India* **53:**525–530.
75. Uhr, J. W., and Papenheimer, A. M., Jr, 1958, Delayed type hypersensitivity. III. Specific desensitization of guinea pigs to protein antigens, *J. Exp. Med.* **108:**891–904.
76. Schlossman, S. F., Levin, H. A., Rocklin, R. E., and David, J. R., 1971, The compartmentalization of antigen-reactive lymphocytes in desensitized guinea pigs, *J. Exp. Med.* **134:**741–750.
77. Rich, A. R., 1951, *The Pathogenesis of Tuberculosis,* Charles C. Thomas, Springfield, Illinois.
78. Wilson, G. S., Schwabacher, H., and Maier, I., 1940, The effect of the desensitization of tuberculous guinea-pigs, *J. Pathol.* **50:**89–109.
79. Hehre, E., and Freund, J., 1939, Sensitization, antibody formation and lesions produced by tubercle bacilli in albino rats, *Arch. Pathol. Lab. Med.* **27:**289–306.
80. Dwyer, J. M., and Kantor, F. S., 1975, *In vivo* suppression of delayed hypersensitivity: Prolongation of desensitization in guinea pigs, *J. Exp. Med.* **142:**588–599.
81. Rook, G. A. W., 1976, Immunological responses to mycobacteria in mice and men, *Proc. R. Soc. Med.* **69:**442–444.
82. Poulter, L. M., 1978, Systemic immunological reactivity in the absence of delayed-type hypersensibility during *Mycobacterium lepraemurium* infection. *Cell. Immunol.* **40:**117–127.
83. Martin, L. N., Gormus, B. J., Wolf, R. H., Gerone, P. J., Meyers, W. M., Walsh, G. P., Binford, C. H., Hadfield, T. L., and Schlager, C. J., 1985, Depression of lymphocyte

responses to mitogens in mangabeys with disseminated experimental leprosy, *Cell. Immunol.* **90:**115–130.

84. Hoffenbach, A., Lagrange, P. H., and Bach, M. A., 1983, Surface Lyt phenotype of suppressor cells in C57BL/6 mice infected with *Mycobacterium lepraemurium*, *Clin. Exp. Immunol.* **54:**151–157.
85. Hoffenbach, A., Lagrange, P. H., and Bach, M. A., 1983, Deficit in interleukin-2 production associated with impaired T cell proliferative responses in *Mycobacterium lepraemurium* infection, *Infect. Immun.* **39:**109–116.
86. Colizzi, V., Fergula, J., Garreau, F., Malkovsky, M., and Asherson, G. L., 1984, Suppressor cells induced by BCG release non specific factors *in vitro*, which inhibit DNA synthesis and interleukin-2 production, *Immunology* **51:**65–71.
87. Rook, G. A. W., 1975, The immunological consequence of antigen overload in experimental mycobacterial infections of mice, *Clin. Exp. Immunol.* **19:**166–177.
88. Zatz, M. M., 1976, Effects of BCG on lymphocytes trapping, *J. Immunol.* **116:**1587–1591.
89. Bullock, W. E., 1976, Perturbation of lymphocyte circulation in experimental murine leprosy. I. Description of the defect, *J. Immunol.* **117:**1164–1170.
90. Bullock, W. E., 1976, Perturbation of lymphocyte circulation in experimental murine leprosy. II. Nature of defect, *J. Immunol.* **117:**1171–1178.
91. Brown, C. A., Brown, I. N., and Sljivic, V. S., 1980, Active suppression masks an underlying enhancement of antibody production *in vitro* by spleen cells from BCG infected mice, *Immunology* **40:**303–309.
92. Bullock, W. E., Carlson, E. M., and Gershon, R. K., 1978, The evolution of immunosuppressive cell populations in experimental mycobacterial infection, *J. Immunol.* **120:**1709–1716.
93. Brown, C. A., and Brown, I. N., 1982, *Mycobacterium bovis* BCG modulation of murine antibody responses: Influence of dose and degree of aggregation of live or dead organisms, *Br. J. Exp. Pathol.* **63:**133–143.
94. Schrier, D. J., Allen, E. M., and Moore, V. L., 1980, BCG induced macrophage suppression in mice: Suppression of specific and nonspecific antibody mediated and cellular mediated immunologic responses, *Cell. Immunol.* **56:**347–356.
95. Watson, S. R., and Collins, F. M., 1981, The specific suppressor T cells induced by chronic *Mycobacterium avium* infection in mice, *Clin. Exp. Immunol.* **43:**10–19.
96. Collins, F. M., and Watson, S. R., 1980, Effect of chemotherapy on suppressor T cells in BCG infected mice, *Immunology* **40:**529–537.
97. Alexander, J., 1979, Adoptive transfer of immunity and suppression by cells and serum in early *Mycobacterium lepraemurium* in mice, *Parasite Immunol.* **1:**159–166.
98. Hoffenbach, A., Lagrange, P. H., and Bach, M. A., 1984, Influence of dose and route of *Mycobacterium lepraemurium* inoculation on the production of interleukin 1 and interleukin 2 in C57BL/6 mice, *Infect. Immun.* **44:**665–671.
99. Hoffenbach, A., Lagrange, P. H., and Bach, M. A., 1985, Strain variation of lymphokine production and specific antibody secretion in mice infected with *Mycobacterium lepraemurium*, *Cell. Immunol.* **91:**1–11.
100. Bach, M. A., Hoffenbach, A., Lagrange, P. H., Wallach, D., and Cottenot, F., 1983, Mechanisms of T cell unresponsiveness in leprosy, *Ann. Immunol. (Paris)* **134D:**75–84.
101. Colizzi, V., 1984, *In vivo* and *in vitro* administration of interleukin 2-containing preparation reverses T-cell unresponsiveness in *Mycobacterium bovis* BCG, infected mice, *Infect. Immun.* **45:**25–28.
102. Orme, I. M., Ratcliffe, M. J. H., and Collins, F. M., 1984, Acquired immunity to heavy infection with *Mycobacterium bovis* Bacilli Calmette Guerin and its relationship to the development of non specific unresponsiveness, *Cell, Immunol.* **88:**285–296.

103. Nakamura, R. M., and Tokunaga, T., 1978, Strain difference of delayed-type hypersensitivity to BCG and its genetic control in mice, *Infect. Immun.* **22:**657–664.
104. Lagrange, P. H., Hurtrel, B., and Thickstun, P. M., 1979, Immunological behavior after mycobacterial infection in selected lines of mice with high or low antibody responses, *Infect. Immun.* **25:**39–47.
105. Nakamura, R. M., and Tokunaga, T., 1980, Induction of suppressor T cells in delayed-type hypersensitivity to *Mycobacterium bovis* BCG in low responder mice, *Infect. Immun.* **28:**331–335.
106. Nakamura, R. M., Tokunaga, T., and Yamamoto, S., 1980, Difference in antigen presenting activity of macrophages between high or low responder mice in delayed-type hypersensitivity to *Mycobacterium bovis* BCG, *Infect. Immun.* **27:**268–270.
107. Stach, J. L., Gros, P., Forget, A., and Skamene, E., 1984, Phenotypic expression of genetically controlled natural resistance to *Mycobacterium bovis* (BCG), *J. Immunol.* **132:**888–892.
108. Denis, M., Forget, A., Pelletier, M., Turcotte, R., and Skamene, E., 1986, Control of the Bcg gene of early resistance in mice to infection with BCG strains and atypical mycobacteria, *Clin. Exp. Immunol.* **63:**517–525.
109. Pelletier, M., Forget, A., Bourrassa, D., Gros, P., and Skamene, E., 1982, Immunopathology of BCG infection in genetically resistant and susceptible mouse strains, *J. Immunol.* **129:**2179–2185.
110. Hurtrel, B., Hurtrel, M., and Lagrange, P. H., 1985, Genetic control of tuberculin DTH time course, correlation with natural and acquired resistance against BCG, in: *Genetic Control of Host Resistance to Infection and Malignancy* (E. Skamene, ed.), pp. 305–312, Liss, New York.
111. Adu, H. O., Curtis, J., and Turk, J. L., 1981, Difference in cell mediated immune responses of "high resistance and low resistance" mice to non pathogenic mycobacterium, *Scand. J. Immunol.* **14:**467–480.
112. Howard, J. G., Hale, C., and Liew, F. Y. 1981, Immunological regulation of experimental cutaneous leishmaniasis. IV. Prophylactic effect of sublethal irradiation as a result of abrogation of suppressor T cell generation in mice genetically susceptible to *Leishmania tropica*, *J. Exp. Med.* **153:**557–568.
113. Liew, F. Y., Hale, C., and Howard, J. G., 1982, Immunologic regulation of experimental cutaneous leishmaniasis. V. Characterization of effector and specific suppressor T cells, *J. Immunol.* **128:** 1917–1922.
114. Liew, F. Y., 1983, Specific suppression of response to *Leishmania tropica* by a cloned T cell line, *Nature (Lond.)* **305:**630–632.
115. Adu, H. O., Curtis, J., and Turk, J. L., 1983, The resistance of C57BL/6 mice to subcutaneous infection with *Mycobacterium lepraemurium* is dependent on both T cells and other cells of bone marrow origin, *Cell. Immunol.* **78:**249–256.
116. Leffort, M. J., and Mackaness, G. B., 1977, Suppression of immunity in *Mycobacterium lepraemurium* infection, *Infect. Immun.* **18:**363–369.
117. Brown, I. N., Glynn, A. A., and Plant, J. E., 1982, Inbred mouse strain resistance to *Mycobacterium lepraemurium* follows the *Ity-Lsh* pattern, *Immunology* **47:**149–156.
118. Lagrange, P. H., and Hurtrel, B., 1979, Local immune response to *Mycobacterium lepraemurium* in C3H and C57BL/6 mice, *Clin. Exp. Immunol.* **38:**461–474.
119. Han, S. H., and Weiser, R. S., 1967, Systemic tuberculin sensitivity in mice. Factors contributing to active tuberculin shock, *J. Immunol.* **98:**1153–1157.
120. Lagrange, P. H., and Stach, J. L., 1985, Strategy control of leprosy *Int. J. Lepr.* **53:**278–288.

9

Anergy in Experimental Mouse Models of Mycobacterial Infection

IAN M. ORME

1. INTRODUCTION

Unresponsiveness, or anergy, is often observed in association with active tuberculosis and other mycobacterial diseases, both in the clinical setting and in experimental animal models of these infections. In both clinical and experimental laboratory settings, various manifestations of anergy can be observed using both *in vitro* and *in vivo* approaches. In the case of the former, most *in vitro* observations of anergy following mycobacterial infection consist of the measurement of a reduced capacity of lymphocytes to undergo blastogenic transformation in response to stimulation by mitogens, by specific antigens associated with the infecting organism, or by alloantigens in a mixed lymphocyte culture. As for *in vivo* expressions of anergy, these are most usually measured as a reduction or loss of the capacity of the infected host to mount a delayed-type hypersensitivity (DTH) response to a cutaneous injection of specific antigen.

The purpose of this chapter is to consider various situations in which anergy may develop, as well as the mechanisms that may underlie this development, in a number of experimental mouse models of mycobacterial infection. It is important to realize, however, that the conditions under which anergy can be measured using such models may dif-

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fer greatly from those existing in the clinical laboratory. For example, mouse models of infection are invariably performed using syngeneic inbred strains, which are usually bred under specific-pathogen free conditions. Furthermore, these animals will be infected with very large numbers of bacteria (in order to induce a rapid state of anergy), usually by routes that do not mirror the natural disease process. In the clinical setting, however, the various parameters contributing to anergy may be much more diverse, involving not only a consideration of the primary infectious disease itself, but many other disposing factors, such as secondary infection, underlying pathology (e.g., neoplasia), the age of the patient, and the like. The clinical aspects of this subject are covered in Chapter 11, this volume and therefore are not considered here.

Mycobacteria are both excellent immunogens and adjuvants; it is not surprising that they induce an extremely powerful acquired cellular immune response in the infected mouse. It is equally unsurprising that the generation and expression of this host response is often associated with considerable perturbation of the immunologic reactivity of the host in general. These perturbations may manifest themselves variously as increases in the capacity of the animals to mount a given immune response, for instance to tumor-specific antigens, or as a reduction or loss in immune reactivity, such as cutaneous anergy to injection of specific antigen.

In a number of experimental situations in which anergy may develop, the roles of various cell populations that contribute to unresponsiveness is well documented and understood and is discussed below. What is much less understood, and regrettably is apparently not even considered in a number of publications on the subject, concerns the precise relevance of the development of anergy to the overall immunocompetence of the animal *in vivo,* in terms of both its ability to generate protective immunity to the infecting organism and its ability to respond to other, unrelated antigens. In this regard, the concept is developed in this chapter, using a number of examples, suggesting that some expressions of anergy that follow from heavy mycobacterial infection of mice in fact represent an increased immunologic reactivity of the animal as a result of the infection, rather than reflecting some form of decrease or loss of specific responsiveness. For example, while it can be shown that T cells harvested from *M. bovis* bacillus Calmette–Guérin (BCG)-infected mice are unable, under certain circumstances, to divide *in vitro* following stimulation with specific antigen or mitogen, this event paradoxically occurs at a time when actively dividing T cells can be passively transferred from these mice that can adoptively mediate increased antituberculous resistance. These types of observations and other examples of increased immunologic reactivity in the anergic host are discussed in greater detail below.

2. MANIFESTATIONS OF ANERGY *IN VITRO*

The possibility that high intravenous (IV) doses of BCG might lead to immunosuppression first emerged more than a decade ago, at a time when the vaccine was being seriously considered as a potentially important new immunomodulator of cancer immunotherapy. Florentin *et al.*[1] found that administration of high doses of BCG ($>10^7$ viable bacteria) resulted in the generation of an adherent macrophage-like population of cells that were able to depress severely the blastogenic response of normal T cells to the mitogen Concanavalin A (Con A) and were also able to inhibit the cytolytic response of such cells. By contrast, the BCG infection had no effect on the capacity of the animal to express contact sensitivity *in vivo* following exposure to picryl chloride.

The ability of inhibitory cells induced by BCG to suppress *in vitro* responses to mitogens has been extensively studied by Turcotte and colleagues,[2–4] who found that BCG infection resulted in the subsequent depression of blastogenic responses of splenic cells both to T-cell mitogens and to lipopolysaccharide. Subsequent analysis of this phenomenon revealed that the primary cell population mediating this inhibition bore the characteristics of a macrophage. Evidence was also provided that a second cell population could also mediate inhibition, albeit at a weaker level, and that this population consisted of a radiation-sensitive T lymphocyte. The functional role of these inhibitory cells was not formally examined, but it was suggested that they might contribute to the regulation of the immune response to the BCG infection and to the anergy observed following the cutaneous injection of tuberculin.

Further evidence for an inhibitory adherent cell population acquired in response to BCG infection was provided by Klimpel and Henney[5] and by Orbach-Arbouys and Poupon.[6] That this phenomenon was not solely restricted to BCG infection was revealed by Bullock *et al.*,[7] who presented evidence for a similar cell population in mice infected with *M. lepraemurium*. Again, although this population was extremely inhibitory *in vitro* and could be detected over a long period of time, a second population of T lymphocytes with inhibitory properties were also identified, similar to the findings of Turcotte and colleagues.

At the time that these publications appeared, our knowledge and understanding of the basic networks that contribute to the regulation of the immune response was expanding rapidly; thus, it was perfectly reasonable at that time to hypothesize that suppressor T cells and suppressor macrophages were acquired by the animal in response to large infectious doses of mycobacteria as part of a negative immunoregulatory mechanism designed to control the mediator cell (T cell) and effector cell (macrophage) antimicrobial response. Other hypotheses were also advanced, including the interesting suggestion that the functional role

of suppressor macrophages was to depress the possible development of autoimmune mechanisms that might result from tissue damage occurring at sites of bacterial implantation.[8]

Other workers at this time were less convinced that the inhibitory cell populations played any functional role in the intact animal. For instance, Rook and King,[9] while noting the inhibitory activity of mycobacteria-induced adherent cells *in vitro,* pointed out that this did not necessarily correlate with immunosuppression *in vivo.* Using another intracellular bacterial infection, Cheers and colleagues,[10,11] similarly noted that although highly inhibitory macrophages were acquired in the spleens of mice infected with *Brucella,* these animals responded in a normal fashion *in vivo* to immunization with sheep erythrocytes or to secondary infection with *Listeria.*

Where mycobacteria-induced suppressor macrophages may play a functional role in experimental animal models *in vivo* may lie in the area of tumor immunotherapy. For example, Wepsic *et al.*[12] presented evidence that immunization with the cell wall of BCG (in an oil-in-water emulsion) can result in the acceleration of the growth of a syngeneic tumor implant. The cell-wall material is highly effective in inducing suppressor macrophages capable of inhibiting mitogen responses *in vitro;* it was speculated that this cell population might act *in vivo* by interfering with the induction of the tumor-specific immune response. This interesting area is clearly in need of much further study, however, as these investigators themselves concede in a recent review on this subject.[13]

3. POSSIBLE MECHANISMS UNDERLYING UNRESPONSIVENESS *IN VITRO*

As a generalization, unresponsiveness *in vitro* following IV mycobacterial infection is a function of the inoculum size; the larger the number of bacteria injected, the more likely that lymphoid cells will become anergic *in vitro* to blastogenic stimulation. Other factors are equally important, however; for instance, the mycobacterial strain in question must be able to colonize the host—even large inocula of organisms such as *M. vaccae* or *M. scrofulaceum,* which are not infectious for mice, are rapidly destroyed and have no detectable perturbative effects on the host-immune response.

A third factor concerns the genetic makeup of the strain of mouse employed, in that the resistance or susceptibility of the mouse strain to a given mycobacterial infection will determine the types of inhibitory cell populations subsequently acquired. For example, if a mouse strain (e.g.,

the A/J, or the B6D2 F_1 hybrid) is employed that is highly resistant to the growth of even very high doses of certain mycobacteria, such as *M. simiae* or *M. avium,*[14] then only adherent cells can be found to subsequently mediate unresponsiveness in *in vitro* assays.[15] By contrast, if an organism such as BCG Pasteur is used that will grow well in all conventional mouse strains,[16] both inhibitory macrophages and T lymphocytes can be detected in *in vitro* assays if the inoculum of BCG is sufficiently large.

To illustrate the first of these factors, Fig. 1 shows the *in vitro* blastogenic responsiveness to the T-cell mitogen phytohemagglutin (PHA) of spleen cells harvested at various times during the course of IV infection with various doses of viable BCG Pasteur. Whereas no perturbation of *in vitro* responsiveness was evident at the lower doses, a moderate reduction in blastogenesis was observed during the second week of infection with 10^6 BCG. By contrast, spleen cells from mice infected with 10^8 BCG rapidly became anergic to blastogenic stimulation and had only partially recovered their responsiveness by day 60 of the experiment.

A further series of experiments were then performed to characterize the cell populations involved in mediating the *in vitro* unresponsiveness induced by IV injection of 10^8 BCG. Spleen cells were harvested and were either unfractionated or depleted of macrophages by adherence to nylon wool, prior to culture in the presence of PHA. It was found (Fig. 2) that unfractionated spleen cells rapidly became anergic to blastogenic stimulation, and that responsiveness was only slowly recovered, returning to close to control values on day 90 of the experi-

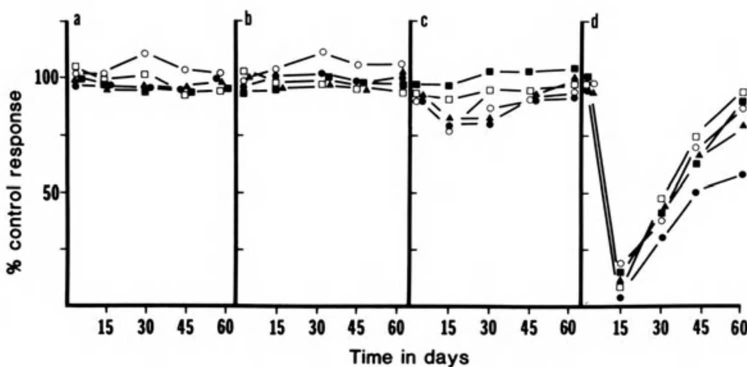


FIGURE 1. Responsiveness, against time, of 5×10^5 spleen cells to blastogenic stimulation with 2.0 μg PHA in mice infected intravenously with (a) 10^4, (b) 10^5, (c) 10^6, or (d) 10^8 viable BCG Pasteur. Strains tested were C57BL/6 (○), BALB/c (□), DBA/2 (■), C3H/He (●), and B6D2 F_1 hybrids (▲). Control responses ranged from 7×10^4 to 1.5×10^5 cpm. Background responses always less than 2000 cpm. Data calculated from responses of triplicate cultures.

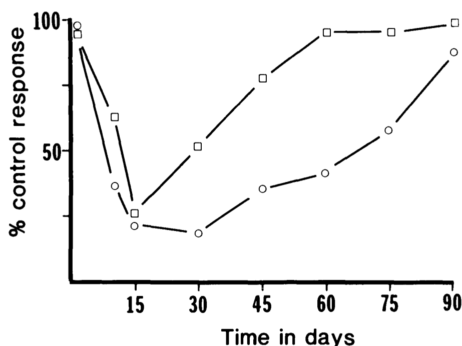


FIGURE 2. Responsiveness to PHA of unfractionated (○) or T-cell-enriched (plastic nonadherent cells passed through nylon-wool columns) (□) spleen cells harvested at indicated timepoints from B6D2 F_1 hybrid mice infected intravenously with 10^8 BCG Pasteur. Control data as in Fig. 1.

ment. Anergy to the PHA mitogen was also observed within the T-cell-enriched population, although unresponsiveness became apparent more slowly (peaking between days 15 and 20) and recovered much more quickly (values returned close to control values by day 45). It was concluded that, while 10^8 BCG could induce unresponsiveness via both macrophage and T-cell mediator populations, the kinetics of emergence and loss of these inhibitory cells was clearly different.

Various strains of mice can be designed as susceptible or resistant on the basis of their ability to restrain the growth of a number of nontuberculous mycobacteria infections. Although the well-defined *Bcg/Lsh/Ity* locus probably plays a role in this event (see Chapter 3, this volume), the resistance trait is clearly under multigene control.[14] The practical consequence of natural resistance to these infections is that in mouse strains expressing the resistant trait, little or no evidence for any substantial expression of acquired mechanisms of cellular immunity can be found.[14] The only exception to this was observed in mice infected with very high numbers (10^8) of nontuberculous mycobacteria, in which moderate levels of macrophage activation *in vivo* (measured as the capacity of the animal to destroy an intravenous inoculum of *Listeria*) could be detected. Under these latter circumstances, spleen cells from such mice which have been depleted of adherent cells respond essentially normally to T-cell mitogens, whereas unfractionated spleen cells are considerably depressed in responsiveness (Fig. 3).

It will be apparent from the above argument that anergy at the T-cell level *in vitro* seems somehow to be associated with the level of acquired T-cell-mediated immunity that is generated in response to the mycobacterial infection. Thus, a number of questions arise (and that appear to have been ignored in a number of publications on this subject): If the T cells that mediate anergy *in vitro* have a functional role *in vivo,* how can this be measured? Would it not be predicted, if it were

hypothesized that these cells represented a negative feedback mechanism to downregulate protective T-cell immunity to the BCG infection, that overall levels of acquired immunity to 10^6 BCG (in which T-cell anergy *in vitro* is negligible) would be expected to be at least as high as, or higher than, levels of acquired immunity generated to 10^8 BCG, in which anergy *in vitro* is substantial? Furthermore, what are the precise temporal relationships between the emergence of inhibitory cells *in vitro* and the emergence of the acquired immune response to BCG *in vivo?*

In order to begin to answer these questions, mice were infected IV with either 10^8 or 10^6 BCG and the development of *in vitro* T cell anergy compared with the emergence of the protective T-cell response *in vivo.* This latter parameter was measured as the capacity of splenic T cells to protect T-cell-deficient recipients adoptively from an IV challenge infection with virulent *M. tuberculosis;* this assay is described in detail elsewhere.[17] It was found (Fig. 4) that substantial levels of protective antituberculous T cells were rapidly generated in mice infected with the higher dose (10^8 IV) of BCG, whereas immunity was generated at a slower rate in mice given the lower dose (10^6 IV). Thus, again, paradoxically, it appeared that the more powerful the acquired response *in vivo,* the more likely that *in vitro* anergy at the T-cell level would ensue.

One possible way of looking at these data would be to hypothesize that the suppressor T cells observed in the *in vitro* assays were acquired to dampen the *in vivo* response; i.e., if they were removed, the levels of acquired immunity generated against 10^8 BCG would actually be higher.

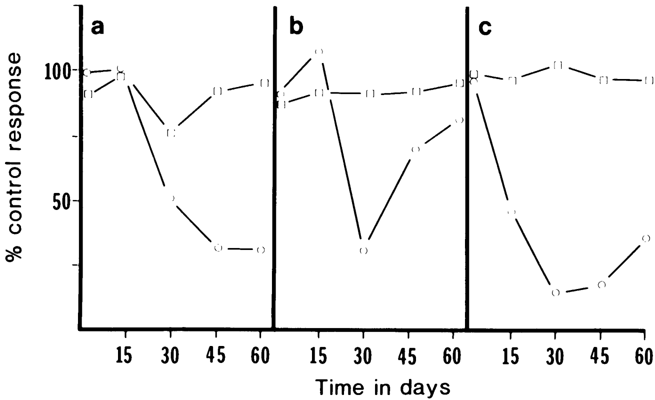


FIGURE 3. Responsiveness to PHA of unfractionated (○) or T-cell-enriched (□) spleen cells from B6D2 F_1 hybrid mice infected intravenously with 10^8 (a) *M. avium* 724, (b) *M. simiae* 1226, or (c) *M. kansasii* 1203. Control responses ranged from 8.5×10^4 to 1.2×10^5 cpm.

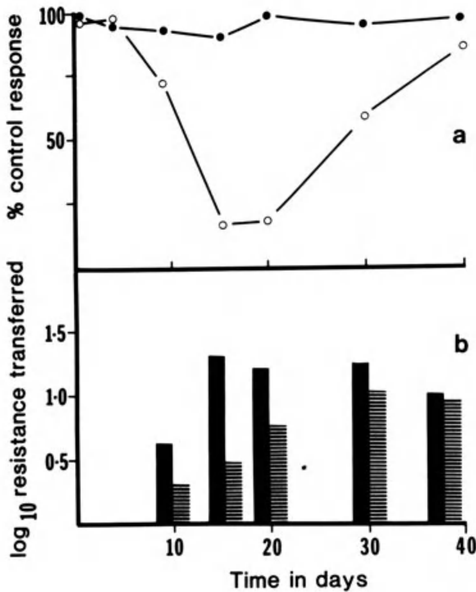


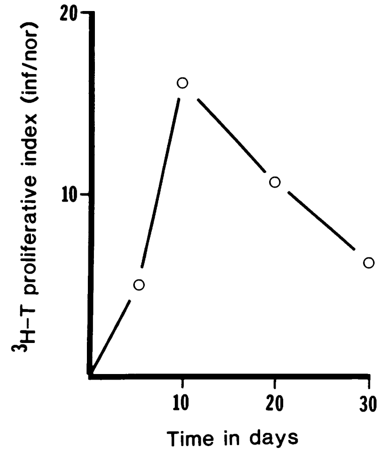
FIGURE 4. Evidence that T-cell unresponsiveness *in vitro* to PHA is temporally associated with the rapid generation of protective T cells, acquired in response to 10^8 BCG infection. (a) Response to PHA of T-cell-enriched spleen cells from mice infected intravenously with 10^8 (○) or 10^6 (●) BCG. (b) Capacity of T cells from such mice to adoptively protect T-cell-deficient recipients from a challenge infection with *M. tuberculosis*. Cell donor mice infected with 10^8 BCG (solid bars) or 10^6 BCG (hatched bars) (n = 4–5 mice per transfer group).

This possibility could be argued as unlikely, however, in that such a hypothesis would have to explain how such cells could mediate a specific negative regulatory role *in vivo* and yet utterly suppress a pan-T-cell proliferative response *in vitro*. Another more direct argument against the hypothesis that such suppressor cells might mediate an antiproliferative function is provided by the results of experiments, showing (Fig. 5) that despite a lack of proliferative activity *in vitro*, substantial levels of blastogenesis were occurring at that time in the intact animal.[18]

A possible explanation for this paradoxical situation was provided by experiments designed to attempt to restore T-cell responsiveness *in vitro*. The rationale for the experiments was as follows. The protective T-cell response to BCG involves both L3T4$^+$ Lyt-2$^-$ and L3T4$^-$ Lyt-2$^+$ protective cell populations (I. M. Orme, to be published); indeed, the Lyt-2$^+$ population is particularly effective in protecting against airborne tuberculosis.[19] Although the kinetics of emergence and loss of the Lyt-2$^+$ protective T cell is only partially known at this time, preliminary data suggests that its activity is maximally expressed between days 15 and 25 in mice infected with 10^8 BCG IV, i.e., at a time when peak T cell anergy can be observed *in vitro*.

Since cells bearing the Lyt-2$^+$ phenotype are believed to be a primary target for the growth hormone interleukin-2 (IL-2), experiments were performed to test the possibility that addition of IL-2 to cultures might reverse the unresponsiveness observed *in vitro*.[18] This indeed was

FIGURE 5. Proliferation *in vivo* of spleen cells in mice infected intravenously with 10^8 BCG Pasteur. Animals (n = 3) pulsed intravenously with 20 μC [^{3}H]thymidine and spleen cells harvested 30 min later. Data expressed as index (uptake of thymidine in cpm per 10^6 cells by infected spleen cells/uptake by normal cells).



found to be the case (Fig. 6) and has been confirmed elsewhere.[20] In addition, subsequent experiments revealed that the apparent lack of available IL-2 in the anergic cultures may have come about as a result of absorption of this material by IL-2-dependent T-cell blasts present in the spleen cells harvested from the BCG-infected mice. This was based on the observation that such cells possessed the capacity to rapidly absorb IL-2 from IL-2-containing supernatants,[18] a finding consistent with a

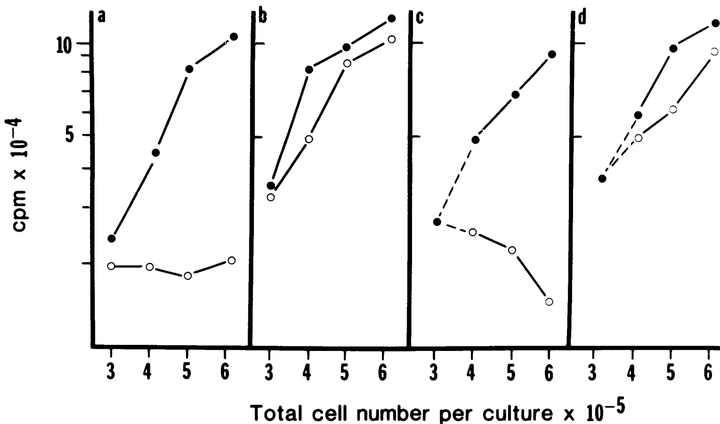


FIGURE 6. Evidence that the addition of IL-2 restores unresponsiveness and ablates suppression of PHA responses *in vitro.* (a) Response of increasing numbers of T-cell-enriched spleen cells from normal (●) or BCG-infected (10^8 IV 15 days prior) mice (○). (b) Effect of addition of 5U IL-2 to cultures. (c) Effect of admixing increasing numbers (1–3 × 10^5) of T cells from infected mice (○) or from normal mice (●) to a fixed number of cells (3 × 10^5) from normal mice. (d) Restoration of responsiveness by addition of IL-2.

high frequency of receptor expression by such cells[18] (M. J. H. Ratcliffe, personal communication). On the basis of these findings, it was hypothesized that BCG-infected mice acquire a population of IL-2-dependent Lyt-2$^+$ T cells capable of adoptively protecting recipient mice against *M. tuberculosis* infection and yet, because of their IL-2 dependency, artifactually behave as suppressor T cells when cultured with mitogens *in vitro*.

Recently, Turcotte and Legault[21] suggested that the anergic response to mitogens in mice infected with 10^7 BCG is not a consequence of IL-2 absorption, but rather reflects a transient defect in IL-2 secretion by IL-2-producing T cells. How this functional defect relates to the immunocompetence of the animal, in which a powerful acquired cellular immune response is ensuing, is not explained by these investigators.

Although supplementation of anergic cultures with IL-2 can restore T-cell responsiveness, it has no effect when added to cultures of normal T cells admixed with macrophages obtained from BCG-infected mice (Fig. 7). Indeed, nothing is known about the mechanisms by which such cells are able to suppress immune responses *in vitro*. Suppressor factors have been implicated, but it must be borne in mind that macrophages obtained from infected mice release a large variety of substances that are capable of perturbing *in vitro* assays of immunity.[22] For instance, such cells can release large quantities of thymidine (which will compete with radiolabelled thymidine added to cultures) and arginase (which by depleting arginine will remove an amino acid required by dividing T cells). Many other inhibitory substances are also released, including polyamine oxidase (which will result in the production of inhibitory aminoaldehydes), prostaglandins, interferons, and toxic oxygen products. It will be realized, in addition, that although many of these materials will have a

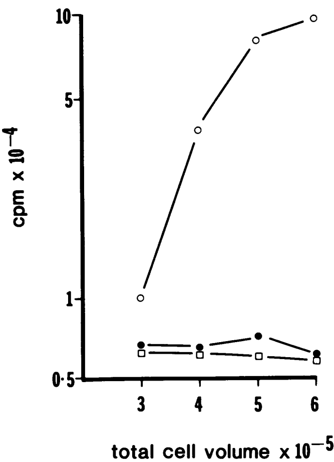


FIGURE 7. Demonstration that addition of IL-2 has no effect on inhibition of responsiveness by adherent cells from infected mice. T-cell-enriched normal cells were cultured with PHA in the presence of adherent spleen cells from normal mice (○) or from BCG-infected mice (●), in medium supplemented with 2.5U IL-2. Addition of IL-2 to unfractionated spleen cells from BCG-infected mice (□) had no effect. In admixtures, 5×10^5 unfractionated cells from normal or infected mice were precultured for 1 hr; wells were then washed to remove nonadherent cells.

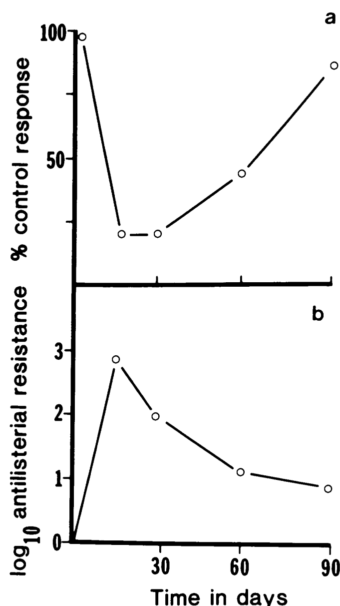


FIGURE 8. Temporal association between the development of unresponsiveness to PHA of unfractionated spleen cells from mice infected intravenously with 10^8 BCG Pasteur (a), and the emergence of nonspecific resistance *in vivo* to infection with *Listeria monocytogenes* (b). Growth of *Listeria* measured in the spleens of normal and infected mice 48 hr after IV infection with 10^5 viable bacteria ($N = 4$ mice).

very short half-life *in vivo, in vitro* they may be present in the culture medium for the entire duration of the assay.

Whatever the precise mechanism involved *in vitro,* one aspect of BCG-induced suppressor macrophages that is quite striking is the similarity between the kinetics of emergence and loss of this population, as measured *in vitro,* and the emergence of an acquired population of activated macrophages *in vivo.* It can be seen in Fig. 8 that this latter population, which can be measured as an increased capacity of the animal to destroy an IV injected inoculum of *Listeria* rapidly, closely follows the kinetics of the depressed responsiveness *in vitro.*

How can these data be interpreted? One possibility is that suppressor macrophages and activated macrophages are in fact the same cell population. This possibility was alluded to in some early papers on the subject[1] but has not been extensively discussed since. Indeed, I believe that the onus is on the workers in this field to dissociate these two populations validly before a functional physiologic role for suppressor macrophages can be ascribed.

4. MANIFESTATIONS OF ANERGY *IN VIVO*

The tuberculin test is the most important and widely used procedure in the diagnosis of tuberculosis; both the emergence and loss of

cutaneous responsiveness to this material have important clinical implications. Indeed, the association between tuberculosis infection and the development of DTH to the cutaneous injection of mycobacterial antigens (tuberculins) has long been recognized.[23]

In experimental animal models of mycobacterial infection, it has invariably been found that moderately sized infectious inocula will result in the generation of the capacity to express strong DTH to tuberculin, while high-dose infection invariably leads to anergy[24,25] (Fig. 9). In the early work on this phenomenon, a correlation was drawn between anergy to tuberculin, such as that induced by the repeated injection of BCG,[26] and desensitization, in which the animal could be rendered specifically unresponsive for a long period of time, and nonspecifically unresponsive for a transient period, by the IV injection of a large dose of the specific antigen.[27,28] In such experiments, cells from sensitized animals could transfer contact sensitivity to control mice but not to desensitized animals, whereas cells taken from unresponsive desensitized animals could transfer sensitivity to controls.[29]

Bullock,[30,31] in reviewing such evidence, suggested that cell sequestration might be an important factor in the loss of sensitivity. Indeed, in his own experiments, infection of rats with *M. lepraemurium* resulted in a severe disturbance of the recirculating lymphocyte pool (measured as a reduction in numbers of lymphocytes present in the thoracic duct lymph). Injection of radiolabeled lymphocytes revealed that the presence of infection caused a considerable sequestration or trapping of such cells within the reticuloendothelial system, particularly within the spleen.[32]

Orme and Collins[33] presented evidence to suggest that a similar mechanism may be operating in tuberculin-anergic mice infected IV with 10^8 BCG. By use of the passive transfer technique, they have revealed that cells capable of mediating DTH can be readily found in the

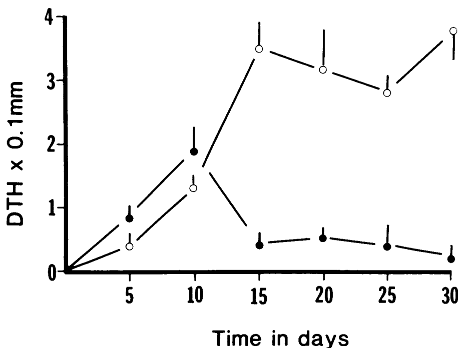


FIGURE 9. Kinetics of emergence of delayed-type cutaneous sensitivity to tuberculin (PPD) in mice infected IV with 10^8 (●) or 10^6 (○) BCG Pasteur. Data expressed as mean DTH response at 24 hr (+SEM; N = 5).

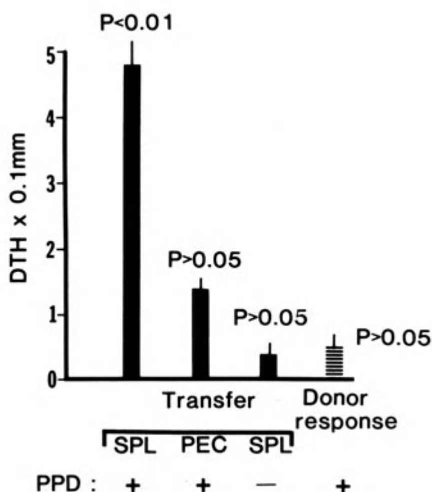


FIGURE 10. Local passive transfer of reactivity to PPD by spleen cells harvested from mice rendered anergic by infection IV with 10^8 BCG Pasteur. (Mean values at 24 hr +SEM; $N = 5$). Normal syngeneic recipients inoculated in hind footpads with 3×10^6 cells + 5 μg PPD.

spleens, but not in the peritoneal exudate, of tuberculin-anergic mice (Fig. 10). These data were interpreted as indicating that an adaptive physiologic change had occurred in the heavily infected mice, in that antigen-reactive cells had become sequestered in the spleen (the site at that time of the generation of a powerful acquired immune response; see above), with a resulting reduction in the number of DTH effector T cells still circulating, and thus with a reduction in the numbers of cells able to extravasate into cutaneous sites of inflammation.

Sequestration of antigen-reactive T cells may not only be involved in anergy to tuberculin but may also contribute to a more general phenomenon: i.e., the finding that moderate doses of BCG, if given subcutaneously, give rise to much higher levels of DTH than a similar inoculum injected intravenously. For instance, if an inoculum of 10^6 BCG is given in the footpad, about 1% of this can be found in the draining popliteal lymph node within 24 hr. By contrast, 10% of an IV inoculum can be found in the spleen within 1 hr; thus, one would predict, assuming no qualitative differences between the spleen and the lymph nodes, that DTH would be better expressed in the IV infected mice. That the reverse is in fact true may reflect the anatomy of the organs involved. Once sensitized, the DTH effector cell population may encounter a large number of lesions within the spleen caused by bacterial implantation, where immunity is being expressed and relevant antigens are being presented. Thus, it is quite possible that following sensitization, a sizable proportion of antigen-reactive cells do not enter the circulation but remain sequestered close to the site of their generation. By contrast, possibly as a result of the more gradual uptake of the infec-

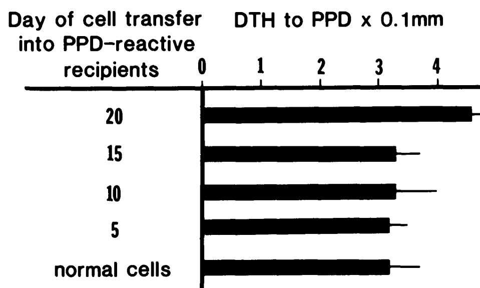


FIGURE 11. Demonstration that intravenous infusion of PPD-reactive recipients (infected with 10^6 BCG IV 20 days prior) with 5×10^7 T-cell-enriched spleen cells from anergic donor mice (infected with 10^8 BCG IV) had no effect on subsequent response of recipients to cutaneous injection of 5 μg PPD. Attempts to block inductive phase of DTH by infusion of cells from anergic mice at earlier stages of (10^6) BCG infection in recipients were also unsuccessful. Means responses +SEM; $N = 5$.

tious organism, DTH effector T cells generated in the draining lymph nodes are much more readily able to pass into the circulation via the efferent lymphatics.

Attempts in this laboratory to correlate tuberculin anergy to an active mechanism of immunosuppression have proved unsuccessful. For example, infusion of mice sensitized to 10^6 BCG (tuberculin-reactive) with spleen cells from tuberculin-anergic mice did not reduce the capacity of the recipients to respond to tuberculin (Fig. 11). Other workers, however, have suggested that if such cells are enriched for B cells, active suppression mediated via an anti-idiotypic mechanism can be demonstrated.[34] By contrast, Turcotte and Forget[25] showed, using two methods believed to ablate suppressor mechanisms (splenectomy and cyclophosphamide chemotherapy), that such methods have no effect on tuberculin anergy in mice.

5. UNRESPONSIVENESS TO UNRELATED ANTIGENS

It can be seen that infection with mycobacteria can perturb a number of important diagnostic assays considerably. In addition, however, many other *in vitro* immune responses and some *in vivo* responses are also considerably modulated in the infected host.

One type of assay system considerably modulated *in vitro* by the presence of infection consists of the host response to alloantigens, such as that measured using the mixed lymphocyte response (MLR). Again, however, caution must be applied in interpreting the meaning of the perturbation of immunity that is observed. For example, the data presented in Fig. 12a show that IV infection with three nontuberculous mycobacteria, *M. kansasii, M. saimiae,* and *M. avium,* resulted in a severe

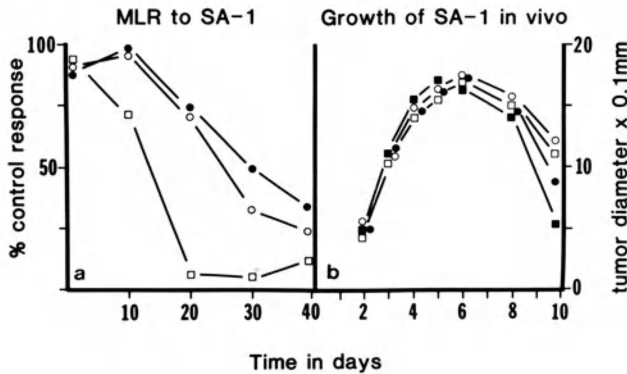


FIGURE 12. (a) Depression of allogeneic responsiveness of B6D2 F_1 hybrid spleen cells (5×10^5) to a similar number of irradiated SA-1 tumor cell (H-2^a) targets in mice infected IV with 10^8 *M. avium* 724 (○), *M. simiae* 1226 (●), or *M. kansasii* 1203 (□). (b) Ability of infected animals to reject implant in the footpad of 5×10^5 untreated SA-1 tumor cells. Implant given on day 30 of infection. Data expressed as mean tumor diameter. $N = 4$; (■), controls.

depression of the MLR of spleen cells to an H-2^a target cell population. This suppression was longlasting and mediated by adherent cells; thus, it bore a strong similarity to suppressor macrophages observed in other assay systems described above. That this inhibitory cell had no apparent relevance to the immunocompetence of the animal *in vivo* was then demonstrated by experiments in which infected animals were tested for their capacity to reject the implant of an H-2^a tumor cell allograft. It was found (Fig. 12b) that the kinetics of rejection of the tumor cell burden in the infected mice was similar to that measured in controls, indicating that the suppressor cells observed *in vitro* played no detectable role in inhibiting or slowing the allogeneic response *in vivo.*

Another assay system widely used to measure the perturbation of immunity that results from mycobacterial infection consists of measurements of immunity to the antigens expressed by sheep red blood cells (SRBC). This antigen is particularly useful because priming of mice with low doses intravenously (or higher doses subcutaneously) results in the acquisition of delayed-type sensitivity, while higher doses given intravenously induce the formation of specific antibody.

Both enhancement and depression of antibody responses to SRBC in BCG-infected mice have been reported; current evidence may suggest that these differing results may reflect the overall viability of the BCG inoculum injected.[35] In the case of the induction of sensitivity, whereas IV BCG infection has little or no effect on levels of DTH generated in

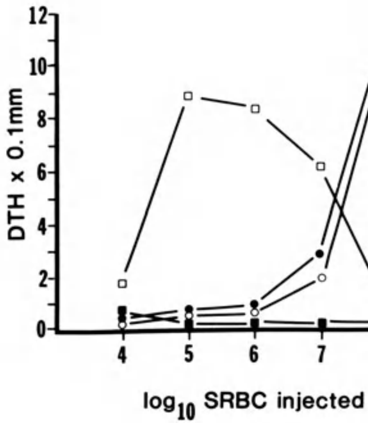


FIGURE 13. Cutaneous DTH response in footpad to challenge with 10^8 SRBC, 5 days after priming with indicated range of doses of antigen. Response in normal mice (□) or BCG-infected (10^8 IV, 15 days prior) mice (■) primed IV, or in normal (●) or infected (○) mice primed SC. Mean values: $N = 5$; SEM range: 0.2–1.0.

response to subcutaneous SRBC priming, the DTH response to this antigen is ablated if both the SRBC and high numbers of BCG are injected IV (Fig. 13). A similar observation has been made in animals injected with high doses of other mycobacteria, such as *M. avium* and *M. simiae.*[15]

A possible explanation for this *in vivo* anergy was provided by experiments in which the dose of SRBC was increased in an attempt to restore responsiveness. It was found (Fig. 14) that if the dose of SRBC administered to infected mice was increased 1000-fold over that required to prime normal mice for DTH (10^5 SRBC IV), DTH responses similar to those expressed by control mice could be obtained. Because these mice were expressing increased antimicrobial resistance in the spleen at this time, it was hypothesized that the requirement for an increased dose of SRBC necessary to prime these mice for DTH re-

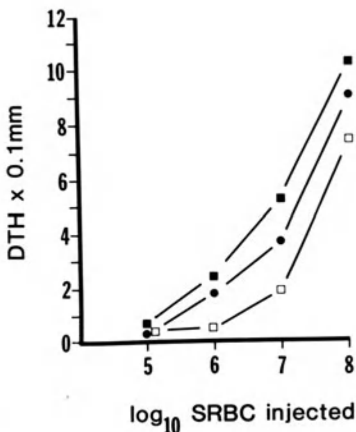


FIGURE 14. Restoration of DTH response to SRBC in mice infected IV with BCG (□), *M. avium* 724, (■) or *M. simiae* 1226 (●) by increasing intravenous SRBC priming dose. Mice primed on day 18 of BCG infection, and on day 30 of nontuberculous mycobacterial infections. Mean values: $N = 5$; SEM range: 0.2–1.1.

flected a lack of T-cell sensitization to the lower SRBC dose. As a possible explanation for this lack of sensitization, since activated macrophages were present in the infected spleens at these times, these cells resulted in the rapid destruction or catabolism of relevant SRBC antigens before they could be adequately presented to T cells, and saturating the system with high numbers of SRBC overcame this effect. That the lack of effector T cells reflected a lack of sensitization, rather than some active mechanism preventing the induction of immunity, was shown by appropriate passive transfer experiments.[15] Once again, an anergic response *in vivo* seemed to be directly associated with high levels of acquired immunity to the infection, rather than reflecting a loss of immunocompetence that a more cursory examination of the data might intimate.

6. CONCLUSIONS

There is a world of difference between controlled laboratory models of anergy in experimental animals and various expressions of anergy observed in the clinical setting. What these experimental models do imply is that there are apparently several situations in which expressions of anergy in *in vitro* assays do not reflect the immunocompetence of the animal when this competence is measured under *in vivo* conditions. Indeed, because of the voluminous literature available concerning the generation *in vitro* of a whole host of various suppressor cells in mycobacterial infections, it is easy to lose sight of the fact that, at the same time, the animal is generating a powerful acquired immune response to the infection. One is faced with the strange scenario in which suppressor macrophages and suppressor T cells totally prevent the division of cells in a pan-T-cell mitogenic response *in vitro;* yet at the same time the animal possesses (1) macrophages that can destroy a lethal inoculum of *Listeria,* and (2) a dividing T-cell population that can adoptively protect recipients against a lethal infection with *M. tuberculosis.*

It is apparent that a number of complicated mechanisms may underlie the development of anergy observed in mice heavily infected with mycobacteria. There is no good evidence at this time that these expressions of anergy reflect a decrease in the ability of the animal to generate adequate levels of acquired immunity to the mycobacterial infection.

REFERENCES

1. Florentin, I., Huchet, R., Bruly-Rosset, M., Halle-Pannenko, O., and Mathe, G., 1976, Studies on the mechanisms of action of BCG, *Cancer Immunol. Immunother.* **1**:31–39.

2. Turcotte, R., Lafleur, L., and Lebreche, M., 1978, Opposite effects of BCG on spleen and lymph node cells: Lymphocyte proliferation and immunoglobulin synthesis, *Infect. Immun.* **21**:696–704.
3. Turcotte, R., 1981, Evidence for two distinct populations of suppressor cells in the spleens of *Mycobacterium bovis* BCG-sensitized mice, *Infect. Immun.* **34**:315–322.
4. Turcotte, R., and Lemieux, S., 1982, Mechanisms of action of *Mycobacterium bovis* BCG-induced suppressor cells in mitogen-induced blastogenesis, *Infect. Immun.* **36**:263–270.
5. Klimpel, G. R., and Henney, C. S., 1978, BCG-induced suppressor cells. I. Demonstration of a macrophage-like suppressor cell that inhibits cytotoxic T cell generation *in vitro, J. Immunol.* **120**:563–569.
6. Orbach-Arbouys, S., and Poupon, M. F., 1978, Active suppression of *in vitro* reactivity of spleen cells after BCG treatment, *Immunology* **34**:431–437.
7. Bullock, W. E., Carlson, E. M., and Gershon, R. K., 1978, The evolution of immunosuppressive cell populations in experimental mycobacterial infection, *J. Immunol.* **120**:1709–1716.
8. Bennett, J. A., and Marsh, J. C., 1980, Relationship of Bacillus Calmette–Guérin-induced suppressor cells to hematopoietic precursor cells, *Cancer Res.* **40**:80–85.
9. Rook, G. A. W., and King, J., 1978, Is the adherent non-specific "suppressor" cell from rodent spleens an *in vitro* artifact?, *Clin. Exp. Immunol.* **32**:466–471.
10. Riglar, C., and Cheers, C., 1980, Macrophage activation during experimental murine brucellosis. II. Inhibition of *in vitro* lymphocyte proliferation by Brucella-activated macrophages, *Cell. Immunol.* **49**:149–154.
11. Cheers, C., Pavlov, H., Riglar, C., and Madraso, E., 1980, Macrophage activation during experimental murine brucellosis. III. Do macrophages exert feedback control during brucellosis?, *Cell. Immunol.* **49**:166–181.
12. Wepsic, H. T., Alaimo, J., Druker, B. J., Murray, W., and Morris, H. P., 1981, The negative systemic effects of BCGcw inoculated intraperitoneally. I. *In vivo* demonstration of intramuscular tumor growth enhancement using Morris hepatomas, *Cancer Immunol. Immunother.* **10**:217–227.
13. Druker, B. J., Wepsic, H. T., 1983, BCG-induced macrophages as suppressor cells, *Cancer Invest.* **1**:151–161.
14. Orme, I. M., Stokes, R. W., and Collins, F. M., 1986, Genetic control of natural resistance to nontuberculous mycobacterial infections in mice, *Infect. Immun.* **54**:56–62.
15. Orme, I. M., and Collins, F. M., 1984, Immune response to atypical mycobacteria: Immunocompetence of heavily infected mice measured *in vivo* fails to substantiate immunosuppression data obtained *in vitro, Infect. Immun.* **43**:32–37.
16. Orme, I. M., and Collins, F. M., 1984, Demonstration of acquired resistance in *Bcg*^{r} inbred mouse strains infected with a low dose of BCG Montreal, *Clin. Exp. Immunol.* **56**:81–88.
17. Orme, I. M., and Collins, F. M., 1983, Protection against *Mycobacterium tuberculosis* infection by adoptive immunotherapy, *J. Exp. Med.* **158**:74–83.
18. Orme, I. M., Ratcliffe, M. J. H., and Collins, F. M., 1984, Acquired immunity to heavy infection with *Mycobacterium bovis* bacillus Calmette–Guérin and its relationship to the development of nonspecific unresponsiveness *in vitro, Cell. Immunol.* **87**:285–296.
19. Orme, I. M., and Collins, F. M., 1984, Adoptive protection of the *Mycobacterium tuberculosis*-infected lung, *Cell, Immunol.* **84**:113–120.
20. Colizzi, V., 1984, *In vivo* and *in vitro* administration of interleukin 2-containing preparations reverses T cell-unresponsiveness in *Mycobacterium bovis* BCG-infected mice, *Infect Immun.* **45**:25–28.
21. Turcotte, R., and Legault, D., 1986, Mechanisms underlying the depressed production of interleukin-2 in spleen and lymph node cell cultures of mice infected with *Mycobacterium bovis* BCG, *Infect. Immun.* **51**:826–831.

22. Allison, A. C., 1978, Mechanisms by which activated macrophages inhibit lymphocyte responses, *Immunol. Rev.* **40**:3–24.
23. Zinsser, H., 1921, Studies on the tuberculin reaction and on specific hypersensitiveness in bacterial infection, *J. Exp. Med.* **34**:495–524.
24. Collins, F. M., and Mackaness, G. B., 1970, The relationship of delayed hypersensitivity to acquired antituberculous immunity. I. Tuberculin sensitivity and resistance to reinfection in BCG-vaccinated mice, *Cell. Immunol.* **1**:253–265.
25. Turcotte, R., and Forget, A., 1983, Cutaneous unresponsiveness to *Mycobacterium bovis* BCG in intravenously infected mice, *Infect. Immun.* **41**:453–461.
26. Lamoreux, G., and Poisson, R., 1974, B.C.G. and immunological anergy, *Lancet* **1**:989–990.
27. Sprent, J., Miller, J. F. A. P., and Mitchell, G. F., 1970, Antigen-induced selective recruitment of circulating lymphocytes, *Cell, Immunol.* **2**:171–181.
28. Schlossman, S. F., Levin, H. A., Rocklin, R. E., and David, J. R., 1971, The compartmentalization of antigen-reactive lymphocytes in desensitized guinea pigs, *J. Exp. Med.* **134**:741–750.
29. Dwyer, J. M., and Kantor, F. S., 1973, Regulation of delayed hypersensitivity. Failure to transfer delayed type hypersensitivity to desensitized guinea pigs, *J. Exp. Med.* **137**:42.
30. Bullock, W. E., 1975, Anergy and infection, *Adv. Intern. Med.* **21**:149.
31. Bullock, W. E., 1978, Leprosy: A model of immunological perturbation in chronic infection, *J. Infect. Dis.* **137**:341–345.
32. Bullock, W. E., 1976, Perturbation of lymphocyte circulation in experimental murine leprosy. I. Description of the defect, *J. Immunol.* **117**:1164–1170.
33. Orme, I. M., and Collins, F. M., 1984, Passive transfer of tuberculin sensitivity from anergic mice, *Infect. Immun.* **46**:850–853.
34. Campa, M., Benedettini, G., and Marelli, P., 1986, B and T lymphocytes regulated by idiotype anti-idiotype interactions inhibit delayed-type hypersensitivity to BCG in mice, *Cell. Immunol.* **98**:93–103.
35. Brown, C. A., Brown, I. N., and Sljivic, V. S., 1979, Suppressed or enhanced antibody responses *in vitro* after BCG treatment of mice: Importance of BCG viability, *Immunology* **38**:481–488.

10

Suppressor Cells in Mycobacterial Infections

REIKO M. NAKAMURA and TOHRU TOKUNAGA

1. INTRODUCTION

Mycobacterium bovis BCG has been widely used as the antituberculosis vaccine and is also a well-known immunopotentiator in both humoral and cell-mediated immune responses. Bacillus Calmette–Guérin (BCG) or *Mycobacterium tuberculosis* is an indispensable component of Freund's complete adjuvant and has been used in various experimental systems to potentiate immune responses. Because of its adjuvant activity, BCG has been used for immunotherapy of cancer, such as acute lymphoid leukemias[1] or melanomas[2] in humans. There are quite a few reports in this field both in animal experiments and in human cases,[3–7] many of which indicated improvement or even cure of the tumor by the immunotherapy with BCG. However, several reports show enhanced tumor growth by treatment with BCG. Likhite[4] reported that in some instances tumor growth was enhanced by BCG injection in rats. Piessens *et al.*[8] showed that injection of a tumor cell–BCG mixture into rats resulted in either suppressed or enhanced growth of the tumor depending on the dose of BCG. Mathé,[9] who had emphasized the immunotherapeutic effect of BCG, even found that a high dose of BCG suppresses antibody production against sheep red blood cells (SRBC) in mice. BCG injected intravenously (IV) induced suppressed immunity to rodent malaria.[10] Thus, it has been known for some time that the famous immunopotentiator

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BCG has double-faced activity, modulating immune responses either into a positive (immunity) or negative (suppression) direction.

Now, various types of suppressor cells are known to be induced by mycobacterial infection. Antigen-specific suppressor T (Ts) cells directed toward mycobacteria are responsible for inhibiting the induction of immunity to the bacilli, and consequently, enhance bacterial growth *in vivo*. Such condition is known in human leprosy. Nonspecific Ts cells and suppressor macrophages inhibit T-cell proliferation in general. Suppressor factors released by T cells or macrophages have been partially characterized, and some molecules were identified to be responsible for the suppression. The development of molecular biology techniques and discovery of a new experimental animal, armadillo, brought advantages in human leprosy study where many investigators have struggle for the absence of assay systems because the bacilli are not cultivable *in vitro*.

This chapter reviews these studies in relation to the suppressor cells induced by mycobacterial infection, the suppressor factors and molecules involved, and the mechanisms operative in the suppressor cell circuit.

2. SUPPRESSOR CELLS INDUCED BY MYCOBACTERIAL INFECTION

2.1. Suppressor Cells in *M. bovis* and *M. tuberculosis* Infection

Two types of cells, T cells and macrophages, are known to induce the anergic state observed in the hosts receiving high doses of BCG or *M. tuberculosis*. Ts cells[11–16] or suppressor macrophages,[17–21] or both, have been reported by many investigators. Mice receiving 10^8 BCG IV do not show the antigen-specific delayed-type hypersensitivity (DTH).[12,13,22] The spleen cells of these mice does not respond to tuberculin purified protein derivative (PPD), phytohemagglutinin (PHA), concanavalin A (Con A), and sometimes to bacterial lipopolysaccharide (LPS) *in vitro*.[16,18,22,23] Neither cytotoxic T lymphocytes nor alloreactive T cells are induced in mixed cultures of spleen cells from BCG-infected mice and γ-irradiated allogeneic stimulator cells.[14,18] To induce the unresponsiveness, doses of BCG as high as 10^7–10^8 are required. Killed BCG are less effective than live BCG. The route of administration is also critical.[13,24] Intravenous injection induces suppressor cells in the spleen most effectively. So does intraperitoneal (IP) injection. The subcutaneous (SC), intradermal (ID), or intramuscular (IM) routes are not effective for the induction of suppressor cells but are good for immunizing the hosts.

Antigen-specific Ts cells in BCG-infected mice were described by Nakamura and Tokunaga.[13] C3H/He mice are genetically low responders to BCG[25] that show weak delayed-type hypersensitivity (DTH) to PPD 2 weeks after the injection of 10^7 BCG SC, by which time BCG-high-responders exhibit significant DTH. Complete suppression of DTH was observed in the mice given 10^7 BCG IV. The reason for this unresponsiveness was attributed to Ts cells, which are detectable in the spleen within 6 days from infection. These Ts cells inhibit the induction of effector T cells in DTH but not the expression of DTH in already immunized mice. Therefore, these Ts are Ts-aff (afferent limb acting) and not Ts-eff (efferent limb acting). Such Ts are induced in another BCG-low-responder strain of mice, A.SW, suggesting that the induction of antigen-specific Ts-aff is the basic mechanism for the low responsiveness to BCG.

Suppressor adherent cells were reported by Ellner[19] in the peripheral blood of active pulmonary tuberculosis patients. The peripheral mononuclear cells (PMNC) from these patients showed depressed proliferation in response to PPD, streptokinase-streptodornase, or *Candida* antigen but responded normally to PHA. Larger numbers of circulating monocytes were observed in low responders than in high-responder patients. Depletion of monocytes resulted in enhanced blastogenic response of the patient lymphocytes to PPD. An enhanced mitogenic response of human peripheral lymphocytes was observed also in normal subjects following depletion of monocytes.[21] The inhibitory effect of monocytes in this case was mediated by prostaglandin secretion.

The time course for the induction of suppressor cells by mycobacterial infection was studied in various systems. Suppressor cells appeared as early as 3[13] or 5 days[16] after BCG injection IV and were still demonstrated as late as 40–90 days postinjection.[12,16] In some instances, Ts cells appear earlier than suppressor macrophages in the spleen.[26,27] But in most cases, both Ts cells and macrophages are detected simultaneously in the spleen.

The suppressor cells induced by the cellular components of BCG. Janss *et al.*[28] reported that IP injection of BCG cell walls (BCG-CW) in oil droplets induces Ts cells that inhibit graft-versus-host reaction. Kato *et al.*[29] reported that *N*-acetyl-muramyl-L-alanyl-D-isoglutamine, or muramyl dipeptide (MDP), the minimum component of BCG-CW having adjuvant activity but no antigenicity, induces suppression of DTH when injected IV into mice prior to immunization with BCG-CW. Tuberculin-associated protein (TAP), an antigenic determinant of BCG, also induces suppression of DTH in BCG-CW immunization. Interestingly, the suppression induced by MDP, which has no antigenicity, is nonspecific, while TAP-induced suppression is antigen specific. Ts cells

induced by MDP or TAP inhibit delayed footpad reaction *in vivo* and macrophage migration inhibition mediated by a lymphokine (LK) *in vitro*. Thus, Ts cells seem to interfere with LK-dependent mechanisms in BCG-CW induced DTH.

2.2. Suppressor Cells in Human Leprosy

From early times of investigation, human leprosy has been known as a disease characterized by defects in cell-mediated immune (CMI) responses. Immunosuppression in leprosy, either antigen specific or nonspecific, was reported by many investigators as reviewed by Turk and Bryceson[30] and Bloom and Mehra.[31] Early studies showed suppressed skin reaction in lepromatous leprosy (LL) or borderline (BL) patients to lepromin, *Dharmendra* antigen, or *Candida* antigen.[32,33] Shepard *et al.*[24] showed that *M. leprae*-induced suppression of DTH is observed in mice injected with 10^7 living bacilli IV or IP. Peripheral blood T cells of LL patients responded poorly to PHA, Con A, or lepromin *in vitro*.[33–35] Lepromin-induced Ts cells and suppressor macrophages were reported in LL or BL patients, but not in tuberculoid-type (TT) patients.[35,36]

Mehra *et al.*[36] described two types of suppressor cells in LL and BL patients, i.e., T cells and adherent cells (monocytes). Ts-cell activity was associated with the $OKT8^+/TH_2^+$ subset of T cells. OKT8-depleted cells from some LL patients (6/21) showed enhanced proliferative responses to lepromin *in vitro*, in spite of the depressed response of the original unfractionated cells. About 50% of the $T8^+$ cells are FcR^+ and Ia^+. The number of FcR^+/Ia^+ T cells increases in LL patients and decreases in patients treated with a mixture of killed *M. leprae* and living BCG (Convit vaccine)[37] along with an improvement of a skin reactivity. These results suggest that T cells with this phenotype could be the index for active suppression in leprosy.

Stoner *et al.*[38] tried to ascertain the presence of suppressor cells in leprosy by mixing the PMNC of LL patients (LL-PMNC) with those of HLA-D-identical healthy siblings in culture in the presence of lepromin. No suppressive effect was observed on lepromin-induced blastogenesis. Mitomycin C-treated normal PMNC activated LL-PMNC to respond to lepromin. These results suggest that there are no active suppressor cells circulating in the blood of LL patients. Because LL-PMNC did not respond to lepromin in the presence of normal antigen-presenting cells (APC), Stoner *et al.* concluded that the depressed response to lepromin in LL patients is due to the lack of sufficient numbers of sensitized T cells. However, the suppressive effect exerted by LL-PMNC could be too weak to overcome the strong blastogenic response of healthy PMNC with lepromin.

2.3. Suppressor Cells in Atypical Mycobacteria and *M. lepraemurium* Infection

The studies on suppressor cells in atypical mycobacteria infection are few. Watson and Collins[26,39] reported that Ts cells are induced in mice infected with *M. habana* or *M. avium*. In these mice, suppression of DTH to PPD and of T-cell proliferation induced by PHA or cytoplasmic protein antigen (CPA) was observed at 30–90 days of infection. In the case of *M. avium* infection, suppressor macrophages were detected in the spleen 90 days after bacterial IV injection.

We have observed that Ts cells are induced in the spleens of mice infected with *M. intracellulare* Mino strain. Mino strain grows continuously in susceptible strains of mice, such as BALB/c or C57BL/6. These mice show no DTH to PPD-intracellulare (PPD-I). Ts cells are detectable by transferring the spleen cells of infected mice into syngeneic recipients, converting the response to PPD-I from positive to negative. Both Ts-aff and Ts-eff were shown to exist in the spleen of infected mice; Ts-aff in the early stages of infection, and Ts-eff at later stages (Y. Goto and R. M. Nakamura, unpublished data).

Macrophage-like suppressor cells are induced in the spleen of mice infected IP with *M. lepraemurium* 5–10 weeks after infection.[40] These cells inhibit direct plaque forming cell response of normal spleen cell cultures to SRBC. At 10–11 weeks of infection, Ts cells become detectable in the spleen. Suppressor cells are present in peripheral lymph nodes at 22–25 weeks of infection.

Hoffenbach *et al.*[41] reported that spleen cells of *M. lepraemurium*-infected mice become less responsive to Con A or alloantigens and their IL-2 production also decreases gradually. Peak depression in Con A and alloantigen response was observed 6 months after infection, but IL-2 production was depressed as early as 1 month after bacterial injection. T cells of infected mice also show a low reactivity to IL-2.

However, results in *M. lepraemurium* are variable. In some cases, *M. lepraemurium*-infected mice show marked DTH to the bacterial antigen and inhibition of bacterial growth *in vivo,* while in some cases DTH is present but no acquired immunity to the bacteria is expressed.[42,43] Peritoneal macrophages of *M. lepraemurium*-infected mice are activated but fewer than normal in number, while spleen macrophages of the same mice are numerous but suppressed in the activity.[44]

In mice *M. lepraemurium* causes a chronic infection, and the bacilli multiply very slowly. The host's immune response should vary along with the stage of infection. Strain variations in susceptibility to *M. lepraemurium* are also known in mice.[45–47] The different results reported above could be due to differences in timing and in the animal strains used.

3. CELL–CELL INTERACTIONS RESPONSIBLE FOR Ts-CELL INDUCTION

3.1. Antigen-Presenting Cells for Ts-Cell Induction

The need for accessory cells for Ts-cell induction was first reported in a system of mycobacterial infection. Nakamura *et al.*[48] developed an *in vitro* system for the induction of Ts against DTH to BCG. Spleen cells of BCG-low-responder mice, C3H/He, cultured with PPD *in vitro* yielded Ts cells which blocked the induction of DTH. The suppressive activity was evaluated by transferring the cultured cells into syngeneic recipients where the immunization process was undertaken *in vivo.* By separating the spleen cells into T cells and plastic adherent cells (macrophages) before culturing with PPD, it has become apparent that I-J$^+$ adherent cells are necessary for the induction of Ts. I-A$^+$ adherent cells, which are known to serve as APC for the induction of helper T (Th) cells, are not required for Ts-cell induction. In the same *in vitro* method, it was found that the precursors for Ts cells are also I-J$^+$. Both APC and Ts cell precursors are cyclophosphamide sensitive. Therefore, in the inductive phase of the Ts pathway in BCG infection an I-J$^+$ precursor Ts cell appears to recognize nominal antigen (PPD) presented on the I-J$^+$ APC in association with an I-J molecule, and differentiate into an effector Ts. In this scheme, the I-J molecule plays the role of "self" as the I-A/E molecule in the Ts-cell circuit. Supporting evidence is that macrophages from B10.A (5R) mice presented PPD to C3H T cells and induced Ts *in vitro,* but macrophages from B10.A (3R) mice did not.[49] Difference between 3R and 5R is only in I-J subregion. 5R shares it (I-J^k) with C3H, but 3R (I-J^b) does not. Thus, I-J compatibility between APC and T cells is required for Ts cell induction. Nakamura *et al.*[50] also showed that an I-J$^+$ macrophage cell line could replace the splenic adherent cells in the induction of Ts cells *in vitro.*

The role of I-J$^+$ APC for the induction of Ts cells was confirmed in hapten-specific DTH or contact sensitivity.[51–53] Although the I-J gene has raised a paradoxical problem in that it is not located within the H-2 complex as originally considered,[54,55] I-J on APC or macrophages is likely a key molecule for the suppressor cell circuit.

3.2. Effect of UV Irradiation on Ts-Cell Induction

Recent studies of Granstein *et al.*[56,57] are suggestive to explain the role of UV irradiation in suppressor cell activation. I-A$^+$ epidermal cells (most probably Langerhans cells) are UV sensitive and decrease following whole-body UV irradiation in mice. On the contrary, I-J$^+$ epidermal

cells are resistant to UV and responsible for Ts induction. Thus, it seems reasonable to postulate that UV irradiation causes skin cancer because of increased Ts cells that depress CMI. Noma *et al.*[52] reported that UV irradiation selectively destroys the APC for the positive limb of immune response but not that for the suppressor pathway.

4. MECHANISMS UNDERLYING THE SUPPRESSION INDUCED BY MYCOBACTERIAL INFECTION

4.1. Suppressor Factors and IL-2

Ts cells and suppressor macrophages produce suppressor factors which inhibit T-cell proliferation induced by PHA, Con A, LPS, or PPD.[22,27,58] As Colizzi[22] described, this suppression is reversed by exogenous IL-2 both *in vivo* and *in vitro*. Suppressor factors from T cells and macrophages have been partially characterized.[58] T-suppressor factor is 50,000–70,000 M_r, while macrophage factor is 10,000–30,000 M_r. These factors are distinct from each other in their effective timing to inhibit DNA synthesis *in vitro;* the T-cell factor acts only when it is added to the culture within 12 hr, but the macrophage factor is effective until 48 hr after culture. Both factors inhibit IL-2 production by T cells.

In many studies on human leprosy, it was shown that T cells of LL patients are incapable of producing IL-2 when stimulated with *M. leprae* antigen.[31,59–61] The lack of IL-2 production is the reason for the unresponsiveness of *M. leprae*-infected patients. Modulin *et al.*[62] determined the distribution of IL-2-producing cells and IL-2-receptor-positive cells (Tac^+ cells) in the tissue of leprosy patients using monoclonal antibodies. IL-2 producers have the phenotype $Leu4^+ Leu3a^+ Leu2a^- Tac^- OKT6^-$ and were fewer in LL patients compared with TT patients. Numbers of Tac^+ cells did not differ in LL and TT patients. The presence of Tac^+ cells in LL patients explains the restoration of DNA synthesis by exogenous IL-2 in such patients,[59,60,63] which is consistent with what has been observed in anergy induced by BCG.[22] But a recent study of Mohagheghpour *et al.*[61] showed that the defect in LL patients does not concern the production of IL-2, but the expression of IL-2 receptors. This observation does not agree with others.[59,60,62,63] The reason for this inconsistency is not clear, however most results seem to indicate that the defect in IL-2 production is the main reason for the unresponsiveness of LL patients and for anergy in BCG or other mycobacterial infection. IL-2 receptor is induced by IL-1, which is secreted normally by the monocytes of LL patients.[64] This evidence is consistent with the fact that the numbers of Tac^+ cells are similar in LL patients and normal controls.[62]

Honda *et al.*[65] reported that a lymphokine inhibiting IL-2-mediated T-cell proliferation is released from Con A-stimulated mouse spleen cells. The suppressive activity of the culture supernatant appeared in two peaks; 10,000–12,000 M_r and 60,000–80,000 M_r. This IL-2 inhibitor is produced by $L3T4^+$ or Ly 2^+ T cells. IL-2 inhibitory molecules are reported by Hardt *et al.*[66] as 50,000 M_r in mouse, and by Fontana *et al.*[67] as 97,000 M_r in human. It is not known whether suppressor factors in mycobacterial infection contain IL-2 inhibitor or not, but it might be worthy to test the hypothesis that any suppressor cell can inhibit the proliferation of other T cells in general by secreting IL-2 inhibitor.

4.2. Phenolic Glycolipid-I, an *M. leprae*-Specific Antigen That Induces Ts-Cell Activation

Search for *M. leprae*-specific antigens has been conducted in many laboratories. The use of the bacteria from armadillos[68] made it possible to raise monoclonal antibodies directed to *M. leprae*. Several monoclonal antibodies were reported specific to *M. leprae* internal proteins of 68,000, 34,000, and 12,000 or 14,000 M_r,[31,69,70] but these are unrelated to suppressor cells.

Hunter *et al.*[71] isolated a specific phenolic glycolipid (gly-I) from *M. leprae*. This molecule is immunologically active, as reacted with rabbit antisera to *M. leprae* and with sera from LL patients. Mehra *et al.*[72] investigated the possibility that unique antigens of *M. leprae* induce specific suppressor cells. They tested *M. leprae* specific antigens identified by monoclonal antibodies and found that the gly-I described by Hunter *et al.*[71] is the only molecule that induces suppression of mitogenic response of lymphocytes from LL patients Gly-I incorporated in liposomes suppressed the Con A response of LL lymphocytes. Ts cells recognize the specific terminal trisaccharide of gly-I. When $T8^+$ suppressor cells were depleted, gly-I-induced suppression was abolished. Monoclonal antibody directed to gly-I restored Con A response of LL lymphocytes.

This glycolipid was titrated in the serum or urine of LL patients by chemical or immunologic method.[73] The quantitation showed a reasonable correlation with the status of the disease and the effect of chemotherapy. This method would also be applicable to the diagnosis of leprosy.

Specific antigens of *M. tuberculosis* and BCG were isolated using monoclonal antibodies and recombinant DNA techniques.[74,75] However, no molecule-activating suppressor cells has yet been found.

4.3. Lipids from Mycobacteria

Macrophages that have ingested *M. tuberculosis* release a suppressor-cell-activating factor.[76] When ^{125}I-labeled bacteria were ingested, most radioactivity was released into the culture supernatant. The radiolabelled material contained phosphatidylethanolamine and phosphatidylinositol of bacterial origin. These molecules were found to activate Ts cells.

4.4. Anti-Idiotypic Antibody

Anti-idiotypic antibody in BCG-immunized mice is responsible for the suppression of DTH.[77] This antibody blocks effector T cells by reacting with T cell receptors and inhibits the interaction with the antigen. The antibody is only absorbed by antigen specific sensitized T cells. Anti-idiotype-induced suppression was reported in DTH to hapten-coupled syngeneic cells in mice.[78] Anti-idiotype antibody injected IV induces Ts-eff, while idiotype antibody induces Ts-aff. Idiotypic regulation was observed in the Ts circuit in azobenzenearsonate-specific DTH response.[79] Thus, anti-idiotypic antibody could be one of the suppressor factors induced by mycobacterial infections, especially in cases in which high-titer antibodies are detected during infection as with atypical mycobacteria or leprosy bacilli.

4.5. Prostaglandins and Interferons

Macrophages release prostaglandins, especially prostaglandin E_2 (PGE_2), which block immune pathways. Intraperitoneal injection of BCG-CW induces suppressor macrophages in the spleen and bone marrow, which secrete prostaglandins that inhibit mitogenic responses or enhance tumor growth in mice.[23,28] The effect was reversed by indomethacin in drinking water. Treatment of human PBMC with indomethacin enhances the mitogenic response to the level of monocyte-depleted blood cells.[21] These results suggest that prostaglandin secretion might be a mechanism of suppression by macrophages in mycobacterial infection as in other forms of suppression.

α-Interferon (IFN_α) produced in response to killed mycobacteria resulted in nonspecific suppression of Con A-induced blastogenesis of normal lymphocytes.[31] Monoclonal antibodies directed to IFN_α eliminate this mycobacteria-induced suppression of lymphocyte proliferation of either normal or LL patients. Suppression of Con A response in LL lymphocytes induced by killed cultivable mycobacteria was also elimi-

nated by anti-IFN antibody, but the suppression induced by lepromin or killed *M. leprae* was not, indicating that the mechanisms are different.

5. FUTURE PROSPECT

Suppressor cells are induced by heavy mycobacterial infection in general. Experimentally, IV injection yields suppressor cells more effectively than ID injection. The reason for this difference seems due to a different location of APC for Ts induction; I-J$^+$ APC would seem to be more numerous in the spleen than in the skin, while I-A$^+$ APC (Langerhans cells) are dominant in the skin. Conversion of the epidermal cell population from I-A$^+$ dominant to I-J$^+$ dominant by UV irradiation is consistent with these results. The role of APC for Ts cell induction should be studied more extensively in the context of regulatory mechanisms in the whole body.

Antigen-specific suppression and nonspecific suppression are both observed in mycobacterial infection but what mechanism is present to educate Ts cells to be antigen specific is not known. The *M. leprae*-specific antigen gly-I induces suppression in LL patients. This elegant study suggests the need for future research on suppressor-inducing molecules and their recognition by Ts cells. Although such molecules are not found in BCG or *M. tuberculosis* infection, evidence that different components of BCG-CW induce discriminately antigen-specific and nonspecific Ts cells is highly suggestive.

Suppression of IL-2 production is a general phenomenon in *Mycobacterium*-infected hosts. Ts cells, antigen specific or nonspecific, and suppressor macrophages all suppress IL-2 production similarly, at least phenotypically. Studies on the suppressive factors that inhibit IL-2 production will be interesting, especially within the context of the triggering mechanisms and the target molecules for suppression.

Suppressor mechanisms are important for the homeostasis of the body. Further analysis of these mechanisms will bring much progress in the study of disseminated infections by *M. leprae* or other microbial parasites, and also in the understanding of autoimmune diseases and tumor immunology.

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REFERENCES

1. Mathé, G., 1976, Surviving in company of BCG, *Cancer Immunol. Immunother.* **1**:3–5.
2. Morton, D. L., Eilber, F. R., Holmes, E. C., Sparks, F. C., and Ramming, K., 1976, BCG immunotherapy as a systemic adjunct to surgery in malignant melanoma. *Med. Clin. North Am.* **60**:431–439.
3. Hawrylko, E., 1977, Influence of BCG-potentiated immunotherapy in tumor-bearing mice, *J. Natl. Cancer Inst.* **59**:359–365.
4. Likhite, V. V., 1976, Experimental cancer Immunotherapy: Comparison of tumor rejection in F344 rats given live *Mycobacterium bovis* (strain BCG) and killed *Corynbacterium parvum, J. Natl. Cancer Inst.* **56**:985–989.
5. Bast, R. C., Zbar, B., Borsos, T., and Rapp, H. J., 1974, BCG and cancer, *N. Engl. J. Med.* **290**:1413–1420.
6. Mathé, G., Shwarzenberg, L., DeVassal, G., Delgado, M., Pena-Angulo, J., Belponne, E., Poullart, P., Machover, D., Misset, J. L., Pico, J. L., Jasmin, C., Hayat, M., Schneider, M., Cattan, A., Amiel, J. L., Musset, M., and Rosenfeld, C., Chemotherapy followed by active immunotherapy in the treatment of acute lymphoid leukemias for patients of all ages, in: *Immunotherapy of cancer: Present status of trials in man* (W. D. Terry and D. W. Windhorst, eds.), pp. 451–469, Raven, New York.
7. Yamamura, Y., Kitagawa, M., and Azuma, I. (eds.), *Cancer Immunology and Its Immunological Basis,* Japan Science Society Press–Tokyo, University Park Press, Baltimore, 1978.
8. Piessens, W. F., Campbell, M., and Churchill, W. H., 1977, Inhibition or enhancement of rat mammary tumors dependent on dose of BCG, *J. Natl. Cancer Inst.* **59**:207–211.
9. Mathé, G., 1976, *Active Immunotherapy of Cancer. Its Immunoprophylaxis and Immunorestoration,* Springer, New York.
10. Smrkovski, L. L., 1981, Effect of route of *Mycobacterium bovis* BCG administration on induction of suppression of sporozoite immunity in rodent malaria, *Infect. Immun.* **31**:408–412.
11. Geffard, M., and Orbach-Arbouys, S., 1976, Enhancement of T suppressor activity in mice by high doses of BCG, *Cancer Immunol. Immunother.* **1**:41–43.
12. Collins, F. M., and Watson, S. R., 1979, Suppressor T-cells in BCG-infected mice, *Infect. Immun.* **25**:491–496.
13. Nakamura, R. M., and Tokunaga, T., 1980, Induction of suppressor T cells in delayed-type hypersensitivity to *Mycobacterium bovis* BCG in low-responder mice, *Infect. Immun.* **28**:331–335.
14. Kendall, L., and Sabbadini, E., 1981, Effect of Bacillus Calmette–Guérin on the *in vitro* generation of cytotoxic T lymphocytes. I. Effect of BCG on the frequency of cytotoxic T lymphocyte precursors and on the production of helper factors, *J. Immunol.* **127**:234–238.
15. Moore, V. L., Mondloch, V. M., Pederson, G. M., Schrier, D. J., and Allen, E. M., 1981, Strain variation in BCG-induced chronic pulmonary inflammation in mice: Control by a cyclophosphamide-sensitive thymus-derived suppressor cell, *J. Immunol.* **127**:339–342.
16. Turcotte, R., 1981, Evidence for two distinct populations of suppressor cells in the spleens of *Mycobacterium bovis* BCG-sensitized mice, *Infect. Immun.* **34**:315–322.
17. Florentin, I., Huchent, R., Bruley-Rosset, M., Halle-Pennenko, O., and Mathé, G., 1976, Studies on the mechanisms of action of BCG, *Cancer Immunol. Immunother.* **1**:31–39.
18. Klimple, G. R., and Henney, C. S., 1978, BCG-induced suppressor cells. I. Demonstra-

tion of a macrophage-like suppressor cell that inhibits cytotoxic T cell generation *in vitro*, *J. Immunol.* **120:**563–569.
19. Ellner, J. J., 1978, Suppressor adherent cells in human tuberculosis, *J. Immunol.* **121:**2573–2579.
20. Allen, E. M., and Moore, V. L., 1979, Suppression of phytohemagglutinin and lipopolysaccharide responses in mouse spleen cells by *Bacillus Calmette–Guérin*, *J. Reticuloendothel. Soc.* **26:**349–336.
21. Novogrodsky, A., Rubin, A. L., and Stenzel, K. H., 1979, Selective suppression by adherent cells, prostaglandin, and cyclic AMP analogues of blastogenesis induced by different mitogens, *J. Immunol.* **122:**1–7.
22. Colizzi, V., 1984, *In vivo* and *in vitro* administration of interleukin 2-containing preparation reverses T-cell unresponsiveness in *Mycobacterium bovis* BCG-infected mice. *Infect. Immun.* **45:**25–28.
23. DeSilva, M. A., Wepsic, H. T., Mizushima, Y., Nikcevich, D. A., and Larson, C. H., 1985, Modification of *in vitro* BCG cell wall-induced immunosuppression by treatment with chemotherapeutic agents or indomethacin, *J. Natl. Cancer Inst.* **74:**917–921.
24. Shepard, C. C., Walker, L. L., Van Landingham, R. M., and Ye, S. Z., 1982, Sensitization or tolerance to *Mycobacterium leprae* antigen by route of injection, *Infect. Immun.* **38:**637–680.
25. Nakamura, R. M., and Tokunaga, T., 1978, Strain difference of delayed-type hypersensitivity to BCG and its genetic control in mice, *Infect. Immun.* **22:**657–664.
26. Watson, S., and Collins, F. M., 1980, The specificity of suppressor T cells induced by chronic *Mycobacterium avium* infection in mice, *Clin. Exp. Immunol.* **43:**10–19.
27. Turcotte, R., and Lemieux, S., 1982, Mechanisms of action of *Mycobacterium bovis* BCG-induced suppressor cells in mitogen-induced blastogenesis, *Infect. Immun.* **36:**263–270.
28. Janss, G., Wepsic, H. T., Mizushima, Y., DeSilva, M. A., and Larson, C. H., 1984, The inhibition of fatal graft-versus-host disease by immunization of donor or host with BCG cell walls, *Transplantation* **38:**407–411.
29. Kato, K., Yamamoto, K., Kimura, T., Azuma, I., and Askenase, P. W., 1984, Suppression of BCG cell wall-induced delayed-type hypersensitivity by pretreatment with killed BCG: Induction of nonspecific suppressor T cells by the adjuvant portion (MDP) and of specific suppressor T cells by the antigen portion (TAP), *J. Immunol.* **132:**2790–2795.
30. Turk, J. L., and Bryceson, A. D. M., 1971, Immunological phenomona in leprosy and related diseases, *Adv. Immunol.* **13:**209–266.
31. Bloom, B. R., and Mehra, V., 1984, Immunological unresponsiveness in leprosy, *Immunol. Rev.* **80:**5–28.
32. Bullock, W. E., 1968, Studies of immune mechanisms in leprosy. 1. Depression of delayed allergic response to skin test antigens, *N. Engl. J. Med.* **278:**298–304.
33. Myrvang, B., Godal, T., Ridley, D. S., Fröland, S. S., and Song, Y. K., 1973, Immune responsiveness to *Mycobacterium leprae* and other mycobacterial antigens throughout the clinical and histopathological spectrum of leprosy, *Clin. Exp. Immunol.* **14:**541–553.
34. Mehra, V. L., Talwar, G. P., Balakrishnan, K., and Bhutani, L. K., 1972, Influence of chemotherapy and serum factors on the mitogenic response of peripheral leucocytes of leprosy patients to phytohaemagglutinin, *Clin. Exp. Immunol.* **12:**205–213.
35. Mehra, V., Mason, L. H., Fields, J. P., and Bloom, B. R., 1979, Lepromin-induced suppressor cells in patients with leprosy, *J. Immunol.* **123:**1813–1817.
36. Mehra, V., Convit, J., Rubinstein, A., and Bloom, B. R., 1982, Activated suppressor T cells in leprosy, *J. Immunol.* **129:**1946–1951.
37. Convit, J., Aranzazu, N., Pinardi, M., and Ulrich, M., 1979, Immunological changes observed in indeterminate and lepromatous leprosy patients and Mitsuda-negative

contacts after the inoculation of a mixture of *Mycobacterium leprae* and BCG, *Clin. Exp. Immunol.* **36:**214–220.

38. Stoner, G. L., Mshana, R. N., Touw, J., and Belehu, A., 1982, Studies on the defect in cell-mediated immunity in lepromatous leprosy using HLA-D-identical siblings. Absence of circulating suppressor cells and evidence that the defect is the T-lymphocyte, rather than the monocyte, population, *Scand. J. Immunol.* **15:**33–48.
39. Watson, S. R., and Collins, F. M., 1979, Development of suppressor T-cells in *Mycobacterium habana*-infected mice, *Infect. Immun.* **25:**497–506.
40. Bullock, W. E., Carlson, E. M., and Gershon, R. K., 1978, The evolution of immunosuppressive cell populations in experimental mycobacterial infection, *J. Immunol.* **120:**1709–1716.
41. Hoffenbach, A., Lagrange, P. H., and Bach, M. A., 1983, Deficit of interleukin 2 production associated with impaired T-cell proliferative responses in *Mycobacterium lepraemurium* infection, *Infect. Immun.* **39:**109–116.
42. Lefford, M. J., Patel, P. J., Poulter, L. W., and Mackaness, G. B., 1977, Induction of cell-mediated immunity to *Mycobacterium lepraemurium* in susceptible mice, *Infect. Immun.* **18:**654–659.
43. Lagrange, P. M., and Hurtrel, B., 1979, Local immune response to *Mycobacterium lepraemurium* in C3H and C57BL/6 mice, *Clin. Exp. Immunol.* **38:**461–474.
44. Ha, D. K. K., Gardner, I. D., and Lawton, J. W., 1983, Macrophage function in *Mycobacterium lepraemurium* infection: morphological and functional changes of peritoneal and splenic macrophages *in vitro*, *Infect. Immun.* **39:**353–361.
45. Løvik, M., Collins, F. M., and Closs, O., 1982, Inbred C3H mouse substrains differences demonstrated in experimental murine leprosy, *Immunogenetics* **16:**607–611.
46. Curtis, J., Adu, H. O., and Turk, J. L., 1982, H-2 linkage control of resistance to subcutaneous infection with *Mycobacterium lepraemurium*, *Infect. Immun.* **38:**434–439.
47. Skamene, E., Gros, P., Forget, A., Patel, P. J., and Nesbitt, M. N., 1984, Regulation of resistance to leprosy by chromosome 1 locus in the mouse, *Immunogenetics* **19:**117–124.
48. Nakamura, R. M., Tanaka, H., and Tokunaga, T., 1982, *In vitro* induction of suppressor T-cells in delayed-type hypersensitivity to BCG and an essential role of I-J positive accessory cells, *Immunol. Lett.* **4:**295–299.
49. Nakamura, R. M., and Tokunaga, T., 1984, I-J positive macrophages in induction of suppressor T cells against delayed-type hypersensitivity to *Mycobacterium bovis* BCG, in: *Microbiology 1984*, pp. 363–365, American Society of Microbiology, Washington, D. C.
50. Nakamura, R. M., Nakamura, Y., Nagayama, A., and Tokunaga, T., 1986, I-J positive cloned macrophages as the accessory cells for the induction of suppressor T cells *in vitro*, *Immunol. Res.* **5:**106–116.
51. Lowy, A., Tominaga, A., Drebin, J. A., Takaoki, M., Benacerraf, B., and Greene, M. I., 1983, Identification of an I-J$^+$ antigen-presenting cell required for third order suppressor cell activation, *J. Exp. Med.* **157:**353–358.
52. Noma, T., Usui, M., and Dorf, M. E., 1985, Characterization of the accessory cells involved in suppressor T cell induction, *J. Immunol.* **134:**1374–1380.
53. Nakamura, Y., Nakamura, R. M., and Tokunaga, T., 1986, The cell–cell interaction responsible for the induction of the first order suppressor T cells in hapten-specific contact sensitivity reaction, *Immunol. Res.* **5:**117–128.
54. Steinmetz, M., Minard, K., Horvath, S., McNicholas, J., Sretinger, J., Wake, C., Long, E., Mach, B., and Hood, L., 1983. A molecular map of the immune response region from the major histocompatibility complex of the mouse, *Nature (Lond.)* **300:**35–42.
55. Kronenberg, M., Steinmetz, M., Kobori, J., Kraig, E., Kapp, J., Pierce, C. W., Sorensen, C. M., Suzuki, G., Tada, T., and Hood, L., 1983, RNA transcripts for I-J polypeptides

are apparently not encoded between the I-A and I-E subregions of the murine major histocompatibility complex, *Proc. Natl. Acad. Sci. USA* **80:**5704–5708.
56. Granstein, R. D., Lowy, A., and Greene, M. I., 1984, Epidermal antigen-presenting cells in activation of suppression: identification of a new functional type of ultraviolet radiation-resistant epidermal cells, *J. Immunol.* **132:**563–565.
57. Granstein, R. D., Tominaga, A., Mizel, S. B., and Greene, M. I., 1985, Defective antigen-presenting cell function and interleukins, in: *Limphokines,* Vol. 12 (E. Pick, ed.), pp. 57–74, Academic, New York.
58. Colizzi, V., Ferluga, J., Garreau, F., Malkovsky, M., and Asherson, G. L., 1984, Suppressor cells induced by BCG release nonspecific factors *in vitro* which inhibit DNA synthesis and interleukin-2 production, *Immunology* **51:**65–71.
59. Haregewoin, A., Godal, T., Mustafa, A. S., Belehu, A., and Yamaneberhan, T., 1983, T-cell conditioned media reverse T-cell unresponsiveness in lepromatous leprosy, *Nature (Lond.)* **303:** 342–344.
60. Haregewoin, A., Mustaga, A. S., Helle, I., Waters, M. F. R., Leiker, D. L., and Godal, T., 1984, Reversal by interleukin-2 of the T cell unresponsiveness of lepromatous leprosy to *Mycobacterium leprae, Immunol. Rev.* **80:**77–86.
61. Mohagheghpour, N., Gelber, R. H., Larrick, J. W., Sasaki, D. T., Brennan, P. J., and Engleman, E. G., 1985, Defective cell-mediated immunity in leprosy: failure of T cells from lepromatous leprosy patients to respond to *Mycobacterium leprae* is associated with defective expression of interleukin 2 receptors and is not reconstituted by interleukin 2, *J. Immunol.* **135:**1443–1449.
62. Modulin, R. L., Hofman, F. M., Horwitz, D. A., Hasmann, L. A., Gillis, S., Taylor, C. R., and Rea, T. H., 1984, *In situ* identification of cells in human leprosy granulomas with monoclonal antibodies to interleukin 2 and its receptor, *J. Immunol.* **132:**3085–3090.
63. Nogueira, N., Kaplan, G., Levy, E., Sarno, E. N., Kushner, P., Granelli-Piperno, A., Vierra, L., Gould, V. C., Levis, W., Steinman, R., Yip, Y. K., and Cohn, Z. A., 1983, Defective γ-interferon production in leprosy. Reversal with antigen and interleukin 2, *J. Exp. Med.* **158:**2165–2170.
64. Sathish, M., Bhutani, L. K., Sharma, A. K., and Nath, I., 1983, Monocyte-derived soluble suppressor factor(s) in patients with lepromatous leprosy, *Infect. Immun.* **42:**890–899.
65. Honda, M., Chan, C., and Shevach, E., 1985, Characterization and partial purification of a specific interleukin 2 inhibitor, *J. Immunol.* **135:**1834–1839.
66. Hardt, C., Röllinghoff, M., Pfizenmaier, K., Mosmann, H., and Wagner, H., 1981, Lyt-23$^+$ cyclophosphamide-sensitive T cells regulate the activity of interleukin 2 inhibitor *in vitro, J. Exp. Med.* **154:**262–274.
67. Fontana, A., Hengartner, H., Tribolet, N., and Weber, E., 1984, Glioblastoma cells release interleukin 1 and factors inhibiting interleukin 2-mediated effects, *J. Immunol.* **132:**1837–1844.
68. Kirchheimer, W. F., and Storrs, E. E., 1971, Attempts to establish the armadillo (*Dasypus nevemcinctus* Lnn.) as a model for the study of leprosy, *Int. J. Lepr.* **39:**693–702.
69. Gillis, T. P., and Buchanan, T. M., 1982, Production and partial characterization of monoclonal antibodies to *Mycobacterium leprae, Infect. Immun.* **37:**172–178.
70. Ivanyi, J., Sinha, S., Aston, R., Cussell, D., Keen, M., and Sengupta, U., 1983, Definition of species specific and cross-reactive antigenic determinants of *Mycobacterium leprae* using monoclonal antibodies, *Clin. Exp. Immunol.* **52:**528–536.
71. Hunter, S. W., Fujiwara, T., and Brennan, P. J., 1982, Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae, J. Biol. Chem.* **257:**15072–15078.

72. Mehra, V., Brennan, P. J., Rada, E., Convit, J., and Bloom, B. R., 1984, Lymphocyte suppression in leprosy induced by unique *M. leprae* glycolipid, *Nature (Lond.)* **308:**194–196.
73. Cho, S-N., Hunter, S. W., Gelber, R. H., Rea, T. H., and Brenna, P. J., 1986, Quantitation of the phenolic glycolipid of *Mycobacterium leprae* and relevance to glycolipid antigenemia in leprosy, *J. Infect. Dis.* **153:**560–569.
74. Young, R. A., Bloom, B. R., Grosskinsky, C. M., Ivanyi, J., Thomas, D., and Davis, R. W., 1985, Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA, Proc. Natl. Acad. Sci. USA **82:**2583–2587.
75. Minden, P., Kelleher, P. J., Freed, J. H., Nielsen, L. D., Brenna, P. J., McPheron, L., and McClatchy, J. K., 1984, Immunological evaluation of a component isolated from *Mycobacterium bovis* BCG with a monoclonal antibody to *M. bovis* BCG, *Infect. Immun.* **46:**519–525.
76. Wadee, A. A., Mendelsohn, D., and Rabson, A. R., 1983, Characterization of a suppressor cell-activating factor (SCAF) released by adherent cells treated with *M. tuberculosis*, *J. Immunol.* **130:**2266–2270.
77. Colizzi, V., Giuntini, M., Garzelli, C., Campa, M., and Falcone, G., 1983, Auto-anti-idiotypic antibodies inhibit T-cell-mediated hypersensitivity in BCG-infected mice, *Cell. Immunol.* **80:**205–210.
78. Monroe, J. G., Gurish, M., Dambrauskas, J., Slaoui, M., Lowy, A., and Greene, M. I., 1985, Genetic and biological characterization of a T suppressor cell induced by anti-idiotypic antibody, *J. Immunol.* **135:**1589–1597.
79. Dietz, M. H., Sy, M-S., Benacerraf, B., Nisonoff, A., Greene, M. I., and Germain, R. N., 1981, Antigen- and receptor-driven regulatory mechanisms. VII. H-2-restricted anti-idiotypic suppressor factor from efferent suppressor T cells, *J. Exp. Med.* **153:**450–463.

11

Immunoregulatory Function of Mononuclear Phagocytes in Tuberculosis

JERROLD J. ELLNER

1. INTRODUCTION

A complete understanding of the interaction of *Mycobacterium tuberculosis* with the host would explain several distinctive epidemiologic and clinical features of human tuberculosis. Following primary infection with *M. tuberculosis,* the usual course is resolution, without specific drug treatment; only 5–15% of infected individuals develop overt tuberculosis. Factors predisposing to progression of the primary infection or delayed reactivation of a latent focus are not well understood. It is clear, however, that major deficits in cell-mediated immunity are associated with increased risk of progressive tuberculosis.

A possible contribution of genetically determined factors to susceptibility to tuberculosis is suggested by reports of associations between HLA type and the occurrence of tuberculosis[1]; both class I and class II major histocompatibility complex (MHC) determinants have been associated with disease. The strongest evidence for genetic factors, however, derives from a study in India that shows excess haplotype sharing among sibs with disease. The existing genetic studies cannot be viewed as definitive and certainly do not show consistent linkage of a specific HLA haplotype to disease. If genetic associations do exist, it would seem rea-

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sonable to speculate that the link reflects an hereditable determinism of the level or regulation of the immune response to mycobacteria. In fact, patients with malignant melanoma and the phenotype HLA-B7 fail to develop delayed-type hypersensitivity (DTH) to tuberculin protein-purified derivative (PPD) following immunotherapy with bacillus Calmette–Guérin (BCG). Moreover, certain inbred strains of mice fail to develop DTH responses following immunization with BCG, apparently because of activation of suppressor cells.

Early in the course of active tuberculosis, 20–25% of patients show depressed DTH skin-test responses to intradermal tuberculin PPD.[2] Anergy is more frequent in miliary than in pulmonary tuberculosis and may imply a poor prognosis. Anergy is also a transient phenomenon. Most of these patients develop skin-test reactivity weeks to months into the course of treatment.[3] Anergy therefore does not represent a genetically determined inability of the host to respond to mycobacterial antigens. By contrast, an initial inappropriate hyporesponsiveness could favor disease progression. This assumes that DTH and protective immunity are related, which seems reasonable despite the controversies ensuing from experimental systems in which they are dissociable.

Within this context, microbial constituents that interfere with the cellular immune response can be considered factors in microbial pathogenicity. Host factors, whether genetically determined or acquired, that lead to patterns of immunoregulation favoring hyporesponsiveness to mycobacterial antigens despite active infection can also be viewed as contributing to host susceptibility.

I would like to review the current understanding of the immunoregulatory function of mononuclear phagocytes in tuberculosis and how this cell may modulate host–parasite interactions to affect the balance between infection and disease.

2. IMMUNE INDUCTION AND EFFECTOR FUNCTIONS OF MONONUCLEAR PHAGOCYTES

The mononuclear phagocyte is unique, certainly among phagocytes, in its dual capacity to serve both as a regulatory cell and as a key effector cell in the cell-mediated immune (CMI) response. Immune induction requires that accessory cells bind, ingest, alter, and present microbes and their derivative antigens to responder lymphocytes. Lymphocytes that react with the monoclonal antibody OKT4 include helper cells and show class II MHC restriction in their activation by antigens. That is, they respond to the nominal antigen to which they are sensitized only when it is presented by an accessory cell within the context of self-class II MHC determinants. The importance of this genetic restriction of cel-

lular interactions remains a mystery, although it is usually considered essential in distinguishing self from foreign antigens. A further role for class II MHC products has been suggested in the direct binding of certain antigens; this, in turn, may explain the relative differences among inbred strains of mice in levels of immune responsiveness to these antigens. Interleukin-1 (IL-1) produced by the accessory cell has an amplifying effect on immune induction, enhancing production of the cytokine interleukin-2 (IL-2) by T lymphocytes and stimulating surface expression of the receptor for IL-2. IL-2 is pivotal in immune induction, as it leads to clonal expansion of activated T cells. Products of these activated T cells include macrophage activation factor (MAF). γ-Interferon (IFN_γ) exhibits important MAF activity, although recently it has been shown that IFN_γ paradoxically enhances intracellular growth of *M. tuberculosis*.[4] MAF activate macrophages to a state in which they can destroy facultative intracellular parasites. Thus, mononuclear phagocytes figure prominently in both the afferent and efferent limbs of the CMI response.

3. IMMUNOSUPPRESSION BY MONONUCLEAR PHAGOCYTES

Much less is known concerning the suppressor function of macrophages. As the ratio of macrophages to lymphocytes is increased in *in vitro* culture systems, suppression of the immune response begins to predominate. The *in vivo* significance of this form of immunoregulation is uncertain; it may be relevant in certain tissues and fluids, such as bronchoalveolar lavage, in which the ratio of macrophages to lymphocytes is 9 : 1, and in diseases characterized by mononuclear cell infiltration of tissue. Activation of macrophages *in vivo,* for example, by treatment with *Corynebacterium parvum,* or induction of tumors or graft-versus-host disease, or *in vitro,* by exposure to *Escherichia coli* lipopolysaccharide, is associated with an increase in their suppressor function.

Suppression by adherent blood mononuclear cell populations, which are mostly monocytes, is a feature of a number of human diseases. Nonspecific suppression has been reported in patients with solid tumors, Hodgkin disease, fungal infections, and systemic lupus erythematosus.[5] Suppression by monocytes is restricted to antigen derived from the causative agent in schistosomiasis, leprosy, filariasis, and tuberculosis.[5] In several situations, dual suppressor mechanisms are operant. For example, in leprosy, suppressor T cells are found in some patients; however, suppression by adherent mononuclear cells predominates in individuals with lepromatous leprosy, having the most extensive infection.

Several types of mediators have been implicated in immunosuppression by adherent mononuclear cells. Prostaglandin E_2 (PGE_2) seems

to be relevant in immunosuppression at high monocyte to T-cell ratios, when monocytes are stimulated by bacterial lipopolysaccharide (LPS),[6] and in Hodgkin disease, and, in some studies, in sarcoidosis. PGE_2 suppresses T-cell responses through interaction with Fc_γ receptor-bearing (Fc_GR^+) cells,[7] which are labile in cell culture. Alternative mediators must be relevant in other disease states, in which suppression by adherent mononuclear cells is not reversed by indomethacin.

4. IMMUNOSUPPRESSIVE MYCOBACTERIAL PRODUCTS

Mycobacterial polysaccharides elicit a humoral but not a CMI response in *M. tuberculosis* infected humans and animals. This finding led to the question of whether mycobacterial polysaccharides, much as bacterial LPS, might be a factor in the inverse relationship between antibody levels and DTH characteristic of many chronic infections, and moreover, might activate monocyte and prostaglandin-dependent immunosuppression.

Initially, we studied the immunosuppressive effects of D-Arabino-D-mannan (AM).[8] D-Arabino-D-galactan (AG) seems to be more potent as an immunosuppressant, however, and contamination of AM with AG might explain our initial observations.[9] AG caused dose-dependent inhibition of antigen- and mitogen-induced blastogenesis in human peripheral blood mononuclear cells (PMNC), with significant suppression first detectable at 30 ng/ml. Inhibition was not due to cytotoxicity; in fact, rather than a direct effect, suppression required the presence of monocytes, was associated with a 5.6-fold increase in production of PGE_2, and was blocked by indomethacin (Fig. 1). These observations closely resembled the effects of LPS but were not due to contamination with LPS, as assessed by assay with lysates of limulus amebocytes.

An immediate question is the relevance of the nonspecific suppressive effects of AG, *in vitro,* to the immune response in tuberculosis. A plasma pool from 28 patients with newly diagnosed tuberculosis (TB plasma) produced parallel functional effects *in vitro* (Fig. 1). Moreover, a goat antibody to mycobacterial polysaccharides blocked the suppressive effects of the plasma pool. The plasma pool contained antibody to AG at a dilution of 1 : 640 by enzyme-linked immunosorbent assay (ELISA). We conclude that nonspecific suppressive effects of tuberculosis plasma are due to immune complexes, in antibody excess and containing mycobacterial polysaccharides. The active fragment of the immune complexes is an exposed polysaccharide residue that induces monocyte production of immunosuppressive PGE_2.

It is unclear whether immunosuppression by mycobacterial products is important in local modulation of the immune response and mi-

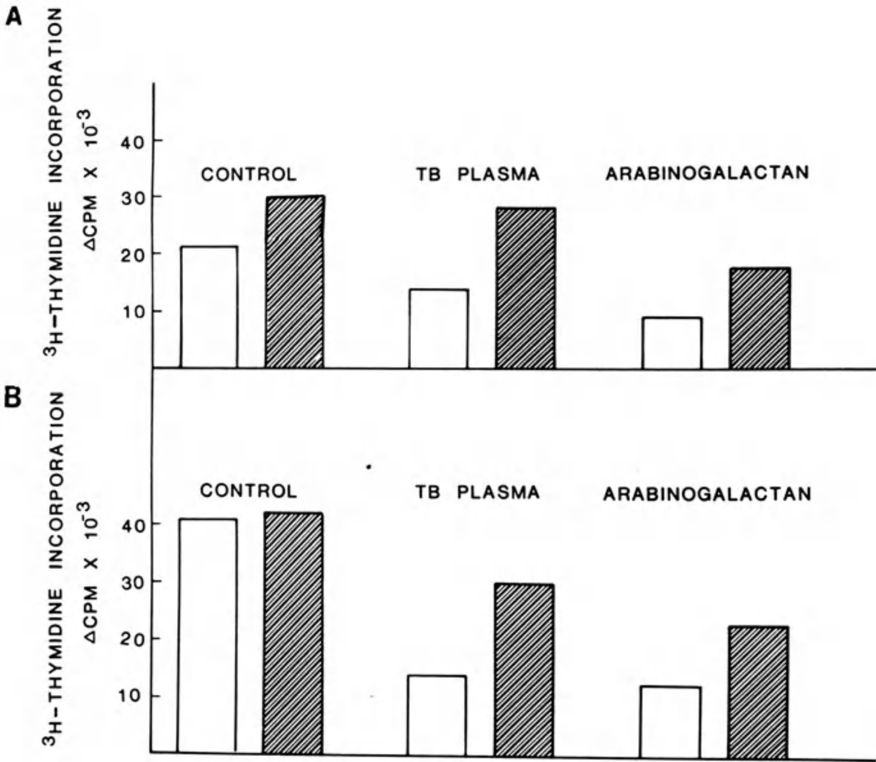

FIGURE 1. Effect of indomethacin on TB plasma and AG suppression of PMNC responses to PPD (A) and to PHA (B). Data are presented as the mean response in Δcpm for PMNC cultured without (open bars) or with (hatched bars) 1.0 μg/ml indomethacin. Data are shown for control cell suspensions and for cells cultured with TB plasma or AG. (From Kleinhenz *et al.*,[9] with permission.)

crobial pathogenesis. Monocyte and prostaglandin-dependent immunosuppression may have a role in the generalized anergy seen in some patients with tuberculosis. It differs mechanistically, however, in several important ways, from the antigen-specific hyporesponsiveness found in patients with tuberculosis.

5. IMMUNOSUPPRESSION BY ADHERENT MONONUCLEAR CELLS IN TUBERCULOSIS

5.1. Regulation of Blastogenesis

Depression of DTH skin tests is selective in some patients with tuberculosis; tuberculin reactivity is impaired, whereas the response to

other microbial antigens is preserved. We found a correlation in patients with pulmonary tuberculosis between negative tuberculin skin tests and depressed *in vitro* responses to PPD.[10] Moreover, tuberculin low responders had a greater proportion of PMNC identifiable as monocytes by cytochemical criteria. Depletion of adherent mononuclear cells from PMNC resulted in a 24.4-fold increment in PPD-induced blastogenesis in nine low responder patients as compared with a 2.8-fold increase in healthy tuberculin reactors and in 11 tuberculous (TB) patients with a normal response to PPD—at least mean −2SD of PPD-induced blastogenesis in healthy tuberculin skin-test reactors (Fig. 2). Reconstitution experiments confirmed that adherent cells from patients with low responses to PPD were activated as antigen-specific suppressor cells (Fig. 3). Thus, individuals with depressed PPD-induced blastogenesis were at double jeopardy. They had more, as well as more active, suppressor adherent cells. In these studies, the blastogenic response to nonmycobacterial antigens also was decreased in comparison to healthy tuberculin reactors. Adherent cell depletion did not, however, affect the response to streptokinase-streptodornase, tetanus toxoid, or *Candida* antigen.

In an unselected series of patients, antigen-specific suppression by adherent cells was found in one third of subjects with pulmonary tuber-

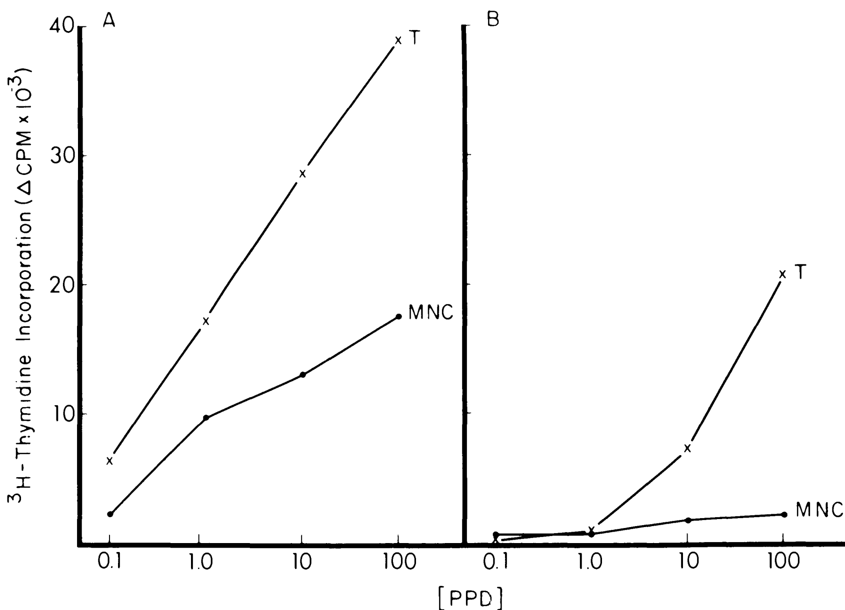

FIGURE 2. PPD-induced ^{3}H-thymidine incorporation of PMNC and T cells for representative high (A) and low (B) responder patients. (From Ellner,[10] with permission.)

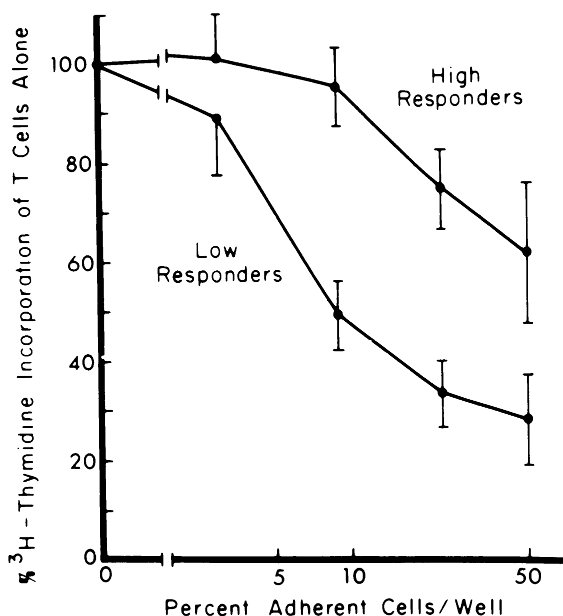

FIGURE 3. Inhibition of PPD-induced T-cell ^{3}H-thymidine incorporation by adherent cells. Data are presented as the mean ±SEM for six high responders and five low responders. Significant differences between low and high responders were noted at 9, 23, and 47% adherent cells ($p<0.01$, Student's *t*-test).

culosis studied within 2 weeks of diagnosis. These individuals were not distinctive as regards the extent of tuberculosis, frequency of underlying disease, or other clinical or laboratory findings. In a study of patients from Japan, adherent cell suppression of blastogenesis was associated with tuberculous pleurisy, a clinical finding that was rare in our patients.

5.2. Regulation of IL-2 Production

Production of the cytokine IL-2 and expression of the receptor for IL-2 are integral to T-cell activation. We studied IL-2 metabolism in tuberculosis.[11] PPD-induced IL-2 production and blastogenesis in PMNC were decreased in patients, whereas streptolysin O-induced responses could not be differented from healthy donors (Table I). Purified exogenous IL-2 was directly mitogenic for PMNC but failed to correct the depressed blastogenic response to PPD of tuberculosis patients. In fact, PMNC from tuberculosis patients showed an antigen-specific defect in expression of the IL-2 receptor identified by reactivity with the mouse monoclonal antibody anti-Tac. After 5 days of culture with PPD, 32 ± 8%

of cells from healthy donors were anti-Tac reactive, whereas 5 ± 3% of cells from tuberculosis patients expressed the IL-2 receptor. Thus, tuberculosis is associated with abnormalities in both production and response to IL-2. These are not necessarily unrelated events, however, since IL-2 can induce expression of its own receptor. Defects in IL-2 metabolism therefore appear to provide the basis for abnormalities in PPD-induced blastogenesis.

Next, we examined regulation of IL-2 production by adherent cells in tuberculosis. By comparison with healthy tuberculin reactors, tuberculosis patients could be divided into those in whom adherent cell depletion increased IL-2 production to levels comparable to those of healthy tuberculin reactors, and low producers in whom it did not (Fig. 4). In the normal IL-2 producers, adherent cell depletion resulted in a 7.2-fold increase in PPD-induced IL-2 production; in healthy tuberculin reactors, the increase was 3.0-fold. Two differences in regulation of IL-2 production by adherent cells emerged between this subset of tuberculosis patients and healthy donors. In patients, the addition of 2% adherent cells to the nonadherent (NA) population produced a 50% drop in IL-2 activity; in healthy subjects, 2% adherent cells did not affect IL-2 production significantly. Indomethacin reversed most of suppression by adherent cells in healthy subjects; its effect in tuberculosis patients was insignificant. Therefore, in some patients with tuberculosis, adherent mononuclear cells suppressed PPD-induced IL-2 production as well as blastogenesis. This regulatory mechanism differed from that observed in health by virtue of its greater level of activity and lack of reversibility by indomethacin. The role of adherent cells in modulating the response to IL-2 and IL-2 receptor expression will also need further study.

In these studies, a clinical difference emerged in the subsets of tuberculosis patients grouped by the level of PPD-induced production of

TABLE I
Antigen-Induced IL-2 Production and Blastogenesis in PMNC from Tuberculosis Patients and Healthy Donors

	IL-2 activity (cpm ± SEM)[a]		Blastogenesis (cpm ± SEM; [^{3}H]-TdR)	
Antigen	TB patients (N = 22)	Healthy donors (N = 14)	TB patients (N = 22)	Healthy donors (N = 14)
PPD	3,571 ± 790	15,947 ± 4,406	6655 ± 1,410	18,187 ± 2,132
Streptolysin O	53,493 ± 16,636	55,781 ± 17,409	36,561 ± 15,813	37,592 ± 15,344

[a] [^{3}H]Thymidine incorporation of CTLL-20 after incubation with a 1 : 2 dilution of supernatants of antigen-stimulated PMNC.

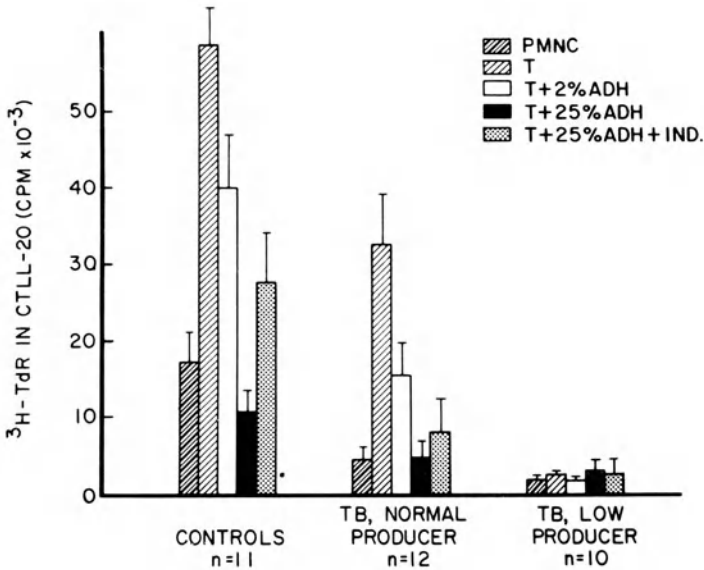

FIGURE 4. PPD-induced IL-2 activity in cultures of PMNC, T cells (T), T cells + 2% adherent cells (ADH), T cells + 25% ADH, and T cells + 25% ADH, in the presence of 1 μg/ml indomethacin. Each bar represents mean ±SEM. (From Toossi *et al.*,[11] with permission.)

IL-2 by NA lymphocytes. Patients with low IL-2 production by NA cells more often had far-advanced tuberculosis than did normal producers of IL-2. Alternate bases for depressed IL-2 production, such as suppression by NA lymphocyte subpopulations, need to be evaluated in this group of patients.

6. ADHERENT MONONUCLEAR CELL SUBPOPULATIONS RESPONSIBLE FOR SUPPRESSION

Further definition of the cellular mechanisms associated with and ultimately responsible for suppression by adherent cells required the development of systems in which suppression was both predictable and reproducible. We found that depletion of $Fc_G R^+$ cells from NA mononuclear cells resulted in a population uniformly sensitive to suppression by adherent mononuclear cells in all TB patients.[12] Suppression of $Fc_G R^-$ NA cells by adherent mononuclear cells retained its antigen specificity; it also differed diametrically from studies in healthy individuals in whom $Fc_G R^+$ depletion actually interfered with susceptibility to suppression by adherent cells.

Adherent mononuclear cell preparations from patients with pulmonary tuberculosis were 89 ± 4% peroxidase-staining monocytes, 7 ± 3% sheep erythocyte- (E-)rosetting T cells, and 79 ± 10% reactive with OKM1, a monoclonal antibody reactive with most circulating monocytes and a small fraction of lymphocytes. The addition of adherent mononuclear cells to autologous Fc_GR^- NA cells at a ratio of 1 : 2, inhibited PPD-induced blastogenesis 42 ± 10% (Fig. 5). The suppressive activity of adherent cells was unaffected by depletion of E-rosetting cells or the presence of indomethacin in culture but was blocked by depletion of OKM1-reactive cells (complement lysis) or γ-irradiation (1500 rad) (Fig. 6). The characterization of the functionally active population in adherent cells is not yet definitive. Although the data point toward suppression by monocytes, we cannot exclude an ancillary or even a major role for OKM1 reactive lymphocytes that would include large granular lymphocytes and natural killer (NK) cells. In other systems, a minor fraction of NK cells within adherent cell preparations proved responsible for much of the effector function of adherent mononuclear cells. The radiosensitivity of suppressor function raises further issue as to

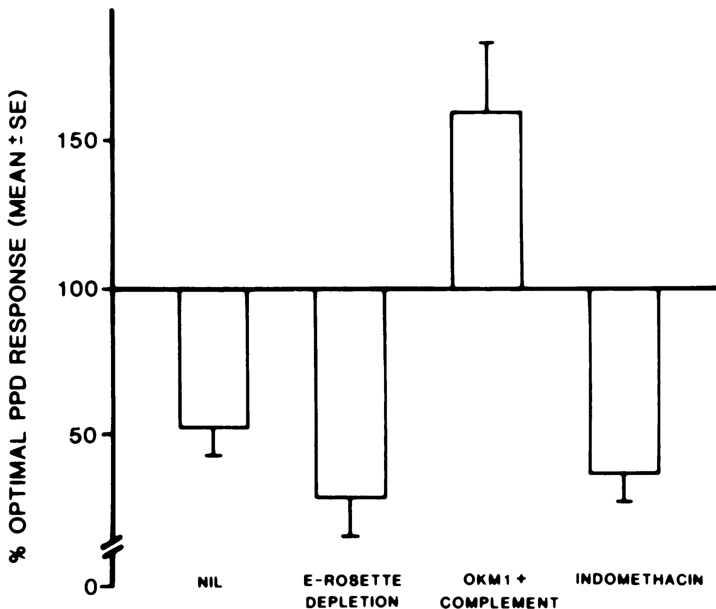

FIGURE 5. Effect of purification of adherent cell populations and indomethacin on T non-γ cell responses. In six experiments, adherent mononuclear cells (AMC) or modified AMC were added to T non-γ cells at a 1 : 2 ratio and incubated in the presence of PPD. Results are expressed as percent optimal response; bars = SE. AMC : T non-γ cell ratio, 1 : 20. (From Kleinhenz and Ellner,[12] with permission.)

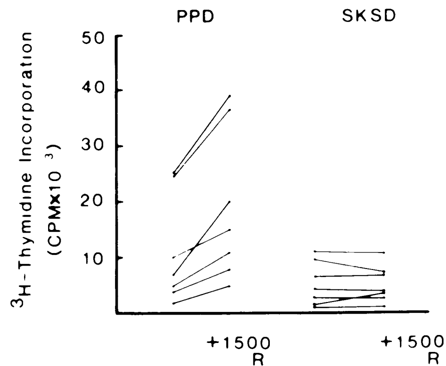

FIGURE 6. Effect of γ-irradiation on adherent cell suppression. Lines connect T non-γ cell responses observed at an AMC:T non-γ cell ratio of 1:2 with those responses observed when AMC were irradiated with 1500 rads (R) before addition to non-γ T cells at the same 1:2 ratio. (From Kleinhenz and Ellner,[12] with permission.)

whether the suppressor cell is a monocyte that is traditionally considered radioresistant in its functions. In fact, γ-irradiation interferes with monocyte phagocytic function and chemotactic function. Therefore, neither the phenotypic studies nor the radiosensitivity of suppressor cells permits unequivocal assignment of the responsible adherent mononuclear cell subpopulation(s). Yet it seems reasonable to ascribe a role to the major constituent of OKM1-reactive, E-rosetting negative, peroxidase-positive adherent cells, i.e., the monocytes.

An issue closely related to the identity of the adherent cell is the basis for the antigen specificity of suppression in tuberculosis. Mononuclear phagocytes lack a specific antigen receptor, although it is possible that cytophilic antibody that binds to Fc_G receptors and that coats the cells functions as a surrogate antigen receptor. Antigen specificity of suppression probably resides in a tuberculin-specific T cell that interacts with the adherent cell in suppressor cell circuits. It is not yet clear whether all the machinery for antigen-specific suppression is contained in the adherent cell population, as in leprosy, or whether suppression is dependent on interactions with nonadherent cells. The only data to this point are negative; i.e., neither Leu $2a^+$ reactive nor Fc_GR^+ NA cells are required for antigen-specific suppression by adherent mononuclear cells in tuberculosis. It remains possible that adherent cells acquire antigen specificity through obligatory interactions with Leu $2a^-$ Fc_GR^- NA lymphocytes; alternatively, PPD-induced responses may show heightened susceptibility to suppression by adherent cells in tuberculosis.

Studies to date support the existence of dual regulatory mechanisms. We studied 14 patients with pulmonary tuberculosis selected by virtue of hyporesponsiveness to PPD *in vitro*.[13] In five of these subjects, depletion of adherent cells normalized PPD-induced blastogenesis; Fc_GR^+ cell depletion had no additional effect on their response to PPD. In five other tuberculosis patients, Fc_GR^+ depletion selectively increased PPD-induced blastogenesis, although not to normal levels. As

noted, Fc_GR^+ cells depletion produced a responder population that was uniformly sensitive to suppression by adherent cells in all patients. Therefore, suppression by adherent mononuclear cells or Fc_GR^+ NA lymphocytes was observed in most anergic patients. Dual regulatory mechanisms have also been identified in other diseases, such as leprosy; in leprosy, the extent of infection is quantifiable and suppression by adherent cells predominates in those patients with most extensive disease. The observations in tuberculosis further indicate an intrinsic abnormality of adherent mononuclear cells in all tuberculosis patients; moreover, an antagonism is suggested since, in most patients, suppression by Fc_GR^+ NA lymphocytes masks suppression by adherent mononuclear cells.

7. CELLULAR PROPERTIES ASSOCIATED WITH IMMUNOSUPPRESSION

Monocyte-enriched adherent PMNC from patients with tuberculosis show activation as regards effector as well as immunoregulatory function. Circulating monocyte-derived macrophages demonstrate increased bactericidal function, hexose monophosphate shunt activity, and receptors for the Fc portion of IgG. However, we found augmentation of only selected monocyte functions in tuberculosis—adherence to plastic and killing of schistosomula of *S. mansoni.*[14] By contrast, basal (Fig. 7) and LPS-stimulated production of PGE_2 and tumoricidal activity were comparable to that of monocytes from healthy tuberculin reactors. Besides selective activation of effector functions, monocytes from some tuberculosis patients are immature; they show increased DNA labeling and increased cytoplasmic peroxidase activity.

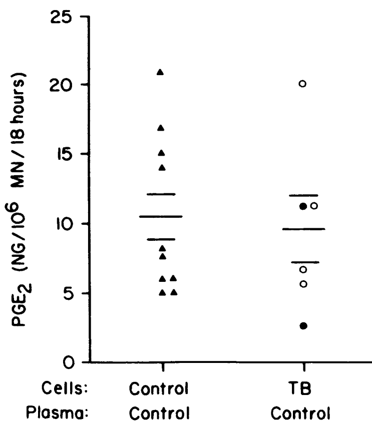

FIGURE 7. Basal production of prostaglandin E_2 (PGE_2) by monocytes (MN) from healthy control subjects and TB patients. The patient group consisted of (○) four patients with low levels of tuberculin PPD-induced incorporation of [^{3}H]thymidine (Δcpm, <2000) and (●) two patients with a Δcpm of >2000. Horizontal lines indicate the mean ±SEM for each group. (From Ellner *et al.*,[14] with permission.)

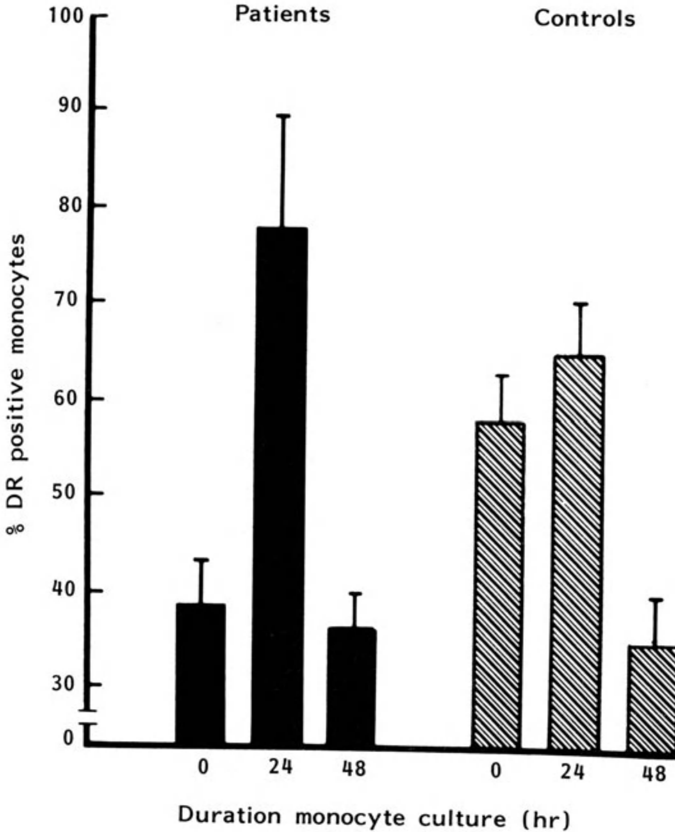

FIGURE 8. DR expression on monocytes (MN) from TB patients and healthy subjects. DR^+ fractions were determined at the time of MN isolation and after 24 and 48 hr of *in vitro* culture. Data are expressed as mean ±SEM values. MN from 10 TB patients and 12 healthy subjects were studied at time 0, from seven TB patients and nine healthy subjects at 24 hr, and from four TB patients and eight healthy subjects after 48 hr of incubation. The fraction of freshly isolated DR^+ MN in TB patients was significantly depressed compared with that in healthy subjects ($p<0.02$) and increased significantly compared with freshly isolated MN during 24 hr of culture ($p<0.005$). (From Tweardy *et al.*,[15] with permission.)

Expression of class II MHC determinants on the surface of mononuclear phagocytes is critical to their immunoregulatory function. We studied HLA-DR-determinant expression on monocytes using OKIa1, a mouse monoclonal antibody to the HLA-DR framework with a ^{51}Cr-release microcytotoxicity assay.[15] Monocytes from tuberculosis patients showed a smaller fraction identifiable as expressing surface HLA-DR by this technique (Fig. 8). During *in vitro* cultivation for 24 hr, a sharp increase was observed in HLA-DR expression by monocytes from tuber-

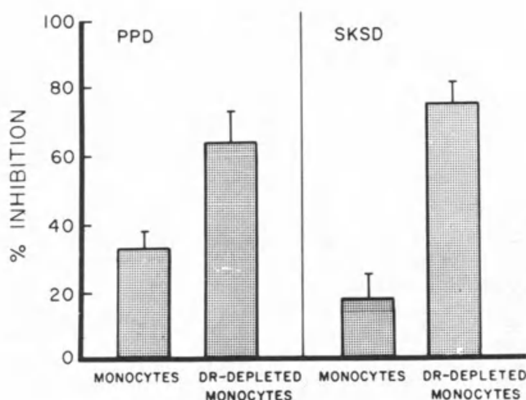

FIGURE 9. Suppression by MN from TB patients of antigen-induced [^{3}H]thymidine incorporation by T lymphocytes. Inhibition by MN of antigen-induced [^{3}H]thymidine incorporation by T lymphocytes at a MN : T-cell ratio of 1 : 2 was determined by comparison to maximal T lymphocyte responses, which occurred at an MN : T-cell ratio of 1 : 100–1 : 20. Data are expressed as mean ±SEM values for determinations in four TB patients. Increased suppression by DR-depleted MN was significant for PPD and SKSD ($p<0.05$ by paired t-statistic). (From Tweardy *et al.*,[15] with permission.)

culosis patients. The increase in HLA-DR expression required protein synthesis and was not blocked by factors present in autologous plasma. To examine the functional significance of the altered expression of class II MHC products on monocytes, the tuberculosis patients were subdivided on the basis of PPD-induced blastogenesis. It was the group of low responders that showed the greatest abnormality in HLA-DR expression; in these cases, 34.6 ± 6.0% of fresh monocytes were reactive with OKIa1. Moreover, the fraction of PMNC identified as monocytes by cytochemical techniques was also higher in the low responders (51.2 ± 3.6% as compared with 38.0 ± 5.0% in the patients with normal PPD-induced blastogenesis). These studies establish an association of low-surface expression of HLA-DR on adherent mononuclear cells with immunosuppression in tuberculosis. HLA-DR-depleted populations of adherent cells from tuberculosis patients, in fact, are more suppressive of antigen-induced blastogenesis than the starting population (Fig. 9). Yet, it is unclear whether a low level of HLA-DR expression is simply a marker for a cell, perhaps indicative of its immaturity, which is suppressive by mechanisms unrelated to class II MHC determinants. Alternatively, adherent cells with less surface HLA-DR could preferentially activate suppressor mechanisms. There is precedence for this in experimental models.

8. MEDIATORS OF IMMUNOSUPPRESSION IN TUBERCULOSIS

We consistently have not observed reversibility of suppression by adherent mononuclear cells in tuberculosis by indomethacin. Nor do we

find evidence for increased production of PGE_2 by monocytes in tuberculosis. A limitation in cell number and suppressive potency of cellular supernatants has prevented direct elucidation of the biochemical nature of functionally active mediators of suppression; in fact, the lack of suppression by supernatants of adherent cells raises the possibility that suppressive molecules may be cell-associated, as in the recent studies of Stallcup *et al.*[16]

We have increasing circumstantial evidence, however, that soluble IL-1 has a role in immunosuppression in tuberculosis. Thus, release of IL-1 into supernatants by adherent mononuclear cells stimulated with LPS or PPD is increased in tuberculosis patients[17] (Table II). Cell lysis with study of cell-associated pools of IL-1 shows that production (rather than simply release) of IL-1 is increased in tuberculosis. Suppression of antigen-induced blastogenesis by adherent cells, in fact, correlates with IL-1 production (Fig. 10). Moreover, exogenous IL-1, at levels simulating those generated *in vitro,* is suppressive of antigen-induced blastogenesis. Immunoabsorbent chromatography using an antibody to purified recombinant IL-1 β removes suppressive activity in parallel with removal of IL-1 activity from supernatants of PPD-stimulated monocytes from healthy individuals (R. S. Wallis and J. J. Ellner, unpublished data). We have yet to examine the effects of removing IL-1 from mono-

TABLE II
LPS- and PPD-Induced IL-1 Production by Monocytes from Tuberculosis Patients[a]

Subjects	Stimulant	IL-1 activity[b] (U/ml)
Pulmonary tuberculosis (n=7)	Nil	<0.8
	LPS	56.1 ± 20.0[c]
	PPD	28.1 ± 7.2[d]
Healthy control (n=7)[e]	Nil	<0.5
	LPS	7.3 ± 1.7
	PPD	9.5 ± 2.9

[a]Blood monocytes from tuberculosis patients or from healthy subjects were cultured at 0.5×10^6 cells/ml in the presence or absence of LPS (10 μg/ml) or PPD (100 μg/ml). After 24 hr, the culture supernatants were aspirated, filtered, and stored at −20°C.

[b]IL-1 activity in monocyte culture supernatants was assayed by direct mitogenic effects on mouse thymocytes. IL-1 activity was transformed into units. Values are mean ±SE.

[c]Differs from LPS-induced IL-1 activity in monocyte supernatants from healthy control subjects; $p < 0.05$ (Student's t-test).

[d]Differs from PPD-induced IL-1 activity in monocyte supernatants from healthy control subjects; $p < 0.05$.

[e]Tuberculin skin-test positive.

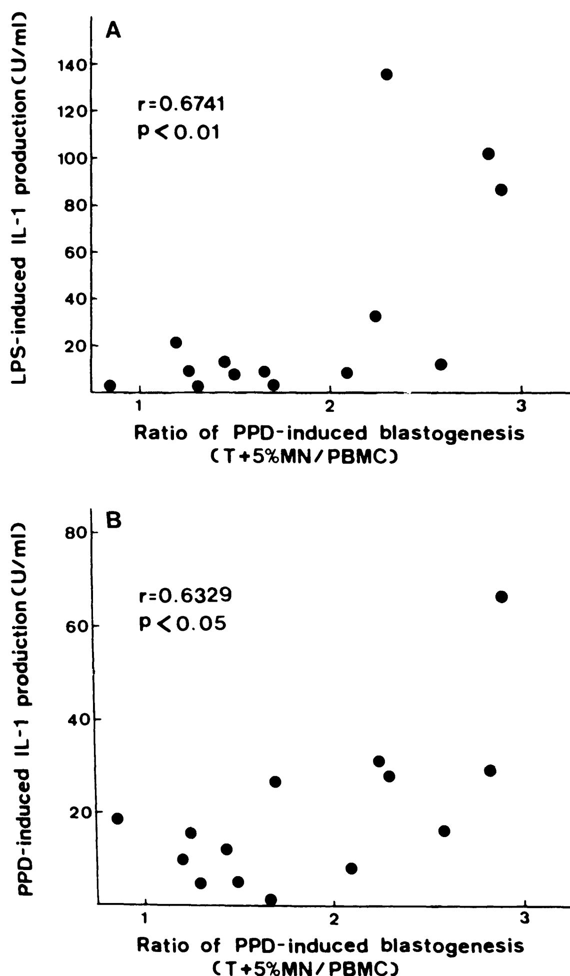

FIGURE 10. Correlations of (A) LPS- and (B) PPD-induced IL-1 production by monocytes with the ratio of blastogenic responses in TB patients and healthy subjects. (From Fujiwara *et al.*[17]).

cyte supernatants on the functional activity of the supernatants[18] in tuberculosis.

Two interesting tangential points emerge from the preliminary studies of IL-1. First, PPD directly induces IL-1 production by monocytes, independent of T cells; this effect is not due to LPS contamination of PPD, nor is it an invariable property of soluble microbial products. Neither tetanus toxoid nor streptolysin-O had comparable effects on

monocytes. Therefore, the antigen specificity of suppression by adherent cells in tuberculosis could be due to stimulation of immunosuppressive levels of IL-1 by PPD, exceeding the quantities of IL-1 present in cultures containing nonmycobacterial antigens. Second, the production of IL-1 by monocytes is sensitive to γ-irradiation (E. A. Rich, H. Fujiwara, and J. J. Ellner, unpublished observations). Therefore, the sensitivity of immunosuppression by adherent cells in tuberculosis to γ-irradiation could reflect impaired production of IL-1.

It is attractive to speculate that other macromolecular mediators could contribute to suppression by mononuclear phagocytes. For example, we have studied a product of the macrophage-like cell line U937. U937 cells spontaneously produce an immunosuppressive factor that appears to be a protein of approximately 85,000 M_r. This factor interferes with the lymphocyte-activating factor activity of IL-1[19] and blocks antigen- and mitogen-induced blastogenesis in human PMNC. The effects on blastogenesis are mediated through interference with IL-2 production and IL-2 receptor expression (H. Fujiwara, Z. Toossi, and J. J. Ellner, unpublished observations). Therefore, this factor produces some of the same defects found in patients with tuberculosis. The U937 factor is probably one of a family of growth inhibitory factors produced by normal and malignant mononuclear phagocytes. It also is of interest that the urine of patients with tuberculosis shows increased levels of an IL-1 inhibitor.[20] The cellular source of such factors and their relevance in immunosuppression in tuberculosis will need to be established.

9. DISCUSSION

The studies discussed provide evidence that products of *M. tuberculosis* are immunosuppressive *in vitro* and may account for suppression by plasma from tuberculosis patients; that tuberculosis is associated with immunosuppression, which is partly attributable to antigen-specific suppression by adherent mononuclear cells.

The immunosuppressive properties of mycobacterial polysaccharides are in keeping with their failure to elicit DTH responses in animals with experimental tuberculosis or tuberculin hypersensitivity. It should be noted that indomethacin reversibility of suppression by AG does not necessarily indicate that a cyclooxygenase product is the final mediator of suppression; indomethacin can modulate production of macromolecular products through effects on cyclic adenosine monophosphate (cAMP). For example, IL-1 production is increased in the presence of indomethacin. It should be noted, however, that AG does not induce monocyte production of IL-1. The effects of AG may be important in

the pathogenesis of disease at local sites and in the nonspecific anergy seen in some TB patients. In fact, Mason and Kirkpatrick and co-workers have provided evidence for indomethacin reversal of depressed *in vitro* response[21] and skin-test reactivity[22] in patients with chronic nontuberculous mycobacterial infections of at least 6 months duration.

Other mycobacterial products could have immunoregulatory effects. For example, Wadee *et al.*[23] showed that following ingestion of *M. tuberculosis,* monocytes release phosphatidyl ethanolamine and phosphatidyl inositol of bacterial origin that activate $OKT8^+$ suppressor cells.

Dual regulatory mechanisms involving adherent mononuclear cells and NA lymphocytes have been described in several diseases, notably leprosy. In leprosy, antigen-specific adherent cell suppression predominates in patients with most extensive disease; existing data do not yet firmly support such a conclusion in tuberculosis. This is not surprising, in view of the greater difficulty in ascertaining extent of disease in tuberculosis.

Suppression by adherent cells is effected by an $OKM1^+$ $E\text{-}R^-$ population that is radiosensitive in its function. We ascribe this activity to monocytes but cannot exclude a role for large granular lymphocytes. Suppression is associated with activation of select effector mechanisms and an immaturity of circulating monocytes. The actual basis for suppression is not clear but could involve altered expression of class II MHC products or increased production of immunoregulatory mediators, such as IL-1.

The explanation for the antigen specificity of suppression by adherent cells remains enigmatic. The existence of suppressor circuits involving antigen-specific adherent or NA lymphocytes is an attractive possibility. The available evidence does not support a role for Fc_GR^+ or $OKT8^+$ NA cells in such adherent cell-dependent circuits. It may be that the missing link is an adherent lymphocyte or a nontraditional NA suppressor cell that is, for example, OKT4 reactive. We cannot currently distinguish between these possibilities. Alternative scenarios also need to be considered. Cytophilic antibody on monocytes could function as a surrogate antigen-specific receptor, altering the uptake and distribution of mycobacterial antigens to lead to apparent specificity of suppression. Also, the ability of PPD to stimulate IL-1 production by monocytes could be a factor in the specificity of immunomodulation.

Existing approaches should permit further unraveling of the basis for immunosuppression in tuberculosis and its significance in terms of host–microbial interactions.

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REFERENCES

1. Pollack, M. S., and Rich, R. R., 1985, The HLA complex and the pathogenesis of infectious diseases, *J. Infect. Dis.* **151:**1–8.
2. Nash, D. R., and Douglas, J. E., 1980, Anergy in active pulmonary tuberculosis, *Chest* **77:**32–37.
3. Rooney, J. J., Jr., Crocco, J. A., Kramer, S., and Lyons, H. A., 1976, Further observations on Tuberculin Reactions in Active Tuberculosis. *Am. J. Med.* **60:**517–522.
4. Douvas, G. S., Looker, D. L., Vatter, A. E., and Crowle, A. J., 1985, Gamma interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria, *Infect. Immun.* **50:**1–8.
5. Ellner, J. J., 1981, Suppressor cells of man, *Clin. Immunol. Rev.* **1:**119–214.
6. Ellner, J. J., and Spagnuolo, P. J., 1979, Suppression of antigen and mitogen-induced T-lymphocyte DNA synthesis by bacterial lipopolysaccharide. Mediation by macrophage activation and production of prostaglandins, *J. Immunol.* **123:**2689–2695.
7. Kleinhenz, M. E., and Ellner, J. J., 1983, Divergent T-gamma cell functions in antigen-induced blastogenesis: facilitatory interaction with T non-gamma cells and participation in monocyte and prostaglandin-mediated suppression, *J. Lab. Clin. Med.* **102:**751–761.
8. Ellner, J. J., and Daniel, T. M., 1979, Immunosuppression by mycobacterial arabinomannan, *Clin. Exp. Immunol.* **35:**250–257.
9. Kleinhenz, M. E., Ellner, J. J., Spagnuolo, P. J., and Daniel, T. M., 1981, Suppression of lymphocyte responses by tuberculous plasma and mycobacterial arabinogalactan: Monocyte dependence and indomethacin reversibility, *J. Clin. Invest.* **68:**153–162.
10. Ellner, J. J., 1978, Suppressor adherent cells in human tuberculosis, *J. Immunol.* **121:**2573–2578.
11. Toossi, Z., Kleinhenz, M. E., and Ellner, J. J., 1986, Defective interleukin-2 production and responsiveness in human pulmonary tuberculosis, *J. Exp. Med.* **163:**1162–1172.
12. Kleinhenz, M. E., and Ellner, J. J., 1985, Immunoregulatory adherent cells in human tuberculosis: Radiation-sensitive antigen-specific suppression by monocytes, *J. Infect. Dis.* **152:**171–176.
13. Kleinhenz, M. E., and Ellner, J. J., 1987, Antigen responsiveness during tuberculosis: Regulatory interactions of T-cell subpopulations and adherent cells, *J. Lab. Clin. Med.* **110:**31–40.
14. Ellner, J. J., Spagnuolo, P. J., and Schacter, B. Z., 1981, Augmentation of selective monocyte functions in tuberculosis, *J. Infect. Dis.* **144:**391–398.
15. Tweardy, D. J., Schacter, B. Z., and Ellner, J. J., 1984, Association of altered dynamics of monocyte surface expression of human leukocyte antigen-DR with immunosuppression in tuberculosis, *J. Infect. Dis.* **149:**31–37.
16. Stallcup, K. C., Liu, Y.-N., Dorf, M. E., and Mescher, M. F., 1986, Inhibition of lymphoid cell growth by a lipid-like component of macrophage hybridoma cells, *J. Immunol.* **136:**2723–2728.
17. Fujiwara, H., Kleinhenz, M. E., Wallis, R. S., and Ellner, J. J., 1986, Increased interleukin-1 production and monocyte suppressor cell activity associated with human tuberculosis, *Am. Rev. Respir. Dis.* **133:**73–77.
18. Wallis, R. S., Fujiwara, H., and Ellner, J. J., 1986, Direct stimulation of monocyte release of interleukin-1 by mycobacterial protein antigens, *J. Immunol.* **136:**193–196.

19. Fujiwara, H., and Ellner, J. J., 1986, Spontaneous production of a suppressor factor by the human macrophage-like cell line U937. I. Suppression of interleukin-1, interleukin-2 and mitogen-induced blastogenesis in mouse thymocytes, *J. Immunol.* **136:**181–185.
20. Liao, Z., Grimshaw, R. S., and Rosenstreich, D. L., 1984, Identification of a specific interleukin-1 inhibitor in the urine of febrile patients, *J. Exp. Med.* **159:**126–136.
21. Mason, U. G. III, Greenberg, L. E., Yen, S. S., and Kirkpatrick, C. H., 1982, Indomethacin-responsive mononuclear cell dysfunction in "atypical" mycobacteriosis, *Cell. Immunol.* **71:**54–65.
22. Mason, U. G. III, and Kirkpatrick, C. H., 1984, Modulation of immunologic responsiveness in nontuberculous mycobacterial infections with indomethacin, *J. Clin. Immunol.* **4:**112–117.
23. Wadee, A. A., Mendelsohn, D., Rabson, A. R., 1983, Characterization of a suppressor-cell activating factor (SCAF) released by adherent cells treated with *M. tuberculosis*, *J. Immunol.* **130:**2266–2270.

12

Role of B Lymphocytes and Antibodies in the Regulation of Cell-Mediated Immune Reactions to BCG in Mice

MARIO CAMPA, PAOLA MARELLI, and GIOIA BENEDETTINI

1. INTRODUCTION

The mechanisms underlying the depression of the specific cell-mediated immune (CMI) reactions during the course of active tuberculous infections are complex and far from being fully understood. As comprehensively discussed in other chapters, in spite of considerable research, a clear picture is still lacking.

Mice infected intravenously (IV) with a large dose of *Mycobacterium bovis* strain BCG (BCG) rapidly develop a state of hyporesponsiveness to cutaneous challenge with the purified protein derivative (PPD) from *M. tuberculosis.* This hyporeactivity has been attributed to the fact that antigen-reactive T lymphocytes become sequestered in the central lymphoid tissues soon after infection and are therefore prevented from reaching the site of challenge.[1] This contrasts with the conclusions of a decade or more of investigation that the anergy observed both in humans and in experimental animals with active tuberculosis is mediated by an acquired immunosuppressive mechanism, i.e., the appearance of suppressor cells.

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A variety of factors, including properties and dose of the strain of the infecting microorganism, route of infection, as well as species and strain of the animal used, may influence the development and nature of the suppressor cells. For instance, whereas IV or intraperitoneal (IP) infections generally give rise to suppressor cells, no such cells can be detected in the spleen of mice infected with bacillus Calmette–Guérin (BCG) subcutaneously (SC), in the lung, or in the footpad.[2–5] Similarly, heat-killed BCG, as opposed to viable BCG, does not induce suppression.[5]

The suppressor cells that arise during BCG infection have been characterized as macrophages by certain investigators[5–7] and as T lymphocytes by others.[3,4,8] The coexistence of both suppressor cell populations has also been recorded.[9–11] Obviously, this does not exclude the involvement of other types of cells.

2. IMMUNOREGULATORY ROLE OF B LYMPHOCYTES AND/OR ANTIBODIES IN NONMYCOBACTERIAL MODEL SYSTEMS

In recent years, a number of observations have called attention to the central role that B lymphocytes and/or antibodies can play in the regulation of the host's immune responses. In mice, *Staphylococcus aureus* induces suppressor B lymphocytes capable of inhibiting the development of the specific delayed-type hypersensitivity (DTH) response to the antigens of the infecting microorganism.[12] B lymphocytes that suppress CMI reactions in an antigen-nonspecific manner were found in mice treated with killed cells of *Candida albicans.*[13] It has also been shown that *Pseudomonas aeruginosa,* the lipopolysaccharide of gram-negative bacteria, *Staph. aureus* strain Cowan I, all induce antigen-specific B lymphocytes that suppress contact sensitivity to oxazolone (Ox), a well-defined CMI response, when given to mice 24–72 hr before sensitization.[14–16]

Taken together, these results suggest that B lymphocytes and/or their products (antibodies) can play a central role in the regulation of CMI reactions. In experiments designed to better define the mechanisms by which B lymphocytes and/or antibodies suppress contact sensitivity to Ox, it was found that suppression is mediated by both idiotype-positive (Id^+) anti-Ox and anti-idiotype (anti-Id) B lymphocytes and T lymphocytes. These different types of cells develop within the lymph nodes that drain the site of sensitization at different times after immunization and interfere with different phases of the immune response. The anti-Ox B lymphocytes can be detected very early after sensitization; their role essentially consists in the induction of anti-Id B lymphocytes. These latter cells, which appear at later phases of the sensitization

process, act by inducing suppressor T (Ts) lymphocytes, which affect the efferent phase of the immune response through the release of soluble factors.[17–19] These studies also demonstrated that the induction of an immunosuppressive circuit of such complexity is due to the ability of the inducing stimulus, be it a microorganism or a bacterial constituent, to produce a polyclonal activation of B lymphocytes. *Staph. aureus* strain Wood 46, which, unlike *Staph. aureus* strain Cowan I, lacks this ability, did not suppress contact sensitivity.[18] In addition, it was found that activation of Ts cells by anti-Id B lymphocytes and induction of these cells by Id^+ anti-Ox B lymphocytes are both mediated by antibodies, respectively, anti-Id and anti-Ox.[19,20] The fact that sensitizer-specific antibodies may inhibit contact sensitivity to Ox has also been reported by Asherson *et al.*,[21] who found that anti-Ox IgM antibodies are detectable on the surface of committed T lymphocytes and limit the immune functions effected by these cells. There is also a suggestion that the suppressive effect exerted by anti-Ox antibodies is mediated by a functional block of the antigen-presenting cells[22] that might limit the expansion and proliferation of committed T lymphocytes.

Recent studies in mice[23] have shown that B and T lymphocytes regulated by idiotypic–anti-idiotypic interactions may also be involved in the suppression of humoral immune responses. Antibody production to the capsular polysaccharide of type III *Streptococcus pneumoniae* was found to be inhibited in an antigen-specific manner by Ts lymphocytes activated in response to the idiotypic determinants of B-cell associated antibody specific for the microbial polysaccharide.[23]

3. REGULATION OF DELAYED-TYPE HYPERSENSITIVITY TO BCG IN MICE

The results summarized in Section 2, together with the observation that B lymphocytes capable of inhibiting *in vitro* CMI responses in an antigen-nonspecific manner had been found in individuals vaccinated with BCG,[24] led us to investigate whether B lymphocytes and/or antibodies may also play a role in the regulation of *in vivo* CMI reactions to BCG. More precisely, we wondered whether a suppressive circuit similar to that seen in the contact sensitivity model could be involved in the suppression of the BCG-specific DTH.

To test this possibility, groups of C57BL/6 mice from our breeding colony, 8–12 weeks old and sex matched, were infected with 2×10^7 colony-forming units (CFU) of BCG strain Pasteur either IV in 0.5 ml saline or SC (in the right hind footpad) in 50 μl saline. Two weeks after infection, the animals were assayed for DTH by challenging the left hind

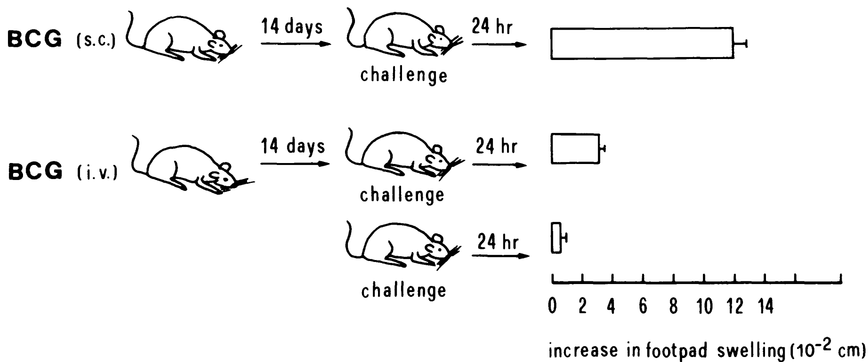

FIGURE 1. Delayed-type hypersensitivity (DTH) response to bacillus Calmette–Guérin (BCG) of mice infected with 2×10^7 CFU of BCG-Pasteur either IV in 0.5 ml saline or SC in the right hind footpad in 50 μl saline. Fourteen days after infection, mice were challenged in the left hind footpad with 20 μg PPD (Statens Seruminstitut, Copenhagen, Denmark) in 50 μl saline. The thickness of the left hind footpad was measured with a dial-gauge caliper before and at 24 and 48 hr after challenge. The increase in the footpad swelling was expressed in units of 10^{-2} cm. Since no significant differences were found between data obtained at 24 or at 48 hr after challenge, only data obtained at 24 hr are reported. Data are expressed as mean ±SD. In all experiments, six to seven mice per group were used, and the significance of the difference between the means was assessed by Student's *t*-test.

footpad with PPD. As shown in Fig. 1, mice infected IV were markedly depressed, whereas animals infected SC were not.

To establish the cellular bases underlying this suppression, multiple-step cell transfer studies were carried out according to the experimental design shown in Fig. 2. The various T- and B-cell-enriched subpopulations were obtained as follows: cell suspensions, prepared from various donors in Eagle's medium supplemented with 10% fetal calf serum (Flow Laboratories), were washed twice with the same medium and then filtered first through a glass-wool column in order to remove macrophages, and subsequently through a nylon wool column. As judged by immunofluorescence techniques, this method yielded (1) effluent lymphocytes with 94% viability, 84–94% of which were θ antigen-bearing cells; and (2) nylon-wool adherent lymphocytes with 95% viability, of which 4–8% bore the θ antigen and 75–85% carried membrane surface immunoglobulins. This fraction also contained macrophages that were generally fewer than 2% of the total count. Further details of this method are given by Campa *et al.*[25] Nylon wool-enriched B- and T-cell subpopulations were further fractionated by the "panning" method described by Wysocki and Sato,[26] with some modifications reported by Campa and co-workers.[25] This method yielded T- and B-

cell subpopulations enriched in anti-PPD specific cells or depleted in anti-Id cells.

In a pilot experiment, anti-PPD B lymphocytes obtained from the spleen of mice infected IV with BCG 18–24 hr earlier (animals destined to become immunodepressed) were infused IV into syngeneic mice infected SC with BCG 3 hr earlier (animals expected to be able to mount a normal DTH response to BCG). These cells were found capable of inhibiting the DTH response in the recipients, and a similar effect was brought about by the anti-Id-depleted B-cell subpopulation. By contrast, the anti-PPD-depleted and the anti-Id-enriched B-cell subpopulations failed to do so.

Since the anti-Ox B lymphocytes in the contact sensitivity model had been found to exert their suppressive effects by inducing anti-Id B lym-

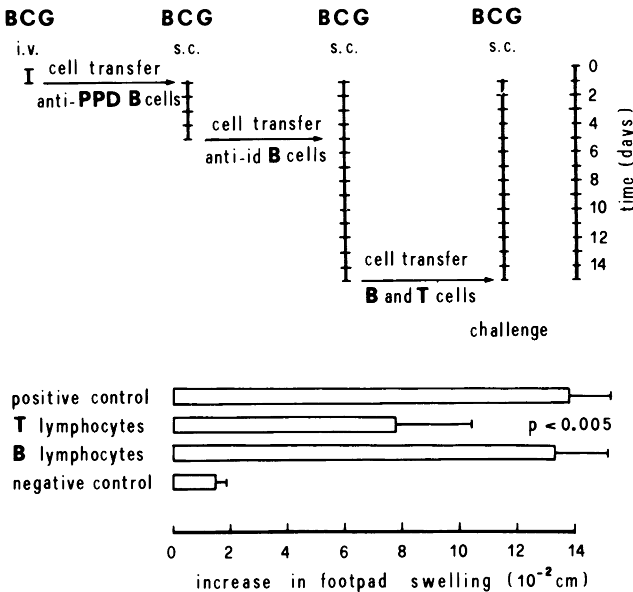

FIGURE 2. Delayed-type hypersensitivity (DTH) response to bacillus Calmette-Guérin (BCG) of mice infected SC (right hind footpad) with 2×10^7 CFU of BCG 14 days earlier and injected IV 3 hr before challenge (20 μg PPD in the left hind footpad) with splenic T or B lymphocytes (3×10^7) obtained from mice infected SC with 2×10^7 CFU of BCG 14 days earlier and injected 4 davs after infection with anti-Id B lymphocytes (1.5×10^7). These cells had been obtained from the spleen of donors infected with 2×10^7 CFU of BCG 4 days earlier and injected 3 hr after infection with anti-PPD B lymphocytes (1.5×10^7). In turn, these cells had been obtained from the spleen of mice infected IV with 2×10^7 CFU of BCG 24 hr earlier.

phocytes (see Section 2), the next step was to investigate whether the anti-PPD B lymphocytes were capable of activating anti-Id B lymphocytes. Anti-PPD B lymphocytes, obtained from the spleen of donors infected IV with BCG 24 hr earlier, were transferred IV into recipients infected SC with BCG 3 hr earlier. Four days later, anti-Id B lymphocytes were found in great numbers in the spleen of these recipients, but not in the spleen of mice injected with B lymphocytes depleted of the anti-PPD B-cell subpopulation.

Once more, by analogy with the contact sensitivity model, anti-Id B lymphocytes turned out to exert their effect by activating Ts lymphocytes. As can be seen in Fig. 2, anti-Id B lymphocytes obtained from the spleen of mice given IV anti-PPD B cells 4 days earlier and also infected SC with BCG 3 hr before receiving these cells were transferred IV into recipients infected SC 4 days earlier with BCG. These recipients were sacrificed 10 days after cell transfer, and their spleen cell suspensions were separated into B and T lymphocytes by filtration through a nylon-wool column. These cell subpopulations were transferred into mice infected SC 14 days earlier with BCG and challenged with PPD 3 hr after cell transfer. Only the recipients of the T-cell-enriched subpopulation turned out to be significantly depressed, indicating that the anti-Id B lymphocytes exert their effect by activating Ts lymphocytes. By contrast, in a similar protocol, mice infected SC with BCG and infused 4 days later with the anti-Id B-cell-depleted subpopulation showed no Ts lymphocytes in their spleen (data not shown). Further studies demonstrated that the Ts lymphocytes that emerge at the end of the circuit involved in the regulation of the DTH response to BCG are Id^+ and act through the release of soluble factors.

The antigenic specificity of these Ts lymphocytes was evaluated by examining whether they inhibited contact sensitivity to Ox. As can be seen in Fig. 3, the splenic T cells involved in the suppression of DTH response to BCG failed to affect the response to Ox when transferred into sensitized recipients 2–3 hr before challenge with the sensitizer. They also turned out to be unable to suppress contact sensitivity development when transferred into mice 2–3 hr before Ox sensitization (Fig. 4).

In conclusion, these results demonstrate that suppression of the DTH response to BCG is mediated by Id^+ anti-PPD B lymphocytes that induce anti-Id B lymphocytes. In turn, such cells activate Ts lymphocytes that affect the efferent phase of the response in an antigen-specific manner. Thus, present data extend previous findings of ours[28] that anti-Id antibodies take part in the suppression of the BCG-specific DTH response in mice infected IV with this microorganism, by demonstrating that specific antibodies are an integral part of a complex immunosuppressive circuit.

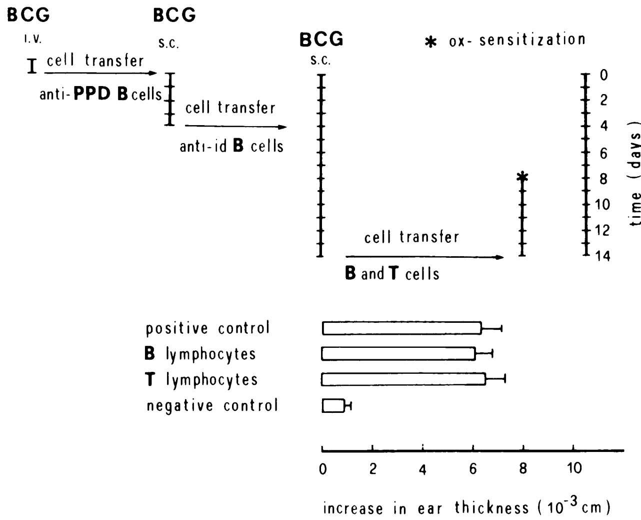

FIGURE 3. Failure of splenic T lymphocytes obtained as described in Fig. 2 to affect contact sensitivity to 4-ethoxymethylene-2-phenyl-oxazolone (Ox; BDH, Poole, UK) when transferred IV at doses of 3×10^7 into sensitized recipients, 2–3 hr before challenge with the sensitizer. Sensitization and detection of contact sensitivity were performed as follows. Mice were sensitized by painting the skin of the abdomen and lower thorax with 0.2 ml 1.5% Ox in absolute ethanol. Sensitized animals were challenged 6 days later by painting their ears with a drop of 1% Ox in olive oil. Contact sensitivity was tested by measuring the increase in ear thickness with a dial-gauge caliper, 24 hr after challenge (1 unit = 10^{-3} cm). For further details, see Campa *et al.*[14]

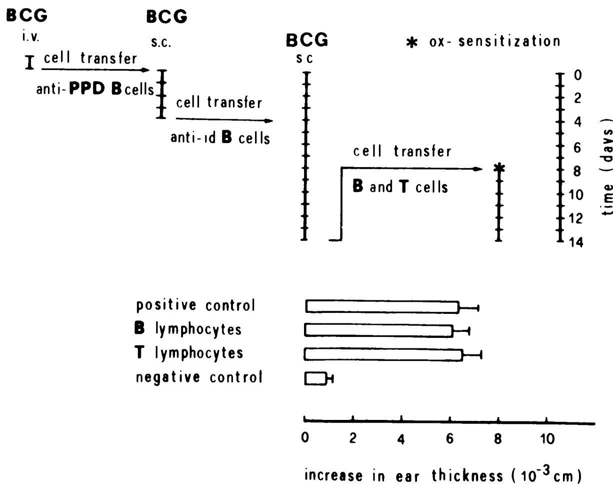

FIGURE 4. Failure of splenic T lymphocytes obtained as described in Fig. 2 to affect contact sensitivity to Ox when transferred IV at doses of 3×10^7 into syngeneic recipients, 2–3 hr before sensitization. Contact sensitivity was established and tested as in Fig. 3.

4. REGULATION OF THE GRANULOMATOUS RESPONSE TO BCG IN MICE

It is generally recognized that activation of macrophages and granuloma formation play a prominent role in acquired resistance to tuberculosis. It was therefore of interest to investigate whether the cells involved in the suppression of the DTH response to BCG are also capable of interfering with the granulomatous response to this microorganism.

Groups of C57BL/6 mice were infected IV with 2×10^7 CFU of BCG and evaluated for the granulomatous response by weighing their spleens and lungs 28 days later. Preliminary studies had shown that a considerable proportion of the increase in spleen and lung weights occurring at this time is attributable to an increase in cellularity and not merely to edema.

B lymphocytes obtained from the spleen of mice infected IV with BCG 24 hr earlier (i.e., animals unable to mount a normal DTH response to BCG) were infused IV into syngeneic recipients infected IV with BCG 7, 14, or 21 days before cell transfer. These cells induced a marked depression of the granulomatous response only in the recipients that had been infected 7 days earlier (Fig. 5). Splenic T lymphocytes,

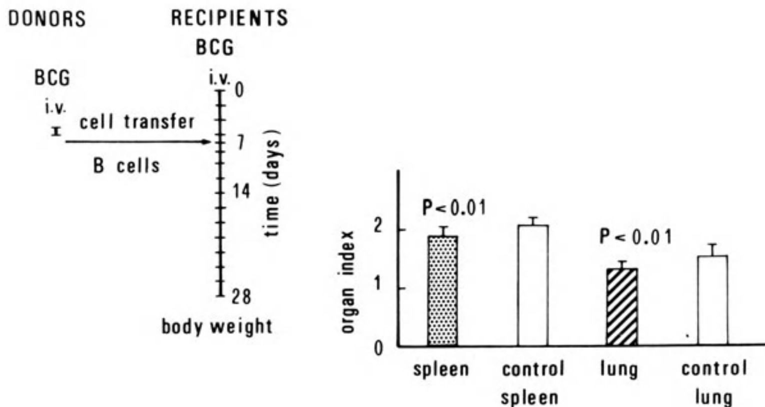

FIGURE 5. Effect of splenic B lymphocytes from mice infected IV with 2×10^7 CFU BCG 24 hr earlier on the granulomatous response to BCG. Recipients had been infected IV with 2×10^7 CFU of BCG 7 days before receiving IV 2×10^7 cells. The granulomatous response was evaluated by determining the increase in spleen and lung weight 28 days after IV infection, which preliminary experiments had shown to be the time of peak response. It was calculated as follows:

$$\text{Organ index} = \frac{\text{organ weight}}{\text{body weight}} \times 100$$

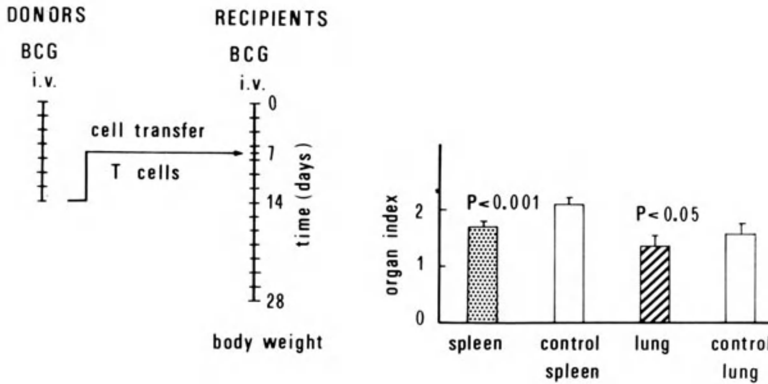

FIGURE 6. Effect of splenic T lymphocytes from mice infected IV with 2×10^7 CFU of BCG 14 days earlier on the granulomatous response to BCG. Recipients had been infected IV with 2×10^7 CFU BCG 7 days before receiving IV 2×10^7 cells. Other details as in Fig. 5.

which in previous experiments had been found to mediate the suppression of the DTH response to BCG at day 14 after IV infection (Fig. 2), also inhibited the granulomatous response only in the recipients infected IV with BCG 7 days earlier (Fig. 6). However, these data provide no insight into the mechanisms by which B lymphocytes suppress the granulomatous response. We are currently investigating whether these cells exert their effect by activating Ts lymphocytes, as previously shown in the case of the DTH response.

These results indicate that B and T lymphocytes that co-purify with those mediating the suppression of the DTH response, also play a role in the regulation of the granulomatous response to BCG. The characterization of such cells, their relationship to those involved in the regulation of the DTH response, and their mechanism of action are under study.

5. CONCLUSION

These results demonstrate that the suppression of the DTH response to BCG in mice infected IV with this microorganism is mediated by at least three types of cells: anti-PPD B lymphocytes, anti-Id B lymphocytes, and T lymphocytes. These cells arise at different times in the infectious process and appear to belong to the same immunosuppressive circuit. The activity of B lymphocytes is due to their products (antibodies); the Ts lymphocytes, which represent the final effectors of the circuit, seem to be antigen specific.

Conflicting evidence has been presented on the antigen specificity

of the T lymphocytes involved in the suppression of the DTH response to BCG. According to some authors, these cells act in an antigen-nonspecific manner,[8,27] while according to others, they are antigen specific.[8] As already seen in other models,[28,29] the possibility that both antigen-specific and antigen-nonspecific Ts lymphocytes coexist and contribute to the suppression should be explored.

It must be emphasized that the results of this investigation prove that the activation of an immunosuppressive circuit may participate in determining the anergy of mice infected IV with large doses of BCG. They do not exclude that additional underlying mechanisms can be operative, such as those reported by Orme and Collins[1] and others[30] of a disturbance in the circulation of the antigen-specific T lymphocytes, as a consequence of their trapping within the foci of infection and/or the draining lymphoid tissues. Nor do these data exclude that other types of cells, such as macrophages, may take part in the suppression, or that the induction of Ts lymphocytes may be the end result of different pathways.

The main point raised by the present study is that B lymphocytes and/or specific antibodies may play a crucial role in the regulation of CMI reactions to tubercle bacilli—an aspect that has long been neglected. Furthermore, by showing that specific antibodies, secreted or B cell associated, trigger a complex circuit that eventually leads to suppression of both DTH and the granulomatous response to BCG, these results may also represent an indirect support to the recent hypothesis that suppression of cell-mediated immunity in lepromatous leprosy is a phenomenon regulated by idiotypic–anti-idiotypic interactions.[31] Finally, these results, together with findings by others that antibody production to type III pneumococcal polysaccharide is suppressed by Ts lymphocytes activated in response to the idiotypic determinants of B-cell-associated antibody specific for the microbial polysaccharide,[23] suggest that the induction of immunosuppressive circuits regulated by idiotypic anti-idiotypic interactions may represent one mechanism whereby parasites interfere with the host's immune reactivity.

Our previous studies in mice[17,18] had shown that a strong and early activation of Id^+ anti-Ox B lymphocytes can generate anti-Id B lymphocytes, which in turn induce Ts lymphocytes, and that this mechanism is responsible for the suppression of contact sensitivity to Ox brought about by several polyclonal B-cell activators (PBA) when administered 24–72 hr before sensitization. In view of the fact that BCG is also a PBA,[4] it is possible that BCG induces an early nonspecific activation of B-cell clones, including anti-Id B lymphocytes. These cells might be stimulated to expand further by the emerging Id^+ anti-PPD B lymphocytes, eventually generating Ts lymphocytes, which represent the final

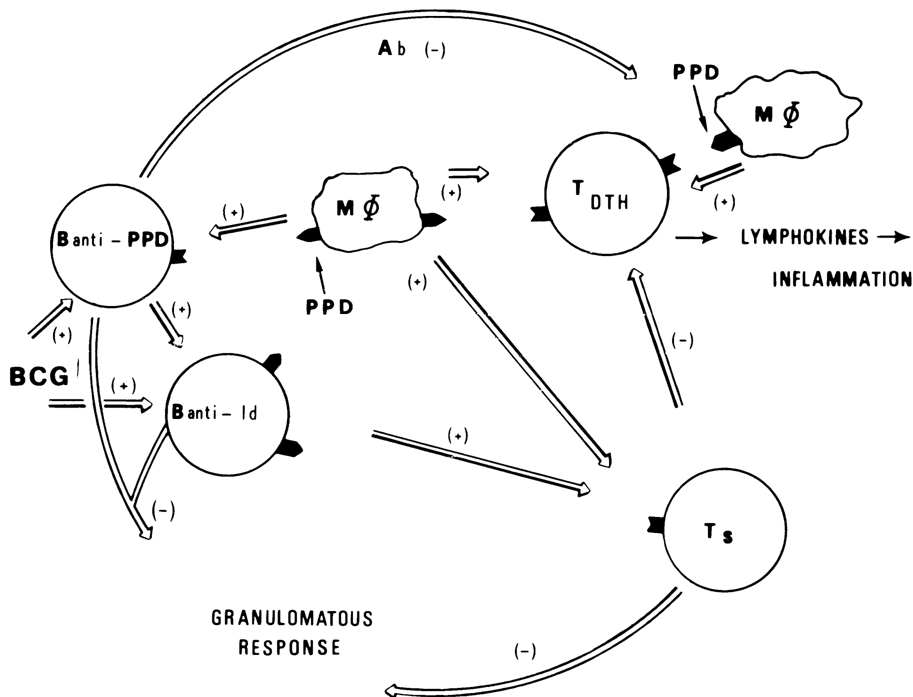

FIGURE 7. Suggested mechanism for the regulation of CMI reactions to BCG in mice. +, activation; −, suppression. See text for discussion.

effectors responsible for the inhibition of DTH and granulomatous responses (Fig. 7). The observation that the lipopolysaccharide of gram-negative bacteria, the best characterized of all PBA, directly induces anti-Id B lymphocytes,[32] lends support to this possibility. However, why viable but not heat-killed BCG and IV and not SC BCG infections induce such a complex immunosuppressive circuit remain critical points to be examined.

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REFERENCES

1. Orme, I. M., and Collins, F. M., 1984, Passive transfer of tuberculin sensitivity from anergic mice, *Infect. Immun.* **46:**850–853.

2. Turcotte, R., Lafleur, L., and Labreche, M., 1978, Opposite effects of BCG on spleen and lymph node cells: Lymphocyte proliferation and immunoglobulin synthesis, *Infect. Immun.* **21**:696–704.
3. Collins, F. M., and Watson, S. R., 1979, Suppressor T-cells in BCG-infected mice, *Infect. Immun.* **25**:491–496.
4. Sultzer, B. M., 1978, Infection with Bacillus Calmette–Guérin activates murine thymus-independent (B) lymphocytes, *J. Immunol.* **120**:254–261.
5. Klimpel, G. R., and Henney, C. S., 1978, BCG-induced suppressor cells. I. Demonstration of a macrophage-like suppressor cell that inhibits cytotoxic T cell generation *in vitro*, *J. Immunol.* **120**:563–569.
6. Tweardy, D. J., Schacter, B. Z., and Ellner, J. J., 1984, Association of altered dynamics of monocyte surface expression of human leukocyte antigen DR with immunosuppression in tuberculosis, *J. Infect. Dis.* **149**:31–37.
7. Schrier, D. J., Allen, E. M., and Moore, V. L., 1980, BCG-induced macrophage suppression in mice: Suppression of specific and nonspecific antibody-medicated and cellular immunologic responses, *Cell. Immunol.* **56**:347–356.
8. Kato, K., Yamamoto, K-I., Kimura, T., Azuma, I., and Askenase, P. W., 1984, Suppression of BCG cell wall-induced delayed-type hypersensitivity by pretreatment with killed BCG: Induction of nonspecific suppressor T cells by the adjuvant portion (MDP) and of specific suppressor T cells by the antigen portion (TAP), *J. Immunol.* **132**:2790–2795.
9. Orbach-Arbouys, S., and Poupon, M-F., 1978, Active suppression of *in vitro* reactivity of spleen cells after BCG treatment, *Immunology* **34**:431–437.
10. Turcotte, R., 1981, Evidence for distinct populations of suppressor cells in the spleens of *Mycobacterium bovis* BCG-sensitized mice, *Infect. Immun.* **34**:315–322.
11. Ellner, J. J., 1978, Suppressor adherent cells in human tuberculosis, *J. Immunol.* **121**:2573–2579.
12. Easmon, C. S. F., and Glynn, A. A., 1979, The cellular control of delayed hypersensitivity to *Staphylococcus aureus* in mice, *Immunology* **38**:103–108.
13. Rivas, V., and Rogers, T. J., 1983, Studies on the cellular nature of *Candida albicans*-induced suppression, *J. Immunol.* **130**:376–379.
14. Campa, M., Garzelli, C., Ferrannini, E., and Falcone, G., 1976, Evidence for suppressor cell activity associated with depression of contact sensitivity in *Pseudomonas aeruginosa* infected mice, *Clin. Exp. Immunol.* **26**:355–362.
15. Campa, M., 1984, Bacterial interference with cell-mediated immunity, in: *Bacterial and Viral Inhibition and Modulation of Host Defenses* (G. Falcone, M. Campa, H. Smith, and G. M. Scott, eds.), pp. 135–157, Academic, London.
16. Benedettini, G., De Libero, G., Mori, L., and Campa, M., 1984, *Staphylococcus aureus* inhibits contact sensitivity to oxazolone by activating suppressor B cells in mice, *Int. Arch. Allergy Appl. Immunol.* **73**:269–273.
17. Campa, M., Benedettini, G., De Libero, G., Mori, L., and Falcone, G., 1984, T suppressor cells as well as anti-hapten and anti-idiotype B lymphocytes regulate contact sensitivity to oxazolone in mice injected with purified protein derivative from *Mycobacterium tuberculosis*, *Infect. Immun.* **45**:701–707.
18. Benedettini, G., De Libero, G., Mori, L., Marelli, P., Angioni, M. R., and Campa, M., 1985, *Staphylococcus aureus*-induced suppression of contact sensitivity in mice. Suppressor cells elicited by polyclonal B-cell activation are regulated by idiotype anti-idiotype interactions, *Cell. Immunol.* **93**:508–519.
19. Campa, M., De Libero, G., Benedettini, G., Mori, L., Angioni, M. R., Marelli, P., and Falcone, G., 1985, Polyclonal B cell activators inhibit contact sensitivity to oxazolone in mice by potentiating the production of anti-hapten antibodies that induce T sup-

pressor lymphocytes acting through the release of soluble factors, *Int. Arch. Allergy Appl. Immunol.* **78:**391–395.
20. Colizzi, V., Giuntini, M., Garzelli, C., Campa, M., and Falcone, G., 1983, Auto-anti-idiotypic antibodies inhibit T-cell-mediated hypersensitivity in BCG-infected mice, *Cell. Immunol.* **80:**205–210.
21. Asherson, G. L., Colizzi, V., and Watkins, M. C., 1983, Immunogenic cells in the regional lymph nodes after painting with the contact sensitizers picryl chloride and oxazolone: Evidence for the presence of IgM antibody on their surface, *Immunology* **48:**561–569.
22. Zubler, R. H., 1981, Epitope-specific antibody feedback regulation of the humoral immune response against sheep erythrocytes *in vitro:* Specific effects of anti-antigen antibody *vs* nonspecific T cell activities, *J. Immunol.* **126:**557–562.
23. Taylor, C. E., Stashak, P. W., Caldes, G., Prescott, B., Chused, T. E., Brooks, A., and Baker, P. J., 1983, Activation of antigen-specific suppressor T cells by B cells from mice immunized with type III pneumococcal polysaccharide, *J. Exp. Med.* **158:**703–717.
24. Bona, C., Audibert, F., Juy, D., and Chedid, L., 1976, Cell suppression in PPD-induced blast specific response of human peripheral blood lymphocytes, *Clin. Exp. Immunol.* **26:**258–266.
25. Campa, M., Benedettini, G., and Marelli, P., 1986, B and T lymphocytes regulated by idiotype anti-idiotype interactions inhibit delayed-type hypersensitivity to BCG in mice, *Cell. Immunol.* **98:**93–103.
26. Wysocki, L. J., and Sato, V. L., 1978, "Panning" for lymphocytes: A method for cell selection, *Proc. Natl. Acad. Sci. USA* **75:**2844–2848.
27. Watson, S. R., and Collins, F. M., 1980, Development of suppressor T cells in mice heavily infected with mycobacteria, *Immunology* **39:**367–373.
28. Zembala, M. A., Asherson, G. L., James, B. M. B., Stein, V. E., and Watkins, M. C., 1982, Anti-haptene T suppressor factor acts through an I-J$^+$, Ly 1$^-$2$^+$, T acceptor cell that releases a nonspecific inhibitor of the transfer of contact sensitivity when exposed to antigen, *J. Immunol.* **129:**1823–1829.
29. Zembala, M. A., Colizzi, V., Asherson, G. L., and Watkins, M. C., 1984, Non-specific inhibitor made by T acceptor cells inhibits both the afferent and efferent stage of the contact sensitivity reaction, *Immunology* **52:**599–606.
30. Rook, G. A. W., Carswell, J. W., and Stanford, J. L., 1976, Preliminary evidence for the trapping of antigen-specific lymphocytes in the lymphoid tissue of "anergic" tuberculosis patients, *Clin. Exp. Immunol.* **26:**129–132.
31. Ferluga, J., Colizzi, V., Ferrante, A., Colston, J., and Holborow, E. J., 1984, Possible idiotypic suppression of cell-mediated immunity in lepromatous leprosy, *Lepr. Rev.* **55:**221–227.
32. Rose, L. M., Goldman, M., and Lambert, P-H., 1982, The production of anti-idiotypic antibodies and of idiotype-anti-idiotype immune complexes after polyclonal activation induced by bacterial LPS, *J. Immunol.* **128:**2126–2133.

13

Polyclonal Lymphocyte Activation by *M. tuberculosis* and Its Products

BARNET M. SULTZER

1. INTRODUCTION

The origin of the term polyclonal activation as applied to lymphocytes can be ascribed to the original finding of Nowell,[1] who was the first to show that the proliferation of human peripheral blood lymphocytes in culture could be stimulated by a plant lectin phytohemagglutinin (PHA). With the development of an adequate culture system for mouse lymphocytes came an expansion of the use of this mitogen and the discovery of others, many of which are bacterial products. The value of these substances derived from the fact that lymphoid cells were stimulated nonspecifically. Consequently, large numbers of cells were activated that permitted examination of the biochemical and immunologic events attendant to this process that was difficult at best to measure when dealing with a cell system based on specific antigenic recognition.

All types of lymphocytes, however, are not stimulated by the same substances, so that the study of the activation of T and B cells became possible by the use of selected polyclonal mitogens. In the case of T cells, concanavalin A (Con A) is an effective stimulant of DNA synthesis that also activates these cells to elaborate lymphokines. By contrast, the lipopolysaccharide (LPS) endotoxin from various gram-negative bacte-

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ria activates murine B cells to proliferate and differentiate into antibody-producing cells of various specificities. As a result, LPS was the first mitogen to be classified as a polyclonal B-cell activator (PBA). Other examples of bacterial products that are polyclonal activators would include endotoxin-associated protein (EP)[2] or lipid A-associated protein (LAP),[3] both of which consist of outer membrane proteins from gram-negative bacteria,[4] a glycoprotein from *Klebsiella pneumoniae,*[5] the lipoprotein and its synthetic lipopeptide analogues from gram-negative bacterial outer membranes,[6] and the staphylococcal peptidoglycan.[7] Not to be excluded from this list are the mycobacteria and their products, including the tuberculin proteins, the mycobacterial cell wall and its components, as well as the whole cells such as the vaccine strain of *Mycobacterium bovis* or bacillus Calmette–Guérin (BCG).

It is the intention of this chapter, then, to review the polyclonal activating properties of the various mycobacterial components. In the process, perhaps some insight will be gained concerning the activation and differentiation processes exhibited by the lymphocytes of the immune system when first exposed to the mycobacteria and, subsequently, after sensitization from either infection or immunization.

2. TUBERCULIN: A POLYCLONAL B-CELL ACTIVATOR

Tuberculin in the form of its purified protein derivative (PPD) has long been used as a model antigen for the induction of delayed-type sensitivity (DTH). At the cellular level, this tuberculin hypersensitivity has been generally regarded as a T-cell-dependent phenomenon[8]; therefore, PPD has been widely used in defining antigen-driven T-lymphocyte activities associated with DTH. Lymphokines, such as migration inhibiting factor (MIF), have been shown to be produced *in vitro* as a result of PPD acting as an antigen on specifically sensitized T cells.[9] Furthermore, from a variety of experiments with mice, evidence has developed to suggest that after infection of the host with either the virulent tubercle bacillus or its attenuated counterpart, *M. bovis* (BCG), the cells that proliferate and may be the specific mediators of tuberculin sensitivity were T cells.[10–13]

Aside from its antigenic properties, however, PPD has been also shown to be a nonspecific mitogen for murine B cells; i.e., such cells cultured from normal, unimmunized, uninfected animals were activated to blastogenesis and DNA synthesis.[14] The response was rapid and occurred optimally at relatively high concentrations of PPD. Immature thymocytes and so-called cortisone-resistant mature thymocytes did not respond to PPD. In addition, splenic lymphocytes from thymectomized

irradiated mice reconstructed with anti-θ treated bone marrow cells (B cells), and spleen cells from congenitally athymic nude mice, which possess only functional B cells, were stimulated to proliferate by PPD. Furthermore, adult and neonatal guinea pig spleen cells responded to PPD with DNA synthesis in culture, so that the basic response was not limited to the mouse. However, the PPD activation of B cells was not restricted to blastogenesis or proliferation. Nonspecific immunoglobulin synthesis by B cells was also shown to be induced by PPD in culture.[15] When normal mouse spleen cells were stimulated to produce antibody as measured by plaque-forming cells against sheep red blood cells (SRBC) and horse red blood cells (HRBC) as well as the determinant dinitrophenacetyl (NNP), the response was again rapid and mainly IgM antibody of low average avidity, as measured by hapten inhibition. Finally, fluorescein-labeled antimouse immunoglobulin antibody applied to cells cultured with PPD showed that a high percentage of the cells contained immunoglobulin,[15] and autoradiography of PPD-stimulated cells using [^{125}I]-anti-IgG also identified the blast cells as B cells.[16] The conclusion drawn from this work was simply that PPD was a polyclonal activator of B cells. Nevertheless, since PPD has been used extensively to activate T cells from specifically sensitized hosts, this finding was greeted with a certain degree of skepticism.

2.1. Nonspecific Lymphocyte Activation by Tuberculin Protein

In order to prove that tuberculin protein in the form of PPD or culture filtrates was not due to an extraneous contaminant such as lipopolysaccharide endotoxin (LPS) or denatured materials, various experiments were conducted. Circumstantial evidence that PPD was not merely a bland antigen but had other intrinsic biologic properties also was considerable.[14] Various lots of PPD from different sources were active.[14,16] The kinetics of the cellular response to PPD were consistently different from those seen with LPS, being earlier and less variable. The B lymphocytes of the C3H/HeJ mouse strain, which do not respond to LPS or its lipid A by proliferation or differentiation, do respond in this manner when cultured with PPD. Also, a tuberculin-active peptide and a polysaccharide fraction obtained from tuberculin were inactive.[14]

Subsequent experiments using sterile unheated tuberculin culture filtrate (CF002-NIAID) demonstrated that CF002 was a mitogen highly potent PBA for mouse spleen cells.[16] These results ruled out the possibility that in the method of preparing PPD from culture filtrates, the heat treatment and salt or acid precipitation procedures produced active altered macromolecules (proteins) that were not mitogenic or stimulating in their original state.

To assess further the importance of the protein components of tuberculin in the activation of normal B lymphocytes, PPD was subjected to digestion with chymotrypsin bound to Sepharose beads.[16] Under various conditions of pH, buffer concentration, and temperature, PPD activity was lost to varying degrees after treatment with this protease, strongly suggesting that the PBA activity was due to tuberculoprotein. In addition, both the *Limulus* amebocyte assay and the actinomycin D mouse lethality test for endotoxin at concentrations of PPD and CF002 10-100 times the mitogenic doses used were negative.[16] Therefore, if any LPS contaminated PPD, insufficient amounts were present to account for the lymphocyte activation, since this form of tuberculin is equivalent to or superior to LPS as a PBA.[15,17,18] Further independent observations on the use of endotoxin-free PPD have been reported, showing this material to be active on human lymphocytes as well.[19,20] Of added interest were the experiments reported by Jacobs and Morrison,[21] who used polymyxin B to inhibit the activity of LPS in experiments directed at studying the relationship between the immunogenicity and mitogenicity of LPS. Polymyxin B blocked the mitogenicity of LPS on mouse spleen cells but did not abrogate the activity of PPD.

Clearly, from all the results described, neither endotoxin contamination nor procedural artifacts are responsible for the nonspecific lymphocyte activating properties of tuberculin and, therefore, it is safe to conlude that tuberculoprotein is responsible for the mitogenic and PBA activity of PPD.

Additional studies in a number of laboratories confirmed and extended the initial finding that PPD is a PBA. Palmer and Smith[22] reported that PPD stimulated DNA synthesis in a T-independent subpopulation of normal C57B1 mouse spleen cells. This proliferative response was augmented by leukocyte lysates from tuberculosis patients. PPD was also found to be mitogenic for the B cells of BALB/c mice *in vitro,* was a PBA, and enhanced the *in vivo* anti-SRBC response when injected shortly before the administration of SRBC.[23,24] However, PPD did not activate C′3, in contrast to poly I : poly C, which was active in all the systems.[23] From the results obtained with these compounds as well as with a variety of others, Diamantstein and Blitstein-Willinger[23] concluded C′3 activation, immunogenicity and B-cell stimulation are not correlated properties of certain molecules, where B-cell mitogenicity and adjuvanticity are related. Lipsky and Rosenthal[20] reported that normal guinea pig B cells are stimulated to proliferate by PPD and that macrophages or factors produced by them inhibited PPD-induced B-cell DNA synthesis. PPD has also been used to monitor the age-related decline in thymic-independent cell function in a long-lived mouse strain.[25] The proliferative response to PPD and LPS of cultured spleen cells was

found to decline with the age of the mouse from which the cells were derived but to a lesser degree and later than a thymic-dependent function measured by the immune response to SRBC.[25]

Whereas the experiments just described were conducted for the most part on cultured lymphocytes, Moatamed *et al.*[26] described early cellular responses in the spleens of mice following intravenous (IV) injection of mitogens and adjuvants. Marked B-cell zone hyperplasia was found in mice injected with either PPD, endotoxin, or dextran sulfate. Similar histologic changes were seen in thymectomized animals. Tritiated thymidine incorporation and the number of immunoglobulin-positive cells were significantly increased by 24 hr. These experiments show that PPD as well as other B-cell mitogens trigger the same cellular components of the immune system *in vivo* as they do *in vitro*. Perhaps tuberculin proteins elaborated in the host by the tubercle bacillus may induce nonspecific B-cell activation and proliferation in localized situations.

2.2. Activation of B-Cell Subpopulations

Because PPD can activate lymphocytes directly without the necessity for presensitizing animals, PPD has been found useful for studying a variety of basic immunologic problems. This is also the case with LPS, which is generally considered the reference mitogen and polyclonal antibody inducer for murine B cells. In this regard, one of the first questions raised was whether PPD and LPS were activating the same population of B cells. Studies directed at answering this question led to the finding that the differentiation of fetal liver cells to mature B cells in irradiated hosts could be followed by the use of LPS, PPD, and dextran sulfate.[27] It was shown that these cells subsequently gained responsiveness to dextran sulfate, LPS, and PPD, in that order, and that the resulting activation depended only on the degree of differentiation at the time they were activated. Thus, more primitive cells can only divide, whereas the response of more differentiated cells is also characterized by a high rate of antibody synthesis.[27]

Further studies in this direction have revealed that subpopulations of mouse B lymphocytes possessing C′3 and Fc receptors were affected by exposure to PPD and LPS.[28] PPD caused a rapid loss of cells capable of binding C′3 and a concomitant increase of Fc-binding cells after 24 hr in culture. By contrast, LPS induced analogous changes, but they were not complete until after 72 hr in culture. These results suggested LPS and PPD were activating different populations of B cells. PPD activated cells at a higher stage of differentiation, whereas LPS activated more immature B cells. In a more recent study, by Kakiuchi and Nariuchi,[29]

responses of B cells with or without receptors for C′3 to polyclonal B-cell activators were examined by means of the PFC assay to TNP–HRBC and protein-coated HRBC. C′3-depleted mouse splenic lymphocytes were responsive to LPS but responded poorly to PPD and were essentially unresponsive to dextran sulfate. By contrast, C′3-enriched lymphocytes responded poorly to LPS but well to both PPD and dextran sulfate. These different responses were not explainable by Ts cells, changes in the kinetics or dose–response profile, or macrophage depletion.

More direct evidence of the selective triggering of distinct subpopulations of B cells by PPD and LPS has been provided by another series of experiments. These trials were based on the degree of stimulation obtained after the simultaneous addition of optimal concentrations of two mitogens to the cell cultures,[16,30] the selective inhibition of the response of one cell population over another,[16] and the elimination of the responding population to one mitogen by a hot pulse of [^{3}H]thymidine while retaining the response of the second population to the second mitogen.[30] The results indicated that the cells responding to LPS and PPD were largely distinct. However, it was also apparent that some cells responded to both mitogens, although this was limited and the extent of overlapping cells varied from one mouse strain to another.[16]

This heterogeneity of B cells has also been observed in other systems. The antibody-induced cell-mediated immunity, which may be a product of B-cell activity, can be enhanced by LPS but not by PPD at any dose or time interval studied.[31] In examining the evolution of the lymphoid system of a phylogenetically primitive telost fish, the rainbow trout, PPD, LPS, and concanavalin A (Con A) were used to measure the capacity of leukocytes from various lymphoid organs to respond to these mitogens.[32] The patterns of responses of cells from the spleen, peripheral blood, and anterior kidney were significantly different, suggesting both heterogeneity and a unique distribution of lymphoid cells. Indeed, LPS activated lymphocytes from the anterior kidney, whereas PPD and Con A did not. By contrast, PPD was a more potent mitogen than LPS or Con A for the peripheral blood lymphocytes.[32]

2.3. Mediator Production

A most important finding related to the nonspecific activation of B lymphocytes by PPD was the report by Yoshida *et al.*,[33] who used PPD to stimulate lymphokine production from guinea pig lymphocytes. In a study to examine the cellular origin of MIF, these workers found that while soluble egg albumin or dinitrophenylated egg albumin induced MIF in sensitized T cells and not B cells, when PPD was used on cells

from animals immunized with the aforementioned antigens in Freund's complete adjuvant containing killed tubercle bacilli, the B cells as well as T cells made MIF. Further experiments done with cells from unimmunized animals demonstrated that PPD could stimulate B cells but not T cells to make MIF. Yoshida and co-workers ascribed the results to the nonspecific activation of B cells by PPD and confirmed these *in vitro* results *in vivo* by the use of the macrophage disappearance reaction.[33] That normal B cells could be activated by PPD led to a re-evaluation of the B cells in cell-mediated immunity.

An example of this change in our understanding of the role of B cells in facets of immunity other than antibody production is provided by the work of Rocklin *et al.*[9] Using purified human T and B cells, MIF activity was found to be a property of both classes of lymphocytes. In fact, MIF production was found to be quantitatively greater in B cells than T cells. Both cell types were stimulated by specific antigens to which the individuals were sensitized, including streptokinase-streptodornase, *Candida,* and PPD. However, cells from tuberculin-negative individuals did not produce MIF when exposed to PPD, in contrast to the results with guinea pig lymphocytes. This apparent discrepancy may well be due to the different cultural conditions employed, with particular reference to serum supplementation, in addition to the fact that the concentration of PPD used for the human cell work was one fifth of that used with guinea pig cells.[9,33] In any event, the general assumption that DTH of the tuberculin type is a pure T-cell phenomenon is still open to question. Thus, while the production of MIF correlates with cutaneous DTH, it reflects not only T-cell function but B-cell function as well.

2.4. Regulation of the Immune Response

Since, by definition, B-cell mitogens stimulate cell division, and proliferation is a necessary step in the full expression of the immune response, it is reasonable to assume that these substances would act to increase the magnitude of B-cell-specific responses. In fact, the adjuvant activity of LPS endotoxin had been known prior to the discovery that LPS activates B cells to DNA synthesis and division.[34] Consequently, PPD has also been investigated from this point of view. PPD can enhance the immune response to SRBC in mice if given 30 min before immunization of the animals with a suboptimal dose of antigen.[23] When injected into mice, PPD also can induce synthesis of autoantibodies. This type of response was detected by complement-dependent cytotoxicity against syngeneic spleen cells.[35] Thus, self-reactive B cells could be activated *in vivo* by PPD. The authors believe that the capacity of self-reactive B cells to secrete antibodies reflects the polyclonal activating capacity of the ac-

tivator used, since a weaker PCA such as dextran sulfate was a weak inducer of the autoantibody response.[35] This novel aspect of B-cell activation was shown to occur *in vitro* as well. PPD could induce the formation of autoantibodies by bovine spleen cells to autologous RBCs.[36] Speculation was raised that perhaps these findings may have clinical significance in chronic infectious diseases caused by organisms having or releasing B-cell polyclonal activating substances and that the induction of autoantibody production might develop as a consequence.[36] In a study designed to examine the possible role of polyclonal B-cell activation in the induction of anti-DNA antibodies, Izui *et al.*[37] showed that the injection of PPD at high doses could induce anti-DNA antibodies but was less effective on a weight basis than LPS. Also PPD did not provoke the release of DNA in the circulating blood, whereas LPS was very active. Thus, Izui *et al.* concluded that the induction of anti-DNA antibodies, although a consequence of polyclonal B-cell activation, is not dependent on the release of DNA into the circulation. These findings conform to the work of Primi *et al.*,[35] who demonstrated that PBA could induce autoantibodies cytotoxic for syngeneic spleen cells and RBCs. Thus, it would appear that bacterial polyclonal activators such as PPD and LPS have a role in the induction of at least some autoantibodies and that perhaps bacterial infections may play a part in the development of autoimmunity.

The *in vitro* adjuvanticity of PPD also has been demonstrated.[17] PPD has been found to enhance the primary immune response to heterologous RBCs by splenic lymphocytes cultured from normal unimmunized mice. The enhanced response was specific for the antigen added and adherent cells were required for the synergistic effect between antigen and PPD. Interestingly, in comparison with other B-cell mitogens such as LPS and SIII, PPD was found to be the most effective agent in increasing the primary immune response *in vitro.*[17]

PPD and other polyclonal activators not only have been shown to enhance antibody responses both *in vitro* and *in vivo,* but recently these materials have been reported to suppress the primary immune response to SRBC *in vivo.* When high doses of PPD were injected 1–3 days before immunization with SRBC, the response as measured by direct (IgM) and indirect (IgG) PFCs was suppressed.[38] In order to obtain an immunosuppressive effect, it was necessary to inject PPD in the absence of the test antigen. When PPD was given with the antigen, enhancement of the immune response developed. Diamantstein *et al.*[38] offer the explanation that PPD and other B-cell mitogens, when introduced into an animal at the appropriate time, may lead to the emergence of a cell population that has lost its ability to respond to a specific antigen.

It would seem apparent from the accumulated evidence that the property of PPD to nonspecifically activate B cells has become useful for

studying various aspects of the immune response both at the level of the cell and of the whole organism.

2.5. Polyclonal Activation of Human Lymphocytes

The use of polyclonal activators as a means of stimulating human lymphocytes to proliferate or differentiate has been extensive. Particular emphasis has been directed to the secretion and regulation of immunoglobulin synthesis stimulated by PBAs. Chief among the PBAs are bacterial products which have been shown to be quite potent for murine lymphocytes including LPS endotoxin, staphylococcal protein A, dextran sulfate, Nocardia water-soluble mitogen (NWSM), pneumococcal polysaccharide type III, selected outer membrane proteins of gram-negative bacteria such as endotoxin-associated protein (EP) and the membrane lipoprotein, and finally PPD tuberculin. At one time or another, all have been tested on human blood lymphocytes or spleen and lymph node cells with various degrees of success. For a review of this topic, the reader may find the article by Waldmann and Broder[39] of interest.

In the particular case of PPD, the interpretation of the results of such studies is complicated by the source of the lymphocytes and the culture conditions used. Depending on geographic factors, the cells may be drawn from individuals previously sensitized to PPD by immunization with the BCG vaccine and either skin-test negative or positive to tuberculin or from individuals not so immunized and tuberculin negative by skin test. In addition, variables such as the concentration of PPD; length of time of the cultures; whether the culture media included fetal calf serum, human serum, or plasma; and finally whether the lymphocytes were derived from the blood, spleen, or lymph nodes all have been shown to affect the results. For example, Nilsson[40] reported that blood lymphocytes from tuberculin negative persons were significantly activated to proliferate when cultured with relatively high concentrations of PPD for six days. These results have been confirmed by Closs[41] and Janicki *et al.*,[42] who showed that tuberculoprotein antigens at low concentrations in contrast to tuberculopolysaccharides stimulated the transformation of lymphocytes from tuberculin-negative volunteers, although to a lesser extent than lymphocytes obtained from tuberculin-positive individuals. Again, at high concentrations of PPD, Ringden *et al.*[43] reported the stimulation of polyclonal antibody and DNA synthesis in spleen, tonsil, adenoid, and mesenteric lymph nodes cells. Using the sensitive protein A plaque assay, Hammarstrom *et al.*[44] demonstrated that PPD was even more effective than LPS in generating PFC of different subclasses from peripheral blood lymphocytes, including IgM, IgG,

and IgA. In addition, in this same study, a marked proliferative response was stimulated by PPD. However, it is important to note that the cells used in the reports cited above were from Swedish adults who had been routinely immunized as infants with BCG, so that there may have been some contribution from the reaction of specifically sensitized cells as well as the transformation of nonimmune lymphocytes. In this regard, PPD-induced polyclonal immunoglobulin production has also been found in lymphocytes drawn from Japanese individuals who were tuberculin positive.[45] In this study, the polyclonal activation of B cells was shown to be dependent on T-helper cell activity. These results support the findings by Blomgren[46] that PPD selectively activates T cells in the blood of BCG-immunized donors, so that T-cell factors can subsequently activate B cells. By contrast, Waldmann and Broder[39] reported that PPD at high concentrations was ineffective in stimulating IgG, IgM, and IgA synthesis by peripheral blood mononuclear cells drawn from normal persons who had not been previously vaccinated with BCG. Nevertheless, these investigators point out that there may be differences in the reactivity of subpopulations of B cells that are found distributed differently in the blood and lymphoid organs. For example, Fauci and Pratt[17] were able to induce PBA in cultured human tonsillar lymphocytes with PPD as well as LPS and keyhole limpet hemocyanin, as measured by antibody-forming cells to SRBC. In fact, PPD was the most potent material on a weight basis. Other B-cell mitogens, such as pneumococcal polysaccharide SIII and dextran sulfate, were inactive. Complicating the picture still further is the finding by Soren[47] that suppressor cells may be stimulated by PPD in culture, which may dampen the polyclonal response.

Culture conditions may also play a role in whether PPD activates lymphocytes. Matthews *et al.*[48] found that when unfractionated peripheral blood leukocytes were used, human plasma was essential for PPD to stimulate DNA synthesis. Human serum was less effective, and fetal calf serum was ineffective. When the lymphocytes were separated by Ficoll-Hypaque gradients which now is done routinely, serum supplementation worked so that in those studies cited above, by Waldmann and Broder, it is unlikely the culture conditions were a factor. Nevertheless, consideration of such variables in the analysis of the results obtained with PPD and human lymphocytes remains critical.

2.6. Genetic Effects on Lymphocyte Responses to PPD

Limited research devoted to the genetics of the lymphocyte responses to PPD as a polyclonal activator has been reported. The classic endotoxin-hyporesponsive C3H/HeJ mouse strain responds normally to

PPD. By using this strain for interbreeding experiments, it has been found that the mitogenic responsiveness to LPS is governed by a single gene locus (*lps*) composed of codominant alleles.[49] Furthermore, Watson *et al.*[50] reported that the *lps* locus regulates a number of immunologic reactions to the lipid A of LPS, including polyclonal and adjuvant responsiveness. Thus, the deficient LPS response in the C3H/HeJ strain is highly selective, serving to underscore the different nature of the polyclonal response of B cells to PPD and various outer membrane proteins of the gram-negative bacteria as well.[49]

In an attempt to define, if possible, a genetic pattern that controlled the polyclonal response to PPD, 15 inbred strains derived from several families of mice were tested for the response of their lymphocytes to the mitogenic effect of PPD.[16] No true low or nonresponder strain was found, although there were some quantitative differences found between the strains, as might be expected. In addition, young mice (neonates to weanlings) responded as well as adults and sex had no influence. In a study by Stiffel *et al.*[51] designed to accomplish a genetic selection for PHA mitogenic responsiveness, both a high- and a low-responder line were separated after six consecutive generations of selective breeding. However, PPD and LPS stimulated the proliferation of the lymph node lymphocytes from these lines equally as well. Also, the results of experiments obtained with PHA and these two lines demonstrated that the responsiveness to PHA was under polygenic control, in contrast to the responsiveness of B cells to their mitogens as expressed by LPS, clearly indicating that the genetic control of the response to T- and B-cell mitogens is unrelated.

Another inbred mouse strain that has received considerable attention because of its marked defect in antibody formation to T-independent antigens, including type III pneumococcal polysaccharide (SIII) and LPS is the CBA/N strain.[52,53] Genetic studies have demonstrated that this defect is inherited as an X-linked recessive trait. In other words, when CBA/N females are crossed to normal male mice, the F1 male progeny are unable to form antibody to SIII, whereas the F1 female progeny form normal amounts of antibody. This X-linked mutation affects a number of B-cell functions. For example, young CBA/N and F1 male mice (3–6 weeks) are relatively low responders to the mitogenic effect of LPS, but the response of B cells from old mice (31–52 weeks) is considerably higher.[54] In experiments using PPD, both the mitogenic and polyclonal antibody responses of the B cells of CBA/N mice were clearly age dependent. Young mice were low responders, whereas aged mice were high responders. Taken together, these results clearly show that the B-lymphocyte defect in this mouse is due to a delay in maturation. In an independent study, Nariuchi and Kakiuchi[55] showed, fur-

thermore, that this hyporesponsiveness was not due to a shift in the dose response or in the kinetics of the responding cells nor to a defect in T cell or macrophage function in the CBA/N strain.

2.7. Other Nonspecific Activities Stimulated by PPD

Polyclonal activators including PPD have been explored for their potential to stimulate a number of different lymphocytes activities besides proliferation and antibody synthesis. Stejskal *et al.*[56] showed that normal CBA mouse lymph node cells could be activated in culture by PPD to be cytotoxic for mouse L cells but not for human live cells (Chang cells). This cytotoxic effect correlated with increased DNA synthesis. Wlodarski *et al.*[57] reported that PPD could induce endogenous spleen colony formation when given subcutaneously in sublethally irradiated mice. This effect was obtained irrespective of whether PPD was given before or after irradiation. Endogenous C-type viruses of mice have been induced in cultures of spleen cells exposed to PPD from a variety of inbred strains.[58] This release of virus emanates from B cells and can be induced by LPS as well, although the genetically deficient B cells of the C3H/HeJ strain do not respond to LPS. Nevertheless, they do contain C-type viruses, as shown by their release when stimulated by PPD.

3. *MYCOBACTERIUM BOVIS*

3.1. Activation of Normal Lymphocytes by Whole Cells and Their Fraction

During the early 1970s BCG received considerable attention because of its property as an immunologic adjuvant and its potential usefulness in cancer immunotherapy. In this context, BCG was used in the experimental treatment of tumors, for the immunotherapy of leukemia and was considered as a method of immunoprophylaxis for tumors. However, there was no understanding of how BCG worked and in the first attempt to gain some insight into the effects of BCG at the cellular level, Mokyr and Mitchell[59] investigated the activation of normal lymphoid cells by BCG. Whole BCG cells were found to stimulate DNA synthesis of C3H/J mouse spleen cells and thymocytes in culture. However, the activation of the thymocytes was dependent on the presence of macrophages, although high levels of these adherent cells actually depressed the DNA synthesis of the splenic lymphocytes. At the same time, BCG stimulated splenic macrophages to produce lymphocyte activating factor (Interleukin-I) which has a direct effect on T-cell proliferation.

Further experiments to define the components of BCG that were responsible for activating these cells revealed that a water-insoluble delipidated cell fraction was as active as BCG on splenic lymphocytes, but less so on the thymic lymphocytes of C3H mice.[60] By contrast, a lipid fraction obtained from BCG by chloroform-methanol extraction was relatively ineffective as a mitogen. Nevertheless, the methanol-extracted residue (MER) from mycobacteria originally described by Weiss and Wells[61] was found to be a potent mitogen for both splenic and thymic lymphocytes, but none of these fractions was as active as whole BCG cells in stimulating IL-1 from splenic macrophages.

In a similar set of experiments, Bekierkunst[62] showed that not only BCG but killed cells of *M. kansasii* and *M. smegmatis* activated DNA synthesis in C3H mouse spleen cells. Interestingly, thymocytes were not stimulated directly by these organisms but with appropriate mixtures of thymocytes and spleen cells, a relatively small but significant synergistic effect was obtained, most likely reflecting the need for the interaction of macrophages and thymocytes.[59] Bekierkunst[62] also tested the cell walls of *M. tuberculosis* H34Ra, the delipidated and deproteinized walls, cord factor (trehalose-6,6′ dimycolate) and the neo water-soluble adjuvant (NWSA), which is a peptidoglycan linked to an arabinogalactan extracted from *M. smegmatis.* Neither NWSA nor cord factor was active, whereas the H37Ra cell wall preparation was a potent mitogen for mouse splenic lymphocytes. As might be expected, the delipidated and deproteinized H37Ra cell walls were much less active, indicating the removal of mitogenic components such as the tuberculin proteins.

The MER of BCG was studied in detail by Ben-Efraim and Diamantstein[63] with respect to its mitogenic and adjuvant activity in mice. MER activated DNA synthesis in Balb/c spleen cells and nu/nu mice which lack functional T cells. This polyclonal mitogenic effect was not dependent on macrophages. Bone marrow and lymph node cells were marginally stimulated, whereas MER was inactive on thymocytes, most likely because of the critical need for macrophage interaction in the case of T-cell activation by mycobacterial products. Activation of antibody synthesis was also demonstrated in cultures of BALB/c and nude spleen cells by MER, as measured by PFCs to SRBCs and TNP–SRBC; i.e., the primary immune response was significantly enhanced, comparing favorably to the *in vitro* adjuvant effect of both PPD and LPS. Likewise MER was found to be active on guinea pig splenic lymphocytes as a polyclonal mitogen.[64] However, spleen cells depleted of B lymphocytes were not stimulated, although these T-enriched cells responded to PHA and Con A, demonstrating that the proliferation obtained was restricted to the B-cell compartment. By contrast, guinea pig spleen cells did not respond with polyclonal antibody synthesis to MER, LPS, or PPD. This may be a

reflection of the fact that guinea pig cells are weak responders to T-independent antigens, many of which are B-cell mitogens, whereas mice that are good responders have spleen cells that are readily activated to polyclonal antibody synthesis by these materials.

Cell-wall preparations from BCG were compared with similar preparations from *Nocardia rubra* and various species of *Corynebacterium* and *Propionibacterium* by Azuma *et al.*[65] in terms of their mitogenic activity for T and B cells from mice. All these chemically complex preparations were active on B- and T-enriched cells as well as the lymphocytes of the congenitally athymic (nu/nu) mice. Macrophage-deleted spleen cells were activated by BCG cell walls, although the depletion was not extensive and residual macrophages were undoubtedly present. Nevertheless, these results support the earlier studies reported above.

Another approach to characterize the immunologic response to BCG was taken by Baker *et al.*,[66] who prepared a water-soluble fraction of sonically disrupted *M. bovis* (BCG) cells containing a mixture of protein, mucopolysaccharides, and polysaccharides. This soluble sonicate activated the proliferation of normal mouse spleen cells *in vitro* and enhanced the PFC response to SRBC in culture and *in vivo*.

3.2. Activation of Lymphocytes from BCG-Infected Mice

The work discussed so far has dealt with the activation of lymphocytes from normal animals by mycobacteria in the form of whole cells or fractions derived from these organisms by one means or another. One question that naturally follows is: What role does nonspecific activation of the immune system play during the *in vivo* sensitization of the host infected with the tubercle bacillus? For example, it is reasonable to assume that B cells are activated in such circumstances in view of the appearance of both specific antibody to tuberculosis antigens and elevated levels of antibody to nontuberculosis antigens. Furthermore, since normal B cells can be activated by tuberculin to produce lymphokines, it is possible that nonspecific activation could participate in the cell-mediated immune reaction consequent to sensitization of the host by products of the tubercle bacillus.

In a series of experiments designed to determine how infection with BCG affects the responsiveness of lymphocytes to PPD as well as B- and T-cell mitogens, Sultzer[67] found that the type of response obtained depended on how BCG was given mice and the organ source of the lymphocytes. The proliferation of splenic lymphocytes was depressed in the presence of PPD *in vitro* when BCG was given intravenously, as compared with such cells taken from uninfected mice. Likewise, the stimulation of DNA synthesis in T cells from the spleens of BCG-infected mice

was depressed when they were cultured with Con A; however, the response of splenic B cells to LPS was enhanced. All these cell types were cultured as mixtures. When the spleen cells were then fractionated, however, B-enriched lymphocytes were more reactive to PPD, and T-enriched cells were more reactive to Con A, suggesting that suppressor cells were activated in the spleens of mice given BCG intravenously. This was not the case if BCG was given subcutaneously, since the splenic lymphocyte responses to PPD and Con A was unchanged, as compared with cells from uninfected animals. In addition, regardless of how the mice were infected with BCG, the lymph node T and B cells were enhanced in their response to PPD.

In a similar manner, washed killed whole BCG cells were used to determine whether other components of these organisms besides the tuberculin proteins would activate normal and sensitized lymphocytes *in vitro.* The results clearly showed that BCG acted as a B-cell mitogen for normal mouse lymphocytes and was inactive on splenic T cells or thymocytes. BCG also functioned to stimulate B-cell differentiation, as measured by polyclonal antibody synthesis using TNP–SRBC as an antigen. Furthermore, when sensitized lymph node cells were taken from BCG-infected mice, a dramatic increase in proliferation occurred when these cells were cultured with BCG, PPD, or LPS. This result suggested that an expansion of various subpopulations of B cells probably occurred within the regional lymph nodes of the infected mice either by recruitment, trapping, or proliferation *in situ,* so that proportionately more B cells were available in culture to be stimulated. In this regard, Meyer *et al.*[68] reported the results of an autoradiographic quantification of the T- and B-cell areas in the spleens and lymph nodes of mice given BCG intraperitoneally. Interestingly, the B-cell areas in the spleen increased, whereas the T-cell areas appeared to be significantly reduced. This may explain the enhanced B-cell proliferative response in cultures when these cells are separated from suppressor cells as described above. By contrast, they also showed that a doubling in size of the T-cell areas of the lymph nodes occurred, which also correlates with the enhanced *in vitro* proliferative response of lymph node cells to PPD.

The induction of suppressor activity by BCG, particularly when high doses are given in a systemic fashion, is discussed at greater length in another chapter of this volume. However, as such suppression applies to polyclonal mitogenic effects, a brief summary is appropriate here. Mitchell *et al.*[69] reported a decreased responsiveness of nonadherent spleen cells (T) to Con A in mice given BCG IV and likewise a decreased response to LPS in unfractionated spleen cells. Geffard and Orbach-Arbouys[70] showed that a lower reactivity of spleen cells from BCG-treated mice in a mixed lymphocyte reaction was at least partly due to a

nonspecific suppression by T cells. In a series of investigation by Turcotte and colleagues,[71–73] it was shown that BCG infection induced either suppression or enhancement of the response of mouse lymphocytes to T- and B-cell mitogens. Their evidence indicated two populations of suppressor cells, including T cells and macrophages. Moreover, both cell types elaborated mediators of suppression in the supernatants of their respective cultures. Klimpel[74] also demonstrated that supernatants from BCG-treated splenic macrophages could inhibit the *in vitro* response of normal mouse C57BL/6 spleen cells to SRBC. Suppressor macrophages from BCG-treated mice were also found to depress both T- and B-mitogen-induced spleen cell DNA synthesis in this strain, but this suppression was not detected in CBA/J mice.[75,76] Consequently, genetic differences exist in the induction by BCG of suppressor cell activity. Finally, Collins and Watson[77] showed that BCG given IV to specific pathogen-free mice in high doses produced a severe depression in the response of spleen cells to both PHA and PPD. This effect was time dependent, appearing about 4 weeks after the BCG was given and persisting as long as 4 months. Again, T cells were implicated in this phenomenon, since anti-θ treatment of the spleen cells abrogated the depressed proliferative response.

4. MURAMYL DIPEPTIDES

Since the isolation, identification, and synthesis of the muramyl dipeptides such as *N*-acetylmuramyl-L-alanyl-D-isoglutamine and its analogues, a considerable literature has developed pertaining to their varied and intriguing biological properties. MDP was the first adjuvant derived from the mycobacterial cell-wall peptidoglycan to be synthesized and shown to be the minimal structure that could substitute for the mycobacteria in Freund's complete adjuvant. In addition, MDP can enhance nonspecific resistance to infection, is pyrogenic and can regulate humoral and cellular immune responses. Because of its simple structure, MDP has offered an attractive opportunity to relate the mechanisms of immune regulation to its chemical structure at the cellular level. However, it is not the intention of this review to cover the broad field of MDP research but rather the discussion will focus on the activity of MDP and its analogues as a mitogen and polyclonal activator.

4.1. Mitogenicity of MDP

Purified peptidoglycans from various gram-negative and gram-positive bacteria as well as from the mycobacteria have been shown to

enhance antibody synthesis and to induce DTH when incorporated in Freund's complete adjuvant as a replacement for the mycobacterial cells. Damais *et al.*[78] showed that such peptidoglycan preparations from *Bacillus megatherium* and *Escherichia coli* were mitogenic for rabbit spleen cells, AKR mice, and the spleen cells of congenitally athymic nu/nu mice. However, a monomer of the *E. coli* peptidoglycan, which was an adjuvant, did not activate these cells in culture, suggesting that a repetitive structure might be a critical requirement for mitogenicity. Likewise, Ciorbaru *et al.*[79] found that *Nocardia* peptidoglycans solubilized by peptidases were mitogenic for the same types of lymphocytes (B cells), but lysozyme digestion produced inactive fractions. Ciorbaru and co-workers suggested that the activity therefore resided in glycan strands substituted by tetra- or tripeptides obtained by the peptidase digestion, whereas the mixture of oligosaccharides substituted by partly crosslinked peptides obtained by lysozyme treatment was incapable of stimulating lymphocyte proliferation.

The first reports of the mitogenic activity of MDP were somewhat conflicting. Azuma *et al.*[80] examined *N*-acetylmuramyl dipeptide and 6-*O*-stearoyl-*N*-acetylmuramyl dipeptide for their ability to stimulate DNA synthesis in C57BL/6J mouse spleen cultures but found no activity at a range of concentrations. The culture conditions included the use of 10% fetal calf serum (FCS) and a 48-hr culture period. By contrast, Igarashi *et al.*[81] used DBA/2 spleen cells, high concentrations (100 μg) of MDP or several analogues, and a culture period of 72 hr with 10% FCS, and obtained low levels of [^{3}H]-Tdr uptake. Specter *et al.*[82] showed that MDP could stimulate low levels of DNA synthesis in BALB/c spleen cells after 48 hr of culture as well as 2–3 times more background PFC to SRBCs after 5 days of culture. Subsequently, Damais *et al.*[83] emphasized that both culture conditions and genetic factors were determinants in the nonspecific activation of mouse spleen cells by MDP. Optimal responses (fivefold increases) in [^{3}H]-Tdr uptake took 4–5 days instead of 2–3 days seen with the classic B-cell mitogen, LPS. FCS at concentration of 10% abolished DNA synthesis, although this did not affect the stimulation induced by LPS, NWSM, or peptidoglycan from *B. megatherium.* That various levels of activation can be obtained in mouse spleen cell cultures stimulated by mitogens has been shown to depend on various lots of FCS that are used, so it is not surprising that results vary from different laboratories.[84]

From the genetic viewpoint, it is clear that mouse strains differ in their responsiveness to MDP, as is the case with other mitogens such as LPS. For example, DBA/2, BALB/c and BALB/c-nu/nu mice have cells that respond to MDP, whereas C6AK mice are weak responders, if at all. In another study, by Damais *et al.*,[85] 14 synthetic MDP analogues were

tested for mitogenicity, using cells from a variety of mouse strains. The results obtained in serum-free medium again emphasized the genetic differences in the response to MDP. C57Bl/6 and C3H/He mice did not respond, but AKR, DBA/2, BALB/c CBA, and (C57Bl/6 × AKR)F1 hybrids did. Furthermore, those MDP analogues that were active as adjuvants (8) were mitogenic, whereas those that were inactive as adjuvants (6) were nonmitogenic. Similar results were obtained using guinea pig spleen cells from individual animals.

Wood and Staruch[86] also conducted a systematic investigation on the mitogenicity of MDP to determine what factors influenced this activity. First, they found that MDP had to be in contact with the spleen cells for at least 6 hr for activation to occur. B cells were the responders. T-cell proliferation was insignificant and did not appear to act as helper cells for B-cell division. From depletion and reconstitution experiments, macrophages were found to function as accessory cells. Genetic experiments indicated that a dissociation existed between the mitogenic response and the adjuvanticity stimulated by MDP and that the LPS gene was not closely linked to the gene(s) controlling the mitogenic response to MDP. Given these results and the fact that the intensity of the mitogen response did not correlate with the intensity of the adjuvant response to bovine serum albumin, Wood and Staruch concluded that the activity of MDP as a mitogen does not make a significant contribution to its adjuvanticity.[86]

4.2. Polyclonal Antibody Synthesis Stimulated by MDP

In continuing studies to elucidate the mechanism of action of MDP at the cellular level, polyclonal antibody synthesis was investigated at some length in a number of laboratories. Specter *et al.*[87] showed that MDP could enhance the PFC response of normal BALB/c mouse spleen cells to SRBC equivalent to an *E. coli* LPS, as well as adjuvant, the antibody response when SRBC were added to the culture. Watson and Whitlock[88] provided evidence that MDP substituted for T-helper cells in the *in vitro* primary immune response to the T-dependent antigen SRBC; moreover, this effect was antigen dependent. Therefore, they concluded that the relatively weak mitogenicity of MDP obtained under the right cultural conditions was not a factor in its adjuvanticity. Confirmatory studies by Leclerc *et al.*[89] demonstrated that adjuvant active analogues of MDP were active as polyclonal activators of antibody synthesis by B cells, whereas those analogues that were inactive *in vivo* were less effective PBAs. Cell viability not due to cell proliferation was enhanced by MDP. Background PFC to both thymus-dependent and thymus-independent antigen were stimulated by MDP in athymic nu/nu spleen cell

cultures,[90] and macrophage depletion did not alter the response to syngeneic bromelain-treated RBCs (br-MRBC) or the autoantigen mouse albumin.[91] However, it should be noted that rigorous exclusion of the macrophages was not accomplished in these experiments, a notoriously difficult task without depleting a substantial number of B cells.

In a survey study of polyclonal B-cell activation by cell-wall preparations from various gram-positive bacteria, Saito-Taki *et al.*[92] also chose to examine MDP using athymic nu/nu mice and their normal counterpart nu/+ mice. As compared with the cell-wall preparations and LPS, the number of PFC obtained against SRBC and TNP-SRBC was 50–90% less, but the PBA induced by MDP in BALB/c cultures compared favorably with both LPS and the bacterial cell walls. More recently, Saito-Taki *et al.*[93] reported that the elimination of T cells and macrophages significantly reduced the PBA of MDP. Supplementation of the purified B-cell cultures with the supernatants of MDP stimulated T-cell cultures restored the response. Recombinant interleukin-2 (IL-2) partially restored the PBA response. By contrast, macrophage supernatant was not as effective, and IL-1 had no effect at all. Nevertheless, the addition of IL-1 and IL-2 to the purified B-cell cultures resulted in full restoration of the PBA response to MDP, which argues for the necessity of T cells and macrophages or their soluble products in order for MDP to nonspecifically activate B cells to differentiation and antibody synthesis. These interesting results await confirmation.

Another intriguing discovery as it pertains to the effect of MDP on polyclonal activation was reported by Lowy *et al.*[94] Paradoxically, MDP was shown to inhibit the stimulation of polyclonal antibody synthesis to TNP–SRBC or anti BR–MRBC by LPS or NWSM. MDP was effective in suppressing the PBA response when added simultaneously or 24 hr after the LPS or NWSM. However, the DNA synthesis activated by these mitogens was actually increased, indicating that the suppression resided in the B-cell differentiation steps, and not the initial proliferative events. Whether this suppression induced by MDP was a direct effect on B cells or involved the activation of suppressor macrophages or T cells by the combination of these compounds remains to be determined.

Finally, the effect of MDP on human lymphocytes has been studied by Bahr *et al.*[95] Peripheral blood lymphocytes from normal volunteers were cultured with MDP as well as its adjuvant inactive sterioisomer MDP (D-D) and the nonpyrogenic adjuvant active analogue, MDP-butyl ester (MDP-BE). Neither DNA synthesis nor immunoglobulin synthesis was induced by these compounds. MDP and MDP-BE, however, did enhance IgA or IgM secretion induced by pokeweed mitogen (PWM). By contrast, MDP could suppress the [^{3}H]-Tdr uptake of T cells induced by optimal concentrations of Con A and was able to reverse or enhance

the lower responses to supraoptimal concentrations of Con A. Clearly, MDP can regulate the responses of human peripheral blood lymphocytes but does not directly activate these cells. This lack of direct stimulation contrasts with the results obtained with mouse lymphocytes; however, it is essential to recall that all the studies with the mouse dealt with spleen cells. Human spleen cells have not been tested with MDP. An analogous situation exists with LPS. Protein-free LPS is essentially inactive on HPBL but stimulates human tonsillar and spleen cells. The cultural conditions used for the human lymphocyte experiments may be critical as well. High concentrations of FCS or autologous serum were used by Bahr *et al.*, conditions that can suppress lymphocyte activation in mouse cell cultures. Whether MDP can activate human lymphocytes from sources other than the blood and how it can regulate the proliferation of T cells and B-cell antibody synthesis induced by the appropriate stimulants are questions waiting to be addressed.

5. VARIOUS MYCOBACTERIAL COMPONENTS

As a forerunner to the discovery of the muramyl dipeptides, research from the laboratory of Edgar Lederer, as reported by Adam *et al.*,[96] described the isolation and adjuvant properties of a water-soluble fraction (WSA) from the cell walls of *M. smegmatis.* WSA contained typical cell-wall constituents, including amino sugars, neutral sugars, and amino acids in an oligomeric form. No mycolic acids were detected. Subsequent experiments described by Chedid *et al.*[97] indicated that WSA was effective in enhancing the immune response to viruses in the absence of toxic reactions. WSA was devoid of pyrogenicity and lacked toxicity in adrenalectomized mice. At the cellular level, Modolell *et al.*[98] found WSA to be an adjuvant, increasing the PFC response to SRBC and other antigens in spleen cell cultures from a variety of mouse strains. However, there was no indication that this adjuvant activity was due at least in part to polyclonal activation. No increase in background PFC to SRBC or DNP–SRBC occurred, nor was there any evidence of cellular proliferation at either low or high concentrations of WSA in the presence of low concentrations of FCS.[98] Given the probability that muramyl dipeptides were components of WSA to some degree and that such materials are relatively weak polyclonal activators, it is likely that the lack of polyclonal responses induced by WSA may have been due to low concentrations of these components in this extract.

In addition to the constituents of WSA, the cell walls of mycobacteria are rich in complex lipids. As reviewed by Goren,[99] the mycobacterial lipids consist of a variety of compounds, such as the toxic glycolipids known as cord factor and the sulfolipids, the peptidoglycolipids (Wax D),

several phospholipids, the mycosides, and mycolic acids. Of these, cord factor (trehalose mycolate) and Wax D have been shown to be adjuvants to various degrees in mice, rats, and guinea pigs.[100] While cord factor has been shown to increase nonspecific resistance to bacterial infections by Parant *et al.*[101] and to increase the phagocytic function of the reticuloendothelial system in mice by Saito *et al.*,[102] it does not appear to be active as a polyclonal activator of cell proliferation.[62] By contrast, a number of other mycobacterial lipids and cell-wall constituents were examined for these properties in spleen cell cultures of BALB/c and athymic mice.[103] The preparations included Wax D, mycolic acid, glycopeptides, and polysaccharide II. None of the adjuvant-active glycopeptides or polysaccharides was active as mitogens or as PBAs. However, the lipid-rich peptidoglycolipid compounds were mitogenic for nu/nu mouse spleen cells but not thymocytes from BALB/c mice. Rook and Stewart-Tull concluded that Wax D was a B-cell mitogen and as well as a PBA, given the increased PFC to TNP–SRBC when cells from both nu/nu and BALB/c spleen cells were cultured with Wax D. When the arabinogalactan and mycolic acid moieties were removed from the Wax D, these activities were lost, although pure mycolic acid was a weak mitogen at best, indicating that the carbohydrate moiety was also essential. Furthermore, Wax D stimulated the release of lymphocyte-activating factor (IL-1) from peritoneal macrophages, a property not shared by the glycopeptides or polysaccharide II. Thus, Wax D resembles the LPS from gram-negative bacteria in its ability to activate proliferation and differentiation of B cells and stimulate the production of IL-1 from macrophages.

6. SUMMARY AND CONCLUSIONS

From the foregoing, it is clear that the tubercle bacillus and several of its defined components are capable of activating lymphocytes in a polyclonal fashion. The tuberculoproteins in the form of PPD and the peptidoglycolipids (Wax D) stimulate B lymphocytes to proliferate and differentiate into antibody-producing cells. The peptidoglycan of the tubercle bacillus and other bacteria can act as mitogens. The muramyl dipeptides originally derived from the mycobacterial cell wall peptidoglycan and now available along with numerous analogues as synthesized products are also polyclonal B-cell activators. In general, those derivatives of MDP that are adjuvants act as PBAs, whereas those that are nonadjuvants cannot nonspecifically activate lymphocytes. However, the polyclonal activity expressed by MDP is by and large less than that seen with most other PBAs, requires special cultural conditions for optimal results, and exhibits genetic restrictions. Also, in contrast to PPD and mycobacterial cells, recent evidence has been reported indicating

that the polyclonal activation of B cells requires the participation of accessory cells in the form of macrophages and T cells. This participation may be mediated by the respective products of these cell types, including both IL-1 and IL-2.

Although most of these studies on polyclonal activation have been accomplished by *in vitro* experiments with murine and human cell systems, there is evidence that this phenomenon may take place *in vivo* when animals are either infected with the tubercle bacillus or given PPD tuberculin. That the host's cells are altered in their response to PBAs, including PPD, after injection with mycobacteria is also apparent. Indeed, in this situation, not only is lymphocyte proliferation enhanced, but under the appropriate circumstances, suppression of polyclonal activation can take place.

With these considerations in mind, it is reasonable to speculate that in the natural process of infection with the tuberculosis organism, both nonspecific as well as specific activation of the immune system occurs as a result of the various constituents of the mycobacteria that act in concert as polyclonal activators. As a consequence, some untoward effects may emerge, such as immune suppression or autoantibody production. Whether polyclonal lymphocyte activation in some manner contributes to the disease process we know as tuberculosis is another intriguing question that remains to be answered.

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REFERENCES

1. Nowell, P. C., 1960, Phytohemagglutinin: An initiator of mitosis in cultures of normal human leukocytes, *Cancer Res.* **20:**462–466.
2. Sultzer, B. M., and Goodman, G. W., 1976, Endotoxin protein: A B-cell mitogen and polyclonal activator of C3H/HeJ lymphocytes, *J. Exp. Med.* **144:**821–827.
3. Morrison, D. C., Betz, S. J., and Jacobs, D. M., 1976, Isolation of a lipid A bound polypeptide responsible for "LPS-initiated" mitogenesis of C3H/HeJ spleen cells, *J. Exp. Med.* **144:**840–846.
4. Goldman, R. G., White, D., and Lieve, L., 1981, Identification of outer membrane proteins, including known lymphocyte mitogens as the endotoxin protein of *Escherichia coli* 0111, *J. Immunol.* **127:**1290–1294.
5. Wood, C. D., and Moller, G., 1984, Influence of RV 41.740, a glycoprotein extract from *Klebsiella pneumoniae,* on the murine immune system. I. T-independent polyclonal B cell activation, *J. Immunol.* **132:**616–621.
6. Bessler, W. G., Cox, M., Lex, A., Suhr, B., Weismuller, K-H., and Jury, G., 1985, Synthetic lipopeptide analogs of bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes, *J. Immunol.* **135:**1900–1905.

7. Dziarski, R., 1980, Polyclonal activation of immunoglobulin secretion in B lymphocytes induced by staphylococcal peptidoglycan, *J. Immunol.* **15:**2478–2483.
8. Greaves, M. F., Owen, J. J. T., and Raff, M. C., 1974, *T and B lymphocytes: Origins, Properties and Roles in Immune Responses,* pp. 81–82, American Elsevier, Inc., New York.
9. Rocklin, R. E., MacDermott, R. P., Chess, L., Schlossman, S. F., and David, J. R., 1974, Studies on mediator production by highly purified T and B lymphocytes, *J. Exp. Med.* **140:**1303–1316.
10. Mackaness, G. B., Auclair, D. J., and Lagrange, P. H., 1973, Immunopotentiation with BCG. I. Immune response to different strains and preparations, *J. Natl. Cancer Inst.* **51:**1655–1667.
11. Miller, T. E., Mackaness, G. B., Lagrange, P. H., 1973, Immunopotentiation with BCG. II. Modulation of the response to sheep red blood cells, *J. Natl. Cancer Inst.* **51:**1669–1676.
12. North, R. J., 1973, Importance of thymus-derived lymphocytes in cell-mediated immunity to infection. *Cell Immunol.* **7:**166–176.
13. North, R. J., Mackaness, G. B., and Elliott, R. W., 1972, The histogenesis of immunologically committed lymphocytes. *Cell. Immunol.* **3:**680–694.
14. Sultzer, B. M., and Nilsson, B. S., 1972, PPD-tuberculin—a B-cell mitogen, *Nature New Biology* (London). **240:**198–200.
15. Nilsson, B. S., Sultzer, B. M., and Bullock, W. W., 1973, Purified protein derivative of tuberculin induces immunoglobulin production in normal mouse spleen cells, *J. Exp. Med.* **137:**127–139.
16. Sultzer, B. M., Nilsson, B. S., and Kirschenbaum, D., 1977, Nonspecific stimulation of lymphocytes by tuberculin, *Infect. Immun.* **15:**799–806.
17. Kreisler, J. M., and Moller, G., Effect of PPD on the specific immune response to heterologous red cells *in vitro, J. Immunol.* **112:**151–161.
18. Coutinho, A., and Moller, G., 1975, Thymus-independent B-cell induction and paralysis, *Adv. Immunol.* **21:**113–236.
19. Fauci, A. S., and Pratt, K. R., 1976, Activation of human B lymphocytes. I. Direct plaque-forming cell assay for the measurement of polyclonal activation and antigenic stimulation of human B-lymphocytes, *J. Exp. Med.* **144:**674–684.
20. Lipsky, P. E., Rosenthal, A. S., 1976, The induction and regulation of guinea pig B-lymphocyte proliferation *in vitro, J. Immunol.* **117:**1594–1602.
21. Jacobs, D. M., and Morrison, D. C., 1975, Dissociation between mitogenicity and immunogenicity of TNP-lipopolysaccharide, T-independent antigen, *J. Exp. Med.* **141:**1453–1458.
22. Palmer, D. W., and Smith, R. T., 1974, Augmentation of PPD and LPS-induced T independent DNA synthesis in normal mouse spleen cells by leukocyte lysates from tuberculosis patients, *Cell. Immunol* **13:**196–206.
23. Diamantstein, T., and Blitstein-Willinger, E., 1975, Relationship between biological activities of polymers. I. Immunogenicity, C3 activation, mitogenicity for B-cells and adjuvant properties, *Immunology* **29:**1087–1092.
24. Ben-Efraim, S., and Diamantstein, T., 1975, Mitogenic and adjuvant activity of a methanol extraction residue (MER) of tubercle bacilli on mouse lymphoid cells *in vitro, Immunol. Commun.* **4:**565–577.
25. Berbase-De Lima, M., Wilkinson, J., Smith, G. S., and Walford, R. L., 1974, Age-related decline in thymic-independent immune function in a long-lived mouse strain, *J. Gerontol.* **29:**261–268.
26. Moatamed, F., Karnovsky, M. J., and Unanue, S. R., 1975, Early cellular responses to mitogens and adjuvants in the mouse spleen, *Lab Invest.* **32:**303–312.
27. Gronowicz, E., Coutinho, A., and Moller, G., 1974, Differentiation of B-cells: Sequen-

tial appearance of responsiveness to polyclonal activators, *Scand. J. Immunol.* **3**:413–421.
28. Moller, G., 1974, Effect of B-cell mitogens on lymphocyte subpopulations possessing C'3 and Fc receptors, *J. Exp. Med.* **139**:969–982.
29. Kakiuchi, T., and Nariuchi, H., 1981, Responses of B cells with or without C3 receptor to polyclonal B cell activators, *J. Immunol.* **127**:954–958.
30. Gronowicz, E., and Coutinho, A., 1976, Heterogeneity of B-cells: Direct evidence of selective triggering of distinct subpopulations by polyclonal activators, *Scand. J. Immunol.* **5**:55–69.
31. Forman, J., and Moller, G., 1973, The effector cell in antibody-induced cell mediated immunity. *Transplant. Rev.* **17**:108–149.
32. Etlinger, H. M., Hodgins, H. O., and Chiller, J. M., 1976, Evolution of the lymphoid system. I. Evidence for lymphocyte heterogeneity in rainbow trout revealed by the organ distribution of mitogenic responses, *J. Immunol.* **116**:1547–1553.
33. Yoshida, T., Sonozaki, H., and Cohen, S., 1973, The production of migration inhibition by B and T cells of the guinea pig, *J. Exp. Med.* **138**:784–797.
34. Johnson, A. G., 1983, Adjuvant action of bacterial endotoxins on antibody formation, in: *Beneficial Effects of Endotoxins* (A. Nowotny, ed.), pp. 249–253, Plenum Press, New York.
35. Primi, D., Hammarstrom, L., Smith, E., and Moller, G., 1977, Characterization of self-reactive B-cells by polyclonal B-cell activators, *J. Exp. Med.* **145**:21–30.
36. Hammarstrom, L., Smith, E., Primi, D., and Moller, G., 1976, Induction of autoantibodies to red blood cells by polyclonal B-cell activators, *Nature* 263:60–61.
37. Izui, S., Kobayakawa, T., Zryd, M-J., Louis, J., and Lambert, P-H., 1977, Mechanism for induction of anti-DNA antibodies by bacterial lipopolysaccharides in mice, *J. Immunol.* **119**:2157–2162.
38. Diamantstein, T., Keppler, W., Blitstein-Willinger, E., and Ben-Efraim, S., 1976, Suppression of the primary immune response *in vivo* to sheep red blood cells by B-cell mitogens, *Immunology* **30**:401–407.
39. Waldmann, T. A., and Broder, S., 1982, Polyclonal B-cell activators in the study of the regulation of immunoglobulin synthesis in the human system, *Adv. Immunol.* **32**:1–63.
40. Nilsson, B. S., 1972, The response of lymphocytes from tuberculin-positive or negative humans to various doses of PPD-tuberculin *in vitro*. *Cell. Immunol.* **3**:493–500.
41. Closs, O., 1975, *In vitro* lymphocyte response to purified protein derivative, BCG, and *Mycobacterium leprae* in a population not exposed to leprosy. *Infect. Immun.* **11**:1163–1169.
42. Janicki, B. W., Aaron, S. A., Schechter, G. P., and McFarland, W., 1972, Tuberculin antigens active in human lymphocyte blastogenesis, *Proc. Soc. Exp. Biol. Med.* **141**:809–813.
43. Ringden, O., Rynnel-Dagoo, B., Kunori, T., Smith, C. I. E., Hammarstrom, L., Freijd, A., and Moller, E., 1979, Induction of antibody synthesis in human B lymphocytes by different polyclonal B cell activators: Evaluation by direct and indirect PFC assays, *Immunol. Rev.* **45**:195–218.
44. Hammarstrom, L., Bird, A. G., and Smith, C. I. E., 1980, Mitogenic activation of human lymphocytes: A protein A plaque assay evaluation of polyclonal B-cell activators, *Scand. J. Immunol.* **11**:1–13.
45. Nishikawa, S-I., Hirata, T., Nagai, T., Mayumi, M., and Izumi, T., 1979, PPD-induced immunoglobulin production in human peripheral blood lymphocytes, *J. Immunol.* **122**:2143–2149.

46. Blomgren, H., 1975, Role of B cells in the expression of human lymphocytes *in vitro, Scand, J. Immunol.* **4:**499–510.
47. Soren, L., 1979, Suppressor cells induced by purified protein derivative of tuberculin (PPD): The suppression is mediated by cells that proliferate in response to stimulation with PPD, *Scand. J. Immunol.* **10:**171–178.
48. Matthews, K. P., Pow, P. M., and Weisberg, S. C., 1977, Lymphocyte transformation by pollen extracts and purified protein derivative (PPD) in leukocyte cultures of normal human subjects, *Cell Immunol.* **32:**120–134.
49. Sultzer, B. M., 1983, Lymphocyte activation by endotoxin and endotoxin protein: The role of the C3H/HeJ mouse, in: *Beneficial Effects of Endotoxins* (A. Nowotny, ed.), pp. 227–248, Plenum, New York.
50. Watson, J., Kelly, K., and Whitlock, C., 1980, Genetic control of endotoxin sensitivity, in: *Microbiology 1980* (D. Schlessinger, ed.), American Society for Microbiology, Washington, D.C., pp. 4–10.
51. Stiffel, C., Liacopoulos-Briot, M., Decruesefond, C., and Lambert, F., 1977, Genetic selection of mice for quantitative responsiveness of lymphocytes to phytohemagglutinin, *Eur. J. Immunol.* **7:**291–297.
52. Amsbaugh, D. F., Hauser, C. T., Prescott, B., Stashak, P., Barthold, D. R., and Baker, P. J., 1972, Genetic control of the antibody response to type III pneumococcal polysaccharide in mice. I. Evidence that an x-linked gene plays a decisive role in determining responsiveness, *J. Exp. Med.* **136:**931–949.
53. Scher, I., Ahmed, A., Strong, D. M., Steinberg, A. D., and Paul, W. E., 1975, X-linked B-lymphocyte immune defect in CBA–HN mice. I. Studies of the function and composition of spleen cells, *J. Exp. Med.* **141:**788–802.
54. Fidler, J. M., Morgan, E. L., and Weigle, W. O., 1980, B lymphocyte differentiation in the CBA/N mouse: A delay in maturation rather than total arrest. *J. Immunol.* **124:**13–19.
55. Nariuchi, H., and Kakiuchi, T., 1981. Responses of spleen cells from mice with X-linked B-cell defect to polyclonal B-cell activators, purified protein derivative of tuberculin, and dextran sulfate, *Cell. Immunol.* **61:**375–385.
56. Stejskal, V., Holm, G., and Perlmann, P., 1973, Differential cytotoxicity of activated lymphocytes and xenogeneic target cells. I. Activation by tuberculin and by *Staphylococcus* filtrate, *Cell. Immunol.* **8:**71–81.
57. Wlodarski, K., Jakobisiak, M., Kassakowska, A., and Zelechowska, M., 1974, Stimulation of endogenous spleen colony formation by B- and T-lymphocyte mitogens, *Folia Biologica* **20:**133–137.
58. Moroni, C., and Schumann, G., 1976, Mitogen induction of murine C-type viruses. II. Effect of B-lymphocyte mitogens, *Virology* **73:**17–22.
59. Mokyr, M. B., and Mitchell, M. S., 1975, Activation of lymphoid cells by BCG *in vitro, Cell Immunol.* **15:**264–273.
60. Mitchell, M. S., Mokyr, M. B., and Kahane, I., 1975, Stimulation of lymphoid cells by components of BCG, *J. Nat. Cancer Inst.* **55:**1337–1343.
61. Weiss, D. W., and Wells, A. Q., 1960, Vaccination against tuberculin with nonliving vaccine. III. Vaccination of guinea pigs with fractions of phenol-killed tubercle bacilli. *Am. Rev. Respir. Dis.* **82:**339–357.
62. Bekierkunst, A., 1976, Stimulation of lymphocyte proliferation by killed mycobacteria and other bacterial species, *Infect. Immun.* **14:**28–32.
63. Ben-Efraim, S., and Diamantstein, T., 1975, Mitogenic and adjuvant activity of a methanol extraction residue (MER) of tubercle bacilli on mouse lymphoid cells *in vitro, Immunol. Commun.* **4:**565–577.
64. Ben-Efraim, S., Ulmer, A., Schmidt, M., and Diamantstein, T., 1976, Differences

between lymphoid cell populations of guinea pigs and mice as determined by the response to mitogen *in vitro, Int. Archs. Allergy Appl. Immunol.* **51:**117–130.
65. Azuma, I., Taniyama, T., Sugimura, K., Aladin, A. A., and Yamamura, Y., 1976, Mitogenicity of the cell walls of mycobacteria, nocardia, corynebacteria and anaerobic coryneforms, *Jap. J. Microbil.* **20:**263–271.
66. Baker, L. A., Sharpton, T., Minden, P., and Campbell, P. A., 1976, Adjuvant and mitogenic properties of a supernatant fraction of sonically treated *Mycobacterium bovis* (BCG), *Infect. Immun.* **14:**83–87.
67. Sultzer, B. M., 1978, Infection with Bacillus Calmette–Guérin activates murine thymus-independent(B) lymphocytes, *J. Immunol.* **120:**254–261.
68. Meyer, E. M., Wilmsmann, K., Schlake, W., and Grundmann, E., 1979, Quantification of BCG-induced reactions of T and B areas in peripheral lymphoid organs of young adult Balb/c mice. A histomorphometrical and authoradiographical study. *Path. Res. Pract.* **164:**127–140.
69. Mitchell, M. S., Kirkpatrick, D., Mokyr, M. B., and Gery, I., 1973, On the mode of action of BCG, *Nature New Biology.* **243:**216–217.
70. Geffard, M., and Orbach-Arbouys, S., 1976, Enhancement of T suppressor activity in mice by high doses of BCG, *Cancer Immunol. Immunother.* **1:**41–43.
71. Turcotte, R., Lafleur, L., and Labreche, M., 1978, Opposite effects of BCG on spleen and lymph node cells: Lymphocyte proliferation and immunoglobulin synthesis, *Infect. Immun.* **21:**696–704.
72. Turcotte, R., 1981, Evidence for two distinct populations of suppressor cells in mitogen-induced blastogenesis, *Infect. Immun.* **34:**315–322.
73. Turcotte, R., and Lemieux, S., 1982, Mechanisms of action of *Mycobacterium bovis* BCG-induced suppressor cells in mitogen-induced blastogenesis, *Infect. Immun.* **36:**263–270.
74. Klimpel, G. R., 1979, Soluble factors from BCG-induced suppressor cells inhibit *in vitro* PFC responses but not cytotoxic responses, *Cell. Immunol.* **47:**218–226.
75. Klimpel, G. R., and Henney, C. S., 1978, BCG-induced suppressor cells. I. Demonstration of a macrophage-like suppressor cell that inhibits cytotoxic T cell generation *in vitro, J. Immunol.* **120:**563–569.
76. Allen, E. M., and Moore, V. L., 1979, Suppression of phytohemagglutinin and lipopolysaccharide responses in mouse spleen cells by Bacillus Calmette-Guérin, *J. Reticuloendo. Soc.* **26:**349–356.
77. Collins, F. M., and Watson, S. R., 1979, Suppressor T-cells in BCG-infected mice, *Infect. Immun.* **25:**491–496.
78. Damais, C., Bona, C., Chedid, L., Fleck, J., Nauciel, C., and Martin, J. P., 1975, Mitogenic effect of bacterial peptidoglycans possessing adjuvant activity, *J. Immunol.* **115:**268–271.
79. Ciorbaru, R., Petit, J-F., Lederer, E., Zissman, E., Bona, C., and Chedid, L., 1976, Presence and subcellular localization of two distinct mitogenic fractions in the cells of *Nocardia rubra* and *Nocardia opaca:* Preparation of soluble mitogenic peptidoglycan fractions, *Infect. Immun.* **13:**1084–1090.
80. Azuma, I., Sugimura, K., Taniyama, T., Yamawaki, M., Yamamura, Y., Kusumoto, S., Okada, S., and Shiba, T., 1976, Adjuvant activity of mycobacterial fractions: Adjuvant activity of synthetic N-acetylmuramyl-dipeptide and the related compounds, *Infect. Immun.* **14:**18–27.
81. Igarashi, T., Okada, M., Azuma, I., Yamamura, Y., 1977, Adjuvant activity of synthetic N-acetylmuramyl-L-ananyl-D-isoglutamine and related compounds on cell-mediated cytotoxicity in syngeneic mice, *Cell. Immunol.* **34:**270–278.
82. Specter, S., Friedman, H., and Chedid, L., 1977, Dissociation between the adjuvant vs

mitogenic activity of a synthetic muramyl dipeptide for murine splenocytes, *Proc. Soc. Exp. Biol. Med.* **155:**349–352.

83. Damais, C., Parant, M., and Chedid, L., 1977, Nonspecific activation of murine spleen cells *in vitro* by a synthetic immunoadjuvant (n-acetylmuramyl-L-alanyl-D-isoglutamine, *Cell. Immunol.* **34:**49–56.
84. Goodman, G. W., and Sultzer, B. M., 1977, Mild alkaline hydrolysis of lipopolysaccharide endotoxin enhances mitogenicity for murine B cells, *Infect. Immun.* **17:**205–214.
85. Damais, C., Parant, M., Chedid, L., Lefrancier, P., and Choay, J., 1978, *In vitro* spleen cell responsiveness to various analogs of MDP (n-acetylmuramyl-L-alanyl-D-isoglutamine), a synthetic immunoadjuvant, in MDP high-responder mice, *Cell. Immunol.* **35:**173–179.
86. Wood, D. D., and Staruch, M. J., 1981, Control of the mitogenicity of muramyl dipeptide, *Int. J. Immunopharmac.* **3:**31–44.
87. Specter, S., Cimprich, R., Friedman, H., and Chedid, L., 1978, Stimulation of an enhanced *in vitro* immune response by a synthetic adjuvant, muramyl dipeptide, *J. Immunol.* **120:**487–491.
88. Watson, J., and Whitlock, C., 1978, Effect of a synthetic adjuvant on the induction of primary immune responses in T cell-depleted spleen cultures, *J. Immunol.* **121:**383–389.
89. Leclerc, C., Lowy, I., and Chedid, L., 1978, Influence of MDP and of some analogous synthetic glycopeptides on the *in vitro* mouse spleen cell viability and immune response to sheep erythrocytes, *Cell, Immunol.* **38:**286–293.
90. Leclerc, C., Bourgeois, E., and Chedid, L., 1979, Enhancement by muramyl dipeptide of *in vitro* nude mice responses to a T-dependent antigen, *Immunol. Commun.* **8:**55–64.
91. Lowy, I., Leclerc, C., and Chedid, L., 1980, Induction of antibodies directed against self and altered-self determinants by a synthetic adjuvant, muramyl dipeptide and some of its derivatives, *Immunology,* **39:**441–450.
92. Saito-Taki, T., Tanabe, M. J., Mochizuki, H., Matsumoto, T., Nakano, M., Takada, H., Tsujimoto, M., Kotani, S., Kusumoto, S., Shiba, T., Yokogawa, K., and Kawata, S., 1980, Polyclonal B cell activation by cell wall preparations of Gram-positive bacteria. *In vitro* responses of spleen cells obtained from Balb/c, nu/nu, nu/+, C3H/HeJ and Hybrid (CBA/Nx Balb/c) F1 mice, *Microbiol. Immunol.* **24:**209–218.
93. Saito-Taki, T., Shinomiya, H., and Nakano, M., 1986, Mechanisms of polyclonal B cell activation by murayl dipeptide, *Sixth Int. Cong. Immunol. Abst.* **3:**14.5.
94. Lowy, I., Leclerc, C., Bourgeois, E., and Chedid, L., 1980, Inhibition of mitogen-induced polyclonal activation by a synthetic adjuvant muramyl dipeptide (MDP), *J. Immunol.* **124:**320–325.
95. Bahr, G. M., Modabber, F. Z., Morin, A., Terrier, M., Eyquem, A., and Chedid, L., 1984, Regulation by muramyl dipeptide (MDP) of the lymphoproliferative responses and polyclonal activation of human peripheral blood mononuclear cells, *Clin. Exp. Immunol.* **57:**178–186.
96. Adam, A., Ciorbaru, R., Petit, J-F., and Lederer, E., 1972, Isolation and properties of a macromolecular water-soluble, immuno-adjuvant fraction from the cell wall of *Mycobacterium smegmatis, Proc. Nat. Acad. Sci.* **69:**851–854.
97. Chedid, L., Parant, M., Parant, F., Gustafson, R. H., and Berger, F. M., 1972, Biological study of a nontoxic, water-soluble immunoadjuvant from mycobacterial cell walls, *Proc. Nat. Acad. Sci.* **69:**855–858.
98. Modolell, M., Luckenbach, G. A., Parant, M., and Munder, P. G., 1974, The adjuvant activity of a mycobacterial water soluble adjuvant (WSA) *in vitro, J. Immunol.* **113:**395–403.

99. Goren, M., 1972, Mycobacterial lipids: Selected topics, *Bacteriol. Rev.* **36:**33–64.
100. Saito, R., Tanaka, A., Sugiyama, K., Azuma, I., Yamamura, Y., Kato, M., and Goren, M., 1976, Adjuvant effect of cord factor, a mycobacterial lipid, *Infect. Immun.* **13:**776–781.
101. Parant, M., Audibert, F., Parant, F., Chedid, L., Soler, E., Polonsky, J., and Lederer, E., 1978, Nonspecific immunostimulant activities of synthetic trehalose-6, 6′-diesters (lower homologs of cord factor), *Infect. Immun.* **20:**12–19.
102. Saito, R., Nagao, S., Takamoto, M., Sugiyama, K., and Tanaka, A., 1977, Adjuvanticity (immunity-inducing property) of cord factor in mice and rats, *Infect. Immun.* **16:**725–729.
103. Rook, G. A. W., and Stewart-Tull, D. E. S., 1976, The dissociation of adjuvant properties of mycobacterial components from mitogenicity, and from the ability to induce the release of mediators from macrophages, *Immunology* **31:**389–396.

14

Mechanisms of Toxicity of Tubercle Bacilli for Macrophages

QUENTIN N. MYRVIK, EVA S. LEAKE, and MAYER B. GOREN

1. HISTORICAL BACKGROUND

The early studies of Lurie[1] clearly established that virulent tubercle bacilli are highly infectious for susceptible hosts. For example, he observed repeatedly that one colony-forming unit (CFU) of the highly virulent H37Rv strain of *Mycobacterium tuberculosis* could develop one tubercle in the lungs of susceptible rabbits. Although not proved at that time, it is likely that even one organism is capable of producing one tubercle in the lungs of NZW rabbits (noninbred). By contrast, the H37Ra strain, an avirulent mutant of the H37Rv strain, is incapable of replicating in the macrophages of normal rabbits. Lurie's findings were particularly important with respect to the multiplication of the attenuated BCG strain of *Mycobacterium bovis*. In this case, even though BCG could not produce progressive infection in the rabbit, it was able to multiply in normal alveolar macrophages (AM) essentially at the same rate as the virulent H37Rv strain prior to the time specific cell-mediated immunity was acquired. All these early studies indicated that normal AM

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are incapable of inhibiting or containing the multiplication of phagocytosed organisms of the virulent H37Rv strain of *M. tuberculosis,* as well as the attenuated BCG strain during the early intervals after infection. Accordingly, Lurie concluded that all the immunity expressed against *M. tuberculosis* is acquired after infection. Lurie also demonstrated that macrophages from immune animals were resistant to the replication of virulent mycobacteria. These observations suggested that pathogenic mycobacteria have some virulence mechanism that enables them to multiply in normal macrophages, whereas immunologically activated macrophages are capable of inhibiting the growth of virulent mycobacteria.

Since normal AM are capable of blocking replication of the avirulent H37Ra strain, as well as many saprophytic species of mycobacteria, it is certain that normal AM are capable of expressing significant antimicrobial activity.[2] This suggests two possible explanations: (1) the H37Rv strain of *M. tuberculosis* is innately resistant to the antimicrobial activities of normal AM, or (2) the virulent H37Rv strain is capable of evading or antagonizing the antimicrobial activities expressed by normal resident AM. The latter case would suggest that a virulence factor was secreted or associated with the mycobacterial cell which could eliminate or make the normal antimicrobial function of normal resident AM nonfunctional.

The concept that virulent mycobacteria might be producing an exotoxin or endotoxin was considered by several investigators dating back to the 1940s. For example, Rich[3] made the statement in his monograph, "In spite of intensive study, there is at present not the slightest basis for linking the virulence of tubercle bacilli with toxin production." He further stated in his monograph that "the only definite thing we know at present about its virulence is that it consists in the relative ability of a given strain to survive and reproduce progressively in the tissues of a normal individual of an animal species that is susceptible to the type from which the strain in question was derived." Most of the early studies on potential toxic components of tubercle bacilli involved examining water soluble extracts of intact and disintegrated tubercle bacilli, as well as culture media of old cultures. The general conclusion was that virulent tubercle bacilli do not secrete or contain water soluble toxic products.

Middlebrook *et al.*[4] noted in 1947 that highly virulent strains of *M. tuberculosis* and *M. bovis* grew in a serpentine cord pattern on artificial media, whereas attenuated strains tended to grow in an irregular pattern. These investigators postulated that virulent tubercle bacilli produced some type of surface substance that caused them to stick together in a parallel fashion following division. Furthermore, they speculated

that this putative substance could be the long sought after virulence factor.

In 1950 Bloch[5] found that the addition of organic solvents like petroleum ether to cord-forming mycobacteria dispersed the cording pattern suggesting that the surface factor was extracted. Subsequently, Bloch isolated a lipid fraction, which he called cord factor. Bloch and collaborators[6,7] identified a toxic component in their petroleum extracts as trehalose 6,6′-dimycolate (TDM) which they believed was the putative cord factor. The cord factor virulence theory suffered a severe set back when it was found that avirulent mycobacteria such as *M. smegmatis* and *M. phlei* also contained TDM.[8] It was also found that the avirulent mutant H37Ra strain of *M. tuberculosis* contained TDM.

The concept that tuberculin hypersensitivity could produce extensive tissue damage was noted about 1891 at the time Robert Koch had prepared Old tuberculin. However, this is not the theme of this chapter, which is concerned with toxic moieties or potential virulence factors associated with the mycobacteria that allow them to express resistance to antimicrobial host factors expressed by normal resident AM. It will be our goal to focus on possible mechanisms that could explain the total lack of resistance of normal AM to one or only a few tubercle bacilli during the course of primary infection. Such a condition demands some type of virulence mechanism that is intimately associated with the organism because of the highly infectious nature of virulent strains of *M. tuberculosis* and the fact that one organism presumably can overcome the antimicrobial activity of a single normal macrophage. If a toxic moiety exists on the surface of virulent mycobacteria, it is likely that it is not water soluble.

2. EVIDENCE THAT VIRULENT MYCOBACTERIA ARE TOXIC FOR MACROPHAGES

During the late 1950s, we became interested in the possibility that virulent tubercle bacilli could express toxicity for rabbit AM based largely on the concept that very few organisms can initiate and overcome the antimicrobial activities of a population of normal AM. In these initial experiments, it took comparatively large multiplicities of the virulent H37Rv strain and incubation periods of 12–24 hr to demonstrate that virulent tubercle bacilli were lethal for AM. For example, these *in vitro* studies revealed that multiplicities of about 200–400 organisms of the virulent H37Rv strain per macrophage produced about 80–90% loss in viability of AM when incubated for about 24 hr (Fig. 1). We noted that the H37Rv strain had to be viable to express this low level of toxicity,

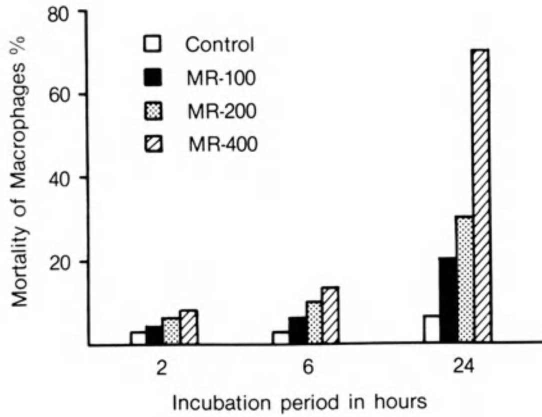


FIGURE 1. Effect of various multiplicity ratios of the virulent H37Rv strain to normal AM, on the viability of the macrophages.

because if the organisms were heat killed, no toxicity was expressed at this multiplicity or even greater multiplicities (Fig. 2). We also observed that if the antimycobacterial drug isoniazid was added to the *in vitro* culture system during the first 6 hr after mixing the virulent H37Rv strain with normal AM, the toxicity was largely abrogated (Fig. 3). Of even greater interest was our observation that the H37Ra strain did not exhibit toxicity for AM, and BCG produced only a low level of toxicity

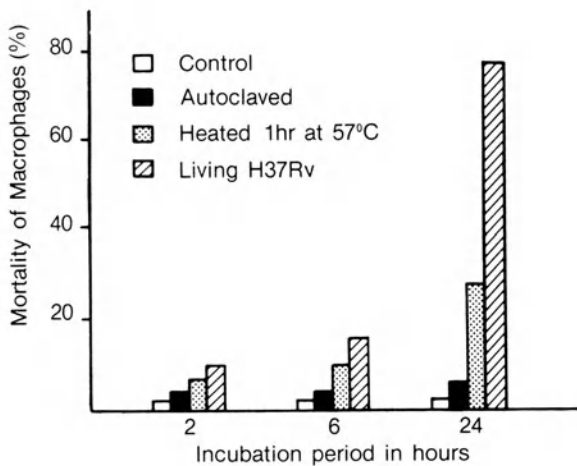


FIGURE 2. Effect of living, inactivated, and heat-killed virulent mycobacteria (H37Rv strain) on the viability of normal AM. Bacteria to macrophage ratio: 400.

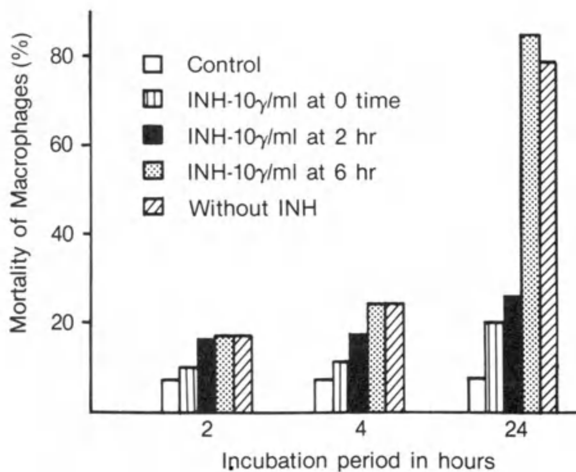


FIGURE 3. Effect of isonicotinic acid hydrazide (INH) on the toxicity of the H37Rv strain of mycobacteria for normal AM. Bacteria to macrophage ratio: 400.

(Fig. 4). These results supported the concept that virulent mycobacteria contain some bacterial-associated moiety that is toxic for normal AM. However, it also demonstrated that this toxicity is not dramatic because of the large numbers of tubercle bacilli required to produce 80–90% loss of viability.

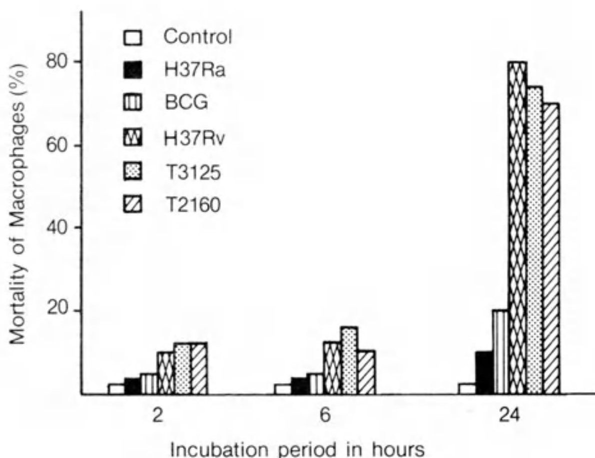


FIGURE 4. Toxicity of virulent mycobacteria H37Rv, T3125 and T2160 human strains, BCG-attenuated bovine strain, and H37Ra human avirulent strain for AM. Bacteria to macrophage ratio: 400.

3. EVIDENCE THAT VIRULENT TUBERCLE BACILLI CAN INTERFERE WITH LYSOSOME–PHAGOSOME FUSION

During the early 1970s, Armstrong and Hart[9] and Draper and Hart[10] reported that the virulent H37Rv strain prevented lysosome–phagosome fusion in normal mouse peritoneal macrophages. The principle of blocking and preventing lysosome–phagosome fusion was previously reported in the case of *Toxoplasma gondii*[11] and certain chlamydia.[12,13] Subsequent studies by Hart and colleagues indicated that the trypanocidal and lysosomotropic drug suramin somehow also could block lysosome–phagosome fusion when it accumulated in macrophage secondary lysosomes. The lysosomes were labeled with either acridine orange or colloidal electron-opaque ferritin. Fusion or its inhibition were studied by subsequently offering the macrophages baker's yeast for phagocytosis and observing whether the markers appeared in the yeast phagosomes. In this manner, sulfatides from virulent *M. tuberculosis* and several anionic polymers, such as dextran sulfate, were found to prevent the transfer of the lysosomal markers to the yeast phagosomes, and presumably therefore to prevent fusion. Thus, the mycobacterial sulfatides seemed to be implicated in the antifusion behavior.[14] The suggestion was made that the dysfunction could be due to ionic interaction between polyanionic micelles of the bacterial sulfatide and the organelle membranes, resulting in a modification of the latter and antagonism of their fusibility.

In subsequent studies, Pesanti[15] reported that suramin-exposed phagocytes challenged with yeast or *Listeria monocytogenes* displayed lysosome–phagosome fusion inhibition when the acridine orange technique was used. However, when ferritin was used as the label in experiments involving electron microscopy, fusion was normal. He concluded that suramin did not block lysosome–phagosome fusion. Goren *et al.*[16] also obtained similar discrepant results in macrophages exposed to sulfatides or dextran sulfate. Although the differences in results obtained with acridine orange and by electron microscopy were ultimately resolved and eliminated, the influence of the polyanionic agents on the behavior of the lysosomal markers has been the subject of an entirely new interpretation.[17] Based on a considerable body of evidence, Goren *et al.* suggested that the hydrosoluble anionic polymers exist in lysosomes in a gelatinous slowly moving form. They do not prevent phagosome–lysosome fusion but transfer to phagosomes only very slowly, if at all. It is suggested that the gelatinous, relatively immobile, polyanionic traps colloidal Thorotrast, ferritin, or gold by a physical process; it also traps acridine orange (a weak base) ionically, therefore, the semblance of fu-

sion inhibition. It is still not certain whether the mycobacterial sulfatides, which can surely interact with membranes, antagonize fusion.

4. DISRUPTION OF THE PHAGOSOMAL MEMBRANE BY THE VIRULENT H37Rv STRAIN OF *M. TUBERCULOSIS*

Subsequent to the studies made by Armstrong and Hart[9] and Draper and Hart,[10] we further evaluated some initial observations in our laboratory which indicated that virulent mycobacteria might perturb or disrupt the phagosomal membrane allowing the mycobacteria to escape to the cytoplasm of the macrophage. This virulence mechanism had

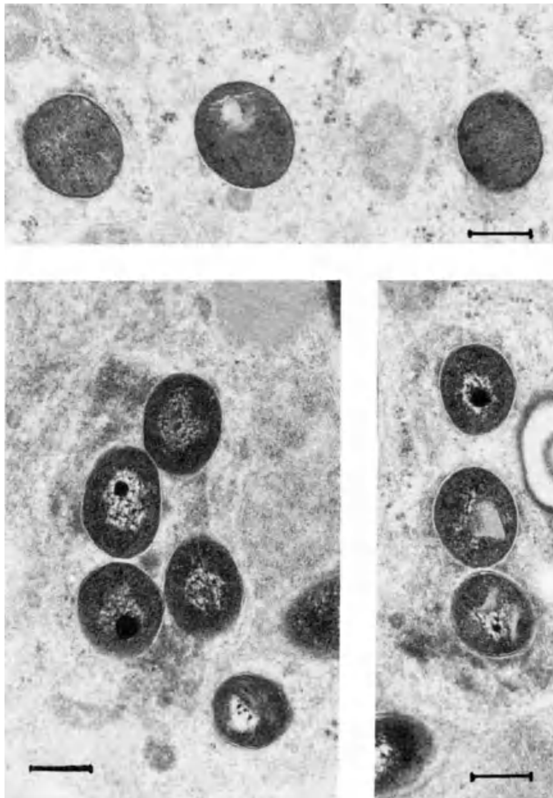


FIGURE 5. Intracellular virulent mycobacteria (H37Rv strain) lacking a demonstrable phagosomal membrane. Normal AM. Bar: 250 nm.

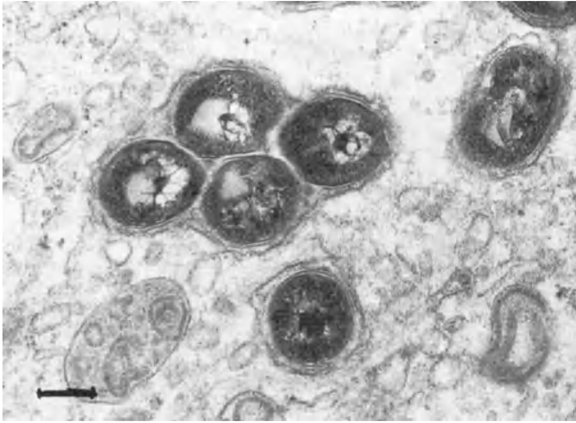


FIGURE 6. Intracellular avirulent mycobacteria (H37Ra strain) surrounded by a well-defined phagosomal membrane. Normal AM. Bar: 250 nm.

been reported previously by Tanowitz *et al.* and Nogueira and Cohn[18,19] with the trypomastigotes of *Trypanosoma cruzi*. In our experiments, after the addition of mycobacteria to normal AM cultures, the mixtures were incubated at 37°C for 16–24 hr. Our initial observations indicated that with the attenuated BCG strain approximately 20–25% of the organisms escaped the phagosome and existed free in the cytoplasm.[20] Subsequently, we noted that the virulent H37Rv strain expressed a high level of toxicity because 60–100% of the organisms were found free in the cytoplasm after 24 hr (Fig. 5). By contrast, 90–100% of the avirulent organisms of the H37Ra strain were retained within intact phagosomes (Fig. 6). These results convincingly demonstrated that there is some

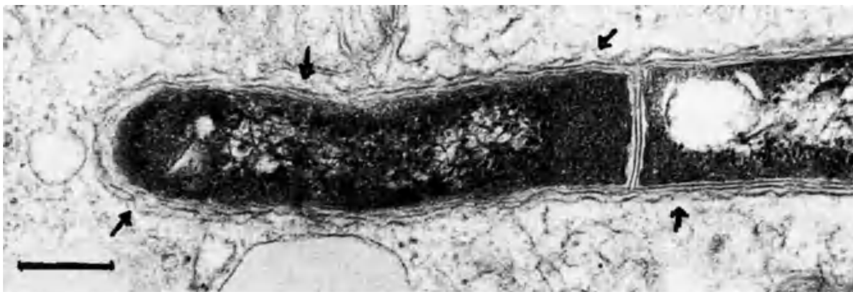


FIGURE 7. Ultrathin section of a virulent mycobacterium in the cytoplasm of a normal AM. Note the breaks in the phagosomal membrane (arrows). Bar: 250 nm.

property of the organisms of the H37Rv strain that permits them to fragment or disrupt the phagosomal membrane allowing the organisms to escape into the cytoplasm. This could be a highly efficient virulence mechanism because a breakdown of the lysosome–phagosome fusion process could occur even if small numbers of mycobacteria were involved. It was of special interest that by 5–6 hr of incubation the phagosome destructive mechanism was already apparent.

We had previously reported that the phagosomal membranes of normal AM adhere to the surface of the virulent H37Rv strain of mycobacteria but not to the surface of the avirulent H37Ra strain. The adherence noted with the H37Rv strain resulted in fragmentation of the membrane and subsequent escape of the tubercle bacilli into the cytoplasm of the normal AM (Fig. 7). These observations strongly suggested that some surface moiety of the virulent mycobacteria interacted with the phagosomal membranes of normal AM.

5. RESISTANCE OF BCG-IMMUNE AM TO PHAGOSOME DESTRUCTION BY THE H37Rv STRAIN

Earlier studies demonstrated that when normal rabbits are given a small dose of heat-killed BCG (0.1 mg) suspended in mineral oil (0.1 ml) via a single IV injection, the cell yields recovered by lung lavage 3 weeks

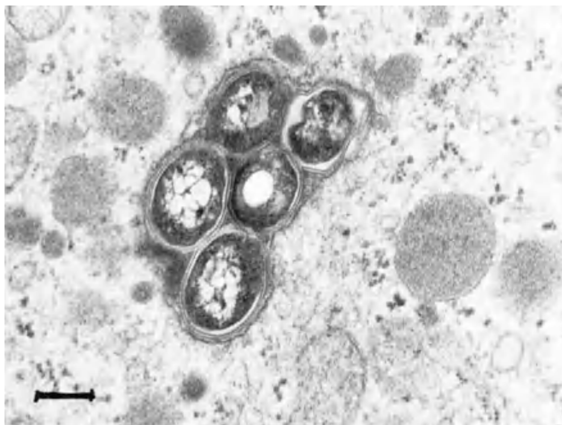


FIGURE 8. Ultrathin section of an AM from a BCG-vaccinated rabbit. Note that in this activated macrophage the intracellular virulent mycobacteria are surrounded by a detectable phagosomal membrane. Bar: 250 nm.

after injection are markedly increased.[22] Furthermore, ultrastructurally these cells differed from the normal resident macrophages by being larger in volume and by exhibiting numerous electron-dense structures often arranged as a rosette.[23] When these macrophages were incubated in the presence of living virulent mycobacteria (H37Rv strain), they avidly engulfed the mycobacteria but, in contrast to what had been observed with normal resident macrophages, most of the intracellular mycobacteria were surrounded by a well-defined phagosomal membrane containing electron-opaque material, which suggested extensive lysosome–phagosome fusion. Whereas other experiments indicated that usually less than 10% of the intracellular H37Rv were intraphagosomal in normal AM, 72–92% of the phagocytosed H37Rv organisms were seen surrounded by apparently intact phagosomal membranes in AM from the BCG vaccinated rabbits (Fig. 8). These observations clearly indicated that the AM from BCG-vaccinated rabbits are highly resistant to the phagosomal-disruptive action of the virulent H37Rv strain of mycobacteria.[24]

6. EFFECT OF ISONICOTINIC ACID HYDRAZIDE (INH) ON THE PHAGOSOME DESTRUCTION POTENTIAL OF THE H37Rv STRAIN

Takayama *et al.*[25,26] noted that INH inhibited the synthesis of unsaturated C_{33}–C_{47} and saturated C_{30}–C_{56} fatty acids; in addition, INH inhibited the synthesis of hydroxylipids (e.g., mycolic acids). Their stud-

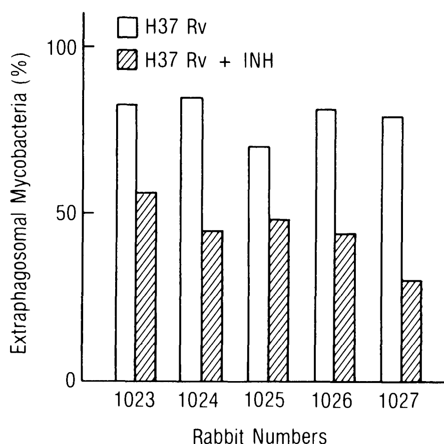


FIGURE 9. Effect of INH on the percentage of extraphagosomal bacteria of the H37Rv strain of *M. tuberculosis* after 18 hr of incubation. Each bar represents the analysis of a minimum of 150 intracellular mycobacteria observed in ultrathin sections with respect to the presence or absence of phagosomal membranes. The final concentration of INH is 5 μg/ml.

ies in effect indicated that INH inhibited the assembly of cord factor (trehalose dimycolate). We evaluated the effect of INH on the expression of the phagosome-destroying property of the virulent H37Rv strain for normal AM. A summary of our observations is presented in Fig. 9. We observed that 70–84% of mycobacteria were extraphagosomal in the control cultures, whereas in cultures containing 5 μg/ml of INH the percent of extraphagosomal bacteria ranged from 30–56%. Some of the bacteria in the AM incubated in the presence of INH possibly had a sufficient amount of the presumed surface "virulence factor" to destroy the phagosome; in other words, these organisms possessed sufficient "virulence factor" at the time of phagocytosis, to escape the phagosome. Accordingly, INH blocked the synthesis of additional "virulence factor" because about one half the organisms failed to express this virulence mechanism.

7. ATTEMPTS TO DEMONSTRATE PHAGOSOME DESTRUCTION UTILIZING LATEX BEADS COATED WITH TREHALOSE DIMYCOLATE AND SULFOLIPIDS

The characteristic adherence of phagosomal membranes to the surfaces of the virulent H37Rv strain, but not to the avirulent H37Ra strain, indicated the presence of some special chemical moiety that could be associated with virulence. These observations prompted a series of experiments to determine if cord factor or the sulfolipids of the cell wall were responsible for the adherence and phagosomal destruction observed with virulent mycobacteria.

Latex beads (5 μm in diameter) were coated with purified fractions of cord factor and/or sulfolipids. In addition, cord factor was dissolved in oil and an oil-in-water emulsion was prepared.

Polystyrene divinylbenzene beads (Dow Diagnostics) 5 μm in diameter were coated with various amounts of cord factor according to the method of Retzinger *et al.*[27] Beads were coated with cord factor or sulfolipid-I from *M. tuberculosis* by mixing a weighed amount of beads with the desired amount of lipids dissolved in a 9 : 1 mixture of hexane–ethanol. The suspensions of beads were sonicated briefly and the solvent was evaporated under a stream of nitrogen.

For example, 55 mg of beads were mixed with 3 ml of solvent containing 160 μg of cord factor and evaporated; this was calculated to provide approximately a collapsed monolayer of cord factor on the bead surface. The above preparations were added to cultures of normal AM and incubated for 24 hr, at which time the AM were harvested and prepared for transmission electron microscopy and phagosome analysis.

TABLE I
Cord Factor and Sulfolipid Preparations Tested in Phagosome Destruction Assays[a]

Experiment number	Latex-bead preparations
1	Cord factor (2.5 μg/mg)
2	Uncoated
	Sulfolipid (5.0μg/mg)
	Sulfolipid/cord factor (2.5 μg/mg)
3	Uncoated
	Sulfolipid (7.5 μg/mg)
	Sulfolipid (5.0 μg/mg)
	Cord factor (2.5 μg/mg)
	Cord factor (2.5 μg/mg)
	Cord factor (5.0 μg/mg)
	Sulfolipid/cord factor (2.5 μg/mg)
4	Cord factor in oil droplets (oil-in-water emulsion)

[a]Latex beads (5 μm) were coated as described in the text.

In a series of four experiments with these preparations we were unable to observe any phagosome membrane destruction. A list of the preparations tested is found in Table I. With respect to phagosomal membrane adherence no consistent adherence was noted. In many instances the phagosomal membrane appeared to be separated from the surface of the coated beads. We were compelled to conclude that cord factor and/or sulfolipid did not effect phagosome membrane destruction under the conditions of these experiments.

8. EVALUATION OF PUTATIVE VIRULENCE FACTORS OF MYCOBACTERIA EXPRESSED IN NORMAL ALVEOLAR MACROPHAGES

The earliest expression of virulence by pathogenic mycobacteria is detected when the phagocytosed organisms begin to multiply inside a normal macrophage from a susceptible species (see Section 8.3). Because normal AM are well equipped to inhibit the growth as well as kill avirulent mycobacteria it must be concluded that normal AM are capable of destroying a variety of mycobacteria.

An examination of this aspect of the problem in the context of the available information suggests that virulent mycobacteria most likely perturb lysosome–phagosome fusion or disrupt the phagosome. This type of virulence mechanism could be highly efficient because it could

abort the entire modus operandi of normal AM in terms of their inherent antimicrobial mechanisms. It is reasonable to assume that inhibition of lysosome–phagosome fusion could be the result of membrane modification that ultimately would allow intraphagosomal mycobacteria to escape to the cytoplasm of AM.

This present data base, although far from complete, supports the concept that some surface moiety on virulent mycobacteria can interact with the interior aspect of the phagosomal membrane and cause adherence of the phagosomal membrane to the surface of mycobacteria, with resulting membrane fragmentation and disintegration of the phagosome. In this regard, the adherence of phagosomal membranes of AM to the surface of endocytosed virulent mycobacteria has been a consistent finding in our experiments. Conversely, avirulent mycobacteria did not show this morphologic correlate of virulence.

The major missing link in this puzzle is the identification of the surface moieties on virulent mycobacteria that cause adherence and phagosomal membrane destruction. The following lipids appear to be the most likely candidates for such a virulence factor.

8.1. Cord Factor

The outstanding biochemical work that has been done on cord factor has established its structure and permitted its synthesis as well as many analogues. The structure of natural occurring cord factor is illustrated in Fig. 10.

Although the biologic activities of cord factor have been studied extensively, little is known about its role in virulence. For example, cord factor inhibits the migration of polymorphonuclear leukocytes, which suggests some toxicity. However, the migration of phagocytes is commonly inhibited when metabolic activators are added to such cultures which also could be the case with cord factor. The most puzzling type of lethal toxicity produced by cord factor was recognized by Bloch, when he gave mice repeated IP injections of cord factor in paraffin oil. However, a single large injection of cord factor was not lethal.[5] These findings have been repeatedly confirmed with highly purified cord factor. Goren and Brennan[28] confirmed Bloch's observation that 10 μg of cord factor injected at 2–3-day intervals caused a marked weight loss that resulted in death in all of the mice within 2 weeks. By contrast, when 50–100 μg was given in a single dose, few, if any, mice died. Mice dying from cord factor intoxication revealed acute pulmonary hemorrhages. The enigma associated with this toxicity is the need for "sensitizing" doses, even though there is no evidence that an underlying immunologic mechanism is involved. More recently, it has been reported that only an

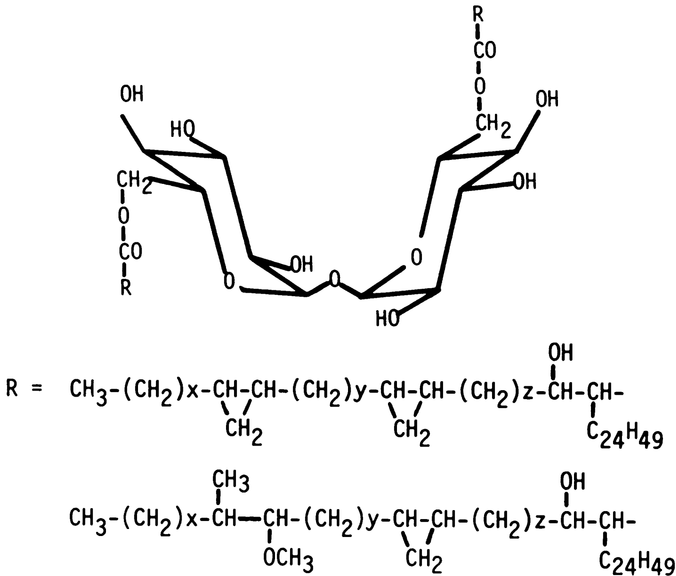


FIGURE 10. Structural formula for 6,6′-trehalose dimycolate (cord factor). In general, *x*, *y*, and *z* are odd, even, and odd, respectively, e.g., 17, 14, and 17.

injection of mineral oil is needed for sensitizing mice to cord factor.[29] How mineral oil sensitizes mice to cord factor remains unknown. This observation suggests that the oil is more important than the cord factor in terms of sensitizing the mice to a lethal dose of cord factor.

Artman *et al.*[30] observed that cord factor stimulated mammalian nicotinamide adenine dinucleotidase activity, which resulted in depressed levels of NAD activity in host tissues. In addition, other investigators noted that the administration of cord factor depressed muscle and liver glycogen metabolism and also perturbed pyruvate metabolism.[31] Kato[32] found that cord factor attacked mitochondrial preparations causing irreversible swelling and fragmentation of the cristae both *in vitro* and *in vivo*. As expected, these pathogenetic effects severely disturbed the electron flow along the mitochondrial respiratory chain and also markedly disrupted oxidative phosphorylation. Structurally modified cord factors suggested that specific reactions between the cord factor hydroxyl groups and target sites on the mitochondrial membranes and probably on respiratory chain enzymes were involved.[33]

Although our preliminary experiments with cord factor-coated beads did not reveal phagosome interaction and subsequent destruction, the problem may be more subtle. For example, it has been proposed by

Hunter and Bennett[34] that the configuration which molecules of TDM assume on the bead surface influences their biologic activity. When TDM was allowed to form a single molecular layer on beads (a collapsed monolayer) the preparations were toxic, whereas if TDM was in excess, it formed cylindrical micelles and was largely devoid of any toxicity. If this is proved the cording versus noncording phenomenon on virulent and avirulent mycobacteria would take on a new and important meaning.

In this regard, Retzinger *et al.*[26] in their provocative studies noted that at low surface concentration of TDM at an air–water interface, much of the lipid–water interface consists of hydrophobic domains that adsorb proteins. Their model system consisted of hydrophobic polystyrene divinylbenzene beads (5-μm diameter) to which various amounts of TDM were allowed to coat the beads after drying. At equilibrium spreading, most of the TDM molecules made contact with their hydrophobic domains with the aqueous interface which facilitated adsorption of protein. When beads were coated with an excess of TDM this excess formed large cylindrical micelles instead of a collapsed monolayer. The authors inferred that surface micelles cause a linking of the beads analogous to cording of mycobacteria. This point needs additional verification because TDM in micellar form is biologically inactive, which is not compatible with the idea that cording is a parameter of virulence.

In a more recent paper, Retzinger[35] reported that TDM-coated beads preferentially bind fibrinogen, which emerges as an apparent cofactor for the expression of the biologic activities. According to this idea, the TDM monolayer adsorbs fibrinogen, so that it can catalyze the formation of thrombin with preferential release of fibrinopeptide B. This peptide is chemotactic for macrophages and neutrophils and thus induces inflammation. In addition, the release of peptide B somehow makes fibrin more resistant to proteases. The authors postulate that the increased stability of the fibrin promotes the persistence of the inflammation. It is noteworthy that multiple injections of TDM-coated beads apparently simulate the pathologic events seen with multiple injections of TDM in oil. Pulmonary hemorrhages are explained by the elevated level of thrombin activity that is generated by the inflammatory cells making contact with the TDM-coated beads. The increased levels of thrombin presumably account for the reduced clotting of the blood with resulting hemorrhages. Retzinger[35] has proposed that the hemorrhaging may be exacerbated by the anticoagulant effects of fibrinogen–fibrin degradation products. A major obstacle in accepting these pathogenetic mechanisms as a generalized view of the pathogenetic events in tuberculosis is that only mice are apparently susceptible to these specialized toxic effects of TDM. However, this does not preclude the idea that

TDM, properly oriented on a virulent mycobacterium, could perturb lysosome–phagosome fusion or disrupt phagosomes allowing mycobacteria to escape to the cytoplasm of a macrophage.

8.2. Sulfatides

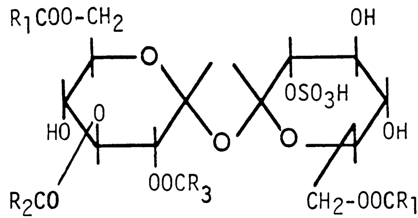
It has been known for decades that virulent mycobacteria stain with neutral red. In a search for the neutral red reactant, which correlates with virulence, Middlebrook *et al.*[36] extracted lipid components with hexane containing a trace of an aliphatic amine for the purpose of solubilizing acidic materials from virulent mycobacteria. The hexane soluble components bound neutral red, thus causing the neutral red to move from the aqueous to the organic phase. The active components concentrated in fractions rich in sulfur and were recognized to be an ester of trehalose sulfate substituted with methyl branched acids. Goren *et al.*[37] found that the sulfolipids were made up of closely related sulfatides. Whereas 5 different sulfatides were recognized (SL-I, SL-I′, SL-II, SL-II′, and SL-III), SL-I was the principal sulfatide, representing about 0.7% of the dry bacillary weight.

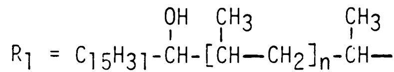
In the case of SL-I the 2, 3, 6, and 6′ positions of trehalose are substituted with 1 mol palmitate/stearate, 1 mole phthioceranate and 2 moles of hydroxyphthioceranate. Accordingly, the complete structure of SL-I is 2-palmitoyl (stearoyl)-3-phthioceranoyl-6,6′-bis-hydroxy-phthioceranoyl trehalose-2′-sulfate (Fig. 11).

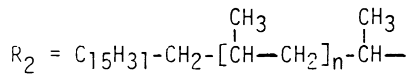
No cord factorlike toxic activity has been observed with the two sulfatides studied, SL-1 or SL-III when multiple doses (100 μg) of these lipids were given IP. Goren and Brennan[28] observed that repeated doses of SL-I markedly sensitized mice so that a subsequent dose (10 μg) of cord factor was lethal. However, if sulfatides were administered in oil, it is possible that oil was the sensitizer, not the sulfatides.[33]

Since the neutral red reaction of virulent *M. tuberculosis* was apparently dependent on their sulfatide content, this lipid automatically became a candidate for one of the virulence factors of *M. tuberculosis.* A significant correlation was reported between sulfatide production and animal virulence. The most virulent strains produced the most strongly acidic lipids of which the sulfatides were dominant, whereas attenuated strains were generally deficient in these components. However, many "aberrant" strains have been recognized, highly virulent but meager in sulfatides.[38]

The early observations made by Goren *et al.*[14] suggesting that sulfatide preparations could block lysosome–phagosome fusion raised the hope that the virulence factor of mycobacteria had been identified. This idea was supported by *in vitro* observations that SL-I attacked liver mito-







$n = 4\text{-}9$

FIGURE 11. Structural formula for the principal sulfatide (SL-I) in *M. tuberculosis* H37Rv.

chondrial membranes to a greater degree than cord factor. The idea that sulfatides, which are strongly acidic and polyanionic, somehow blocked lysosome–phagosome fusion was aggressively pursued by testing many different polyanions.

These exciting observations became suspect when Goren *et al.*[16] later noted that the acridine orange technique was not reliable for reasons already given (trapping by gelatinous polyanionics).

In spite of many uncertainties, the sulfatides could still be involved in phagosomal membrane adherence and membrane disruption due to the highly negatively charged nature of these molecules and their amphipathic properties. It should be pointed out, however, that we did not observe phagosome destruction in the experiments using sulfatide-coated beads. Nevertheless, additional experiments with both cord factor and the sulfatides would be well justified.

8.3. Other Mycobacterial Components that Have Possible Relevance to Virulence

Other lipids that have been considered possible virulence factors include phthienoic and mycolipenic acids, mycocerosic acids, pthiocerol

dimycocerosate, and the mycosides (A, B, and G).[28] However, none of these components provide a good case as phagosome membrane disrupting agents. If they are involved in pathogenesis, it would most likely be during the clinical stage of disease. In this regard, the mycosides do not have a ubiquitous distribution and can be ruled out as putative virulence factors of the H37Rv strain.

9. SUMMATION AND PERSPECTIVES

In spite of spectacular progress on the chemistry of mycobacteria by numerous investigators, we are still in a quandary concerning the identification of virulence factors. We purposely restricted our discussion to the early events following infection of AM by virulent *M. tuberculosis* for the purpose of separating complex pathogenetic mechanisms that are seen in apparent infections as distinct from the microbial determinants that permit the virulent mycobacteria to overcome the innate antimicrobial activities of normal resident AM. Two sets of observations support the principle of an abrogation of innate antimicrobial mechanisms: (1) inhibition of lysosome–phagosome fusion, and (2) destruction of the phagosomal membrane. Because of the marked antimicrobial activities of normal resident AM in the rabbit against "avirulent" mycobacteria, it is reasonable to assume that some virulence factor allows endocytosed virulent organisms to overcome the innate or natural resistance of AM. Since one virulent organism can allegedly overcome the resistance of a single AM, the expression of virulence factor(s) should be readily demonstrable. We are convinced that the virulent H37Rv strain can escape the phagosome of resident rabbit AM by destroying or disrupting the membrane. We are equally convinced that BCG-immune AM are highly resistant to this virulence mechanism. The tight adherence of phagosomal membranes to the virulent H37Rv strain and the lack of adherence to the avirulent H37Ra strain provides a visual display of this interaction, including evidence for membrane fragmentation leading to membrane destruction.

With respect to the identification of putative virulence factors that are responsible for membrane fragmentation and destruction, the uncertainties begin to mount. If cord factor is responsible, we must propose that the TDM molecules have to be properly oriented to present an exterior hydrophobic surface on the mycobacteria. Accordingly, a carefully prepared carrier particle with an appropriate coating of TDM could theoretically produce phagosomal membrane destruction. To our knowledge this is yet to be demonstrated. It is apparent that the presence of random-oriented TDM molecules on the surface of mycobac-

teria cannot explain virulence because the H37Ra strain contains TDM but is totally avirulent. The tempting assumption would be that when TDM is properly oriented on virulent mycobacteria, serpentine cord-forming growth occurs but in the case of the avirulent H37Ra strain TDM is randomly laid down and an irregular growth pattern occurs. However, there is no general agreement that cord factor is responsible for cord formation.

Of the remaining lipids, the sulfur-containing lipids remain as attractive candidates, largely because they are negatively charged and appear to be responsible for the binding of neutral red, a surprisingly good correlate of virulence. These sulfolipids also could explain the adherence of the phagosomal membrane to virulent mycobacteria. Additional experiments with beads properly coated with sulfolipids such as sulfatide I are very much in order.

Lastly, the hypothesis that polyanionics in general can cause inhibition of lysosome–phagosome fusion has been judged to be invalid, particularly with those that are hydrocolloids because of the probably viscous nature of these polyanionics in lysosomes and their slow transfer.[17] Our take-home lesson is to remind us again of the unpredictable difficulties that emerge when artificial modeling is used to explain complex intracellular mechanisms.

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REFERENCES

1. Lurie, M. B., 1964, *Resistance to Tuberculosis: Experimental Studies in Native and Acquired Defensive Mechanisms,* Harvard University Press, Cambridge, Massachusetts.
2. Leake, E. S., and Myrvik, Q. N., 1966, Digestive vacuole formation in alveolar macrophages after phagocytosis of *Mycobacterium smegmatis* in vivo, *J. Reticuloendothel. Soc.* **3:**83–100.
3. Rich, A. R., 1951, *The Pathogenesis of Tuberculosis,* 2nd ed., Charles C Thomas, Springfield, Illinois.
4. Middlebrook, G., Dubos, R. J., and Pierce, C., 1947, Virulence and morphological characteristics of mammalian tubercle bacilli, *J. Exp. Med.* **86:**175–184.
5. Bloch, H., 1950, Studies on the virulence of tubercle bacilli. Isolation and biological properties of a constituent of virulent organisms, *J. Exp. Med.* **91:**197–218.
6. Bloch, H., Sorkin, E., and Erlenmeyer, H., 1953, A toxic lipid component of the tubercle bacillus ("cord factor"). I. Isolation from petroleum ether extracts of young bacterial cultures, *Am. Rev. Tuberc.* **67:**629–643.
7. Noll, H., Bloch, H., Asselineau, J., and Lederer, E., 1956, The chemical structure of the cord factor of *Mycobacterium tuberculosis, Biochem. Biophys. Acta* **20:**299–309.

8. Nagasuga, T., Terai, T., and Yamamura, Y., 1961, Studies on the toxic lipid of isoniazid-resistant human tubercle bacilli, *Am. Rev. Respir. Dis.* **83:**248–254.
9. Armstrong, J. A., and Hart, P. D., 1971, Response of cultured macrophages to *Mycobacterium tuberculosis,* with observations on fusion of lysosomes with phagosomes, *J. Exp. Med.* **134:**713–740.
10. Draper, P., and Hart, P. D., 1975, Phagosomes, lysosomes and mycobacteria, in: *Mononuclear Phagocytes in Immunity Infection and Pathology* (R. van Furth, ed.), pp. 575–589, Blackwell Scientific Publications, Oxford.
11. Jones, T. C., and Hirsch, J. G., 1972, The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites, *J. Exp. Med.* **136:**1173–1194.
12. Friis, R. R., 1972, Interaction of L cells and *Chlamydia psittaci:* Entry of the parasite and host response to its development, *J. Bacteriol.* **110:**706–721.
13. Wyrick, P. B., and Browndridge, E. A., 1978, Growth of *Chlamydia psittaci* in macrophages, *Infect. Immun.* **19:**1054–1060.
14. Goren, M. B., Hart, P. D., Young, M. R., and Armstrong, J. A., 1976, Prevention of phagosome–lysosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis, Proc. Natl. Acad. Sci. USA* **73:**2510–2514.
15. Pesanti, E. L., 1978, Suramin effects on macrophage phagolysosome formation and antimicrobial activity, *Infect. Immun.* **20:**503–511.
16. Goren, M. B., Swendsen, C. L., and Henson, J., 1980, Factors modifying the fusion of phagosomes and lysosomes: Art, fact, and artefact, in: *Mononuclear Phagocytes.* Part II (R. van Furth, ed.), pp. 999–1038, Martinus Nijhoff, Boston.
17. Goren, M. B., Vatter, A. E., and Fiscus, J., 1987, Polyanionic agents as inhibitors of phagosome–lysosome fusion in cultured macrophages: Evolution of an alternative interpretation, *J. Leukocyte Biol.* **41:**111–121.
18. Tanowitz, H., Wittner, M., Kress, Y., and Bloom, B., 1975, Studies of in vitro infection by *Trypanosoma cruzi.* I. Ultrastructural studies on the invasion of macrophages and L-cells, *Am. J. Trop. Med. Hyg.* **24:**25–33.
19. Nogueira, N., and Cohn, Z. A., 1976, *Trypanosoma cruzi:* Mechanism of entry and intracellular fate in mammlian cells, *J. Exp. Med.* **143:**1402–1420.
20. Myrvik, Q. N., Leake, E. S., and Tenner-Racz, K., 1983, Interaction of mycobaceria with normal and immunologically activated alveolar macrophages, *Adv. Exp. Med. Biol.* **162:**83–98.
21. Myrvik, Q. N., Leake, E. S., and Wright, M. J., 1984, Disruption of phagosomal membranes of normal alveolar macrophages by the H37Rv strain of *Mycobacterium tuberculosis.* A Correlate of virulence, *Am. Rev. Respir. Dis.* **129:**322–328.
22. Leake, E. S., and Myrvik, Q. N., 1968, Changes in morphology and in lysozyme content of free alveolar cells after the intravenous injection of killed BCG in oil, *J. Reticuloendothel. Soc.* **5:**33–53.
23. Leake, E. S., and Myrvik, Q. N., 1972, Rosette arrangement of electron-dense structures in granulomatous alveolar macrophages, *J. Reticuloendothel. Soc.* **12:**305–313.
24. Leake, E. S., Myrvik, Q. N., and Wright, M. J., 1984, Phagosomal membranes of *Mycobacterium bovis* BCG-immune alveolar macrophages are resistant to disruption by *Mycobacterium tuberculosis* H37Rv, *Infect. Immun.* **45:**443–446.
25. Takayama, K., Schnoes, H. K., Armstrong, E. L., and Boyle, R. W., 1975, Site of inhibitory action of isoniazid in the synthesis of mycolic acids in *Mycobacterium tuberculosis, J. Lipid Res.* **16:**308–317.
26. Takayama, K., Qureshi, N., and Davidson, L. A., 1980, Studies of the effects of isoniazid on the biosynthesis of nonmycolic C_{30}-C_{56} fatty acids of *Mycobacterium tuber-*

culosis H37Ra, in: *Fifteenth U.S.–Japan Tuberculosis Research Conference, Tokyo, Japan, October 6–8*, pp. 71–95.

27. Retzinger, G. S., Meredith, S. C., Takayama, K., Hunter, R. L., and Kezdy, F. J., 1981, The role of surface in the biological activities of the mycobacterial glycolipid trehalose, 6,6'dimycolate. I. Surface properties of trehalose 6,6'dimycolate, *J. Biol. Chem.* **256**:8208–8216.
28. Goren, M. B., and Brennan, P. J., 1979, Mycobacterial lipids: chemistry and biologic activities, in: *Tuberculosis* (G. P. Youmans, ed.), pp. 63–193, WB Saunders, Philadelphia.
29. Seggev, J., Goren, M. B., Carr, R. I., and Kirkpatrick, C. H., 1982, Interstitial and hemorrhagic pneumonitis induced by mycobacterial trehalose dimycolate. *Am. J. Pathol* **106**:348–355.
30. Artman, M., Bekierkunst, A., and Goldenberg, I., 1964, Tissue metabolism in infection: Biochemical changes in mice treated with cord factor, *Arch. Biochem Biophys.* **105**:80–85.
31. Shankaran, R., and Venkitasubramanian, T. A., 1970, Effect of cord factor on carbohydrate metabolism in mice, *Am. Rev. Respir. Dis.* **101**:401–407.
32. Kato, M., 1969, Studies of a biochemical lesion in experimental tuberculosis in mice. XI. Mitochondrial swelling induced by cord factor in vitro, *Am. Rev. Respir. Dis.* **100**:47–53.
33. Goren, M. B., 1982, Immunoreactive substances of mycobacteria, *Am. Rev. Respir. Dis.* **125**(pt 2):50–69.
34. Hunter, R. L., and Bennett, B., 1985, Glycolipids in the pathogenesis of tuberculosis: The production of serpentine cords by trehalose 6,6'dimycolate, in: *Twentieth U.S.–Japan Tuberculosis Res. Conf.*, Bethesda, MD, August 15–16, pp. 41–49.
35. Retzinger, G. S., 1984, An experimental model of miliary tuberculosis and diffuse intravascular coagulation, in: *Nineteenth U.S.–Japan Tuberculosis research Conference, Tokyo, Japan, August 30–31*, pp. 295–313.
36. Middlebrook, G., Coleman, C. M., and Schaefer, W. B., 1959, Sulfolipid from virulent tubercle bacilli, *Proc. Natl. Acad. Sci. USA* **45**:1801–1804.
37. Goren, M. B., Brokl, O., Roller, P., Fales, H. M., and Das, B. C., 1976, Sulfatides of *Mycobacterium tuberculosis:* The structure of the principal sulfatide (SL-I), *Biochemistry* **15**:2728–2735.
38. Goren, M. B., Grange, J. M., Aber, V. R., Allen, B. W., and Mitchison, D. A., 1982, Role of lipid content and hydrogen peroxide susceptibility in determining the guinea-pig virulence of *Mycobacterium tuberculosis. Br. J. Exp. Pathol.* **63**:693–700.

15

Mycobacterium-Induced Suppressor Cells and Their Clinical Importance

S. ORBACH-ARBOUYS

1. INTRODUCTION

The importance of suppressor cells in mycobacterium-bearing hosts arises from the fact that immunization against tuberculosis and leprosy using BCG vaccines protects in certain areas and fails to give protection in others.[1,2] It has thus been suggested that the mycobacterium injection, rather than inducing a protective response, enhances the formation of suppressor cells[3,4] which prevent the effector cells from either being formed or from expressing their activity.

A similar mechanism has also been proposed to explain the situation in leprosy patients, particularly with lepromatous leprosy, the anergic form. Because of the consistency of the results, we shall review in this chapter all the observations made in animals and humans, whatever the strains of mycobacteria used.

2. EXPERIMENTAL INDUCTION OF SUPPRESSOR CELLS

Mice injected with high doses of BCG undergo some 2 weeks later, a decrease in immune reactivity. *In vivo,* the number of plaque-forming

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cells (PFC) enumerated in their spleen after injection of sheep red blood cells (SRBC) is lower than in controls.[5] The graft-versus-host reaction they induced when their spleen cells are injected into incompatible recipients, is diminished. *In vitro* the same spleen cells respond poorly to phytohemagglutinin (PHA),[6] to histocompatibility antigens, to mixed lymphocyte reaction (MLR), or to SRBC and DNP Ficoll.[5] The phenomenon is an active process since mixing spleen cells from treated and nontreated mice dramatically depresses their *in vitro* responsiveness.[5,7] BCG administration may also lower host defenses against infection; mice injected with BCG and *Listeria* have a greater mortality and higher spleen *Listeria* counts than mice given *Listeria* alone.[8]

The intensity of the depression varies from strain to strain: C57 Bl/6 mice easily develop the suppression, CBA do not. C3H/He mice do not develop a delayed-type hypersensitivity (DTH) response to BCG because of the appearance of suppressor T (Ts) cells against it, while SWM/Ms are high responders to BCG administration.[9]

An important point can be underlined: immune reactivity and suppressive response develop concomitantly. The two phenomena may not strictly overlap neither in time nor in intensity, and enhancement may alternatively occur depending on the precise conditions employed.[10] For example, it has been reported that on day 14 after the injection of BCG, *M. habana* or *M. simiae,* thymidine incorporation by spleen cells was significantly enhanced and this was followed by a profound depression, indicating that spleen cell multiplication is stimulated then repressed. Ts cells are then present in the spleen.[11] In the case of *M. habana*-infected mice, the suppressor cell population develops at the same time as DTH reactivity to the specific mycobacterial sensitizers disappears. Although neither BCG nor *M. simiae*-infected animals developed detectable DTH responses early in infection, the splenic T lymphocytes were still capable of responding specifically *in vitro*. The latter responsiveness was later lost as the infection progressed.

An underlying enhancement of antibody production *in vitro* by spleen cells from BCG-infected mice may be masked by an active suppression,[5] as revealed after removal of the suppressor cells.

Different patterns of response may also be observed when different strains of the same bacterium are used. Not all the strains may suppress. As an example, when the *in vivo* growth of five strains of *M. kansasii* was correlated with the level of DTH they may induce against a cytoplasmic protein antigen injected into the footpad,[12] the strain numbers 1201 and 1203 from the Trudeau Mycobacterial Culture Collection gave rise to persisting systemic infections with an early DTH response (day 7), followed by a profound anergy to the cytoplasmic protein antigen injections. Spleens from strain 1203-injected mice contained T-cell popula-

tions capable of suppressing the MLR between [C57Bl/6 × DBA/2] and C3H/He cells. Strains 1201, 1214, and 1217 declined in viability relatively rapidly and failed to induce detectable levels of DTH. Spleen cells from 1214-infected mice enhanced the MLR.

The duration of suppression is more or less correlated with the presence of the bacteria in the animals; 2–12 months after infection (the longest experimental time period tested), *M. habana*- and *M. simiae*-infected spleen cells were capable of suppressing both specific and nonspecific responses, presumably because there was little, if any, decrease in the bacterial load within the spleen.[11] Spleen cells taken from BCG-infected animals later recovered their ability to respond to both the specific and the nonspecific mitogen at a time when the number of mycobacteria present in the spleen dropped to about 10^4 viable organisms. A comparable conclusion may be drawn from experiments in which heavily BCG-infected mice were given isoniazid plus rifampin; the viable BCG population present within the lungs and spleen declined to undetectable levels, and a decline in Ts-cell activity was demonstrated *in vitro* by the restoration of the blastogenic responses to PHA and protein-purified derivatives (PPD).[13]

The observation that early drug therapy of BCG-infected mice completely prevented the induction of Ts cells suggests that the latter are induced only after the full mobilization of cell-mediated defenses by the metabolically active mycobacteria within the tissues.

These observations give us some information about the size of the metabolically active mycobacterial populations required to induce suppression; a stimulus greater than 10^6 viable bacilli is required to generate suppressor cells within the spleen. In the case of nontuberculous mycobacteria, the suppression is permanent.[11] This is analogous to human lepromatous leprosy, where the anergy persists, even if chemotherapy has drastically reduced the bacillary load within the tissues.[14]

2.1. Induction of Suppressor Cells by Killed BCG or by Its Cell Fractions

The experimental protocols using live BCG are of privileged value since the information they give are of great relevance to clinical situations. However, since it has been observed that the injection of killed BCG[15,16] or of its cell walls[17] induced suppression, additional studies could be designed to define the bacterial structure responsible for its generation. Thus, an intravenous (IV) injection of killed BCG in oil-in-saline emulsion in C57Bl/6 mice induces the development of intense chronic granulomatous inflammation in the lungs and spleen accompanied by a diminished responsiveness to PHA and bacterial lipopoly-

saccharide (LPS), and a suppression of antibody and DTH responses to SRBC.[18]

Both the adjuvant and the antigen portion of the BCG might be involved in the induction of the suppressor cells. Mice treated IV with muramyl dipeptide (MDP), a minimum adjuvant constituent of BCG, or with tuberculin-active peptide (TAP), the antigenic peptide from *M. tuberculosis,* have a suppressed DTH response as their spleen cells suppress the migration inhibition of peritoneal effector cells from mice immunized by BCG cell walls.[15,16]

2.2. The Effector Cells of Suppression

Two cells have been described as responsible for the suppression: adherent macrophages and nonadherent Thyl$^+$ T cells. This lack of concordance is likely to be due to the kinetics of the response, where concomitant or successive activation of one population or the other occurs. This is due also to variations between experimental protocols, which may favor the development or the identification of different types of cell. The suppressive function is attributed to macrophages described as adherent, Thy$^-$ Ig$^+$ spleen cells in most of the literature on experiments done with IV injected BCG cell walls. These cells may suppress the *in vitro* responses to histocompatibility antigens,[7] SRBC, and DNP Ficoll,[5] *in vitro* cytotoxic T cell generation[19,20] PFC response[19] and the DTH to SRBC[18,21] Suppressor adherent cells are also responsible for the reduced resistance to *Listeria* infection of live BCG-injected mice.[8] Spleen cells from killed BCG-[18] or cell-wall injected[17] mice suppress PHA-induced mitogenesis, anti-LPS antibody synthesis, and *in vivo* DTH.

Live BCG may induce Ts cells[9] in some strains of mice (C3H/He) in which the antigen-presenting capacity of macrophages is modified by the treatment.[22] It has thus been shown[23] that I-J$^+$ macrophages of C3H/He mice induce *in vitro* Ts cells which suppress DTH to BCG. It is suggested that strain differences in BCG responsiveness may, at least in part, reside in the ability of macrophages to induce Ts cells against DTH.

Heat-killed BCG induces antigen-nonspecific Ts cells of the DTH. Ts seem to be stimulated more easily by other strains of mycobacterium. *M. lepraemurium* injection suppresses the *in vitro* MLR due to the generation of two distinct types of Ts cells.[24] *M. simiae* and *M. habana* injection generates Ts cells in the spleen that inhibit PHA, PPD and specific cytoplasmic protein antigen responses, while spleen cells of *M. kansasii* 1203-injected animals contained a T-cell subpopulation capable of supressing MLR, and spleens from *M. kansasii* 1214-injected mice enhanced the response.[12] It is not surprising to find the two populations active in some circumstances.[25–28]

Cells from mice pretreated with MDP suppress nonspecifically, while cells of mice pretreated with TAP suppress specific responses. An evolution has also been observed in the immunosuppressive cell populations, whereby macrophages appeared early and T lymphocytes later.[29] In addition, suppressor macrophage-like cells, and not Ts cells, are sensitive to methotrexate.[30–32]

2.3. Suppressor Mediators

Attempts to isolate the mediators from fresh, nonincubated splenocytes failed.[26] Maximal production of suppressor mediators occurred during the first 24 hr in culture, and their production ceased after 72 hr. Supernatants prepared from cultures of spleen cells from mice infected 14 days earlier showed higher suppressive activity than did those obtained 28 days after infection. Two populations of BCG-induced suppressor cells, i.e., T lymphocytes and macrophage-like cells, are able to release suppressor mediators and to retain their suppressive activity thereafter.

The chronic granulomatous inflammation induced by the IV injection of killed BCG in an oil-in-saline emulsion is associated with diminished responsiveness of spleen cells.[18] The latter cells *in vitro,* elaborate factors able to inhibit PHA response and DTH and to diminish the intensity of BCG-induced inflammation in the lung and spleen.

Supernatants that suppressed the *in vitro* PFC response to SRBC by normal C57Bl/6 spleen cells did not suppress *in vitro* cytotoxic response. This suggests either that BCG-induced macrophage-like suppressor cells inhibit these *in vitro* responses via different mechanisms or that different types of suppressor cells are involved.[19] In some circumstances, two factors have been identified.[33] Macrophages and T cells from spleens of mice injected IV with live BCG produce inhibitory factors *in vitro.* The macrophage factor has a molecular weight of 10,000–30,000, while the T-cell factor is 50,000–70,000 M_r. The kinetics of their action is not the same. The T-cell factor acts only when given within 12 hr of stimulation with concanavalin A (Con A), the macrophage factor is active even when given after 48 hr.

It has also been observed that culture supernatants from guinea pig lymph nodes with mycobacterial granuloma contain nondialyzable factors that stimulate [^{14}C]proline and [^{14}C]leucine incorporation by fibroblasts and depress their [^{3}H]thymidine uptake.[34]

2.4. Mechanism of the Suppression

Alterations of the immune reactivity have been studied at the level of both macrophages and T cells, by measuring the interleukins secreted by these cells.

It was first observed that the *in vitro* immunosuppressive activity of Ts cells from BCG-infected mice could be ablated by supplementing their culture medium with a supernatant containing high titers of interleukin-2 (IL-2).[35] T cells have the capacity to absorb or consume IL-2 from these supernatants. It has been hypothesized that mice heavily infected with BCG develop an IL-2-dependent T-cell population and this would explain the poor responsiveness of their spleen cells.

Since IL-2 is produced by cells which respond to allogeneic Ia determinants[35] and concomitantly to interleukin 1 (IL-1),[37] the poor IL-2 production might be due to a defect in IL-1 production. This mechanism seems unlikely, since treatment with BCG increases IL-1-like activity in strains where BCG potentiates the immune responses,[38] as well as in those where it is suppressive.[20] The release of IL-1 like activity by spleen cell cultures stimulated with LPS or Con A is increased by previous BCG treatment of the cell donors. In MLR supernatants, to demonstrate increased IL-1-like activity was difficult due to the presence of suppressor factors. The two mediators can be separated by gel filtration. The addition of IL-1 to BCG-suppressed cultures did not restore normal activity and actually further suppressed cytotoxic T lymphocyte (CTL) formation.

3. CLINICAL OBSERVATIONS

Suppressor cells induced by BCG can be observed in the peripheral blood mononuclear cells of normal individuals, particularly in southern India, where no protection by BCG vaccination could be obtained.[39] When re-exposed to BCG, the cells failed to proliferate, while they retained the ability to respond to Con A and allogeneic cells. These cells inhibit the proliferation of fresh cells to other mycobacterial antigens and, less consistently, the response to other nonmycobacterial-soluble antigens such as tetanus toxoid or diphtheria toxoid. The suppression was mediated by OKT4$^+$ cells. IL-1 production from nonadherent cells in response to BCG was not affected, but IL-2 production by T cells was considerably reduced. Suppressor cells might have inhibited BCG-induced IL-2 receptor expression on other T cells.[40]

The cellular immunity of PPD skin-test-negative patients with "atypical" mycobacteriosis as measured by the *in vitro* proliferative responses to PPD, candida (CAN) and PHA was much lower than in skin-test-positive healthy controls. Such responses can be improved to a variable extent by the addition of indomethacin (inhibitor of the cyclooxygenase pathway of the arachidonic acid metabolism) to PPD-stimulated cultures in 6 out of 9 cases. On the contrary, the addition of nordihydro-

guaiarectic acid, a preferential inhibitor of lipoxygenase pathway, produced a greater suppression in 5 of 9 cultures stimulated with PPD, in 6 of 9 cultures stimulated with CAN, and in all the PHA-stimulated cultures. These observations suggest an abnormal immunoregulation which may be mediated by an imbalance of the metabolic products of arachidonic acid.

The above results may be compared to those observed in the human disease caused by *M. fortuitum* a rapidly growing atypical nontuberculous *Mycobacterium,* common saprophyte found in water and soil. The immunologic studies reported in a patient with a chronic *M. fortuitum* infection show a significant *in vitro* proliferative response to *M. fortuitum* antigen but a poor bactericidal activity against *M. fortuitum. M. fortuitum* antigen-activated suppressor cells contributed to the bactericidal defect. The suppressor cells were antigen specific, both adherent and nonadherent. *In vitro* treatment with a combination of a cholinergic agonist (bethanechol chloride) and indomethacin, but not with either of them alone, eliminated the activity of the suppressor cells. Moreover, the administration of the two drugs to the patient resulted in reversal of the bactericidal defect and in a dramatic clinical improvement.[41]

3.1. Cellular Immune Response of Leprosy Patients

The last part of this chapter is devoted to a short review of the immunology of leprosy patients, in order to suscitate comparisons between *M. tuberculosis-* and *M. leprae*-induced diseases, which could help explain the mechanism of anergy and develop means of overcoming it.

Leprosy affects roughly 15 million people and, despite an effective chemotherapy that was introduced 40 years ago in the form of sulfones, there is little evidence to suggest a decrease in its incidence. It is not clear yet why leprosy disappeared from Europe by the end of the nineteenth century, and why it is currently increasing in some developing countries. Moreover, a maximum of 5% of the exposed population develop manifest clinical leprosy, even in the most hyperendemic areas in the world, as if the initial encounter with the leprosy bacillus would have led to immune protection of the other 95%. The study of staff contacts in a leprosarium demonstrated that these persons developed high lymphocyte transformation responses to *M. leprae,* suggesting that most developed subclinical infection, a situation analogous to tuberculosis.[42] The mode of spread of the organism is likely to be from respiratory tract to respiratory tract, again a situation analogous to tuberculosis.[43]

The bacterium causes a whole spectrum of diseases, which are not dependent on variability of the microorganism, but on variability of the host's immune response.

Mycobacterium leprae has an estimated doubling time of 10–20 days, while that of *M. tuberculosis* is 20 hr and that of *E. coli* 20 min. This slow growth rate may explain to some extent the slow progression of the infection. It is not known, however, whether the growth rate of the microorganism is slow though relatively constant during the subclinical phase of the infection, or microorganism persists for certain periods in a dormant state. The ability of *M. tuberculosis* to persist in a dormant state for months and years is well recognized,[44] and most of the clinical cases of tuberculosis that occur today among old people in developed countries represent an endogenous reactivation of a primary infection occurred many years earlier. The same may be true in leprosy and a second activating event may be required to produce clinical leprosy.

The microorganism may multiply in a number of human cells, mainly the tissue macrophages and the Schwann cells of peripheral nerves. The fate of infected macrophages is still not well known as the pathogen might be capable of actively diverting their metabolism by interfering with cell regulation, e.g., by rendering the cell incapable of responding to exogenous mediator signals which result in activation. Furthermore, the pathogen may also disturb the communication between the infected macrophage and the lymphocyte compartment, or make it generate suppressor signals.[45]

Very early in infection, *M. leprae* is readily phagocytized by host cells, usually macrophages. Since the bacterium depends on an intracellular environment, normal phagocytosis is a prerequisite for the parasite to survive in the host. Phagocytosis of *M. leprae* seems to be equally efficient in healthy individuals and leprosy patients. The next important step is macrophage activation. This activation is not an all-or-nothing phenomenon. Mycobacteria will induce a certain amount of nonspecific activation in macrophages by means of the adjuvant activity of their cell wall components, but this does not seem to result in the destruction of the leprosy bacillus.

Specific sensitization of T lymphocytes requires the presentation of antigen by macrophages. It is not clear yet whether this step is inadequate. Another possibility is the failure of other host cells which contain *M. leprae* to collaborate with T lymphocytes in the immune response. The absence of histocompatibility antigens on the Schwann cells might explain the lack of collaboration. In addition, DTH does not correlate with the ability of the patient to resist bacterial multiplication in his tissues.[46] Since the bacteria are intracellular, defense mechanisms depend to a considerable extent on CTL when they are harbored in Schwann cells. In the massive infection of lepromatous patients, immunosuppressive mechanisms probably play an important role in counteracting immune responses to *M. leprae* antigens.

Leprosy patients fall in two categories with all the intermediates: (1) tuberculoid leprosy with few acid-fast bacilli in the tissues, and high levels of cell-mediated immunity, which ultimately kills and clears the bacilli from the tissues, although often with concomitant immunologic damage to nerves; (2) lepromatous leprosy, with selective unresponsiveness to antigens of *M. leprae,* and the microorganisms ineluctably multiplying in the skin. Antibodies to *M. leprae* occur in all forms of the disease but attain the highest levels in the lepromatous disease, suggesting that they have little to do with protection.[47,48] Indeed, one of the major clinical problems in this form is erythema nodosum leprosum, presumed to be caused by immune complexes in the tissues.

3.2. Specific Unresponsiveness in Lepromatous Leprosy

By and large, no generalized anergy is observed, but only a poor response to lepromin. Lymphocyte transformation to PPD is high. Genetic factors have been claimed, but are unlikely to be the principal determinant of the unresponsiveness.[49]

One or a small number of unique antigenic determinants of *M. leprae* is capable of inducing suppressor cells with the ability to block the responsiveness of helper T cells to other specific or cross-reactive determinants. The suppression may be seen *in vitro* in cultures of lymphocytes from leprosy patients with Con A alone or in presence of lepromin. A total of 84% of the patients had a nonadherent cell capable of suppressing the Con A responses of normal donors' lymphocytes and 64% an adherent suppressor cell. Most lepromatous patients have both adherent and nonadherent suppressor cells. The adherent suppressor cell is presumably a monocyte that secretes a suppressor factor in culture.[50] The nonadherent suppressor cell is $OKT8^{+}$, with a remarkable specificity for the terminal disaccharide or trisaccharide of the complex phenolic glycolipid of *M. leprae,* which is its unique antigen.[51] There is no convincing evidence that T cells may recognize polysaccharide antigens or sugars. The determinants recognized could be the idiotype on the IgM antibody initially formed,[52] although very little antibody is present.

3.3. Relationship of T-Suppressor Cells to the Disease State in Leprosy

Leprosy is a localized disease and the immune reaction takes place where the *M. leprae* is located, primarily in the skin and nerves. In lepromatous patients, Ts cells are predominant in the lesions to control the extent of DTH and macrophages are loaded with bacilli. T4 cells predominate in tuberculoid lesions.[53,54] Both types of lesions might

eventually be observed in the same individual. Generally, lepromatous patients remain leprosis-negative and unresponsive to antigens of *M. leprae* for their lifetime.

3.4. Immunoregulation in Humans

The ultimate mechanism by which suppressor cells prevent competent lymphocytes from functioning remains presently unsolved. Suppressor cells might inhibit the proliferation of T lymphocytes by blocking the production of IL-2.[55,56] Lymphocytes from lepromatous patients cultured with *M. leprae* failed to produce IL-2. The addition of IL-2 partially restores the ability of lymphocytes of patients to respond to *M. leprae*, indicating that some T-helper cells reactive to *M. leprae* exist in the blood of lepromatous patients.[57] Lymphocytes from lepromatous leprosy patients also failed to produce IFN_{γ}[58] the lymphokine required for activating the macrophages to produce O_2^- and H_2O_2.[59] It is not known whether there is a defect in IL-1 production in lepromatous lesions or whether the primary defect is a failure to produce IL-2.

3.5. Overcoming the Anergy by Combined Vaccination

An immune reactivity in lepromatous patients has been obtained by the administration of live BCG plus killed *M. leprae*. A granulomatous response to BCG is produced, and all the acid-fast bacilli are degraded. This vaccine induces a state of both specific and nonspecific reactivity to *M. leprae* antigens.[3,60–64] The mechanism is yet unknown; either tolerance is broken by a crossreactive antigen, or BCG amplifies BCG-reactive clones that produce the IL-2 necessary for the expansion of *M. leprae* specific T-helper cells, or BCG enhances the expression of IL-2 gene products.[65]

Such a protocol of immunization may prompt satellite trials in an attempt to overcome unresponsiveness whenever it is observed, in bacterial, parasitic, viral infections, or even toward tumor cells.

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REFERENCES

1. Brown, J. A. K., Stone, M. M., and Sutherland, I., 1968, BCG vaccination of children against leprosy in Uganda: Results at the end of second follow up, *Br. Med. J.* **1**:24–27.
2. Bechelli, L. M., Garbajosa, P. G., Gyi, M. M., Uemura, K., Sundaresan, T., Dominguez, V. M., Matejka, M., Tamondong, C., Quagliato, R., Engler, V., and Altmann, M., 1973,

BCG vaccination of children against leprosy: Seven-year findings of the controlled WHO trial in Burma, *Bull. WHO* **48:**323–334.

3. Mehra, V., Convit, J., Rubinstein, A., and Bloom, B. R., 1982, Activated suppressor T cells in leprosy, *J. Immunol.* **129:**1946–1951.
4. Piessens, W. F., Partono, F., Hoffman, S. L., Ratiwayanto, S., Piessens, P. W., Palmieri, J. R., Koiman, I., Dennis, D. T., and Carney, W. P., 1982, Antigen specific suppressor T lymphocytes in human lymphatic filariasis, *N. Engl. J. Med.* **307:**144–147.
5. Brown, C. A., Brown, I. N., and Sljivic, V. S., 1980, Active suppression masks an underlying enhancement of antibody production in vitro by spleen cells from BCG-infected mice, *Immunology* **40:**303–309.
6. Orbach-Arbouys, S., and Poupon, M. F., 1978, Active suppression of in vitro reactivity of spleen cells after BCG treatment, *Immunology* **34:**431–437.
7. Orbach-Arbouys, S., and Castes, M., 1980, Suppression of T-cell responses to histocompatibility antigens by BCG pretreatment, *Immunology* **39:**263–268.
8. Wing, E. J., 1981, Bacillus Calmette–Guérin (BCG) decreases resistance to *Listeria monocytogenes* infection in mice, *Immunology* **44:**509–515.
9. Nakamura, R. M., Tanaka, H., and Tokumaga, T., 1982, Strain difference in delayed-type hypersensitivity to BCG in mice: Role of splenic adherent cells in the primary immune response, *Immunology* **47:**729–731.
10. Florentin, I., Huchet, R., Bruley-Rosset, M., Halle-Panenko, O., and Mathé, G., 1976, Studies on the mechanisms of action of BCG, *Cancer Immunol. Immunother.* **1:**31–37.
11. Watson, S. R., and Collins, F. M., 1980, Development of suppressor T cells in mice heavily infected with mycobacteria, *Immunology* **39:**367–375.
12. Collins, F. M., and Cunningham, D. S., 1981, Systemic *Mycobacterium kansasii* infection and regulation of the alloantigenic response, *Infect. Immun.* **32:**614–624.
13. Collins, F. M., and Watson, S. R., 1980, Effect of chemotherapy on suppressor T cells in BCG-infected mice, *Immunology* **40:**529–537.
14. Bullock, W. E., 1978, Leprosy: A model of immunological perturbation in chronic infection, *J. Infect. Dis.* **137:**141–147.
15. Kato, K., Yamamoto, K., Kimura, T., Azuma, I., and Askenase, P. W., 1984, Suppression of BCG cell wall-induced delayed-type hypersensitivity by pretreatment with killed BCG: Induction of nonspecific suppressor T cells by the adjuvant portion (MDP) and of specific suppressor T cells by the antigen portion (TAP), *J. Immunol.* **132:**2790–2795.
16. Kato, K., and Yamamoto, K., 1982, Suppression of BCG cell wall-induced delayed-type hypersensitivity by BCG pretreatment. II. Induction of suppressor T cells by heat-killed BCG injection, *Immunology* **45:**655–661.
17. Mizushima, Y., Wepsic, H. T., Yamamura, Y., DeSilva, M. A., Janns, G., and Larson, C. H., 1984, Negative and positive immunobiological responses in mice pretreated with Bacillus Calmette-Guérin cell wall, *Cancer Res.* **44:**20–24.
18. Allen, E. M., Sternick, J. L., Schrier, D. J., and Moore, V. L., 1981, BCG-induced chronic pulmonary inflammation and splenomegaly in mice: Suppression of PHA-induced proliferation, delayed hypersensitivity to sheep erythrocytes, and chronic pulmonary inflammation by soluble factors from adherent spleen cells. *Cell. Immunol.* **58:**61–71.
19. Klimpel, G. R., 1979. Soluble factors from BCG-induced suppressor cells inhibit in vitro PFC responses but not cytotoxic responses, *Cell. Immunol.* **47:**218–226.
20. Mellow, L., and Sabbadini, E., 1985, Effect of Bacillus Calmette–Guérin on the in vitro generation of cytotoxic T lymphocytes. II. Role of interleukin-I-like factors and soluble suppressor factors, *Immunology* **56:**235–243.
21. Kato, K., Yamamoto, K.-I., Kakinuma, M., Ishihara, C., and Azuma, I., 1981, Suppres-

sion of BCG cell wall induced delayed-type hypersensitivity by BCG pre-treatment. I. Induction of adherent suppressor cells by live BCG injection and their characterization, *Immunology* **42:**259–266.
22. Nakamura, R. M., and Tokunaga, T., 1980, Induction of suppressor T cells in delayed-type hypersensitivity to *Mycobacterium bovis* BCG in low-responder mice, *Infect. Immun.* **28:**331–339.
23. Nakamura, R. M., Tanaka, H., and Tokunaga, T., 1982, In vitro induction of suppressor T cells in delayed-type hypersensitivity to BCG and an essential role of I-J positive accessory cells, *Immunol. Lett.* **4:**295–299.
24. Hoffenbach, A., Lagrange, P. H., and Bach, M. A., 1983, Surface Lyt phenotype of suppressor cells in C57 Bl/6 mice infected with *Mycobacterium lepraemurium, Clin. Exp. Immunol.* **51:**151–157.
25. Turcotte, R., 1981, Evidence for two distinct populations of suppressor cells in the spleens of *Mycobacterium bovis* BCG-sensitized mice, *Infect. Immun.* **34:**315–322.
26. Turcotte, R., and Lemieux, S., 1982, Mechanisms of action of *Mycobacterium bovis* BCG-induced suppressor cells in mitogen-induced blastogenesis, *Infect. Immun.* **36:**263–270.
27. Collins, F. M., and Watson, S. R., 1980, Effect of chemotherapy on suppressor T cells in BCG-infected mice, *Immunology* **40:**529–537.
28. Watson, R. S., Auclair, L. K., and Collins, F. M., 1981, The effect of combined chemotherapy of suppressor T-cell activity in *Mycobacterium simiae*-infected mice, *Immunology* **43:**459–465.
29. Bullock, W. E., Carlson, E. M., and Gershon, R. K., 1978, The evolution of immunosuppressive cell populations in experimental mycobacterial infection, *J. Immunol.* **120:**1709–1716.
30. Orbach-Arbouys, S., and Castes, M., 1979, Augmentation of immune responses after methotrexate administration. *Immunology* **39:**265–269.
31. Kato, K., and Yamamoto, K., 1982, Restoration of suppressed delayed-type hypersensitivity induced in mice by live BCG after methotrexate administration, *Int. J. Immunopharmacol.* **4:**463–467.
32. Kato, K., Yamamoto, K., and Kimura, T., 1985, Migration of natural suppressor cells from bone marrow to peritoneal cavity by live BCG, *J. Immunol.* **135:**3661–3668.
33. Collizzi, V., Ferluga, J., Gareau, F., Malkovsky, M., and Asherson, G. L., 1984, Suppressor cells induced by BCG release non-specific factors in vitro which inhibit DNA synthesis and interleukin-2 production, *Immunology* **51:**65–71.
34. Narayanan, R. B., Curtis, J., and Turk, J. L., 1981, Release of soluble factors from lymphe nodes containing mycobacterial granulomas and their effect on fibroblast function in vitro, *Cell. Immunol.* **63:**93–102.
35. Orme, I. M., Ratcliffe, M. J. H., and Collins, F. M., 1984, Acquired immunity to heavy infection with *Mycobacterium bovis* bacillus Calmette–Guérin and its relationship to the development of nonspecific unresponsiveness *in vitro, Cell. Immunol.* **88:**285–296.
36. Wagner, H., Hardt, C., Heeg, K., Pfizenmaier, K., Solbach, W., Bartlett, R., Stockinger, H., and Röllinghoff, M., 1980, T-T cell interactions during cytotoxic T lymphocyte (CTL) responses: T cell derived helper factor (interleukin 2) as a probe to analyze CTL responsiveness and thymic maturation of CTL progenitors, *Immunol. Rev.* **51:**215–255.
37. Mizel, S. B., 1982, Interleukin 1 and T cell activation, *Immunol. Rev.* **63:**51–60.
38. Mitchell, M. S., Kirkpatrick, D., Birnbaum-Mokyr, M., and Gery, I., 1973, On the mode of action of BCG, *Nature New Biol.* **243:**216–228.
39. Mustafa, A. S., and Godal, T., 1983, In vitro induction of human suppressor T cells by mycobacterial antigens. BCG-activated OKT4$^+$ cells mediate suppression of antigen-induced T cell proliferation, *Clin. Exp. Immunol.* **52:**29–37.

40. Mustafa, A. S., and Godal, T., 1985, BCG-induced suppressor T-cells: Optimal conditions for in vitro induction and mode of action, *Clin. Exp. Immunol.* **62**:474–481.
41. Gardner, J. D., Ousley, M., Godfrey, W., Lindsey, N. J., and Abdou, N. I., 1982, *Mycobacterium fortuitum* infection: Evidence of bactericidal defect due to hyperactive antigen-specific suppressor cells. Correction in vitro and in vivo by cholinergic agonist and indomethacin. *Am. J. Med.* **73**:756–764.
42. Godal, T., and Negassi, K., 1973, Subclinical infection in leprosy, *Br. Med. J.* **3**:557–559.
43. Sheppard, C. C., 1962, The nasal excretion of *Mycobacterium leprae* in leprosy, *Int. J. Lepr.* **30**:10–16.
44. Fenner, F., Martin, S. P., and Pierce, S. P., 1949, The enumeration of viable tubercle bacilli in cultures and infected tissues, *Ann. N.Y. Acad. Sci.* **52**:751–758.
45. Rook, G. A. W., 1975, The potentiating mytogenic and inhibitory effect on lymphocytes in vitro of macrophages in the lymph nodes of mice "overloaded" with mycobacterial products, *Clin. Exp. Immunol.* **21**:163–169.
46. Bjune, G., and Barnetson, R. StC., 1976, Plasma factors in delayed-type hypersensitivity. Augmentation of lymphocyte responses in borderline leprosy reactions, *Clin. Exp. Immunol.* **26**:397–403.
47. Abe, M., Izumi, S., Saito, T., and Mathur, S. K., 1976, Early serodiagnosis of leprosy by indirect imunofluorescence, *Lepr. India* **48**:272–276.
48. Young, D. B., and Buchanan, T. M., 1983, A serological test for leprosy with a glycolipid specific for *Mycobacterium leprae*, *Science* **221**:1057–1059.
49. Chakarvartti, M. R., and Vogel, F., 1973, A twin study in leprosy, in: *Topics in Human Genetics*, Vol. 1, pp. 1–123. Thieme, Stuttgart.
50. Sathish, M., Bhutani, L. K., Sharma, A. K., and Nath, I., 1983, Monocyte-derived soluble suppressor factor(s) in patients with lepromatous leprosy, *Infect. Immun.* **42**:890–899.
51. Hunter, S. W., Fugiwara, T., and Brennan, P. J. (1982) Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*, *J. Biol. Chem.* **257**:15072–15078.
52. Taylor, C. E., Stashak, P. W., Caldes, G., Prescott, B., Chused, T. E., Brooks, A., and Baker, P. J., 1983, Activation of antigen-specific suppressor T cells by B cells from mice immunized with type III pneumococcal polysaccharide, *J. Exp. Med.* **158**:703–717.
53. Van Voorhis, W. C., Kaplan, G., Sarno, E. N., Horwitz, M. A., Steinman, R. M., Levis, W. R., Nogueira, N., Nair, L. S., Gattass, C. R., Arrick, B. A., and Cohn, Z. A., 1982, The cutaneous infiltrates of leprosy. Cellular characteristics and the predominant T cell phenotypes, *N. Engl. J. Med.* **307**:1593–1597.
54. Modlin, R. L., Hofman, F. M., Taylor, C. R., and Rean, T. H., 1983, T lymphocyte-subsets in the skin lesions of patients with leprosy, *J. Am. Acad. Dermatol.* **8**:181–189.
55. Guilberg, M., and Larsson, E. L., 1982, Studies on the induction and effector functions of concanavalin A-induced suppressor cells that limit TCGF production, *J. Immunol.* **128**:747–750.
56. Kramer, M., and Koszinowski, U., 1982, T cell-specific supressor factor(s) with regulatory influence on interleukin 2 production and function, *J. Immunol.* **128**:784–790.
57. Haregewoin, A., Godal, T., Mustafa, A. S., Belehu, A., and Yemaneberhan, T., 1983, T-cell conditioned media reverse T-cell unresponsiveness in lepromatous leprosy, *Nature (Lond.)* **302**:342–344.
58. Nogueira, N., Kaplan, G., Levy, E., Sarno, E. N., Kushner, P., Granelli-Piperno, A., Vieira, L., Colomer Gould, V., Levis, W., Steinman, R., Yip, Y. K., and Cohn, Z. A., 1983, Defective γ interferon production in leprosy. Reversal with antigen and interleukin 2, *J. Exp. Med.* **158**:2165–2170.

59. Nathan, C. F., Murray, H. W., Wiebe, M. E., and Rubin, B. Y., 1983, Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity, *J. Exp. Med.* **158:**670–689.
60. Convit, J., Aranzazu, N., Pinardi, M., and Ulrich, M., 1979, Immunological changes observed in indeterminate and lepromatous leprosy patients and Mitsuda-negative contacts after the inoculation of a mixture of *Mycobacterium leprae* and BCG, *Clin. Exp. Immunol.* **36:**214–220.
61. Convit, J., Aranzazu, N., Ulrich, M., Pinardi, M. E., Reyes, O., and Alvarado, J., 1982, Immunotherapy with a mixture of *Mycobacterium leprae* and BCG in different forms of leprosy and in Mitsuda-negative contacts, *Int. J. Lepr.* **50:**415–424.
62. Convit. J., Pinardi, M. E., Rodriguez-Ochoa, G., Ulrich, M., Avila, J. L., and Goihman-Yahr, M., 1974, Elimination of *Mycobacterium leprae* subsequent to local in vivo activation of macrophages in lepromatous leprosy by other mycobacteria, *Clin. Exp. Immunol.* **17:**261–265.
63. Mehra, V., Mason, L. H., Fields, J. P., and Bloom, B. R., 1979, Lepromin-induced suppressor cells in patients with leprosy, *J. Immunol.* **123:**1813–1817.
64. Mehra, V., Mason, L. H., Rothman, W., Reinherz, E., Schlossman, S. F., and Bloom, B. R., 1980, Delineation of a human T cell subset responsible for lepromin-induced suppression in leprosy patients, *J. Immunol.* **125:**1183–1188.
65. Bloom, B. R., and Mehra, V., 1984, Immunological unresponsiveness in leprosy, *Immunol. Rev.* **80:**5–28.

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The Protective Effects of BCG Vaccination against Tuberculosis

DONALD W. SMITH, ERNST H. WIEGESHAUS, and MARK L. EDWARDS

1. INTRODUCTION

An international conference, sponsored by the Fogarty International Center (FIC) of the National Institutes of Health in Washington, D.C., held in November 1987, entitled Research Toward the Global Control and Prevention of Tuberculosis, with an Emphasis on the Development of New Vaccines, would be expected to lead to an international research effort toward the eradication of tuberculosis. The research objectives of the FIC conference are similar to those of a conference organized in 1982 as the WHO Immunology of Tuberculosis program[1] and therefore with the principal emphasis on the application of the technologies of genetic engineering and monoclonal antibody to the development of new antituberculosis vaccines. The objectives of these two research conferences suggest that tuberculosis control specialists are not satisfied with the protective effect of available BCG vaccines and the degree of their dissatisfaction is sufficiently great that scientists are being encouraged to develop new vaccines.

Given this background, we believe that this chapter will serve a

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useful purpose by summarizing current information about the protective effects of BCG and provides a rationale for the selection of animal models to assess a new generation of antituberculosis vaccines. As a basis for selecting effects of vaccines relevant to protection against tuberculosis, the next section provides a review of some aspects of the pathogenesis of tuberculosis.

2. KEY EVENTS IN THE PATHOGENESIS OF TUBERCULOSIS

Observations of the common form of cavitary pulmonary tuberculosis in humans[2] and knowledge derived from studies in a guinea pig model of experimental airborne tuberculosis are consistent with the paths illustrating endogenous reactivation and exogenous reinfection tuberculosis (Figs. 1 and 2). Tuberculosis can be viewed as progressing through four distinct stages. The first stage involves introduction and implantation into the host, via the airway, of a droplet nucleus containing a viable tubercle bacillus. This initiates the events leading to primary infection. As the bacillus replicates and the primary lesion and associated lymph node (primary complex) develops, the cell-mediated immune (CMI) response is activated. The second stage, the stage at which the two paths differ, begins with the implantion of tubercle bacilli into an apical–subapical site (abbreviated A-SA site) in the lungs. Some tubercle bacilli survive here in a dormant state after the onset of the CMI response and can be reactivated by an immunosuppressive event. Bacilli reach this site by one of two paths. Path A is via the bloodstream, as a consequence of the bacillemia that accompanies development of the primary complex. Because progeny of bacilli from the primary infection are often implanted in an A-SA site, path A requires only a single episode of infection. Disease developing via path A is referred to as *endogenous reactivation tuberculosis*. By contrast, path B requires a new transmission, via the airstream, of bacilli from another host. Because the A-SA area is not well ventilated[3] and the number of droplet nuclei inhaled is small, several exposures may be required to initiate a focus of infection in an A-SA site. Disease developing via path B is referred to as *exogenous reinfection tuberculosis*. This is not a totally satisfactory descriptor because both types of tuberculosis (Fig. 1) involve reactivation of dormant bacilli. Once a bacillus is established in the upper lung zone it multiplies about 1000-fold before replication is arrested by the CMI response. The third stage in the development of cavitary pulmonary tuberculosis, is a period of dormancy during which a few bacilli survive in an A-SA site[4]; however, other sites including the primary complex[5] and presumably metastatic foci in the mid to lower lung are gradually sterilized. The fourth stage is

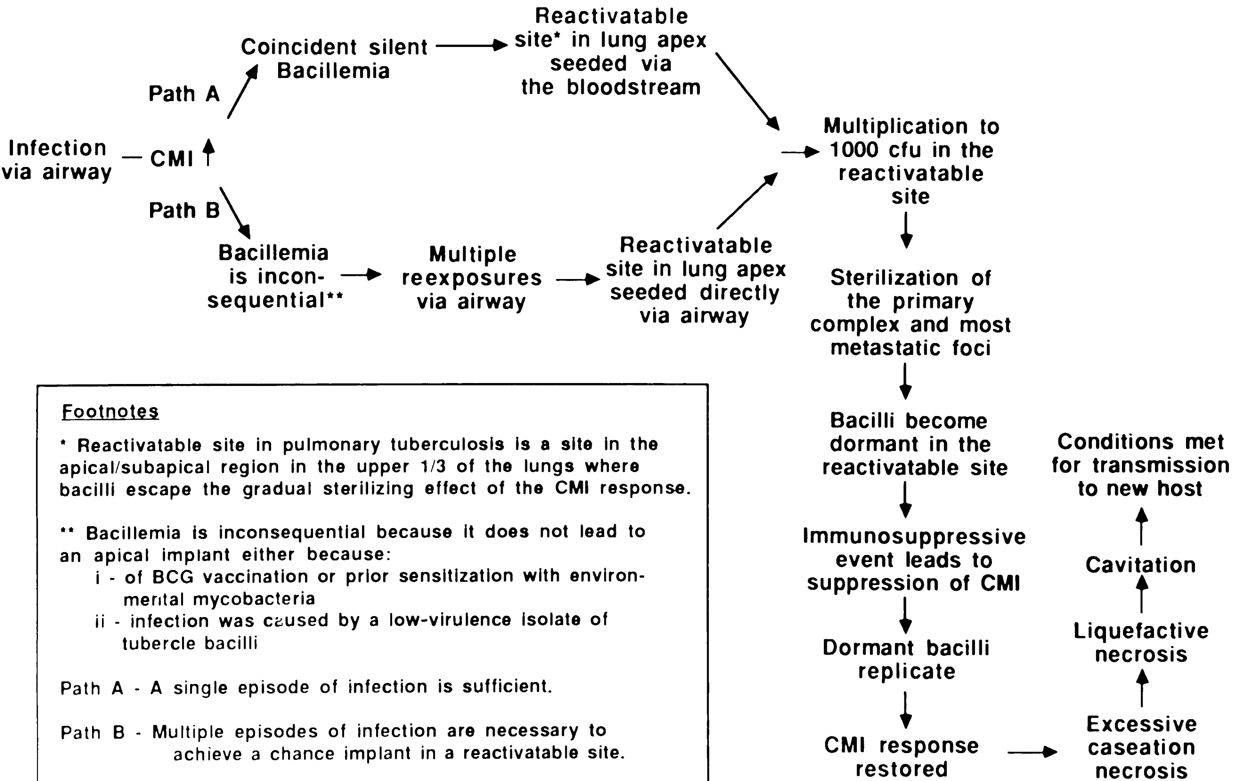
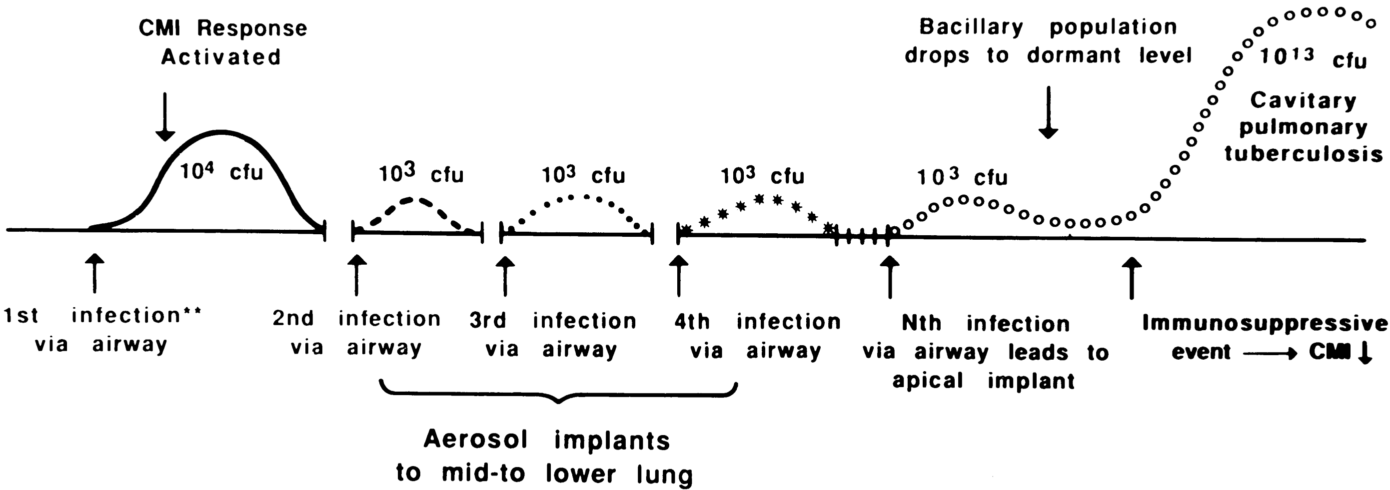


FIGURE 1. Two hypothetical paths leading to cavitary pulmonary tuberculosis.

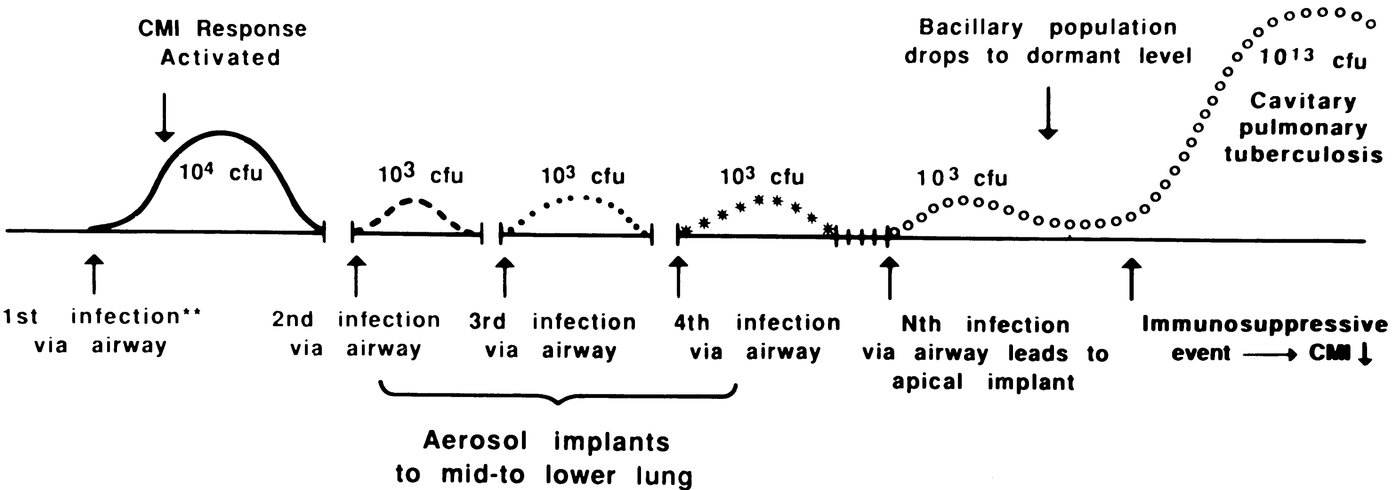


**<u>Necessary Conditions</u>

1- Prior BCG vaccination or infection with environmental mycobacteria carrying cross-reactive epitopes

2- or infection with low virulent tubercle bacilli

FIGURE 2. Change with time in the number of tubercle bacilli in the lungs for two paths leading to cavitary pulmonary tuberculosis.



**<u>Necessary Conditions</u>

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FIGURE 2. Change with time in the number of tubercle bacilli in the lungs for two paths leading to cavitary pulmonary tuberculosis.

initiated by a transient immunosuppressive event: the bacilli in the A-SA site, regardless of whether they arrived via the bloodstream or the airstream, are reactivated and begin to replicate. When the CMI response is restored, the interaction of high levels of mycobacterial antigen with immune T cells results in excessive caseous necrosis, in turn leading to liquefaction and cavitation, ultimately permitting transmission to a new host. In summary, the four stages we have described are as follows: infection, transportation (to an A-SA site), dormancy, and reactivation.

Tubercle bacilli, shown by the standard assay procedure to be of low virulence of guinea pigs, exhibited a reduced capacity to disseminate from the site of implantation in the lung[6] or the site of intramuscular injection.[6] Assuming that virulence for guinea pigs predicts events in tuberculosis in man, these observations suggest that tubercle bacilli of low virulence would cause disease primarily by exogenous reinfection. The low virulence of tubercle bacilli causing tuberculosis in a given region, together with frequent infection with mycobacteria carrying cross reactive epitopes[7] (see Figs. 1 and 2) and high risk of infection, increases the likelihood of exogenous reinfection. In contrast, in areas of the world, where the risk of infection is low and the tendency of the tubercle bacillus to disseminate hematogenously is high, with few exceptions,[8,9] pulmonary tuberculosis is presumed to be primarily of the endogenous reactivation type.[2]

It is also true that since the pathways of endogenous reactivation and exogenous reinfection are not mutually exclusive, organisms in the sputum could reflect experience with both pathogenetic pathways. For example, the first infection in an individual living in an area with a high infection rate may have involved a tubercle bacillus of high virulence, the progeny of which disseminated hematogenously, leading to at least one metastatic focus that survived in an A-SA site. Later, this individual could have been reinfected exogenously one or more times with tubercle bacilli of high or low virulence such that at least one droplet nucleus was implanted directly in an A-SA site. Later, when this individual experienced an immunosuppressive event, bacilli would begin to multiply at each focus containing viable organisms. Thus, the sputum culture isolated from this person could contain a mixed population of tubercle bacilli representing each infective episode that led to an implant in an A-SA site. Evidence has recently been obtained indicating that a proportion of sputum isolates from tuberculous individuals living in south India contain a mixture of biovars, i.e., tubercle bacilli differing in several properties: virulence for guinea pigs, phage type, and resistance to a panel of inhibitors.

To summarize this section on the pathogenesis of tuberculosis, Table I shows the relationship between virulence, immune status of the host, and the path of transmission of tubercle bacilli to an A-SA site.

TABLE I
Relationship between Virulence and Immune Status of the Host to the Transport Path of Tubercle Bacilli to a Reactivatable Site in an Upper Lung Zone in an Immunocompetent Host that Progresses to Cavitary Pulmonary Tuberculosis

	Path to a reactivatable site in an upper lung zone	
Virulence of the primary infecting strain	Immune host	Nonimmune host
High virulence	Airstream[b]	Bloodstream[a]
Low virulence	Airstream[b]	Airstream[b]

[a] If the tubercle bacillus is virulent and the host is not immune, a single infection is sufficient to lead to a bacillemia and upper lung zone seeding.
[b] If the tubercle bacillus is of low virulence *or* the host is immune, multiple infections are necessary to achieve an upper lung zone implant via the airway.

3. EFFECT OF BCG ON TUBERCULOSIS IN HUMANS

Evidence regarding the protective effect of BCG in humans is derived from a series of 10 prospective field trials (Fig. 3). These trials have been summarized in three excellent reviews.[10–12] The following section

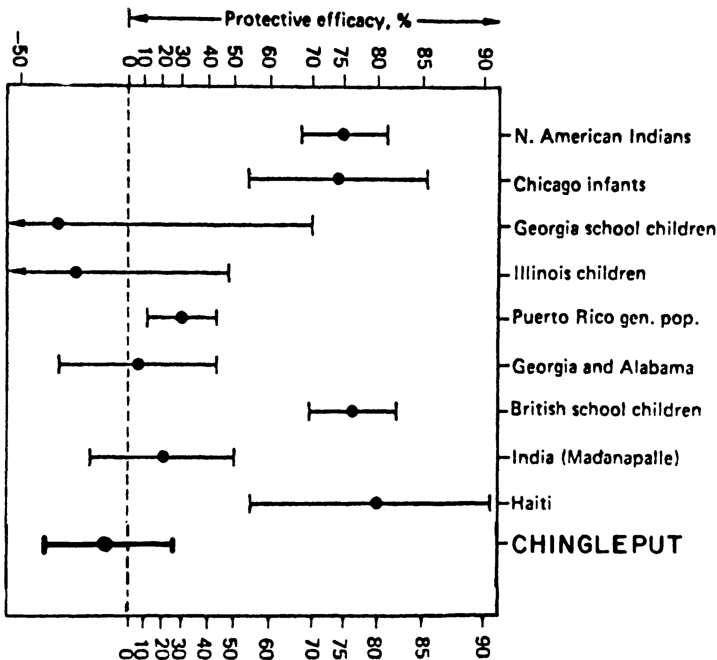


FIGURE 3. Ten prospective BCG field trials.

provides information about three of the trials which are representative of the full range of protective effects of BCG.

3.1. Field Trials

3.1.1. Aronson Trial—North American Indians

The Aronson trial[13] examined the protective effect of the Phipps strain of BCG administered between Dec. 1935 and Jan. 1938 to 1550 tuberculin-negative persons among eight different Indian tribes. As prescribed in the double-blind protocol, an equal number of persons received diluent in place of BCG. Follow-up was by means of annual skin tests and chest radiographs for the first 9–11 years and then at 5-year intervals. A case of tuberculosis was defined by the results of chest radiography and by clinical findings. The effectiveness of BCG vaccination was judged by comparing mortality among the vaccinated and nonvaccinated groups. By 1956, a total of 13 (0.84%) of the vaccinated group had died of tuberculosis as compared with 68 (4.7%) of controls, for a protective efficacy of approximately 75%.

3.1.2. British Medical Research Council Trial

The British Medical Research Council trial[14] was carried out in a population of 5472 children 14–16 years of age who were nonreactors to 100 TU of purified protein derivative (PPD). The BCG vaccine used was a fresh liquid product prepared from the Danish strain of BCG. Follow-up of recipients of vaccine or placebo, administered to randomly selected individuals, consisted of active case findings by means of chest radiographic surveys conducted at intervals of approximately 14 months. The data, over a 20-year period, revealed pulmonary tuberculosis in 248 recipients of placebo and in 62 recipients of BCG, for a protective efficacy against pulmonary tuberculosis of approximately 70%. Records[15] of the number of cases of miliary tuberculosis or of tuberculous meningitis, revealed eight in the group given placebo and none in the group given BCG for a protective efficacy against these hematogenous forms of tuberculosis of 100%.

3.1.3. Tuberculosis Prevention Trial—Chingleput District South India[7,16]

Those eligible for the Chingleput trial included the entire population of the Chingleput District over the age of 1 year; approximately 350,000 persons. Intake began in July 1968, covered a period of 2½ years and for a particular village, included simultaneous skin tests with 3

TABLE II
Number of Tuberculosis Cases in Three Vaccination-Treatment Groups for the Entire Population Who Were Nonreactors at Intake[a]

Vaccination-treatment group	Number of TB cases
0.1 mg BCG	59
0.01 mg BCG	65
Placebo	47

[a]Data were obtained 7½ years postintake.

TU of PPD-S and 5 TU of PPD-B, chest radiography, and administration of vaccine or placebo. Freeze-dried BCG-Pasteur-1173-P2 and BCG Copenhagen, strain 1331, were reconstituted and given in two different doses, 0.1 or 0.01 mg. The placebo consisted of dilute Sauton medium. Because skin tests and vaccination were done on the same day, this trial design included groups that permitted observation of the effect of vaccination on those already infected with *M. tuberculosis*. This was the first trial in which the identification of a case of tuberculosis was based on sputum culture rather than on chest radiography and/or clinical findings. Restricting consideration to the 140,000 Chingleput residents who were PPD-S negative at intake, i.e., the group comparable to that studied in Aronson's trial[13] and in the MRC trial,[14] the data at 7½ years (Table II) revealed no evidence of a protective effect of BCG.

Data after a 12½-year period[17] of follow-up (Table III), for the group who were 0–14 years old at intake, showed a protective effect of 28%. More recently, Tripathy[18] reported the 20-year follow-up data that revealed little change from the protective effect observed at 12½ years.

TABLE III
Number of Tuberculosis Cases per Vaccination-Treatment Group and the Protection Observed, for Two Population Groups[a]

	Number of persons in each treatment group with at least one positive culture			
	Vaccination-treatment group			
Population group	0.1 mg BCG	0.01 mg BCG	Placebo	Protection observed (%)
Total uninfected population (0–7 mm reactors)	93	99	93	0
Age group 0–14 years	47	44	63	28

[a]Data were obtained 12½ years postintake.

3.2. Factors Determining Whether a Protective Effect of BCG Is Observed in Humans

These three BCG field trials illustrate the full range of protective effects of BCG in man; 0–80% protection against pulmonary tuberculosis and, in one trial, 100% protection against tuberculous meningitis. The multiple, possibly interacting factors, which play a role in determining whether a protective effect of BCG is or is not observed include:

1. Protective potency of the vaccine
2. Infection with environmental mycobacteria
3. Virulence of the tubercle bacilli responsible for the infection(s)
4. Main pathogenetic path leading to the development of tuberculosis

3.2.1. Protective Potency of the Vaccine

It is possible that the inherent protective potency of the BCG vaccine used is a major determinant in the outcome of field trials; however, the only vaccine for which animal model data on protective potency have been published is BCG Tice (substrain BL).[19] Even here, no conclusion can be drawn because the producer of the vaccine sent different lots to the field[20–22] and to investigators requesting vaccine for study in laboratory animals.[23,24] We believe this situation illustrates the poor quality of our knowledge of the contribution of the inherent protective potency of vaccines to the outcome of field trials. Because the Chingleput trial was the first in which freeze-dried vaccine was administered, this factor was listed[25] as one of the hypotheses explaining the low protective effect of BCG observed in this trial.

3.2.2. Infection with Environmental Mycobacteria

The factor that has received the most attention with respect to its impact on the outcome of BCG field trials is the degree to which the population was infected (sensitized) with environmental (atypical) mycobacteria.[26,27] Repeated natural infections with environmental mycobacteria can induce a protective immune response comparable to that resulting from BCG vaccination. If this natural immunization were widespread in the population, it means that a trial conducted in such a population will have no true unvaccinated group against which to measure the number of cases of tuberculosis prevented by BCG vaccination. Another hypothesis, proposed by Stanford *et al.*,[28] is that first infection with some types of environmental mycobacteria interferes with the development of a protective response subsequent to BCG vaccination. En-

vironmental mycobacteria, as a factor explaining the low protective effect of BCG in a field trial, was first invoked in relationship to the USPHS trials in Georgia-Alabama[20] and Puerto Rico,[29] areas in which skin tests revealed that infection by environmental mycobacteria was relatively common. Comstock and Edwards[29] examined the data from the Puerto Rico trial with respect to a difference in the number of tuberculosis cases in the group who were nonreactors to 5 TU, but were reactors to 100 TU of PPD, i.e., evidence of nonspecific sensitivity (sensitivity to environmental mycobacteria) and those that were nonreactors to both tests. These latter investigators[29] concluded that no protective effect accompanied infection with environmental mycobacteria.

Infection with environmental mycobacteria has been given renewed stress as a factor in the Chingleput trial because the intake data show that by the age of 10 years, 95% of the trial population were reactors to PPD-B, intracellularin, a tuberculin prepared from *M. avium-intracellulare*. Because of the unexpectedly low total number of cases of tuberculosis and the predominance of sensitization to PPD-B in the population, it has not been possible to factor out the impact on the trial outcome of infection with environmental mycobacteria. Data[27] obtained in guinea pigs indicate that two intradermal injections of *M. avium-intracellulare* induced a protective effect nearly equal to that achieved by BCG vaccination. Assuming that protection in animals predicts the protective effect of *M. avium-intracellulare* in humans, the Chingleput trial findings are best described as a paradox. Ninety-five percent of the population over the age of 10 years were reactors to PPD-B; animal model data show a level of immunity comparable to that given by BCG vaccination, yet one reason for selection of the Chingleput district as the site for the trial was that this district had one of the highest tuberculosis prevalence rates in India. Thus, the paradox is that a region with a high rate of "natural vaccination with environmental mycobacteria" is coincidentally a region with a high prevalence of tuberculosis.

3.2.3. Virulence of the Tubercle Bacilli Responsible for the Infection(s)

An underlying hypothesis (presently being tested) for this section is that virulence of tubercle bacilli measured in guinea pigs reflects events in human disease. We have defined[6] a tubercle bacillus as being virulent if, in nonvaccinated but immunocompetent guinea pigs, it disseminates from the site of implantation or injection. We believe it is significant that BCG vaccinated animals, infected with virulent tubercle bacilli,[27] demonstrate the same characteristic primary lung lesions[30] (see Fig. 8) and the same reduced capacity for hematogenous dissemination that is observed in nonvaccinated guinea pigs infected with tubercle bacilli of low

virulence. Assuming that a relationship is demonstrated between dissemination of tubercle bacilli in guinea pigs and in humans, a strain of tubercle bacilli that has a reduced capacity to disseminate beyond the site of implantation in the lungs, is low virulent and will be restricted to causing cavitary pulmonary tuberculosis via exogenous reinfection. In several regions in south India; Madras,[31] the Chingleput District[6] and in Bangalore and nearby rural areas,[32] the majority of isolates of tubercle bacilli obtained from sputum specimens showed a significantly reduced tendency to spread via the bloodstream in guinea pigs, a finding that could mean that, in these regions, exogenous reinfection is the predominant path to cavitary pulmonary tuberculosis.

3.2.4. Main Pathogenetic Path Leading to the Development of Cavitary Pulmonary Tuberculosis

ten Dam and Pio[33] examined the relationship between the pathogenetic path leading to pulmonary tuberculosis and the results of several BCG field trials. They concluded that BCG vaccination was most effective in trials where most cases of tuberculosis developed within the first 5–10 years after vaccination. Trials in which BCG showed an inferior protective effect were those in which most cases of tuberculosis developed after a longer interval postvaccination. From these observations, ten Dam and Pio[33] proposed the hypothesis that the failure to detect a protective effect of BCG in the Chingleput trial was because tuberculosis in the trial area was caused by exogenous reinfection rather than endogenous reactivation. They reasoned that in exogenous reinfection tuberculosis, the first infection with *M. tuberculosis* would have given as strong a protective effect as was possible to achieve with any BCG vaccine currently available.

The following explanation of the failure to detect a protective effect of BCG in the Chingleput trial is based on our present understanding of the two paths leading to cavitary pulmonary tuberculosis (Figs. 1 and 2; Table I). Evidence indicates that BCG protects against endogenous reactivation disease by interfering with the bacillemia that accompanies primary infection,[34] a bacillemia needed to implant tubercle bacilli in an A-SA site. However, in exogenous reinfection tuberculosis, as a consequence of one of the several episodes of infection, bacilli are deposited in the upper lung zones directly via the airway (Fig. 1). Thus, the failure to observe a protective effect of BCG in the Chingleput trial was presumably because the predominance of infection with tubercle bacilli of low virulence and the predominance of infection with environmental mycobacteria, reduced the likelihood of a significant bacillemia and accordingly reduced the risk of endogenous reactivation tuberculosis.

3.3. Conclusion of Observations on the Protective Effect of BCG in Humans

The evidence is clear that in some trials, BCG vaccination resulted in as much as 80% protection against pulmonary tuberculosis and as high as 100% protection against tuberculous meningitis and miliary tuberculosis. These observations form the basis for the hypothesis that BCG vaccination protects against endogenous reactivation tuberculosis because it interferes with the bacillemia accompanying first infection with virulent tubercle bacilli.

Several factors unrelated to the inherent protective potency of the vaccine can play a decisive role in BCG field trials. The degree of protection observed is the resultant of the interaction among these several factors. For example, because BCG vaccination protects by interfering with the bacillemia, and bacillemia is not part of the pathogenesis of exogenous reinfection tuberculosis, neither BCG vaccination nor sensitization with environmental mycobacteria would be expected to contribute to protection in those trials carried out in regions of the world in which exogenous reinfection is the predominant pathogenetic pathway. These considerations provide one explanation for the low protective effect of BCG observed in the Chingleput trial.[7,16]

4. EFFECT OF BCG ON TUBERCULOSIS IN ANIMAL MODELS

An animal model is defined by the specific choices of test species, route of vaccination, route of challenge, challenge level, and other factors and, as the name implies, within the present context is intended to model, mimic, or predict events in human tuberculosis. With the possible exception of a tissue culture method reported by Crowle,[35] it is not currently possible to measure serum or cellular responses of an individual vaccinated with BCG that quantitate the level of protection against tuberculosis in that individual; i.e., there is no measurement comparable to the level of toxin-neutralizing antibody for diphtheria or of virus-neutralizing antibody for rubella. Accordingly, considerable effort has been directed toward the measurement of protective responses* to BCG vaccination in one or another animal model of tuberculosis. Although the measurement of "protective immunity" in an animal model would seem to be a straightforward task, several reviews of

*Because many different host responses have been measured and arbitrarily defined as protection, such terms as *protection* and *protective effect,* are included in quotation marks, except where the host response measured was inhibition of hematogenous seeding.

the literature on this topic[36–38] indicate that, in the absence of exact knowledge of what to measure as the protective response, many different measurements have been made and arbitrarily defined as "immunity," including lung density,[39] percentage of mice surviving longer than 30 days,[40] number of lung lesions,[41] and extent of gross lesions at necropsy.[42]

Other important variables in animal models are listed in Table IV. Assuming only three specific assignments for each of these variables, there are 3^9 possible combinations—a total of nearly 20,000 different animal models. This is why a survey of the relevant literature[37] demonstrates that no two laboratories in the world have selected the same animal model. Several groups of investigators[36–38] have asked: Do animal models agree on the ranking for potency of a panel of BCG vaccines? The report by Frappier *et al.*,[36] an interlaboratory study involving investigators in 12 different laboratories, demonstrated a disagreement on the ranking of vaccines for potency. Smith *et al.*[43] examined the potency of a panel of live attenuated vaccines (3 BCG and H37Ra) in mice and guinea pigs and in multiple animal models; however, all were carried out in one laboratory, eliminating the between-laboratory effect. The results of this study agreed with those obtained by Frappier *et al.*[36] in showing disagreement among different animal models in the ranking for potency of a series of BCG vaccines. Thus, these studies showed that each of the nine major variables in an animal model (see Table IV), in which BCG vaccine is assayed for "protective activity," played a deciding role in the potency assigned to a vaccine, indicating that the apparent protective potency of a BCG vaccine (strain or seed lot) was a product of

TABLE IV
Three Possible Specific Assignments For The Nine Major Variables in Animal Models[a]

Variable	Assignments
Animal species	Mouse, guinea pig, rabbit
Route of vaccination	ID, SC, IP[b]
Amount of vaccine	Low, medium, high
Vaccination-infection interval	4 weeks, 6 weeks, 8 weeks
Route of infection	IV, IP, respiratory
Level of infection	Low, medium, high
Infection-sacrifice interval	4 weeks, 6 weeks, 8 weeks
Virulence of infecting strain	Low, medium, high
Response measured	Survival, spleen weight, microbial enumeration

[a]Possible combinations = 3^9 = 19,683
[b]ID, intradermal; SC, subcutaneous; IP, intraperitoneal.

the animal model in which it is measured. Therefore, by selecting a specific set of experimental conditions, i.e., a particular animal model, it is possible to demonstrate many different host responses to vaccination with BCG. Some of these responses, however, may be artifacts of the set of experimental conditions selected and would therefore fail to give information relevant to events in human tuberculosis.

Given that different animal models disagree on the potency ranking of a panel of BCG vaccines, how does an investigator wishing to study protective effects of BCG vaccination against tuberculosis with a view toward better understanding of tuberculosis in humans, select a relevant animal model?

4.1. Approaches to the Selection of a Relevant Animal Model

4.1.1. Joint Field Trial–Laboratory Model Assay

Smith and co-workers[43–45] proposed a solution, which they termed a Joint Field Trial–Laboratory Model Assay (JFT–LMA), in which a panel of BCG vaccines were to be ranked for potency in a field trial in human subjects and simultaneously in a number of animal models. The animal models that ranked the BCG vaccines for potency in the same order as their ranking in humans were to be declared valid models for assay of the potency of BCG. The fallacy in the JFT–LMA approach became obvious after the publication of the first findings of the Chingleput trial,[7,16] which showed no evidence of a protective effect of two BCG vaccines. If this trial had been chosen for the field trial segment of the JFT–LMA, every animal model that showed a protective effect for the BCG vaccines used in the trial would have been declared invalid. The fallacy in the JFT–LMA approach is that it failed to recognize that an animal model assay is a relatively pure test of the inherent protective potency of a BCG vaccine, measured under controlled laboratory conditions in which the only variable is vaccination treatment. This is in marked contrast to a field trial of BCG vaccine in humans, in which the techniques for random allocation of individuals to receive vaccine or placebo are used in an attempt to eliminate the impact of other uncontrolled variables by making certain that they are equally represented in the several vaccination treatment groups. However, as indicated by the data from the Chingleput trial, one or more of these uncontrolled variables, even though equally represented in the different vaccination treatment groups, was apparently responsible for the outcome—i.e., no difference in the number of cases of tuberculosis in individuals given BCG or placebo.

4.1.2. Rational Animal Model

As set forth in a recent review,[38] a rational animal model is constructed on the premise that the greater the similarity between the conditions for vaccination and infection of humans and the conditions for vaccination and infection of the animal species selected for the experimental model, the smaller the likelihood that the investigator has inadvertently introduced an artifact that could result in findings unrelated to events in the pathogenesis of tuberculosis in humans. Because evidence[15] indicates that BCG vaccination protects humans by interfering with the hematogenous transport of bacilli, a rational animal model would be one in which interference with hematogenous transport of bacilli would be measured in BCG-vaccinated and -nonvaccinated animals infected by the respiratory (pulmonary, aerosol) route with small numbers of bacilli, simulating the conditions under which humans are infected. Interference with hematogenous transport could be judged from data on the quantitative recovery of bacilli from the spleen or, better still, from the quantitative recovery of bacilli from previously uninvolved areas of the lungs. Thus, a rational animal model for study of the protective potency of BCG would include the specific variables listed in Table V.

To emphasize the crucial importance of the specific animal model used, we will consider primarily those investigations that involved study of the protective effect of BCG in rational animal models (see Section

TABLE V
Specific Assignments for the Major Variables Constituting Rational Animal Models

Animal species: most often guinea pigs, mice, or primates
Vaccination route: ID
Vaccination dose: minimum number of BCG inducing protection
  Guinea pigs: 50–100 CFU
  Mice: approximately 10^4 CFU
Vaccination-challenge interval minimum of 6 weeks (to avoid the early period of nonspecific resistance)
Challenge route: respiratory
Challenge dose: minimum number of virulent bacilli needed to infect each animal
  Guinea pigs: 3–5 CFU of bacilli inhaled and retained capable of developing primary lesions on the lungs
  Mice: 5–10 CFU of bacilli inhaled and retained
Response to infection selected as the criterion of protection: quantitative assay of the extent to which vaccination has interferred with the spread of bacilli via the bloodstream from sites of implantation in the lungs.

4.2). The papers included that do not fit the definition of a rational animal model are those in which different criteria of "protection" have been selected, criteria that may or may not correlate with the criterion of protection in a rational model.

4.2. Studies of the Protective Effect of BCG in Rational Animal Models

4.2.1. Primate Models

Ribi *et al.*[46] reported that viable BCG given by the intravenous (IV) route to rhesus monkeys completely inhibited hematogenous spread when these animals were challenged via the respiratory route with approximately 20 colony-forming units (CFU) of *M. tuberculosis*, strain H37Rv. In a subsequent report from the same group (Anacker *et al.*[47]), the subcutaneous (SC), intramuscular (IM), and IV routes of vaccination were compared for their effect against subsequent challenge via the respiratory route. The maximum "protective effect" (extent of gross tuberculosis) accompanied IV administration of BCG. Barclay *et al.*[48] compared intradermal (ID), IV, and respiratory routes of vaccination and reported that IV vaccination led to superior "protection" (gross organ involvement), while ID vaccination afforded the least "protection." Good and McCarroll[49] vaccinated rhesus monkeys with BCG-Chicago by the ID or by the respiratory route and challenged with *M. tuberculosis* (Erdman strain) 8, 29, or 72 weeks after vaccination. "Protection," judged from the number of survivial animals, showed no difference in the response of animal vaccinated by the ID or by the respiratory route.

The absence of agreement on the importance of route of vaccination in primate models could result from the differences in the criteria of "protection" used or to other unspecified differences in the animal models.

4.2.2. Mouse Models

Rather than investigate the inherent protective effect of BCG, most protection studies in rational mouse models have compared the protective effect of BCG with that of some experimental nonliving vaccine. Two reports by Ribi *et al.*[50,51] exemplify this type of study.

The following series of reports are cited because they provide information about the protective effect of BCG in mice. Outbred Swiss mice. vaccinated by the ID or SC route and infected via the respiratory route 5 weeks after vaccination, showed no evidence[52] of a "protective" re-

sponse (judged by the number of bacilli recovered from lung or spleen) when killed at intervals ranging from 2 to 19 weeks after challenge with *M. tuberculosis* Erdman strain. Schell *et al.*[53] examined the change with time in the number of virulent tubercle bacilli recovered from the lungs and spleen of BCG-vaccinated and -nonvaccinated mice. The mice had been challenged via the respiratory route with an inoculum, leading to the inhalation and retention of 5–10 CFU of *M. tuberculosis* strain H37Rv capable of initiating primary lesions on the lungs of nonvaccinated mice. Groups of animals were killed at intervals 1–14 weeks after challenge; the data in Fig. 4 compare the number of tubercle bacilli recovered from the lungs of nonvaccinated mice and mice given BCG by the ID or IV route. Analyses showed that a significant difference (10-fold) between vaccinated and nonvaccinated animals was evident only 3–7 weeks after challenge. Figure 5 shows the time course of recovery of H37Rv from the spleen of the same three groups of mice. Analyses of these data showed a significant reduction in the number of virulent bacilli recovered from the spleen only for the group vaccinated by the IV route. Thus, the data of Schell *et al.*[53] indicate that mice vaccinated ID (stipulation of a rational animal model) with 10^5 BCG-Tice (substrain BLP) were not protected, as judged by a delay in onset or a reduction in the extent of hematogenous seeding, but were "protected" as judged by the number of bacilli recovered from the lungs. Anacker *et al.*[54] compared five different routes of vaccination [intraperitoneal (IP), IV, IM, ID, SC] for

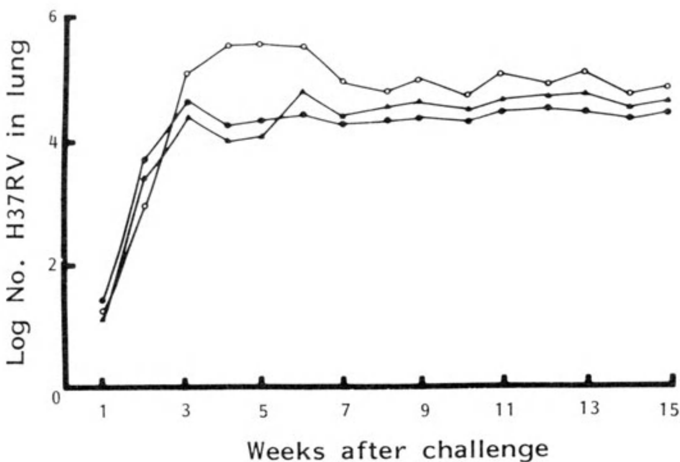


FIGURE 4. Change with time in the number of tubercle bacilli recovered from the lungs of vaccinated and nonvaccinated mice challenged via the respiratory route. (▲) Mice vaccinated IV; (●) mice vaccinated ID; (○) nonvaccinated mice. (From Schell *et al.*,[53] with permission.)

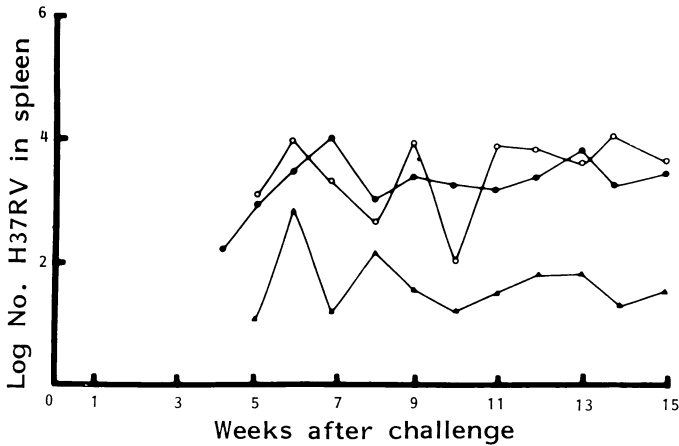


FIGURE 5. Change with time in the number of tubercle bacilli recovered from the spleen of vaccinated and nonvaccinated mice challenged via the respiratory route (from Schell *et al.*,[53] with permission). The key to the treatment groups is the same as for Fig. 4.

their ability to "protect" mice against aerosol challenge and reported that only the ID route failed to "protect" as judged by the number of primary lesions and the number of bacilli recovered from the lungs.

Lefford[55] examined the influence of route of immunization on the active and adoptive immune response of mice to challenge with virulent tubercle bacilli. Lefford concluded that a "protective response" (as judged by the number of tubercle bacilli recovered from lung, spleen, or footpad) to challenge via the respiratory route depended on a population of resident immune T lymphocytes. In another report, Lefford[56] compared three different BCG strains for their ability to influence active or adoptive immunity to challenge via the respiratory route with *M. tuberculosis*, strain R1Rv. He reported that BCG strains that grew best in the lungs imparted the greatest degree of "protection" (as judged from the number of R1Rv recovered from lung and spleen) against challenge via the respiratory route. Hank *et al.*[57] reported that the "protective effect" (as judged from the number of bacilli recovered from primary lung lesions and primary lesion-free lung lobes of BCG in guinea pigs) was influenced by the virulence of the challenge strain. This observation disagrees with a conclusion in a recent report by Collins,[58] in which the criteria of "protection" was microbial enumeration of the lungs and spleen of mice. This disagreement could relate to the observation that isolates of tubercle bacilli differing in virulence for guinea pigs do not differ in virulence for mice.[59]

It has been shown in mice[60] and in guinea pigs[26,27] that the protective effect of BCG is not suppressed in animals sensitized with environmental mycobacteria prior to BCG vaccination.

4.2.3. Guinea Pig Model

A preliminary report of early studies with a rational guinea pig model[52] indicated that animals could be challenged in an aerosol infection chamber under conditions leading to the inhalation and retention of 5–10 virulent tubercle bacilli capable of multiplying and developing primary lesions on the pleural surface of the lungs. This report also describes significant differences in the number of bacilli recovered from the lungs and spleen of BCG vaccinated and nonvaccinated animals killed 3–20 weeks postchallenge. A problem was noted in this early study with regard to the reproducibility of the infection when large numbers of animals were to be exposed. This problem was solved in the next report,[61] with the discovery that single-cell suspensions of tubercle bacilli, to be used for the nebulizer fluid for the aerosol infection chamber, could be stored at constant viability at −70°C. This observation. together with careful monitoring of the operating parameters of the infection chamber (airflow rates, exposure time) permitted the infection of large groups of guinea pigs via the respiratory route, leading to the induction of a reproducible and predictable number of primary lesions on the lungs of nonvaccinated animals.

The next topic considered in this series of studies, that by Wiegeshaus *et al.*[62], was the development of a rationale for the selection of a nonarbitrary measure of acquired resistance. Because the purpose of our research is to gain knowledge relevant to acquired resistance to tuberculosis in humans, acquired resistance was operationally defined as follows: An animal has acquired resistance if after vaccination by regimens used in man and after challenge under conditions which simulate those under which humans are infected, the vaccinated animal lives significantly longer than a nonvaccinated animal. Because determination of survival time in guinea pigs infected via the respiratory route with small numbers of tubercle bacilli is a time-consuming method, this study also asked: What measurement made on the tissues of vaccinated and nonvaccinated animals killed 3 or 5 weeks after challenge is correlated with the survival time of the animals? The survival curves shown in Fig. 6 are for groups vaccinated with (1) BCG, (2) a preparation of nonliving (defatted) tubercle bacilli given in paraffin oil, or (3) placebo. Statistical analyses showed a high correlation between survival time and the number of bacilli recovered from the lungs of guinea pigs killed 3 or 5 weeks after challenge. Other studies[63] have shown that at 3 weeks, bacilli recovered from the lungs essentially reflect bacilli in primary lung lesions. Accordingly, the data from Wiegeshaus *et al.*[62] can be interpreted to indicate that there is a high correlation between survival time and the number of bacilli recovered from excised primary lung lesions.

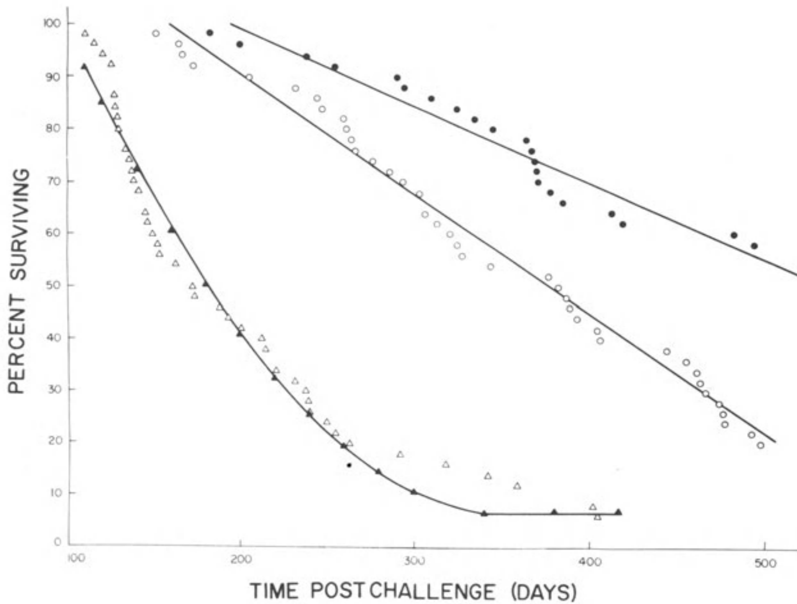


FIGURE 6. Least squares lines showing the change with time in the number of vaccinated and nonvaccinated guinea pigs surviving after respiratory challenge with a small number of virulent tubercle bacilli. (From Weigeshaus *et al.*,[62] with permission.)

The fourth report in this series[64] examined the time course of infection with virulent tubercle bacilli in BCG-vaccinated and -nonvaccinated animals and reported the following observations:

1. The development of virulent tubercle bacilli in the lungs (Fig. 7) followed the same time course in BCG-vaccinated and -nonvaccinated animals during the first 10–14 days after challenge with 3–5 CFU. This suggests that in BCG-vaccinated guinea pigs, tuberculin reactors at the time of challenge, the inhaled bacilli were ingested by nonactivated macrophages. It also means that BCG vaccination did not prevent infection with virulent bacilli.
2. In BCG-vaccinated guinea pigs that were tuberculin reactors at the time of challenge, the exponential increase in the number of tubercle bacilli in the lungs (Fig. 7) did not change until after the number of tubercle bacilli had reached approximately 10^3 CFU in each primary lung lesion.
3. In nonvaccinated (tuberculin-negative) guinea pigs, the exponential increase in the number of tubercle bacilli continued 4–5 days longer (Fig. 7) than in vaccinated animals. Thus, the max-

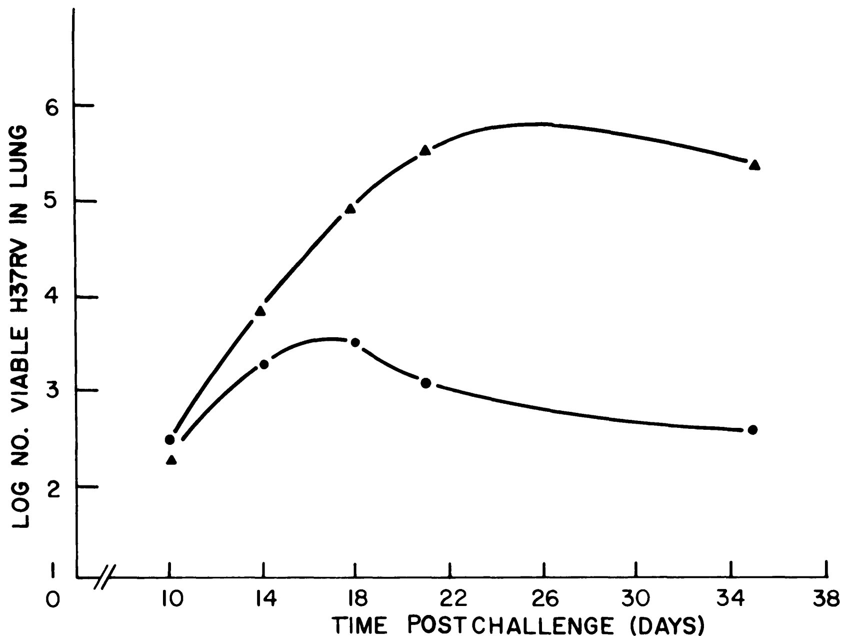
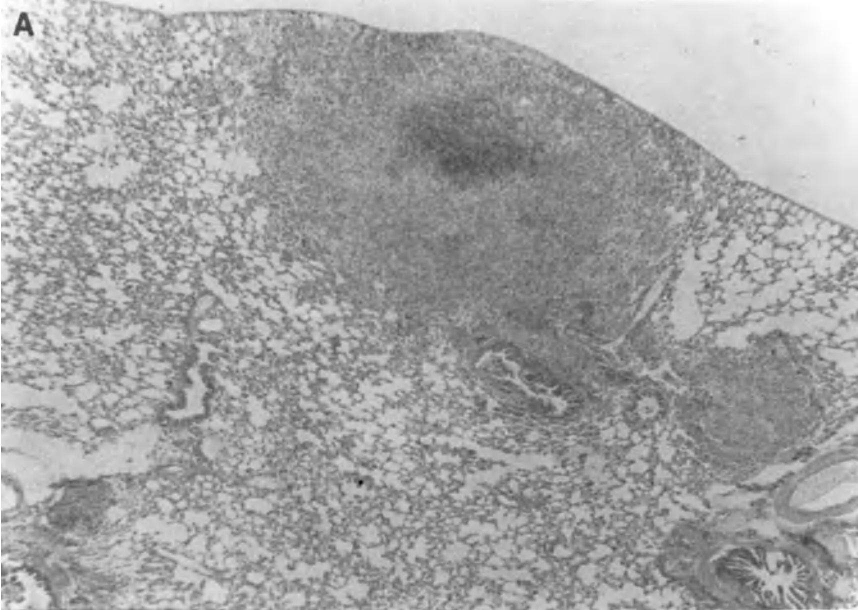


FIGURE 7. Change with time in the number of tubercle bacilli recovered from the lungs of vaccinated (●—●) and nonvaccinated (▲—▲) guinea pigs challenged via the respiratory route. (From Smith *et al.*,[64] with permission.)

imum number of bacilli in the lungs of nonvaccinated animals was approximately 10^5 CFU per primary lesion, 100-fold higher than the maximum number of bacilli in primary lesions in guinea pigs vaccinated with BCG.

4. The difference in the maximum number of bacilli recovered from the lungs of vaccinated and nonvaccinated animals was accompanied by a difference in the extent of tissue damage in primary lung lesions. Whereas extensive caseous necrosis was evident in the primary lesion in a nonvaccinated animal (Fig. 8A), the primary lesion in a lung lobe of a guinea pig vaccinated with BCG (Fig. 8B) only showed evidence of chronic inflammatory changes.
5. In contrast to the prompt bactericidal events accompanying the onset of cell-mediated immune (CMI) and macrophage activation against *Listeria monocytogenes*,[65] macrophage activation against tubercle bacilli leads to bacteriostasis. A significant reduction in the number of bacilli recovered from the lungs was not observed until the 10th week of infection in guinea pigs challenged via the



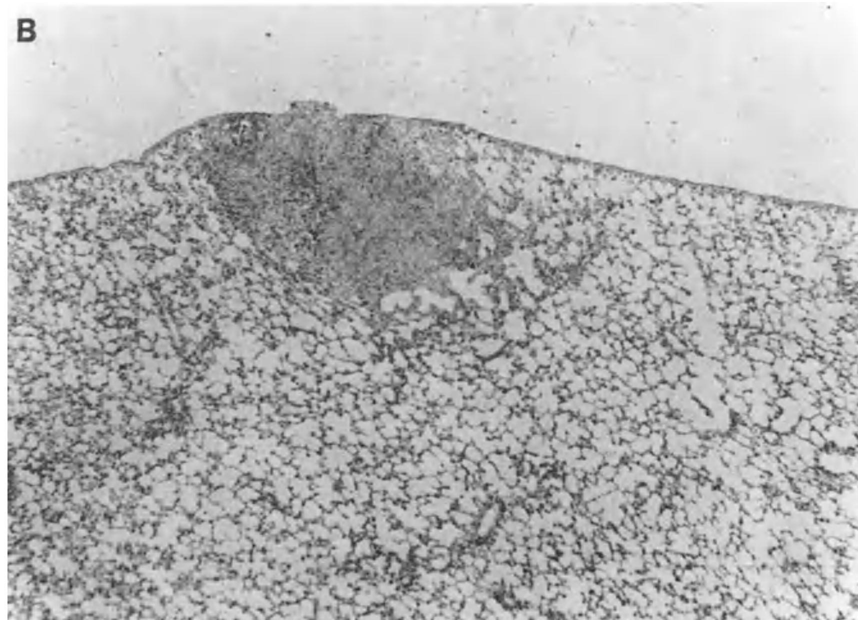


FIGURE 8. Hematoxylin–eosin stained sections of the lungs of guinea pigs killed 24 days after respiratory infection with 3–5 virulent tubercle bacilli. (A) Primary lesion in the lung of a nonvaccinated guinea pig. (B) Primary lesion in the lung of a BCG-vaccinated guinea pig. (From Smith *et al.*,[30] with permission.)

respiratory route with 3–5 CFU of virulent tubercle bacilli.[66] A study by Ho *et al.*[63] indicated that macrophage activation was a local rather than a systemic event.

6. Hematogenous transport of virulent bacilli to the spleen, observed on the 21st day, is coincident with the first detection of delayed-type hypersensitivity in nonvaccinated animals and was delayed in onset and reduced in extent in BCG-vaccinated guinea pigs.

Because of the importance of hematogenous seeding in the pathogenesis of endogenous reactivation tuberculosis, a subsequent series of reports was devoted to quantitative evaluation of the influence of BCG vaccination on hematogenous dissemination. Fok *et al.*[67] asked whether vaccination prevented hematogenous seeding or whether it induced a bactericidal event against tubercle bacilli transported to metastatic sites, e.g., in the lungs and spleen. The results of the study provided no evidence in support of the hypothesis of a prompt bactericidal event; accordingly, the data support the hypothesis that BCG vaccination influences hematogenous dissemination by retarding the release of bacilli from primary lung lesions. This hypothesis was confirmed and observations extended in a subsequent study[68] showing that vaccination influenced both the onset and the extent of hematogenous seeding. The influence of BCG vaccination on hematogenous seeding of fully virulent tubercle bacilli to previously uninvolved regions of the lungs was such that transport of significant numbers of bacilli to the lungs did not occur before 60–80 days postchallenge. Of potential significance to the failure to observe a protective effect of BCG in the Chingleput trial is the observation of Edwards *et al.*[27] that in guinea pigs challenged with a recent isolate of low virulence for guinea pigs, hematogenous seeding of previously uninvolved lung lobes was insignificant, even in nonvaccinated animals.

4.2.4. Protein-Deficient Guinea Pig Model

In order to study the interaction between nutritional status of the host and response to BCG vaccination and challenge with *M. tuberculosis* strain H37Rv, McMurray *et al.*[69,70] developed a protein-deficient rational guinea pig model in which challenge was administered via the respiratory route. Protein-deficient animals developed smaller reactions to skin tests with PPD, and an inferior protective effect was evident from the data showing the number of H37Rv recovered from lungs and spleen. The most recent report by this group[70] included an additional factor, i.e., the use of strain 2 and strain 13 inbred guinea pigs. This

study showed that strain 13 guinea pigs were more strongly affected by protein deficiency—an interaction between protein-deficiency and genetic pedigree.

5. CONCLUDING REMARKS

Evidence indicates BCG vaccination protects against tuberculosis in humans by the induction of changes in the vaccinated person that lead to interference with the bacillemia accompanying the first infection with virulent tubercle bacilli in a nonvaccinated person. For endogenous reactivation tuberculosis, this bacillemia is a key event for the transition from tuberculous infection to clinically apparent tuberculosis. A corollary of these observations is that BCG vaccination has no influence on exogenous reinfection tuberculosis, because it is independent of a bacillemia.

Data obtained in a rational guinea pig model designed to mimic the conditions of vaccination and infection in humans demonstrate the following protective effects of BCG vaccination:

1. There is an earlier onset of bacteriostasis in the lungs of BCG vaccinated animals, leading to a maximum bacillary population in the lungs 100-fold lower in vaccinated than in nonvaccinated animals.
2. The tissue damage due to a CMI response is significantly reduced in the lungs of vaccinated as compared to nonvaccinated animals and is not accompanied by caseous necrosis.
3. The dissemination of virulent bacilli via the bloodstream to the spleen and to previously uninvolved areas in the lungs is significantly delayed in onset and reduced in extent in the tissues of BCG vaccinated animals.
4. BCG alters the host response of a vaccinated guinea pig so that it responds to challenge with high virulent tubercle bacilli in the same way as a nonvaccinated animal responds to challenge with bacilli of low virulence.

A rationale has been presented for the selection of animal models for the assay of the protective potency of the new generation of antituberculosis vaccines currently under development. The authors urge that the protective effect of these vaccines be assayed in rational animal models. Vaccines expected to protect against endogenous reactivation tuberculosis must be able to interfere with some event in path A shown in Fig. 1. BCG vaccine inhibits the bacillemic phase of infection, an event that can be determined by measuring the number of virulent bacilli

recovered from the spleen or from primary lesion-free lung lobes. One of the new vaccines may interfere at an earlier stage in the infection; e.g., vaccination could induce bacteriostasis after only a few cycles of replication of the infecting bacilli. Such a protective response could be quantitated by measuring the number of bacilli recovered from the lungs of vaccinated and nonvaccinated animals killed 3–5 days after challenge via the respiratory route.

Vaccines designed to protect against exogenous reinfection, a much more demanding requirement, must interfere with some event in path B (Fig. 1). This would appear to mean that the vaccine would have to induce an altered state such that a droplet nucleus landing in any alveolar space in the lung would be ingested by a macrophage that has already been activated, i.e., a state of systemic macrophage activation.

A further complication with regard to the ability of vaccines to protect against exogenous reinfection is that evidence obtained with BCG vaccine indicates that activated macrophages in the upper lung zones can reduce the bacillary population in a lesion to a low level but cannot sterilize the lesion. When this individual later experiences an immunosuppressive event, bacilli escape from dormancy to go on to produce disease. These observations suggest that efforts should be directed toward the development of vaccines that produce an immune response of sufficient intensity that sterilization of metastatic foci also occurs in the apical–subapical area of the lungs and in other areas in which bacilli survive in a dormant state. These considerations indicate that a rational animal model designed to detect protective responses to new antituberculosis vaccines should measure not only inhibition of hematogenous transport of bacilli to primary lesion-free lung lobes and spleen but events in excised primary lung lesions and in lung lobes excised 1–2 weeks after aerosol challenge with 3–5 tubercle bacilli as well.

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REFERENCES

1. Memorandum from a WHO meeting, 1982, Immunological research in tuberculosis, *Bull. WHO* **60:**723–727.
2. Stead, W. W., 1967, Pathogenesis of a first episode of chronic pulmonary tuberculosis in man: Recrudescence of residuals of the primary infection or exogenous reinfection?, *Am. Rev. Respir. Dis.* **95:**729–745.

3. West, J. B., 1962, Regional differences in gas exchange in the lungs of erect man, *J. Appl. Physiol.* **17:**893–898.
4. Opie. E. L., and Aronson, J. D., 1927, Tubercle bacilli in latent tuberculous lesions and in lung tissue without tuberculous lesions, *Arch. Pathol. Lab. Med.* **4:**1–21.
5. Canetti, G., 1972, Endogenous reactivation and exogenous reinfection: Their relative importance with regard to the development of non-primary tuberculosis, *Bull. Int. Natl. Union Tuberc.* **47:**116–122.
6. Prabhakar, R., Venkataraman, P., Vallishayee, R. S., Reeser, P., Musa, S., Hashim, R., Dimmer, C., Wiegeshaus, E., Edwards, M.,and Smith, D. W., 1987, Virulence for guinea pigs of tubercle bacilli isolated from the sputum of persons included in the BCG trial, Chingleput district, south India, *Tubercle* **68:**3–17.
7. Tuberculosis Prevention Trial, Madras, 1980, Trial of BCG vaccines in south India for tuberculosis prevention, *Indian J. Med. Res.* **72**(suppl.)**:**1–74.
8. Raleigh, J. W., Wichelhausen, R. H., Rado, T. A., and Bates, J. H., 1975, Evidence for infection by two distinct strains of *Mycobacterium tuberculosis* in pulmonary tuberculosis: report of nine cases, *Am. Rev. Respir. Dis.* **112:**497–503.
9. Nardell, E., McInnis, B., Thomas, B., and Weidhaas, S., 1986, Exogenous reinfection with tuberculosis in a shelter for the homeless, *N. Engl. J. Med.* **313:**1570–1575.
10. ten Dam, H. G., 1984, Research on BCG vaccination, *Adv. Tuberc. Res.* **21:**79–106.
11. ten Dam, H. G., Toman, K., Hitze. K. L., and Guld, J., 1976, Present knowledge of immunization against tuberculosis, *Bull. WHO* **54:**255–269.
12. Eickhoff, T. C., 1977, The current status of BCG immunization against tuberculosis, *Annu. Rev. Med.* **28:**411–423.
13. Aronson, J. D., Aronson, C. F., and Taylor, H. C., 1958, A twenty-year appraisal of BCG vaccination in the control of tuberculosis, *AMA Arch. Intern. Med.* **101:**881–893.
14. Hart, P. D., and Sutherland, I., 1977, BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life. Final report to the Medical Research Council, *Br. Med. J.* **12:**293–295.
15. Tuberculosis Vaccines Clinical Trials Committee, 1959, BCG and Vole bacillus vaccines in the prevention of tuberculosis in adolescents. Second report to the Medical Research Council, *Br. Med. J.* **12:**379–396.
16. Tuberculosis Prevention Trial, 1979, Trial of BCG vaccines in south India for tuberculosis prevention: first report, *Bull. WHO* **57:**819–827.
17. Tripathy, S. P., 1983, The case for BCG, *Ann. Natl. Med. Sci. (India)* **19:**11–21.
18. Tripathy, S. P., 1986, The Chingleput trial—A 15 year report. Presented at the XXVIth World Conference of the International Union Against Tuberculosis, Singapore, November, 1986.
19. Rosenthal, S. R., 1972, Methods of BCG vaccination, *Fogarty Int. Ctr. Proc.* **14:**179–190.
20. Palmer, C. E., Shaw, L. W., and Comstock, G. W., 1958, Community trials of BCG vaccination, *Am. Rev. Tuberc. Respir. Dis.* **77:**877–907.
21. Comstock, G. W., and Palmer, C. E., 1966, Long-term results of BCG vaccination in the southern United States, *Am. Rev. Respir. Dis.* **93:**171–183.
22. Comstock, G. W., and Webster, R. G., 1969, Tuberculosis studies in Muscogee County, Georgia. VII. A twenty-year evaluation of BCG vaccination in a school population, *Am. Rev. Respir. Dis.* **100:**839–845.
23. Jespersen, A., and Weis-Bentzon, M., 1967, Relationship between tuberculin sensitivity and acquired resistance in guinea pigs vaccinated with BCG strains of different virulence, *Acta Pathol. Microbiol. Scand.* **71:**114–131.
24. Dubos, R. J., and Pierce, C. H., 1956, Differential characteristics in vitro and in vivo of several substrains of BCG. IV. Immunizing effectiveness, *Am. Rev. Tuberc.* **74:**699–717.
25. ICMR/WHO Scientific group, 1980, Vaccination against tuberculosis, *WHO Tech. Rep. Ser.* **651:**1–21.

26. Palmer, C. E., and Long, M. W., 1966, Effects of infection with atypical mycobacteria on BCG-vaccination and tuberculosis, *Am. Rev. Respir. Dis.* **94**:553–568.
27. Edwards, M. L., Goodrich, J. M., Muller, D., Pollack, A., Ziegler, J. E., and Smith, D. W., 1982, Infection with *Mycobacterium avium-intracellulare* and the protective effects of Bacille Calmette Guerin, *J. Infect. Dis.* **145**:733–741.
28. Stanford, J. L.. Shield, M. J., and Rook, G. A., 1981, How environmental mycobacteria may predetermine the protective efficacy of BCG. Hypothesis 1, *Tubercle* **62**:55–62.
29. Comstock, G. W.. and Edwards, P. Q., 1972, An American view of BCG vaccination, illustrated by results of a controlled trial in Puerto Rico, *Scand. J. Respir. Dis.* **53**:207–217.
30. Smith, D. W., Fok, J. S., Ho, R. S., Harding, G. E., Wiegeshaus, E., and Arora, P. K., 1975, Influence of BCG vaccination on the pathogenesis of experimental airborne tuberculosis, *J. Hyg. Epidemiol. Microbiol. Immunol.* **19**:407–417.
31. Mitchison, D. A., 1964, The virulence of tubercle bacilli from patients with pulmonary tuberculosis in India and other countries, *Bull. Int. Union Tuberc.* **35**:287–306.
32. Naganathan, N., Mahadev, B., Challu, V. K., Rajalakshmi, R., Jones, B., and Smith, D. W., 1986, Virulence of tubercle bacilli isolated from patients with tuberculosis in Bangalore, India, *Tubercle* **67**:261–267.
33. ten Dam, H. G., and Pio, A.. 1982, Pathogenesis of tuberculosis and the effectiveness of BCG vaccination, *Tubercle* **63**:225–233.
34. Ladefoged, A., Bunch-Christensen, K., and Guld, J., 1970, The protective effect in bank voles of some strains of BCG, *Bull. WHO* **53**:435–443.
35. Crowle, A. J., and May, M., 1981, Preliminary demonstration of human tuberculoimmunity in vitro, *Infect. Immunol.* **31**:453–464.
36. Frappier, A., Portelance, V., St. Pierre, J., and Pannisset, M., 1972, BCG strains: Characteristics and relative efficacy, *Fogarty Int. Ctr. Proc.* **14**:157–178.
37. Smith, D. W., Grover, A. A., and Wiegeshaus, E., 1968, Nonliving immunogenic substances of mycobacteria, *Adv. Tuberc. Res.* **16**:191–227.
38. Smith, D. W., 1985, Protective effect of BCG in experimental tuberculosis *Adv. Tuberc. Res.* **22**:1–97.
39. Crowle, A. J., 1961, Tubercle bacillary extracts immunogenic for mice. 2. Water-soluble proteinaceous extracts, *Tubercle* **42**:479–486.
40. Youmans, G. P., and Youmans, A. S., 1957, The measurement of the response of immunized mice to infection with *M. tuberculosis* var. hominis, *J. Immunol.,* **78**:318–329.
41. Ribi, E., Anacker, R. L., Brehmer, W., Goode, G., Larson, C. L., List, R. H., Milner, K. C., and Wicht, W. C., 1966, Factors influencing protection against experimental tuberculosis in mice by heat-stable cell wall vaccines, *J. Bacteriol.* **92**:869–879.
42. Smith, D. W., Fregnan, G. B., Delaquerriere-Richardson, L., and Valdivia, E., 1964, Induction of acquired resistance in guinea pigs with defatted *M. tuberculosis* vaccines, *J. Bacteriol.* **88**:87–92.
43. Smith, D. W., Wiegeshaus, E. H., Stark, R. H., and Harding, G. E., 1972, Models for potency assay of tuberculosis vaccines, *Fogarty Int. Ctr. Proc.* **14**:205–218.
44. Smith, D. W., Harding, G. E., Wiegeshaus, E. H., Grover, A. A., and McMurray, D. N., 1970, Variables in tuberculosis vaccines test systems; a preliminary report, *Symp. Ser. Immunobiol. Std.* **17**:259–268.
45. Smith, D. W., and Harding, G. E., 1977, Approaches to the validation of animal test systems for assay of protective potency of BCG vaccines, *J. Biol. Std.* **5**:131–138.
46. Ribi, E., Anacker, R. L., Barclay, W. R., Brehmer, W., Harris, S. C., Lief, W. R., Simmons, J., and Smith, A. W., 1971, Efficacy of mycobacterial cell walls as a vaccine against airborne tuberculosis in the rhesus monkey, *J. Infect. Dis.* **123**:527–538.
47. Anacker, R. L., Brehmer, W., Barclay, W. R., Leif, W. R., Ribi, E., and Simons, J. H.,

1972, Superiority of intravenously administered BCG cell walls in protecting rhesus monkeys (*Macaca mulatta*) against airborne tuberculosis, *Z. Immun.forsch.* **143:**363–376.
48. Barclay, W. R., Busey, W. M., Dalgard, D. W.. Good, R. C., Janicki, B. W., Kasik, J. E., Ribi, E., Ulrich, C. E., and Wolinsky, E., 1973, Protection of monkeys against airborne tuberculosis by aerosol vaccination with bacillus Calmette–Guérin, *Am. Rev. Respir. Dis.* **107:**351–358.
49. Good, R. C., and McCarroll, N. E., 1977, BCG vaccination in rhesus monkeys: study of skin hypersensitivity and duration of protective immunity, in: *Mycobacterial Infections of Zoo Animals* (R. J. Montali, ed.), pp. 115–121, Smithsonian Institution Press, Washington, D.C.
50. Ribi, E., Larson, C., Wicht, W., List, R., and Goode, G., 1966, Effective nonliving vaccine against experimental tuberculosis in mice, *J. Bacteriol.* **91:**975–983.
51. Ribi, E., Meyer, T. J., Azuma, I., Parker, R., and Brehmer, W., 1975, Biologically active components from mycobacterial cell walls. IV. Protection of mice against aerosol infection with virulent *Mycobacterium tuberculosis, Cell Immunol.* **16:**1–10.
52. Smith, D. W., Wiegeshaus, E., Navalkar, R., and Grover, A. A., 1966, Host-parasite relationships in experimental airborne tuberculosis. I. Preliminary studies in BCG-vaccinated and nonvaccinated animals, *J. Bacteriol.* **91:**718–724.
53. Schell, R. F., Ealey, W. F., Harding, G. E., and Smith, D. W., 1974, The influence of vaccination on the course of experimental airborne tuberculosis in mice, *J. Reticuloendothelial Soc.* **16:**131–138.
54. Anacker, R. L., Barclay, W. R., Brehmer, W., Goode, G., List, R. H., Ribi, E., and Tarmina, D. F., 1969, Effectiveness of cell walls of *Mycobacterium bovis* strain BCG administered by various routes and in different adjuvants in protecting mice against airborne infection with *Mycobacterium tuberculosis* strain H37Rv, *Am. Rev. Respir. Dis.* **99:**242–248.
55. Lefford, M. J., 1977, Induction and expression of immunity after BCG immunization, *Infect. Immun.* **18:**646–653.
56. Lefford, M. J., 1978, Immunization of mice after airborne infection with various strains of BCG, *Am. Rev. Respir. Dis.* **117:**103–109.
57. Hank, J. A., Chan, J. K., Edwards, M. L., Muller, D., and Smith, D. W., 1981, Influence of the virulence of *Mycobacterium tuberculosis* on protection induced by bacille Calmette–Guérin in guinea pigs, *J. Infect. Dis.* **143:**734–738.
58. Collins, F. M., 1985, Protection to mice afforded by BCG vaccines against an aerogenic challenge by three mycobacteria of decreasing virulence, *Tubercle* **66:**267–276.
59. Wallace, J. G., Mitchison, D. A., Rees, R. J. W., Bhatia. A. L., and Gangadharam, P. R. J., 1961, The virulence of south Indian tubercle bacilli in mice and guinea pigs infected by the intravenous route, *Tubercle* **42:**212–217.
60. Orme, I. M., Roberts, A. R., and Collins, F. M., 1986, Lack of evidence for a reduction in the efficacy of subcutaneous BCG vaccination in mice infected with nontuberculous mycobacteria, *Tubercle* **67:**41–46.
61. Grover, A. A., Kim, H. K., Wiegeshaus, E. H., and Smith, D. W., 1967, Host–parasite relationships in experimental airborne tuberculosis. II. Reproducible infection by means of an inoculum preserved at −70°C, *J. Bacteriol.* **94:**832–835.
62. Wiegeshaus, E. H., McMurray, D. N., Grover, A. A., Harding, G. E., and Smith, D. W., 1970, Host–parasite relationships in experimental airborne tuberculosis. III. Relevance of microbial enumeration to acquired resistance in guinea pigs, *Am. Rev. Respir. Dis.* **102:**422–429.
63. Ho, R. S., Fok, J. S., Harding, G. E., and Smith, D. W., 1978, Host-parasite relationships in experimental airborne tuberculosis. VII. Fate of *Mycobacterium tuberculosis* in primary lung lesions and in primary lesion-free lung tissue infected as a result of bacillemia, *J. Infect. Dis.* **138:**237–241.

64. Smith, D. W., McMurray, D. N., Wiegeshaus, E. H., Grover, A. A., and Harding, G. E., 1970, Host–parasite relationships in experimental airborne tuberculosis. IV. Early events in the course of infection in vaccinated and nonvaccinated guinea pigs, *Am. Rev. Respir. Dis.* **102:**937–949.
65. Mackaness, G. B., 1964, The immunological basis of acquired cellular resistance, *J. Exp. Med.* **120:**105–120.
66. Chan, J. K., 1977, Site of control of hematogenous seeding to the lungs in experimental airborne tuberculosis in guinea pigs, Ph.D. thesis, University of Wisconsin, Madison, Wisconsin.
67. Fok, J. S., Ho, R. S., Arora, P. K., Harding, G. E., and Smith, D. W., 1976, Host–parasite relationships in experimental airborne tuberculosis. V. Lack of hematogenous dissemination of *Mycobacterium tuberculosis* to the lungs in animals vaccinated with bacille Calmette–Guérin, *J. Infect. Dis.* **133:**137–144.
68. Harding, G. E., and Smith, D. W., 1977, Host–parasite relationships in experimental airborne tuberculosis. VI. Influence of vaccination with bacille Calmette–Guérin on the onset and/or extent of hematogenous dissemination of virulent *Mycobacterium tuberculosis* to the lungs, *J. Infect. Dis.* **136:**439–443.
69. McMurray, D. N., Mintzer, C. L., Tetzlaff, C. L., and Carlomagno, M. A., 1986, The influence of dietary protein on the protective effect of BCG in guinea pigs, *Tubercle* **67:**31–39.
70. Cohen, M. K., Bartow, R. A., Mintzer, C. A., and McMurray, D. N., 1987, Effects of diet and genetics on *Mycobacterium bovis* BCG vaccine efficacy in inbred guinea pigs, *Infect. Immun.* **55:**314–319.

17

Changing Faces of Clinical Tuberculosis

WILLIAM W. STEAD and ASIM K. DUTT

1. INTRODUCTION

Throughout recorded and archeologic history, tuberculosis has affected humans in every part of the world. Even today, tuberculosis is a major cause of morbidity and mortality in many countries, despite extensive understanding of its cause, epidemiology, prevention, and therapy.[1] There has been considerable change in the face of the disease, with a shift from the young and female to the old and male.[2] In densely populated developing countries, it remains a major killer of the young.

According to estimates of the World Health Organization (WHO), there are approximately 20 million active cases in the world today. Mortality due to disease is approximately 3 million annually. From 50 to 100 million persons become infected annually. Eighty percent of deaths are in developing countries.

The causative organism, *Mycobacterium tuberculosis,* was discovered more than 100 years ago by Robert Koch. Much remains to be learned about the nature of the organism, variation in virulence, and the host–parasite interaction. Unlike smallpox, there is little prospect of eradicating the disease so long as overcrowding, malnutrition, and poverty prevail in a large part of the world.

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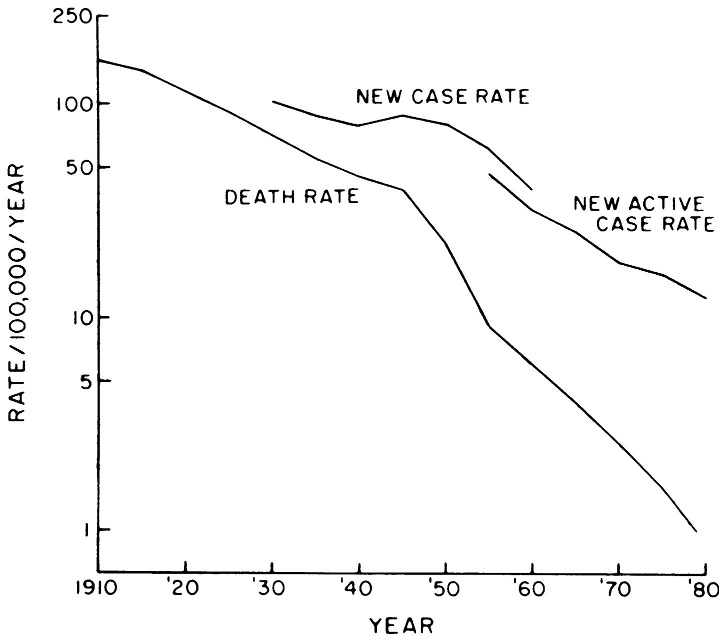

FIGURE 1. Decline in morbidity and mortality rate due to tuberculosis, 1910–1980.

2. EPIDEMIOLOGY OF DISEASE

The decline in incidence of disease in developed countries began even before the scientific advances of the past century (Fig. 1). Mortality from the disease has declined faster than the incidence of disease. With the introduction of chemotherapy in about 1950, there was a much sharper drop in mortality than in incidence.

3. INCIDENCE AND MORBIDITY

In the United States, the average decline in morbidity during the past 32 years has been about 5% annually, save for the period of 1978–1981 when it dropped only 1.4% annually due to the influx of refugees from Southeast Asia.[3] However, since 1985, the rate of decline has again slowed appreciably for reasons that are not yet clear. This coincides temporally with the rapid rise in incidence of acquired immunodeficiency syndrome (AIDS), and there is some speculation that tuberculosis may be a harbinger of a much more serious problem. Four of the five states with the largest population of AIDS cases had the greatest increase

in tuberculosis cases and their metropolitan areas with the largest number of AIDS cases also reported the greatest increase of tuberculosis cases.[4]

4. TUBERCULOSIS IN CHILDREN

Reports from 1985 cases show an increase in morbidity in all age groups, sexes, and races (Fig. 2). An increase in incidence of tuberculosis in children is a clear indication of transmission of infection in the population. Implementation of preventive measures and adherence to the guidelines for screening and preventive therapy are necessary to prevent development of disease. A recent study documents poor compliance by physicians themselves in following the recommendations for the prevention of tuberculosis. Health departments need to redouble their efforts at preventive measures, particularly among children. Both physician and patient compliance are necessary, if the desired results are to be attained in prevention of tuberculosis among infected persons.

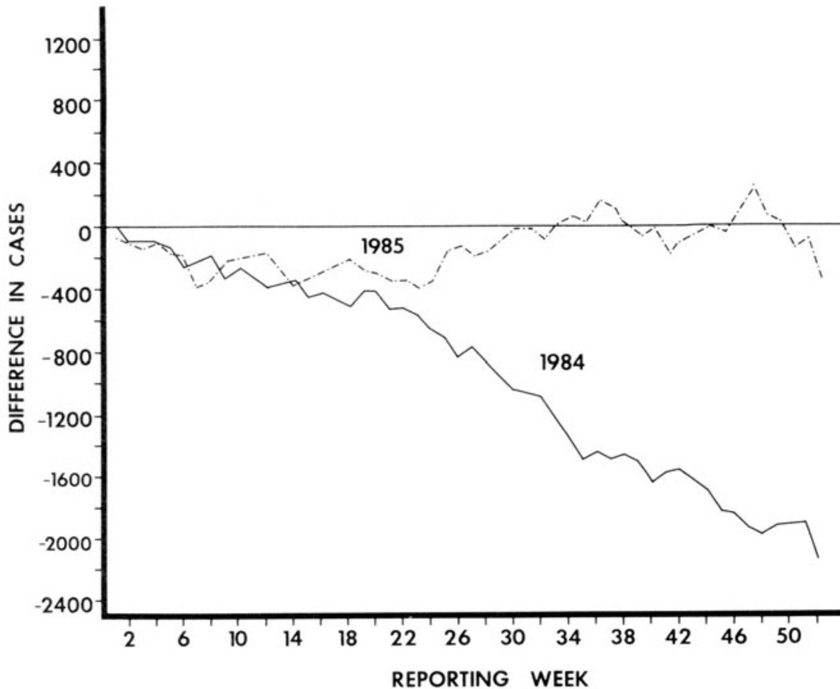

FIGURE 2. Difference in cumulative tuberculosis cases between 1985 and 1984 in the United States.

5. TUBERCULOSIS IN OLD AGE

With the decline of cases in developed countries, tuberculosis is progressively becoming a disease of the elderly population.[7] Many states have experienced an increased incidence among the elderly. There has been a dramatic shift in the age of cases during the past half-century from the young to old age. Figure 3 shows the shift for Arkansas. The age shift in the population has given rise to another problem that needs further attention. About 5% of the elderly population are confined to nursing homes, of whom 80–85% are tuberculin nonreactors. The majority are still immunocompetant, 10–15% are anergic and more susceptible to new infection.[32] Reactivation of an ancient tuberculosis infection

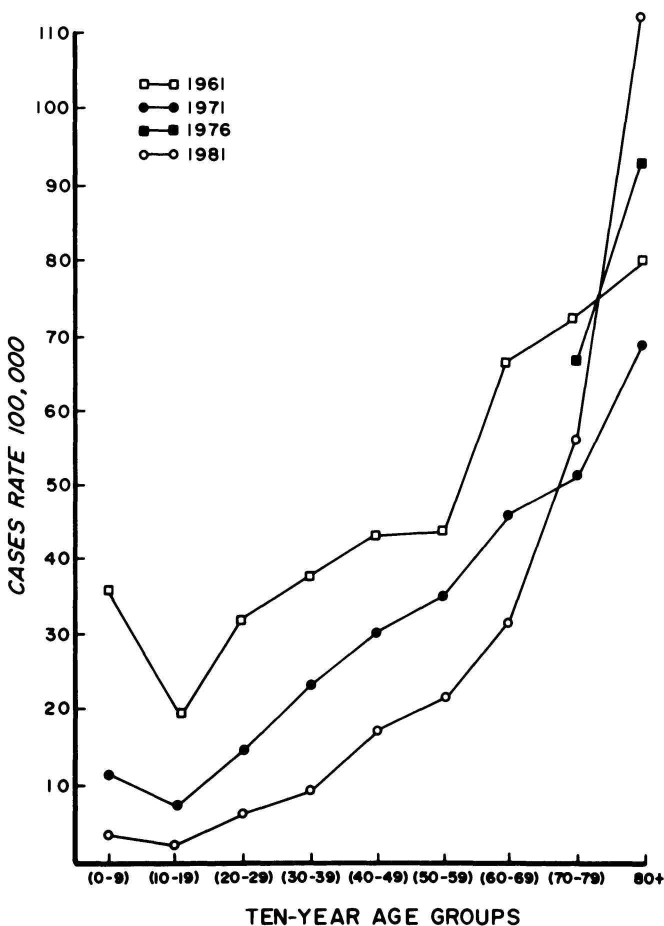

FIGURE 3. Change in case rates for Arkansas during the 1970s and 1980s. Rates are decreasing up to age 70 but increasing beyond that age.

in an elderly resident of a nursing home may give rise to new infection and disease among other susceptible residents who are in close contact for a protracted period. Such newly infected elderly subjects may develop disease in 7% (women) to 13% (men). Occasionally, this may reach epidemic form in the closed environment of a nursing home. The same may occur among inmates in the closed environment of prisons and crowded shelters of homeless, often with added risk of exposure to HIV infection. Prevention consists of identifying infected persons on entry (tuberculin reactors), study of all cases of pneumonia for active tuberculosis, and application of preventive therapy for new infections with isoniazid (INH) according to the guidelines recommended by the American Thoracic Society and Centers for Disease Control.

6. TUBERCULOSIS IN IMMUNOCOMPROMISED HOSTS

Progress in therapy of several diseases in recent years has entailed use of long term costicosteroids, immunosuppressive drugs and sometimes radiation therapy. These immunosuppressed patients are at increased risk of tuberculosis, whether from new infection or from reactivation of healed foci. The latter are likely to reactivate under long-term immunosuppression, producing pulmonary or even disseminated tuberculosis. Clinical symptoms and signs of the disease may be suppressed producing an unusual delay in diagnosis.

Many authors have documented that persons with malignancies are at increased risk of developing clinical tuberculosis. A tuberculin test and chest radiograph should be done before initiation of such therapy. Preventive chemotherapy with INH should be given to those exhibiting a significant (≥ 10 mm) tuberculin reaction. Awareness of the risk of tuberculosis in immunosuppressed subjects and application of appropriate measures for chemoprophylaxis and chemotherapy should eliminate the risk of tuberculosis in these patients.

7. TUBERCULOSIS IN HOMELESS AND ALCOHOLICS

It is well recognized that the prevalence of tuberculosis among vagrant, alcoholic and homeless persons is considerably higher compared with other groups. In advanced countries, homelessness is increasing in most cities. The homeless are generally malnourished, in poor general health, and often crowded into cramped quarters. When tuberculosis reactivation occurs in a crowded shelter for the homeless, widespread transmission may occur. Recently, it has been reported that not only the disease spreads to those who have never been infected before but also to previously infected persons whose immunity is impaired.[6,8] The ex-

ogenous reinfection with tuberculosis in a shelter for the homeless is a serious problem as transmission of drug resistant tuberculosis has even been reported. The high prevalence of infection and disease among the homeless is akin to developing countries where exogenous reinfection plays an important role in the development of disease. Stead and Lofgren[10] also observed tuberculosis from exogenous reinfection among nursing home elderly residents but believed it was generally due to reversion of the skin test to negative, rendering the subjects more susceptable to reinfection.

8. TUBERCULOSIS IN HEALTH CARE WORKERS

Tuberculosis is a recognized occupational hazard among health care workers. With the great decline in prevalence of the disease in developed countries, the hazard to medical students, physicians, and nurses is less than ever but greatly increased over that for the general population. The shift in prevalence of the disease to the elderly means that persons working in hospitals and nursing homes cannot escape inadvertent and unrecognized exposure to tuberculosis. An epidemiologic survey among the practicing physicians in California in 1979 revealed 3.5% of them had disease treated within 10 years of entering medical school. A recent report of a survey in a large medical school showed the incidence of tuberculosis to be higher in physicians than in the general population.[18] The risk of tuberculosis was found to be 140 per 100,000 person-years within 6 years of graduation. As many as 63% of initially tuberculin negative students whose tuberculin test converted to positive during medical school or clinical training developed clinical tuberculosis. These observations are supported by another study that showed a 4% annual conversion rate among house officers.

Increased risk of infection as an occupational hazard among health care workers should not be surprising. Undiagnosed infections in patients present the greatest hazard. Younger physicians who take care of patients from lower socioeconomic strata often work long hours. Inadvertent nosocomial spread of infection may occur among health care workers through special procedures such as bronchoscopy of persons with undiagnosed pulmonary disease. As the disease is encountered less often, alertness to its possibility is declining.

Unexpected exposure to tuberculosis is likely to continue for years to come. The most important preventive measure is to follow the recommendation of regular tuberculin testing and taking preventive INH therapy when a definite conversion to positive occurs. Unfortunately, compliance of health care workers for preventive measures is often very poor.[19,20]

9. TUBERCULOSIS AMONG IMMIGRANTS

Tuberculosis continues to be a major problem in developed countries among immigrants and refugees from areas of the world that have a high prevalence of tuberculosis. Tuberculosis morbidity in the United States from 1978 to 1981 clearly reflected the impact of the large number of cases among Southeast Asian refugees. In many developing countries, virtually the entire population is infected with tubercle bacilli before adulthood. Such immigrants are likely to break down with disease under the stress of moving to a new environment. Development of active disease in the immigrant population is often associated with a high incidence of drug resistant organisms due to misuse of antituberculosis drugs, especially INH, which is often included in over the counter cough syrups. Treatment regiments should include at least two bactericidal drugs besides INH for Southeast Asian refugees.

10. CLINICAL PRESENTATION

With the declining incidence and changing epidemiology of tuberculosis in the United States, there have been changes in the clinical presentation of the disease. The likelihood of misdiagnosis increases with unusual presentations. Several studies have revealed that the diagnosis is missed in 40–50% at the time of hospital admission. Many cases must go undiagnosed, because about 5% of cases are unsuspected until discovered by autopsy.

Over the years, the incidence of pulmonary tuberculosis has been falling in the United States, but the incidence of extrapulmonary tuberculosis has remained almost constant for reasons unknown. Extrapulmonary manifestations of tuberculosis are seen more often in patients with immunosuppression. Acquired immunodeficiency syndrome (AIDS) patients show extrapulmonary disease, particularly lymphatic and disseminated (miliary). The incidence may range from 60–75%. Patients may have an infiltrate in any lung zone often associated with mediastinal and/or hilar adenopathy, although cavitation is uncommon due to inadequate T cells.

In patients with chronic renal failure receiving chronic dialysis, the incidence of tuberculosis is 10–15 times higher than in the general population. The disease presents very often in extrapulmonary sites such as lymphatic, pleural, and skeletal. Diagnosis of the disease becomes more confusing because of confusion of symptoms with those of the renal disease. Disseminated tuberculosis often occurs in immunosuppressed

states, the diagnosis of which may be missed if a high index of suspicion for the disease is not maintained.

Tuberculin negative elderly patients may contract initial infection in nursing homes, which may progress to produce cavitary tuberculosis or even death.[10] There may be tuberculous plural effusion which may be passed off as due to congestive heart failure. The occurrence of tuberculin conversion and progressive primary tuberculosis has been reported among very elderly tuberculin-negative persons in nursing homes. Dissemination of tuberculosis may occur at any stage of disease and may produce miliary tuberculosis or meningitis. A manifestation that is more commonly seen among the elderly is tuberculous endobronchitis which leads to segmental atelectasis. It is often mistaken for a tumor. The diagnosis is made only if bronchoscopy is performed with appropriate biopsy and culture of the material.

For the past decade, several authors have drawn attention to the fact that radiological abnormalities of tuberculosis may be atypical.[12,13] It is estimated that 30–40% of cases may have unusual presentations, such as lower lobe infiltrates, nodules, reticulonodular infiltrates, pleural effusion, and lymphadenopathy. Thus, absence of apical involvement should not dissuade one from considering tuberculosis when the etiology of pulmonary disease is not readily apparent. Tuberculosis must be considered when the chest roentgenogram is not characteristic of nontuberculous pneumonia and fails to resolve in response to antibiotics. Clinicians should remain alert to the possibility of tuberculosis because it is a curable disease that can be devastating if not treated.

11. TUBERCULOSIS THERAPY

The most exciting change in tuberculosis during the past two decades has been in the therapy of tuberculosis. With a better understanding of the effects of drugs on the mycobacteria in tuberculous lesions,[24] the therapy has been considerably simplified and its duration shortened.[25]

A few years ago, the standard therapy of tuberculosis used to be INH and ethambutol (EMB) with or without streptomycin (SM) during initial 1–3 months of treatment. This therapy was effective, provided it was taken for 18–24 months. Consequently, there were great problems of compliance from patients in completing the full course of therapy. Due to availability of another oral bactericidal drug, rifampin (RIF), and revival of interest from better understanding of action of an old drug, pyrazinamide (PZA), during the past one to two decades, it has become possible to complete curative therapy much more rapidly. With the use

of a combination of INH and RIF, the therapy is completed in 9 months or in 6 months if PZA plus SM or EMB are added to the regimen for the first 2 months.

Current chemotherapy is based on evidence from many *in vitro* and *in vivo* studies that have increased our understanding of the bacterial populations in tuberculosis lesions and the effect of drugs on them.[24] Tubercle bacilli are obligate aerobes which thrive best in an environment having a high oxygen tension. There appear to be four distinct bacterial populations in a tuberculous lesion (Fig. 4). The largest population (ranging 10^7–10^9 organisms) replicate actively in a neutral or slightly alkaline medium with high oxygen tension. A much smaller population (range 10^2–10^5 organisms) is harbored in a neutral or slightly alkaline milieu of closed caseous or noncaseous lesions, in which they are metabolically less active. A third population of similar size replicates slowly in the acid environment inside macrophages. Finally, few organisms are dormant and metabolically inactive.

The number and rate of replication of the organisms are important because drug resistance develops at a predictable rate by mutation, irrespective of the presence of drug(s) in the environment. Drug-resistant mutants develop independently of each other, approximately once in 10^5–10^6 divisions. A lung cavity containing 10^9 organisms may contain hundreds of thousands of bacilli resistant to one antituberculous drug, and very few or none may exhibit double drug resistance, since resistance to two drugs occurs in about every 10^{12} divisions. Since active tuberculous lesions harbor a mixture of drug sensitive and a few resistant bacilli, the use of two drugs is mandatory. Antituberculous drugs are effective in killing the bacilli only when they are metabolically active and replicating. Tubercle bacilli replicate infrequently, about every 16–20 hr. Thus, antituberculous drugs are best given in a single daily dose and for a prolonged period, permitting killing of the entire population, including semidormant organisms, which replicate only intermittently.

Rapid killing of the large, actively multiplying extracellular population of bacilli prevents emergence of drug-resistant organisms and eventual treatment failure. Elimination of slowly multiplying organisms or persisters with continued treatment prevents late relapse after discontinuation of therapy.

Modern treatment consists of the use of at least two bactericidal drugs: the major ones that may be considered in this group are SM/Capreomycin (CAP), INH, RIF, PZA, and EMB in doses of 25 mg/kg. The bacteriostatic drugs are EMB (15 mg/kg), ethionamide (ETA), cycloserine (CS), *p*-aminosalicylic acid (PAS), thiacetazone (TZ) which are not often used except in treatment failure and drug resistant cases.

The bactericidal drugs are highly active on rapidly multiplying ex-

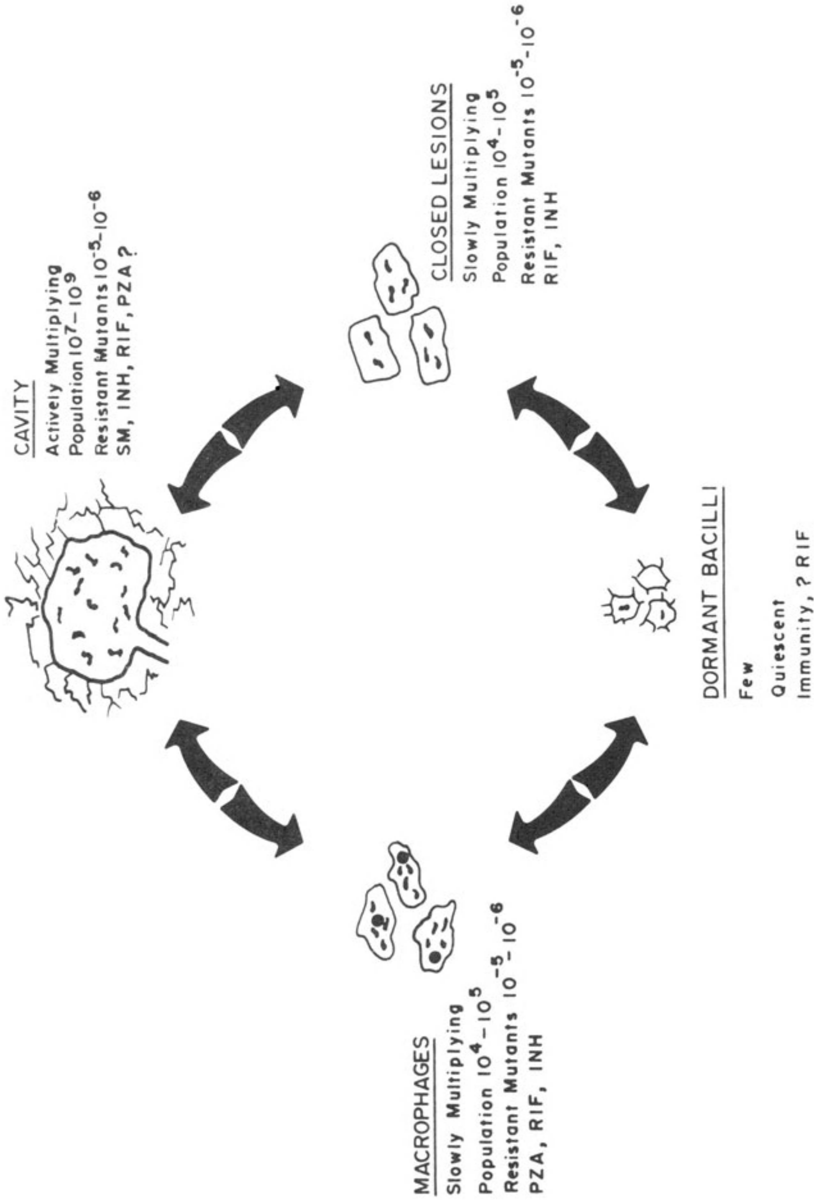

FIGURE 4. Bacterial populations in tuberculous lesions.

tracellular organisms (Fig. 5). Intensive initial daily therapy rapidly eliminates the bacilli. The emergence of drug-resistant mutants is avoided with rapid conversion of sputum smears to negative. Inadequate therapy either due to wrong doses or to irregular compliance may result in therapy failure with emergence of drug resistance.

RIF, INH, and PZA are effective against slowly multiplying bacilli inside macrophages or extracellularly. PZA is particularly effective in the acid milieu of macrophages. The drug is found more effective when given during the initial period of therapy. RIF is a useful drug during the continuation phase of therapy because it is particularly effective against organisms that show even the slightest intermittent activity. Adequate duration of therapy eliminates the persisters, with eventual cure of the disease. Inadequate duration of therapy may result in late relapse due to late replication of some surviving persisters. However, such relapses are usually due to drug-sensitive organisms. It has also been observed that the drugs can be given twice weekly after initial daily administration for 1–2 months. The supplement of SM or EMB and PZA during the initial period of therapy with INH and RIF is not generally necessary in areas with few drug-resistant cases. However, such intensive initial therapy with four drugs for 2 months reduces the necessary duration of treatment to 6 months, with the additional advantage of being effective even in the presence of INH resistance.

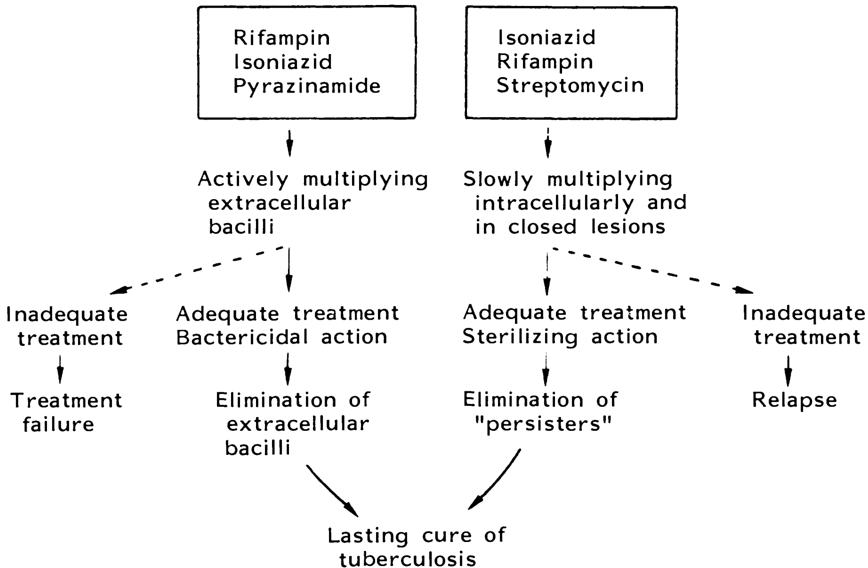

FIGURE 5. Basis of chemotherapy for tuberculosis.

The drug combination of choice for newly diagnosed tuberculosis is INH and RIF, except in areas of increased prevalence of INH resistance. In the United States, newly diagnosed tuberculosis in patients who had not been exposed to antituberculosis drugs in the past almost always harbor drug-sensitive bacilli. INH and RIF are given in a single daily dose for 9 months or daily for 1 month, followed by twice weekly for another 8 months. The regimen has been successful in more than 95% of the 1795 cases in Arkansas.[26] Some recommend the addition of SM or EMB (25 mg/kg) initially for 2–3 months, which, although adding nothing to the bactericidal activity of INH and RIF, ensures against failure due to drug resistant organisms. Dosage of INH is 300 mg and RIF 600 mg during daily therapy. During twice-weekly therapy, the dose of INH is increased to 900 mg, and RIF remains the same at 600 mg. Preferably, the drugs should be given as combination capsules, e.g., Rifamate (Merrell-Dow). Each capsule contains INH, 150 mg, and RIF, 300 mg. Thus, two capsules a day, early in the morning, supplies the full dose. For twice weekly administration, the addition of two tablets of INH, 300 mg each, to two capsules of Rifamate furnishes the adequate dose.

The same regimen (based on weight) is effective in pediatric disease, in all forms of extrapulmonary disease[29] and also when the tuberculosis is associated with additional risk factors, e.g., long-term corticosteroid therapy, diabetes, malignancies, and immunosuppressive stages. In recent studies, the therapy has also been effective in AIDS patients and is the recommended mode of therapy.[4]

However, the addition of at least two other drugs to the regimen is essential in patients with suspected or proven drug-resistant disease. Drug resistant disease should be suspected when antituberculosis drugs were given in the past, disease is acquired in countries with high prevalence of the disease, e.g., Southeast Asia, Africa, and Latin America, acquired infection from a known case with drug resistant disease, or if the prevalence of INH resistance disease is more than 5% in the community. Under such circumstances, therapy should be initiated with INH 300 mg, RIF 600 mg, (SM −0.5–1.0 GM 5 days a week, or EMB 25 mg/kg) and PZA 25–30 mg/kg until drug susceptibility results are known, usually 2 months, when the regimen may be altered. If the organisms are found susceptible to INH and RIF, additional drugs are discontinued and INH and RIF are given daily or twice-weekly for the remaining 6 months of treatment. When the organisms are resistant to INH or RIF, the drug is discontinued and the remaining three drugs are given twice-weekly for the remaining period of 9 months. In INH-resistant cases, INH may be continued for its action against an INH-sensitive population of slowly replicating persisters. The regimen ensures effectiveness of at least two bactericidal drugs against the resistant bacilli,

e.g., SM/EMB and RIF, on the rapidly multiplying extracellular bacilli and RIF and PZA on the slowly replicating organisms. When resistance to both INH and RIF is found, there is no short-course chemotherapy that is effective, and the therapy must be given for a period of 18–24 months with three or four new drugs to which the bacilli are sensitive.

Tuberculosis due to organisms resistant to both INH and RIF requires therapy with three or four other antituberculosis drugs. SM (or CAP, if SM resistant) is given in doses of 0.5–1.0 g/day, 5 days a week, for at least 3 months along with another two sensitive drugs: EMB 25 mg/kg, PZA 25–30 mg/kg, ETA 500–750 mg on the tolerance, CS 0.75–1 g along with pyridoxine 100 mg. ETA and CS are given in divided doses. As these drugs are not bactericidal, the duration of therapy should be 18–24 months.

12. ADVERSE DRUG REACTIONS

The principal side effects of antituberculosis drugs are shown in Table I. In a large number of experiences, hepatic toxicity has occurred among 2–4% of cases, even when PZA is included along with INH and RIF in the regimen. In our experience, hepatic toxicity has occurred in 2.5% of 2000 patients treated with INH and RIF.[31] The series included many elderly patients, 60% over age 65 years, and many alcoholics. It is evident that two hepatotoxic drugs should be avoided in patients with active hepatitis. Usually, the therapy in such patients should be initiated with INH and EMB (25 mg/kg), supplemental with initial SM in smear-positive cases. If RIF is instituted to replace SM and EMB after the hepatitis has subsided, the therapy can still be finished in 9 months. Minor side effects, e.g., rashes, gastrointestinal (GI) disturbances, occur in 5% of cases. In them, the drugs can be reintroduced one at a time without recurrence of the symptoms. PZA regularly causes hyperuricemia but gout rarely occurs.

Intermittent use of RIF may cause thrombocytopenia, but the problem usually arises with higher doses of RIF (900–1200 mg once or twice a week). When RIF is given in a dose of 600 mg twice a week, thrombocytopenia is rare and occurred in less than 1% of our patients. A hypersensitivity reaction termed flulike syndrome may also develop with intermittent use of RIF but is infrequent (2%) with a 600-mg dose twice weekly. More serious effects, e.g., shock, acute renal failure, and hemolytic anemia, have been reported with intermittent RIF, but we have not encountered these problems yet in treating more than 2000 patients in this way.

SM may cause vestibular or auditory damage, but the side effect is minimized by administering it 5 days a week and also reducing the dose

TABLE I
Antituberculosis Drugs: Mode of Action, Dosage, and Side Effects

Drug		Dosage		
Drug	Activity	Daily	Twice weekly	Side effects
Bactericidal				
Streptomycin	Active against actively multiplying bacilli in neutral or slightly alkaline medium, usually extracellular	0.75–1.0 g usually 5 days/week IM	1.5–3.0 g IM	Vestibular or auditory nerve (VIII) damage, dizziness, vertigo ataxia, nephrotoxicity, allergic fever, rash
Capreomycin		0.75–1 g 5 days/week IM	1–1.5 g IM	Nephrotoxicity, allergic fever, rash
Isoniazid	Acts on actively dividing and weakly on slowly multiplying extracellular and intracellular bacilli	5–10 mg/kg usually 300 mg PO or IM	15 mg/kg usually 900 mg PO	Peripheral neuritis, hepatotoxicity, allergic fever and rash, lupus erythematosus phenomenon
Rifampin	Acts both on actively and slowly multiplying bacilli, either extracellular or intracellular, particularly on slowly multiplying persisters	10 mg/kg usually 450–600 mg PO	10 mg/kg usually 450–600 mg PO	Hepatotoxicity, nausea and vomiting, allergic fever and rash, flulike syndrome, petechiae with thrombocytopenia or acute renal failure during intermittent therapy
Pyrazinamide	Active in acid pH medium on intracellular bacilli	30 mg/kg usually 2–3 g PO	50 mg/kg usually 3–4 g PO	Hyperuricemia, hepatotoxicity, allergic fever and rash

Bacteriostatic				
Ethambutol	Weakly active against both extracellular and intracellular bacilli to inhibit the development of resistant bacilli	15–25 mg/kg usually 800–2000 mg PO	50 mg/kg PO	Optic neuritis, skin rash
Ethionamide	As above	15–20 mg/kg usually 0.75–1.0 g in divided doses PO	—	Nausea, vomiting, anorexia hepatotoxicity, allergic fever and rash, loss of diabetes control
Cycloserine	As above	10–20 mg/kg usually 1–1.5 g in divided doses with pyridoxine PO	—	Personality changes, psychosis, convulsions, rash
p-Aminosalicylic acid	Weak action on extracellular bacilli, inhibits development of drug-resistant organisms	150 mg/kg usually 10–12 g in divided doses PO	—	Nausea, vomiting, diarrhea, hepatotoxicity, allergic rash and fever
Thiacetazone (not available in the United States)	As above	150 mg/day PO		Allergic rash and fever, Stevens-Johnson syndrome, blood disorders, nausea and vomiting

of medication with advancing age and impaired renal function. EMB in doses of 25 mg/kg may give rise to visual symptoms in one to three percent of the cases.

Monitoring of side effects of the drug is accomplished by remaining in close contact with the patients who should have easy access to the health care team for reporting unusual signs and symptoms.[31] Transient abnormalities in liver function are common during early periods of chemotherapy in asymptomatic patients, usually subsiding even when chemotherapy is continued. Some authorities recommend monthly laboratory studies in asymptomatic patients, but we believe that this approach leads to more confusion than enlightenment. We recommend performing a baseline study of liver function before initiation of therapy and to repeat only if the patient develops symptoms of hepatitis (nausea and vomiting). The patients are informed about the symptoms and asked to report their occurence promptly.

Health teams must monitor the compliance of patients in ingesting the medication as prescribed by frequent personal interviews, by checking regularly the attendance for clinic appointments, by surprise pill counting, and by random examination of the urine for the metabolites of INH and RIF.

13. PREVENTIVE THERAPY

Since the release of INH in 1952, prevention of tuberculosis became a possibility among infected individuals. Six randomized trials of INH preventive therapy for one year by the U.S. Public Health Service among 70,000 infected persons during the 1960s showed protection in 55–83% of participants. Isoniazid chemoprophylaxis has become a recognized and recommended preventive therapy by the Centers for Disease Control (CDC), which is now widely practiced in the country.[28] However, the pitfall of the therapy is noncompliance in pill taking for 12 months among infected individuals who are otherwise healthy. The CDC estimated that 57% of infected contacts were started on preventive therapy, and approximately 25% of them did not complete the full course of therapy. It is estimated that only 43% of individuals at risk of developing tuberculosis completed the full course of therapy. Stead and co-workers[10,23] reported remarkable protection of 99% among tuberculin converters among nursing home residents compared with 7–13% of those who were not treated.

The protective effect of INH depends on regular ingestion of the drug for a prolonged period. Shortening the duration of chemoprophylaxis has become an issue of considerable importance in achieving

greater success in prevention and also in reducing the high costs of supervision, particularly in developed countries. Moreover, in recent years, the therapy of active disease has been reduced to 6–9 months, while, paradoxically, the duration of INH preventive therapy for infection continues to remain for 12 months. This question was addressed in a large trial in six Eastern European countries.[21] In those persons who ingested 80% of the prescribed INH, the result of 6 months of therapy is nearly as effective as 12 months. The recent recommendation in this country is to reduce the duration of preventive therapy to 6 months for most patients. This is expected to improve the compliance rates. To reduce the duration further, short-course chemotherapy with combination of several drugs is being evaluated at present. A combination of four drugs; SM, INH, RIF, and PZA for 3 months has shown promising results in smear-negative, culture-negative suspected tuberculosis. For 350 such patients, we have used a combination of INH and RIF for only 4 months, daily for 1 months, followed by twice-weekly ingestion for another 3 months. During a median follow-up of 45 months, there has been only 1% relapse. Although this is encouraging, more follow-up in a larger number of patients is needed for a final conclusion. Thus, it seems likely that the duration of preventive treatment may be considerably shortened in the future.

REFERENCES

1. Kass, E. H., 1971, Infectious diseases and social changes, *J. Infect. Dis.* **123:**100–14.
2. Anonymous, 1986, Tuberculosis—United States 1985, *MMWR* **35:**699–703.
3. Anonymous, 1986, Tuberculosis—United States 1985—and the possible impact of Human T-Lymphotropic versus Type III/lymphodenopathy-associated virus infection, *MMWR* **35**(5)**:**74–76.
4. Anonymous, 1986, Prognosis and management of Mycobacterial infection and disease in persons with Human T-Lymphotropic versus Type III/Lymphadenopathy-associated virus infection, *MMWR* **35:**448–452.
5. Anonymous, 1986, Case records of the Massachusetts General Hospital, *N. Engl. J. Med.* **314:**167–174.
6. Nardell, E., McInnis, B., Thomas, B., and Weidhass, S., 1986, Exogenous reinfection with tuberculosis in a shelter for the homeless, *N. Engl. J. Med.* **315:**1570–1575.
7. Stead, W. W., and Lofgren, J. P., 1983, Does the risk of tuberculosis increase in old age?, *J. Infect. Dis.* **147:**951–955.
8. Anonymous, 1985, Drug resistant tuberculosis among the homeless—Boston, *MMWR* **34:**429–431.
9. Patel, K. R., 1985, Pulmonary tuberculosis in residents of lodging houses, night shelters, and common hostels in Glasgow: A 5-year prospective study, *Br. J. Dis. Chest* **79:**60–66.
10. Stead, W. W., Lofgren, J. P., Warren, E., and Thomas, C., 1985, Tuberculosis as an endemic and nosocomial infection among the elderly in nursing homes, *N. Engl. J. Med.* **312:**1483–1487.

11. Greenbaum, M. B., Beyt, B. E., and Murray, P. R., 1980, The accuracy of diagnosing pulmonary tuberculosis at a teaching hospital, *Am. Rev. Respir. Dis.* **121**:477–481.
12. Khan, M. A., Kovnat, D. M., Bachus, B., Whitcomb, M. E., Brody, J. S., and Snider, G. L., 1977, Clinical and roentgenographic spectrum of pulmonary tuberculosis in the adults, *Am. J. Med.* **62**:31–38.
13. Farman, D. P., and Speir, W. A., 1986, Initial roentgenographic manifestations of bacteriologically proven Mycobacterium tuberculosis: Typical or atypical? *Chest* **89**:75–77.
14. Bloch, A. B., Snider, D. E., Jr., 1986, How much tuberculosis in children must we accept?, *Am. J. Public Health* **76**:14–15.
15. Millar, J. W., and Horne, N. W., 1979, Tuberculosis in immunosuppressed patients, *Lancet* **1**:1176–1178.
16. Pitchenik, A. E., Cole, C., Russell, B. W., Fischl, M. A., Spira, T. J., and Snider, D. E., Jr., 1984, Tuberculosis, atypical mycobacterosis and the acquired immunodeficiency syndrome among Haitian and non-Haitian patients in South Florida, *Ann. Intern. Med.* **101**:641–645.
17. Sunderam, G., McDonald, R. J., Maniatis, T., Oleska, J., Kapila, R., and Reichman, L. B., 1986, Tuberculosis as a manifestation of the acquired immunodeficiency syndrome (AIDS), *JAMA* **256**:362–366.
18. Geiseler, P. J., Nelson, K. E., Crispen, R. G., and Moses, V. K., 1986, Tuberculosis in physicians: A continuing problem, *Am. Rev. Respir. Dis.* **133**:773–778.
19. Geiseler, P. J., Nelson, K. E., and Crispen, R. G., 1987, Tuberculosis in physicians: Compliance with preventive measures, *Am. Rev. Respir. Dis.* **135**:3–9.
20. Miller, B., and Snider, D. E., Jr., 1987, Physicians noncompliance with tuberculosis preventive measures. *Am. Rev. Respir. Dis.* **135**:1–2.
21. International Union Against Tuberculosis Committee on prophylaxis, 1982, Efficacy of various durations of isoniazid preventive therapy for tuberculosis: Five years of follow up in the IUAT trial, *Bull WHO* **60**:555–564.
22. Snider, D. E., and Farer, L. S., 1984, Preventive therapy for tuberculosis infection: An intervention in need of improvement, *Am. Rev. Respir. Dis.* **130**:355–356.
23. Stead, W. W., 1981, Tuberculosis among elderly persons: An outbreak in a nursing home, *Ann. Intern. Med.* **94**:606–610.
24. Grosset, J., 1980, Bacteriologic basis for short course chemotherapy for tuberculosis, *Clin. Chest Med.* **1**:231–241.
25. Fox, W., 1981, Whither short course chemotherapy?, *Br. J. Dis. Chest* **75**:331–357.
26. Dutt, A. K., Moers, D., and Stead, W. W.. 1984, Short course chemotherapy for tuberculosis with mainly twice-weekly isoniazid and rifampin: Community physicians with mainly outpatients, *Am. J. Med.* **77**:233–242.
27. Singapore Tuberculosis Source/British Medical Research Council, 1986, Long-term follow-up of a clinical trial of six month and four month regimens of chemotherapy in the treatment of pulmonary tuberculosis, *Am. Rev. Respir. Dis.* **133**:779–783.
28. American Thoracic Society, 1986, Treatment of tuberculosis and tuberculosis infection in adults and children, *Am. Rev. Respir. Dis.* **134**:355–363.
29. Dutt, A. K., Moers, D., and Stead, W. W., 1986, Short course chemotherapy for extrapulmonary tuberculosis: Nine years experience, *Ann. Intern. Med.* **104**:7–12.
30. Girling, D. J., 1978, The hepatic toxicity of antituberculosis regimens containing isoniazid, rifampin and pyrazinamide, *Tubercle* **59**:13–32.
31. Dutt, A. K., Moers, D., and Stead, W. W., 1983, Undesirable side effects of isoniazid and rifampin in largely twice-weekly short course chemotherapy for tuberculosis, *Am. Rev. Respir. Dis.* **128**:419–424.
32. Stead, W. W., and To, T., 1987, The significance of the tuberculin test in elderly persons, *Ann. Int. Med.* **107**:837–842.

18

Mycobacterium avium-Complex Infections and Immunodeficiency

FRANK M. COLLINS

1. INTRODUCTION

The atypical mycobacteria constitute a diverse and heterogeneous group of acid-fast bacilli that are widespread in distribution throughout the natural environment.[1] They also include a number of important opportunistic human and animal pathogens.[2] Most organisms in this group are not considered pathogenic for immunocompetent adults, although they do occasionally establish residence within normal tissues, where they give rise to persistent infections that can be difficult to eliminate, due to their high resistance to most antituberculosis drugs.[3] Such systemic infections are usually associated with some other underlying disease, such as silicosis, emphysema, or Hodgkin disease. A few species (e.g., *M. kansasii, M. avium,* and *M. ulcerans*) are considered overt human pathogens, causing progressive pathologic changes within the infected tissues.[4] Although most cases of pulmonary tuberculosis are caused by *M. tuberculosis,* small numbers of *M. kansasii* and *M. avium* infections are reported each year. When combined, these pathogens are responsible for at least 2 million deaths worldwide each year,[5] with the atypical mycobacterial infections constituting perhaps 5% of the total patient population.[6] These organisms are generally considered much less virulent for humans than *M. tuberculosis,*[7] although recently they have be-

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come a matter for concern due to the increasing acquired immunodeficiency syndrome (AIDS) epidemic.[8]

The *M. avium* complex consists of at least 31 different serotypes that can be readily distinguished one from another both on the basis of their animal virulence patterns[8] and their serology.[9,10] *M. avium* was first isolated from birds but has long been recognized as being virulent for both man and animals.[11] By contrast, the closely related *M. intracellulare* serotypes are of much lower virulence for humans, as well as for most experimental animals. For many years, this difference was the only practical means of differentiating between *M. avium* and *M. intracellulare.* Except for *M. kansasii* and *M. avium,* little significance was attached to the presence of atypical mycobacteria in clinical specimens collected from apparently normal individuals.[4] Now, with the increasing number of immunosuppressed individuals in the community (both naturally occurring and iatrogenically induced), this assumption is having to be reevaluated.[12] It appears that many atypical mycobacteria are able to colonize the nasopharyngeal, bronchial, and intestinal mucosae of normal immunocompetent individuals and epidemiologic data suggest that many of these individuals go on to develop limited tissue involvement, especially when the normal cellular defenses are temporarily depressed.[13] Colonization probably follows the ingestion of contaminated drinking water or the inhalation of aerosols generated from natural waters[14] rather than being the result of animal or person-to-person infections.[15] Widespread exposure to such environmental mycobacteria could explain the continued persistence of atypical mycobacterial infections in the face of a steady decline in the incidence of *M. tuberculosis* infections throughout most of the Western world.[16]

This chapter discusses the increasing public health importance of this group of atypical mycobacteria, which only a few years ago were looked upon as little more than taxonomic curiosities.[13] Ironically, it was only 20 years ago that the prediction was made that pulmonary tuberculosis would be virtually eliminated, at least from the United States and Europe, by the end of the twentieth century.[17] This forecast is now generally acknowledged to have been hopelessly overoptimistic[18] and, if anything, the incidence of tuberculosis is actually on the increase again.[8] Although the effect of AIDS on both tuberculous and nontuberculous disease in this country cannot be accurately assessed at this time, it is bound to be substantial.

2. *M. AVIUM*-COMPLEX INFECTIONS IN THE NORMAL POPULATION

Tuberculosis was first shown to be caused by *M. tuberculosis* in a classic series of papers published by Robert Koch in 1882, in which he

described the isolation and cultivation of the tubercle bacillus in the laboratory and proposed his now-famous set of postulates to establish its pathogenicity for humans.[29] By the turn of the century, a number of other mycobacteria (including *M. avium*) had been described, some of which were later shown to be capable of causing disease in both humans and animals.[1,2] However, it was not until the second half of this century that a number of pigmented atypical or anonymous mycobacteria were first described in the literature.[20] These organisms were resistant to the newly developed antituberculous drugs and were for this reason examined systematically as potential human pathogens. Most of these early studies were methodologic and taxonomic in nature,[21] but gradually a substantial body of information has been built up about the immunogenicity and pathogenicity of the four so-called Runyon groups of nontuberculous mycobacteria.[2] Even so, most members of the *M. avium* complex were ignored in clinical practice because they did not result in progressive disease when inoculated into the guinea pigs routinely used for virulence testing in the laboratory.[22] However, several reports exist in the early literature that atypical mycobacteria could be recovered from routine cultures prepared from gastric washings taken from both tuberculous and nontuberculous patients.[23–25] In the former group, the atypical mycobacteria often persisted long after chemotherapy had successfully eliminated the *M. tuberculosis* from the tissues. In some cases, they may have been secondary invaders.[13] However, their presence in apparently normal control specimens raised the question of their significance as potential secondary invaders versus their accidental presence as benign environmental contaminants.[26] Most of these atypical mycobacteria normally exist as free-living environmental saprophytes, but there is a substantial body of skin test data to suggest that the more virulent members of this group may be capable of temporarily colonizing the bronchial or intestinal mucosae leading to sensitization of the host tissues to specific mycobacterial antigens.[27] In most cases, such colonization results in, at most, a localized, self-limiting lymphadenitis and rarely lead to the development of progressive pulmonary disease.[28] Thus, it was only after the repeated isolation of the same atypical mycobacterial species from a patient showing clinical or radiological evidence of active lung disease, that a positive significance could be attributed to their presence in sputum or gastric washings.[12]

With the increased understanding of the taxonomic and antigenic relationships existing between *M. tuberculosis* and the slow-growing atypical mycobacteria,[4] a number of detailed epidemiologic studies were carried out using normal individuals and tuberculous patients, both in this countrv and overseas.[7,16] The availability of carefully standardized preparations of purified protein derivative (PPD-S) made it possible to compare exposure rates to tuberculous infection in widely separated

populations.[31] Less success was observed for patients infected with atypical mycobacteria, however, most of whom responded poorly (if at all), giving skin-test responses of less than 10-mm in duration measured 72 hr after an injection of 5 TU of PPD-S.[7] Somewhat better reactions were reported when PPD-A (prepared from *M. avium*) or PPD-B (prepared from the Battey bacillus or *M. intracellulare*) were included in these tests.[30] Careful assessment of an extended series of skin tests carried out in children and military inductees in the United States and in Europe suggested that localized infection (or colonization) by various members of the *M. avium* complex may be far more widespread than might otherwise be suspected on the basis of clinical and pathologic findings.[31] Tuberculin skin sensitivity to PPD-A and PPD-B usually declines relatively rapidly with time, suggesting that such local colonization was short-lived and did not result in ongoing infections. Responses by naval recruits coming from the southern Atlantic States were usually stronger and occurred more frequently than in individuals coming from other parts of the country,[29] suggesting a more widespread exposure to the opportunistic pathogens in hot and dusty climates and this generally agrees with the pattern of isolations for the *M. avium* complex across the United States.[32]

While it is generally agreed that the environmental strains of *M. avium* complex constitute the main source of both human and animal infections caused by these organisms, there is also epidemiological evidence that these diseases occur more frequently in individuals coming from a farming background where they may be more heavily exposed to colonization by these environmental strains than most urban dwellers.[13] Various atypical mycobacteria can be readily isolated from tap water samples collected in many parts of this country,[33] as well as from soil and seawater.[34] The main portal of entry for these organisms is presumed to be intragastric or aerogenic, since a variety of atypical mycobacterial species have been recovered from pharyngeal and gastric washings collected from apparently normal individuals.[2] Most of these organisms will be eliminated from the intestinal tract before they can establish a foothold among the commensals inhabiting the normal mucosal surfaces. However, the more virulent serotypes of *M. avium* and *M. intracellulare* may be able to colonize the host producing self-limiting mucosal infections temporarily, leading to sensitization of the host tissues. Young children tend to develop a localized cervical lymphadenitis in which the causative organism turns out to be one of the *M. scrofulaceum* serotypes.[35] The infection pattern seen in these children again strongly suggests an oral infection route and when this is combined with the presence of *M. avium* complex in the intestinal mucosae of many AIDS patients, the principal portal of entry for most of these organisms must

be oral rather than aerogenic in nature.[36] However, this remains a matter for speculation.[37]

The incidence of pulmonary tuberculosis in the Western world has declined steadily for at least the last 100 years.[5] It now stands at around 9 cases per 100,000 in the United States. This represents more than 28,000 new cases per year, with more than 1800 deaths directly ascribable to this disease annually.[18] Approximately 5% of these patients are infected with atypical mycobacteria, although the precise makeup of this population has been difficult to establish.[31] Until recently, up to 50% of the isolates were identified as *M. kansasii,* with various members of the *M. avium* complex making up about one half the other strains.[38] However, over the past decade, this ratio has shifted considerably, with the *M. avium* complex and *M. fortuitum* now making up the major proportion of these isolates.[36] This distribution pattern varies considerably in different parts of the country, with *M. kansasii* infections still predominating in the Midwest, while the *M. avium* complex is found increasingly along the East Coast, in the Southeast and on the West Coast.[31,32] These infections are usually relatively indolent, unless there is evidence of some other form of prior lung damage, such as silicosis, emphysema, or cavitary tuberculosis.[4] Thus, most of the atypical mycobacteria appear to be opportunistic pathogens, at best, although they may be able to persist indefinitely on the mucosal membranes of young children, heavy smokers, and sufferers of chronic bronchitis or asthma.[2] Such infections also tend to remain silent for long periods of time, so that the *M. avium*-complex infection may produce little or no sign of tissue destruction or progressive systemic disease.[39] Heavily infected organs show little sign of a cellular response or tissue destruction, and the lesions can be difficult to detect radiologically or bronchoscopically, even when the disease has become far advanced.[40,41]

The incidence of *M. avium*-complex infections in any community probably depends on the level of available laboratory expertise.[22] Even so, unexplained and extensive variations in the number and type of atypical mycobacterial isolates have been reported for the same hospital over extended periods of time.[46] Most of these infections could not be related to any obvious index source, occupational hazard, or other predisposing factor.[45] In general, adult *M. avium*-complex infections occurred in elderly white males, although, over the past decade, an increasing number (up to one third) of isolates have come from younger men with no sign of coexisting disease.[28] Although most of these patients show little evidence of progressive disease beyond the primary lesion, surgical intervention and aggressive chemotherapy may be required in order to cure the patient completely of this infection.[46] By contrast, children in whom a localized lymphadenitis develops usually

TABLE I
Mycobacterial Serotypes, Isolated from Normal and Immunosuppressed Patients

Mycobacterium sp.	Serovar no.	Old name	Normals		Transplant and cancer patients	AIDS patients
M. avium	1	*M. avium* I	31[a]	8[b]	1[c]	2[c]
M. avium	2	*M. avium* II	24	29	—	—
M. avium	3	*M. avium* III	11	—	—	—
M. intracellulare	4	*M. avium* IV	51	5	—	18
M. intracellulare	6	*M. avium* VI	26	2	1	—
M. intracellulare	8	Davis	92	25	—	3
M. intracellulare	9	Watson	50	9	—	—
M. intracellulare	10	*M. avium* IIIa	14	1	—	—
M. intracellulare	12	Howell	30	5	—	—
M. intracellulare	13	Chance	20	—	—	—
M. intracellulare	14	Boone	28	3	—	—
M. intracellulare	16	Yandle	66	3	—	—
M. intracellulare	18	Altman	14	1	1	—
M. intracellulare	20	Arnold	4	2	—	—
M. scrofulaceum	41	*M. scrofulaceum*	29	—	—	—
M. scrofulaceum	42	Lunning	32	—	—	—
M. scrofulaceum	43	Gause	23	—	—	—
Total			545	93	3	23
			545/690 = 78%	93/98 = 94%	3/9 = 33%	23/26 = 88%

[a]Data taken from McClatchy.[10]
[b]Data taken from Meissner and Anz.[15]
[c]Data taken from Kiehn *et al.*[76]

show involvement only in a single lymph node, with no obvious lung involvement, and the infection resolves spontaneously with time.[35,45]

While there is little demographic or epidemiologic evidence for obvious predisposing factors for these *M. avium*-complex infections, it seems likely that some form of temporary depression to the cell-mediated defenses must be involved.[47] *M. avium*-complex serotypes 1, 4, 8, 12, and 16 are most frequently isolated from these patients (Table I), and it is generally agreed that these serotypes are relatively more virulent for both humans[10] and experimental animals,[4] compared with the other serotypes. Most *M. avium* and *M. intracellulare* isolates can also be readily differentiated into flat translucent, domed opaque, and rough colony variants, when grown on solid media.[48] The translucent colony type appears to be substantially more virulent for experimental animals,[49,50] as well as being more resistant to most antituberculous drugs.[51] Mice infected with a mixture of the two colony variants develop lung infections that yield increasing numbers of the virulent translucent colony forms with time (Fig. 1), a finding that may have important implications for the therapy of chronic lung infections in humans as well.[51] Even when challenged with the translucent colony variant, however, the severity of the infection varies considerably, depending on the strain of organism,[52] the route of inoculation (Fig. 2), and the genetic makeup of the host.[53] Some translucent colony variants of *M. intracellulare* are virtually avirulent for the susceptible BALB/c strain of mouse, even when large numbers of bacilli are inoculated (Fig. 3). More susceptible strains develop chronic *M. intracellulare* infections that often persist until death occurs

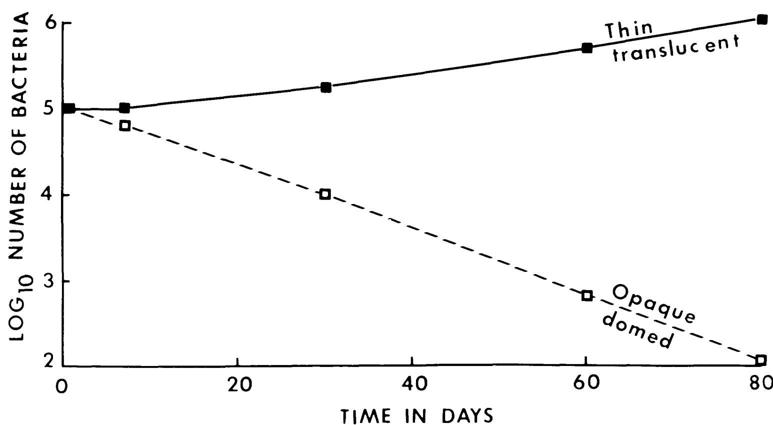

FIGURE 1. Recovery of thin translucent (virulent) and opaque domed (attenuated) colonies of *M. intracellulare* from the spleens (and lungs, not shown) of intravenously challenged B6D2 mice receiving a mixture of the two colony types.

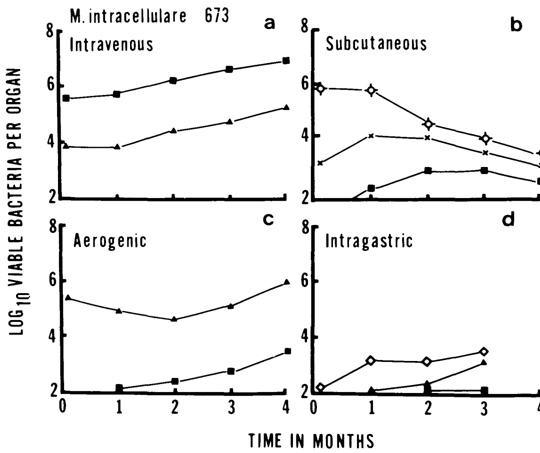

FIGURE 2. Growth of the mouse virulent *M. intracellulare* 673 in B6D2 mice following challenge by the intravenous (a), subcutaneous (b), aerogenic (c), or intragastric (d) routes. Spleen (■), lungs (▲), footpad (○), popliteal lymph nodes (X), mesenteric lymph node (◇).

as a result of old age.[52] However, the more virulent *M. avium* serotypes give rise to life-threatening infections in both the susceptible and resistant strains of mouse, whether introduced by the intravenous, aerogenic, or intragastric routes.[53] Mice infected with *M. scrofulaceum* or avirulent serotypes of *M. intracellulare* eliminate them rapidly, even when inocu-

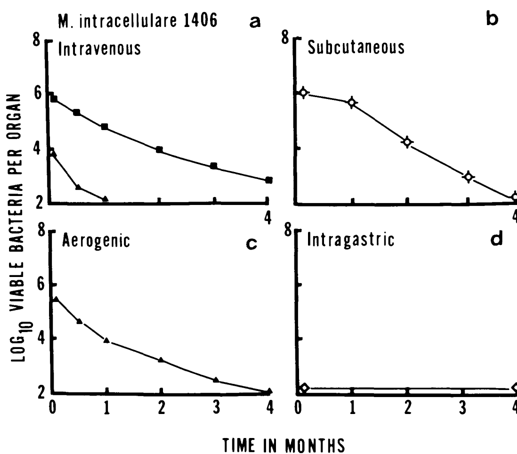

FIGURE 3. Growth of the mouse avirulent *M. intracellulare* TMC #1406 in B6D2 mice. See legend to Fig. 2 for further details.

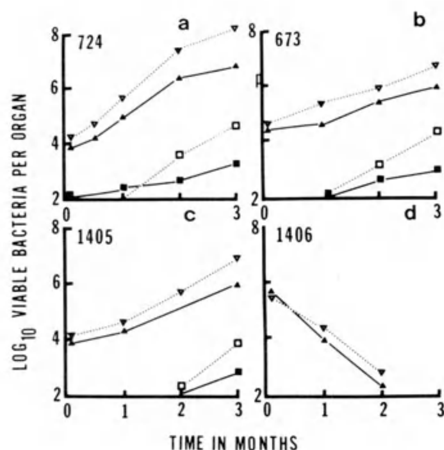

FIGURE 4. Growth of virulent *M. avium* TMC #724 (a), virulent *M. intracellulare* 673 (b), less virulent *M. intracellulare* TMC #1405 (c), or avirulent *M. intracellulare* TMC #1406 (d), following aerogenic challenges to T-cell-depleted BALB/c mice (· · ·) or normal BALB/c mice (——). Lungs (▼), spleen (■,□).

lated into T-cell-depleted BALB/c mice (Fig. 4). By contrast, the growth of the virulent serotypes (*M. intracellulare* 673 and 1405) appears to be potentiated by T-cell depletion. This finding suggests that the avirulent *M. avium*-complex serotypes may be eliminated from the tissues by a non-T-cell-mediated mechanism, whereas the virulent strains can only be killed by immunologically activated macrophages and thus are potentiated by depletion of the T-cell defenses.[37,53] It is clear that the host–parasite interactions responsible for controlling the growth of the *M. avium*-complex serotypes within normal tissues are both complex and diverse; we need to learn a lot more about the mechanisms these opportunistic pathogens use to establish themselves within the normal host.

3. *M. AVIUM*-COMPLEX INFECTIONS IN IMMUNODEPLETED PATIENTS

Although virulent *M. tuberculosis* multiplies freely within the lungs and spleens of normal mice for several weeks (Fig. 5), eventually there will be evidence of an immune response as the rate of growth begins to decline and large numbers of activated macrophages enter the developing tubercles.[19] Despite this immune response, large numbers of viable mycobacteria may persist within the tissues for many months, providing an ongoing antigenic stimulus that results in a chronic granulomatous

response (especially within the lung), eventually leading to cavitation and death.[54] The less virulent *M. avium* may survive within the spleen for many months with no sign of an immune decline equivalent to that seen in the *M. tuberculosis*-infected animals (Fig. 5). Avirulent species, such as *M. intracellulare* 1406 and *M. scrofulaceum* 1306, are eliminated progressively immediately after challenge, with no sign of an early growth phase. Paradoxically, virulent *M. avium* and *M. intracellulare* strains can persist within the spleen despite evidence that both immune T cells and immunologically activated macrophages are also present in the lesions.[55]

Host resistance to a variety of intracellular parasites can be enhanced or depressed by a number of extraneous factors,[56] some natural (intercurrent infections, tumor loads) and others artificial (irradiation, chemotherapy). Most normal individuals (95% of those exposed) develop sufficient cell-mediated immunity to the primary tuberculous infection to limit its growth to subclinical proportions.[19] The sequence of events responsible for the inability of apparently immunocompetent individuals to develop an effective cell-mediated response to the pathogen is not well understood. Some of these factors are nonspecific in nature, involving overcrowding, poor housing, malnutrition, alcoholism, and

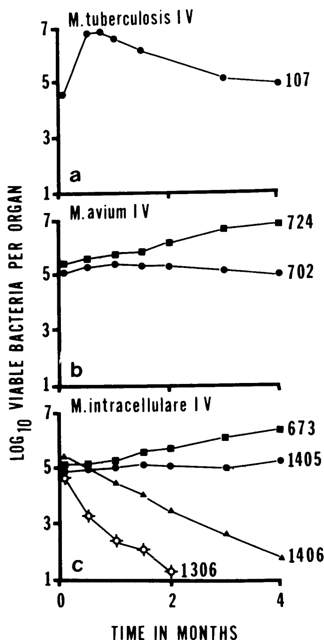

FIGURE 5. Growth of *M. tuberculosis* Erdman (a), *M. avium* (b), *M. intracellulare* or *M. scrofulaceum* (c), in the spleens of intravenously challenged B6D2 mice. 107 = *M. tuberculosis* Erdman; 724 and 702 = *M. avium;* 673, 1405, 1406 = *M. intracellulare;* 1306 = *M. scrofulaceum.*

drug abuse. It is also possible that genetic factors may be involved, although this is still far from proven.[57] Infection by a number of systemic viral pathogens can temporarily reduce the ability of the cellular defenses to function properly.[56] For instance, cytomegalovirus, measles, and Sendal virus infections are all known to cause a marked reduction in tuberculin hypersensitivity and other mitogenic responses *in vitro,* as well as temporarily potentiating the severity of several experimental bacterial lung infections *in vivo.*[58] Further discussion of this virally induced immunosuppression is found in Section 4.

Although tuberculosis traditionally attacks individuals from all socioeconomic levels throughout the community, some individuals appear to be particularly susceptible to mycobacterial lung disease.[16] These include patients suffering from silicosis and pneumoconiosis, especially sand blasters, quarry men, coal miners, and other industrial workers exposed to heavy dust loads.[59,60] The enhanced virulence of the normally opportunistic mycobacteria for these groups can be explained by the known toxic effects of silica on both pulmonary and peritoneal macrophages.[61] Experimentally, even the growth of an attenuated organism, such as BCG, can be substantially potentiated by silica treatment.[62] In addition, age seems to be an important factor. since most adult *M. tuberculosis* infections are now seen in elderly men.[63] On the other hand, nontuberculous disease occurs equally in males and females at all age levels. The sharp increase in the number of geriatric tuberculosis cases appears to be primarily due to the reactivation of healed lesions, presumably as a result of an age-related decline in cell-mediated immune responsiveness.[64] The source of atypical mycobacterial infections in this age group is unknown.

A second group of immunosuppressed patients known to be at increased risk of developing atypical mycobacterial infections includes those suffering from terminal renal disease and kidney transplantation patients.[65,66] While there appears to be a preponderance of *M. tuberculosis* and *M. kansasii* infections in these patients, a substantial number of *M. avium*-complex isolates have also been reported. This seems to be true even in communities in which atypical mycobacterial infections are normally rare,[67] an anomaly reported in other patients subjected to long-term immunosuppressive therapy.[68] Repeated doses of cytoxan, methotrexate, vinblastine, and corticosteroids reduce the ability of the host to defend itself against a number of facultative intracellular pathogens[69]; in particular, heavy cortisone treatment has long been recognized as a potential reactivator of dormant tuberculosis lesions, both in experimental animals and in humans.[70,71] Some antituberculous drugs have even been reported to be immunosuppressive when given at maximum tolerated doses, although much of this evidence is circumstantial

at best.[72] However, this may become an increasingly important factor when massive combined chemotherapy is required to treat acquired immunodeficiency syndrome (AIDS) patients infected with *M. avium* complex.[73]

There are considerable data to indicate that many cancer patients are especially susceptible to severe *M. tuberculosis* and *M. kansasii* lung infections.[74,75] The number of *M. avium*-complex infections seen in this group of patients seems to be relatively much lower,[76] with relatively larger numbers of *M. simiae, M. gordonae,* and *M. fortuitum* isolates, all of which were thought to be virtually avirulent in humans until a few years ago.[2,4] This trend is perhaps due to the increased number of patients in the community who are receiving combined immunosuppressive therapy and in whom systemic *M. tuberculosis* and atypical mycobacterial infections frequently develop as a result.[77] However, this is probably an oversimplification, since these infections also occur in hemophiliacs, who seem to be as susceptible to tuberculous infections as the cancer patients.[78] As a result of the increasing number of immunodeficient individuals in the community, the presence of even small numbers of atypical mycobacteria in sputum, urine, or bone marrow specimens should be viewed with concern and the patient treated immediately in an attempt to eliminate the colonizing organisms before a life-threatening infection develops.

4. *M. AVIUM*-COMPLEX INFECTIONS IN AIDS PATIENTS

Acquired immunodeficiency syndrome was first recognized in 1981 as a new immunologic disease afflicting homosexual men and drug addicts in New York and San Francisco.[73] The disease is caused by the human immunodeficiency virus (HIV-I), which parasitizes the T4 cell population of the host, affecting its ability to mount an effective cell-mediated immune response against a number of facultative intracellular parasites.[79] Many of the AIDS-related complex (ARC) patients (20–30%) develop disseminated mycobacterial infections, and a surprisingly high proportion of these isolates have been shown to be *M. avium* or *M. intracellulare*.[37] In more than 40 separate investigations involving 1000 AIDS patients, at least 20% were infected with *M. avium* complex (Table II), with smaller numbers of *M. tuberculosis* infections (mainly Haitians), together with a few isolates of *M. gordonae* and *M. fortuitum*. In one recent study carried out in New York City, almost 50% of AIDS patients were infected with *M. avium* complex, mainly serotype 4, with smaller numbers of serotypes 1 and 8.[76] Those patients suffering from disseminated *M. tuberculosis* infections[80] were mainly Haitians living in Miami

TABLE II
Mycobacterial Infections in AIDS Patients in the United States [a,b]

Patient population	Number of patients	*M. tuberculosis*	*M. avium* complex	*M. gordonae*
Homosexual	76	0	32	1
Drug abuse	12	0	6	—
Hemophiliac	2	0	2	—
Female consorts	12	—	7	—
Unspecified	819	46	162	4
Haitian	102	53	6	—
Total	1023	99	215	5

[a]Based on 41 reports. 319/1023 = 31% incidence of *M. tuberculosis* and MAC infections.
[b]—, not specified.

and New York City.[81] The increased number of cases of *M. avium*-complex infection may be partly due to better diagnostic procedures, since many of the early AIDS patients were not suspected to be suffering from disseminated mycobacterial disease, largely because of the benign nature of the *M. avium*-complex infections.[82] Specialized staining, cultural, and serologic methods are needed to identify these organisms fully in biopsy and postmortem specimens[10,22] and, since these atypical mycobacteria do not usually induce cavitary lung disease, few acid-fast bacilli will be present in either sputum or bronchial lavage samples or even in gastric washings.[83] However, many of these patients develop heavy intestinal infections with large numbers of viable *M. avium* complex in both intestinal biopsy specimens[36] and fecal samples.[76] Greater success has also been reported for cultures[84] prepared from bone marrow and blood samples collected from both ARC and AIDS patients[85,86] and, with the increasing incidence of these patients in the community, it is now recommended that all high-risk and HIV-positive individuals be routinely skin tested with PPD-S, PPD-A, and PPD-B[87] and that blood and bone marrow specimens be examined for the presence of *M. tuberculosis* or *M. avium* complex, which can now be considered as diagnostic for terminal AIDS.[88] The presence of the *M. tuberculosis* infection often precedes the HIV infection by months or years,[90] but the exact relationship between the *M. avium*-complex infection and AIDS remains more of a matter of speculation.[90]

In many of the early reports, mycobacterial involvement was based simply on the presence of acid-fast bacilli in histologic material prepared at postmortem examination. The lesions had an appearance strongly reminiscent of that seen in lepromatous leprosy or miliary tuber-

culosis,[91,92] consisting of large aggregations of foamy macrophages, often packed with acid-fast bacilli.[93] Massive involvement of the lungs, gut-associated lymphoid tissues, spleen, and bone marrow is frequently observed, together with *Pneumocystis carinii, Candida albicans,* cytomegalovirus, and Epstein–Barr (EBV) lung infections.[94,95] Most of these cases occurred in homosexual and drug abuse patients, although recently there has been an increasing involvement of consorts of drug abusers suffering from AIDS, prostitutes, and other highly promiscuous heterosexuals in the community.[96,97]

Another group of patients of largely unknown size and importance are the transfusion patients who received blood or blood products before effective screening was instituted.[98] These patients form a relatively small component of the overall AIDS population but, because of the long incubation period for this disease, they may turn out to be a critical factor in the final analysis. The fact that they are mainly heterosexuals makes them more analogous to the African and Haitian AIDS populations which also involve equal numbers of males and females.[100] Evidence is accumulating from both central Africa and Haiti that heterosexual spread due to sexual promiscuity seems to be the major factor in promoting this epidemic in those countries. This mode of spread may also become an increasing factor in the United States and Europe. The preponderance of *M. tuberculosis* infections in Haitians and African patients[101] may reflect genetic differences[57] but is more likely to be due to the extremely high prevalence of pulmonary tuberculosis in these countries,[102] where more than 90% of adult Haitians are known to be tuberculin positive by the time they are 20 years old. On the other hand, Americans suffer a much lower rate of exposure to *M. tuberculosis* and a more frequent involvement with environmental opportunistic mycobacteria, including the *M. avium* complex. It is difficult to explain why Americans and Haitians now living side by side in Miami or New York City do not develop mixed *M. tuberculosis* and *M. avium*-complex infections more frequently than they do.[101]

Early detection of systemic *M. avium*-complex infections in ARC patients poses a number of technical problems, mainly because the lesions are inapparent until late in the disease. Careful examination of radiologic evidence has revealed signs of disseminating tuberculous disease in some pre-AIDS patients,[103] and cultures prepared from stool and rectal biopsy specimens have revealed the presence of acid-fast bacilli within the submucosa, usually within large foamy macrophages.[36] The histologic picture seen in many of these patients resembles Whipple's disease in humans[104] or Johne's disease in cattle,[105] and much the same has been reported for *M. tuberculosis*-infected Haitian AIDS patients.[106] Such extensive involvement of the intestinal mucosae, Peyer's

patches, and mesenteric lymph nodes seems to point to an intragastric infection pathway, although direct transfer via rectal trauma may also occur in some homosexual AIDS patients.[36] This has been made the more plausible by the presence of mixed intestinal infections involving several opportunistic gut pathogens in some of these patients.[107]

The alternative route of infection for these organisms involves the nasopharyngeal mucosae, and many AIDS patients do develop severe lung infections relatively early in their disease.[108] However, there is little evidence for direct aerogenic spread by members of the *M. avium* complex, even from heavily immunosuppressed patients.[109] In most patients, sputum samples are consistently negative for acid-fast bacilli at a time when both bone marrow and blood cultures are strongly positive, again suggesting a nonpulmonary infection route of infection for most of these patients.[110,111] Blood cultures also provide a convenient tool for monitoring the effectiveness of the chemotherapeutic regimen,[112] since the presence of only one or two colony-forming units per milliliter (CFU/ml) of blood indicates the presence of many millions of viable mycobacteria elsewhere in the host tissues.[113] Positive blood cultures from an ARC patient must now be considered diagnostic for AIDS.[81]

In addition to playing a relatively passive secondary role in the tissues of the ARC patient, *M. avium* complex may later play a more direct role in the evolution of the terminal phases of this infection.[37] Both splenic and lymph node infections involving virulent *M. avium*-complex serotypes affect the T-cell-dependent periarteriolar regions of the lymphoid organs, thereby contributing to a progressive loss of T-cell function as the infection develops.[114] Eventually, the *M. avium*-complex infection may resemble that seen in lepromatous leprosy,[115] a chronic mycobacterial infection also characterized by a persistent loss of specific T-cell reactivity.[116] This similarity provides further circumstantial evidence that the systemic *M. avium*-complex infection may contribute to the progressive immunosuppression seen in the ARC patient.[117] It is well known that only a proportion of HIV-infected individuals go on to develop clinical AIDS unless they are subjected to some type of secondary antigenic or infectious stimulus, which seems to be required in order to drive the disease into its final stages.[73] A number of opportunistic pathogens can provide this type of stimulus, but it appears that the *M. avium* complex may have a special propensity for doing this to the HIV-infected patient.[90] It should be noted that the distribution of opportunistic mycobacterial infections in AIDS patients is quite different from that seen in other immunosuppressed cancer and transplantation patients.[76] The dominance of *M. avium*-complex serotypes 1, 4, and 8 in the AIDS population suggests the existence of some sort of causal relationship between the two phenomena.[82,118] T-cell depletion is known to

enhance the growth of virulent mycobacteria within the lung.[53] However, the virtual absence of *M. simiae* infections in the American AIDS patient population argues against simple T-cell depletion as the only factor involved.[37] *M. avium*-complex serotypes 1, 4, and 8 are known to be relatively more virulent than other serotypes for humans[10] and might therefore be expected to persist on the mucosal membranes of the normal host more effectively than less virulent strains.[13] However, one would still expect *M. simiae* infections to occur more frequently than they do in the AIDS population, since these organisms are also commonly found in the environment.[4]

One possible explanation for this paradox could be the possession by these three *M. avium*-complex serotypes of unique surface receptors that enable them to colonize the nasopharyngeal and/or intestinal mucosal cells more effectively than other mycobacteria. The flat translucent (virulent) colony variant of *M. avium* is known to possess surface antigens (or receptors) not found on the domed less virulent colony variants.[119] Thus, the presence of these antigens on the translucent variant may be important simply by permitting persistent colonization of the gut-associated lymphoid tissues of the normal host, resulting in an earlier and more substantial local infection and the induction of an ongoing state of immune tolerance to the specific mycobacterial sensitin(s) of these organisms. Much the same process has been postulated to occur in miliary tuberculosis and lepromatous leprosy.[120] Such heavy mycobacterial infections may also induce the formation of suppressor cells within the lymphoreticular organs of the host, which then remains unresponsive to specific antigenic stimulation for many months (Fig. 6). Such an infection may also serve as a polyclonal B-cell activator, resulting in a persistent hyperglobulinemia and excess antigen–antibody complex formation.[121] This B-cell response will have little protective value for the tuberculous host but may divert an already depleted T-cell resource into an essentially unprofitable humoral response. The AIDS virus is also known to act as a potent B-cell mitogen, and the combined effect of these two pathogens may induce an immunologic defect similar to that seen in systemic lupus erythematosus (SLE).[90]

One final contribution by the developing *M. avium*-complex infection to this disease could be its ability to provide an ongoing stimulus to the T-cell population within the virally infected spleen.[122] This would provide an increasing number of activated T cells for the AIDS virus to parasitize,[123] thereby enhancing the growing immunodepletion of the ARC patient.[124] The end result of these multiple antigenic and mitogenic stimuli would be a deepening level of immunosuppression, slowly ablating both humoral and cellular responsiveness until one or other of the opportunistic pathogens within the host tissues assumed life-threat-

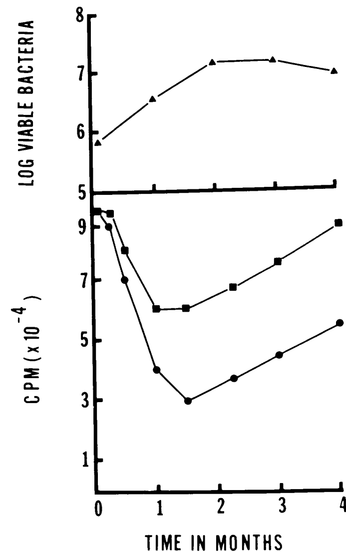

FIGURE 6. Growth of *M. avium* (▲) in the spleens of intravenously infected B6D2 mice compared with the blastogenic responsiveness by whole spleen cells (●) or T-cell-enriched suspensions *in vitro* (■) exposed to phytohemagglutinin.

ening proportions, leading to a diagnosis of clinical AIDS.[125] The nature and hierarchy of these life-threatening infections may provide clues to their role in the evolution of this immunologic disease.[79,84]

The prognosis for the AIDS patient remains poor, with a 2-year survival rate of virtually zero. The outlook for AIDS patients suffering from disseminated *M. avium*-complex disease is even more dismal.[73] While AIDS patients with *M. tuberculosis* infections may respond effectively to conventional antituberculous chemotherapy,[125] those with *M. avium*-complex disease seldom do, partly because of the inherent drug resistance shown by these atypical mycobacteria, and partly because the infections frequently reach massive proportions before their existence is even suspected.[126] Some clinical improvement has been reported in some *M. avium*-infected AIDS patients subjected to combined therapy with ansamycin, clofazimine, ethambutol, cycloserine, amakacin, and ethionamide, but most show a bacteriostatic response at best, with growth resuming once the therapy is discontinued.[73] This poor clinical response may be partly explained by the ablation of the T-cell-mediated defenses, suggesting that a combined chemotherapy and immunotherapy should be provided for the treatment of all ARC patients.[112] Attempts to reconstitute the depleted host defenses in AIDS patients by means of bone marrow transplants and IL-2 and gamma interferon (IFN_{γ}) infusions have not been particularly successful, although this approach will undoubtedly be continued in the future.[127]

5. CONCLUSIONS

Until recently, most atypical mycobacteria have been of interest to bacteriologists more for their taxonomic obscurity and their immunologic complexity than for their public health significance. While some species have been recognized as potential human and animal pathogens, most have been thought of as opportunistic pathogens at best, of doubtful virulence for normal healthy adults. Some species have been shown to function as troublesome secondary invaders in tuberculosis patients, although the resulting infection is usually indolent and relatively limited in nature. Occasionally, they result in disseminated systemic disease, usually when some form of immunosuppression is also present. Atypical mycobacterial infections show a distinctive regional distribution, with some occupational factors involved as well. However, the primary human isolation patterns for most of these opportunistic pathogens seem to conform to the distribution of these organisms in the environment and to the ability of the different species to survive in aerosols generated from infected natural waters. Direct person-to-person spread by these atypical mycobacteria does not appear to be an important public health factor, nor is there any strong evidence for infection from animal sources. However, skin-test data obtained using PPD-A and PPD-B indicate surprisingly widespread colonization of the nasopharyngeal and intestinal mucosae of apparently normal individuals. Some of these organisms establish self-localizing infections within the draining lymphoid tissues of the throat and gut, and the more virulent of these organisms may eventually become part of the commensal membrane flora. This probably results in intermittent involvement of the Peyer's patches and the mesenteric lymph nodes, although the normal T-cell defenses will limit the spread of these organisms beyond the mucosal surface. However, this balance is upset by the introduction of the HIV infection. T-cell depletion allows these organisms to reach the bloodstream, and the infection begins to disseminate. Detection of localized *M. avium*-complex infections at this time can be technically difficult due to the small number of mycobacteria present and the large number of contaminants. Greater success has been achieved using gastric washings, bone marrow biopsies, and blood cultures.

Infection studies using susceptible inbred mice indicate the growth of some but not all *M. avium*-complex serotypes following aerogenic and intragastric inoculation. These infections could be potentiated by T-cell depletion. However, even massive doses of avirulent *M. avium*-complex serotypes failed to induce progressive disease in T-cell-deficient mice, suggesting that only the more virulent serotypes were likely to be recovered from the AIDS-virus infected patient. On the other hand, virulent *M. tuberculosis* can persist within healed lesions in the lung and

elsewhere for many years and so could be expected to reactivate in the AIDS patient population roughly in proportion to the exposure rates for the population as a whole. While this may explain the dominance of *M. tuberculosis* infections in Haitian AIDS patients, the virtual absence of *M. tuberculosis, M. kansasii,* and *M. simiae* from the American AIDS patient population suggests that other factors may be involved as well. The striking difference that exists in the distribution of *M. avium*-complex serotypes in AIDS versus cancer and transplant patients again suggests some unexplained specificity for the HIV-infected individual. Possibly the *M. avium* complex possesses factors (nutritional, metabolic, or antigenic) that render it particularly suited to growth within the tissues of the ARC patient. Once established within the lymphoreticular organs of that host, the *M. avium* complex may contribute to the deepening immunosuppression characteristic of the ARC patient, helping to drive the disease into its terminal phase. Under these circumstances, the *M. avium* complex may no longer be considered a mere casual opportunist but rather a contributory cofactor to this important new immunologic disease. Regardless of the truth or otherwise of this hypothesis, it would seem prudent to determine the nature of the factors governing the growth and spread of these opportunistic human pathogens in both normal and immunodepleted hosts. At present, we know very little about the distributional patterns for these different serotypes in the different organs of the immunosuppressed host or how various immuno- and chemotherapeutic strategies might be able to affect their ability to contribute to this rapidly spreading epidemic of human immunodeficiency disease.

6. SUMMARY

The *Mycobacterium avium* complex consists of 31 serotypes that vary extensively in their distribution within the environment and in their virulence for man, as well as domestic and laboratory animals. Most can be considered opportunistic human pathogens, able to cause disease only when the normal host defenses have been ablated in some way. Some species can colonize the normal nasopharyngeal and intestinal mucosae, producing self-limiting lymph node infections. They can also induce systemic disease in congenitally and iatrogenically immunosuppressed patients, as well as in some renal, transplant and cancer patients. Patients suffering from acquired immunodeficiency syndrome (AIDS) develop life-threatening *M. tuberculosis* and *M. avium*-complex lung infections. Up to 50% of some AIDS populations have been reported to have systemic *M. avium*-complex infections, mostly serotypes 1, 4, and 8.

The existence of these relatively rare opportunistic mycobacterial pathogens in so many AIDS patients suggests some sort of linkage between their presence in the HIV-infected individual and the subsequent development of clinical AIDS. This possibility deserves further detailed investigation that could also lead to improved chemotherapeutic and immunotherapeutic procedures for the treatment of ARC patients before they develop life-threatening disease caused by the more virulent members of this *M. avium* complex.

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REFERENCES

1. Barksdale, L., and Kim, K-S., 1977, Mycobacterium, *Bacteriol. Rev.* **41:**217–372.
2. Wolinsky, E., 1979, Nontuberculous mycobacteria and associated diseases, *Am. Rev. Respir. Dis.* **119:**107–159.
3. Davidson, P. T., 1979, The management of disease with atypical mycobacteria, *Clin. Notes Respir. Dis.* **18:**3–13.
4. Chapman, J. S., 1982, The atypical mycobacteria, *Am. Rev. Respir. Dis.* **125:**S119–124.
5. Anonymous, 1982, Centennial: Koch's discovery of the tubercle bacillus, *M.M.W.R.* **31:**121–123.
6. Farer, L. S., Lowell, A. M., and Meador, M. P., 1979, Extrapulmonary tuberculosis in the United States, *Am. J. Epidemiol.* **109:**205–267.
7. Robakiewicz, M., and Grzybowski, S., 1974, Epidemiological aspects of nontuberculous mycobacterial disease and of tuberculosis in British Columbia, *Am. Rev. Respir. Dis.* **109:**613–620.
8. Anonymous, 1986, Tuberculosis—United States, 1985—and the possible impact of human T-lymphotrophic virus type III/Lymphadenopathy-associated virus infection, *M.M.W.R.* **35:**74–76.
9. Schaefer, W. F., 1979, Serological identification of atypical mycobacteria, *Methods Microbiol.* **13:**323–343.
10. McClatchy, J. K., 1981, The seroagglutination test in the study of nontuberculous mycobacteria, *Rev. Infect. Dis.* **3:**867–870.
11. Thoen, C. O., Himes, E. M., and Karlson, A. G., 1984, *Mycobacterium avium* complex, *Microbiol. Series* **15:**1251–1275.
12. Anonymous, 1981, Diagnostic standards and classification of tuberculosis and other mycobacterial diseases, *Am. Rev. Respir. Dis.* **123:**343–358.
13. Chapman, J. S., 1971, The ecology of the atypical mycobacteria, *Arch. Environ. Health* **22:**41–46.
14. Gruft, H., Katz, J., and Blanchard, D. C., 1975, Postulated source of *M. intracellulare* (Battey) infection, *Am. J. Epidemiol.* **102:**311–318.
15. Meissner, G., and Anz, W.. 1977, Sources of *M. avium*-complex infection resulting in human disease, *Am. Rev. Respir. Dis.* **116:**1057–1064.

16. Comstock, G. W., 1982, Epidemiology of tuberculosis, *Am. Rev. Respir. Dis.* **125:**S8–15.
17. Anonymous, 1972, What needs to be done to eradicate tuberculosis, *Am. J. Public Health* **62:**127–128.
18. Anonymous, 1985, Tuberculosis—United States 1984, *MMWR* **34:**299–307.
19. Collins, F. M., 1982, The immunology of tuberculosis, *Am. Rev. Respir. Dis.* **125:**S42–49.
20. Timpe, A., and Runyon, E. H., 1954, Relationship of "atypical" acid-fast bacilli to human disease, *J. Lab. Clin. Med.* **44:**202–209.
21. Wayne, L. G., 1984, Mycobacterial speciation, *Microbiol. Ser.* **15:**25–65.
22. Runyon, E. H., Karlson, A. G., Kubica, G. P., and Wayne, L. G., 1974, Mycobacterium, in *Manual of Clinical Microbiology*, 2nd ed. (E. H. Lennette, E. H. Spaulding, J. P. Truant, eds.), pp. 148–174, American Society of Microbiology, Washington, D.C.
23. Edwards, L. B., and Palmer, C. E., 1959, Isolation of "atypical" mycobacteria from healthy persons, *Am. Rev. Respir. Dis.* **80:**747–749.
24. Atwell, R. J., and Pratt, P. C., 1960, Unclassified mycobacteria in gastric contents of healthy personnel and patients of tuberculosis hospital, *Am. Rev. Respir. Dis.* **81:**888–892.
25. Tsukamura, M., 1973, Background factors for casual isolation of *M. intracellulare* from patients with tuberculosis, *Am. Rev. Respir. Dis.* **108:**679–683.
26. Wolinsky, E., 1981, Editorial. When is an infection disease?, *Rev. Infect. Dis.* **3:**1025–1027.
27. Edwards, L. B., Acquaviva. F. A., Livesay, V. T., Cross, F. W., and Palmer, C. E., 1969, An atlas of sensitivity to tuberculin, PPD-B and histoplasmin in the United States, *Am. Rev. Respir. Dis.* **99:** S1–132.
28. Rosenzweig, D. Y., 1979, Pulmonary mycobacterial infections due to *Mycobacterium intracellulare-avium* complex, Chest **75:**115–119.
29. Snider, D. E., 1982, The tuberculin skin test, *Am. Rev. Respir. Dis.* **125:**S108–118.
30. Anonymous, 1966, Further studies of acquired sensitivity to human and avian tuberculins at successive tests in school children, *Tubercle* **47:**157–177.
31. Good, R. C., 1980, Isolation of nontuberculous mycobacteria in the United States, 1979. *J. Infect. Dis.* **142:**779–783.
32. Good, R. C., and Snider, D. E., 1982, Isolation of non-tuberculous mycobacteria in the United States, 1980, *J. Infect. Dis.* **146:**829–833.
33. Goslee, S., and Wolinsky, E., 1976, Water as a source of potentially pathogenic mycobacteria, *Am. Rev. Respir. Dis.* **113:**287–292.
34. Falkinham, J. O., Parker, B. C., and Gruft, H., 1980, Epidemiology of infection by nontuberculous mycobacteria. 1. Geographic distribution in the Eastern United States, *Am. Rev. Respir. Dis.* **121:**931–937.
35. Saitz, E. W., 1981, Cervical lymphadenitis caused by atypical mycobacteria, *Pediatr. Clin. North Am.* **28:**823–839.
36. Damsker, B., and Bottone, E. J., 1985, *Mycobacterium avium–M. intracellulare* from the intestinal tracts of patients with acquired immunodeficiency syndrome: Concepts regarding acquisition and pathogenesis, *J. Infect. Dis.* **151,** 179–181.
37. Collins, F. M., 1986, *M. avium*-complex infections and development of the acquired immunodeficiency syndrome: Casual opportunist or causal cofactor?, *Int. J. Lepr.* **54:**458–474.
38. Jenkins, P. A., 1981, Nontuberculous mycobacteria and disease, *Eur. J. Respir. Dis.* **62:**69–71.
39. Horsburg, C. R., Mason, V. G., Farlin, D. C., and Iseman, M. D., 1985, Disseminated infection with *M. avium-intracellulare:* A report of 13 cases and a review of the literature, *Medicine (Baltimore)* **64:**36–48.

40. Fischer, D. A., Lester, W., and Schaefer, W. B., 1968, Infections with atypical mycobacteria. Five years experience at the National Jewish Hospital, *Am. Rev. Respir. Dis.* **98**:29–34.
41. Rosenzweig, D. Y., and Schlueter, D. P., 1981, Spectrum of clinical disease in pulmonary infection with *M. avium-intracellulare, Rev. Infect. Dis.* **3**:1046–1051.
42. Ahn, C. H., Lowell, J. R., Onstad, G. D., Shurford, E. H., and Hurst, G. A., 1979, A demographic study of disease due to *M. kansasii* or *M. intracellulare-avium* in Texas, *Chest* **75**:120–125.
43. Codias, E. K., and Reinhardt, D. J., 1979, Distribution of serotypes of the *M. avium–intracellulare–scrofulaceum* complex in Georgia, *Am. Rev. Respir. Dis.* **119**:965–970.
44. Wiesnethal, A. M., Powell, K. E., Kopp, J., and Splinter, J. W., 1982, Increase in *M: avium*-complex isolates among patients admitted to a general hospital, *Public Health Rep.* **97**:61–65.
45. Lincoln, E. M., and Gilbert, L. A., 1972, Disease in children due to mycobacteria other than *M. tuberculosis, Am. Rev. Respir. Dis.* **105**:683–714.
46. Yeager, H., and Raleigh, J. W., 1973, Pulmonary disease due to *M. intracellulare, Am. Rev. Respir. Dis.* **108**:547–552.
47. Cook, P. L., Riddell, R. W.. and Simon, G., 1971, Bacteriological and radiographic features of lung infection by opportunist mycobacteria: A review, *Tubercle* **52**:232–241.
48. Schaefer, W. B., Davis, C. L., and Cohn, M. L., 1970, Pathogenicity of translucent, opaque and rough variants of *M. avium* in chickens and mice, *Am. Rev. Respir. Dis.* **123**:343–358.
49. Watson, S. R., Morrison, N. E., and Collins, F. M., 1979, Delayed hypersensitivity responses in mice and guinea pigs to *M. leprae, M. vaccae* and *M. nonchromogenicum* cytoplasmic proteins. *Infect. Immun.* **25**:229–236.
50. Moehring, J. M.. and Soloterovsky, M. R., 1965, Relationship of colonial morphology to virulence for chickens of *M. avium* and the nonphotochromogens, *Am. Rev. Respir. Dis.* **92**:704–713.
51. Moulding, T., 1978, The relative drug-susceptibility of opaque colonial forms of *M. intracellulare-avium:* Does it affect therapeutic results?, *Am. Rev. Respir. Dis.* **117**:1142–1143.
52. Collins, F. M., Morrison, N. E., and Montalbine, V., 1978, Immune response to persistent mycobacterial infection in mice. *Infect. Immun.* **19**:430–438.
53. Collins, F. M., and Stokes, R. W., 1987, *M. avium*-complex infections in normal and immunosuppressed mice, *Tubercle* **68**:127–136.
54. Collins, F. M., 1979, Cellular antimicrobial immunity, *CRC Crit. Rev. Microbiol.* **7**:27–91.
55. Hepper, K. P., and Collins, F. M., 1984, Immune responsiveness in mice heavily infected with *M. kansasii, Immunology* **53**:357–364.
56. Kantor, F. S., 1975, Infection, anergy and cell-mediated immunity, *N. Engl. J. Med.* **292**:629–634.
57. Fine, P. E. M., 1981, Immunogenetics of susceptibility to leprosy, tuberculosis and leishmaniasis. An epidemiological perspective, *Int. J. Lepr.* **49**:437–454.
58. Jakab, G. J., 1981, Interactions between Sendai virus and bacterial pathogens in the murine lung: A review, *Lab. Anim. Sci.* **31**:170–177.
59. Bailey, W. C., Brown, M. Beuchner, H. A., 1974, Silico-mycobacterial disease in sandblasters, *Am. Rev. Respir. Dis.* **110**:115–125.
60. Schepers, G. W., Smart, R. H., Smith, C. R., *et al.*, 1958, Fatal silicosis with complicating infection by an atypical acid-fast photochromogenic bacillus. *Indust. Med. Surg.* **27**:27–32.

61. Allison, A. C., and Hart, P. D., 1968, Potentiation of the growth of *M. tuberculosis* in macrophage cultures, *Br. J. Exp. Pathol.* **49:**465–476.
62. Steenken, W., Raleigh, J. W., and Smith, M. M., 1964, Pathogenicity of attenuated and avirulent tubercle bacilli, *Lancet* **1:**85–88.
63. Comstock, G. W., 1975, Frost revisited: The modern epidemiology of tuberculosis, *Am. J. Epidemiol.* **101:**363–382.
64. Anonymous, 1983, Tuberculosis in old age. *Tubercle* **64:**69–71.
65. Andrew, O. T., Schoenfeld, P. Y., Hopewell, P. C., and Humphreys, M. H., 1980, Tuberculosis in patients with end-stage renal disease, *Am. J. Med.* **68:**59–65.
66. Fraser, D. W., Buxton, A. E., Naji, A., Barker, C. F., Rudnick, M., and Weinstein, 1975, Disseminated *M. kansasii* infection presenting as cellulitis in a recipient of a renal graft, *Am. Rev. Respir. Dis.* **112:**125–129.
67. Abbott, M. R., and Smith, D. D., 1981, Mycobacterial infections in immunosuppressed patients, *Med. J. Aust.* **1:**351–353.
68. Millar, J. W., and Horne, N. W., 1979. Tuberculosis in immunosuppressed patients, *Lancet* **1:**1176–1178.
69. Dannenberg, A. M., 1984, Chemical and enzymatic host factors in resistance to tuberculosis, *Microbiol. Ser.* **15:**721–760.
70. McCune, R. M., Feldman. F. M., Lambert, H. P., and McDermott, W., 1966, Microbial persistence. 1. The capacity of tubercle bacilli to survive sterilization in mouse tissues, *J. Exp. Med.* **123:**455–468.
71. Dannenberg, A. M., 1979, The anti-inflammatory effects of glucocorticosteroids: A brief review of the literature, *Inflammation* **3:**329–343.
72. Kasik, J. E., Monick, M., and Thompsen, J. S., 1976, Immunosuppressant activity of the ansamycins, *Antimicrob. Agents Chemother.* **9:**430–473.
73. Fauci, A. S., 1984, Acquired immunodeficiency syndrome: Epidemiologic, immunologic and therapeutic considerations, *Ann. Intern. Med.* **100:**92–106.
74. Feld, R., Bodey, G. P., and Groschel, D., 1976, Mycobacteriosis in patients with malignant disease, *Arch. Intern. Med.* **136:**67–70.
75. Ortbals, D., and Marr, J.. 1978, A comparative study of tuberculosis and other mycobacterial infections and their association with malignancy, *Am. Rev. Respir. Dis.* **117:**39–45.
76. Kiehn, T. E., Edwards, F. F., Brannon, P., Tsang A., Maio, M., Gold, J., Whinbeg, E., Wang, B., McClatchy, J. K., and Armstrong, D., 1985, Infections caused by *M. avium*-complex in immunocompromised patients: Diagnosis by blood culture and fecal examination, antimicrobial susceptibility test and morphological and serological characteristics, *J. Clin. Microbiol.* **21:**168–173.
77. McGeady, S. J., and Murphey, S. A., 1981, Disseminated *M. kansasii* infection, *Clin. Immunol. Immunopathol.* **20:**87–98.
78. Beddall, A. C., Hill, F. G., and George, R. H., 1983, Hemophilia and tuberculosis, *Lancet* **1:**1226.
79. Lane, H. C., and Fauci, A. S., 1985, Immunologic abnormalities in the acquired immunodeficiency syndrome, *Annu. Rev. Immunol.* **3:**477–500.
80. Anonymous, 1984, Update: Acquired immunodeficiency syndrome (AIDS)—Europe, *MMWR* **33:**21–31.
81. Pitchenik, A. E., and Fischi, M. A., 1983, Disseminated tuberculosis and the acquired immunodeficiency syndrome, *Ann. Intern. Med.* **97:**112.
82. Chester, A. C., and Winn, W. C., 1986, Unusual and newly recognized patterns of nontuberculous mycobacterial infection with emphasis on the immunocompromised host, *Pathol. Annual* **21:**251–270.
83. Stover, D. E., White, D. A., Romano, P. A., and Gellene, R. A., 1984, Diagnosis of

pulmonary disease in acquired immunodeficiency syndrome (AIDS). Role of bronchoscopy and bronchoalveolar lavage, *Am. Rev. Respir. Dis.* **130**:659–662.

84. Berlin, O. G., Zakowski, P.. Bruchner, D. A., and Johnson, B. L., 1984, New biphasic culture system for isolates of mycobacteria from blood of patients with acquired immune deficiency syndrome, *J. Clin. Microbiol.* **20**:572–574.
85. Cohen, R. J., Samozuk, M. K., Busch, D., 1983, Occult infections with *M. intracellulare* in bone marrow biopsy specimens from patients with AIDS, *N. Engl. J. Med.* **308**:1475–1476.
86. Macher, A. M., Kovacs, J. A., Gill, V., *et al.*, 1983, Bacteremia due to *M. avium-intracellulare* in the acquired immunodeficiency syndrome, *Ann. Intern. Med.* **99**:782–785.
87. Pitchenik, A. E., Burr, J., and Cole, C. N., 1986, Tuberculin testing for persons with positive serologic studies for HTLV-III, *N. Engl. J. Med.* **314**:447.
88. Sunderam, G., McDonald, R. J., Maniatis, T., *et al.*, 1986, Tuberculosis as a manifestation of the acquired immunodeficiency syndrome (AIDS), *JAMA* **256**:357–361.
89. Pitchenick, A. E., Cole, C., Russell, B. W., *et al.*, 1984, Tuberculosis, atypical mycobacteriosis and the acquired immunodeficiency syndrome among Haitian and non-Haitian patients in South Florida, *Ann. Intern. Med.* **101**:641–645.
90. Lane, H. C., and Fauci, A. S., 1985, Immunologic aspects of acquired immunodeficiency syndrome, *Adv. Host Defense Mech.* **5**:131–148.
91. Welch, K., Finkbeiner, W., Alpers, C. E., *et al.*, 1984, Autopsy findings in the acquired immune deficiency syndrome, *JAMA* **252**:1152–1159.
92. Sohn, C. C., Schroff, R. W., Kliewer, K. E., *et al.*, 1982, Disseminated *M. avium-intracellulare* infection in homosexual men with acquired cell-mediated immunodeficiency: A histologic and immunologic study of two cases, *Am. J. Clin. Pathol.* **79**:247–252.
93. Dryjanski, J., and Gold, J. W. M., 1984, Infections in AIDS patients, *Clin. Hematol.* **13**:709–726.
94. Blaser, M. J., and Cohn, D. L., 1986, Opportunistic infections in patients with AIDS: Clues to the epidemiology of AIDS and the relative virulence of pathogens, *Rev. Infect. Dis.* **8**:21–30.
95. Kovacs, J. A., and Mason, H., 1985, Opportunistic infections, *Adv. Host Defense Mech.* **5**:35–58.
96. Masur, H., Michelis, M. A., Wormser, G. P., 1982, Opportunistic infection in previously healthy women. Initial manifestations of a community acquired cellular immunodeficiency, *Ann. Intern. Med.* **97**:533–539.
97. Plot, P., Quinn, T. C., Taelman, H., *et al.*, 1984, Acquired immunodeficiency syndrome in a heterosexual population in Zaire, *Lancet* **2**:65–69.
98. Poon, M. C., Landay, A., Prasthofer, E. F., and Stagao, S., 1983, Acquired immunodeficiency syndrome with *P. carinii* and *M. avium-intracellulare* infection in a previously healthy patient with classical hemophilia, *Ann. Intern. Med.* **98**:287–290.
99. Landesman, S. H., Ginzburg, H. M., and Weiss, S. H., 1985, The AIDS epidemic. *N. Engl. J. Med.* **312**:521–525.
100. Clumeck, N., Sonnet, J., Taelman, H., *et al.*, 1984, Acquired immunodeficiency syndrome in African patients, *N. Engl. J. Med.* **310**:492–497.
101. Pitchenik, A. E., Fischi, M. A., Dickinson, G. M., Becker, D. M., Fournier, A. M., O'Conell, M. T., Colton, R. M., and Spira, T. J., 1983, Opportunistic infections and Kaposi's sarcoma amongst Haitians: Evidence of a new acquired immunodeficiency state, *Ann. Intern. Med.* **98**:277–284.
102. Pitchenik, A. E., Russell, B. W., Cleary, T., *et al.*, 1982, The prevalence of tuberculosis and drug resistance among Haitians, *N. Engl. J. Med.* **307**:162–165.

103. Pitchenik, A. E., and Rubinson, H. A., 1985, The radiographic appearance of tuberculosis in patients with acquired immune deficiency syndrome (AIDS) and pre-AIDS, *Am. Rev. Respir. Dis.* **131**:393–396.
104. Roth, R. J., Owen, R. L., and Keren, D. F., 1983, AIDS with *M. avium-intracellulare* lesions resembling those of Whipple's Disease, *N. Engl. J. Med.* **309**:1234–1235.
105. Merkel, R. S., 1984, Paratuberculosis, *Microbiol. Ser.* **15**:1237–1250.
106. Malebranche, R., Arnoux, E.. Guerin, J. M., Pierre, G. D., Laroche, A. C., 1983, Acquired immunodeficiency syndrome with severe gastrointestinal manifestations in Haiti, *Lancet* **2**:873–877.
107. Caya, J. G.. Cohen, E. B., Allendorph, M. M., Hogan, W. J., Henderson, L. A., Reedy, M. T., and Pelligrini, J., 1984, Atypical mycobacterial and cytomegalovirus infection of the duodenum in a patient with acquired immunodeficiency syndrome: Endoscopic and histopathologic appearance, *Wisc. Med. J.* **83**:33–36.
108. Hopewell, P. C., and Luce, J. M., 1985, Pulmonary involvement in the acquired immunodeficiency syndrome, *Chest* **87**:104–112.
109. Nash, G., and Fligiel, S., 1984, Pathologic features of the lung in the acquired immune deficiency syndrome (AIDS): An autopsy study of seventeen homosexual males, *Am. J. Clin. Pathol.* **81**:6–12.
110. Osborne, B. M., Guarda, L. A., and Butler, J. J., 1984, Bone marrow biopsies in patients with the acquired immunodeficiency syndrome, *Hum. Pathol.* **15**:1048–1053.
111. Graham, B. S., Hinson, M. V., Bennett, S. R., Gregory, D. W., Schaffner, W., 1984, Acid-fast bacilli on buffy coat smears in the acquired immunodeficiency syndrome: A lesson from Hansen's bacillus, *South. Med. J.* **77**:246–248.
112. Armstrong, D., Gold, J. W., Dryjanski, J., *et al.*, 1985, Treatment of infections in patients with the acquired immunodeficiency syndrome, *Ann. Intern. Med.* **103**:733–743.
113. Wong, B., Edwards, F. F., Kiehn, T. E., *et al.*, 1985, Continuous high-grade *M. avium-intracellulare* bacteremia in patients with the acquired immune deficiency syndrome, *Am. J. Med.* **78**:35–40.
114. Seshi, B., 1985, Two cases of AIDS with florid *M. avium-intracellulare* infection in the T-cell areas of the spleen, *Hum. Pathol.* **16**:964–965.
115. Cole, G. W., and Gebhard, J., 1979, *M. avium* infection of the skin resembling lepromatous leprosy, *Br. J. Dermatol.* **101**:71–74.
116. Bloom, B. R., and Mehra, V., 1984, Immunological unresponsiveness in leprosy, *Immunol. Rev.* **80**:5–28.
117. Sinkovics, J. G., Gyorkey, F., Melnick, J. L., and Gyorkey, P., 1984, Acquired immune deficiency syndrome (AIDS): Speculations about its etiology and comparative immunology, *Rev. Infect. Dis.* **6**:745–760.
118. Masur, H., 1982, *Mycobacterium avium-intracellulare:* Another scourge for individuals with acquired immunodeficiency syndrome. *JAMA* **248**:3013.
119. Thorel, M-T., and David, H. L., 1984, Specific surface antigens of SMT variants of *M. avium, Infect. Immun.* **43**:438–439.
120. Strikland, N. H., 1985, The influence of immunosuppression and immunodeficiency on infections with leprosy and tuberculosis, *Int. J. Lepr.* **53**:886–100.
121. Lane, H. C., Masur, H., Edgar, L. C., *et al.*, 1983, Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome, *N. Engl. J. Med.* **309**:453–458.
122. Watson, S. R., and Collins, F. M., 1981, The specificity of suppressor T-cells induced induced by chronic *M. avium* infection in mice, *Clin. Exp. Immunol.* **43**:10–19.
123. Mosier, D. E., Yetter, R. A., and Morse, H. C., 1985, Retroviral induction of acute

lymphoproliferative disease and profound immunosuppression in adult C57BL/6 mice, *J. Exp. Med.* **161**:766–784.
124. Singer, A., and Shearer, G. M., 1986, AIDS therapy by blocking CD4+ cells, *Nature (Lond.)* **320**:113.
125. Anonymous, 1986, Diagnosis and management of mycobacterial infection and disease in persons with human T-lymphotrophic virus type III/Lymphadenopathy-associated virus infection, *MMWR* **35**:448–452.
126. Polis, M. A., and Tuazon, C. V., 1985, Clues to early diagnosis of *M. avium-intracellulare* infection in patients with acquired immunodeficiency syndrome, *Arch. Pathol. Lab. Med.* **109**:465–466.
127. Brandt, E. N., 1984, AIDS research: Charting new directions, *Public Health Rep.* **99**:433–435.

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