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Isolation and Partial Characterization of Major Protein Antigens in the Culture Fluid of *Mycobacterium tuberculosis*

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Five actively secreted proteins (MPT32, MPT45, MPT51, MPT53, and MPT63) and the MPT46 protein were purified to homogeneity from *Mycobacterium tuberculosis* culture fluid and compared with proteins previously purified by ourselves and other investigators. Antisera were obtained by immunization of rabbits with all of the newly isolated proteins identified to be immunogenic. Two-dimensional electrophoresis of culture fluids obtained each week for 2 to 10 weeks of culturing of *M. tuberculosis* revealed characteristic changes, permitting identification of two distinct groups of proteins being actively secreted from the mycobacterial cells or appearing later in the culture fluids as a result of the release of soluble proteins from the cytosol after lysis of bacteria. The N-terminal amino acid sequences of five MPTs were shown to be identical to those of proteins previously isolated by other investigators and given different designations, and five new sequences are given. These sequences and the use of the antisera may serve to identify these proteins with mycobacterial constituents isolated by other investigators. The previously identified but not isolated MPT45 protein was shown to correspond to the C component of the antigen 85 complex. The 27-kDa MPT51 protein was demonstrated to cross-react with the three components of the antigen 85 complex, and the N-terminal amino acid sequences of MPT51 and MPT59 showed 60% homology. This finding and the extensive cross-reactivity between the components of the antigen 85 complex may indicate that there is a family of closely related secreted proteins in mycobacteria.

Extension of our knowledge of immune responses after mycobacterial infections, improved understanding of their significance, and development of new methods for the diagnosis and prevention of these infections depend on work with isolated immunogenic components of the bacilli. In many instances, particularly with regard to cell-mediated immune responses, these components are required for detailed characterization of the specificity of immune responses. Many observations also indicate that isolated components are essential in basic studies for developing improved methods of vaccination against mycobacterial diseases.

Various approaches are in current use for the preparation and subsequent study of mycobacterial antigens. In the case of noncultivable mycobacteria, recombinant DNA technology forms the basis for marked development during recent years (38), and this is also the case for studies of *Mycobacterium tuberculosis* (25) and *Mycobacterium bovis* BCG (28). So far, many of these studies have depended on the use of monoclonal antibodies for the selection of suitable clones from the mycobacterial gene libraries (38) or for the isolation of antigens by affinity chromatography (14). Such a technique implies that the antigens selected for study depend on the degree of restriction of their immunogenicity in the few inbred strains of mice used so far for the generation of monoclonal antibodies. It is not known whether such antigens are of particular importance in relation to the development of protective immunity or clinical diseases in humans.

Individual immunogenic protein antigens of *M. tuberculosis* and *M. bovis* BCG have also been isolated directly by chromatography of culture fluids and characterized by biochemical and immunological techniques. We have previously described the purification to homogeneity of MPB57 (37), MPB59 (34), MPB64 (12), MPB70 (11, 22), and MPB80 (12) from *M. bovis* BCG, and the corresponding genes have been cloned and sequenced (2, 19, 26, 27, 36, 37).

The antigen 85 complex is a major constituent of *M. bovis* BCG and *M. tuberculosis* culture fluids and binds readily to human fibronectin (1). Its components were originally identified by crossed immunoelectrophoresis (CIE) (4, 31), and we previously described the isolation of the BCG 85A and 85B components (31) as well as the BCG 85C component (35). The major 85A and 85B components were also isolated independently by various groups (5, 7, 9, 14) and therefore provided with different sets of designations (see Table 1). In subsequent work with *M. tuberculosis* culture fluid antigens, MPT44 and MPT59 were shown to correspond to 85A and 85B, respectively (33). The genes corresponding to the 85A (3) and 85B (19) components have been cloned and sequenced (35).

The present work was undertaken to extend our work with the chromatography of *M. tuberculosis* culture fluid. Here, we report the isolation of eight additional proteins from *M. tuberculosis* culture fluid and their partial characterization by biochemical and immunological techniques. The extensive current work on mycobacterial genes also needs to be supplemented with information concerning the isolation and behavior of the corresponding native proteins, with direct determination of their amino-terminal amino acid sequences, and with data on protein expression corresponding to (independently) demonstrated genes.

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MATERIALS AND METHODS

Mycobacteria. *M. bovis* BCG Copenhagen substrain 1331 was obtained from Statens Seruminstitut, Copenhagen, Denmark. *M. tuberculosis* H37Rv (ATCC 27294) was obtained from the National Institute of Health, Tokyo, Japan. The bacilli were grown in wholly synthetic Sauton medium (23) for up to 10 weeks at 37°C without shaking and harvested by centrifugation. For the isolation of proteins, the culture supernatant of a 5-week-old culture was concentrated by ammonium sulfate precipitation (80% saturation), and the salt was removed by dialysis. The bacilli were washed three times in phosphate-buffered saline and subjected to sonification in a rosette cooling cell with a model B12 Sonifier (Branson Sonic Power Co., Danburry, Conn.) as described previously (4). Protein concentrations were determined by the Folin method (18). For the analysis of changes in the protein patterns in culture fluids, samples were taken each week for 2 to 10 weeks of culturing for testing by two-dimensional electrophoresis (2D-E) (24).

Purification of proteins from culture fluids. The term MPB was introduced by Nagai et al. (22) for the designation of a protein purified from *M. bovis* BCG, with a number denoting the relative mobility in 7.7% polyacrylamide gel electrophoresis (PAGE) gels at a running pH of 9.5 by the method of Davis (6). The corresponding term MPT is used to denote a protein isolated from *M. tuberculosis*.

The purification procedure for MPTs was developed on the basis of the technique for the purification of MPB70 and other MPBs as described in detail previously (12, 22, 34). In the first step (see Fig. 1), protein concentrate (4.8 g) from 5-week-old *M. tuberculosis* culture fluid was applied to a DEAE–Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) column (bed volume, 550 ml) with 30 mM Tris hydrochloride buffer (Tris buffer) (pH 8.7) containing 3% methylcellosolve. The concentration of sodium chloride was increased linearly from 50 to 300 mM. Every fraction of the eluate was analyzed by PAGE at a running pH of 9.5 (6) to determine the relative mobility (22) and by sodium dodecyl sulfate (SDS)-PAGE (17) with a 12.8% polyacrylamide gel to determine the apparent molecular weight (MW). The main MPTs in the culture fluid were eluted from the column (see Fig. 1 and 2) and concentrated separately by ultrafiltration with a YM-5 membrane (Amicon Corp., Lexington, Mass.). For further purification to homogeneity, the fractions containing the MPTs were subsequently applied serially to columns of DEAE–Sepharose CL-6B, Sephacryl S-200 HR (Pharmacia), or phenyl–Sepharose CL-4B (Pharmacia) (see Fig. 3).

2D-E. After dialysis of *M. tuberculosis* H37Rv culture fluid against 30 mM Tris buffer (pH 8.7), 150 μg of protein concentrate (18) was subjected to 2D-E (24). The first dimension (see Fig. 4), from right to left at the top of the gel, was isoelectric focusing in 9 M urea with a 2% ampholyte mixture (Bio-Lyte; Bio-Rad; mixture of 3/10:3/5:5/7 = 4:1:1). The second dimension, from the top to the bottom, was SDS-PAGE with a 12.8% polyacrylamide gel. The gel was stained with Coomassie brilliant blue (Fluka Chemie AG, Buchs, Switzerland) and with a silver stain reagent (Wako Chemicals, Osaka, Japan). The MW scale was calibrated with standard proteins (Pharmacia), and the isoelectric point (pI) scale was calibrated from the pHs in individual vials containing 5-mm sections of the isoelectric focusing gel and 2 ml of degassed water.

pI determination by isoelectric focusing. Isoelectric focusing was performed with polyacrylamide gels containing a 2% ampholyte mixture of the same composition as that described above and with a model 111 Mini IEF Cell apparatus (Bio-Rad). The pIs of the isolated MPTs were determined by direct comparison with the pI calibration markers of a low-pI calibration kit (Pharmacia) at 24°C. The gels did not contain urea.

Determination of enzyme reactivity. Superoxide dismutase (SOD) reactivity was assayed by the method of McCord and Fridovich (20).

Antisera. Polyvalent anti-BCG immunoglobulin was kindly provided by DAKO Immunoglobulins, Copenhagen, Denmark (code B124; lot 063B). Anti-*M. tuberculosis* was kindly provided by J. Bennedsen, Statens Seruminstitut (30). Anti-*M. tuberculosis* culture fluid was produced as described previously (32), and antisera against purified MPTs were obtained by our standard immunization procedures (10). Mouse polyclonal antiserum to purified catalase from a cell sonicate of *M. tuberculosis* was kindly provided by K. Karaya, Research Institute of Tuberculosis, Japan Antituberculosis Association.

Immunochemical techniques. CIE (4) and SDS-PAGE (17) with immunoblotting (32) were performed as described elsewhere.

Sensitization of guinea pigs and skin testing. Sensitization of guinea pigs and skin testing have been described in detail previously (22). In brief, guinea pigs were sensitized by intramuscular injection of a suspension of heat-killed dried cells of *M. tuberculosis* in liquid paraffin and skin tested 8 to 13 weeks later. The combined fractions from DEAE column chromatography, the isolated MPTs, or protein purified derivative (PPD) (Japan BCG Laboratory, Tokyo, Japan) was diluted in physiological saline and injected intradermally on shaved flanks of guinea pigs. The mean diameter of the area of induration was measured 24 h after injection.

Determination of amino acid sequences. Amino-terminal sequences were determined by automatic Edman degradation by use of an Applied Biosystems 477A gas-phase sequencer with an on-line 120A phenylthiohydantoin amino acid analyzer.

RESULTS

Chromatography of *M. tuberculosis* culture fluid. Figure 1 shows the elution profile after the first chromatography of *M. tuberculosis* culture fluid protein on DEAE–Sepharose CL-6B. Individual fractions of the eluate were tested by PAGE (6) and SDS-PAGE (17), and eluate fractions were pooled as indicated in Fig. 1 to obtain eight major fractions with an optimal primary localization of individual proteins. The analysis of these separately pooled fractions by PAGE and SDS-PAGE is shown in Fig. 2A and 2B. Some proteins, such as SOD, catalase, and MPT57, did not show clear bands but usually showed diffuse bands in PAGE. In SDS-PAGE, these proteins showed sharper bands, but the catalase band remained broad, indicating a molecular mass of 80 to 84 kDa. While all of the pooled fractions contained several proteins, they were valuable in further chromatography to separate individual protein fractions. Figure 3 shows a summary of the flow charts used for serial chromatographic procedures to obtain the individual proteins in pure form.

Methylcellosolve was used in the first DEAE chromatography step to obtain optimal conditions for the separation of proteins. The use of 3 M urea or 10% ethylene glycol in the elution buffers was quite effective in separating proteins in many cases, but the concentration of urea had to be main-

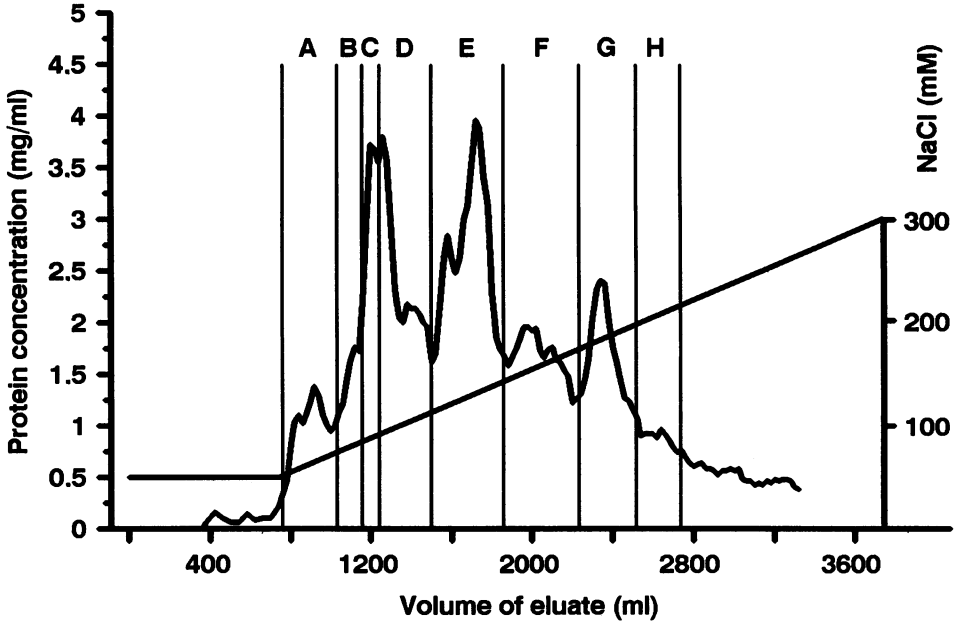

FIG. 1. Elution profile after the first chromatographic separation of *M. tuberculosis* culture fluid protein (4.8 g) on a DEAE–Sepharose CL-6B column with a bed volume of 550 ml. For elution, 30 mM Tris hydrochloride buffer (pH 8.7) containing 3% methylcellosolve and a linear gradient of 50 to 300 mM NaCl were used. The protein concentration in each fraction was determined by the Folin method. A 30-μl sample of each fraction of the eluate was analyzed by PAGE and SDS-PAGE, and eluate fractions were pooled into eight major fractions (A to H) with an optimal primary localization of individual proteins.

tained at less than 4 M to avoid the irreversible denaturation of proteins.

The protein fractions were tested for purity by several partly independent techniques. All isolated proteins except for SOD and catalase, showed a single band after loading of 10-μg samples in SDS-PAGE and appeared in a single spot in 2D-E. The SOD and catalase preparations showed a major band and several additional faint bands in SDS-PAGE. SOD showed three neighboring spots of the same MW in 2D-E. The spot to the left had the highest SOD specific activity, and all three spots had an orange color with a broad optical absorbancy at 320 to 440 nm, indicating the presence of the Fe^{2+} ion (16). The catalase preparation had a red-brown color with a maximum absorbancy at 404 nm, and a single precipitation line was observed in double-diffusion tests in agarose against antiserum to the catalase of *M. tuberculosis*. Testing for purity by immunological techniques is described below.

Figure 4 shows the patterns obtained in 2D-E of proteins in Sauton medium after 5 weeks of culturing of *M. tuberculosis*. The labeled spots were identified by adding individual purified protein fractions to separate samples of the culture fluid and testing by 2D-E, which demonstrated increased protein staining in the area of one spot without significant changes elsewhere in the pattern.

The culture fluids were analyzed every week for 2 to 10 weeks of cultivation by 2D-E to compare the patterns of the major spots. The gels were usually stained with Coomassie brilliant blue and then with silver stain. The spots of MPT32, MPT45, MPT57, and MPT64 were most strongly stained with Coomassie brilliant blue, while the spots of MPT44, MPT46, MPT51, and SOD appeared more distinct after silver staining. The spots of MPT32, MPT44, MPT45, MPT51, MPT53, MPT59, MPT63, and MPT64 were observed as major spots in the pattern after 2 or 3 weeks of culturing, decreased with longer cultivation, and were diminished considerably after 7 weeks of culturing. MPT32 was most labile and, after only 4 weeks of cultivation, significant changes in this spot could be observed. In the pattern of the 3-week culture, the spots of MPT46, MPT57, SOD, and catalase were small, but they increased gradually in size with longer cultivation. These findings are taken to indicate that a series of soluble proteins in the cytosol are released into the culture medium after prolonged culturing, probably because of lysis of dying or dead bacilli; however, even in very young cultures, trace amounts could be seen in some spots, particularly corresponding to proteins of low MW. Conversely, the major spots in the pattern of the 2- or 3-week culture corresponded to proteins being actively secreted into the medium from the mycobacterial cells. The data obtained by other investigators in gene cloning studies have shown that MPT44 (P32), MPT59 (the α antigen), and MPT64 are each synthesized with a signal peptide characteristic of secreted proteins, while the MPT57 gene has no signal peptide. These observations suggest that the culture fluid after 3 weeks of culturing of *M. tuberculosis* is the best source for the primary identification of secreted proteins.

The properties of a series of isolated proteins are recorded in (Table 1). The N-terminal amino acid sequences for five residues of the isolated proteins are shown. Compared with the gene cloning studies revealing the complete configurations of P32 (3), MPB57 (2, 26, 37), the α antigen (19), and MPB64 (36), the analysis of the N-terminal amino acid sequences for the MPT counterparts MPT44, MPT57, MPT59, and MPT64 revealed identity with the published sequences for 40, 20, 45, and 30 amino acid residues, respectively.

Immunological studies of isolated proteins. To study the purity of the protein preparations by immunological tech-

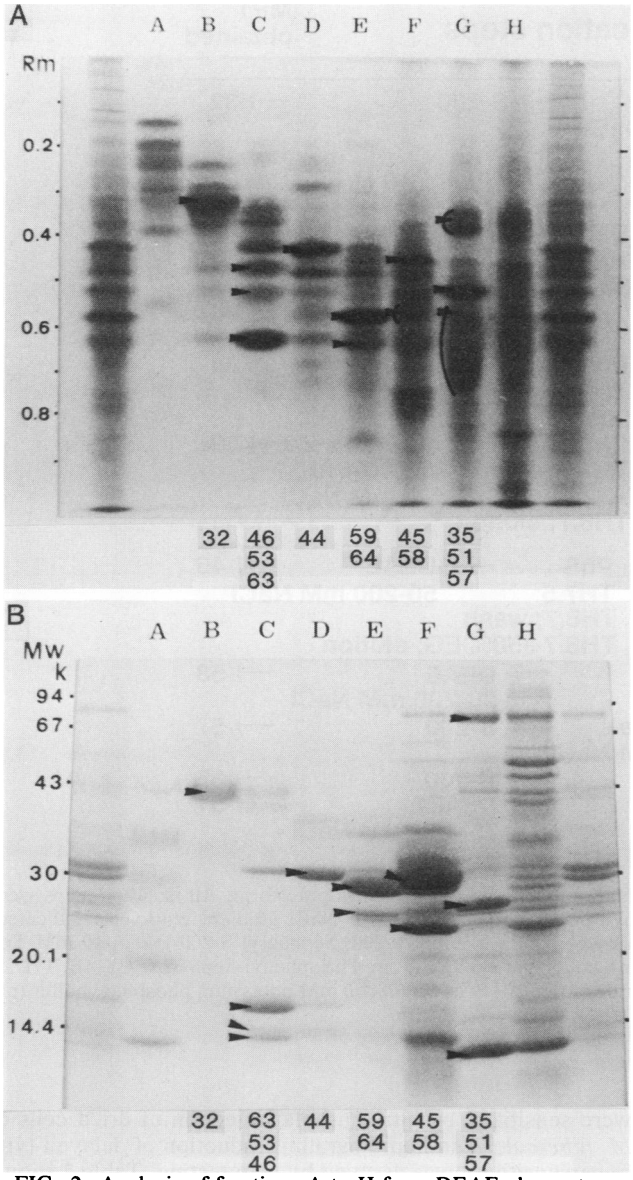

FIG. 2. Analysis of fractions A to H from DEAE chromatography shown in Fig. 1. (A) Protein (50 μg) from each fraction (lanes A to H) and whole *M. tuberculosis* culture fluid (75 μg) (flanking lanes) subjected to PAGE at a running pH of 9.5. (B) SDS-PAGE of 30 μg of culture fluid (flanking lanes) and 15 μg of each fraction (lanes A to H). The gel was stained with Coomassie brilliant blue. The main MPTs are indicated with arrowheads and corresponding numbers below each lane. Rm, Relative mobility.

niques and to characterize their immunogenicity, we raised antisera and studied the preparations by CIE.

The purified antigens were initially tested in CIE with the previously described polyvalent anti-BCG (4) and anti-*M. tuberculosis* (30) in the top gel. Single precipitates were obtained with MPT35, MPT44, MPT51, MPT57, MPT59, and MPT64, showing that they are immunogenic in rabbits and also indicating purification to homogeneity. The SOD preparation gave a major precipitate extending out of the top gel and a few additional minor precipitates in the intermediate gel.

The other protein preparations gave no precipitation lines in similar experiments.

After immunization of rabbits with eight different purified antigens, we obtained precipitating antibodies against MPT32, MPT46, MPT51, MPT53, MPT57, MPT59 (33), MPT63, and MPT64 (12) that gave single precipitates with the corresponding antigens in quantitative immunoelectrophoresis. The anti-MPT46 antibodies were weak, but all of these proteins were immunogenic under the conditions used for immunization.

These antisera were incorporated into the intermediate gel of corresponding CIE plates with *M. tuberculosis* culture fluid in the antigen well and a recently prepared antiserum to *M. tuberculosis* culture fluid in the top gel. Antisera against MPT32, MPT51, MPT53, MPT59, MPT63, and MPT64 gave single precipitates in the intermediate gel that could be identified as corresponding to individual precipitates in the reference pattern. The antiserum against MPT46 did not give a distinct precipitate in the intermediate gel, corresponding to its rather weak reactivity described above.

The antiserum against MPT57 did not precipitate any antigen in the *M. tuberculosis* culture fluid preparation. However, a distinct precipitate was obtained with an *M. tuberculosis* sonicate as well as with reference BCG culture fluid and sonicate preparations. Its reactivity could easily be matched to a single precipitate in these patterns. The antiserum had strong reactivity, and these observations correspond closely to the prior identification of the antigen as a component of the bacterial cytosol that is not released into the culture medium except in instances of bacterial lysis during culturing (32).

Figure 5 shows the results obtained with one particular antigen (MPT63) and the corresponding monospecific rabbit antiserum produced by immunization with the isolated protein.

Figure 5A shows the pattern obtained with an *M. tuberculosis* antigen in the circular well, polyvalent anti-*M. tuberculosis* culture fluid in the top gel, and buffer only in the intermediate gel. Evident is the characteristic pattern with a multitude of precipitates corresponding to individual components of the culture fluid preparation, against which antibodies were present in the pooled immunoglobulin preparation incorporated in the top gel. Note that the antigen concentration in the culture fluid was relatively high for several components, resulting in almost vertical precipitates extending out of the top gel. Most of the precipitates seen in this pattern corresponded to secreted antigens in the culture fluid, while the "conventional" precipitate pattern in the BCG system (4) shows a few secreted antigens (mainly the antigen 85 complex and antigen 78) and a multitude of cytoplasmic antigens being released from dying or dead bacilli because of prolonged culturing and, in most cases, a greater tendency for bacterial lysis in BCG than in *M. tuberculosis* cultures.

Figure 5B shows the pattern obtained with isolated MPT63 in the circular antigen well and the same polyvalent anti-*M. tuberculosis* culture fluid in the top gel. A single precipitate was formed, illustrating the general feature that each of the isolated antigens give a single precipitate with this polyvalent antiserum.

Figure 5C shows the findings obtained with *M. tuberculosis* culture fluid in the circular well, polyvalent anti-*M. tuberculosis* in the top gel, and anti-MPT63 in the intermediate gel. The antiserum was monospecific, giving a single precipitate in comparison with the control plate (Fig. 5A). One of the major precipitates with lines extending out of the

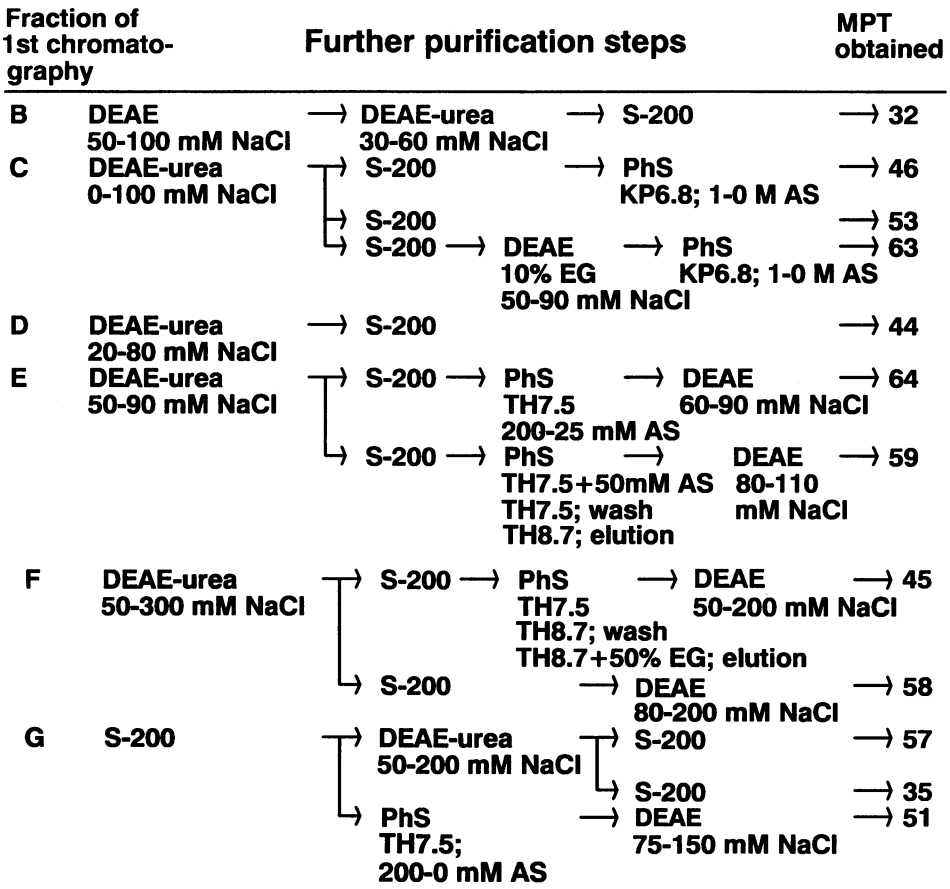

FIG. 3. Flow charts of serial chromatographic procedures used to obtain the individual proteins in pure form. All isolation steps were performed at 4°C. DEAE: DEAE–Sepharose CL-6B in 30 mM Tris hydrochloride (pH 8.7), with NaCl gradient elution as indicated. DEAE-urea: DEAE–Sepharose CL-6B in 30 mM Tris hydrochloride (pH 7.5) with 3 M urea. S-200: Sephacryl S-200 HR in 10 mM Tris hydrochloride (pH 7.5) with 10% ethylene glycol (EG) and 300 mM NaCl in a column of 2.6 by 100 cm. PhS: phenyl-Sepharose CL-4B. TH7.5: 10 mM Tris hydrochloride buffer (pH 7.5). TH8.7: 10 mM Tris hydrochloride buffer (pH 8.7). KP6.8: 50 mM potassium phosphate buffer (pH 6.8). AS: Ammonium sulfate.

top gel showed a definite extension down into the intermediate gel.

Identification of this antigen by incorporation into the intermediate gel is shown in Fig. 5D. A horizontal line fusing with the vertical line corresponding to MPT63 appeared in the pattern.

Figure 5E shows immunoblotting after PAGE of culture fluids and sonicates from *M. bovis* BCG and *M. tuberculosis* in lanes 1 to 4. After being blotted onto nitrocellulose, the gel was exposed to anti-MPT63, and bound antibody was visualized by reaction with peroxidase-labeled antirabbit immunoglobulin. A single, strong band was seen at 18 kDa with both culture fluid preparations (lanes 1 and 3). In lanes 2 and 4, containing the corresponding sonicate preparations, a barely visible band appeared at the same position. In *M. tuberculosis* culture fluids, two weak bands were seen further down, probably corresponding to degradation products of the protein. The staining close to the origin of the gel was an artifact. The gel was subjected to conditions yielding strong staining to illustrate the specificity of the antibody. In other gels exposed to lower concentrations of the antibody, a single band at 18 kDa was observed with both culture fluid preparations.

Induction of delayed-type hypersensitivity. Guinea pigs were sensitized by intramuscular injection of dried cells of *M. tuberculosis* in liquid paraffin. Induction of delayed-type hypersensitivity was assayed by skin testing. Table 2 shows that all of the combined fractions eluted from the DEAE–Sepharose CL-6B column showed similar reactivity to PPD, with slight variations between different fractions, fraction E showing the highest reactivity and fractions C and H showing lower reactivity.

Table 3 shows that the isolated MPTs varied markedly in their ability to induce delayed-type hypersensitivity reactions in sensitized guinea pigs. MPT59 and MPT64 showed strong reactivity, with a potency very similar to that the PPD preparation used for comparison. The results with MPT64 corresponded closely to those of a previous study (12) in which we showed that the corresponding protein isolated from *M. bovis* BCG (MPB64) and PPD induced reactions of similar strength in guinea pigs sensitized with *M. bovis* BCG Tokyo or *M. tuberculosis* H37Rv. The results obtained with isolated MPT64 may also explain the higher reactivity of fraction E than of other fractions shown in Table 2 because fraction E contains both MPT59 and MPT64. MPT32 and MPT35 showed distinct but lower reactivity than PPD at 2 μg, while most of the other proteins did not induce skin test reactions even at this concentration.

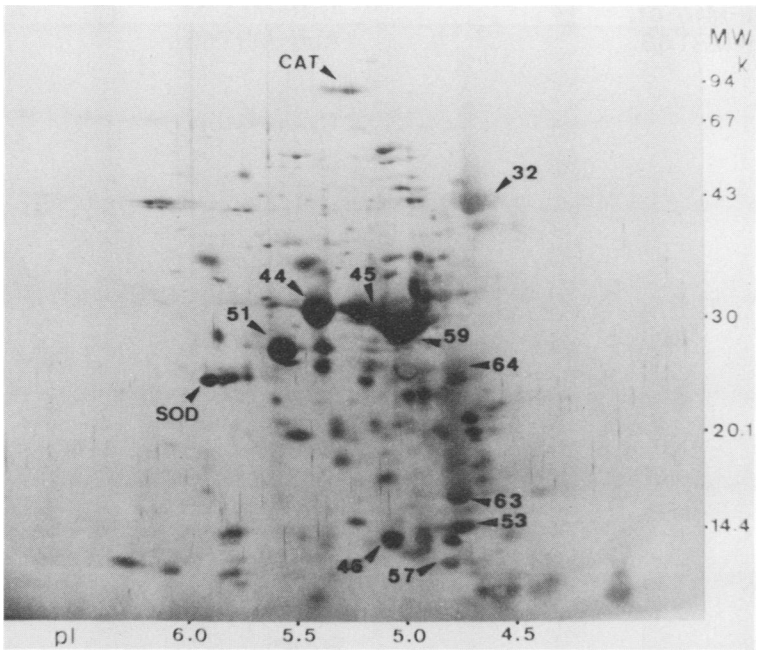

FIG. 4. 2D-E of proteins in Sauton medium after 5 weeks of culturing of *M. tuberculosis*. Concentrated culture fluid containing 150 μg of protein was applied. After being stained with Coomassie brilliant blue, the slab was submitted to silver staining. MW scale was calibrated with standard proteins, and the pI scale was calibrated from the pHs in individual vials containing 5-mm sections of the isoelectric focusing gel and 2 ml of degassed water. The arrows and numbers indicate the positions of individual proteins. CAT, Catalase.

Cross-reaction between MPT59 and MPT51. The antigen 85 complex is a major constituent of the secreted proteins of *M. bovis* BCG and *M. tuberculosis*. The three constituent components show extensive immunological cross-reactivity (31, 33), and structural studies have revealed extensive homology as well as characteristic differences among the individual components (35). In view of these observations, we searched for cross-reactivity with other secreted proteins.

Figure 6 illustrates immunoblotting with anti-MPT51 after SDS-PAGE of the isolated protein compared with culture fluids and sonicates of *M. bovis* BCG and *M. tuberculosis*. The isolated protein in lane 1 gave a single, sharp band at 27 kDa. BCG culture fluid gave a strong band at the same position (lane 2). In addition, a doublet with bands at 31 kDa and 30 kDa was seen. These bands corresponded to the components of the antigen 85 complex. 85A (MPB44) and 85C (MPB45) were both localized in the upper band, while the sharper, lower band corresponded to the 85B component (MPB59). In the corresponding sonicate (lane 3) there were no visible bands around 30 kDa and 31 kDa and only a barely visible band at 27 kDa because of the markedly lower concentration of these proteins in sonicates of washed bacilli. In *M. tuberculosis* culture fluid in lane 4, there was a

TABLE 1. Physiochemical properties of proteins isolated from *M. tuberculosis* culture fluid

MPT	CIE designation	Other designation(s)	MW (10^3) (determined by SDS-PAGE)	pI determined by:		N-terminal amino acid sequence	Secreted	Comments
				2D-E	Isoelectric focusing			
32			41	4.7	4.2	DPAPA	Yes	
35	56	Catalase	80–84	5.2			No	With peroxidase activity; maximum absorbance at 404 nm
44	85A	P32 (7)	31	5.4	4.7	FSRPG	Yes	
45	85C		31.5	5.15	4.6	FSRPG	Yes	
46			14	5.1	4.9	RDSEK	No	
51			27	5.55	5.1	APYEN	Yes	
53			15	4.8	4.0	DECIQ	Yes	
57		BCGa (21), 10-kDa antigen (2)	12	4.9	4.6	AKVNI	No	
58	62	SOD	25	5.9	4.5		No	Broad absorbancy at 320 to 440 nm
59	85B	Antigen 6 (30), α antigen (9, 35)	30	5.05	4.2	FSRPG	Yes	
63			18	4.8	4.2	AYPIT	Yes	
64			26	4.8	4.5	RIKIF	Yes	

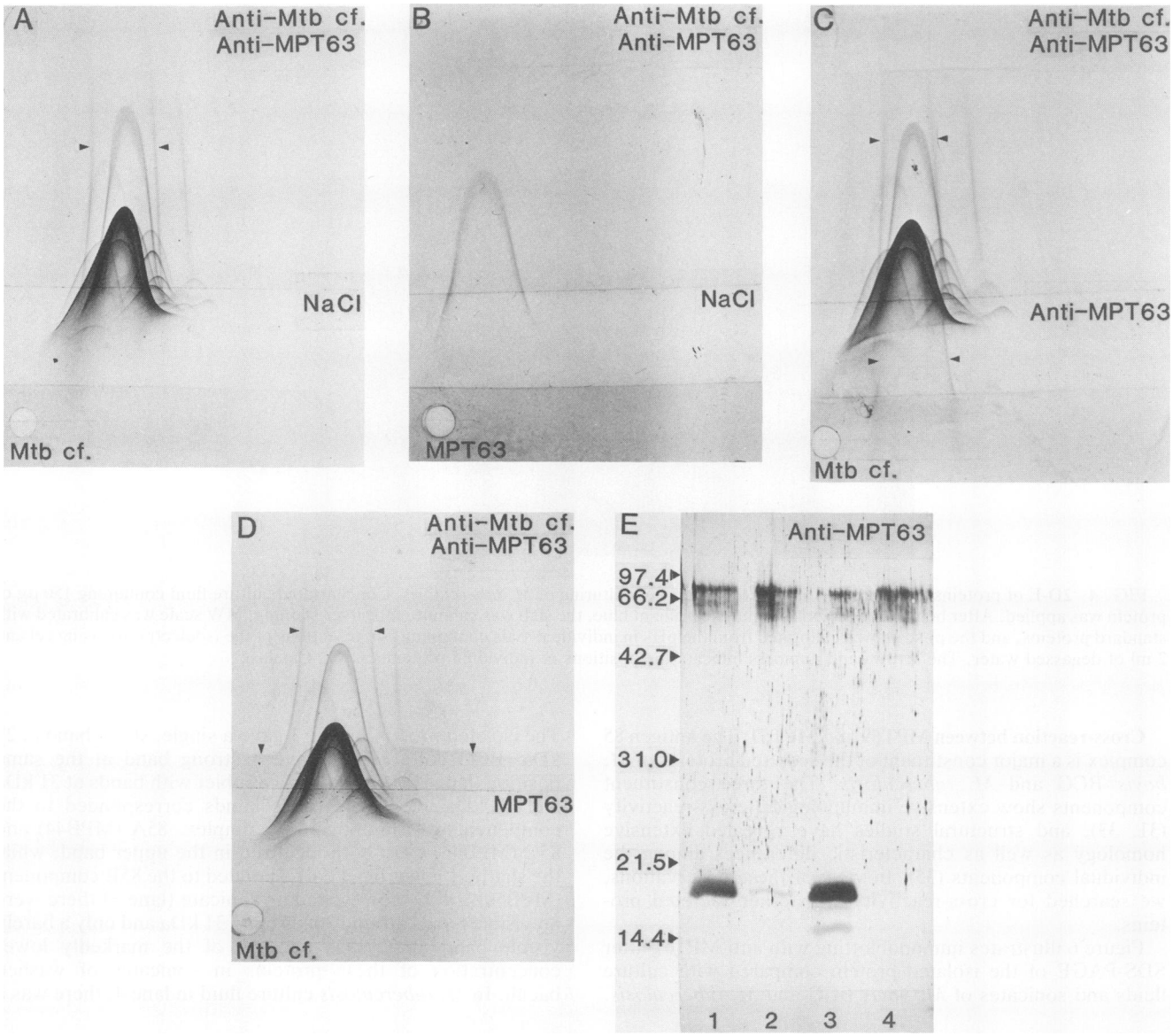

FIG. 5. CIE and immunoblotting to illustrate the behavior of a selected antigen (MPT63) and the corresponding rabbit antiserum produced by immunization with the isolated protein. (A) Control pattern with 10 μg of *M. tuberculosis* culture fluid in the circular antigen well and 200 μl of polyvalent anti-*M. tuberculosis* culture fluid in the top gel. Arrowheads show the precipitate of MPT63. (B) 1 μg of isolated MPT63 in the antigen well and 200 μl of polyvalent anti-*M. tuberculosis* culture fluid in the top gel. A single precipitate is seen. (C) 200 μl of rabbit anti-MPT63 in the intermediate gel. A single precipitate of MPT63 was affected. (D) 1 μg of isolated MPT63 in the intermediate gel. A horizontal precipitate fusing with the vertical precipitate corresponding to MPT63 is formed. (E) Immunoblotting with anti-MPT63 (1:200) after PAGE of culture fluids (lanes 1 and 3) and sonicates (lanes 2 and 4) from *M. bovis* BCG and *M. tuberculosis*, respectively.

strong band at 27 kDa and a single broad band above it because of the higher concentration of the antigen 85 components in *M. tuberculosis* culture fluids, causing the bands to fuse to each other. The bands at the lower MWs probably corresponded to degradation products but could have been due to other cross-reactive proteins. Again, a much lower concentration of protein was detected by anti-MPT51 in the sonicate of washed bacilli (lane 5).

Anti-MPT51 reacted with all three purified components of the antigen 85 complex, 85A (MPT44), 85B (MPT59), and 85C (MPT45), in SDS-PAGE with immunoblotting and in enzyme-linked immunosorbent assay experiments (data not shown).

Figure 7 shows the results of N-terminal amino acid sequence determinations for isolated proteins. The top line shows the N-terminal sequence as deduced from the α antigen gene cloned and sequenced by Matsuo et al. (19). This deduced sequence is identical to the sequence determined for the isolated MPT59 protein for 45 residues in the present study. The bottom line shows the N-terminal sequence determined for the isolated MPT51 protein for the first 45 residues. After alignment, 27 of the 45 N-terminal residues of MPT51 were identical to residues in MPT59, corresponding to 60% homology in this region. Thus, the immunological cross-reactivity corresponds to the extensive structural homology between these proteins.

TABLE 2. Skin test reactivity of the fractions from DEAE column chromatography

Antigen (0.2 μg[a]/0.1 ml)	Diam (mm) of the area of induration 24 h after injection[b] (six guinea pigs)[b]
Before fractionation	17.2 ± 1.8
Fraction	
A	14.5 ± 2.0
B	14.6 ± 2.0
C	13.5 ± 1.8
D	15.8 ± 2.4
E	16.8 ± 2.2
F	15.5 ± 1.9
G	14.1 ± 3.0
H	13.2 ± 3.0
PPD[c]	14.0 ± 2.7

[a] The protein concentration was estimated by the Folin method.
[b] Mean ± standard deviation.
[c] 12 tuberculin units, corresponding to 0.2 μg/0.1 ml.

DISCUSSION

Electrophoresis was used as the main technique to monitor the separation of individual proteins during the various chromatographic procedures. PAGE was initially used (Fig. 2) to separate proteins on the basis of molecular size as well as charge, but some of the proteins showed diffuse bands. Separation by SDS-PAGE is based on molecular sieving, and more distinct bands were observed. Separation of proteins in one dimension is generally not sufficient to resolve a large number of constituents. 2D-E as described by O'Farrell (24) is very powerful, since two different principles of protein separation are applied in the first and second dimensions, permitting a precise definition of individual spots (Fig. 4). The application of these methods guided the development of the successive chromatographic procedures that purified 12 different proteins from the *M. tuberculosis* culture fluid, 10 of them to homogeneity.

The individual proteins showed different pIs in 2D-E and after isoelectric focusing (Table 1). The discrepancy observed in pIs depended on the presence or absence of urea. Under the conditions used for 2D-E, the standard marker proteins did not work well, as they were denatured by 9 M urea. Thus, the pIs in 2D-E were those of proteins denatured by 9 M urea. However, both types of pIs were useful for the identification of individual proteins.

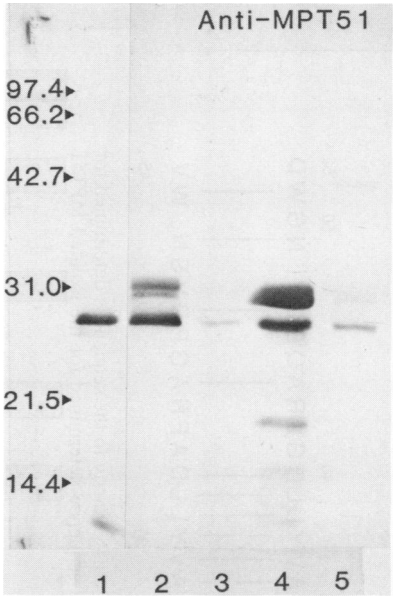

FIG. 6. Immunoblotting with anti-MPT51 (1:500) after SDS-PAGE. Lanes: 1, isolated MPT51 (1 μg of protein); 2 to 5, culture fluids (lanes 2 and 4) and sonicates (lanes 3 and 5) from *M. bovis* BCG and *M. tuberculosis*, respectively (40 μg of total protein in each lane).

2D-E of culture fluids obtained every week for 2 to 10 weeks of culturing of *M. tuberculosis* revealed very characteristic changes, some protein spots appearing early, being particularly strong in these patterns and gradually becoming less prominent. This result was taken to indicate that these proteins were actively secreted from the mycobacterial cells. Other spots appeared later, becoming stronger as bacterial lysis occurred in the culture. These proteins were taken to be

TABLE 3. Skin test reactivity of the isolated MPTs

Antigen	Diam (mm) of the area of induration 24 h after injection[a] in expt:				
	1 (three guinea pigs; protein, 0.2 μg/0.1 ml)	2 (three guinea pigs; protein, 2 μg/0.1 ml)	3 (six guinea pigs) with protein at:		
			0.05 μg/0.1 ml	0.1 μg/0.1 ml	0.2 μg/0.1 ml
MPT					
32	0	10.8 ± 1.8	—[b]	—	—
35 (catalase)	0	9.4 ± 0.3	—	—	—
44	0	0	—	—	—
46	—	0	—	—	—
51	0	0.8 ± 1.5	—	—	—
53	—	0	—	—	—
57	0	0.5 ± 1.0	—	—	—
59	16.8 ± 1.6	—	15.6 ± 0.7	18.1 ± 0.7	20.1 ± 0.5
63	0	0	—	—	—
64	19.8 ± 1.8	—	15.2 ± 1.2	16.8 ± 0.8	18.8 ± 2.1
PPD	15.5 ± 0.7	17.0 ± 0.1[c]	16.3 ± 0.9	19.0 ± 1.1	—

[a] Mean ± standard deviation. Protein concentration is given as dry weight.
[b] —, Not done.
[c] 0.2 μg/0.1 ml (12 tuberculin units).

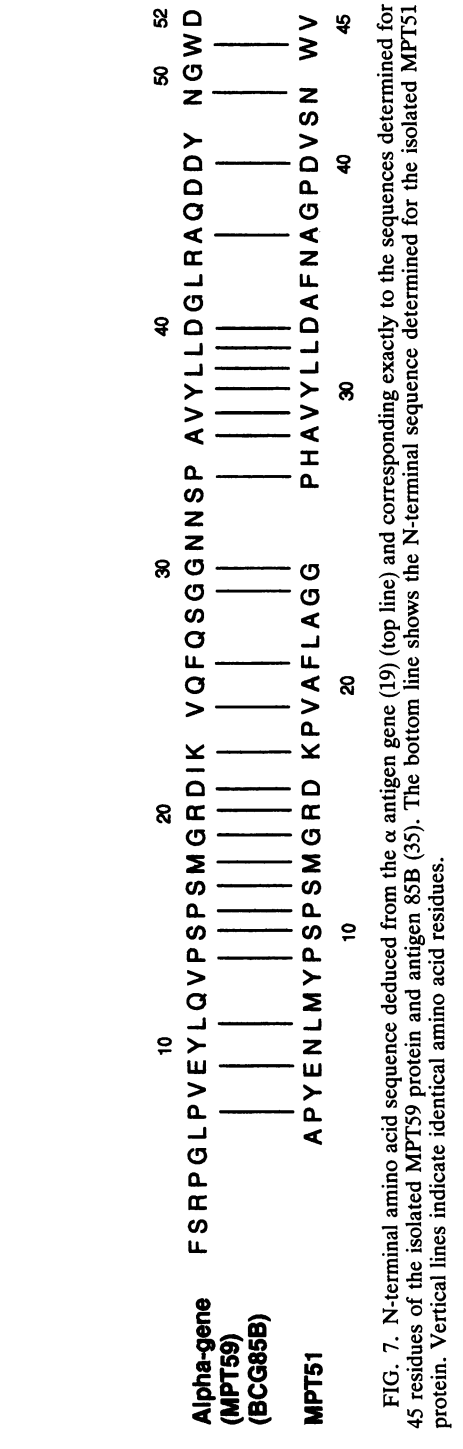

FIG. 7. N-terminal amino acid sequence deduced from the α antigen gene (19) (top line) and corresponding exactly to the sequences determined for 45 residues of the isolated MPT59 protein and antigen 85B (35). The bottom line shows the N-terminal sequence determined for the isolated MPT51 protein. Vertical lines indicate identical amino acid residues.

nonsecreted (Table 1), the interpretation being that they were soluble proteins present in the cytosol and appearing in culture fluid as a result of the lysis of bacteria. Purification of secreted proteins is best obtained by use of early cultures, in which these proteins are quantitatively dominating. The total protein content is much lower (about 1/3) in 3-week culture fluid than after 5 weeks of culturing of *M. tuberculosis*. For isolation, the initial amount of protein is also an essential factor, and the use of 5-week *M. tuberculosis* cultures was found to be optimal. Older cultures were avoided to prevent possible denaturation of secreted proteins. The majority of the secreted proteins appeared, however, to be rather stable, and evidence for substantial degradation was not observed, except for MPT32.

In a separate study (32), we quantified a series of mycobacterial antigens by quantitative immunoelectrophoresis and enzyme-linked immunosorbent assays with monospecific antisera against proteins purified from *M. tuberculosis* and *M. bovis*. The ratio between the relative concentrations of each protein in culture fluid and a sonicate of washed bacilli was calculated and termed the localization index. An index of 0 was found for several proteins confined to the bacterial cytoplasm, while secreted proteins had indexes varying from 4.6 to 1,050, probably indicating different degrees of secretion efficiency or different tendencies of secreted proteins to adhere to the bacterial surface.

Classification of proteins as secreted or nonsecreted on the basis of 2D-E in the present study gave results identical to those given by classification on the basis of immunological quantification and calculation of localization indexes of individual proteins (32). The presence or absence of signal peptides in those genes that have been cloned also corresponds fully to our classification of proteins as secreted or nonsecreted by biochemical or immunological techniques, respectively.

Analysis of N-terminal amino acid sequences revealed distinct differences among the A, B, and C components of the BCG antigen 85 complex (35). Determination of the N-terminal sequences of the corresponding proteins (MPT44, MPT59, and MPT45) from *M. tuberculosis* yielded identical results. All of the MPT proteins in *M. tuberculosis* listed in Table 1 were also demonstrated in *M. bovis* BCG at identical positions by 2D-E analysis. These findings indicated extensive similarities in the primary structures of individual proteins in these two species.

It is noteworthy that only five of the *M. tuberculosis* proteins listed in Table 1 have been given numbers in the reference system for BCG antigens by CIE (4), although all of them have been demonstrated in BCG by 2D-E. Identification of a protein by CIE as well as by immunoblotting is critically dependent on the specificity of the antibody used, and it is now evident that the polyvalent anti-BCG antibody permitting the establishment of a reference system by CIE (4) reacts mainly with cytoplasmic antigens of BCG and *M. tuberculosis*. For improved immunological studies of secreted antigens of BCG and *M. tuberculosis*, new polyvalent antibodies had to be produced by immunization of rabbits with concentrated culture fluids after a few weeks of culturing of *M. tuberculosis*. By a combination of biochemical and immunological techniques, a series of new secreted protein antigens of *M. tuberculosis* have now been identified and purified. Among secreted proteins, MPB70 is immunogenic in vivo in bovine tuberculosis (8, 13), and the proteins of the antigen 85 complex are major immunigens in vivo in leprosy, human and bovine tuberculosis, and paratuberculosis (13, 15, 29, 34). They should be further studied, since secreted

protein antigens may be of particular significance in the induction of protective immune responses as well as in the development of tissue damage in cases with particularly strong delayed-type hypersensitivity reactions following infection.

The induction of cellular immune responses to individual antigens of microorganisms is often interpreted on the basis of delayed-type hypersensitivity skin test reactions. The immunogenicities of individual antigens should also be evaluated in relation to the procedure used for the sensitization of animals, which will influence the sensitizing doses for individual antigens. We expect that considerable differences may occur when killed versus live bacteria are used for sensitization. Dead bacteria probably contain relatively small quantities of secreted antigens as compared with live bacteria, which may continue to produce and release secreted antigens for various times after the sensitizing procedure. Our results indicate that there is a tendency for quantitatively major secreted antigens to induce delayed-type hypersensitivity reactions even after the sensitization of animals with killed bacilli, as exemplified by the strong reactions to MPT59 (antigen 85B) and MPT64 (Table 3). For antigens that are negative in the skin test, low quantities in the killed mycobacteria may be the explanation for the failure of sensitization.

One marked exception was observed. Both MPT44 (antigen 85A) and MPT59 (antigen 85B) are major secreted antigens of *M. tuberculosis*. MPT59 manifested itself as one of the most potent antigens in the series of purified antigens tested. However, no delayed-type hypersensitivity response was found with MPT44. This observation corresponds to the finding of De Bruyn et al. (7) that P32 (MPT44) produced poor delayed-type hypersensitivity responses. There is extensive sequence homology between these two proteins (3). Attempts to localize the T-cell-reactive epitopes on MPT59 would be particularly interesting in view of the marked difference in immunogenicity of the two structurally closely related proteins. It should also be noted that the localization index is considerably higher for MPT44 than for MPT59 (32), implying that MPT44 is more efficiently released from the bacilli, and the dose of this antigen may therefore be markedly reduced by use of killed cells for sensitization.

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