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Comparative Studies with Various Substrains of *Mycobacterium bovis* BCG on the Production of an Antigenic Protein, MPB70

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A protein, isolated and purified from the unheated culture filtrate of *Mycobacterium bovis* BCG (substrain Tokyo 172) and designated MPB70, elicited a delayed skin reaction in guinea pigs sensitized with viable cells of BCG but not in those sensitized with heat-killed cells. The skin reaction reached the maximum 4 to 8 weeks after the inoculation of the BCG and then decreased gradually, resulting in conversion to negative after 20 weeks, whereas the skin reaction to purified protein derivative (PPD) continued to be positive. Guinea pigs immunized with viable cells of various substrains of BCG were skin tested with MPB70 and PPD. Guinea pigs immunized with the BCG substrain Tokyo 172 and the substrain Moreau (Brazil) showed strong delayed skin reactions to both MPB70 and PPD. On the other hand, guinea pigs immunized with the Pasteur substrain 1173P2, the Glaxo substrain 1077, the Copenhagen substrain 1331, the Tice substrain, or the Beijing substrain 64-42 showed negative skin reactions to MPB70, whereas they were strongly positive to PPD. In a two-dimensional acrylamide gel electrophoretic analysis of proteins from the culture filtrates of the BCG substrains, the culture filtrates of the Tokyo and Moreau substrains showed the spot of MPB70 on the gel slabs, whereas those of the other BCG substrains did not.

A protein (MPB70) has been isolated from an unheated culture filtrate of substrain Tokyo 172 of *Mycobacterium bovis* BCG (BCG-Tokyo), and the properties of this high-yield protein have been studied in detail (10). MPB70 has been reported as a unique, BCG-specific antigen; among guinea pigs sensitized with heat-killed cells of various species of mycobacteria emulsified in liquid paraffin oil, only those sensitized with BCG show strongly positive skin reactions to MPB70 (10).

All of the BCG substrains currently available for vaccination against tuberculosis throughout the world were derived from the strain maintained and distributed by A. Calmette of the Pasteur Institute in Paris. However, different methods of growing, processing, and preserving BCG have resulted in the selection of variants; the various substrains are known to differ in, for instance, their immunogenic and antigenic properties (2, 3, 5, 8, 9), virulence (13, 14), and biological or biochemical characteristics (6, 12).

In this study, we examined delayed-type hypersensitivity (DTH) by the skin reaction to MPB70 in guinea pigs sensitized with various substrains of BCG. During the study, we found that the DTH reaction to MPB70 became positive when guinea pigs were sensitized with viable cells of BCG-Tokyo but not when they were sensitized with heat-killed cells without oil. Accordingly, we immunized guinea pigs with viable cells of various substrains of BCG and tested the skin reactions. The results indicated that only two of seven substrains tested induce positive reactions to MPB70. Furthermore, a two-dimensional electrophoretic analysis of the proteins of the unheated culture filtrates of these BCG substrains also clearly showed that only the same two substrains produce detectable amounts of MPB70.

MATERIALS AND METHODS

Animals. Hartley female guinea pigs weighing 350 to 480 g were grouped five per cage and fed commercial pellets.

BCG. Under the program of the Inter-Four Laboratories Study designed in 1976, the BCG substrains of the Pasteur Institute, Paris (Pasteur substrain 1173P2; BCG-Pasteur), of the Glaxo Laboratories Ltd., London (Glaxo substrain 1077; BCG-Glaxo), and of the Statens Serum Institute, Copenhagen (Copenhagen substrain 1331; BCG-Copenhagen) were sent to the Japan BCG Laboratory, Tokyo. Cells of these sub-

TABLE 1. Delayed skin reaction to PPD or MPB70 in guinea pigs[a] sensitized with BCG-Tokyo

BCG-Tokyo[b]	Delayed skin reaction[c] at (wk after sensitization):						
	4				8		
	PPD (μg)		MPB70 (μg)		PPD (μg)	MPB70 (μg)	
	0.1	1.0	0.1	1.0	0.1	0.1	1.0
Viable BCG (0.5 mg)	12.5 ± 1.7	19.2 ± 1.4	15.7 ± 1.3	20.6 ± 1.6	16.0 ± 0.9	17.2 ± 0.8	20.8 ± 3.0
Heat-killed BCG (10 mg)	10.3 ± 0.5	16.1 ± 1.8	2.0 ± 1.8	4.6 ± 1.1	14.0 ± 2.6	0.9 ± 1.0	2.3 ± 0.8
Dried BCG (0.1 mg) in oil	11.6 ± 1.4	18.1 ± 1.4	7.0 ± 4.4	13.4 ± 2.4	15.8 ± 2.5	15.3 ± 3.3	13.9 ± 3.8

[a] Five guinea pigs were used for each group.
[b] Injected s.c.
[c] Values indicate the mean diameter (millimeters) of induration with the standard deviation, measured 24 h after antigen injection.

strains, in addition to those of the seed-lot substrain of Tokyo 172 (BCG-Tokyo), were grown on the surface of Sauton liquid medium, dispersed, distributed into ampoules at 2.5 mg (semidry weight) per ampoule, and freeze-dried at the Japan BCG Laboratory in September 1976. These lyophilized BCG preparations were provided by T. Sawada (Japan BCG Laboratory).

BCG of the substrains from Chicago (BCG-Tice), from Brazil (BCG-Moreau) and from Beijing (Beijing substrain 64-42; BCG-Beijing) were lyophilized in our laboratory by the same procedures as described above between 1976 and 1980.

Sensitization of guinea pigs with BCG. The lyophilized BCG cells were suspended in physiological saline and diluted adequately, and viable units were counted by incubating cells on Ogawa egg slants. A 0.1-ml quantity of the BCG suspensions, containing a definite weight of cells or a definite number of viable cells, was injected subcutaneously (s.c.) into the hind limbs of guinea pigs. In some experiments, the lyophilized cells suspended in saline were heated at 120°C for 20 min (heat killed) and then injected s.c. In one experiment, the heat-killed cells were dried, emulsified with paraffin oil (dried cells in oil), and injected s.c.

Skin test. Guinea pigs were skin tested on a shaved flank by the intradermal injection of 0.1 ml of purified protein derivative (PPD) (Japan BCG Laboratory, Tokyo), which was prepared from the heated culture filtrate of *M. tuberculosis* Aoyama B, or of MPB70 dissolved in physiological saline. The mean diameter of induration was measured 24 h after injection.

Preparation of culture filtrate. The lyophilized BCG cells were suspended in physiological saline and inoculated onto Ogawa slants. Cells grown on the slants were inoculated into Sauton liquid medium without further passage on Ogawa medium, and the culture filtrates were obtained after 2 to 4 weeks of incubation at 37°C by filtration through a membrane filter with a pore size of 0.2 μm. The filtrates were dialyzed exhaustively against 0.03 M ammonium bicarbonate in cellulose tubing (Visking Co., Chicago, Ill.) at 4°C and then lyophilized.

Two-dimensional electrophoresis. Proteins in the culture filtrate were analyzed by two-dimensional polyacrylamide gel electrophoresis by virtually the same method as that of O'Farrell (11): isoelectric focusing in the first dimension and electrophoresis with sodium dodecyl sulfate (SDS) in the second dimension.

As the carrier Ampholite, 2% Bio-lyte (Bio-Rad Laboratories, Richmond, Calif.), comprised of 1.33% pH range 3 to 10, 0.33% pH range 3 to 5, and 0.33% pH range 5 to 7, was used in glass tubing (inside diameter, 130 by 2.0 mm). After a prerun, the lyophilized preparation of culture filtrate (300 to 400 μg [dry weight]), which was dissolved in lysis buffer (11), was applied on the gel and submitted to electrophoresis at 400 V for 16 h. After the run, the gels were stored in a frozen state. Before electrophoresis in the second dimension, the gels were extruded from the tubes and equilibrated sufficiently in SDS sample buffer (11) to remove the carrier Ampholite, which disturbs the final staining. The gel was loaded on the top of the slab plate and submitted to electrophoresis in the second dimension in the discontinuous SDS-gel system of Laemmli (7) with a slab which had a resolving gel (14.5 by 12 by 0.1 cm) of 10% acrylamide. The electrophoresis was performed at 15 mA for several hours. After staining with Coomassie blue and destaining with methanol-acetic acid solution, the slab was dried and pressed on a filter paper at 90°C.

RESULTS

Skin reaction to MPB70 in guinea pigs sensitized with viable and dead cells of BCG-Tokyo. Guinea pigs were sensitized with BCG-Tokyo by the s.c. injection of either 0.5 mg of viable cells, 10 mg of heat-killed cells, or 0.1 mg of dried cells in oil. At 4 and 8 weeks after sensitization, the animals were skin tested with 0.1 and 1.0 μg of PPD or MPB70. The results are shown in Table 1. PPD elicited typical DTH skin reactions in all of the animals. On the other hand, MPB70 elicited strong DTH skin reactions in animals sensitized with viable cells, but not in those sensitized with heat-killed cells. Guinea pigs sensitized with the dried cells in oil, which was the method of sensitization used in the previous study (10), showed positive reactions to MPB70, but they were weaker than those in the animals sensitized with viable cells. Based on these results, we used viable cells of BCG for the sensitization of animals in further experiments.

Skin reaction to MPB70 in animals sensitized with various substrains of BCG. Guinea pigs were sensitized with 0.5 mg of lyophilized cells of either BCG-Pasteur, BCG-Copenhagen, BCG-Glaxo, or BCG-Tokyo, which had been

TABLE 2. Delayed skin reaction to PPD and MPB70 in guinea pigs immunized with various substrains of BCG

Expt[a]	BCG substrain	Delayed skin reaction[b] to:			
		PPD (0.2 μg) at (wk after sensitization):		MPB70 (0.2 μg) at (wk after sensitization):	
		4	6	4	6
1	Pasteur	17.2 ± 1.9	20.0 ± 0.5	2.2 ± 0.4	6.8 ± 1.1
	Copenhagen	16.2 ± 2.1	21.3 ± 0.6	0	0
	Glaxo	12.6 ± 2.6	16.6 ± 1.1	0	2.6 ± 2.6
	Tokyo	17.0 ± 2.0	16.7 ± 2.5	20.7 ± 2.3	19.3 ± 1.2
	None	0	3.6 ± 1.3	0	1.2 ± 1.1
2	Tice	10.4 ± 2.7	16.5 ± 1.2	0	4.5 ± 3.4
	Moreau	14.5 ± 3.5	18.0 ± 3.0	18.5 ± 0.5	18.3 ± 4.6
	Beijing	6.0 ± 1.0	13.5 ± 3.0	1.7 ± 2.9	1.0 ± 1.7
	Pasteur	20.3 ± 2.8	16.5 ± 0.5	0	5.3 ± 4.7
	Tokyo	12.8 ± 0.8	15.5 ± 1.3	14.2 ± 1.3	14.0 ± 2.3

[a] Guinea pigs (five for each group) were immunized s.c. with 0.5 mg of lyophilized cells of each substrain of BCG in experiment 1 or with 10^4 viable U in experiment 2.
[b] Values indicate the mean diameter (millimeters of induration with the standard deviation, measured 24 h after antigen injection.

prepared under the program of the Inter-Four Laboratories Study in 1976. A 1-mg amount of these lyophilized samples contained 5.2×10^6 viable U in BCG-Pasteur, 1.6×10^5 viable U in BCG-Copenhagen, 6.2×10^5 viable U in BCG-Glaxo, and 6.0×10^7 viable U in BCG-Tokyo. At 4 to 6 weeks after sensitization, the animals were skin tested with 0.2 μg of PPD or MPB70. The results are shown in experiment 1 of Table 2. MPB70 could elicit positive skin reactions only in the animals sensitized with BCG-Tokyo, whereas PPD induced strong skin reactions in all of the animals except the unsensitized controls.

Then we compared the skin reactions to PPD and MPB70 in animals sensitized with the other BCG substrains—BCG-Tice, BCG-Moreau, and BCG-Beijing—with those of animals sensitized with BCG-Pasteur and BCG Tokyo. A 1-ml amount of a suspension of each of the BCG substrains, containing approximately 10^4 viable U, was injected s.c. After 4 to 6 weeks, the animals were skin tested with 0.2 μg of PPD or MPB70. The results are shown in experiment 2 of Table 2. All of the animals showed strong DTH reactions to PPD. On the other hand, strong DTH reactions to MPB70 were observed in the animals sensitized with either BCG-Moreau or BCG-Tokyo, but not in those sensitized with BCG-Tice, BCG-Beijing, or BCG-Pasteur.

Time course of skin reactivity to MPB70 after sensitization with various substrains of BCG. A time course experiment on DTH skin reactivity to MPB70 in animals sensitized with three substrains of BCG was designed. The lyophilized cells of BCG-Tokyo, BCG-Pasteur, and BCG-Glaxo were suspended in physiological saline at a concentration of 1 mg/ml, and 1 ml of each suspension was inoculated s.c. into the hind limbs of separate guinea pigs. At 2, 4, 6, 8, and 20 weeks after the inoculations, guinea pigs were skin tested with 0.1 μg of PPD or MPB70. The results are shown in Table 3. DTH reactions to

TABLE 3. Time course of delayed skin reaction to PPD or MPB70 in guinea pigs immunized with viable BCG of three different substrains

BCG substrain used for immunization[a]	Eliciting antigen (0.1 μg)	Delayed skin reaction[b] at (wk after immunization with BCG):				
		2	4	6	8	20
Tokyo	PPD	9.7 ± 0.6	11.5 ± 4.5	13.0 ± 4.8	15.3 ± 4.4	16.1 ± 1.1
	MPB70	5.5 ± 0.9	10.4 ± 4.9	13.3 ± 0.4	14.2 ± 2.8	10.3 ± 3.3
Pasteur	PPD	11.3 ± 7.5	21.8 ± 1.3	17.5 ± 0.5	17.7 ± 2.1	17.4 ± 0.8
	MPB70	0	3.3 ± 1.2	5.3 ± 4.2	0	2.0 ± 0.1
Glaxo	PPD	7.0 ± 6.2	7.3 ± 4.5	14.2 ± 4.0	16.5 ± 2.2	14.3 ± 1.7
	MPB70	0	2.3 ± 0.6	1.3 ± 1.2	0	1.5 ± 1.9

[a] Guinea pigs (five for each group) were immunized with 1 mg of viable cells of each substrain by s.c. injection, and at the indicated intervals the delayed skin reactions to 0.1 μg of PPD or MPB70 were measured.
[b] Values indicate the mean diameter (millimeters) of induration with the standard deviation, measured 24 h after antigen injection.

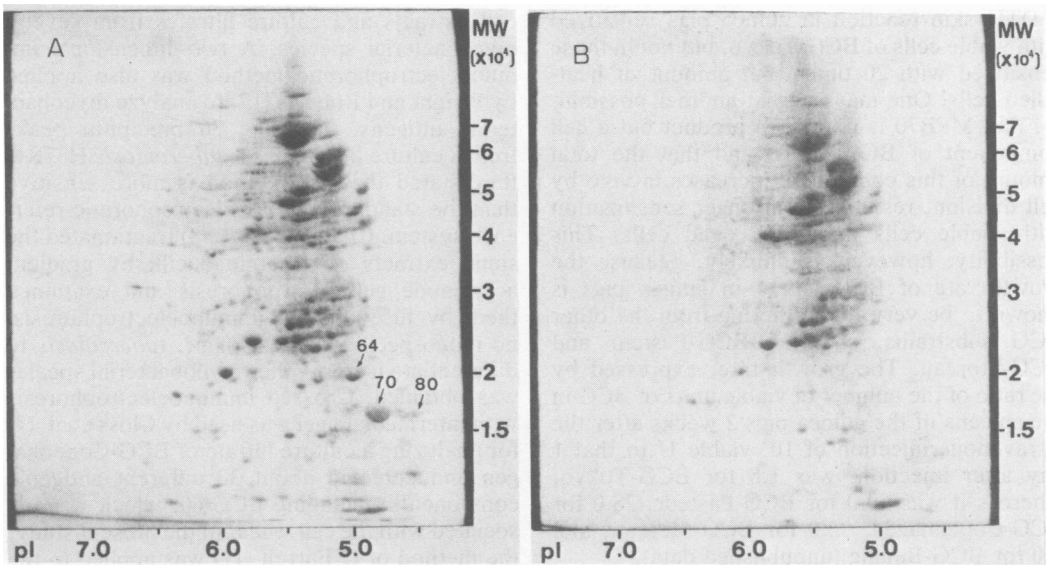

FIG. 1. Two-dimensional electrophoretic patterns of the culture filtrate of (A) BCG-Tokyo and (B) BCG-Pasteur. The scale of the approximate molecular weight (MW) was calibrated with the following standard proteins: bovine serum albumin (molecular weight, 67,000), ovalbumin (molecular weight, 45,000), α-chymotrypsinogen A from porcine pancreas (molecular weight, 25,700), and cytochrome *c* from horse heart (molecular weight, 11,740). The scale of the isoelectric point (pI) was calibrated from the pH values in individual vials, which contained 5-mm sections of the isoelectric focusing gel and 2 ml of degassed water. Arrows indicate the positions of MPBs which were confirmed by the isolated MPB64, -70, and -80.

PPD converted to positive (induration of more than 10 mm in diameter) around 2 weeks after sensitization with BCG-Tokyo, within 2 weeks after sensitization with BCG-Pasteur, and around 4 weeks after sensitization with BCG-Glaxo. On the other hand, the reaction to MPB70 converted to positive only in the guinea pigs sensitized with BCG-Tokyo; in the animals sensitized with either BCG-Pasteur or BCG-Glaxo, it was negative throughout the experimental period. The skin reaction to MPB70 in these guinea pigs decreased gradually with time after about 10 weeks after the BCG inoculation and converted to negative after 20 weeks.

Two-dimensional electrophoresis of the culture filtrates of various BCG substrains. The above results strongly suggested that the two substrains BCG-Tokyo and BCG-Moreau produced and secreted MPB70 in vivo and sensitized the hosts to this protein, whereas the other five substrains did not. To examine this possibility, analysis by two-dimensional polyacrylamide gel electrophoresis was performed on the culture filtrates of these BCG substrains, except BCG-Beijing.

Proteins from the culture filtrates of the BCG substrains generally scattered as about 300 spots on the gel slabs, and about 20 main spots appeared to be common among the BCG substrains. The particular spot of MPB70, however, was detected only in BCG-Tokyo and BCG-Moreau. The culture filtrates of BCG-Tokyo and BCG-Moreau showed two more distinct spots, designated MPB64 and MPB80 (10), but the other BCG substrains did not. Two typical patterns obtained from the culture filtrates of BCG-Tokyo and BCG-Pasteur are shown in Fig. 1. The molecular weights estimated by gel electrophoresis containing SDS (15) were 18,000 for both MPB70 and MPB80 and 23,000 for MPB64.

DISCUSSION

Numerous reports have indicated that purified proteins obtained from mycobacterial cells or their culture filtrates elicit a DTH skin reaction in humans or animals infected with *M. tuberculosis* or BCG. Among those proteins, MPB70 is a unique protein produced by viable cells of BCG-Tokyo and released into the culture filtrate in an amount of more than 10% of the total protein secreted while they are growing (10). The concentration of MPB70 of BCG-Tokyo in the culture filtrate was found to be much higher than that in the cell sonic extracts (M. Harboe, personal communication). It was also evident that a small amount of the protein exists inside the cells, because dried cells of BCG-Tokyo emulsified in paraffin oil sensitized guinea pigs to MPB70 (Table 1). The present data suggest that this is also true in vivo, because MPB70 elicited

a DTH skin reaction in guinea pigs sensitized with viable cells of BCG-Tokyo, but not in those sensitized with 20 times that amount of heat-killed cells. One may suggest another possibility, that MPB70 is not a cell product but a cell component of BCG-Tokyo and that the total amount of this component increases in vivo by cell division, resulting in stronger sensitization with viable cells than with dead cells. This possibility, however, is unlikely, because the growth rate of BCG-Tokyo in guinea pigs is known to be very low, differing from the other BCG substrains, such as BCG-Pasteur and BCG-Moreau. The growth rate, expressed by the ratio of the number of viable units of BCG in the spleens of the guinea pigs 2 weeks after the intravenous injection of 10^4 viable U to that 1 day after injection, was 1.8 for BCG-Tokyo, whereas it was 50.0 for BCG-Pasteur, 28.0 for BCG-Copenhagen, 36.0 for BCG-Moreau, and 1.0 for BCG-Beijing (unpublished data).

Many differences in biological and biochemical characteristics have been reported among the BCG substrains (2, 5, 6, 8, 9, 12–14), although all of the substrains stem from the original Bacille Calmette-Guérin established in the Pasteur Institute, Paris. For the antigenic components, however, only a few papers have discussed such a difference. Lind (8) found, with the double-diffusion-in-gel technique of Ouchterlony, that the preparations made from the cultures of BCG substrains of Copenhagen and Pasteur lacked one antigenic factor (designated c), whereas Swedish, Moreau, and Russian substrains contained this factor. On the other hand, Chaparas and Hedrick (2) reported that unheated culture filtrates from each of 11 substrains of BCG, including BCG-Pasteur, BCG-Tokyo, BCG-Tice, BCG-Glaxo, and BCG-Copenhagen, gave equivalent reactions in homologously and heterologously sensitized guinea pigs. In addition, the results obtained by antigen-antibody immunoelectrophoresis showed that at least 10 of 11 demonstrable antigens were shared in unheated culture filtrates of 12 substrains of BCG (2). Our present data indicate that BCG-Tokyo and BCG-Moreau massively produce at least three antigenic proteins, which are not detected on the gel slabs of the other BCG substrains tested. It is unknown yet whether or not one of these antigens corresponds to the factor c of Lind (8).

Two-dimensional acrylamide gel electrophoresis of a mycobacterial preparation was first used by Augier and Augier-Gibory (1); however, the separation of the components of PPD appeared incomplete. Wright et al. (16) used two-dimensional acrylamide gel electrophoresis in a 2 to 30% linear gradient gel slab, and more than 200 protein-staining spots were detected from cell extracts and culture filtrates from several mycobacterial species. A two-dimensional immunoelectrophoretic method was also applied by Wright and Roberts (17) to analyze mycobacterial antigens, detecting 36 precipitin peaks from a culture filtrate of *M. tuberculosis* H37Rv; they stated that the method is more sensitive than the standard immunoelectrophoretic reference system. Chaparas et al. (3) fractionated the sonic extracts of tubercle bacilli by gradient acrylamide gel electrophoresis and examined them by fused rocket immunoelectrophoresis; no monospecific antigen for *M. tuberculosis* to differentiate it from other mycobacterial species was obtained. Crossed immunoelectrophoresis with intermediate gel was used by Closs et al. (4) for analyzing a culture filtrate of BCG-Copenhagen and detected about 30 different antigenic components, including BCG-60, which was associated with the cell walls. In the present study, the method of O'Farrell (11) was applied to the analysis of mycobacterial proteins secreted into the culture filtrates of various substrains of BCG. Because of the method's powerful resolving ability, by using each of two different parameters in two dimensions, the proteins in the culture filtrates were clearly differentiated as more than 300 spots on a slab, by Coomassie blue staining.

The spot of MPB70, which was the largest one on the gel slabs in the case of the culture filtrates of BCG-Tokyo and BCG-Moreau, could not be found in either BCG-Pasteur, BCG-Copenhagen, BCG-Glaxo, or BCG-Tice. The culture filtrate of BCG-Beijing also may not produce the spot as judged by skin reaction. Furthermore, two more main spots, which were identified as MPB64 and MPB80 (10), were always accompanied by MPB70 in our analyses of the substrains of BCG.

It should be noted, although the data are not shown, that the culture filtrate of *M. bovis* Ravenel also contained spots at the positions which corresponded to MPB70, MPB64, and MPB80 and that *M. tuberculosis* H37Rv and Aoyama B possessed one spot corresponding to that of MPB64 but not to those of MPB70 or MPB80. In this respect, the pattern of Ravenel was quite similar to that of BCG-Tokyo and BCG-Moreau, although these strains differ greatly in their pathogenicity; Ravenel is highly virulent for guinea pigs and mice, BCG-Moreau is intermediate, and BCG-Tokyo is very weakly pathogenic. Since the original strain of BCG was isolated from a strain of *M. bovis*, it can be expected that the substrains of BCG produce proteins similar to those of Ravenel. It is curious, however, that four substrains of BCG possess no or much less ability to produce MPB70, MPB64, and MPB80, which were found as main

components in the other two substrains and Ravenel. In the case of BCG-Copenhagen, in the culture filtrate and in the ultrasonic extracts of the cells, a very small amount of MPB70 (less than 1% of that of BCG-Tokyo) could be detected by a more sensitive method, using ^{125}I-labeled MPB70 and the anti-MPB70 antibody (M. Harboe, personal communication). It is possible that some or all of the BCG substrains other than BCG-Tokyo and BCG-Moreau produce much smaller amounts of MPB70 or a related protein(s) which induce some reactivity.

Three proteins were discussed in this paper because they were identified with the isolated preparations and clearly defined in the slab patterns, but some other small spots could be differentiated in the patterns of the BCG substrains. Studies on the distribution of the three antigens described in this paper in various mycobacterial species and various strains of *M. bovis* are desirable. The possible role of such a protein released from viable cells in vivo as a protective antigen against infection would be interesting to investigate because viable vaccines are generally more efficient than killed vaccines for inducing cell-mediated immunity.

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