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Properties of Proteins MPB64, MPB70, and MPB80 of *Mycobacterium bovis* BCG

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The immunogenic proteins MPB64 and MPB80 of *Mycobacterium bovis* BCG were purified to homogeneity and compared with MPB70. MPB70 and MPB80 showed a similar distribution in substrains of BCG, both being present in high concentrations in culture fluids of BCG substrain Tokyo, BCG Moreau, BCG Russia, and BCG Sweden and in only very small amounts in BCG Glaxo, BCG Tice, BCG Copenhagen, and BCG Pasteur. In various physicochemical properties MPB70 and MPB80 were closely similar, but MPB80 had a distinctly lower pI value. The N-terminal amino acid sequence was identical for the first 30 residues. In reactions with anti-MPB70 antibodies and delayed-type hypersensitivity skin reactions, MPB70 and MPB80 also had very similar properties. These results show that MPB70 and MPB80 are two closely similar forms of the same gene product, and postsynthetic changes probably explain the observed differences. By contrast, MPB64 had a higher molecular weight. The N-terminal amino acid sequence showed no homology with MPB70, and these two proteins showed no immunologic similarity. MPB64 and MPB70 showed only very restricted cross-reactivity with other species of mycobacteria but cross-reacted with *Nocardia asteroides*. The similar occurrence in eight different substrains of BCG indicated that the two proteins are influenced by similar control mechanisms, but in contrast to MPB70, MPB64 occurred in sufficient concentration in two strains of *Mycobacterium tuberculosis* to give a distinct spot in two-dimensional polyacrylamide gel electrophoresis of their culture fluids.

Two-dimensional polyacrylamide gel electrophoresis (PAGE) has revealed the complexity of the protein content in the wholly synthetic Sauton medium after culture of *Mycobacterium bovis* BCG. It contains a multitude of different proteins which give a very characteristic dotted pattern in the two-dimensional gels (19). Crossed immunoelectrophoresis (CIE) has greater resolving power than previously used immunological techniques to characterize the antigenic content of mycobacterial cells and culture fluids. For BCG a reference system based on CIE has been described (2) in which about 30 different antigenic components were selected as reference antigens and numbered. Most of these antigens are also present in the cytosol of the mycobacterial cell and in extracts prepared from BCG by ultrasonication or bacterial press extraction. Similar complex CIE patterns have been found in *Mycobacterium tuberculosis* (26) and several other mycobacterial species, but in these instances the individual precipitate lines have not been numbered, and there is little information available for exact identification of precipitate lines in the patterns of other mycobacterial species that correspond to identified antigens in BCG.

MPB70 is a protein selected for purification from BCG culture fluid by Nagai et al. (20) because of its presence in high concentration in the culture fluid. It gives a strong spot in the two-dimensional PAGE pattern of BCG substrain Tokyo culture fluid and was purified to homogeneity (19, 20). When various substrains of BCG were tested by two-dimensional PAGE, the remarkable observation was made that the MPB70 spot was very marked in some strains but lacking in several other BCG substrains (19). Immunological studies revealed that the MPB70 protein was highly specific for strains of *M. bovis* BCG and *M. bovis* Ravenel, whereas it did not cross-react with several other species of mycobacteria. A definite cross-reaction was, however, demonstrated with *Nocardia asteroides* (12). By the use of a radioimmunoassay (RIA) inhibition assay for demonstration and quantification of MPB70 protein in different strains of BCG and species of mycobacteria, similar findings were made as in the two-dimensional PAGE experiments. The protein was found in high concentrations in those strains giving a marked MPB70 spot upon two-dimensional PAGE. In BCG culture fluids giving no MPB70 spot, the protein could be demonstrated by this immunological technique, but it occurred in less than 6% of the concentration in BCG Tokyo (12).

MPB80 is a protein which also gives a distinct spot in the two-dimensional PAGE pattern of BCG Tokyo culture fluid. When the culture fluids of different substrains of BCG and species of mycobacteria were tested, these two spots either occurred together or were both absent.

MPB64 is another distinct spot in the two-dimensional PAGE pattern of BCG Tokyo culture fluid. When different substrains of BCG and species of mycobacteria were tested by two-dimensional PAGE, very similar observations were made, as with the comparison of MPB70 with MPB80. The MPB64 spot occurred together with the MPB70 and MPB80

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spots in all instances tested except two. In two strains of *M. tuberculosis*, the MPB64 spot was distinct, whereas both strains lacked the MPB70 and MPB80 spots. The present paper describes purification of the MPB64 and MPB80 proteins of *M. bovis* BCG substrain Tokyo to homogeneity and their physicochemcial and immunological properties, compared with those of MPB70.

MATERIALS AND METHODS

Mycobacteria. BCG substrain Copenhagen was kindly provided by Statens Seruminstitut, Copenhagen, Denmark, after being grown on Sauton medium. These bacteria were used for immunization and for preparation of polyvalent anti-BCG antisera and BCG antigen for CIE as described in detail previously (10). Other mycobacteria were obtained and grown in Japan as described elsewhere (19). Lyophilized BCG cells were suspended in physiological saline and inoculated onto Ogawa slants and subsequently into Sauton liquid medium. Culture filtrates for preparation of proteins were obtained after 2 to 4 weeks of incubation at 37°C. Three additional strains were included in the present study: *M. tuberculosis* Aoyama B, *Mycobacterium kansasii* NIHJ 1614, and *M. intracellulare* Tasaka were provided by the National Institute of Health, Tokyo, Japan.

Purification of MPB80 and MPB64. The purification procedure for MPB80 and MPB64 was developed on the basis of the technique for purification of MPB70 described in detail previously (20). The protein in the culture fluid of BCG Tokyo was concentrated by ammonium sulfate precipitation (75% saturation), and the salt was removed by dialysis. The first step, on DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden), was the same as for purification of MPB70. The second step, on DEAE-Sepharose CL-6B (Pharmacia), was also the same, but the buffers contained 2% butanol. Every fraction of the eluate was analyzed by PAGE at a running pH of 9.5 (6) to determine the relative mobility. The large peak containing MPB70 appeared first, and then the peaks which contained MPB80, MPB64, and other main MPBs in the culture fluid were eluted from the column (see Fig. 2). Fractions of each MPB were concentrated separately by ultrafiltration with a YM-5 membrane (Amicon Corp., Lexington, Mass.) The fraction with MPB70 was treated further as described previously (20).

The fraction containing MPB80 was applied to a DEAE-Sepharose CL-6B column in 30 mM Tris hydrochloride buffer (Tris buffer) (pH 7.5) containing 6 M urea with a linear concentration gradient of NaCl from 30 to 70 mM. After the desired fraction was concentrated by ultrafiltration, the protein was treated further in a DEAE-Sepharose CL-6B column with 30 mM Tris buffer (pH 8.7) containing a linear concentration gradient of NaCl from 60 to 90 mM. The concentrated protein solution was finally applied to a column of Sephacryl S-200 (Pharmacia) with 30 mM Tris buffer (pH 8.7) containing 0.5 M NaCl and 2% butanol. The fraction with MPB80 was dialyzed extensively against water and then lyophilized.

The fractions from the DEAE-Sepharose CL-6B column of the second step described above containing MPB64 were pooled and concentrated by the use of an ultrafiltration cell and then applied to a DEAE-Sepharose CL-6B column in 30 mM Tris buffer (pH 7.5) containing 3 M urea with a linear concentration gradient of NaCl from 40 to 90 mM. Based on the PAGE results, the fractions containing MPB64 were pooled, concentrated, and passed through a DEAE-Sepharose CL-6B column with 30 mM Tris buffer (pH 8.7) with a linear concentration gradient of NaCl from 60 to 110 mM. Fractions containing MPB64 were again pooled, concentrated, and finally applied to a column of Sephacryl S-200 with 30 mM Tris buffer (pH 7.5) containing 0.5 M NaCl. The fractions with MPB64 were pooled, dialyzed extensively against water, and then lyophilized.

The MPB80 and MPB64 proteins were stored in a desiccator over silica gel at 4°C. The yields of MPB80 and MPB64 were 0.8 and 0.1%, respectively, of the total protein content in the original ammonium sulfate concentrate. MPB64 was more unstable than MPB70 and MPB80 and more easily denatured in butanol and urea, tending to precipitate out of the buffer. Concentrations of butanol and urea higher than 2% and 3 M, respectively, should be avoided.

Two-dimensional PAGE. Two-dimensional PAGE was performed as described previously (19) by virtually the same method as that of O'Farrell (22).

Estimation of molecular weight. The molecular weights of MPB80 and MPB64 were estimated by electrophoresis in sodium dodecyl sulfate-containing gels (15), and MPB80 was also analyzed by the method of sedimentation equilibrium, assuming the partial specific volume of MPB80 was 0.74.

Sugar analysis. With 10 mg of the lyophilized preparation of the purified MPB80 at each analysis, the neutral sugar content was determined with phenol-sulfuric acid (7), and the hexosamine content was analyzed by the method of Elson and Morgan (8) after hydrolysis by 2 N HCl for 16 h at 100°C (1). The possibility of sialic acid binding to MPB70 and MPB80 was tested by PAGE analysis after treatment with neuraminidase (EC 3.2.1.18) (Nakarai Chemicals, Japan) from *Arthrobacter ureafaciens*. The incubation was performed at 37°C for 3 h with 20 μg of MPB70 or MPB80 and 20 mU of the enzyme at pH 5.0.

Determination of amino acid composition and sequence. Amino acid composition was determined as described previously (20). For determination of the amino acid sequence, the automated Edman-Begg degradation process was used with a Beckman 890C Sequencer updated with a cold trap, applying 10 nmol of the protein on the spinning cup. The modified 0.1 M Quadrol program (no. 121078, Beckman) was used. The anilin thiazolinone residues were converted into phenylthiohydantoin derivatives with 0.2 ml of 1 N HCl at 80°C for 10 min. The identification of the phenylhydantoin amino acids was performed on an Altex Ultrasphere-ODS column (internal diameter, 4.5 mm; length, 250 mm) protected with a precolumn of the same material (internal diameter, 4.5 mm; length, 45 mm) with an Altex high-performance liquid chromatography instrument (model 332) equipped with a microprocessor. On the mobile phase, a gradient of two solvents was used: A, 5% tetrahydrofuran in 5.57 mM acetate buffer (pH 5.0); and B, acetonitrile-tetrahydrofuran (90:10). Each sequence was repeated at least twice.

Antisera. Rabbit anti-BCG Copenhagen immunoglobulin was kindly provided by DAKO Immunoglobulins, Copenhagen, Denmark (code B124, lot 068 B), and used throughout the experiments. Antisera against other mycobacteria and *N. asteroides* have been described previously (10, 11). Production of anti-MPB70 is described elsewhere (12). Anti-MPB64 was produced as a part of the present study by the same technique previously used to make anti-MPB70.

CIE. CIE was carried out on glass plates (5 by 7 cm) as described in detail previously (2, 3, 9), and an intermediate gel was used in all experiments. The analysis and designation of individual precipitate lines were made as in the BCG

reference system (2). The line given by MPB70 in the BCG Tokyo pattern has been localized, and the protein is referred to as BCG antigen 70 (12). Similarly, the MPB80 protein is referred to as BCG antigen 80.

RIA. Protein A-containing staphylococci were used as the solid phase to separate antibody-bound antigen from free antigen by centrifugation (13), and the technical setup for demonstration of antibody activity against mycobacterial antigens, cross-reactivity, and the RIA inhibition assay have all been described previously (12).

Immunofixation and autoradiography. Imprint immunofixation was performed by method of Vartdal and Vandvik (24). Thin-gel plates were made of 1.2% agarose (Litex HSB, Glostrup, Denmark) in barbiturate buffer (pH 8.6) on plastic sheets (GelBond film; FMC Corp., Marine Colloids Div., Rockland, Maine) and were kept in a humid chamber until they were to be used. After two-dimensional PAGE of BCG Tokyo culture fluid, the proteins in the acrylamide gel were transferred to the agarose plates by sequential gel-to-gel overlays, each for 3 min. After imprinting, the polyacrylamide gel was stained with Coomassie brilliant blue (CBB) to serve as a reference for the protein spots. Protein imprints in the agarose gels were chemically fixed by incubating the gels for 30 min in a solution of 11.5% trichloroacetic acid and 2.9% sulfosalicylic acid in 30% methanol-water. The agarose gels were washed twice in 0.3 M phosphate-buffered saline (pH 7.6), incubated overnight at 4°C in anti-MPB70 rabbit antiserum diluted 1:1,000, washed in phosphate-buffered saline (15 min), and pressed (10 min) three times between filter papers to remove unbound antibodies. The gels were subsequently incubated in ^{125}I-labeled, affinity-purified sheep anti-rabbit immunoglobulin G for 2 h, washed and pressed as above, and dried. Autoradiography was carried out on Kodak X-Omat S film for 67 h at −70°C.

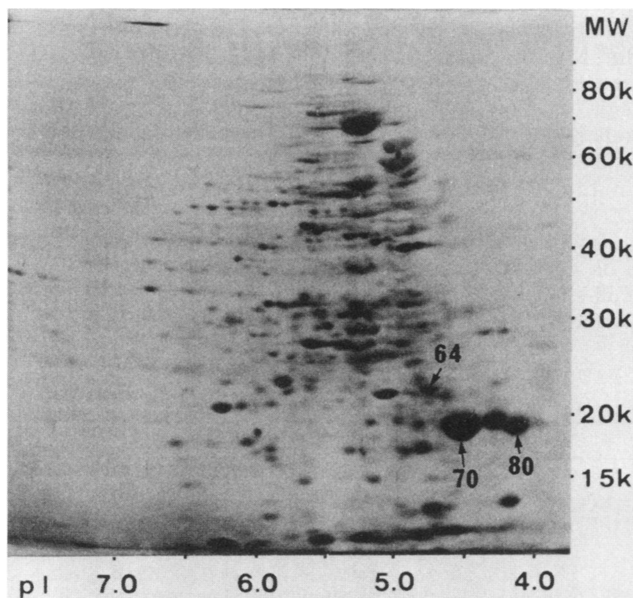

FIG. 1. Two-dimensional PAGE pattern of BCG Tokyo culture fluid. Concentrated culture fluid containing 300 μg of protein was applied. After being stained with CBB, the slab was submitted to silver staining. The molecular weight (MW) scale was calibrated with standard proteins, and the scale of the isoelectric point (pI) was calibrated from the pH values 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 MPB64, MPB70, and MPB80 which were confirmed by the isolated proteins.

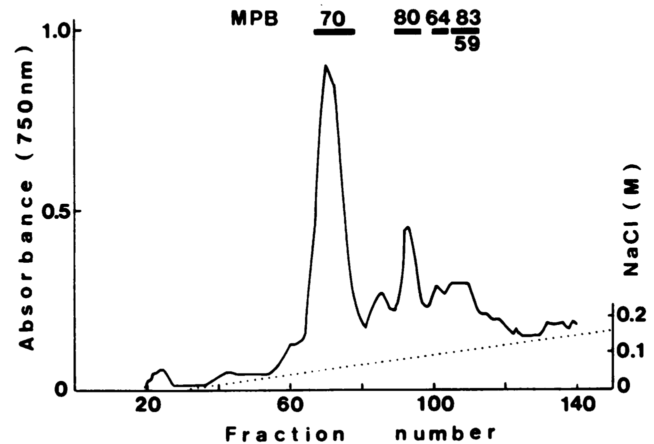

FIG. 2. Separation of the main MPBs through a column of DEAE-Sepharose CL-6B. The bed volume of the column was 475 ml. The applied amount of protein was 5.07 g in 104 ml of solution. For elution, 30 mM Tris buffer (pH 8.7) containing 2% butanol and a linear gradient of 0 to 0.2 M NaCl was used. Total volume of the buffer was 3 liters. A 30-μl sample of each fraction (20 ml) of the eluate was used for analysis of protein content (17) and the relative mobility during PAGE (6). The bars indicate the fractions that contained the respective MPBs.

Sensitization of guinea pigs and skin testing. These methods have been described in detail previously (20). Briefly, guinea pigs were sensitized by intramuscular injection of a suspension of dried mycobacterial cells in liquid paraffin. For testing, MPB70, MPB80, MPB64, or tuberculin purified protein derivative (PPD) (Japan BCG Laboratory, Tokyo, Japan) prepared from heated culture filtrates of *M. tuberculosis* Aoyama B dissolved in 0.9% NaCl without addition of Tween 80 was injected intradermally on a shaved flank of female guinea pigs. The mean diameter of induration was measured 24 h after injection.

RESULTS

MPB64, MPB70, and MPB80 spots shown by two-dimensional PAGE. Figure 1 shows the two-dimensional PAGE pattern of BCG Tokyo culture fluid, and the spots corresponding to MPB64, MPB70, and MPB80 are indicated with arrows and numbered. The MPB70 spot has been identified previously (19). The MPB80 spot also appears as one of the main protein components in the culture fluid. MPB70 and MPB80 showed almost the same molecular weight in the pattern, but a distinct difference was observed in pI, being 4.8 for MPB70 and 4.5 for MPB80. Between MPB70 and MPB80 a spot of an unidentified protein component was also seen. These three spots were pale and somewhat vague when stained with CBB (19), but when stained with silver stain they were prominent, clearly showing the substantial amounts of protein in them. When culture fluids of different species of mycobacteria and different strains of BCG were examined by two-dimensional PAGE, the MPB80 and MPB70 spots were either both missing or occurred together. These findings suggested a close relationship between these two proteins. MPB64 was stained strongly by CBB, giving a very distinct spot by this staining technique after two-dimensional PAGE, in contrast to MPB70 and MPB80,

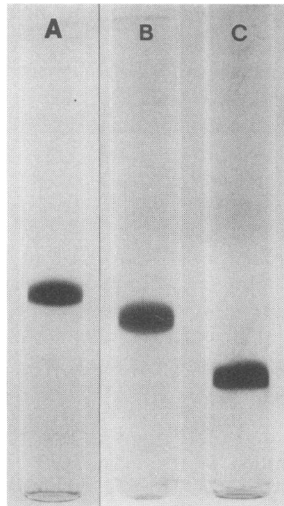

FIG. 3. PAGE illustrating homogeneity of the isolated proteins. Protein (200 μg) (17) was subjected to electrophoresis at a running pH of 9.5 (6, 20), and the gel (7.7% polyacrylamide) was stained with CBB. Lanes: A, MPB64; B, MPB70; C, MPB80.

which were more weakly stained by CBB than by silver staining.

Purification of MPB80 and MPB64 to homogeneity. During purification, MPB70 and MPB80 were clearly separated by passage through the DEAE-Sepharose column. Figure 2 shows that MPB80 was eluted at a higher concentration of NaCl as the second-largest peak after MPB70. To remove minor contaminants from the MPB80-containing fractions, some additional steps were needed compared with purification of MPB70. The amounts of the final preparations of MPB80 and MPB64 were about 1/10 and 1/100, respectively, of the amount of MPB70 obtained from the same culture fluid. Figure 3 shows that each of these three proteins gave a single band upon PAGE. The preparation of MPB64 shown in Fig. 3 was used for the immunization of two rabbits. The resulting antisera gave a single, distinct precipitate line against the protein used for immunization as well as against concentrated culture fluid of BCG Tokyo. Thus both PAGE and CIE indicated that MPB64 had been purified to homogeneity. The protein was called MPB64 on the basis of its relative mobility of 0.64 during PAGE under the conditions specified in Materials and Methods. The terms MPB70 and MPB80 were originally constructed on the same basis, these two proteins having relative mobilities of 0.70 and 0.80, respectively (20). The number 70 had not been used previously in the BCG reference system. For the sake of simplicity and conformity with previous use, the line given by MPB70 in the BCG Tokyo CIE pattern was therefore denoted 70, and the protein was referred to as BCG antigen 70 (12). Similarly, MPB80 is referred to as BCG antigen 80. A component present in the bacterial press extract of BCG Copenhagen giving a distinct precipitate line with anti-BCG Copenhagen was given a number 64 in the BCG reference system described by Closs et al. (2). It is stressed that this component, BCG antigen 64, does not correspond to the MPB64 detected as a major spot in the two-dimensional PAGE pattern of BCG Tokyo and described in this paper.

Physicochemical properties. MPB70 and MPB80 were first compared. The sedimentation coefficient ($s_{20,w}$) of MPB80 was 1.94, the same value as for MPB70 (20). The molecular weights of MPB70 and MPB80 were the same by sodium dodecyl sulfate-PAGE analysis which provided a value of 18,000. By sedimentation equilibrium analysis, the molecular weights were slightly different, 15,100 and 15,600 for MPB70 and MPB80, respectively. Because of the similarity in their molecular weights, the difference in relative mobility during PAGE (Fig. 3) appears to be a reflection of the charge difference of the two proteins. The pI of MPB80 was 4.5, compared with 4.8 for MPB70 (Fig. 2). The amino acid composition of MPB80 was determined and compared with the data previously published for MPB70 (20). The values obtained were very similar indeed, with no significant differences. The slightly more acidic nature of MPB80 might be explained by charge differences in the side chains of some amino acids; some of the residues of glutamine or asparagine in MPB70 might be changed to glutamic acid or aspartic acid in MPB80. Postsynthetic changes of these kinds would not be reflected in the analysis of the amino acid composition. Table 1 shows the amino acid sequence for the first 30 residues from the amino-terminal end, which was identical in MPB70 and MPB80. Using 10 mg of the lyophilized preparation of MPB80, we analyzed its sugar content. There was no significant amount of neutral sugars and hexosamines since the content was less than 0.06 and 0.07%, respectively. These results are the same as for MPB70 (20). Treatment with neuraminidase did not affect the mobility of MPB70 or MPB80 during PAGE. MPB70 and MPB80 thus showed extensive similarities in physicochemical properties. The only difference observed was a charge difference between the two proteins, which gave two clearly distinct spots upon two-dimensional PAGE (Fig. 1) and completely separated positions in the elution pattern (Fig. 2). By contrast, MPB64 showed several distinctly different properties. The molecular weight of MPB64 was estimated to be 23,000 on the basis of sodium dodecyl sulfate-PAGE, a value distinctly higher than the molecular weight of 18,000 found by the same technique for MPB70 and MPB80. Table 1 shows the amino acid sequence for the first 30 residues from the amino-terminal end, which was identical in MPB70 and MPB80. On the other hand, there was no identical residues between MPB64 and MPB70 up to position 30. Thus, there is no homology in the N-terminal amino acid sequence of MPB64 and MPB70.

Immunological relationship between MPB70 and MPB80. MPB80 was precipitated by anti-MPB70 antisera, giving a bell-shaped precipitate with similar appearance to MPB70 in

TABLE 1. N-terminal amino acid sequence of MPB64, MPB70, and MPB80

Proteins	Amino acid sequence								
	1				5				
MPB64	Ala	Pro	Lys	Thr	Tyr	His/Arg	Glu	Glu	
MPB70 and MPB80	Gly	Asp	Leu	Val	Gly	Pro	Gly	Val	
		10					15		
MPB64	Leu	Lys	Gly	Gly/Thr	Asp	Thr	Gly	Gln	
MPB70 and MPB80	Ala	Glu	Tyr	Ala	Ala	Ala	Asn	Pro	
				20					25
MPB64	Ala	Tyr	Gln	Ile	Gln	Met	Ser	Asp	Pro
MPB70 and MPB80	Thr	Gly	Glu	Ala	Ser	Val	Gln	Gly	Met
					30				
MPB64	Ala	Tyr	Asn	Ile	Asn				
MPB70 and MPB80	Glu	Gln	Asp	Pro	Val				

TABLE 2. Comparison of skin test reactivity of MPB70, MPB80, and PPD

Protein concn (μg/0.1 ml)[a]	Diam (mm) of induration 24 h[b] after injection in guinea pigs sensitized with:							
	BCG Tokyo (n = 6)			*M. tuberculosis* H37Rv (n = 6)			Not sensitized (n = 4)	
	MPB70	MPB80	PPD	MPB70	MPB80	PPD	MPB80	PPD
0.025	—[c]	—	12.5 ± 1.8	—	—	12.1 ± 2.0	—	—
0.05	—	—	13.7 ± 0.7	—	—	12.8 ± 1.6	—	—
0.1	—	—	15.7 ± 0.9	—	—	14.3 ± 2.0	—	2.0 ± 1.4
0.2	—	13.7 ± 3.1	—	—	—	—	—	—
0.4	10.8 ± 1.5	14.8 ± 1.7	—	—	—	—	—	—
0.8	12.5 ± 1.3	17.0 ± 1.3	—	—	—	—	—	—
1.6	14.8 ± 2.4	—	—	—	10.8 ± 4.5	—	—	—
3.2	—	—	—	4.3 ± 1.2	11.5 ± 2.1	—	—	—
6.4	—	—	—	5.3 ± 1.6	14.5 ± 3.5	—	0.7 ± 0.9	—
12.8	—	—	—	7.3 ± 2.3	—	—	—	—

[a] For MPB70 and MPB80, the values were calculated from the amount of nitrogen determined by the Kjeldahl method, and for PPD, the values were calculated from the amount corresponding to the standard potency.
[b] No reaction was observed 3 h after injection.
[c] —, Not done.

CIE but with a slight difference in electrophoretic mobility as expected from their different mobility during PAGE. The proteins gave a reaction of identity in double diffusion tests. Due to the low concentration of MPB80, it did not give a separate peak in CIE of BCG Tokyo culture fluid and is probably contained within the huge peak of MPB70 (Fig. 1D in reference 12). RIA inhibition tests were then made with our most potent anti-MPB70 antiserum and labeled isolated MPB70. MPB70 and MPB80 had equal inhibiting capacity in this system. When labeled MPB80 was used with three other anti-MPB70 antisera, the same finding was made. Further, labeled MPB80 was used in experiments with antibodies against other species of mycobacteria to test for cross-reactions, and the same findings were made as in previous experiments with labeled MPB70 (12). Thus, with the presently available antibodies, we could not detect any antigenic differences between MPB70 and MPB80.

Figure 4 shows the results of autoradiography after transfer of protein by the blotting of a two-dimensional polyacrylamide gel onto an agarose gel reacted with anti-MPB70 and ^{125}I-labeled, affinity-purified sheep anti-rabbit immunoglobulin. Three large spots are seen, corresponding to the positions of MPB70 and MPB80 and that of the intermediate spot shown in another two-dimensional PAGE gel in Fig. 2. A vague radioactive spot was observed to the right of MPB80, but this spot was not reproducible. These three major spots were consistently found in blotting experiments with antisera from four different rabbits immunized with MPB70 purified to homogeneity and found to be monospecific by extensive CIE experiments. These experiments showed that MPB70, MPB80, and the protein giving the spot between these in the two-dimensional PAGE pattern are, if not identical, immunologically closely similar. The delayed-type skin reactivity of MPB80 was compared with that of MPB70 and PPD in guinea pigs sensitized with dried cells of either BCG or *M. tuberculosis* H37Rv in oil suspension (Table 2). MPB80 showed a marked specificity like that of MPB70 (20) in guinea pigs sensitized with BCG, with somewhat stronger reactions than with MPB70, and some weak reactions were observed in the guinea pigs sensitized with *M. tuberculosis*. MPB80 is present in *M. tuberculosis* culture fluid but only at a low concentration, and we presume there is a very low content of MPB80 in the cells, as is true for MPB70 in BCG Tokyo (12). Therefore, the immunogenicity and antigenicity of MPB80 in delayed-type hypersensitivity reactions appear to be stronger than those of MPB70.

Immunological properties of MPB64. Immunization with purified MPB64 resulted in rapid production of antibodies detectable by RIA. The anti-MPB64 antibody precipitated the corresponding antigen in double diffusion tests and in CIE. Figure 5 shows the identification of the MPB64 precipitate line in CIE of BCG Tokyo culture fluid. In Fig. 5A, the upper gel contained anti-BCG Copenhagen and showed the basic precipitate pattern corresponding to components which are present in both BCG Tokyo and BCG Copenhagen. In Fig. 5B, the top gel contained both anti-BCG Copenhagen and anti-MPB64, and the additional precipitate line observed thus corresponds to the MPB64 antigen.

The immunological relationship between MPB64 and

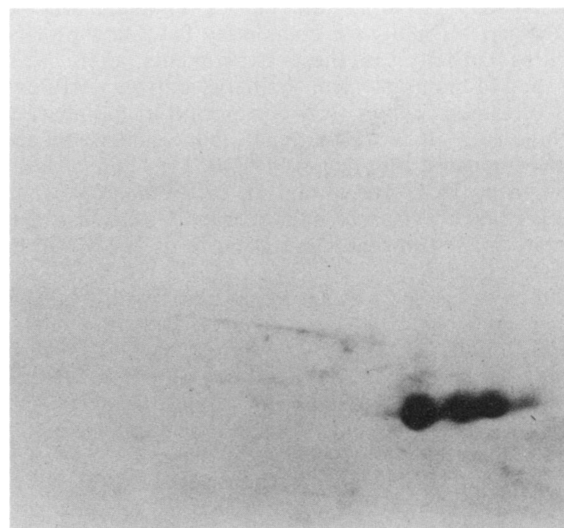

FIG. 4. Autoradiogram after immunoblotting. After two-dimensional PAGE of BCG Tokyo culture fluid, the protein in the acrylamide gel was imprinted to a thin plate of agarose gel. After fixation, the agarose plate was incubated with anti-MPB70 rabbit antiserum and ^{125}I-labeled, affinity-purified sheep anti-rabbit immunoglobulin G, followed by autoradiography.

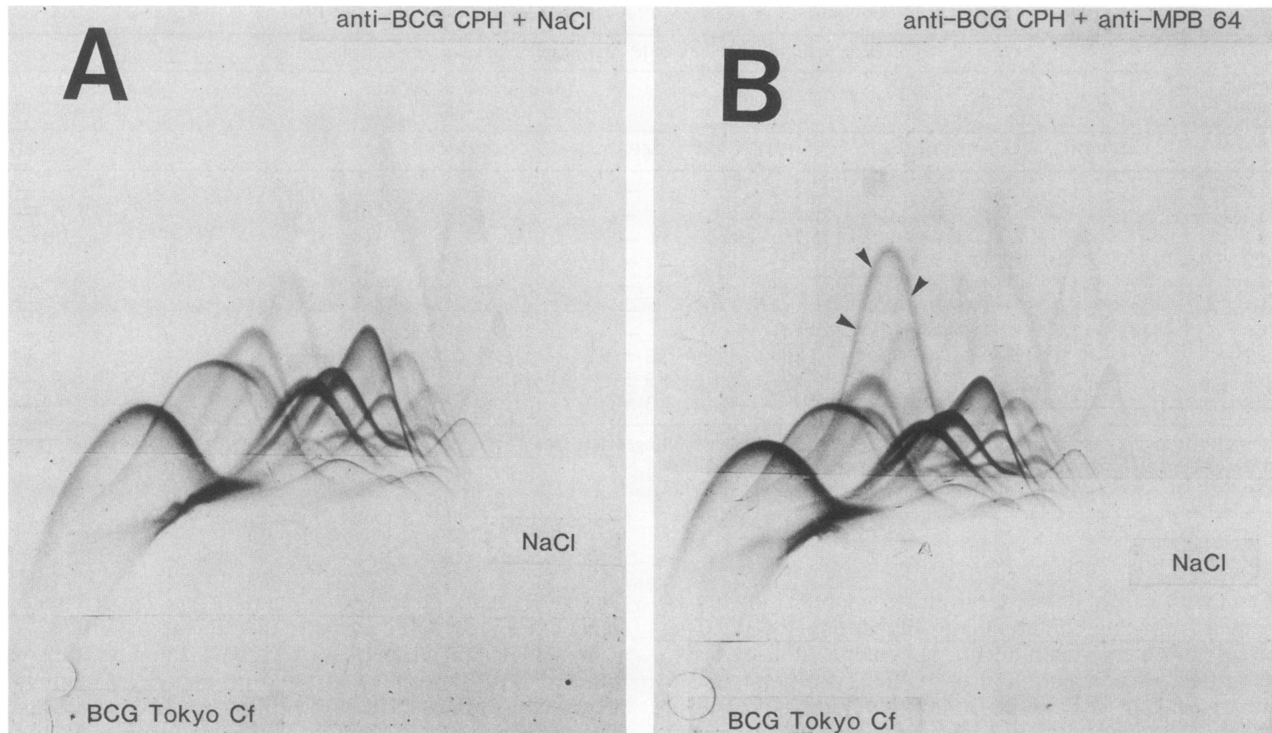

FIG. 5. Identification of the MPB64 line in BCG Tokyo culture fluid in the CIE pattern. (A) Anti-BCG Copenhagen (CPH) plus NaCl; (B) anti-BCG Copenhagen plus anti-MPB64 in the top gel.

MPB70 was tested by RIA inhibition assays. In the RIA based on the use of labeled MPB70 and anti-MPB70, unlabeled purified MPB70 had a marked inhibiting capacity as expected, whereas no inhibition was obtained by addition of purified MPB64. The findings made in RIA based on the use of labeled MPB64 and anti-MPB64 are shown in Fig. 6. In this system, unlabeled purified MPB64 had a marked inhibiting capacity, whereas the curves obtained with both MPB70 and MPB80 were completely flat, showing lack of inhibiting capacity. In these experiments, there was no indication of immunological similiarity between MPB64 and MPB70. These findings thus correspond to the absence of any homology in the N-terminal amino acid sequence of MPB64 compared with that of MPB70. The figure also shows inhibition by BCG Tokyo and *M. tuberculosis* Aoyama B culture fluids and lack of inhibition by *M. kansasii* culture fluid due to the presence and absence of MPB64 in these species, respectively.

Binding of labeled antigen by antisera against other species of mycobacteria is a sensitive indicator of cross-reactivity. Labeled MPB64 was therefore tested for reactivity with antibodies raised against MPB64, MPB70, two substrains of BCG, six different species of mycobacteria, and *N. asteroides*. All antisera were used diluted to 1:10, and the data given in Table 3 represent the percentage of total added labeled MPB64 bound by antiserum in this dilution. The top row shows that the pre-immune samples of two rabbits later immunized with MPB64 bound less than 2% of the labeled antigen. After being immunized for the third time, the samples bound 67 and 61% of the labeled antigen, whereas two anti-MPB70 antisera bound less than 2% of the labeled MPB64. Four antisera against BCG Tokyo showed marked reactivity, whereas some of the antisera against BCG Copenhagen showed low but significant binding of labeled MPB64 corresponding to the occurrence of small amounts of MPB64 in this substrain of BCG (cf. Table 5). The experiment gave no indication of cross-reactivity with the other six species of mycobacteria, whereas binding of 11% of labeled MPB64 by anti-*N. asteroides* is a definite indicator of cross-reactivity with this species. These findings are very similar to those made previously with MPB70 (12).

To study the reactivity of MPB64 in cell-mediated immune reactions, guinea pigs sensitized with five different species of mycobacteria were tested for delayed-type hypersensitivity by skin testing with MPB64 and tuberculin PPD. MPB64 and PPD induced reactions of similar strength in guinea pigs sensitized with BCG Tokyo or *M. tuberculosis* H37Rv (Table 4). In animals sensitized with *M. tuberculosis* Aoyama B, the reaction to MPB64 was weaker but distinctly positive. In contrast, no significant reaction to MPB64 was observed in guinea pigs sensitized with *M. kansasii* or *M. intracellulare*. Thus, in tests for cell-mediated immune reactions, evidence for occurrence of MPB64 in *M. tuberculosis* and limited cross-reactivity was also obtained.

Occurrence of MPB64, MPB70, and MPB80 in different substrains of BCG and species of mycobacteria. Table 5 summarizes data from biochemical and immunological studies of *M. bovis* Ravenel, seven different substrains of BCG, two strains of *M. tuberculosis, M. kansasii,* and *M. intracellulare*. In these strains, the MPB70 and MPB80 spots were either both missing or occurred together. These findings obtained by two-dimensional PAGE corresponded closely to the findings obtained by RIA inhibition tests with labeled MPB70 or labeled MPB80 with anti-MPB70. In the various substrains of BCG, occurrence or absence of the

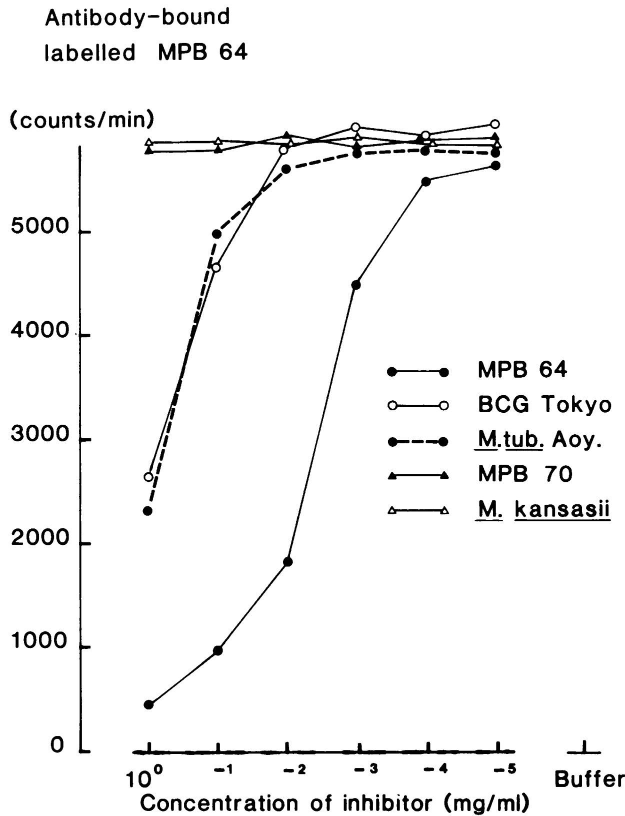

FIG. 6. RIA inhibition test illustrating antigenic nonidentity of MPB64 and MPB70. Serial dilutions of MPB64, MPB70, and three culture fluids were tested for inhibiting capacity. They were mixed with ^{125}I-labeled MPB64, a constant dilution of rabbit anti-MPB64 antiserum, and then protein A-containing staphylococci. The radioactivity in the bacterial pellet was counted. The control value without inhibitor (●) is shown above the section of the horizontal axis marked "Buffer."

MPB64 spot corresponded to the behavior of the MPB70 spot. This was also true of the tests with the other species of mycobacteria with the exception of *M. tuberculosis*, in which the MPB64 spot was distinct in two different strains whereas the MPB70 spot did not occur in these two strains.

The MPB64 content in these strains was then investigated by the RIA inhibition assay with labeled MPB64 and anti-MPB64. The culture fluids fell into two entirely distinct groups, the first with a marked inhibiting capacity in the test and the second with very low or no inhibiting capacity at all in this test system. There was a complete correspondence between the findings by the RIA inhibition test and by two-dimensional PAGE. The culture fluids with inhibiting capacity in RIA all showed a distinct MPB64 spot upon two-dimensional PAGE, and this spot was absent in the culture fluids with very low or no inhibiting capacity in the RIA. Anti-MPB64 antibody was then incorporated into agarose gels, followed by testing of the same culture fluids by rocket immunoelectrophoresis. The results in this assay corresponded fully to the findings in the RIA inhibition assay and two-dimensional PAGE.

Table 5 also shows that there is a striking correlation in the occurrence of the MPB64 and the MPB70 spots. In 10 of the 12 culture fluids, these two proteins occurred together or were both absent, the notable exceptions being two strains of *M. tuberculosis* giving markedly positive reactions for MPB64, whereas the MPB70 content was very low in RIA inhibition, and there was no MPB70 spot upon two-dimensional PAGE.

DISCUSSION

Isolation of several individual components from mycobacterial cells or culture fluid has been described by many authors, but purification to homogeneity has met with considerable difficulties (4, 5, 18, 20, 21, 23). The culture fluids contain a wide variety of different components, resulting in low yields in most instances when purification of a given component is attempted. Further, there is often a tendency toward molecular interactions between different components, frequently resulting in contamination of a selected component with other constituents of the culture fluid. Heating procedures should not be used to avoid these interactions since they lead to extensive changes of the antigenicity of several components (9). Addition of urea to the buffers reduces this tendency toward molecular interactions (21), greatly facilitating purification of MPB70 from unheated BCG Tokyo culture fluid (20). This principle was therefore also used in the presently described procedures for purification of MPB64 and MPB80. The use of butanol in some steps facilitated purification for the same reason. MPB64 was found to be more unstable than MPB70 and MPB80, but with the inclusion of 2% butanol or 3 M urea in the buffers very effective separation from other components was obtained.

Different, independent criteria for purification to homogeneity should be and were fulfilled. Exemplified by MPB64, the preparation gave a single band upon PAGE (Fig. 3). Further, upon two-dimensional PAGE it gave a single spot in a position corresponding to that of MPB64 in Fig. 1. By immunization of two rabbits with the isolated protein, antibodies were obtained which gave a single precipitate line upon CIE or BCG Tokyo culture fluid. Various physicochemical properties and the amino-terminal amino acid sequence were determined as described above, but due to a 0.1% yield of the total protein content in culture fluid, we were unable to perform additional analyses on MPB64 to determine the

TABLE 3. Binding of ^{125}I-labled MPB64 by rabbit antisera

Antibody	% Binding of labeled MPB64 by serum no.[a]			
	1	2	3	4
Normal rabbit serum[b]	<2	<2		
Anti-MPB64[c]	67	61		
Anti-MPB70	<2	<2		
Anti-BCG Tokyo	23	62	28	40
Anti-BCG Copenhagen	5	3	4	8
Anti-*M. avium*	3			
Anti-*M. leprae*	<2			
Anti-*M. lepraemurium*	<2			
Anti-*M. smegmatis*	<2			
Anti-*M. phlei*	3			
Anti-*M. nonchromogenicum*	2			
Anti-*N. asteroides*	11			

[a] Sera from individual animals were diluted 1:10.
[b] These were pre-immune samples of two rabbits later immunized with MPB64.
[c] Same as described in footnote *b* except the sample was immunized a third time.

TABLE 4. Skin test reactivity of MPB64 and PPD in guinea pigs sensitized with five species of mycobacteria

Antigen	Protein concn (μg/0.1 ml)[a]	Diam (mm) of induration 24 h[b] after injection in guinea pigs sensitized with organism shown					
		BCG Tokyo (n = 11)	M. tuberculosis H37Rv (n = 9)	M. tuberculosis Aoyama B (n = 6)	M. kansasii (n = 7)	M. intracellulare (n = 7)	None (n = 4)
MPB64	0.025	—[c]	12.6 ± 2.5	—	—	—	—
	0.05	11.7 ± 3.4	14.8 ± 2.4	—	—	—	—
	0.1	13.4 ± 3.1	16.3 ± 2.1	—	—	—	—
	0.2	14.4 ± 2.9	—	—	—	—	—
	0.4	—	—	7.8 ± 2.6	—	—	—
	0.8	—	—	8.8 ± 3.3	2.8 ± 0.9	—	—
	1.6	—	—	11.8 ± 3.2	2.4 ± 0.5	2.6 ± 0.7	—
	3.2	—	—	—	2.6 ± 0.7	3.7 ± 1.3	—
	6.4	—	—	—	—	4.6 ± 1.2	2.0 ± 1.4
PPD[d]	0.025	10.9 ± 2.2	10.1 ± 1.5	11.9 ± 1.8	—	—	—
	0.05	13.1 ± 1.9	12.7 ± 1.4	12.5 ± 1.9	—	—	—
	0.1	14.6 ± 1.4	13.2 ± 2.8	15.4 ± 1.4	11.3 ± 1.3	7.7 ± 0.9	—
	0.2	—	—	—	12.6 ± 0.5	10.0 ± 1.1	—
	0.4	—	—	—	14.1 ± 0.6	11.4 ± 0.7	2.0 ± 1.2

[a] Protein concentration for MPB was calculated from the amount of nitrogen determined by the Kjeldahl method; that for PPD was calculated from the amount corresponding to the standard potency.
[b] No reaction was observed 3 h after injection.
[c] —, Not done.
[d] PPD was from *M. tuberculosis* Aoyama B.

molecular weight by sedimentation equilibrium ultracentrifugation and the sugar content.

The similarities between MPB70 and MPB80 are striking. No significant differences were found in molecular weight or amino acid composition, and the N-terminal amino acid sequence is identical for the first 30 residues. These similarities in physicochemical properties are paralleled by the immunological studies. In the tests carried out so far with monospecific anti-MPB70 antibodies, the two proteins were indistinguishable, and in immunoblotting experiments four different anti-MPB70 antisera reacted both with the MPB70 and the MPB80 spot as well as with a third protein situated in between these two (Fig. 4). By contrast, MPB70 and MPB80 have distinctly different electrophoretic mobilities. Similar features are evident at various sites in the two-dimensional PAGE pattern shown in Fig. 1. In many instances several distinct spots are aligned in a fashion suggesting microheterogeneity in a protein revealed by distinct differences in electrophoretic mobility but similar if not identical molecular weight. This pattern has been observed upon two-dimensional PAGE of concentrated culture fluids of many different species of mycobacteria (S. Nagai, unpublished observations) and several other complex biological fluids, e.g., human serum.

Microheterogeneity resulting in different electrophoretic mobility may be induced by different mechanisms. Various degrees of glycosylation, and particularly in the amount of sialic acid, are a frequent cause in proteins synthesized by eucaryotic cells but cannot explain the charge difference between MPB70 and MPB80, since significant amounts of neutral and amino sugars have not been found in these two proteins, and treatment with neuraminidase did not affect the electrophoretic mobility of MPB70 or MPB80.

Charge differences in the side chains of amino acid residues are a typical result of postsynthetic changes occurring after release of homogeneous protein products from procaryotic and eucaryotic cells. This appears to be the simplest explanation for the differences observed between MPB70 and MPB80. Alternatively, small peptides might be split off from the parent molecule by enzymes active inside the mycobacterial cell or in the culture fluid.

The observations with MPB70 and MPB80 underline the importance of combining biochemical and immunological techniques to establish the structural relationship between proteins giving different bands in one-dimensional PAGE

TABLE 5. Occurrence of MPB70, MPB80, and MPB64 in culture fluids of mycobacteria

Culture fluids	Occurrence as indicated by following method for:				
	MPB70		MPB80	MPB64	
	2D-PAGE[a]	RIA inhibition[b]	2D-PAGE[a]	2D-PAGE[a]	RIA inhibition[c]
M. bovis Ravenel	+	1.5	+	+	+
BCG Tokyo	+	1.0	+	+	+
BCG Moreau	+	0.7	+	+	+
BCG Russia	+	0.7	+	+	+
BCG Sweden	+	0.5	+	+	+
BCG Glaxo	−	0.02	−	−	−
BCG Tice	−	0.01	−	−	−
BCG Copenhagen	−	0.007	−	−	−
BCG Pasteur	−	0.006	−	−	−
M. tuberculosis H37Rv	−	0.008	−	+	+
M. tuberculosis Aoyama B	−	0.005	−	+	+
M. kansasii	−	<0.001	−	−	−
M. intracellulare	−	<0.001	−	−	−

[a] + and −, Occurrence and absence, respectively, of a distinct spot at the same site as the MPB70, MPB80, or MPB64 spots, respectively, in two-dimensional PAGE (2D-PAGE) of BCG Tokyo culture fluid. All culture fluids were tested directly after concentration by ammonium sulfate precipitation at 75% saturation.
[b] The values indicate relative concentrations in RIA inhibition test with ^{125}I-labeled MPB70 and anti-MPB70 referring to BCG Tokyo and the cultures fluids being tesed at equal total protein concentrations. Data are from Harboe and Nagai (12).
[c] +, Distinct inhibition; −, lack of inhibition of binding of ^{125}I-labeled MPB64 by anti-MPB64. The findings obtained by this test corresponded completely with findings obtained by rocket immunoelectrophoresis in anti-MPB64-containing agarose gel.

and different spots in two-dimensional PAGE. The relationship between MPB64 and MPB70 is complex and of the greatest interest. Structurally, we found no evidence of similarity. The amino acid sequence showed no homology in the first 30 residues from the amino-terminal end (cf. Table 3). Further, antisera against MPB70 showed no evidence of reactivity with MPB64 and vice versa. MPB64 and MPB70 shared the property of being markedly species specific. In our experience, this is quite unusual. It is well established that polysaccharides, such as arabinogalactan and arabinomannan, show extensive cross-reactivity between different species of mycobacteria. The same is true of many protein constituents of mycobacteria (3, 10), and when species-specific reactivity is sought, this is much easier to obtain at epitope than at component level. In fact, it is questionable whether species-specific protein components have been demonstrated at all when sufficiently strict criteria are used for their definition. MPB70 is quite close to a species-specific protein (12, 19, 20); MPB64 behaves similarly but is shared between *M. bovis* BCG and *M. tuberculosis*. Cross-reactivity with *N. asteroides* occurred with both proteins and is reminiscent of a similar, strange cross-reactivity between *Mycobacterium leprae* and *Nocardia* species (25).

The significance of the similar occurrence of MPB64 and MPB70 in different substrains of BCG should be further studied, as it indicates that both proteins may be under the influence of similar control mechanisms. Lind (16) has previously demonstrated a distinct difference between BCG substrains by double diffusion in gel studies regarding the antigen factor designated "c," defined by a reference system in neighboring wells containing anti-*M. tuberculosis* and *M. tuberculosis* culture fluid. Antigen c was demonstrated in the culture fluids of BCG Sweden, BCG Russia, and BCG Moreau but not in the BCG substrains of Danish and French origin. This pattern concerning the occurrence of antigen c is identical to the data concerning MPB64 shown in Table 2. Whether they are identical cannot be concluded or excluded, due to the incomplete data on physicochemical properties of antigen c. In further studies on antigens of *M. tuberculosis* and BCG, there is an obvious need for exchange between the different investigators of purified antigens and antibodies of defined specificity so that their behavior can be directly compared in presently available detection systems of high resolving power, such as CIE and two-dimensional PAGE, and corresponding blotting systems.

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LITERATURE CITED

1. **Boas, N. F.** 1953. Method for the determination of hexosamines in tissues. J. Biol. Chem. **204**:553–563.
2. **Closs, O., M. Harboe, N. H. Axelsen, K. Bunch-Christensen, and M. Magnusson.** 1980. The antigens of *Mycobacterium bovis* strain BCG, studied by crossed immunoelectrophoresis: a reference system. Scand. J. Immunol. **12**:249–264.
3. **Closs, O., M. Harboe, and A. M. Wassum.** 1975. Cross-reactions between mycobacteria. I. Crossed immunoelectrophoresis of soluble antigens of *Mycobacterium lepraemurium* and comparison with BCG. Scand. J. Immunol. **4**(Suppl. 2):173–185.
4. **Daniel, T. M.** 1978. The isolation by immunoabsorbent affinity chromatography and physicochemical characterization of *Mycobacterium tuberculosis* antigen 5. Am. Rev. Respir. Dis. **117**:533–539.
5. **Daniel, T. M., and B. W. Janicki.** 1978. Mycobacterial antigens: a review of their isolation, chemistry and immunological properties. Microbiol. Rev. **42**:84–113.
6. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
7. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
8. **Elson, L. A., and W. T. J. Morgan.** 1933. Colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J. **27**:1824–1828.
9. **Harboe, M.** 1981. Antigens of PPD, old tuberculin and autoclaved *Mycobacterium bovis* BCG studied by crossed immunoelectrophoresis. Am. Rev. Respir. Dis. **124**:80–87.
10. **Harboe, M., O. Closs, B. Bjorvatn, G. Kronvall, and N. H. Axelsen.** 1977. Antibody response in rabbits to immunization with *Mycobacterium leprae*. Infect. Immun. **18**:792–805.
11. **Harboe, M., O. Closs, K. Svindahl, and J. Deverill.** 1977. Production and assay of antibodies against one antigenic component of *Mycobacterium bovis* BCG. Infect. Immun. **16**:662–672.
12. **Harboe, M., and S. Nagai.** 1984. MPB70, a unique antigen of *Mycobacterium bovis* BCG. Am. Rev. Respir. Dis. **129**:444–452.
13. **Jonsson, S., and G. Kronvall.** 1974. The use of protein A containing *Staphylococcus aureus* as a solid phase anti-IgG reagent in radioimmunoassays as exemplified in the quantitation of α-fetoprotein in normal human adult serum. Eur. J. Immunol. **4**:29–33.
14. **Kronvall, G., G. Bjune, J. Stanford, S. Menzel, and D. Samuel.** 1975. Mycobacterial antigens in antibody responses of leprosy patients. Int. J. Leprosy **43**:299–306.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Lind, A.** 1960. Serological studies of mycobacteria by means of diffusion-in-gel techniques. III. A difference in precipitogenic content found in substrains of BCG. Int. Arch. Allergy **17**:1–9.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
18. **Minden, P., P. J. Kelleher, J. H. Freed, L. D. Nielsen, P. J. Brennan, L. McPheron, and J. K. McClatchy.** 1984. Immunological evaluation of a component isolated from *Mycobacterium bovis* BCG with a monoclonal antibody to *M. bovis* BCG. Infect. Immun. **46**:519–525.
19. **Miura, K., S. Nagai, M. Kinomoto, S. Haga, and T. Tokunaga.** 1983. Comparative studies with various substrains of *Mycobacterium bovis* BCG on the production of an antigenic protein, MPB70. Infect. Immun. **39**:540–545.
20. **Nagai, S., J. Matsumoto, and T. Nagasuga.** 1981. Specific skin-reactive protein from culture filtrate of *Mycobacterium bovis* BCG. Infect. Immun. **31**:1152–1160.
21. **Nagai, S., T. Nagasuga, and J. Matsumoto.** 1980. Tuberculin peptide from culture filtrate of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **121**:551–559.
22. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
23. **Reich, M., L. F. Affronti, and G. L. Wright, Jr.** 1982. Isolation and partial characterization of the most immunologically reac-

tive antigen from *Mycobacterium tuberculosis* H37Ra culture filtrate. Tubercle **63**:99–106.

24. **Vartdal, F., and B. Vandvik.** 1983. Imprint immunofixation of electrofocused immunoglobulins. J. Immunol. Methods **62**: 23–29.
25. **Widebäk, K., G. Kronvall, B. Bjorvatn, O. Closs, and M. Harboe.** 1980. Comparative studies of antigen 21 in *Mycobacterium* and *Nocardia* species: possible taxonomic relationships with *Mycobacterium leprae*. Infect. Immun. **30**:413–420.
26. **Wright, G. L., Jr., and D. B. Roberts.** 1974. Two-dimensional immunoelectrophoresis of mycobacterial antigens. Comparison with a reference system. Am. Rev. Respir. Dis. **109**:306–310.