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Protein G-Based Enzyme-Linked Immunosorbent Assay for Anti-MPB70 Antibodies in Bovine Tuberculosis

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MPB70 is a highly species specific protein which is secreted from *Mycobacterium bovis* during culture. To investigate whether antibodies against MPB70 can be used as an indicator of infection with *M. bovis*, an enzyme-linked immunosorbent assay was developed, based on the use of biotinylated protein G, to provide a common indicator for antibody formation in different species. During experimental infection with *M. bovis* in cattle, a characteristic pattern of anti-MPB70 antibody production was observed with an initial flat plateau followed by a marked rise 18 to 20 weeks after infection. Skin testing with bovine tuberculin purified protein derivative (PPD), which was shown to contain antibody-reactive MPB70, was a potent stimulator of antibody production in infected animals. In experimentally infected cattle, we observed an inverse relationship between antibody activity and delayed-type hypersensitivity skin test reactions. In natural *M. bovis* infections, skin testing with PPD was also a potent stimulator of anti-MPB70 formation. Comparison between the enzyme-linked immunosorbent assay for antibodies to MPB70 and that for antibodies to the widely cross-reacting *M. bovis* BCG antigen 85B in animals with *M. bovis*, *Mycobacterium avium*, *Mycobacterium paratuberculosis*, and *Corynebacterium pseudotuberculosis* infections showed that formation of antibody to MPB70 was highly specific for infection with *M. bovis*. The use of an MPB70-containing PPD preparation for skin testing followed by this anti-MPB70 assay is a highly specific indicator of *M. bovis* infection. Adjustment of the test conditions is expected to provide an increased sensitivity of the procedure for the diagnosis of natural *M. bovis* infections.

Demonstration of immune responses to a causative organism is widely used for the diagnosis and evaluation of mycobacterial infections.

Delayed-type hypersensitivity (DTH) reactions are important indicators of mycobacterial infections in humans (43) as well as animals (17, 18). While the DTH reaction is indicative of infection or exposure, it is less informative concerning bacterial multiplication and progression to disease. Because of extensive cross-reactions, these assays also often show insufficient specificities for a given type of mycobacterial infection (17, 18).

Antibody formation occurs regularly in response to mycobacterial infections and often appears to be more closely related to the extent of bacterial multiplication and antigenic load in the infected individual (6, 15, 29). The lack of specificity has been a major problem in serological assays. The major mycobacterial polysaccharides, arabinomannan and arabinogalactan, cross-react extensively in different mycobacterial species (14), but the phenolic glycolipid 1 of *Mycobacterium leprae* is highly specific for this species. A terminal trisaccharide is immunodominant in this macromolecule, and synthetic analogs coupled to bovine serum albumin provided the basis for the development of enzyme-linked immunosorbent assays (ELISAs) for corresponding antibodies (4). Immunoglobulin M (IgM) anti-phenolic glycolipid 1 antibodies occur regularly in patients with leprosy, showing a definite relationship to the extent of bacterial multiplication and antigenic load in the infected individual (5). A species-specific glycolipid has also been demonstrated recently in *Mycobacterium bovis* (8).

Protein antigens of mycobacteria are also characterized by extensive cross-reactions (11, 22). The general feature is that species specificity can be demonstrated at the epitope level (7) but rarely at the component level, implying that species-specific proteins are very rare in mycobacteria.

The MPB70 protein of *M. bovis* that was purified to homogeneity by Nagai et al. (33) is of great interest. The protein shows a striking species specificity. In antibody assays we could not detect any cross-reaction with several species of mycobacteria, but the protein cross-reacted with a corresponding component in *Nocardia asteroides* (23). MPB70 also induces DTH reactions in skin tests of guinea pigs sensitized with *M. bovis* and the purified protein, but not other mycobacterial species, again demonstrating its species specificity (23–25, 31, 33). Different substrains of *M. bovis* BCG vary markedly in the production and secretion of this protein. High-producer and low-producer strains have been identified by biochemical (31) and immunological (23) techniques, and the delineation of two groups of BCG strains based on these protein patterns corresponds to different mycolic acid patterns (30) and a restriction fragment length polymorphism at the DNA level (13). In high-producer strains, the protein is secreted in large amounts, constituting at least 10% of the total protein content in the culture fluid (33). Active transport of MPB70 across the bacterial cell membrane is also indicated by the occurrence of a typical signal sequence deduced from the cloned gene (37). The pathogenic *M. bovis* Ravenel strain is a high producer (23). Immune responses to MPB70 might therefore be expected to be a sensitive indicator of bacterial growth in vivo after infection and of bovine tuberculosis.

Because of these properties of MPB70, the present investigation was undertaken to explore whether anti-MPB70 antibodies would be a useful indicator of infection with *M.*

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TABLE 1. Groups of animals from which serum or plasma samples were taken

Group	Species	No. of animals	Age range (yr)[a]	Health status and treatment
1	Cattle	15	3–8	Minimal disease herd; healthy animals skin tested with johnin PPD
2	Cattle	4	0.5	Healthy animals skin tested with bovine tuberculin PPD
3	Cattle	4	ND[b]	Sensitized with nonviable *M. bovis* 110 in oil adjuvant; skin tested with bovine tuberculin PPD
4	Cattle	11	0.5	Experimentally infected[c] with *M. bovis* BM228; skin tested four times with bovine tuberculin PPD
5	Cattle	5	0.5	Experimentally infected[d] with *M. bovis* BM228; skin tested twice with bovine tuberculin PPD
6	Cattle	44	1–10	Natural infection with *M. tuberculosis* diagnosed in 11 animals (mostly between 5 and 10 yr old); skin tested with bovine tuberculin PPD
7	Calf/goats	1/15	0.5/0.4–1.1	Experimentally infected[e] with *M. avium* C2789 serotype 2
8	Cattle	20	ND	Natural infection with *M. paratuberculosis*; seven animals were skin tested with bovine tuberculin PPD, avian PPD, and johnin PPD
9	Goats/sheep	12/1	1–7/ND	Naturally infected with *M. paratuberculosis* or *Corynebacterium pseudotuberculosis* (caseous lymphadenitis); one serum sample pooled from several goats belonging to a single farm
10	Sheep (single flock)	74	2–10	Natural infection with *M. paratuberculosis* (17 animals), caseous lymphadenitis (10 animals), or both (5 animals); *Corynebacterium pyogenes* isolated from abscesses in 2 animals

[a] Approximate age at first bleeding.
[b] ND, Not determined; most animals were adults, but the exact ages of some animals could not be determined.
[c] Inoculated intratracheally with 1.0 ml containing 5 μg of mycobacteria in saline.
[d] Inoculated intratracheally with 1.0 ml containing 30 μg of mycobacteria in saline.
[e] The calf was orally inoculated with 0.1 mg (wet weight) of cells on 5 alternate days; 14 goats were inoculated similarly but each animal was given a total of 10 mg; one other goat was inoculated once subcutaneously with 2 mg.

bovis and bovine tuberculosis. Since the streptococcal protein G binds IgG of a wide variety of animal species with a high affinity (3), we developed an ELISA based on this protein to obtain a single final indicator system for the demonstration of anti-MPB70 antibodies in various animal species that are susceptible to infection with *M. bovis* and related mycobacteria.

All sera were also tested for anti-BCG85B antibodies by the same technique. Antigen 85 has been defined in crossed immunoelectrophoresis (CIE) reference systems for BCG and *Mycobacterium tuberculosis* antigens (10, 39). The BCG85 antigen complex consists of three closely related proteins (40) encoded by separate genes (42). As for MPB70, they are actively secreted from the mycobacterial cell into the culture medium (1, 10, 28, 40). The proteins of this complex are immunogenic in vivo (41) and widely cross-react (22; H. G. Wiker, M. Harboe, S. Nagai, and J. Bennedsen, Am. Rev. Respir. Dis., in press). Anti-BCG85B antibodies were therefore expected to be useful as a more general indicator of mycobacterial infection.

MATERIALS AND METHODS

Animals. Serum and plasma samples were selected from cattle, sheep, and goats that differed with respect to their presumptive mycobacterial infection and sensitization status. The groups were tabulated for direct evaluation of the anti-MPB70 assay. Thus, groups 1 and 2 served as negative controls; groups 3, 4, and 5 consisted of animals that were experimentally infected or sensitized with *M. bovis*; and groups 6 to 10 served to evaluate the specificity and sensitivity of the assay. Details of the sampling times, skin tests, and other relevant features of the animals are included in Table 1 and the Results section.

Preparation of bovine tuberculin purified protein derivative. Bovine tuberculin purified protein derivative (PPD) was prepared essentially as described previously (19). *M. bovis* AN-5 was grown at the Animal Diseases Research Institute in a modified Watson-Ried Medium (a list of components in a complex medium available upon request) for 14 to 21 days and was sterilized by autoclaving. Following sedimentation of mycobacterial cells, the culture fluid was filtered, dialyzed against phenolized phosphate buffer, and concentrated. PPD was obtained from the concentrated culture fluid by ammonium sulfate precipitation and dialysis and was sterilized by filtration. Essentially the same extraction procedure was used to prepare avian and johnin PPDs. Avian PPD was obtained from a mixture of three strains of *Mycobacterium avium* that were grown in Bureau of Animal Industry (a list of components in complex medium is available upon request) synthetic medium for approximately 10 days, while johnin PPD was obtained from a mixture of seven strains of *Mycobacterium paratuberculosis* grown on Long synthetic medium for approximately 21 days.

Skin testing. Tuberculin PPD was injected intradermally in the neck region by alternating between the right and left sides on four occasions; injections were spaced in time as shown in Fig. 1. Comparative skin tests on the negative control calves (group 2) and experimentally infected animals (groups 4 and 5) were performed with three injections each time. The injections consisted of 0.06 mg of protein in 0.1 ml of European Economic Community Standard, 0.12 mg of protein in 0.1 ml of the proposed international standard, and 0.12 mg of protein in 0.1 ml of the Canadian *M. bovis* PPD.

Calves that were experimentally infected with *M. bovis* received three different *M. bovis* PPDs at two different doses; thus, six different skin sites were tested on each occasion.

Preparation of nonviable tubercle bacilli for sensitization. *M. bovis* 110 was grown in Animal Diseases Research Institute liquid synthetic medium for 2 weeks. The culture was autoclaved, and the bacilli were removed by filtration and ground with saline before they were mixed with adjuvant (see group 3).

Preparation of *M. avium* for challenge. *M. avium* C2789

was grown for 3 weeks in Middlebrook 7H9 medium supplement with albumin, glucose, and catalase enrichment.

ELISA. Immunoplate II (lot 0984; Nunc, Copenhagen, Denmark) was used in the ELISAs. The coating step was performed at 4°C for at least 48 h in a humidified chamber with phosphate-buffered saline (pH 7.4) containing 0.02% NaN_3. Phosphate-buffered saline with 0.2% Tween 20–0.05% bovine serum albumin was used in the blocking step and in all other incubations, and phosphate-buffered saline with 0.1% Tween 20 was used as the washing buffer. The plates were washed three times in a Dynawasher (Dynatech Laboratories, Inc., Alexandria, Va.) between each incubation. All incubations were performed with 100 μl per well at 37°C for 30 min, except the blocking step lasted 60 min. As a substrate for horseradish peroxidase (lot 10854923-09; Boehringer GmbH, Mannheim, Federal Republic of Germany), 2,2′-azino-di(ethyl-benzthiazolinesulfate) was used, and the optical density was read at 405 nm after 10 to 15 min on a reader (MR 580; Dynatech).

For the assay of anti-MPB70 antibodies, the protein was purified to homogeneity from culture fluid after the culture of BCG strain Tokyo on Sauton medium as described previously (33), using 0.5 μg per well. Serum was added in the second step. For the positive control, rabbit anti-MPB70 (23) was used at dilutions of 1:100, 1:1,000, and 1:10,000. For the negative control, normal bovine serum was added to six wells at a dilution of 1:50. Serum samples to be tested for antibody content were diluted 1:50. The third layer consisted of biotinylated protein G diluted 1:1,000. Protein G was obtained from Pharmacia LKB Biotechnology AB, Uppsala, Sweden (lot no. nm 05680), and biotinylated as described by Subba Rao et al. (36). Streptavidin (lot 28F-6837; Sigma Chemical Co., St. Louis, Mo.) was coupled to horseradish peroxidase (2); the conjugate was diluted 1:5,000.

For assay of antibodies to BCG antigen 85B, protein 85B was purified as described elsewhere (40). The wells were coated with 0.5 μg of protein per well. Rabbit anti-BCG85 K1020A was used as a positive control, and the rest of the procedure was identical to the ELISA for anti-MPB70 antibodies described above.

RESULTS

Development of the ELISA. For the assay of anti-MPB70 antibodies in different species, a system was needed that had a common indicator that reacted with immunoglobulins of various species that were susceptible to infection with *M. bovis* and related mycobacteria. The streptococcal protein G was selected since it binds with a higher affinity and to more animal species than does the staphylococcal protein A (3), which we have used previously (23) for the radioimmunoassay of anti-MPB70 antibodies. The procedure described in Materials and Methods was developed on the basis of initial experiments that were performed to establish the optimal conditions and dilutions of reagents in the various steps of the procedure.

Each plate contained a positive control serum sample. Two triplicate sets of rabbit anti-MPB70 K216 diluted 1:100 provided the reference value that was used to determine the correction coefficient. The correction coefficient was used to adjust all test values to correspond to the positive control serum sample giving an optical density of 1.000 at this dilution. When this rabbit antiserum was diluted 1:1,000, it consistently showed a weaker, but still strongly positive reaction (median corrected optical density, 0.704), while a dilution of 1:10,000 showed a considerably weaker, but still positive, reaction (median corrected optical density, 0.109). The reactions were read against a reagent control on the same plate that was subjected to all procedures and that contained all reagents except the test serum. Serum samples to be tested were diluted 1:50; initial tests showed that this dilution did not increase the risk of false-positive reactions, while it gave an increased sensitivity for detecting anti-MPB70 antibodies. Each plate also contained two triplicate sets of a selected serum sample from a normal cow; this serum sample was diluted 1:50. In 50 consecutive plates, this serum sample gave a median corrected optical density value of 0.008 with an interquartile range of 0.002 to 0.010.

MPB70 in *M. bovis* culture fluid and bovine tuberculin PPD. MPB70 occurs in markedly different concentrations in different substrains of BCG (23), and little is known about its occurrence in virulent strains of *M. bovis*.

The presence of MPB70 in *M. bovis* culture fluids was tested by inhibition tests based on the ELISA by using specific rabbit anti-MPB70 as the indicator antibody. A marked inhibition capacity was observed with culture fluids of *M. bovis* Ravenel and AN5 as well as with PPD from *M. bovis* AN5 and old tuberculin prepared from *M. bovis* 110. All six different *M. bovis* PPD preparations tested in Japan in a dot blot assay with anti-MPB70 showed a distinctly positive reaction. The presence of MPB70 in the bovine tuberculin PPD preparation used in the present study was also directly confirmed by CIE as described below.

Antibodies in healthy cattle (groups 1 and 2). Healthy cattle were delivered by cesarean section and were raised in isolation from the other cattle. They were skin tested once with the johnin PPD. The serum samples from this group of cattle included a set from 15 animals that was taken before skin testing and another set that was obtained 2 weeks after skin testing. A third set was obtained from five animals prior to slaughter, when they were examined to exclude tuberculosis, and selected tissue samples were collected to exclude the presence of paratuberculosis. At the time these tests were performed, there was no evidence of mycobacterial disease. Thirty-four serum samples from this group of cattle were negative in the ELISAs for anti-MPB70 and anti-BCG85B antibodies, while one serum sample showed a weakly positive reaction. There was no indication of any effect of skin testing with the johnin PPD on the antibody specificities in this group of cattle.

The effect of repeated skin testing with bovine tuberculin PPD in healthy, noninfected cattle was further tested in four healthy group 2 calves (age, 6 months). These calves were skin tested four times (at 0, 10, 14, and 16 weeks) by using a schedule and PPD doses corresponding to the protocol used for tuberculin testing of the animals in group 4 infected with *M. bovis* (see below). The skin tests were read at 48 h. No increase in skin thickness or any other significant reaction was detected at the inoculation sites. Eighteen serum samples were taken from each of these animals pre- and post-skin testing. Skin testing had no effect on anti-MPB70 levels in this group of animals. In three of the animals, the optical densities fluctuated around 0.025, while one animal had slightly higher values, but these values were consistently below 0.050.

Based on these observations, an optical density value of 0.050 was chosen as the cutoff point between negative and positive results in the assay for anti-MPB70 antibodies. Similar values were obtained in the anti-BCG85B assay, and the same cutoff point was chosen in this test.

Antibodies in cattle after sensitization and experimental inoculation with *M. bovis* (groups 3 to 5). Group 3 consisted of

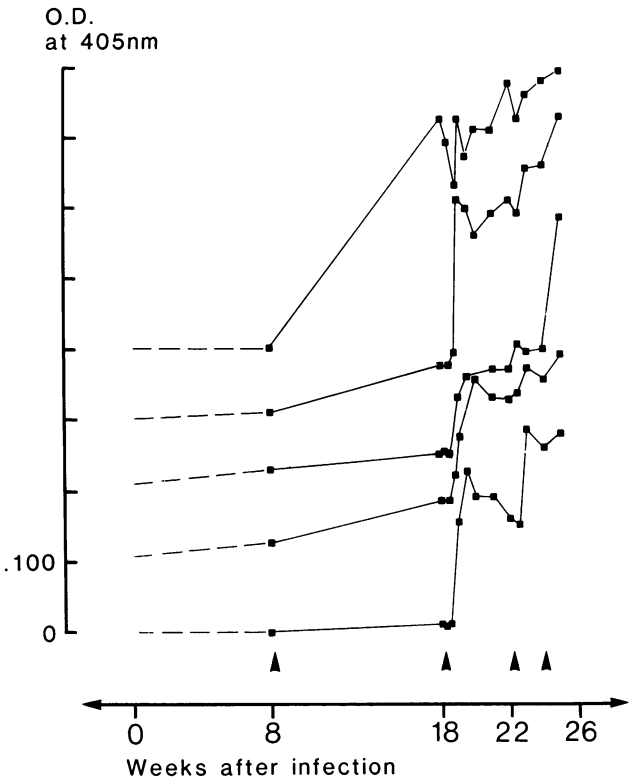

FIG. 1. Anti-MPB70 antibodies in five cattle of group 4 after experimental inoculation with *M. bovis*. Thirteen serum samples from each animal were tested. The timing of the PPD skin test is indicated with arrowheads. The distance between each horizontal indicator on the ordinate corresponds to 0.100 optical density (OD) unit at 405 nm, with the origin of each curve being at zero and ordered stepwise above each other to facilitate reading.

four mature castrated male cattle immunized with 50 or 100 mg of heat-killed *M. bovis* 110 in an oil adjuvant. Serum samples taken before immunization were all negative. Skin testing at 4.5 weeks after immunization showed a conversion to positive reactions to both bovine and avian tuberculins. Serum samples taken 10 weeks after immunization, which corresponded to 5.5 weeks after the skin test, were positive for anti-MPB70 in three of the animals (optical densities, 0.375, 0.222, and 0.113) and negative in one animal (optical density, 0.022).

Serum samples were taken from six cattle of group 4 that were experimentally inoculated with live *M. bovis* BM228 8 weeks after infection and prior to the first skin test, 18 weeks postinfection and prior to the second skin test, 22 weeks postinfection and prior to the third skin test, and 25 weeks after infection and 2 weeks after the final skin test. Each animal received a combined dose of 0.3 mg of protein in 0.6 ml injected at six sites for each skin test. Anti-MPB70 activity showed a very similar pattern in these animals. At 18 weeks after infection there was no or only a slight antibody rise in five animals, while one animal showed fairly high antibody activity. Then, there was a marked rise in anti-MPB70 activity from 18 to 22 weeks in five animals, and in four of the six animals there was a further increase after 25 weeks.

Figure 1 shows the anti-MPB70 activity in the five remaining cattle of group 4; 13 serum samples from each animal were tested. The animals were skin tested with *M. bovis*

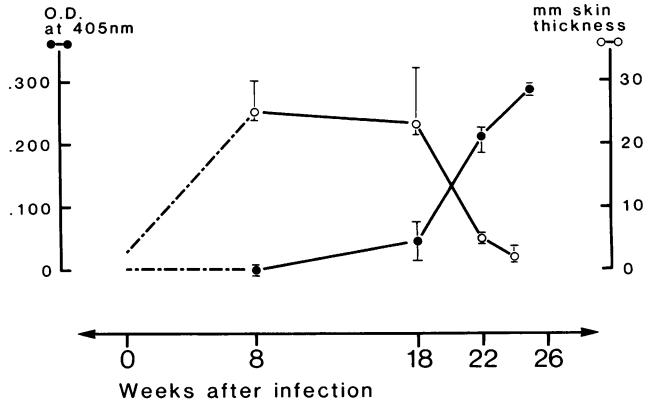

FIG. 2. Relation between anti-MPB70 and DTH. Symbols: ●, median values of ELISA for anti-MPB70 in 11 cattle of group 4; ○, skin tests with *M. bovis* PPD. The initial dotted lines depict the curve expected from previous experience concerning PPD conversion following inoculation with *M. bovis*. The interquartile range is shown at each point. OD, Optical density.

PPD four times. In four of the animals the form of the curves was very similar, with an initial flat plateau and a slight rise in antibody activity at 18 weeks after infection. In these animals one sample taken before and two samples taken shortly after the second skin test showed very similar antibody activities followed by an abrupt rise 1 week after skin testing. For the first animal (Fig. 1, bottom curve) the antibody activity then fell slowly, increasing markedly once more after the next skin test. Three of the other four animals showed a tendency toward the same pattern. The curve at the top of Fig. 1 shows a slightly more rapid increase in anti-MPB70 antibodies after inoculation, with there being a marked variation in antibody content in five samples taken 18 to 20 weeks after infection. All animals showed a further rise in antibody after the third skin test.

These results indicated that skin testing with PPD affects anti-MPB70 formation. This can be considered from various points of view. MPB70 has previously been shown to be one of the more thermostable proteins in mycobacteria. After MPB70 was heated to 120°C for 15 min, it had the same inhibiting capacity in radioimmunoassay inhibition tests as that of unheated MPB70 and gave a similar precipitate line in CIE (23). The *M. bovis* PPD preparation used in this study was therefore examined by CIE by using polyvalent anti-BCG in the top gel. The pattern was basically similar to those of other PPD preparations (21), with there being a distinct skewed antigen 60 line dominating the pattern and a few weak additional precipitate lines because of the reaction with partially denatured proteins. When anti-MPB70 was added in the top gel, an additional precipitate appeared, with the typical position of MPB70 directly demonstrating the presence of MPB70 in *M. bovis* PPD.

Relation between anti-MPB70 and DTH. Figure 2 shows the median values of ELISA for anti-MPB70 in the 11 cattle of group 4. The results of skin tests with *M. bovis* PPD are also shown in Fig. 2. The initial dotted lines in Fig. 2 depict the curve that was expected based on extensive previous experience concerning PPD conversion following inoculation with *M. bovis* (18), while the remaining solid lines depict our observations in the present study. After 8 weeks following infection, the mirror image of the DTH reactions compared with the antibody assay was striking.

Relation between anti-MPB70 and anti-BCG85B in cattle experimentally infected with *M. bovis* (groups 4 and 5). Figure

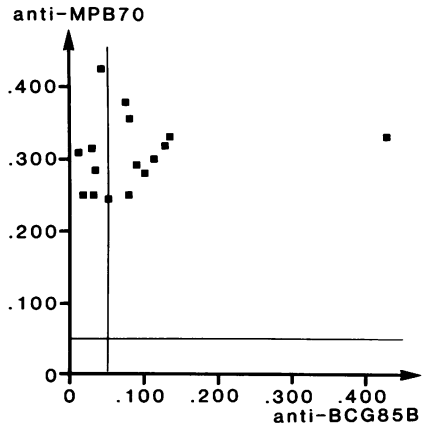

FIG. 3. Cattle (groups 4 and 5) infected with live *M. bovis*. Anti-MPB70 activity is shown along the ordinate, and anti-BCG85B activity is shown on the abscissa. Each point represents one animal. The thin lines indicate the cutoff value for positive reactions (at 0.050).

3 shows anti-MPB70 and anti-BCG85B activities. A single serum sample taken 22 weeks after inoculation of all animals is recorded. For both assays, an optical density of 0.050 was recorded as the cutoff point between negative and positive reactions.

All 16 animals that were experimentally inoculated with live *M. bovis* had a positive anti-MPB70 antibody assay result 22 weeks after inoculation.

In the anti-BCG85B assay, the majority of the animals (10 of 16) reacted positively, but all of these reactions were weaker than those in the anti-MPB70 assay. Six of the animals had a negative anti-BCG85 assay, even though they had extensive tuberculous lesions at the time of necropsy. Thus, antigen 85 is immunogenic in vivo in *M. bovis*-infected animals, as is the case in animals with leprosy (41) and in human tuberculosis (26). MPB70 may be a more dominant immunogen in experimental bovine tuberculosis. On the other hand, the presence of MPB70 in bovine PPDs may have caused a boosting effect on anti-MPB70 antibody production.

Cattle with naturally occurring *M. bovis* infection (group 6). Table 2 provides information on 44 animals. Following the finding of lesions consistent with bovine tuberculosis in one animal that was sold to slaughter from the Holstein dairy herd, a blood sample was taken from each animal, and the herd was subsequently subjected to an intradermal skin test by using *M. bovis* tuberculin (0.1 mg of protein in 0.1 ml) in the region of the caudal tail fold. From the first set of blood samples, one single serum sample (animal 27) showed a value (optical density, 0.048) close to the cutoff point. The rest were negative in the anti-MPB70 assay. Many of the animals showed a positive reaction to skin testing with bovine PPD, and the entire herd was sent to slaughter 2 weeks later, when a second serum sample was taken. The mean ELISA optical densities for anti-MPB70 and anti-BCG85B before and after skin testing were 0.006 versus 0.049 and 0.029 versus 0.024, respectively. This was a clear indication of an anamnestic response with anti-MPB70 and an absence of an anamnestic response with anti-BCG85B. Table 2 shows the occurrence of gross lesions that was consistent with tuberculosis in 11 of the animals. Tissue samples from those animals suspected of having tuberculosis were taken for histological examination. *M. bovis* was recovered by culturing tissue samples from animal 29.

TABLE 2. Skin test; gross and histopathological observations on a herd naturally infected with *M. bovis* (group 6)

Animal no.	Age (yr)	Skin test[a]	Pathology		anti-MPB70[b]
			Gross[c]	Histo[d]	
1	2	P5			–
2	2	N			–
3	2	P1			–
4	2	P3			–
5	2	P1	T	n	–
6	2	P3			–
7	2	P1			–
8	2	P1	T	n	–
9	1	N			–
10	1	N			–
11	4	N			0.039
12	6	P2	B	++	0.040
13	8	P2			–
14	10	P1			–
15	8	N			0.076
16	8	P4			–
17	3	N			–
18	4	P1			–
19	6	P8	B,M,Me	+	0.047
20	8	P3	B	+	0.044
21	10	N			0.582
22	10	N	Me	+	–
23	8	P5	Me	++	–
24	8	P2			0.046
25	6	P4	M,I	+	–
26	6	P4			–
27	6	P6	M,B,I	+	0.128
28	8	P3			–
29[e]	8	P4	Me,B,M,L	++	0.069
30	8	P8	M,B	++	0.131
31	6	P1			–
32	10	P4	B	++	0.035
33	10	P1			–
34	5	P1			–
35	5	P3	M,L	++	0.512
36	5	N			–
37	10	P3			–
38	5	N			–
39	2	P1			–
40	2	P7			–
41	3	P1			–
42	3	P1			–
43	2	P7			–
44	1				–

[a] Skin reactions were graded by increasing size from P1 to P8, with P1 equal to 3/16. N, Negative.

[b] Results are those of samples that were obtained after skin testing. Optical densities higher than 0.025 are given. A minus sign indicates an optical density of <0.025.

[c] Locations in which gross lesions were observed at slaughter: B, bronchial lymph node; M, mediastinal lymph node; Me, mesenteric lymph node; I, inguinal lymph node; L, lung; T, tonsil.

[d] n, An abscess or granuloma of nonmycobacterial origin; +, a lesion consistent with *M. bovis* infection and no mycobacteria demonstrated by histopathology; ++, a lesion consistent with *M. bovis* infection and the presence of mycobacteria demonstrated by histopathology.

[e] *M. bovis* was isolated from this animal.

Table 3 provides a summary of findings in the anti-MPB70 antibody assay of the second blood sample from all animals in group 6 compared with the findings by histological examination of tissue samples recovered from the 11 animals showing signs of tuberculous infection by gross examination at the time of necropsy. There was a definite correlation between ++ histological evidence of tuberculous lesions and the presence of anti-MPB70 antibodies. One of the

TABLE 3. Anti-MPB70 antibody assay results and histological examination of cattle naturally infected with *M. bovis* (group 6)

Histology[a]	No. of animals with the following anti-MPB70 assay results:		
	Positive	Marginal	Negative
++	3	2	1
+	1	2	2
No evidence of infection	2	2	29

[a] See footnote *d* of Table 2 for explanations of ++ and + histologies.

animals in this group, however, was negative, as determined by the antibody assay. It is noteworthy that two of the three animals with a positive histology and negative antibody findings had lesions that were confined to the mesenteric lymph nodes.

Tissue samples from animal 21, which had high antibody activity, were not examined microscopically. This animal may have had a focus of infection which was missed by gross examination at the time of necropsy.

Exposure to *M. avium* (group 7). Fifteen goats were inoculated with live *M. avium* cells. They were not skin tested. Since the animals were sacrificed at defined intervals postinoculation (28 to 252 days), they varied with respect to the degree of bacterial load and the duration of exposure to *M. avium*. Lesions consistent with *M. avium* infection and containing acid-fast bacilli were observed in the intestines or mesenteric lymph nodes of all orally inoculated animals. Serum samples from all of these animals were negative in the anti-MPB70 assay, while four serum samples were positive for anti-BCG85B antibodies, which was an indicator of the established mycobacterial infection.

The calf that was orally inoculated with *M. avium* showed negative results in both antibody assays.

Cattle with paratuberculosis (group 8). Twenty animals of various beef and dairy cattle breeds were naturally infected with *M. paratuberculosis*. The infection levels varied markedly, but in many animals the lesions were extensive and contained large numbers of acid-fast bacilli. Comparative cervical skin testing was conducted by using bovine, avian, and johnin PPDs on seven of the animals prior to serum collection, with two samples being collected at the same interval after the skin test (2 weeks) as the interval used for group 6 animals. Figure 4 shows the anti-MPB70 and anti-BCG85B activities in this group. All sera were negative in the anti-MPB70 assay, while 15 serum samples were positive in the anti-BCG85B assay. Antigen 85 is a major antigen complex that is secreted by *M. bovis* BCG and *M. tuberculosis*, and cross-reacting antigen has been demonstrated in many mycobacterial species (22, 41; Wiker et al., in press). During the present study, a corresponding antigen was directly demonstrated in *M. paratuberculosis* C286 by CIE of culture fluid. A purified antigen from *M. paratuberculosis* designated A (6) fused with BCG85B in a tandem CIE with spur formation. Figure 4 illustrates the in vivo immunogenicity of cross-reactive antigen 85B (antigen A) in animals with paratuberculosis, as well as the high specificity of the MPB70 assay for bovine tuberculosis.

Sheep and goats with paratuberculosis or caseous lymphadenitis (groups 9 and 10). Group 9 consisted of 11 serum samples from goats that were naturally infected with *M. paratuberculosis* or *Corynebacterium pseudotuberculosis*, 1 pooled serum sample from several *M. paratuberculosis*-infected goats from a single farm, and 1 serum sample from an infected male sheep. The goats were mature females of

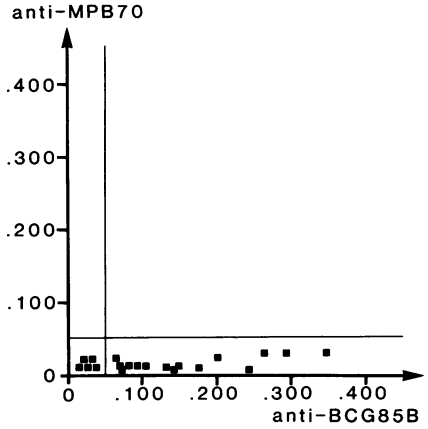

FIG. 4. Anti-MPB70 and anti-BCG85B activities in cattle (group 8) with paratuberculosis. Details of the figure are as described in the legend to Fig. 3.

the Toggenburg breed. Comparative cervical skin tests were conducted on six animals, as described above for animals in group 8, from 4 to 36 days prior to sample collection. With the exception of three goats, all animals had paratuberculosis or caseous lymphadenitis, with the latter being confirmed by culture of *C. pseudotuberculosis* from typical lesions. All of these sera were negative for anti-MPB70 antibodies. Seven of them were positive for anti-BCG85, and four showed very strong reactions. The four serum samples showing very strong reactions were tested further by incorporating them in the intermediate gel of CIE plates, which demonstrated a marked retention of the 85 antigen complex in the intermediate gel as well as precipitating antibodies against several other antigenic constituents of *M. paratuberculosis* and BCG. These four serum samples were selected because of previous demonstration of high antibody activity against *M. paratuberculosis* by immunodiffusion and complement fixation (unpublished data). The lack of anti-MPB70 antibodies in all sera from animals in this group that received comparative cervical skin tests further documents the high specificity of the anti-MPB70 assay for infection with *M. bovis* compared with infection with *M. paratuberculosis*.

Group 10 consisted of sera obtained at the time of postmortem examination from 74 sheep culled from one flock over a 3.5-year period on the basis of positive immunological tests for paratuberculosis or maedi visna or poor performance. Skin testing was not done. Results of the histological and serological examinations of these animals have been described elsewhere (6). In the present study, 23 of these serum samples showed a positive result in the anti-BCG85B assay as a further indicator of the widespread occurrence of mycobacterial infection in this flock. Two serum samples were positive in the anti-MPB70 assay as well. Animal 907, which was found to be marginally positive by the anti-MPB70 assay (optical density, 0.053), was positive in the complement fixation test for paratuberculosis and it had masses of acid-fast bacilli in the lesions, as determined by histological examination. One animal that was distinctly positive by the anti-MPB70 assay (optical density, 0.099) was negative in the complement fixation assay for *M. paratuberculosis* and negative by culture for this organism. While there was no history of skin testing in these sheep, we have no data to establish the reason for the induction of antibody against MPB70. It is possible that *Nocardia* species exist in the environment in which the animals live.

DISCUSSION

The ELISA was developed, by using streptococcal protein G, to have a common indicator for the demonstration of antibodies in different animal species, since protein G reacts with immunoglobulins of more animal species than does staphylococcal protein A, which was previously used in our radioimmunoassay for anti-MPB70 antibodies (23). Since there is some evidence for different reactivities of protein G with different subclasses of bovine IgG (32), additional experiments were performed by using a mouse monoclonal antibody to bovine IgG1, a mouse monoclonal antibody to bovine immunoglobulin light chains, and a polyclonal antiserum to bovine IgG $F(ab')_2$ as part of the indicator system. The assays based on these antisera did not provide essential additional information compared with that provided by the protein G ELISA described here, other than indications that animals probably differ in their isotype distributions of antibody to MPB70. This may be of some importance in optimizing the differential diagnostic aspect of an assay in animals with different stages and degrees of infection.

The pattern of anti-MPB70 antibody formation in experimental *M. bovis* infections (Fig. 1 and 2) is characterized by a flat initial plateau followed by a rather steep rise. The latency period before there is an increase in antibody activity is long compared with that in animals with other acute infections. The latency period was even longer in experimental *M. leprae* infections in armadillos (20). In the latter case it was probably related to the longer generation time of *M. leprae* than that of *M. bovis*. It is also appreciated that a test based on the use of protein G to detect IgG antibodies would probably take a longer period of time to reach positivity than would tests based on the detection of IgM antibodies and primary immune responses.

The influence of skin testing with PPD on the formation of anti-MPB70 antibodies depends on several different sets of factors. The findings in group 2 animals indicate that repeated skin testing does not induce a significant increased production of anti-MPB70 antibodies in uninfected animals. In animals with experimental *M. bovis* infection, there was a rapid increase of anti-MPB70 activity at a certain stage of infection, and it was clearly affected by PPD skin testing, as illustrated in Fig. 1. The effect was marked following the second skin test and was less evident after the third test, when the infection apparently had progressed to a stage at which it stimulated anti-MPB70 production on its own.

Similar observations were made in group 6 animals, which consisted of a herd of cattle with natural infection. Under these conditions, the extent of infection may, in many instances, be less and may probably be more variable than that under experimental conditions. Prior to skin testing, only one animal showed a marginal value in the ELISA for anti-MPB70 antibodies. After skin testing this animal showed a distinctly positive value in the assay. In group 6 as a whole, several animals showed distinctly positive values in the second blood sample, which was taken 2 weeks after skin testing with PPD.

Table 3 shows a comparison between the findings in the anti-MPB70 antibody assay and evidence of infection obtained at the time of necropsy. There was a striking correlation, but when considering the procedure as a diagnostic test for bovine tuberculosis, there was evidence of both false-negative and false-positive reactions. Two of the animals with false-negative reactions had lesions confined to the mesenteric lymph nodes, which suggests that the location of the lesion may determine the nature of the ensuing immune response in that it invokes different immunoregulatory pathways. In lymphocyte stimulation tests (LSTs), animal 23 showed a stronger response to *M. bovis* PPD than it did to *M. avium* PPD. The latter observation was also made in animal 25, which was the third false-negative reaction (data not shown). Two of the three animals with false-negative reactions (animals 23 and 25) had positive skin tests (P4 and P5) and positive LST (stimulation index of >2.5; stimulation was stronger with bovine PPD than with avian PPD; data not shown), indicating that they were sensitized to *M. bovis* but did not respond anamnestically to the skin test.

Two animals (animals 15 and 21) showed distinctly positive findings in the antibody assay, but tissues were not taken for histological examination, as gross lesions were not observed. While both of these animals had negative skin tests, one had a positive and preferential response to *M. bovis* PPD on LST (data not shown), and it is evident that animals with *M. bovis* infection may have negative skin tests (see Fig. 2). We assume that the most probable explanation is the occurrence of a focus of infection which was missed by gross examination at the time of necropsy.

Based on previous observations in other species, the marked increase in anti-MPB70 activity following skin testing with PPD was unexpected. An increase in anti-mycobacterial antibody activity is usually not observed after skin testing with PPD in mice and humans. However, in these species, testing has usually been performed with PPD prepared from *M. tuberculosis*, which produces only minimal amounts of MPB70 (23), and most of the other antigens are extensively denatured during the preparation of PPD so they do not react with antibodies against native mycobacterial antigens (21). In cattle, several previous studies have demonstrated an increase in anti-mycobacterial antibodies following skin testing with bovine tuberculin PPD (34, 35, 38, 44). In the present study, we demonstrated that anti-MPB70 antibodies were reactive with MPB70 protein in the bovine tuberculin PPD preparations that we tested. The MPB70 antigen was thus present in the bovine PPD preparations that were used for skin testing in a form which may directly induce antibody formation, particularly in an anamnestic response situation in infected animals. In this connection, it should also be noted that the amount of tuberculin used in skin tests in cattle is much higher (on the order of 5,000 to 10,000 tuberculin units) than it is in humans (on the order of 1 to 10 tuberculin units) with a single intradermal skin test.

Figure 2 shows a striking inverse relationship between the amount of anti-MPB70 antibodies and the skin test reactions in group 4 animals. This observation is of considerable interest in relation to other mycobacterial infections, in which a negative skin test is a characteristic feature of extensive infection. There is considerable discussion on the mechanisms behind this phenomenon. In mice, at least, it appears that several different mechanisms may account for this phenomenon. In C3H mice with *Mycobacterium lepraemurium* infections, the negative skin test responses are due to a primary genetic predisposition, which causes a lack of resistance and unimpaired multiplication of *M. lepraemurium* (12, 27). In C57BL mice, similar footpad inoculation with *M. lepraemurium* usually leads to the development of systemic immunity and positive skin test responses (9). If large amounts of bacilli are used for inoculation, the infection proceeds in these mice and large amounts of mycobacteria are generated in the lesions and, concomitantly, the skin test responses are entirely negative (9). So, under these conditions, the negative skin test reaction is a property acquired by infected animals. In the cattle of group 5, it is

evident that the negative skin test reaction was an acquired feature that was seen as a result of repeated exposure to the antigen and extensive infection.

Assays for anti-MPB70 antibodies probably have insufficient sensitivities to provide important diagnostic information for cattle and other animal species with natural *M. bovis* infections. Skin testing with bovine tuberculin stimulates the immune system of infected animals, and under certain conditions it induces a rapid and marked increase in anti-MPB70 antibodies in serum. The bovine PPD preparations probably boost a secondary immune response with IgG antibody formation in the infected animals. Naive animals would only be able to mount a primary immune response upon skin testing with production of IgM antibodies, which would not be detected by the protein G ELISA described here. The risk of having false-positive reactions in the MPB70 assay upon skin testing is thus small. Since advanced mycobacterial infection often is associated with negative skin test results, as shown in Fig. 2, it appears that it is essential to combine skin testing with an assay for antibody activity which is more closely related to bacterial multiplication and antigen load in the tissues than it is to infection. We would also expect that the specificity of antibody assays would be further improved compared with the present protocol if skin tests were also performed by using MPB70 protein isolated from *M. bovis* rather than convential bovine tuberculin PPD preparations which contain several other mycobacterial proteins in addition to MPB70; the majority of those other proteins are strongly cross-reactive between different mycobacterial species. Furthermore, the MPB70 protein gives positive results in lymphocyte stimulation tests, with cells obtained from tuberculous cattle giving higher specificity than bovine tuberculin PPD in this assay (16).

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LITERATURE CITED

1. **Abou-Zeid, C., I. Smith, J. M. Grange, T. L. Ratliff, J. Steele, and G. A. W. Rook.** 1988. The secreted antigens of *Mycobacterium tuberculosis* and their relationship to those recognized by the available antibodies. J. Gen. Microbiol. **134**:531–538.
2. **Avrameas, S., and T. Ternynck.** 1971. Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry **8**:1175–1179.
3. **Bjørck, L., and G. Kronvall.** 1984. Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. J. Immunol. **133**:969–974.
4. **Brennan, P. J.** 1986. The carbohydrate-containing antigens of *Mycobacterium leprae*. Lepr. Rev. **57**(Suppl. 2):39–51.
5. **Brett, S. J., P. Draper, S. N. Payne, and R. J. W. Rees.** 1983. Serological activity of a characteristic phenolic glycolipid from *Mycobacterium leprae* in sera from patients with leprosy and tuberculosis. Clin. Exp. Immunol. **52**:271–279.
6. **Brooks, B. W., R. H. Robertson, A. H. Corner, B. S. Samagh, M. M. Garcia, C. Turcotte, and J. R. Duncan.** 1988. Evaluation of the serological response of sheep in one flock to *Mycobacterium paratuberculosis* by crossed immunoelectrophoresis. Can. J. Vet. Res. **52**:199–204.
7. **Buchanan, T. M., H. Nomaguchi, D. C. Anderson, R. A. Young, T. P. Gillis, W. J. Britton, J. Ivanyi, A. H. J. Kolk, O. Closs, B. R. Bloom, and V. Mehra.** 1987. Characterization of antibody-reactive epitopes on the 65-kilodalton protein of *Mycobacterium leprae*. Infect. Immun. **55**:1000–1003.
8. **Chatterjee, D., C. M. Bozic, C. Knisley, S.-N. Cho, and P. J. Brennan.** 1989. Phenolic glycolipids of *Mycobacterium bovis*: new structures and synthesis of a corresponding seroreactive neoglycoprotein. Infect. Immun. **57**:322–330.
9. **Closs, O.** 1975. Experimental murine leprosy: induction of immunity and immune paralysis to *Mycobacterium lepraemurium* in C57BL mice. Infect. Immun. **12**:706–713.
10. **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.
11. **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.
12. **Closs, O., and O. A. Haugen.** 1975. Experimental murine leprosy. 3. Early local reaction to *Mycobacterium lepraemurium* in C3H and C57/BL mice. Acta Pathol. Microbiol. Scand. Sect. A **83**:51–58.
13. **Collins, D. M., and G. W. de Lisle.** 1987. BCG identification by DNA restriction fragment patterns. J. Gen. Microbiol. **133**: 1431–1434.
14. **Daniel, T. M., and B. W. Janicki.** 1978. Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. Microbiol. Rev. **42**:84–113.
15. **de Lisle, G. W., B. S. Samagh, and J. R. Duncan.** 1980. Bovine paratuberculosis. II. A comparison of fecal culture and the antibody response. Can J. Comp. Med. **44**:183–191.
16. **Fifis, T., P. Plackett, L. A. Corner, and P. R. Wood.** 1989. Purification of a major *Mycobacterium bovis* antigen for the diagnosis of bovine tuberculosis. Scand. J. Immunol. **29**:91–101.
17. **Francis, J., C. L. Choi, and A. J. Frost.** 1973. The diagnosis of tuberculosis in cattle with special reference to bovine PPD tuberculin. Aust. Vet. J. **49**:246–251.
18. **Francis, J., R. J. Seiler, I. W. Wilkie, D. O'Boyle, M. J. Lumsden, and A. J. Frost.** 1978. The sensitivity and specificity of various tuberculin tests using bovine PPD and other tuberculins. Vet. Rec. **103**:420–435.
19. **Green, H. H.** 1946. Weybridge P.P.D. tuberculins. Br. Vet. J. **102**:267–278.
20. **Harboe, M.** 1981. Radioimmunoassay and other serologic tests and their application in epidemiological work. Lepr. Rev. **52** (Suppl. 1):275–288.
21. **Harboe, M.** 1981. Antigens of PPD, old tuberculin and autoclaved *Mycobacterium bovis* BCG studied by crossed immunoelectrophoresis. Am. Rev. Respir. Dis. **124**:80–87.
22. **Harboe, M., R. N. Mshana, O. Closs, G. Kronvall, and N. H. Axelsen.** 1979. Cross-reaction between mycobacteria. II. Crossed immunoelectrophoretic analysis of soluble antigens of BCG and comparison with other mycobacteria. Scand. J. Immunol. **9**:115–124.
23. **Harboe, M., and S. Nagai.** 1984. MPB70, a unique antigen of *Mycobacterium bovis* BCG. Am. Rev. Respir. Dis. **129**:444–452.
24. **Harboe, M., S. Nagai, M. E. Patarroyo, M. L. Torres, C. Ramirez, and N. Cruz.** 1986. Properties of proteins MPB64, MPB70 and MPB80 of *Mycobacterium bovis* BCG. Infect. Immun. **52**:293–302.
25. **Hasløv, K., Å. B. Andersen, and M. W. Bentzon.** 1987. Biological activity in sensitized guinea pigs of MPB70, a protein specific for some strains of *Mycobacterium bovis* BCG. Scand.

J. Immunol. **26**:445–454.

26. **Kaplan, M. H., and M. W. Chase.** 1980. Antibodies to mycobacteria in human tuberculosis. II. Response to nine defined mycobacterial antigens with evidence for an antibody common to tuberculosis and lepromatous leprosy. J. Infect. Dis. **142**: 835–843.
27. **Løvik, M., and O. Closs.** 1989. Local reactivity, local resistance and systemic dissemination in *Mycobacterium lepraemurium* (MLM) infection. Clin. Exp. Immunol. **75**:461–465.
28. **Matsuo, K., R. Yamaguchi, A. Yamazaki, H. Tasaka, and T. Yamada.** 1988. Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular α antigen. J. Bacteriol. **170**: 3847–3854.
29. **Miller, R. A., S. Dissanayake, and T. M. Buchanan.** 1983. Development of an enzyme-linked immunosorbent assay using arabinomannan from *Mycobacterium smegmatis*: a potentially useful screening test for the diagnosis of incubating leprosy. Am J. Trop. Med. Hyg. **32**:555–564.
30. **Minnikin, D. E., J. H. Parlett, M. Magnusson, M. Ridell, and A. Lind.** 1984. Mycolic acid patterns of representatives of *Mycobacterium bovis* BCG. J. Gen. Microbiol. **130**:2733–2736.
31. **Miura, K., S. Nagai, M. Kinomoto, S. Haga, and T. Tokunaga.** 1983. Comparative studies of various substrains of BCG on the production of an antigenic protein, MPB70. Infect. Immun. **39**:540–545.
32. **Myhre, E. B., and G. Kronvall.** 1981. Specific binding of bovine, ovine, caprine and equine IgG subclasses to defined types of immunoglobulin receptors in gram-positive cocci. Comp. Immunol. Microbiol. Infect. Dis. **4**:317–328.
33. **Nagai, S., J. Matsumoto, and T. Nagasuka.** 1981. Specific skin-reactive protein from culture filtrate of *Mycobacterium bovis* BCG. Infect. Immun. **31**:1152–1160.
34. **Richards, W. D., E. M. Ellis, H. S. Wright, and R. A. van Deusen.** 1966. The stimulating effect of tuberculin skin tests on precipitin levels in cattle. Am. Rev. Respir. Dis. **93**:912–918.
35. **Ritacco, V., I. N. de Kantor, L. Barrera, A. Nader, A. Bernadelli, G. Torrea, F. Errico, and E. Fliess.** 1987. Assessment of the sensitivity and specificity of enzyme-linked immunosorbent assay (ELISA) for the detection of mycobacterial antibodies in bovine tuberculosis. J. Vet. Med. Ser. B **34**:119–125.
36. **Subba Rao, P. V., N. L. McCartney-Francis, and D. D. Metcalf.** 1983. An avidin-biotin micro-ELISA for rapid measurement of total and allergen-specific human IgE. J. Immunol. Methods **57**:71–85.
37. **Terasaka, K., R. Yamaguchi, K. Matsuo, A. Yamazaki, S. Nagai, and T. Yamada.** 1989. Complete nucleotide sequence of immunogenic protein MPB70 from *Mycobacterium bovis* BCG. FEMS Microbiol. Lett. **58**:273–276.
38. **Thoen, C. D., M. R. Hall, T. A. Petersburg, and R. Harrington, Jr.** 1983. Detection of mycobacterial antibodies in sera of cattle experimentally exposed to *Mycobacterium bovis* by use of a modified enzyme-linked immunosorbent assay, p. 25–38. 26th Annu. Proc. Am. Assoc. Vet. Lab. Diagnosticians. American Association Veterinary Laboratory Diagnosticians, Las Vegas, Nev.
39. **Wiker, H. G., M. Harboe, J. Bennedsen, and O. Closs.** 1988. The antigens of *Mycobacterium tuberculosis*, H37Rv, studied by crossed immunoelectrophoresis. Comparison with a reference system for *Mycobacterium bovis*, BCG. Scand. J. Immunol. **27**:223–239.
40. **Wiker, H. G., M. Harboe, and T. E. Lea.** 1986. Purification and characterization of two protein antigens from the heterogenous BCG85 complex in *Mycobacterium bovis* BCG. Int. Arch. Allergy **81**:298–306.
41. **Wiker, H. G., M. Harboe, S. Nagai, M. E. Patarroyo, C. Ramirez, and N. Cruz.** 1986. MPB59, a widely cross-reacting protein of *Mycobacterium bovis* BCG. Int. Arch. Allergy **81**: 307–314.
42. **Wiker, H. G., K. Sletten, S. Nagai, and M. Harboe.** 1990. Evidence for three separate genes encoding the proteins of the mycobacterial antigen 85 complex. Infect. Immun. **58**:272–274.
43. **Youmans, G. P.** 1979. Tuberculosis. The W. B. Saunders Co. Philadelphia.
44. **Yugi, H., and C. Nozaki.** 1972. Serologic diagnosis of bovine tuberculosis. Am. J. Vet. Res. **33**:1377–1384.