INFECTION AND IMMUNITY, Nov. 1992, p. 4517–4527
0019-9567/92/114517-11$02.00/0

Vol. 60, No. 11

Identification and Characterization of Epitopes Shared between the Mycobacterial 65-Kilodalton Heat Shock Protein and the Actively Secreted Antigen 85 Complex: Their In Situ Expression on the Cell Wall Surface of *Mycobacterium leprae*

ANURA RAMBUKKANA,[1] PRANAB K. DAS,[1]* JAN D. BURGGRAAF,[1] WILLIAM R. FABER,[1] PETER TEELING,[1] SUZE KRIEG,[1] JELLE E. R. THOLE[2] AND MORTEN HARBOE[3]

Departments of Dermatology and Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam,[1] and Department of Immunohaematology and Bloodbank, University Hospital Leiden, 2300 RC Leiden,[2] The Netherlands, and Institute of Immunology and Rheumatology, University of Oslo, N-0172 Oslo 1, Norway[3]

Received 7 May 1992/Accepted 27 August 1992

Both mycobacterial hsp65 and the actively secreted antigen 85 complex of 30-kDa region proteins are considered to be major immune targets in mycobacterial diseases. In this study, by using a novel series of monoclonal antibodies (MAbs) directed to these antigens, we identified and partially characterized three unique epitopes (Rb2, Pe12, and A2h11) that are shared between mycobacterial hsp65 and the individual components of the antigen 85 complex. Dot blot assays with native purified proteins revealed that all three MAbs are strongly bound to hsp65 and antigens 85A (MPT44) and 85B (MPT59), while a weak reaction or no reaction was found with antigen 85C (MPT45). Immunoblotting showed that MAb Rb2 reacted strongly with both hsp65 and the antigen 85 complex proteins, whereas MAbs Pe12 and A2h11 reacted strongly with the former but weakly with the latter. Moreover, these MAbs did not react with other closely related MPT51 and MPT64 secreted proteins. Further characterization of these epitopes was performed by using recombinant fusion and truncated proteins of *Mycobacterium bovis* BCG hsp65 (MbaA) and the *M. leprae* 30- and 31-kDa antigen 85 complex fusion proteins. In hsp65, Rb2-Pe12- and A2h11-reactive epitopes were found to reside in the C-terminal region of amino acid residues 479 to 540 and 303 to 424, respectively. In the *M. leprae* 30- and 31-kDa antigen 85 complex, all three epitopes were located in an N-terminal region of amino acid residues 55 to 266, one of the known fibronectin-binding sites of the *M. leprae* antigen 85 complex. Comparison of these MAb-reactive amino acid sequence regions between mycobacterial hsp65 and the components of the antigen 85 complex revealed that these regions show certain amino acid sequence identities. Furthermore, by immunoperoxidase and immunogold ultracytochemistry, we demonstrated that Rb2-, Pe12-, and A2h11-reactive epitopes are expressed both on the cell wall surface and in the cytosol of *M. leprae* bacilli within the lesions of lepromatous leprosy patients and in *M. leprae*-infected armadillo liver tissue.

The mycobacterial 65-kDa heat shock protein (hsp65) and the antigen 85 complex of 30-kDa region antigens are known to be major constituents of the somatic and actively secreted proteins of pathogenic mycobacteria, respectively (14, 48). These proteins have been implicated as important target antigens in both leprosy and tuberculosis (43, 48, 49). Antibody and T-cell responses to these antigens can be detected in patients with leprosy and tuberculosis, as well as in individuals sensitized with mycobacteria (11, 12, 26, 32, 39, 40, 42, 43). The human immune responses to these antigens appear to be directed mostly against cross-reactive epitopes, since they are widely distributed among many mycobacterial species (3, 6, 11, 26, 27). These cross-reactive antigenic determinants, like the species-specific ones, have been shown to be important in both the pathogenesis of and the protective immune response to mycobacterial infections (10, 33, 35, 36).

Proteins associated with the mycobacterial cell wall and proteins actively secreted by live mycobacteria have been shown to be potentially important in protective immunity and in immunopathological events that occur during mycobacterial infections (2, 4, 14, 18, 23, 24, 44). Cell wall proteins of *Mycobacterium leprae* have been shown to contain powerful immunologic activity and are the predominant antigens recognized by T cells (21, 23). Several protein antigens, including the 28-kDa protein and hsp65, have been shown to be associated with the *M. leprae* cell wall (15). Cell wall-reactive T-cell clones have also been shown to react with mycobacterial 28-, 30-, and 65-kDa proteins (4).

A number of antigens actively secreted from mycobacteria have been identified in culture fluids of *M. tuberculosis* and *M. bovis* BCG (14, 24, 45, 46). These studies have revealed that the antigen 85 complex of the 30-kDa region is the major secreted protein in mycobacterial culture fluid (14, 45). This complex has been further characterized as three internally cross-reacting components, namely, 85A, 85B, and 85C, that are encoded by three separate genes (47). The genes that code for these three components have been cloned and sequenced (5, 9, 20). Interestingly, human fibronectin readily binds to all three components of the antigen 85 complex. This property has been implicated as the possible role of the antigen 85 complex in the pathogenesis of mycobacterial diseases (1). In a recent study using a monoclonal antibody (MAb) specific for the components of the antigen 85 complex, we have demonstrated the localization of this antigen in various forms of leprosy lesions (28). Furthermore, by

* Corresponding author.

TABLE 1. Species specificity of MAbs Rb2, Pe12, and A2h11 as analyzed by immunoblotting using sonicates of different mycobacterial species

Mycobacterial species	MAb reactivity[a]		
	Rb2	Pe12	A2h11
M. bovis BCG	3+	2+	3+
M. tuberculosis	3+	2+	3+
M. microti	3+	2+	3+
M. africanum	3+	2+	3+
M. leprae	3+	2+	3+
M. avium	2+	2+	3+
M. intracellulare	+	+	+
M. scrofulaceum	−	−	+
M. nonchromogenicum	−	−	−
M. xenopi	+	+	2+
M. vaccae	−	−	2+
M. marinum	+	+	+
M. duvali	+	+	+
M. kansasii	−	−	2+
M. smegmatis	−	−	+
M. terrae	−	−	−
M. gordonae	−	−	−
M. fortuitum	−	−	+
M. flavescens	−	−	−

[a] In all positive cases, MAbs showed a 65-kDa band whereas a weak 30-kDa region band(s) was detected only in some mycobacterial species. Reactivity was graded as negative (−) or from 1+ to 3+ in order of increasing intensity of the 65-kDa band.

screening of an *M. leprae* genomic library with lepromatous leprosy sera, the gene that encodes an *M. leprae* homolog of the fibronectin-binding antigen 85 complex has been cloned and sequenced (39).

These culminative data further indicate the importance of both mycobacterial hsp65 and the 30-kDa region proteins as immune targets in mycobacterial infections. Therefore, we searched for possible target epitopes of these proteins in relation to leprosy pathogenesis by using a novel series of MAbs. We identified three unique epitopes shared between the native proteins of mycobacterial hsp65 and actively secreted components of the antigen 85 complex. Here we report their immunological characterization and their intracellular and cell wall surface localization in *M. leprae* within skin lesions of lepromatous leprosy patients and in *M. leprae*-infected armadillo tissue.

(A part of this study was presented at the 8th International Congress of Immunology, Budapest, Hungary, 23 to 28 August 1992.)

MATERIALS AND METHODS

Mycobacteria. The different mycobacterial species, including armadillo-derived *M. leprae*, used in this study are listed in Table 1. Sonic extracts of each of these mycobacterial species were prepared as described previously (11). Culture fluids were prepared from stationary-phase cultures of *M. tuberculosis* H37Rv (ATCC 27294) and *M. bovis* BCG substrain 1331 (Statens Serum Institute, Copenhagen, Denmark) as described previously (24, 45, 47).

Isolation, fractionation, and purification of antigens. Antigens in the 65- and 30-kDa regions were isolated from the whole sonicate of *M. tuberculosis* (RIVM strain 7114 from the National Institute of Public Health, Bilthoven, The Netherlands) by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (11). In addition, the cytosol fraction from the same *M. tuberculosis* sonicate was prepared as described in our earlier report (27).

The actively secreted antigen 85 complex, i.e., 85A (MPT44), 85B (MPT59), and 85C (MPT45), as well as the closely related proteins MPT51 and MPT64, were purified from stationary-phase culture fluid of *M. tuberculosis* H37Rv by a combination of ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography on DEAE-Sepharose as described by Nagai et al. (24).

Recombinant fusion proteins. The native purified recombinant *M. bovis* BCG hsp65 (MbaA) protein and the Cro–β-galactosidase–MbaA fusion or truncated proteins carrying either all or defined parts of MbaA that were expressed by recombinant plasmids pRIB1404-pRIB1453 and pRIB1300-pRIB1302 were prepared as described by Thole et al. (38, 40). Preparation of *M. leprae* 30- and 31-kDa antigen 85 complex fusion proteins expressed by pIHB1004 and pIHB1006 has also been described by Thole et al. (39).

Preparation, purification, and labeling of MAbs. Antibody-producing hybridomas were prepared as described previously (27, 30) and were based on the standard protocol (7). Briefly, spleen cells from female BALB/c mice immunized with gel-purified 65- and 30-kDa region antigen fractions from the *M. tuberculosis* sonicate were fused with the Ag8-653 BALB/c myeloma cell line at a ratio of 2:1 in the presence of 5% polyethelene glycol 4000 (Serva Feinbiochemica, Heidelberg, Germany) in RPMI 1640. Antibody activity in hybridoma supernatants was detected by using gel-purified mycobacterial 65-kDa region, hsp65, and 30-kDa region proteins in an enzyme-linked immunosorbent assay (ELISA) and by using both the sonicate and the culture fluid of *M. tuberculosis* in immunoblotting as described below. Selected hybridomas were cloned by limiting dilution and grown in bulk cultures. Most of the antibody-containing culture supernatants were concentrated by the Minitan Ultrafiltration system (Millipore Corp., Bedford, Mass.), whereas ascitic fluids were made from some clones. The isotypes of immunoglobulins produced by the stable hybridomas were determined by an ELISA-based isotype kit (Zymed Laboratories, South San Francisco, Calif.). Immunoglobins were either partially purified from concentrated culture supernatants by 45% ammonium sulfate precipitation or affinity purified by using a protein G-Sepharose 4 column (MAb Trap G; Pharmacia, Uppsala, Sweden) in accordance with the instructions of the manufacturer. Affinity-purified MAbs were labeled with horseradish peroxidase (HRP) as reported earlier (25, 28).

In addition, the previously characterized anti-65-kDa MAb F67-2 (3, 6) and anti-30-kDa MAb HYT27 (44) used in this study were kindly provided by A. H. J. Kolk, Royal Tropical Institute, Amsterdam, The Netherlands, and J. Bennedsen, Statens Seruminstitut, Copenhagen, Denmark, respectively.

ELISAs. ELISAs were performed as described in our earlier reports, by using either gel-purified 65- or 30-kDa region antigens (11), affinity-purified recombinant mycobacterial hsp65 (30), or secreted MPT44 (85A), MPT59 (85B), and MPT45 (85C) protein antigens (13). In addition, an inhibition ELISA with HRP-labeled and unlabeled MAbs using mycobacterial hsp65 was performed as described previously (30).

SDS-PAGE, immunoblotting, and dot blot assay. SDS-PAGE was performed on vertical slab gels (12%) in a discontinuous Tris buffer system as described by Laemmli (17). Sonicated mycobacterial antigen preparations and the

cytosol fraction of *M. tuberculosis* were applied at a protein concentration of 15 μg per slot, whereas 1 to 2 μg of proteins from purified mycobacterial hsp65 and secreted MPT antigens was loaded. In the case of recombinant fusion proteins of the *M. leprae* 30- and 31-kDa antigen 85 complex, protein extract from *Escherichia coli* POP2136 carrying recombinant plasmids was analyzed by SDS–6% PAGE (39). Separated proteins were electroblotted onto nitrocellulose paper (Schleicher & Schuell, Inc.) as described by Towbin et al. (41) incubated either with partially purified MAbs from concentrated supernatants or with ascitic fluid at a dilution of 1:500, and processed for immunoblotting (27).

Dot blot assays with purified native mycobacterial hsp65 and MPT proteins were performed as described previously (46). Briefly, 1.5 μg of proteins in a 3-μl volume was spotted onto nitrocellulose paper and air dried. Nonspecific binding was blocked by 2% bovine serum albumin with 0.2% Tween 20 and 1% gelatin in phosphate-buffered saline (PBS), and nitrocellulose papers were incubated with MAbs (1:500) overnight at room temperature. After washing, nitrocellulose papers were incubated with HRP-labeled sheep anti-mouse immunoglobulin G $F(ab')_2$ (1:2,000) and further processed to visualize the reaction by using standard diaminobenzidine tetrahydrochloride as an indicator system.

Mapping of epitopes on mycobacterial hsp65 and the *M. leprae* antigen 85 complex. Mapping of MAb-defined epitopes on mycobacterial hsp65 was performed by using Cro–β-galactosidase–MbaA fusion or truncated proteins in a dot blot ELISA (40). Mapping of MAb-reactive epitope-containing regions on the *M. leprae* 30- and 31-kDa antigen 85 complex was determined by immunoblotting analysis with recombinant *M. leprae* fusion proteins as described in our earlier report (39).

Patients and tissue specimens. The biopsies from skin lesions of lepromatous leprosy patients (classified by the clinicopathological criteria of Ridley and Joplin [31]) used in this study were similar to those used in our previous study (28). A liver specimen from an armadillo experimentally infected with *M. leprae* was obtained from T. A. Eggelte, Royal Tropical Institute, Amsterdam, The Netherlands. All specimens were stained with hematoxylin and eosin for histological diagnosis, and a portion of each biopsy was snap frozen for immunoperoxidase and immunogold labeling studies.

Immunoperoxidase staining. A two-step MAb–peroxidase-conjugated rabbit anti-mouse immunoglobulin staining system with 3-amino-9-ethylcarbazole as the staining reagent was used as previously described (29). Briefly, 6-μm-thick cryostat serial sections were cut, dried, acetone fixed, and incubated with MAbs at a predetermined optimum dilution of 1:10 or 1:50 for 60 min. This step was followed by incubation with peroxidase-conjugated rabbit anti-mouse immunoglobulin for 30 min (Dako, Glostrup, Denmark). Peroxidase activity was visualized with 3-amino-9-ethylcarbazole, and sections were counterstained lightly with hematoxylin. Control experiments were performed by substituting either the primary antibody with an irrelevant antibody of a similar isotype or with PBS (pH 7.4). Positive staining was evaluated by at least two independent investigators.

The specificity of staining with mycobacterial antibody was also confirmed by preabsorbing the optimally diluted MAbs with excess antigens which were either purified mycobacterial hsp65 (38), antigen 85B, or total culture fluid of *M. tuberculosis* (24). Absorbtion was carried out for 1 h at 37°C and then overnight at 4°C. Absorbed MAb was then used in immunoperoxidase staining with frozen sections of either armadillo tissue or leprosy lesions and compared with the unabsorbed MAbs.

Immunogold labeling and electron microscopy. Frozen 6-μm-thick sections of *M. leprae*-infected armadillo tissue and lesional skin biopsies of lepromatous leprosy patients were placed on cellotape-covered microscopic slides and fixed in ice-cold acetone for 10 min. After fixation, the slides were incubated with MAbs overnight at 4°C in a humid chamber and washed extensively with PBS for 2 h at room temperature, with changing of the buffer at least four times. Then slides were incubated with rabbit anti-mouse immunoglobulin G (DAKO, Glostrup, Denmark) diluted 1:25 in PBS for 2 h and washed with a special PBS (PBSS) which contained 0.02 M glycine and 0.002% Triton X-100 (19). This step was followed by 3 h of incubation with 1-nm gold-labeled goat anti-rabbit immunoglobulin G (Aurion, Wageningen, The Netherlands) diluted 1:25 in PBSS. After overnight washing with PBSS at 4°C, slides were postfixed in 2.5% glutaraldehyde for 10 min and again washed thoroughly with ultrapure distilled water. Silver enhancement of the gold particles was performed for 10 min by using the enhancement kit provided by the manufacturer (Aurion). Following dehydration with ethanol and impregnation with Epon, a BEEM capsule (LADD, Burlington, Vt.) filled with Epon was placed over the section and allowed to polymerize overnight at 80°C. After cooling, the inverted BEEM capsule, along with the section, was popped off and 50- to 80-nm sections were cut and placed on Formvar-coated grids. Sections were then stained with uranyl acetate and then lead citrate and viewed in a Philips CM 10 electron microscope. Control experiments with PBS or irrelevant MAbs were performed to monitor nonspecific gold labeling.

RESULTS

Specificity and characterization of MAbs. Three hybridoma clones that reacted specifically with both mycobacterial hsp65 and the 30-kDa region protein–antigen 85 complex were isolated by screening over 200 antibody-producing hybridomas. Initial screening of these clones was performed by ELISA by using the same immunization agents and affinity-purified mycobacterial hsp65, followed by immunoblotting with both sonicate and stationary-phase culture fluid of *M. tuberculosis*. The clones were further characterized and established as stable hybridomas. The MAbs designated Rb2 [IgG1(κ)] and Pe12 [IgG1(κ)] were isolated from a fusion experiment after immunization with the 65-kDa antigen fraction, whereas MAb A2h11 [IgG1(κ)] was obtained from a separate fusion after immunization with the 30-kDa antigen fraction.

The reactivity patterns of MAbs Rb2, Pe12, and A2h11 with different antigen preparations are illustrated in Fig. 1 and 2. All MAbs showed strong reactivity with 65-kDa proteins from sonicated *M. tuberculosis*, *M. bovis* BCG, and *M. leprae* but not with that from *E. coli* (Fig. 1A). Only the reactivity of Rb2 with 30-kDa region antigens could be visualized weakly with all three sonicated mycobacteria, whereas Pe12 and A2h11 did not show any detectable reaction with 30-kDa region proteins. Further, these MAbs showed limited or broad cross-reactivity with the different sonicated mycobacterial species tested (Table 1). In all positive cases, reaction with the 65-kDa proteins was invariably seen, whereas weak 30-kDa bands were observed only with some mycobacterial species.

It is known that 30-kDa region proteins are present in different concentrations inside and outside the bacteria and

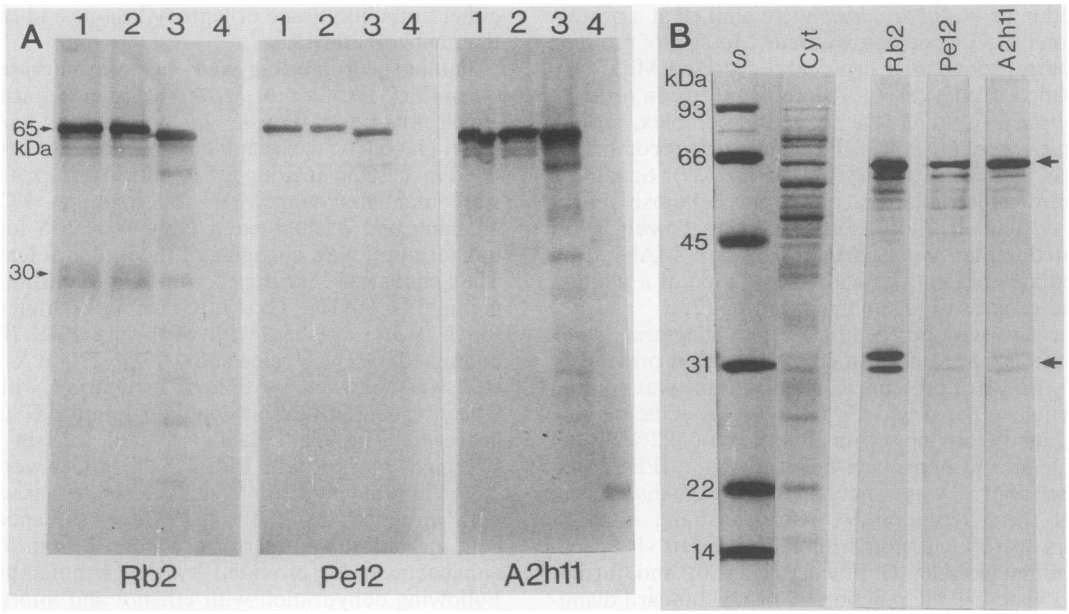

FIG. 1. (A) Immunoblot analysis of the reactivity of MAbs with sonic extracts of *M. bovis* BCG (lane 1), *M. tuberculosis* (lane 2), *M. leprae* (lane 3), and *E. coli* (lane 4). Note the reactivity only with the mycobacterium-specific 65-kDa protein by all three MAbs and weak binding to 30-kDa region proteins by MAb Rb2 alone. (B) Immunoblot analysis showing the reactivity of MAbs Rb2, Pe12, and A2h11 with the cytosol fraction (Cyt) of *M. tuberculosis*. An amido black-stained Western blot containing the cytosol fraction is shown in the left. Note the strong reactivity of MAb Rb2 with the 30-kDa region doublet protein (lower arrow) in the cytosol fraction compared with Pe12 and A2h11. The upper arrow indicates the 65-kDa band. Molecular size markers were in lane S.

also constitute the major proteins in culture fluid. Therefore, we tested the MAb reactivity with a well-characterized *M. tuberculosis* cytosol fraction and culture fluid in immunoblotting. Figure 1B illustrates the reactivities of MAbs with the cytosol fraction. It can be seen that MAb Rb2 reacted strongly with both the mycobacterial 65-kDa protein and 30-kDa doublet with similar intensities, whereas Pe12 and A2h11 showed a very weak reaction with 30-kDa doublet proteins but strongly bound to the 65-kDa protein. The binding patterns of these MAbs to culture fluid prepared from stationary-phase cultures of *M. tuberculosis* H37Rv is described below (Fig. 2, lane 4).

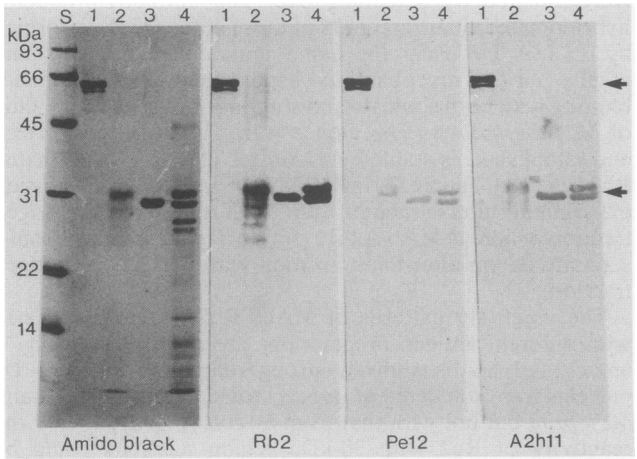

FIG. 2. Immunoblot analysis of MAbs that recognize mycobacterial hsp65 and the components of the antigen 85 complex. (A) Amido black-stained Western blots of purified mycobacterial hsp65 MbaA protein (lane 1), antigen 85A (lane 2), antigen 85B (lane 3), and the total culture fluid of *M. tuberculosis* H37Rv (lane 4) from which the antigen 85 complex was derived. (B) Reactivity of MAbs with Western blots containing the same antigens. Note the strong binding of all MAbs to mycobacterial hsp65 (upper arrow) and the strong reactivity of MAb Rb2 with the antigen 85 complex and corresponding 30-kDa proteins (lower arrow) from culture fluid compared with Pe12 and A2h11. Lane S contained molecular size markers.

Relationship between mycobacterial hsp65 and the secreted antigen 85 complex. The 30-kDa region proteins and the antigen 85 complex of *M. tuberculosis* and *M. bovis* BCG culture fluids correspond to the major mycobacterial secreted proteins. Therefore, we studied the reactivities of these MAbs with purified individual components of the antigen 85 complex, namely, 85A (MPT44), 85B (MPT59), and 85C (MPT45), and with mycobacterial hsp65 at the epitope level.

(i) **Immunoblotting.** Figure 2 illustrates the relationship between mycobacterial hsp65 and the secreted antigen 85 complex. Western blots (immunoblots) containing purified mycobacterial hsp65, MPT44 (85A), and MPT59 (85B), as well as total *M. tuberculosis* H37Rv culture fluid, were separately reacted with all three MAbs. Rb2 showed a strong reaction with both mycobacterial hsp65 and 85A-85B, as well as with the 30-kDa doublet proteins from the total culture fluid corresponding to the components of the antigen 85 complex. In contrast, the binding of Pe12 and A2h11 to purified MPT proteins and culture filtrate antigens was found to be rather weak while the reaction to mycobacterial hsp65 was strong. The reactivity of Pe12 and A2h11 to mycobacterial hsp65 showed an intensity similar to that of MAb Rb2. The results indicate that all three MAb-defined epitopes are shared between mycobacterial hsp65 and the antigen 85 complex. Although these MAbs bind to the same proteins,

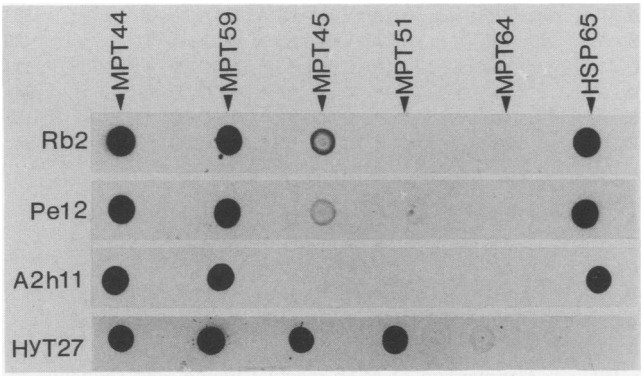

FIG. 3. Dot blot ELISA showing binding of MAbs to purified native proteins of the secreted antigen 85 complex (MPT44 [85A], MPT59 [85B], and MPT45 [85C], MPT51 (27 kDa), and MPT64 (26 kDa), as well as mycobacterial hsp65. Note the strong reactivity of MAbs Rb2, Pe12, and A2h11 with native hsp65 and the MPT44 (85A) and MPT59 (85B) components but not with the closely related MPT51 and MPT64 proteins. The reactivity of previously characterized MAb HYT27 is shown as a positive control for the antigen 85 complex and MPT51 (44, 46). Note the nonreactivity of HYT27 with hsp65.

TABLE 2. Reactivity of MAbs with Cro-LacZ-MbaA *M. bovis* BCG hsp65 fusion proteins and truncated MbaA derivatives

Protein-expressing plasmid	Amino acid residues of MbaA expressed[a]	MAb reactivity[b]		
		Rb2	Pe12	A2h11
pRIB1404	1–540	+	+	+
pRIB1421	62–540	+	+	+
pRIB1418,1419	66–540	+	+	+
pRIB1424	84–540	+	+	+
pRIB1422	109–540	+	+	+
pRIB1425	110–540	+	+	+
pRIB1427	124–540	+	+	+
pRIB1426	171–540	+	+	+
pRIB1451	276–540	+	+	+
pRIB1430	280–540	+	+	+
pRIB1444	303–540	+	+	+
pRIB1453	424–540	+	+	−
pEX2	None	−	−	−
pRIB1300	1–540	+	+	+
pRIB1301	1–14, 235–540	+	+	+
pRIB1302	1–481	−	−	+

[a] The amino-terminal methionine was taken as residue 1 in MbaA hsp65, which has a length of 540 amino acids (38, 40).
[b] Reactivities of MAbs with total proteins from induced cells carrying various recombinant plasmids were determined in a dot blot ELISA as described previously (40).

there was a difference at the individual epitope level, as Pe12 and A2h11 bound weakly to the antigen 85 complex alone but strongly to hsp65.

(ii) MAb reactivity with purified native proteins. Binding of MAbs to a particular protein is known to vary significantly in accordance with the native or denatured form of the protein and the system used for detection. The individual identities of the components of the antigen 85 complex cannot be accurately determined by immunoblotting, as these components do not resolve properly in SDS-PAGE (44). Therefore, we employed a dot blot assay and a solid-phase ELISA using previously characterized purified native proteins of the antigen 85 complex (24) and mycobacterial MbaA hsp65 (38, 40) to study the binding pattern of these MAbs. In contrast to the reactivity pattern in immunoblotting, all three MAbs, bound strongly to the native proteins of the antigen 85 complex and mycobacterial hsp65 with similar intensities in a dot blot assay (Fig. 3). Interestingly, MAb A2h11 reacted specifically with 85A and 85B alone, whereas Rb2 and Pe12 bound strongly to 85A and 85B and less strongly to 85C (Fig. 3). None of these MAbs showed a positive reaction with MPT51 or MPT64, although these proteins show extensive immunological cross-reactivity and sequence homology with the antigen 85 complex, indicating that these MAb-defined epitopes are specific for either the 85A or the 85B component alone or only all three components of the antigen 85 complex.

In the solid-phase ELISA, only MAb Rb2 showed a high optical density in response to 85A, 85B, and hsp65, but Pe12 and A2h11 bound very weakly to 85A and 85B while showing a high ELISA titer to hsp65 (data not shown). These results indicate that the crucial epitopes on the antigen 85 complex when coated in ELISA plates are not accessible for the latter two MAbs. In this study, the above reactivity patterns in both the dot blot assay and the ELISA were compared with that of previously characterized MAb HYT27 (1, 44). HYT27 did not show any reactivity with mycobacterial hsp65 but reacted strongly with the 85 complex and MPT51 and weakly with MPT64, indicating that the HYT27 epitope is present not only in the three components of the antigen 85 complex but also in MPT51 and MPT64 (Fig. 3). These data further confirm the specific binding of MAbs Rb2, Pe12, and A2h11 to mycobacterial hsp65 and to the secreted components of the antigen 85 complex alone.

Mapping of epitopes on *M. bovis* BCG hsp65 and the *M. leprae* antigen 85 complex. To identify the epitope-containing regions shared between mycobacterial hsp65 and the antigen 85 complex, MAbs were tested with the fusion proteins of *M. bovis* BCG hsp65 (40) and the *M. leprae* 30- and 31-kDa antigen 85 complex fusion proteins (39). The reactivity patterns of MAbs with fusion or truncated proteins of mycobacterial hsp65 are shown in Table 2. These results indicate that both Rb2 and Pe12 epitopes reside in the carboxy-terminal region of amino acid residues 479 to 540, whereas the A2h11 epitope-containing region is located in amino acid residues 303 to 424. The reactivities of these MAbs with the *M. leprae* 30- and 31-kDa fusion proteins are shown in Table 3. Interestingly, all three MAbs reacted only with the fusion protein expressed by pIHB1004, which expresses the amino acid sequence from 55 to 266, indicating the presence of these shared hsp65 epitopes on the N-terminal region of the *M. leprae* homolog of the antigen 85 complex.

TABLE 3. Reactivity of MAbs with the recombinant fusion proteins of the *M. leprae* 30- and 31-kDa antigen 85 complex

Fusion protein[a]-expressing plasmid	Amino acid sequence expressed	MAb reactivity[b]		
		Rb2	Pe12	A2h11
pIHB1004	55–266	+	+	+
pIHB1006	265–237	−	−	−
pEX2	None	−	−	−

[a] Expressed by recombinant plasmids containing two different parts of the 30- and 31-kDa antigen 85 complex gene of *M. leprae* (39).
[b] Determined by immunoblotting analysis using protein extracts from *E. coli* POP2136 carrying recombinant plasmids.

TABLE 4. Amino acid sequence similarities between *M. tuberculosis* and *M. bovis* BCG hsp65 and the *M. leprae* 30- and 31-kDa antigen 85 complex in the MAb-reactive regions of the two proteins

Compared amino acid sequences[a]		No. of amino acid similarities/total no. of amino acids
hsp65	Antigen 85 complex	
307–318	163–174	5/12
367–375	201–209	5/9
375–383	124–132	5/9
499–505	169–175	5/7

[a] From references 38 and 39.

To investigate the epitope specificities of these MAbs further, especially for Rb2 and Pe12 epitopes, as they reside in the same region of mycobacterial hsp65, we performed a cross-inhibition ELISA by using HRP-labeled, affinity-purified MAbs (Table 4). These experiments revealed that MAbs Rb2 and Pe12 partially inhibited each other for binding to mycobacterial hsp65, indicating a certain overlap between these two epitopes. On the other hand, A2h11 did not inhibit the binding of the latter two MAbs to mycobacterial hsp65 and thus provided further evidence that the location of the A2h11 epitope on mycobacterial hsp65 is different from that of the Rb2 and Pe12 epitopes. Furthermore, binding of Rb2 and Pe12 to hsp65 was not inhibited by previously characterized MAb F67-2, which has been mapped to a region within the Rb2-Pe12 epitope on mycobacterial hsp65 (3, 40). These results therefore indicate the occurrence of at least three different epitopes of mycobacterial hsp65 that are shared with the secreted antigen 85 complex of *M. tuberculosis*, *M. bovis* BCG, and *M. leprae*.

Amino acid sequence comparison. The epitope specificity of these MAbs showed that two regions of hsp65 and a region of the antigen 85 complex are involved in the cross-reactivity. To determine whether specific sequences are involved in this phenomenon, we searched the sequence homologies between these specific regions of the two proteins. Table 5 depicts the comparison of different sequences within the MAb-reactive regions of hsp65 from *M. tuberculosis* and *M. bovis* BCG and the 30- and 31-kDa antigen 85 complex of *M. leprae*. A number of amino acid similarities were found within these stretches. Figure 4 shows the best homologies between *M. tuberculosis* and *M. leprae* hsp65 and the proteins of the antigen 85 complex, 85A, 85B, and 85C. These similarities at the amino acid level might explain the locations of MAb-reactive epitopes common to these proteins.

In situ localization of shared epitopes in *M. leprae*. To ascertain the possible expression of these epitopes in *M. leprae*, we performed immunohistochemical analysis of bacilliferous lepromatous leprosy lesions and *M. leprae*-infected armadillo tissues by using MAbs.

TABLE 5. Specificity of MAb-defined epitopes on mycobacterial hsp65 as analyzed by inhibition ELISA

HRP-labeled MAb[a]	% inhibition produced by unlabeled MAb[b]:			
	Rb2	Pe12	A2h11	F67-2
Rb2	92	58	15	18
Pe12	50	87	10	20

[a] MAb dilutions corresponding to 50% of the maximum optical density at 450 nm were used; the dilution of both labeled MAbs was 1:2,000.

[b] Inhibition of binding of labeled MAbs to purified mycobacterial hsp65-coated ELISA plates (0.5 μg per well) in the presence of predetermined concentrations of unlabeled competitor MAbs.

```
A  375  A V I K A G A A T  383
B  422  A V I K A G A A T  430
C  124  * C G * * * C T *  132
D   86  * C R * * * C Q *   94
E  116  * C G * * * C Q *  124
F   86  * C G * * * C Q *   94

A  499  S A L Q N A A  505
B  546  S A L Q N A A  552
C  169  * * * I L * *  175
D  131  * * * T L * I  137
E  161  * * M I L * *  167
F  131  * * * I L * *  137
```

FIG. 4. Comparison of MAb-reactive amino acid sequence regions among mycobacterial hsp65 and the proteins of the antigen 85 complex. The best-homology-containing regions of MAbs A2h11 (top) and Rb2-Pe12 (bottom) are shown. The sequences of hsp65 of *M. tuberculosis* and *M. bovis* BCG (A; reference 38) and *M. leprae* (B; reference 33) are depicted in the single-letter code and compared with those of the *M. leprae* 30- and 31-kDa antigen 85 complex (C; reference 39), antigen 85A of *M. tuberculosis* (D; reference 5), antigen 85B of *M. bovis* BCG (E; reference 20), and antigen 85C of *M. tuberculosis* (F; reference 9). Identical residues between hsp65 and the components of the antigen 85 complex are indicated by asterisks.

(i) Immunoperoxidase staining. All of the MAbs showed clear rod-shaped bacillary staining in both bacilliferous lepromatous leprosy lesions and *M. leprae*-infected armadillo liver tissue. Figure 5 illustrates a representative bacterial staining pattern of MAb Rb2 with liver tissue of an armadillo infected with *M. leprae*. A similar staining pattern was observed with MAbs Pe12 and A2h11. These results indicate strong expression of all three epitopes on the cell surface of *M. leprae*. Detailed comparative immunohistochemical analysis of various forms of leprosy lesions with these MAbs, together with a panel of anti-30-kDa MAbs, will be described elsewhere (manuscript in preparation).

(ii) Immunogold ultracytochemistry. In view of the immunoperoxidase staining of *M. leprae* by MAbs and their reactivity with the bacterial cytosol fraction in immunoblotting, we further studied the ultrastructural localization of these MAb-defined epitopes within the bacilli present in human lepromatous leprosy skin lesions and armadillo tissue. As expected, in situ gold labeling (represented by the numerous black dots of 1-nm gold particles) on the cell surface of *M. leprae* bacilli was clearly demonstrated by all three MAbs in both *M. leprae*-infected armadillo tissue and lepromatous leprosy lesions. Representative examples with MAb Rb2 are shown in Fig. 6. Close examination of electron micrographs showed that the cell surface of the bacilli showed the highest labeling density (Fig. 6A), while a significant number of gold particles were also localized within the cytoplasm of the bacilli (Fig. 6B and C). Our data further provide evidence that these epitopes are expressed not only on the cell surface but also in the cytosol of *M. leprae*. Moreover, a few gold spheres were also observed within the tissue milieu but in the vicinity of bacteria (Fig. 6A). These labelings are probably due to the reactivity of MAbs with either cell wall debris, leaked cytoplasmic components, or secreted products of *M. leprae* within tissues.

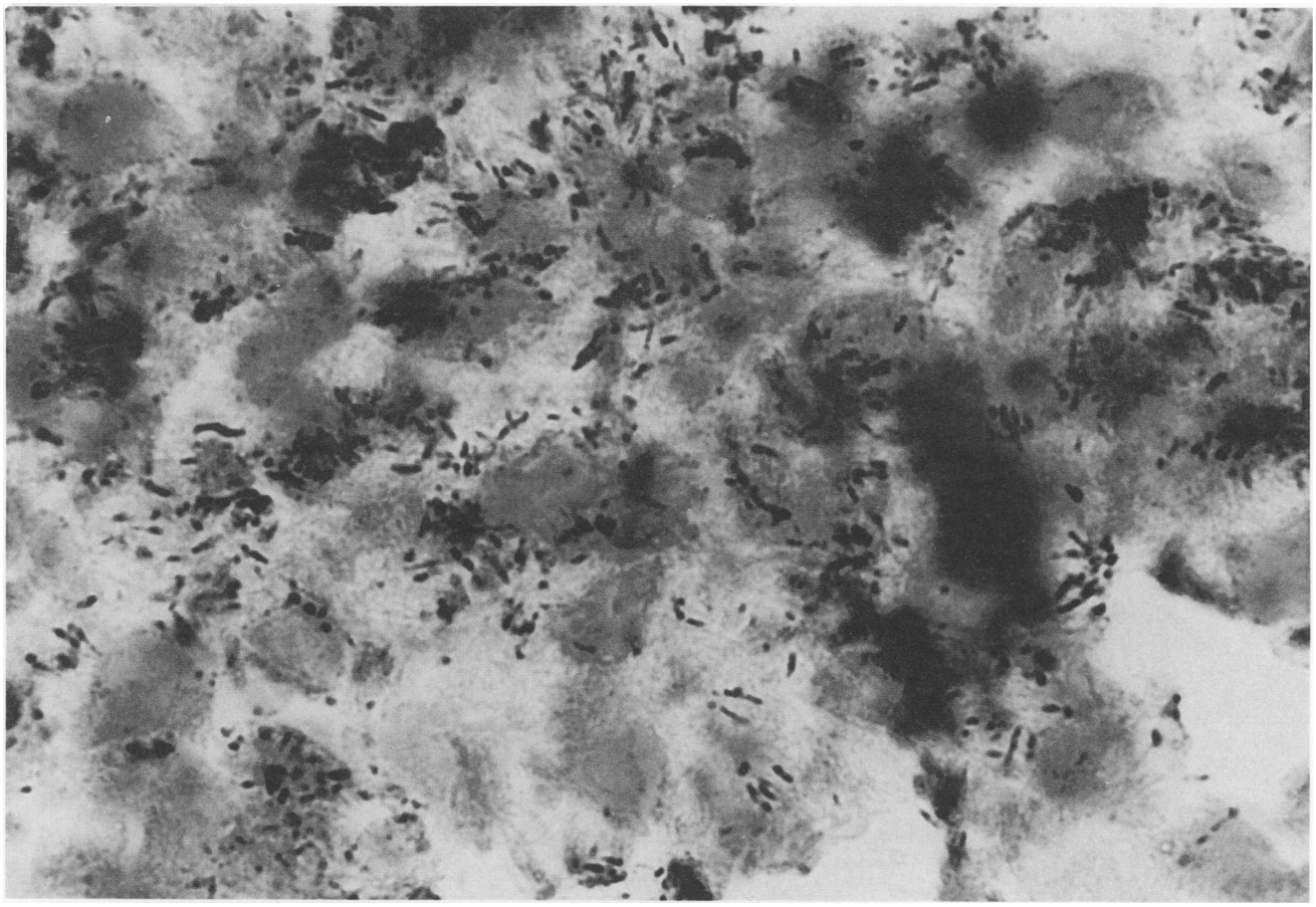

FIG. 5. Representative example showing immunoperoxidase staining by MAb Rb2 of liver tissue from an armadillo infected with *M. leprae*. Note the staining of rod-shaped bacilli in the tissues, indicating the presence of the Rb2 epitope at high density on the *M. leprae* surface. Both MAbs Pe12 and A2h11 showed staining identical to that of Rb2. Sections were counterstained with hematoxylin. Magnification, ×2,000.

DISCUSSION

The present study describes the identification and immunological characterization of three unique epitopes shared between mycobacterial hsp65 and the secreted components of the antigen 85 complex by three novel MAbs, Rb2, Pe12, and A2h11. We also describe the specific expression of all three epitopes on the cell surface of *M. leprae* in lepromatous leprosy lesions and in the liver tissue of armadillos infected with *M. leprae*.

The 65-kDa antigen is one of the most extensively studied antigens of *M. tuberculosis* and *M. leprae* and corresponds to the product of the conserved bacterial and human 60-kDa heat shock or stress protein family (16, 33, 34, 38, 48). Despite its evolutionary conservation and its postulated involvement in autoimmune diseases (8), mycobacterial hsp65 is still considered to be a source of a potential immune target in leprosy and tuberculosis (49). Several MAb-reactive epitopes and T-cell epitopes of this protein have been identified (3, 6, 12, 22, 23, 40). The three epitopes on *M. tuberculosis* and *M. leprae* hsp65 presented in this study are different from known epitopes defined by previously characterized MAbs. By using fusion and truncated proteins of the MbaA molecule, we mapped the Rb2-Pe12 and A2h11 epitopes to the C-terminal region of the 479 to 540- and 303 to 424-amino-acid sequences, respectively. A number of MAb-reactive epitopes in this particular region of *M. tuberculosis* and *M. leprae* have been previously identified (23, 33, 40), and some of them have been precisely localized by using synthetic peptides (3). Within this region, two epitopes have been mapped to *M. leprae*-specific sequences (3, 33) whereas one epitope has been mapped to an *M. tuberculosis*-specific sequence (30). In the A2h11 epitope-containing region of *M. tuberculosis* and *M. leprae* hsp65, at least five MAb-defined epitopes have been previously mapped (22, 33, 40). None of these anti-hsp65 MAbs have demonstrated any evidence of reactivity with the secreted antigen 85 complex or the presence of their epitopes on the *M. leprae* cell surface or cell wall (15, 43, 48). In addition, the species specificity of these MAbs is different from that of MAb A2h11, and some of these MAbs (IT32 [22], F67-13, and F67-18 [6]) also reacted with *E. coli* and other nonmycobacterial species (37), while A2h11 did not show any reactivity with *E. coli* (Fig. 1A). On the other hand, three well-characterized MAbs have been mapped to a part of the region of the Rb2-Pe12 epitope (amino acid residues 479 to 540) of *M. tuberculosis* and *M. leprae* hsp65 (3, 33, 40); the MAb F67-2 and IIC8 epitopes have been precisely mapped to a single epitope (3), whereas the MAb T-23 epitope was located in a region next to the F67-2–IIC8 epitope (22, 33). We confirmed by an ELISA inhibition experiment that the

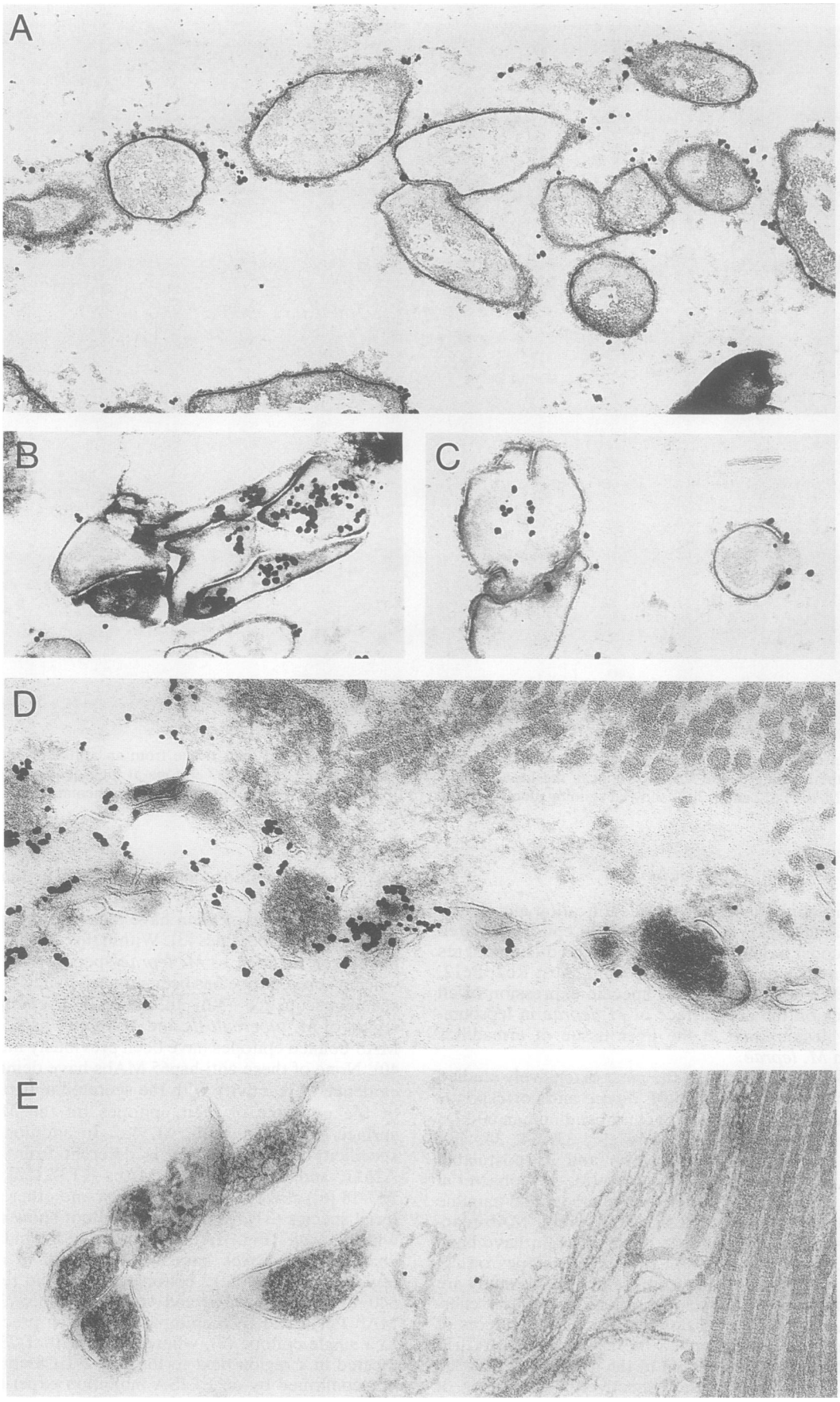
A
B
C
D
E

FIG. 6. Electron micrograph showing in situ immunogold labeling of *M. leprae* bacilli by MAb Rb2 in liver tissue of an armadillo infected with *M. leprae*. (A) Overview showing gold labeling mostly on the cell surface of *M. leprae*. Note the presence of few gold particles in the tissue milleu only in the vicinity of the bacilli, probably indicating labeling of either cell wall debris or degraded or secreted products of *M. leprae*. (B) Heavily labeled clusters of *M. leprae* showing bound gold particles along the characteristic electron-dense double layer of the cell wall and also within the cytoplasm. (C) Clear cytoplasmic labeling showing the presence of the Rb2 epitope in the cytosol of *M. leprae*. (D) In situ gold labeling of *M. leprae* in a human skin lesion from a patient with lepromatous leprosy (bacillary index [BI] = 6+) by MAb Rb2. Note the specific labeling only in association with bacilli in the lesion compared with the negative control with PBS (E). Magnifications: A, ×34,000; B, ×37,000; C, ×40,000; D and E, ×50,000.

F67-2–IIC8 epitope is different from the Rb2-Pe12 epitope as MAb F67-2 did not inhibit the binding of labeled MAbs Rb2 and Pe12 to mycobacterial hsp65. Interestingly, MAb IIC8 has been shown to react with the cell wall protein complex purified from *M. leprae* (15), but when compared with Rb2 and Pe12, the epitope of F67-2–IIC8 was not expressed in situ on the *M. leprae* cell surface. This was demonstrated by immunohistochemical analysis of bacilliferous lepromatous lesions and *M. leprae*-infected armadillo tissues by using MAb F67-2 (unpublished data). Moreover, when the published data on previously described MAb T-23 (21, 33) were compared with those on presently described MAbs Rb2 and Pe12, it was also found that the characteristics of the latter are different from those of the former. On the basis of these comparative analyses, we conclude that the three MAb-reactive epitopes described in the present study are newly identified epitopes on the C-terminal region of *M. tuberculosis* and *M. leprae* hsp65 that are shared with the components of the actively secreted antigen 85 complex.

By using fusion proteins of the *M. leprae* 30- and 31-kDa antigen complex, we confirmed the localization of the Rb2-Pe12 and A2h11 epitopes to the N-terminal region of amino acid residues 55 to 266 of the *M. leprae* antigen 85 complex. This amino acid region of the *M. leprae* antigen 85 complex was previously identified by using sera from lepromatous leprosy patients (39). Interestingly, the same *M. leprae* protein fragment of the antigen 85 complex has also been shown to be one of the binding sites for fibronectin (39). On comparing these MAb-reactive amino acid regions of mycobacterial hsp65 and the antigen 85 complex, we found that hsp65 showed certain amino acid sequence similarities to the proteins of the antigen 85 complex of *M. tuberculosis*, *M. bovis* BCG, and *M. leprae*. The specific cross-reactivity of these MAbs with hsp65 and the antigen 85 complex is probably due to recognition of these common amino acid sequences.

Further confirmation of shared epitopes between mycobacterial hsp65 and the antigen 85 complex was obtained by testing the reactivity of MAbs with purified antigens. An interesting finding is the different behavior of these epitopes with hsp65 and the antigen 85 complex under native and denatured conditions. Immunoblotting showed that MAb Rb2 reacted strongly with both hsp65 and the antigen 85 complex, as well as with the corresponding secreted 30-kDa region doublet proteins from culture fluid. On the other hand, while MAbs Pe12 and A2h11 reacted strongly with hsp65, they bound weakly to the antigen 85 complex and the 30-kDa doublet from culture fluid in the same Western blot. In contrast, all three MAbs strongly bound to the native proteins of mycobacterial hsp65, as well as to the 85A and 85B components, while only MAbs Rb2 and Pe12 showed a weak positive reaction with the 85C component. Since the strong signals produced by MAbs in immunoblotting is known to be due to recognition of linear or continuous epitopes, it is probable that the Rb2 epitope exists as a linear epitope on both mycobacterial hsp65 and the components of the antigen 85 complex. For the same reason, it is possible that while both Pe12 and A2h11 do exist as linear epitopes in two different regions of mycobacterial hsp65, they seem to be present as conformational epitopes in the antigen 85 complex. It is striking that a single epitope shared between two distinct mycobacterial proteins may exist in two different configurations. This behavior provides further evidence of the complexity of the antigen 85 complex at the epitope level.

Both hsp65 and the corresponding antigen 85 complex are considered to be immunodominant antigens of *M. leprae* (3, 6, 39, 43, 45, 49). In the present study, by using immunohistochemical analysis, we demonstrated the expression of these three shared epitopes on the cell wall surface of *M. leprae* within macrophages in bacilliferous lepromatous lesions and in *M. leprae*-infected armadillo liver. Further analysis of the in situ expression of these epitopes in lesions from leprosy patients across the clinical spectrum showed that these epitopes are differentially expressed within the lesions (data not shown). Furthermore, ultrastructural cytochemical analysis of these tissues demonstrated gold labeling on the cell surface of *M. leprae*. Interestingly, detection of significant gold particles within the cytoplasm of the bacilli may provide evidence that the antigen 85 complex is a cytoplasmic protein of *M. leprae*. However, since these MAbs reacted with both the antigen 85 complex and hsp65, it is not clear which antigen is present on the *M. leprae* cell surface or cytosol. Nevertheless, immunohistochemical inhibition experiments showed that staining of *M. leprae* in the lesions was abolished after preincubation of MAbs with hsp65 and the antigen 85 complex or the total culture filtrate of *M. tuberculosis*. These data provide evidence for the intracellular and surface localization of both the 30-kDa antigen 85 complex and hsp65 in *M. leprae*.

In conclusion, our results demonstrate that at least three novel MAb-defined epitopes of mycobacterial hsp65 are shared with the secreted proteins of the antigen 85 complex. Strong in situ expression of these epitopes was found on intact *M. leprae* within macrophages in lepromatous leprosy lesions and in *M. leprae*-infected armadillo tissue. Since both secreted proteins and the polypeptides associated with the *M. leprae* cell wall have powerful immunologic activity, it is likely that these epitope-containing peptides are important during *M. leprae* infection.

ACKNOWLEDGMENTS

This work was carried out under project GNK/DA/004 (AHG 49) of the Faculty of Medicine, University of Amsterdam, and Dutch Medical Research Council project 511-022. We thank Q. M. Gastmann-Wichers Stichting for partial financial support during this investigation.

We also thank J. D. A. van Embden (National Institute of Public Health, Bilthoven, The Netherlands) for providing purified mycobacterial hsp65 and the fusion proteins, S. Nagai and H. G. Wiker

for provision of purified MPT proteins, and J. D. Bos and J. J. Weening for continuing support during this investigation. We are also grateful to S. Yong, A. Chand, Y. E. Cornelisse, and Gunni Ulvund for excellent technical assistance.

REFERENCES

1. **Abou-Zeid, C., T. L. Ratliff, H. G. Wiker, M. Harboe, J. Bennedsen, and G. A. W. Rook.** 1988. Characterization of fibronectin-binding antigens released by *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. Infect. Immun. **56:** 3046–3051.
2. **Andersen, P., D. Askgaard, L. Ljungqvist, M. W. Bentzon, and I. Heron.** 1991. T-cell proliferative response to antigens secreted by *Mycobacterium tuberculosis*. Infect. Immun. **59:**1558–1563.
3. **Anderson, D. C., M. E. Barry, and T. M. Buchanan.** 1988. Exact definition of species specific and crossreactive epitopes of the 65-kilodalton protein of *Mycobacterium leprae* using synthetic peptides. J. Immunol. **141:**607–613.
4. **Barnes, P. F., V. Mehra, G. R. Hirschfield, S. Fong, C. Abou-Zeid, G. A. W. Rook, S. W. Hunter, P. J. Brennan, and R. L. Modlin.** 1989. Characterization of T cell antigens associated with the cell wall protein-peptidoglycan complex of *Mycobacterium tuberculosis*. J. Immunol. **143:**2656–2662.
5. **Borremans, M., L. De Wit, G. Volckaert, J. Ooms, J. De Bruyn, K. Huygen, J.-P. Van Vooren, M. Stelandre, R. Verhofstadt, and J. Content.** 1989. Cloning, sequence determination, and expression of a 32-kilodalton-protein gene of *Mycobacterium tuberculosis*. Infect. Immun. **57:**3123–3130.
6. **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.
7. **Campbell, A. M.** 1984. Monoclonal antibody technology, p. 1–215. *In* R. H. Burdon and P. H. Knippenberg (ed.), Laboratory techniques in biochemistry and molecular biology. Vol. 14. Elsevier, Amsterdam.
8. **Cohen, I. R., and D. B. Young.** 1991. Autoimmunity, microbial immunity and the immunological homunculus. Immunol. Today **12:**105–110.
9. **Content, J., A. de la Cuvellerie, L. De Wit, V. Vincent-Levy-Frébault, J. Ooms, and J. De Bruyn.** 1991. The genes coding for the antigen 85 complexes of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG are members of a gene family: cloning, sequence determination, and genomic organization of the gene coding for antigen 85-C of *M. tuberculosis*. Infect. Immun. **59:**3205–3212.
10. **Convit, J., C. Sampson, M. Zuniga, P. G. Smith, J. Plata, J. Silva, J. Molina, M. E. Pinardi, B. R. Bloom, and A. Salgado.** 1992. Immunoprophylactic trial with combined *Mycobacterium leprae*/BCG vaccine against leprosy: preliminary results. Lancet **339:**446–450.
11. **Das, P. K., A. Rambukkana, J. G. Baas, D. G. Groothuis, and M. Halperin.** 1990. Enzyme-linked immunosorbent assay for distinguishing serological responses of lepromatous and tuberculoid leprosies to the 29/33-kilodalton and 64-kilodalton antigens of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **28:**379–382.
12. **Emmrich, F., J. E. R. Thole, J. D. A. Van Embden, and S. H. E. Kaufmann.** 1986. A recombinant 65-kDa protein of *Mycobacterium bovis* BCG specifically stimulates human T4 clones reactive to mycobacterial antigens. J. Exp. Med. **163:**1024–1029.
13. **Harboe, M., H. G. Wiker, J. R. Duncan, M. M. Garcia, T. W. Dukes, B. W. Brooks, C. Turcotte, and S. Nagai.** 1990. Protein G-based enzyme-linked immunosorbent assay for anti-MPB70 antibodies in bovine tuberculosis. J. Clin. Microbiol. **28:**913–921.
14. **Harboe, M., H. G. Wiker, and S. Nagai.** 1992. Protein antigens of mycobacteria studied by quantitative immunologic techniques. Clin. Infect. Dis. **14:**313–319.
15. **Hunter, S. W., M. McNeil, R. L. Modlin, V. Mehra, B. R. Bloom, and P. J. Brennan.** 1989. Isolation and characterization of the highly immunogenic cell wall-associated protein of *Mycobacterium leprae*. J. Immunol. **142:**2864–2872.
16. **Jindal, S., A. K. Dudani, B. Singh, C. B. Harley, and R. S. Gupta.** 1989. Primary structure of a human mitochondrial protein homologous to the bacterial and plant chaperonins and to the 65-kilodalton mycobacterial antigen. Mol. Cell. Biol. **9:** 2279–2283.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **226:**680–685.
18. **Lovik, M., and O. Closs.** 1984. Induction of immunity against live *Mycobacterium lepraemurium*: a requirement for viable bacilli. Immunology **53:**165–173.
19. **Manuela, M. M. G., and K. T. Tokuyasu.** 1988. A pre-embedding immunolabeling technique for basal lamina and extracellular matrix molecules. J. Histochem. Cytochem. **36:**453–488.
20. **Matsuo, K., R. Yamaguchi, A. Yamazaki, H. Tasaka, and T. Yamada.** 1988. Cloning and expression of the *Mycobacterium bovis* BCG gene for the extracellular α antigen. J. Bacteriol. **170:**3847–3854.
21. **Mehra, V., B. R. Bloom, V. K. Torigian, D. Mandich, M. Reichel, S. M. M. Young, P. Salgame, J. Convit, S. W. Hunter, M. McNeil, P. J. Brennan, T. H. Rea, and R. L. Modlin.** 1989. Characterization of *Mycobacterium leprae* cell wall-associated proteins with the use of T lymphocyte clones. J. Immunol. **142:**2873–2878.
22. **Mehra, V., D. Sweetser, and R. A. Young.** 1986. Efficient mapping of protein antigenic determinants. Proc. Natl. Acad. Sci. USA **83:**7013–7017.
23. **Melancon-Kaplan, J., S. W. Hunter, M. McNeil, C. Stewart, R. L. Modlin, T. H. Rea, J. Convit, P. Salgame, V. Mehra, B. R. Bloom, and P. J. Brennan.** 1988. Immunological significance of *Mycobacterium leprae* cell walls. Proc. Natl. Acad. Sci. USA **85:**1917–1921.
24. **Nagai, S., H. G. Wiker, M. Harboe, and M. Kinomoto.** 1991. Isolation and partial characterization of major protein antigens in the culture fluid of *Mycobacterium tuberculosis*. Infect. Immun. **59:**372–382.
25. **Nakana, P. K., and A. Kawaoi.** 1974. Peroxidase-labeled antibody. A new method of conjugation. J. Histochem. **22:**1084–1091.
26. **Pessolani, M. C. V., F. D. Rumjanek, M. A. de Melo Marques, F. S. F. De Melo, and E. N. Sarno.** 1989. Serological response of patients with leprosy to a 28- to 30-kilodalton protein doublet from early cultures of *Mycobacterium bovis* BCG. J. Clin. Microbiol. **27:**2184–2189.
27. **Rambukkana, A., P. K. Das, A. Chand, J. G. Baas, D. G. Groothuis, and A. H. J. Kolk.** 1991. Subcellular distribution of monoclonal antibody defined epitopes on *Mycobacterium tuberculosis* proteins in the 30-kDa region: identification and localization of 29/33-kDa doublet proteins on mycobacterial cell wall. Scand. J. Immunol. **33:**763–775.
28. **Rambukkana, A., P. K. Das, S. Krieg, and W. R. Faber.** 1992. Association of mycobacterial 30-kDa region proteins with the cutaneous infiltrates of leprosy lesions: evidence for the involvement of the major secreted proteins of mycobacteria in the local immune response of leprosy. Scand. J. Immunol. **36:**35–48.
29. **Rambukkana, A., P. K. Das, S. Krieg, I. C. Poole, and J. D. Bos.** 1992. Mycobacterial 65-kilodalton heat shock protein shares a carboxy terminal epitope with human epidermal cytokeratin 1/2. Immunology **77:**267–276.
30. **Rambukkana, A., S. Yong, and P. K. Das.** 1991. Identification of a novel B cell epitope of restricted specificity on the hsp 65-kDa protein of *Mycobacterium tuberculosis*. FEMS Microbiol. Immunol. **76:**39–46.
31. **Ridley, D. S., and W. H. Joplin.** 1986. Classification of leprosy according to immunity. A five group system. Int. J. Leprosy **34:**255–273.
32. **Rumschlag, H. S., T. M. Shinnick, and M. L. Cohen.** 1988. Serological responses of patients with lepromatous and tuberculoid leprosy to 30-, 31-, and 32-kilodalton antigens of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **26:**2200–2202.
33. **Shinnick, T. M., D. Sweetser, J. Thole, J. D. A. van Embden, and R. A. Young.** 1987. The etiologic agents of leprosy and

tuberculosis share an immunoreactive protein antigen with the vaccine strain *Mycobacterium bovis* BCG. Infect. Immun. **55:** 1932–1935.

34. **Shinnick, T. M., M. H. Vodkin, and J. C. Williams.** 1988. The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to *Escherichia coli* GroEL protein. Infect. Immun. **56:**446–451.
35. **Standford, J. L.** 1984. Leprosy research present and future. Acta Leprol. **II:**421.
36. **Stanley, S. J., C. Howland, M. M. Stonne, and I. Sutherland.** 1981. BCG vaccination of children against leprosy in Uganda. Final results. J. Hyg. **87:**233–248.
37. **Thole, J. E. R., P. Hindersson, J. De Bruyn, F. Cremers, J. Van Der Zee, H. De Clock, J. Tommassen, W. Van Eden, and J. D. Van Embden.** 1988. Antigenic relatedness of a strong immunogenic 65-kDa mycobacterial protein antigen with a similarly sized ubiquitous bacterial common antigen. Microb. Pathog. **4:**71–83.
38. **Thole, J. E. R., W. J. Keulen, A. H. J. Kolk, D. G. Groothuis, L. G. Berwald, R. H. Tiesjema, and J. D. van Embden.** 1987. Characterization, sequence determination, and immunological properties of a 65-kilodalton protein of *Mycobacterium bovis* BCG expressed in *Escherichia coli* K-12. Infect. Immun. **55:** 1466–1475.
39. **Thole, J. E. R., R. Schoningh, A. A. M. Janson, T. Garbe, Y. E. Cornelisse, J. E. Clark-Curtiss, A. H. J. Kolk, T. H. M. Ottenhoff, R. R. P. De Vries, and C. Abou-Zeid.** 1992. Molecular and immunological analysis of fibronectin-binding protein antigen secreted by *Mycobacterium leprae*. Mol. Microbiol. **6:**153–163.
40. **Thole, J. E. R., W. C. A. van Schooten, W. J. Keulen, P. W. M. Hermans, A. A. M. Janson, R. R. P. de Vries, A. H. J. Kolk, and J. D. A. van Embden.** 1988. Use of recombinant antigens expressed in *Escherichia coli* K-12 to map B-cell and T-cell epitopes on the immunodominant 65-kilodalton protein of *Mycobacterium bovis* BCG. Infect. Immun. **56:**1633–1640.
41. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
42. **Turneer, M., J. P. Van Vooren, J. De Bruyn, E. Serruys, P. Dierckx, and J.-C. Yernault.** 1988. Humoral immune response in tuberculosis: immunoglobulins G, A, and M directed against the purified P32 protein antigen of *Mycobacterium bovis* bacillus Calmette-Guérin. J. Clin. Microbiol. **26:**1714–1719.
43. **Watson, J. D.** 1989. Leprosy: understanding protective immunity. Immunol. Today **10:**218–221.
44. **Wiker, H. G., M. Harboe, S. Nagai, and J. Bennedsen.** 1990. Quantitative and qualitative studies on the major extracellular antigen of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG. Am. Rev. Respir. Dis. **141:**830–838.
45. **Wiker, H. G., M. Harboe, S. Nagai, M. E. Patarroyo, C. Ramirez, and N. Cruz.** 1986. MPB59, a widely crossreacting protein of *Mycobacterium bovis* BCG. Int. Arch. Allergy Appl. Immunol. **81:**307–314.
46. **Wiker, H. G., S. Nagai, M. Harboe, and L. Lingqvist.** 1992. A family of crossreacting proteins secreted by *Mycobacterium tuberculosis*. Scand. J. Immunol. **36:**307–319.
47. **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.
48. **Young, D. B.** 1988. Structure of mycobacterial antigens. Br. Med. Bull. **44:**562–583.
49. **Young, D. B., R. Lathigra, R. Hendrix, D. Sweetser, and R. Young.** 1988. Stress proteins are immune targets in leprosy and tuberculosis. Proc. Natl. Acad. Sci. USA **85:**4267–4270.