The Antigen 85 Complex: a Major Secretion Product of Mycobacterium tuberculosis

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INTRODUCTION

The mycobacterial cell contains a large number of different proteins, which are beginning to be identified in considerable detail. To describe and study these proteins, it is useful to classify them into major groups with common features, e.g., in physical and chemical properties, function, or localization as exemplified by lipoproteins, heat shock proteins, and cytoplasmic, membrane-bound, and actively secreted proteins, respectively.

The group of secreted proteins is of great current interest in relation to the immune response to infection since these proteins are candidates of particular importance for development of protective immunity as well as clinical symptoms and complications of the disease.

The proteins of the antigen 85 complex are major secretion products of Mycobacterium tuberculosis and Mycobacterium bovis BCG and have been studied by independent investigators for at least three decades. The major findings by these investigators were not considered to be related until recently. In this review this antigen complex is considered as an important example of the secreted mycobacterial proteins in relation to their particular role in the interaction between the bacilli and the infected host.

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Table 1 provides information about the components of the antigen 85 complex and compares them with a series of other antigens of M. tuberculosis and M. bovis BCG that are classified into major groups.

The nomenclature of mycobacterial antigens is complicated, and various designations are in current use for individual proteins (Table 1). Precise identification of individual proteins is necessary, often needing a set of criteria. Molecular mass, shown in the first column, is often used but is clearly insufficient as a single criterion. Available molecular mass standards often provide inconsistent results, implying that an individual protein has been given different molecular masses in different laboratories. The 12-kDa heat shock protein illustrates this. It was initially designated BCG-a (67) and independently as MPT57 and assigned a mass of 12 kDa (70). Baird et al. (12) referred to it as the 10-kDa antigen of M. tuberculosis, and this term is often used today. The molecular mass of an individual protein may also vary among different species of mycobacteria; in this case the corresponding protein is 14 kDa in Mycobacterium leprae (121). Until final characterization is provided by cloning of the gene, a combination of molecular mass, immunological specificity, and N-terminal amino acid sequence determination should provide consistent and sufficiently precise identification in most instances. This principle is followed in...
Table 1, which provides information on reference antibodies for several of the components.

Secreted proteins are listed according to their MPB and MPT numbers. The term MPB was introduced by Nagai et al. (69) for designation of a protein purified from \textit{M. bovis} BCG, with a number denoting its relative mobility in polyacrylamide gel electrophoresis (PAGE) (7.7% acrylamide) at a running pH of 9.5 by the method of Davis. The corresponding term MPT is used to denote a protein isolated from \textit{M. tuberculosis}. In the proteins examined so far, no difference at the N-terminal amino acid sequence level has been demonstrated between a given protein isolated from these two mycobacterial species.

The localization index is an expression of secretion efficiency (103). Cytoplasmic proteins should, by definition, have a localization index of zero. The heat shock proteins are cytoplasmic proteins in \textit{M. tuberculosis}, and the values given are those determined in a particular \textit{M. tuberculosis} culture fluid with minimal bacterial lysis (103).

The last column in Table 1 shows the five N-terminal amino acids, conveniently used for identification of isolated proteins. In closely related proteins, distinct differences may appear only farther along the polypeptide chain, as illustrated and described further below for the antigen 85 complex.

### Heat Shock Proteins

Heat shock proteins were first described and later named to reflect their production by cells exposed to sudden elevations in temperature (97). It is now apparent that heat shock proteins are induced in virtually all kinds of cells during stress and enable the cells to survive otherwise unfavorable conditions (74). Although cellular stress increases the synthesis of heat shock proteins, many are also constitutively expressed and play an essential role in normal cell function.

Heat shock proteins are classified according to molecular weight into families termed Hsp90, Hsp70, Hsp60, and small Hsps (63); they function by associating with, and functionally influencing, other proteins. Several recent reviews deal extensively with these proteins, some concentrating on those of mycobacteria (95, 118) and some dealing in more general terms (43, 60, 63). Only limited information is available on heat shock proteins belonging to the Hsp90 family in mycobacteria (118); however, proteins of the three other families in mycobacteria have been extensively studied and are listed in Table 1.

A prominent characteristic of heat shock proteins is the highly conserved nature, with extensive sequence homology between different species (55, 95, 118). The bacterial "common antigen" originally discovered as a protein antigen of \textit{Escherichia coli} that cross-reacted with an antigen in more than 50 other bacterial species in gel precipitation experiments (50, 56) was later shown to correspond to the 65-kDa heat shock protein of \textit{M. tuberculosis} (95). Homologous proteins are also present in eukaryotes, and the human homolog has 40 to 50% sequence identity with the \textit{M. tuberculosis} 65-kDa protein and a further 20% conservative replacements (55).

This 65-kDa protein in \textit{M. tuberculosis} and related mycobacteria has been extensively studied by using a series of monoclonal antibodies defining at least 12 different antibody-
reactive epitopes along the surface of the molecule (18, 39, 66, 95, 96). Several of these monoclonal antibodies cross-react with different species of mycobacteria. Others are apparently species specific, particularly those that react with *M. leprae* (18), whereas monoclonal antibodies with high specificity for the 65-kDa protein of *M. tuberculosis* also react with the protein in the other species of the *M. tuberculosis* complex.

**Lipoproteins**

Young and Garbe (120) observed that a significant amount (but less than 5%) of the total protein in soluble extracts of *M. tuberculosis* was recovered in the detergent phase during Triton X-114 phase separation. Two-dimensional electrophoresis and autoradiography following metabolic labeling with [3H]palmitate indicated that four antigens with subunit molecular masses of 38, 27, 26 and 19 kDa are lipoproteins. Few data are available on the 27- and 26-kDa proteins, and only the other two are included in Table 1.

The 38-kDa protein is one of the most intensively studied mycobacterial antigens. Early on, it was designated as antigen 5 in the U.S.-Japan reference system, which is based on immunoelectrophoresis (54), and later it was studied under different designations by independent investigators, largely without exchange of reagents. The identity of these antigens was then demonstrated by crossed immunoelectrophoresis (CIE) (44, 101).

The cloned gene (6) contains a signal sequence that is characteristic of proteins actively secreted from mycobacteria. Young and Garbe (120) pointed out that the sequence contains a lipoprotein consensus element, suggesting that the N-terminal cysteine is acetylated with a lipid tail that is probably responsible for partial attachment of the protein to the lipid-rich mycobacterial surface. This feature would also explain the blocked N terminus and the earlier failure to sequence the protein isolated from culture fluids.

The 38-kDa protein is a major constituent of *M. tuberculosis* culture fluid. It is also present in *M. bovis* BCG culture fluid, but in far lower concentrations (44, 101). Thus, it is at least a quantitatively specific antigen of *M. tuberculosis* compared with *M. bovis* BCG. Extensive experiments with polyclonal and monoclonal antibodies have failed to demonstrate a cross-reacting protein in other species of mycobacteria. Antibodies to the protein occur in a high percentage of patients with tuberculosis, showing high specificity for tuberculosis when tested at the epitope level in inhibition assays based on monoclonal antibodies and in enzyme-linked immunosorbent assays (ELISAs) with intact protein of high purity as summarized in extensive reviews (21, 44).

The deduced amino acid sequence of the cloned gene shows about 30% homology with the PhoS protein of *E. coli* and 51% homology when conservative substitutions are considered (6). PhoS is essential for phosphate transport in *E. coli*. Homologous proteins are therefore also expected to occur in other mycobacteria, although this has not yet been conclusively demonstrated outside the *M. tuberculosis* complex. Phosphate starvation during culture enhances the expression of the protein, which has been directly demonstrated by immunogold electron microscopy to be located mainly in the wall and on the surface of the bacterial cell (30).

The gene encoding the 19-kDa lipoprotein (11) contains an open reading frame coding for a 159-amino-acid protein with a calculated molecular mass of 15.1 kDa. The gene contains a signal sequence and a lipoprotein consensus element (120), indicating that cysteine 22 is the N-terminal amino acid in the mature protein. Attachment of a lipid tail to this acylated cysteine residue would explain the difference between the calculated and observed molecular masses of the mature protein.

**Secreted Proteins**

Nine proteins are listed in Table 1 according to their MPT numbers and other designations under this common heading. Differentiation between antigens that are actively secreted from the mycobacterial cell and intracellular antigens released during the autolytic process during culture has met with considerable difficulty in the past, although these two groups are conceptually quite distinct. The use of marker enzymes such as isocitrate dehydrogenase (9) or other cytoplasmic marker proteins with simultaneous quantification of these and other individual proteins in early culture fluids and sonicates of the washed bacilli (47, 103) has been essential in defining this group of proteins. The genes of five of them have been cloned, as shown in Table 1; all of them contain a typical signal sequence.

This review concerns in particular the components of the antigen 85 complex as main constituents of the group. In fact, it is now appreciated that these proteins have been studied for more than three decades and illustrate essential aspects of the immune response after mycobacterial infection and the current emphasis on the role of secreted proteins in development of protective immunity and in complications of the disease.

**Enzymes**

Of the large group of mycobacterial enzymes, two are included in Table 1. For both, the genes have been cloned.

On the basis of sequence homology and functional analysis, Andersen et al. (5) demonstrated that a 40-kDa protein from *M. tuberculosis* was an L-alanine dehydrogenase (EC 1.4.1.1). Although no consensus signal sequence was identified in the gene, the enzyme was found in early culture fluids at a time of minimal bacterial lysis, indicating that the enzyme is transported across the cell membrane.

The same feature has been demonstrated for the 23-kDa superoxide dismutase (93, 122). We expect, therefore, that signal peptide-independent transport mechanisms across the cell membrane and lipid-rich cell wall exist in mycobacteria, analogous to the family of closely related ATP-binding proteins associated with membrane transport in several bacterial species (49). Extensive homology has been demonstrated at both the gene and amino acid levels among the members of this protein family, although each is specific for a different substrate. In none of these cases does the transported polypeptide have a typical hydrophobic signal sequence.

Young et al. (121) have recently provided a compilation of mycobacterial antigens with extensive referencing.

**EFFICACY OF LIVE AND DEAD MYCOBACTERIAL VACCINES**

Bloch and Segal (15) studied the effect of various vaccines on the survival time of mice after challenge with virulent *M. tuberculosis* and found that phenol-killed *M. bovis* BCG was less effective than live BCG. The nonmultiplying and avirulent *M. tuberculosis* H37Ra vaccine was slightly but consistently superior to the multiplying BCG vaccine. These
observations have been confirmed by several independent authors (117). Bloch and Segal concluded that the immunity achieved by use of killed organisms was low and did not stand comparison with the vaccination effect of a comparable dose of live organisms of the same strain; they also concluded that the immunizing effect did not require multiplication of the bacilli in vivo. These conclusions still appear to be valid, although the field has been beset by conflicting views for several decades.

Comparing different strains of BCG, Dubos et al. (27–29) found that the immunizing capacity of BCG was related to the extent of its multiplication in vivo after inoculation. Considering these findings, Weiss (100) emphasized that after injection of similar numbers of live and killed attenuated mycobacterial cells into experimental animals, the living cells multiply for a sufficiently long time to produce far more immunizing substance than could be present in the dead cells. However, Youmans (117) confirmed the findings of Bloch and Segal (15) with M. tuberculosis H37Ra. When the respective dose-response curves for killed (by heat, acetone, or phenol) and live M. tuberculosis H37Ra cells were compared, living cells appeared to be significantly more effective. It was possible to obtain a protective immune response with dead cells equivalent to that produced by living cells only when the immunizing dose of dead cells was several hundred times larger. As anticipated from the findings of Bloch and Segal (15), these experiments clearly show that the difference between live and dead vaccine is not related to different amounts of bacilli, as suggested by Weiss (100), but that it is qualitative.

The finding of Dubos et al. (29) that the effect of BCG correlates with in vivo multiplication is not necessarily in conflict with the finding of Bloch and Segal (15) that nonmultiplying M. tuberculosis H37Ra is as effective as multiplying M. bovis BCG. In this respect it may be important that M. tuberculosis has a longer generation time than BCG. Other differences, such as the pattern and quantity of expressed antigens, may also account for the efficacy of nonmultiplying M. tuberculosis H37Ra. In an attempt to unify the findings of Bloch and Segal (15) and of Dubos et al. (27–29), it is suggested that the similar vaccination effect of BCG and H37Ra is due to a similar product of the number of bacilli and the bacillary survival time. Bacillary multiplication thus affects this relationship by giving rise to a larger number of bacilli.

It is of great interest to note that Bloch and Segal (15) stated, "Acquired immunity in tuberculosis is based on the ability of the host to live in a state of equilibrium with the tubercle bacillus rather than on its power to eradicate the bacillus from its organism. Under natural conditions in man, this ability is often acquired during a minimal infection in which the host acquires the ability to check the infection at the same rate as the bacillus multiply. Under experimental conditions, it can be shown that virulent tubercle bacilli often survive in the animal for long periods of time without causing manifest disease. It is suggested that, during this phase in which the bacilli survive in a resting stage without increasing continuously in number, they produce those antigens which are concerned in causing this type of immunity. It is conceivable, but not postulated, that the resting stage is essential for the production of these antigens."

INITIAL REPORTS OF SECRETED ANTIGENS

In a series of reports Fukui and Yoneda (34, 112, 113) described the purification of antigens from M. tuberculosis H37Rv culture fluid. In 1965 more refined results were presented on the alpha and beta antigens of M. tuberculosis H37Rv (33). The alpha antigen corresponds to one or more of the antigen 85 components, most probably antigen 85A or 85B or both (102). The identity of the beta antigen is uncertain. By careful immunoprecipitation, they calculated that alpha antigen constituted 41% of the protein in the unheated culture fluid while beta antigen made up 28%. Assuming that the antisera were sufficiently specific, these high percentages indicate that the culture fluids contained almost exclusively secreted antigens. The content of alpha and beta antigens in the supernatant of disintegrated washed cells was not exactly determined by quantitative immunoprecipitation. However, by rough calculations it was estimated that they constituted less than 1% of this protein, confirming their secreted nature. For further characterization, the antisera were absorbed with thoroughly washed bacterial cells. No detectable amounts of antibodies reacting with alpha or beta antigens remained in the absorbed serum. The absorption was specific in that anti-bovine serum albumin activity was not removed in a control experiment. The heat lability of alpha and beta antigens was demonstrated by exposing the cells to 80°C for 30 min. After being heated, the cells did not absorb the antibody activities. Furthermore, alpha and beta antigens were liberated from thoroughly washed cells by agitation for a short period (3 to 5 minutes) and were detected in the supernatant after removal of the cells. DNA and RNA were not detected in the supernatant.

The last experiments demonstrated that alpha and beta antigens are released from washed intact bacterial cells during a brief incubation is particularly interesting in view of the position of Bloch and Segal (15), indicating that secreted antigens are released from cells which are not in the division cycle.

The work of Yoneda and Fukui (33, 34, 112–114), which is only occasionally referred to in current literature, provides a clue to why a live mycobacterial vaccine is far more efficient than a dead one. However, the connection between the work of Bloch and Segal and that of Yoneda and Fukui was apparently not recognized for several decades. This connection was neglected partly because Youmans (116, 117) maintained that the live bovine tubercle bacillus vaccine could be explained by a labile factor which could be isolated from live cells. In our view this "metabolic antigen concept" would in current terminology correspond closely to the concept that secreted antigens are of particular importance for development of protective immunity. Large amounts of this group of antigens would be expected to be released from actively dividing bacilli following inoculation, whereas washed dead bacilli would contain far less of these antigens to stimulate the immune system.

RECENT DEVELOPMENTS

To our knowledge, only little information on secreted mycobacterial antigens appeared in the subsequent literature until Nagai et al. (69) purified the skin test-reactive antigen MPB70. It constituted more than 10% of the total protein in culture fluid after growth of M. bovis BCG substrain Tokyo on the wholly synthetic Sauton medium. Nagai et al. concluded that "MPB70 is probably not an autolysed protein, but one secreted into the culture medium as a natural product when the cells are growing actively." The quantitative dominance of MPB70 in the culture fluid compared with the sonicate of washed bacilli was further established by Harboe and Nagai (42), using CIE.
To avoid bacterial lysis and release of intracellular antigens, an alternative method for identification of secreted antigens was applied; this involved using metabolic labeling of proteins with $^{35}$S]methionine in short-term cultures of exponentially multiplying mycobacteria (3). MPB70 and MPB64 were subsequently identified as secreted antigens of BCG by this method (1), and the genes coding for these two proteins have typical signal sequences. By contrast, the monoclonal antibodies L7 (17), D2D (119), and SA12 (67) are directed against intracellular antigens as judged by the lack of a signal peptide-encoding segment in their corresponding genes (12, 35, 103, 111, 122). Abu-Zeid et al. (4) tested these monoclonal antibodies for identification of secreted antigens in M. tuberculosis by using the metabolic labeling approach in short-term cultures and found them to be positive. The demonstration of intracellular antigens in short-term culture fluids may indicate that there is low-grade ongoing bacterial lysis even during the exponential growth phase, which results in the release into the culture medium of intracellular antigens available for detection by highly sensitive and specific methods. Some intracellular proteins may also be released from living cells by other mechanisms than the conventional signal peptide-mediated one, as demonstrated for superoxide dismutase and discussed in relation to Table 1 (93, 122).

When intracellular proteins are released into the culture medium from dead cells, they are probably exposed to proteolytic enzymes. Intracellular antigens detected in early culture fluids may be inherently resistant to such proteolytic degradation and may thereby accumulate in parallel with the increase in bacterial mass. Antigens which are readily degraded, such as antigen 82 (the 65-kDa heat shock protein), are detected at a later stage when lysis becomes more prominent, as shown by Wiker et al. (103). The presence of antigens in log-phase mycobacterial cultures is thus not an appropriate single criterion for identification of secreted antigens, and proper identification of true secreted antigens is most reliably obtained by simultaneous specific quantitation in sonicates of washed bacterial cells and the corresponding culture fluid (47, 103). When quantifying soluble antigen from disintegrated washed cells, it is important to ensure that the washing procedure is carried out very carefully. If this is not taken care of, too many antigens will appear to be secreted (103).

The proteins of the antigen 85 complex represent a major part of the secreted proteins of M. tuberculosis and are certainly the most intensively studied. They are therefore used to illustrate important features of this group of mycobacterial antigens.

**RELATIONSHIP OF ANTIGEN 85 COMPLEX WITH ANTIGENS DESCRIBED BY OTHER INVESTIGATORS**

The antigen 85 complex consists of three internally cross-reacting antigens encoded by three genes located at separate sites in the mycobacterial genome (20, 107). It has not been possible to identify precisely which of these Yoneda and Fukui worked with, but the purified M. tuberculosis alpha antigen from recent years (89) identifies with antigen 85B in two-dimensional PAGE (107) performed by the method of O'Farrell. Antigen 6 of the U.S.-Japan reference system in immunoelectrophoresis (54) also corresponds to antigen 85 (101). More recently, purified antigen 6 has identified with antigen 85B in tandem CIE (82). The P32 antigen purified from BCG was identified as corresponding to antigen 85A by tandem CIE (25). In Nagai's terminology, MPT44/MPB44, MPT59/MPB59, and MPT45/MPB45 correspond to antigens 85A, 85B, and 85C, respectively.

**IMMUNOPRECIPITATION STUDY OF BEHAVIOR OF ANTIGEN 85 COMPLEX**

Antigen 85 was selected for further study on the basis of observations in the CIE reference system for BCG antigens (19). Most of the BCG culture fluid antigens could also be identified in sonicates and cell pressates of washed BCG bacilli. Antigen 85 behaved differently. It gave a major precipitate line in the CIE pattern of culture fluid, but this precipitate was barely or not detectable in extracts of washed bacilli. Furthermore, it did not form a typical symmetric bell-shaped precipitate line characteristic of a homogeneous component. Instead, the precipitate line showed internal spurs with three descending anodic legs. Other patterns of the antigen 85 complex may also be observed in CIE. The pattern formed depends on the specificity of the antibodies in the top gel and may also be influenced by the quantitative relationship between the individual components in the particular culture fluid. Frequently, the antigen complex forms a doubly-peaked precipitate line with a spur in the middle (104). When antibodies raised by immunization with purified components are used, the individual components are better resolved (102). The heterogeneous pattern formed by the antigen 85 complex in CIE is characteristic, making it easily recognizable in CIE of mycobacterial antigens published by other investigators.

By double diffusion in gel, purified antigen 85A and antigen 85B gave a pattern of partial identity with spur formation (102). It is a true spur because the two antigens have very similar molecular weights.

Similar observations have occasionally been published, but without further attempts to pursue them. Initial crude antigen fractions of M. tuberculosis culture fluid with the alpha antigen as the major component were further purified by DEAE-cellulose column chromatography (34). The fractions were heterogeneous, giving three peaks in the optical-density chromatogram. Double diffusion in gel indicated that the first eluted peak contained antigens showing partial as well as complete identity with the alpha antigen in the major peak. Kniker and LaBorde (61) also found that two antigens that eluted in different fractions after DEAE-cellulose column chromatography showed partial identity in double diffusion.

**APPEARANCE OF ANTIGEN 85 COMPLEX IN SDS-PAGE AND STRUCTURAL FEATURES**

The antigen 85 complex is often referred to as the 30/31-kDa doublet, and slightly different molecular mass designations have been used. In our hands the molecular masses of the individual components of the antigen 85 complex as determined by sodium dodecyl sulfate (SDS)-PAGE are as follows: antigen 85B, 30 kDa; antigen 85A, 31 kDa; antigen 85C, 31.5 kDa. Antigen 85C appears to be slightly heavier than antigen 85A, and in most SDS-PAGE runs these two components are not properly resolved. Thus, the pattern in Western immunoblotting usually reveals a doublet in the 30-kDa region, as exemplified by using the monoclonal antibody HYT27 (86) which reacts with all three components of the complex (104, 106). The components are clearly resolved in isoelectric focusing, providing three distinct bands (99).

We have recently studied a fourth protein purified from M.
tuberculosis H37Rv culture fluid (70), termed MPT51 from its relative mobility in PAGE. This 27-kDa antigen revealed 60% homology with antigen 85B in the N-terminal amino acid sequence. Polyclonal rabbit antibodies raised by immunization with highly purified MPT51 cross-reacted extensively with the antigen 85 complex in Western blotting. Cross-reactivity was also observed with MPT64, which showed significant but lower homology. In addition, a striking internal homology was demonstrated between two different stretches within the 85B sequence and indicated between three stretches within the MPT64 molecule. Similar internal homologies have been essential in defining other protein families. Thus, a family of at least four secreted proteins with common structural features has been found in mycobacteria. MPT64 may also belong to this family (106).

As evaluated by CIE, most of the precipitable antigens disappear from the pattern when mycobacterial culture fluids are heated to 120°C (40). In a study of the denaturation of mycobacterial proteins during preparation of tuberculin purified protein derivative (PPD), radiolabeled purified antigen 85B was added to unlabeled BCG culture fluid, heated at 120°C for 30 min, centrifuged, and analyzed by SDS-PAGE and autoradiography (46). The characteristic pattern of BCG culture fluid with a series of distinct, sharp bands after protein staining disappeared on heating. A small fraction of radioactive material remained in the supernatant as soluble material, while most of the radioactivity was recovered in the insoluble pellet. On SDS-PAGE with autoradiography, the soluble fraction gave a band at about 10 kDa and the insoluble fraction revealed the intact molecule, degraded components, aggregated material remaining in the application gel, and a smear. The heat-stable antigen MPB70 (42) was used for comparison. The major fraction of MPB70 was found as an intact molecule in the supernatant and as a distinct but weaker band at lower molecular mass. No significant radioactivity was found in the insoluble pellet fraction. MPB70 is thus a heat-stable protein antigen, in contrast to antigen 85 complex, which consists of heat-labile proteins.

SPECIES-SPECIFIC AND CROSS-REACTIVE EPITOPES OF ANTIGEN 85 COMPLEX IN VARIOUS MYCOBACTERIA

Antigen cross-reactive with the antigen 85 complex of M. tuberculosis and M. bovis BCG has been found in all mycobacterial species tested so far by double-diffusion precipitation tests in gel (22, 89–91, 114), CIE (41), ELISA (104), and Western blotting (7, 24, 26, 76).

In most of these studies, it is not possible to determine with certainty which components of the antigen 85 complex are present as cross-reactive antigen. By isoelectric focusing and Western blotting with a panel of nine monoclonal antibodies to BCG antigen 85, Drowart et al. (26) detected distinct components probably corresponding to antigens 85A, 85B, and 85C in five of eight mycobacterial species tested. Only limited attempts have been made to demonstrate antigen 85-related antigen in bacteria outside the genus Mycobacterium (41).

Species-specific epitopes have been found on cross-reactive antigen 85 from several mycobacterial species. This was first reported by Yoneda et al. (115). Daniel and de Muth (22) found that antigen 6 from M. tuberculosis and Mycobacterium szulgai contained species-specific epitopes. In two studies Tasaka et al. (90, 91) raised antisera to alpha antigen from Mycobacterium avium-intracellulare, Mycobacterium scrofulaceum, Mycobacterium gordonae, M. szulgai, Mycobacterium kansasi, and Mycobacterium marinum. Species-specific epitopes were found in all of these species. The various polyclonal antisera were rendered species specific by absorption with partially purified alpha antigen from M. tuberculosis and could subsequently be used for identification of these mycobacterial species. In view of the heterogeneity and cross-reactivity within the antigen 85 complex, it is mandatory that future studies of this kind include strict controls to ascertain that corresponding proteins are compared and that apparently species-specific epitopes are not present on related proteins in the other species in question.

Different monoclonal antibodies to antigen 85 of M. tuberculosis and BCG show a striking variation in reaction patterns when tested with other species of mycobacteria (26, 76), and a high species specificity of new monoclonal antibodies is certainly expected to be obtained provided that adequate selection procedures are applied after fusion and generation of clones. Rambukkana et al. (76) have recently demonstrated that a mouse monoclonal antibody to the 30-kDa region protein of M. tuberculosis also reacts with a related 27-kDa protein (probably corresponding to MPT51) with specificity for the M. tuberculosis complex in the latter reaction.

LOCALIZATION OF ANTIGEN 85

The antigen 85 complex is the major secreted protein constituent of mycobacterial culture fluids, but it is also found in association with the bacterial surface.

The antigen 85 complex is the quantitatively major secreted constituent during culture of mycobacteria on the synthetic, liquid Sauton medium. By quantitative assays which did not discriminate among the individual components, Fukui et al. (33) found that they made up 41% of M. tuberculosis H37Rv culture fluid protein. De Bruyn et al. (24) determined by a dot blot assay that these proteins constituted 14 to 15% of the total protein in M. bovis or M. tuberculosis culture fluids. The 30-kDa proteins are major constituents of 3-day-old M. tuberculosis culture fluid as judged from the autoradiography pattern of [35S]methionine-labeled proteins (4). These studies show that the antigen 85 complex is a major constituent of the culture fluid protein. The variation in the results is probably due to different degrees of bacterial lysis and contamination of the culture fluids with intracellular components, reducing the relative prominence of secreted proteins. We found that in M. tuberculosis H37Rv culture fluid obtained from a batch with minimal cell lysis, the quantitatively dominating components of the antigen 85 complex, antigens 85A and 85B, constituted about 60% of total protein and that there was slightly more antigen 85B than antigen 85A. The assays were performed by using rocket immunoelectrophoresis with purified proteins as standards (100a).

Although antigen 85 complex is a major constituent of the mycobacterial culture fluid, it is also found in association with the bacterial surface. Soluble extracts of washed disrupted cells, prepared by sonication under careful temperature control, contain only small amounts of the antigen 85 complex. However, there are various reports supporting the association of one or more of the members of the antigen 85 complex with the mycobacterial surface. Schou et al. (86) found that monoclonal antibody HYT27 gave a positive signal in ELISA with whole M. tuberculosis cells. In a publication describing the binding of purified components of the antigen 85 complex to human fibronectin (FN), Abou-
Zeid et al. (2) studied the interaction of \(^{3}H\)-labeled whole BCG cells with FN-coated plastic surfaces. Binding of BCG bacilli was completely inhibited when FN was preincubated with anti-FN antibodies or purified antigen 85A or 85B or when labeled BCG was preincubated with anti-antigen 85 antibodies. This indicates that FN-binding molecules, i.e., antigen 85 components, are associated with the bacterial surface. Recently, Rambukkana et al. (77) showed by electron microscopy with immunogold labeling that a monoclonal antibody (3A8) specific for components of the antigen 85 complex bound exclusively to the surface of \(M.\,\text{tuberculosis}\) and not to intracellular structures.

An important question relates to whether components of the antigen 85 complex are destined for the bacterial surface or as soluble proteins in the vicinity of the bacteria. The large quantities found as soluble antigen in the culture fluid favor the latter alternative, suggesting that soluble secreted antigens are only transiently associated with the bacterial surface on their way out of the cells. However, the answer is probably more complex. It is not contradictory that an antigen may be a soluble secreted antigen as well as being partly associated with the bacterial cell wall or surface, particularly when considering the heterogeneity of the antigen 85 complex. Several observations indicate that one or more members of the antigen 85 complex may have a close association with the bacterial surface or the cell wall. The localization index for antigen 85A has consistently been found to be higher than for antigen 85B (103), indicating that antigen 85B may be less efficiently secreted and more closely associated with the bacterial surface.

Young and Garbe (120) found that a 31-kDa antigen, supposedly a member of the antigen 85 complex, partitioned with detergent in phase separation experiments on soluble extracts of disintegrated \(M.\,\text{tuberculosis}\) cells. In this connection the findings of George and Falkinham (36), who analyzed cytoplasmic membranes isolated from representative strains of the \(M.\,\text{avium-M.}\,\text{intracellulare-M.}\,\text{scrofulaceum}\) (MAIS) group, are of interest. A protein with a molecular mass of 31 kDa predominated, constituting up to 50% of the total membrane protein, and was designated the major cytoplasmic membrane protein. Their immunoblots revealed a doublet, indicating that this may be the antigen 85 complex of MAIS, although additional antibody cross-reactivity data or protein or nucleotide sequence comparisons are needed to substantiate this view. There is also one report (13) indicating that at least one member of the antigen 85 complex, or some cross-reactive peptide, is part of the protein-peptidoglycan complex (PPC). PPC was purified by a vigorous extraction procedure, designed to remove all soluble constituents from the high-molecular-weight PPC. A T-cell clone which proliferated in response to PPC also reacted with a 30-kDa fraction prepared from nitrocellulose blots of SDS-PAGE-separated mycobacterial antigen. The antigen fraction was positive for antigen 85 when reacted with anti-antigen 85 antibodies. Further studies with purified antigens of the antigen 85 complex are needed to establish whether proteins of this complex or peptides thereof are linked to the PPC. Western blotting is not reliable as the sole technique for identification of these antigens. An illustrative example was presented by Worsaae et al. (108). The monoclonal antibody HBT7 reacted with a molecule having the same position as HYT27-defined antigen (the antigen 85 complex). However, HBT7 did not bind to the affinity-purified antigen 85 complex.

It is essential to work with isolated proteins purified to homogeneity and precisely identified. Additional complexity will affect studies of this kind, as exemplified by the demonstration by Rambukkana et al. of 30-kDa proteins that were present at different relative amounts in the cytosol and culture fluid compartments and of unexpected cross-reactions between monoclonal anti-30-kDa protein antibodies and the 65-kDa heat shock protein (77).

**FUNCTION AND ROLE OF FIBRONECTIN BINDING**

Human FN binds readily to purified antigen 85 components (2). As judged from Western blotting, FN binds more strongly to antigen 85A than to 85B. Soluble antigen 85A is a better inhibitor than 85B of attachment of BCG to FN-coated surfaces. Monoclonal antibody HYT27 does not appear to react with the relevant site for FN binding.

Efficient release of antigen 85 from bacilli may inhibit FN binding to bacilli in two ways. First, released antigen 85 can bind to FN, resulting in “inactivation” of FN before it is bound to the bacillary surface. Second, FN bound to antigen 85 at the surface may subsequently be released by secondary release of antigen 85. For the latter mechanism to be valid, it must be determined whether antigen 85 is permanently fixed to or only transiently associated with the bacillary surface. The release of antigen 85 into culture medium shows that antigen 85 is transiently associated with the surface. However, a fraction may be permanently fixed, making it possible for FN to bind “irreversibly” to bacilli. In treatment of bladder carcinoma with BCG, FN binding appears to play an important role in attachment of the bacilli to the bladder epithelium (78).

In general, intracellular parasites evade extracellular host defense mechanisms by intracellular habitation. Apparently it would be advantageous for such parasites to enter cells quickly after infection, and the mechanism by which they do so is of great interest. Recently it has been shown that complement receptors mediate ingestion of \(M.\,\text{tuberculosis}\) and \(M.\,\text{leprae}\) by host cells (84, 85). Is it, however, possible that FN plays a role in phagocytosis of mycobacteria? There is little support for a direct mechanism in which FN mediates ingestion of bacilli via FN receptors, because it is unclear whether FN is an opsonin. Another more indirect role of FN in phagocytosis has some support in the literature. FN enables monocytes to phagocytose C3b- or immunoglobulin G (IgG)-sensitized erythrocytes without binding to the opsonized particles (75). This mechanism may be relevant and is of great interest in relation to the observations of Schlesinger et al. (84, 85). When FN is bound to gelatin, it activates complement receptors, enhancing complement-mediated phagocytosis more strongly than does free FN (109), which may suggest a possible role for antigen 85 in phagocytosis of mycobacteria if antigen 85 exerts a similar effect to gelatin.

Antibodies are not considered to play a significant role in protective immunity against mycobacterial infections. The interaction between antigen 85 and FN implies that antibodies to antigen 85 which block this interaction may affect early events and increase resistance against mycobacterial infection.

Observations by Godfrey et al. (37, 38) provide another intriguing possibility for the role of antigen 85 in the immunopathology of mycobacterial disease, which is discussed in the section on delayed-type hypersensitivity to antigen 85 (below).

Apart from FN binding, few data concerning the primary function of the antigen 85 complex proteins are available. In \(Bacillus\) species, several secreted proteins are cleavage enzymes (83), and enzyme activities such as protease, mu-
cinase, lipase, and RNase have been detected in mycobacterial culture fluids (57).

The major cytoplasmic membrane protein of MAIS organisms, which is probably related to the antigen 85 complex (36), was not detected in detergent-grown cells. Such cells also lack the normal MAIS permeability barrier (68). George and Falkinham (36) found that cells grown in medium containing Tween 80 had a long lag period following transfer to other non-Tween 80-containing mycobacterial media in which the cells grow well normally. From these data they suggested that the major cytoplasmic membrane protein may provide a porine function to supply the cells with nutrients. The requirement for this protein in normal media may thus explain the lag period after transfer from Tween 80-containing medium.

**CONSERVATION OF INDIVIDUAL COMPONENTS OF ANTIGEN 85 COMPLEX AMONG SLOW-GROWING MYCOBACTERIA**

Slow-growing mycobacteria have only one set of genes coding for rRNA (87). This fact has previously been used as an argument against the duplication of other genes in slow-growing mycobacteria. The components of the antigen 85 complex are encoded by separate genes, which probably arose by duplication and subsequent mutations (20, 107). Considering that the components of the antigen 85 complex are major secreted antigens, gene multiplication may be used in mycobacteria for proper expression of proteins which are needed in large amounts. However, the structural heterogeneity of the individual components of the antigen 85 complex is probably an expression of a corresponding functional heterogeneity. This position is supported by the apparent conservation of individual components in different mycobacterial species. Western blotting with *M. kansasii* or *M. marinum* antigen and the monoclonal antibody HYT27 (8) and with *M. kansasii*, *M. avium*, or *Mycobacterium phlei* antigen and polyclonal antibodies to antigen 85A (24) revealed double bands in the 30-kDa region of all of these species. Although immunological identity at the individual-component level cannot be determined with certainty in Western blotting, the data indicate that the heterogeneity observed in the *M. tuberculosis* antigen 85 complex is expressed in several mycobacterial species. Components immunologically related to antigens 85A and 85B have been observed in CIE of *M. kansasii* and *M. avium* (104), and components probably corresponding to 85A, 85B, and 85C have been demonstrated in six of nine mycobacterial species by isoelectric focusing followed by Western blotting (26).

In the *M. tuberculosis* antigen 85 complex, the gene for antigen 85A encodes a 294-amino-acid mature protein (16) which shows 79% homology with the deduced 285-amino-acid sequence of antigen 85B (65). The *M. kansasii* gene for antigen 85B (64) also encodes a 285-amino-acid mature protein which shows 89% homology with the BCG antigen 85B protein (Table 2). These data strongly suggest that there are greater differences between the components of the antigen 85 complex in a given species than between the individual components in different species. Recently an *M. leprae* gene coding for an FN-binding protein more closely related to 85B than 85A has been cloned (94).

**ASSAYS FOR ANTIBODIES TO ANTIGEN 85**

Antibodies to proteins of the antigen 85 complex are among those widely recognized to be present in healthy individuals. Increased formation of antibodies to these proteins after experimental (45, 105) and natural infection with *M. tuberculosis*, *M. leprae* or other mycobacteria shows that they are strongly immunogenic in vivo. This observation indicates that one or more of these proteins are released from the bacilli in vivo and that active secretion is not merely an in vitro phenomenon that appears in cultures of mycobacteria on artificial media.

**Assay Based on Incorporation of Human Sera into the Intermediate Gel in CIE**

With polyclonal antibodies in the top gel, assays based on incorporation of sera into the intermediate gel in CIE do not distinguish among antibody activities against the individual components of the antigen 85 complex. The sensitivity is also lower than in ELISA and Western blotting techniques, since precipitating antibodies are needed for positive results.

In a study of 52 patients with active tuberculosis, Kaplan and Chase (59) found 4 (8%) with antibodies to the antigen 85 complex (termed antigen 6 in their study), the precipitate legs being pulled down in the intermediate gel. In the study by Jagannath et al. (53), precipitate line 12 probably represents the antigen 85 complex as judged by its characteristic form and position. They used BCG sonicate as the antigen, which explains why the precipitate line is less dominant than is usually the case with BCG culture fluid preparations. Ten of 54 patients with tuberculosis (19%), 4 of 30 patients with tuberculoid leprosy (13%), and 10 of 30 patients with lepromatous leprosy (33%) were positive for antibodies to this antigen. As judged by the percentage of positive responders to individual antigens studied in these two reports, antibodies to the antigen 85 complex had an intermediate sensitivity in these patients. A higher percentage of responders in patients with lepromatous leprosy than in those with tuberculoid leprosy is expected from the dominating humoral immune response and high load of bacilli in the tissues of the former group of patients.

**ELISA for Antibodies to Purified Antigen 85**

In a study in Hong Kong, Stroebel et al. (88) measured IgG antibodies to affinity-purified antigen 85 complex (antigen 6) in ELISA. Patients with tuberculous infection of the hip or knee, tendosynovitis, or spondylitis were divided in two groups. Of 16 patients on therapy, 15 (94%) were positive for anti-antigen 85 antibodies with titers of 64 or more. Twenty patients in whom all therapy for tuberculosis had been discontinued for 3 months or longer, with no clinical or radiological signs of reactivation, all gave negative results. All 21 subjects in a control group of BCG-vaccinated patients with acute trauma or osteomyelitis due to other organisms than *M. tuberculosis* also gave negative results.
Less specific and less sensitive results were reported by Benjamin and Daniel (14) in a comparison of the occurrence of antibodies to the antigen 85 complex (antigen 6) in various groups. If a titer of 80 or more in serum is applied as the cutoff level, the following frequencies of positive responders were observed: 11 of 27 (41%) patients with pulmonary tuberculosis; 2 of 10 (20%) patients with M. kansasi infection; 6 of 11 (55%) patients with M. intracellulare infection; 3 of 23 (13%) patients with sarcoidosis; 4 of 23 (17%) healthy PPD+ controls; and 2 of 22 (9%) healthy PPD− controls.

Turneer et al. (98) studied IgG, IgA, and IgM antibodies to antigen 85A in 102 patients with tuberculosis and in a total of 221 tuberculin-positive and -negative control subjects. The sensitivity was 55% for IgG antibodies and 40% for IgA antibodies. The IgM antibody titers were generally low. The specificity was 95% for both the IgG and IgA antibodies. More treated than untreated patients gave positive results. Smear-positive patients with tuberculosis had higher antibody titers than smear-negative patients did, and patients with advanced tuberculosis had higher antibody titers than patients with minimal disease did.

Harboe et al. (45) used detection of antibodies to antigen 85B as a general indicator of mycobacterial infection. Antibodies to antigen 85B were detected in sera from cattle infected with M. bovis (9 of 16 animals [56%]) or Mycobacterium paratuberculosis (15 of 20 animals [75%]), in goats infected with M. avium or M. paratuberculosis, and in sheep infected with M. paratuberculosis.

These studies indicate a sensitivity of about 50% for antibodies to the antigen 85 complex. With regard to specificity, it must be considered that antigen 85 is highly cross-reactive and that positive responses are also expected in healthy controls, particularly in areas where exposure to atypical mycobacteria is common. The different degree of specificity is thus highly dependent on the kind of control subjects included. BCG vaccination does not appear to induce significant antibody production.

Another observation is also of considerable interest. Kaplan and Chase (58) found that antibodies to mycobacterial antigens increased when chemotherapy was administered against tuberculosis. Turneer et al. (98) also detected higher antibody titers in treated than untreated patients; the difference was, however, not significant. They found that antibody levels were more closely related to disease activity. The highest antibody titers were thus found in the most severely ill patients receiving chemotherapy. It may be difficult to distinguish raised antibody levels due to chemotherapy from raised antibodies due to the severity of the disease. The observation that individuals with active tuberculosis in the past are negative for anti-antigen 85 antibodies (88) has yet to be confirmed.

Antibody Response to Antigens in the 30-kDa Region in Western Immunoblotting

Rumschlag et al. (81) used Mycobacterium tuberculosis culture fluid as the antigen in their study. Antibodies to 30- and 31-kDa proteins were present in 88 and 81%, respectively, of 16 patients with lepromatous leprosy but were not detected in 16 patients with tuberculoid leprosy. Pessolani et al. (73) found that the 28- to 30-kDa protein doublet of 7-day-old BCG culture fluid was recognized by serum samples from 51 of 56 (92%) patients with polar and borderline lepromatous leprosy, whereas essentially negative results were obtained with serum samples from 33 patients with borderline tuberculoid and indeterminate leprosy, 110 household leprosy contacts, 30 sputum-positive tuberculosis patients under treatment, 27 Leprosy Department staff, and 123 randomly chosen hospitalized patients. In both studies it is suggested that antibodies to these antigen bands may be useful for distinguishing between tuberculoid and lepromatous leprosy. If this is so, it should be shown that antibody titers to these antigens give more reliable information than other criteria used to distinguish various forms of leprosy. The picture is complicated by the observation of Jagannath et al. (53) that there were positive responders to antigen 85 in the group of patients with tuberculoid leprosy. Somewhat contradictory to the results of Pessolani et al. (73) are the ELISA results showing reactivity of sera from patients with tuberculosis to antigen 85 in ELISA (see above). Espitia et al. (31) found antibodies to a 30/31-kDa doublet band in 55.9% of sera from patients with tuberculosis and in 56.5% of sera from patients with lepromatous leprosy. Reactivity with sera from healthy individuals was also observed, but judged from their immunobLOTS, the patients gave significantly stronger responses than the control subjects did. In a more quantitative assay, application of a high cutoff level is expected to considerably increase the specificity for mycobacterial infection.

An isoelectric focusing technique followed by Western blotting for assay of IgG antibodies has been reported to provide increased specificity (99). The 85A component reacted with sera from tuberculous as well as nontuberculous individuals. By contrast, the 85B and 85C components of the complex did not react with the control sera but with 20 of 28 (71%) serum samples from tuberculous patients.

Prospects for the future of anti-antigen 85 serology involve investigation of antibodies to component-specific epitopes and in particular to species-specific epitopes. Inhibition assays based on monoclonal antibodies are valuable but are critically dependent on the immunogenicity of the individual species-specific epitopes. The extensive cross-reactivity of antigen 85 in different species of mycobacteria indicates that tests of this kind will attain sufficient sensitivity since previous exposure to cross-reactive epitopes would provide ample opportunity for T-cell help. Generation and selection of suitable monoclonal antibodies would be essential for further development of tests for infection with Mycobacterium tuberculosis as well as atypical mycobacteria. A more detailed analysis of antibodies at different stages of disease is also needed.

DELAYED-TYPE HYPERSENSITIVITY AND OTHER BIOLOGIC ACTIVITIES

Yoneda and Fukui (114) found that "Considering their purity, we thought that these antigen preparations would prove to have a high grade of tuberculin activity. However, contrary to our expectation, these antigens behaved very peculiarly with respect to tuberculin activity." They found that even 100 μg of alpha and beta antigen preparations failed to elicit skin test reactions in guinea pigs sensitized with heat-killed M. tuberculosis H37Rv, whereas Old Tuberculin or PPD-S elicited strong positive tuberculin reactions in the same animals. In preliminary studies they reported that positive responses could be elicited by the purified alpha and beta antigens in guinea pigs infected with viable units of H37Rv bacilli, suggesting as an explanation that alpha and beta antigens are absent in heat-killed bacilli.

Several other independent investigations found positive skin test responses. Worsaae et al. (108) performed skin testing with the antigen 85 complex affinity purified with
monoclonal antibody HYT27. Significant skin test responses (13 to 15 mm) were observed in guinea pigs sensitized with live BCG or phenol-killed or heat-killed \textit{M. tuberculosis}. Guinea pigs sensitized with \textit{M. intracellulare}, \textit{M. kansasii}, or \textit{M. scrofulaceum} gave moderate responses (6 to 8 mm). Hashimoto et al. (48) performed skin tests with HYT27-purified antigen 85 complex on several inbred guinea pig strains sensitized with glutaraldehyde-killed \textit{M. tuberculosis}. When compared with other affinity-purified antigens, this complex was observed to have an intermediate reactivity. The responses varied considerably among the guinea pig strains. Daniel and Ferguson (23) isolated a component termed a2 (antigen 6) from \textit{M. tuberculosis} culture fluid by conventional purification methods. Positive skin test responses were obtained in guinea pigs sensitized with heat-killed \textit{M. tuberculosis}, and a2 had 20% of the potency of PPD-S. This result has been confirmed. Preliminary skin testing with recently purified \textit{M. tuberculosis} antigen 6 (82) showed that 0.5 μg of antigen 6 was bioequivalent to 0.1 μg of PPD-S. This antigen was identified as antigen 85B in tandem CIE.

Nagai et al. (70) also found significant responses in guinea pigs sensitized with heat-killed \textit{M. tuberculosis} and challenged with MPT59 (\textit{M. tuberculosis} antigen 85B). On the other hand, MPT44 (\textit{M. tuberculosis} antigen 85A) gave a negative response, which is strange considering the close structural relationship between the two components. Interestingly, De Bruyn et al. (25) also found that purified antigen 85A (P32) was negative in delayed-type hypersensitivity tests in C57BL/6 mice sensitized with live BCG and observed only weak reactions in BCG-sensitized guinea pigs. A similar explanation to that of Yoneda and Fukui (114) may be applied to the unexpected negative skin test results. A higher localization index of antigen 85A than antigen 85B (103) may indicate that not enough antigen 85A is present in killed mycobacteria to be able to sensitize the animals. However, this does not explain the findings of De Bruyn et al. (25), since the animals were sensitized with live BCG.

Results presented by Godfrey et al. (38) provide another explanation. A purified antigen preparation containing 90% antigen 85A and 10% antigen 85B inhibited skin test responses to PPD in sensitized guinea pigs by about 75%. FN produced by T cells is a potent inflammatory lymphokine which appears to play a critical role in initiating delayed-type hypersensitivity responses (37). Since purified antigen 85 components bind to plasma FN (2) and also to FN produced by T cells (38), proteins of the antigen 85 complex might inhibit delayed-type hypersensitivity responses by interacting with T-cell FN. This explanation opens an interesting new perspective into the study of immunity to mycobacterial infection. One may speculate that bacilli survive by releasing antigen 85, which inhibits induction of protective immunity locally at the infection focus by inhibiting T-cell activation.

The available results indicate that antigen 85B is a relatively good skin test reagent whereas antigen 85A is not. There is no information on antigen 85C as yet.

Although antigen 85A appears to be a poor skin test agent, positive responses in in vitro lymphoproliferation tests have been reported. Huygen et al. (52) found that 71% of 21 patients treated for active tuberculosis and all 12 tuberculin-negative healthy volunteers responded to purified antigen 85A. Twelve tuberculin-negative control subjects gave negative responses. The same study also reported significant gamma interferon (IFN-γ) responses to antigen 85A in the patient group and the tuberculin-positive controls. IFN-γ production in response to antigen 85A was also seen in cultured spleen cells from BCG-sensitized mice (51) and in peripheral blood mononuclear cells from patients with leprosy (62).

**EPILOGUE**

Other authors have also claimed that secreted antigens are of particular importance in the development of protective immunity (4, 71, 72, 80, 118). Future experiments should aim at providing direct evidence that proteins that are actively secreted in vitro are also secreted in vivo, which would be essential if they are to have a particular role in contributing to the development of protective immunity and thus to the greater efficacy of live vaccines.

Recent evidence appears particularly relevant in this regard. Andersen et al. established an infection model of human tuberculosis in C57BL/6j mice (10). The lymphoproliferative responses to antigens from \textit{M. tuberculosis} were investigated during the course of infection and compared with results obtained with a group of mice immunized with large amounts of killed bacteria. The two groups responded similarly to a number of mycobacterial antigens, but marked differences were found in their responses against secreted antigens; only mice infected with live \textit{M. tuberculosis} responded vigorously to these.

In an extension of this study (8), short-term culture filtrate, which is a complex mixture of secreted proteins, was fractionated by a modified preparative SDS-PAGE technique. The ability of each fraction to be recognized by T cells isolated from infected mice was evaluated by quantifying cellular proliferation and IFN-γ production in the cultures. Two molecular mass regions of 4 to 11 and 26 to 35 kDa were found to possess markedly stimulatory properties. Four potent single antigens were mapped within the stimulatory regions. These purified antigens stimulated T cells isolated from mice at the height of a tuberculous infection to produce large amounts of IFN-γ, which is considered to be of particular importance for increasing the ability of macrophages to inhibit the growth of or kill intracellular \textit{M. tuberculosis} (32, 79). Two of these stimulatory antigens belonged to the antigen 85 complex.

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