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Heterogeneity of Monoclonal Antibody-Reactive Epitopes on Mycobacterial 30-Kilodalton-Region Proteins and the Secreted Antigen 85 Complex and Demonstration of Antigen 85B on the *Mycobacterium leprae* Cell Wall Surface

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Proteins of the antigen 85 complex in the 30-kDa region secreted by live mycobacteria are important in the immune response against mycobacterial infections and may play an important biological role in the host-parasite interaction. In the present study, we have characterized epitopes of the 30-kDa-region proteins and the antigen 85 complex by using a panel of 13 monoclonal antibodies (MAbs) reacting with these antigens, 6 of which have not been described before. By using five previously characterized related secreted proteins of *Mycobacterium tuberculosis*, MPT44 (85A), MPT59 (85B), MPT45 (85C), MPT51 (27 kDa), and MPT64 (26 kDa), we have identified at least 10 different MAb-reactive epitopes on the proteins of the antigen 85 complex. A heterogeneous distribution of epitopes was observed within the components of the antigen 85 complex. Two distinct epitopes specific for antigen 85B and two other epitopes restricted to the 85A and 85B components were recognized. Two of them were shared with a previously unidentified 27-kDa protein present in *M. tuberculosis* culture fluid from which all MPT proteins were derived. The rest of the MAb-reactive epitopes were found to be present mostly in antigens 85A and 85B and to a lesser extent in antigen 85C. None of these MAbs recognized component 85C alone nor did they bind to the related MPT51 and MPT64 proteins. Interestingly, most of the MAbs reacted with purified native proteins of the antigen 85 complex but not to them in their denatured forms. In contrast, reactivity of the MAbs with the cytosol fraction of *M. tuberculosis* in immunoblotting revealed that they bound to a closely related cytosolic 30-kDa protein(s) even when they were denatured. Heterogeneity of these MAb-reactive epitopes of the antigen 85 complex was further evident as they were found to be distributed in various patterns among 19 different mycobacterial species. By using fusion proteins of the *Mycobacterium leprae* 30/31-kDa antigen 85 complex, we have localized at least six different epitopes within amino acid residues 55 to 266 of the *M. leprae* antigen 85 complex. Finally, by immunohistochemical analysis, we have demonstrated the in situ expression of one of the novel MAb-reactive epitopes specific for antigen 85B on the cell wall surface of *M. leprae* within macrophages in lepromatous leprosy lesions and thus provide direct evidence for the presence of the B component of the antigen 85 complex on the surface of intact *M. leprae*.

Detailed analyses of immunodominant mycobacterial antigens and their epitopes are important to understand the pathogenic events occurring during mycobacterial infections and in the development of new diagnostic tools and subunit vaccines. Proteins in the 30-kDa region (30/31-kDa), being the major secreted antigens of mycobacteria which have been characterized as the antigen 85 complex, are considered to be important targets in immunopathological and/or protective responses during infections with *Mycobacterium leprae* and *Mycobacterium tuberculosis* (1, 3, 6, 14, 21, 23, 25, 34, 35, 37, 40). Antibody and T-cell responses to 30-kDa-region proteins and the antigen 85 complex of *M. tuberculosis*, *Mycobacterium bovis* BCG, and *M. leprae* have been shown in patients with leprosy and tuberculosis as well as in animals infected with these mycobacteria (3, 11–13, 22, 28, 34, 36, 37, 41). High-affinity binding of the 30/31-kDa antigen 85 complex to fibronectin further highlighted its potential relevance in the pathogenesis of mycobacterial diseases (1).

The extensive study of the antigen 85 complex has revealed that its individual components, 85A, 85B, and 85C, are closely related and possess significant amino acid sequence homologies (5, 8, 19–21, 43). The immunological importance of these antigens of similar molecular sizes and similar physical characteristics has long been realized, and the antigens have been described under a variety of names such as α antigen (20, 45), antigen a_2 (10), antigen 6 (16), and P32 (5, 36). More recently, a 30-kDa protein has also been purified and characterized from *M. tuberculosis* culture fluid (29). All of these antigens have been shown to identify immunologically with the components of the antigen 85 complex (16, 20, 21, 29, 41). Although structural analyses of the antigen 85 complex proteins have provided important clues concerning the location of their epitopes (19, 20), detailed characterizations of epitopes of these closely related proteins have not been carried out. It is considered that the components of the antigen 85 complex contain common antigenic epitopes as well as epitopes distinguishing them from one another (19, 40). The identification of such epitopes is of significance for the development of new diagnostic and protective tools as well as characterization of immune re-

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sponses to mycobacterial infections. Thus far, an exact definition of such epitopes and their location within the mycobacterium, especially in *M. leprae*, have not been determined. Moreover, in spite of the recent interest in the immunological role of the 30-kDa-region proteins and the antigen 85 complex, virtually nothing is known about the functional aspects of these proteins and their antigenic epitopes.

In the present study, by using a panel of 13 monoclonal antibodies (MAbs) reacting with 30-kDa-region proteins, we carried out detailed analyses of the individual secreted components of the antigen 85 complex and the 30-kDa-region somatic proteins at the epitope level. Here we report the identification and partial characterization of at least 10 different MAb-reactive epitopes on the antigen 85 complex, their relationship with 30-kDa cytosolic proteins as well as other related proteins, and their mapping on the recombinant *M. leprae* 30/31-kDa antigen 85 complex. We also report the in situ presence of an antigen 85B-specific epitope on the cell wall surface of *M. leprae* in the skin lesions of lepromatous leprosy patients.

MATERIALS AND METHODS

Mycobacterial antigens. Sonic extracts of 19 different mycobacterial species used in this study were prepared as described previously (24). They are listed in the legend to Fig. 3. Culture fluids were prepared from stationary-phase cultures of *M. tuberculosis* H37Rv (ATCC 27294) and *M. bovis* BCG substrain 1331 (Statens Seruminstitut, Copenhagen, Denmark) (14, 21).

Antigens in the 30-kDa region were isolated from a whole sonicate of *M. tuberculosis* by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11, 24).

Preparation of cytosol fraction. The preparation and biochemical characterization of the cytosol fraction of *M. tuberculosis* used in this study have been described in detail previously (24). Briefly, mycobacteria were harvested from 3- to 4-week-old stationary-phase cultures and washed with cold phosphate-buffered saline (PBS; pH 7.4). The bacterial mass was broken in a chilled French press and centrifuged for 2 h at 145,000 × *g* and 4°C. The supernatant was centrifuged twice at the same speed and dialyzed against PBS to obtain a cytosol fraction free of particulate materials. Finally, the purity, enzyme activities, and other biochemical characteristics of this cytosol fraction were evaluated (24).

Purification of secreted antigen 85 complex and other MPT proteins. The individual components of the actively secreted antigen 85 complex, i.e., 85A (MPT44), 85B (MPT59), and 85C (MPT45), as well as the other closely related proteins MPT51 (27-kDa) and MPT64 (26-kDa) were purified from concentrated *M. tuberculosis* H37Rv culture fluid by a combination of ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography on DEAE-Sepharose as described in detail previously (21). The term MPT has been used to denote the proteins purified from *M. tuberculosis* by their relative mobility in SDS-PAGE (21).

Recombinant fusion proteins of *M. leprae* 30/31-kDa antigen 85 complex. *M. leprae* antigen 85 complex fusion proteins expressed by pIHB1004 and pIHB1006 that span amino acid residues 55 to 266 and 265 to 327, respectively, of the molecule were prepared as described previously (34).

Preparation of MAbs. The procedure for the generation of MAbs to 30-kDa-region mycobacterial proteins was based on the standard protocol (7). Immunization, fusion, screening, and selection of antibody-producing hybridomas were performed as described in our earlier report (24). Briefly, spleen cells from female BALB/c mice immunized with gel-purified 30-kDa-region antigen fractions from *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 demonstrated by a previously established enzyme-linked immunosorbent assay (ELISA) by using gel-purified 30-kDa-region proteins (11) and by immunoblotting with both a sonicate and culture fluid of *M. tuberculosis* as described below. In addition, mycobacterial hsp65 (33) or gel-purified 65-kDa (11) proteins were also used for the screening of these hybridomas to determine possible antibody cross-reactivity since a 65-kDa protein(s) is known to be immunodominant in BALB/c mice (47). 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, 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.).

In addition, previously characterized antimycobacterial hsp65 MAbs Rb2 and Pe12 have also been included in this study because they cross-reacted with the antigen 85 complex (23). Anti-30-kDa MAb HYT27 (30, 40) was kindly provided by J. Bennedsen, Statens Seruminstitut.

SDS-PAGE and immunoblotting. SDS-PAGE was performed in slab gels (12%) in a discontinuous Tris-buffer system by the method of Laemmli et al. (18). Sonic extracts from various mycobacterial species 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 was loaded on purified, secreted MPT antigens. In the case of recombinant fusion proteins of the *M. leprae* antigen 85 complex, the fusion protein carrying *Escherichia coli* lysates was analyzed by 6% SDS-PAGE as reported earlier (34). Separated proteins were electroblotted overnight onto nitrocellulose papers (Schleicher & Schuell, Inc., Dassel, Germany) by the method of Towbin et al. (35) and incubated either with partially purified MAbs from concentrated supernatants or ascitic fluid at a preevaluated dilution of 1:200 to 1:500 and processed for immunoblotting as described previously (24). All of the secondary antibodies and their corresponding conjugates were tested for the presence of any antimycobacterial antibody by both ELISA and immunoblotting and found to be negative.

Dot blot assay. A dot blot assay with purified native and denatured MPT proteins was performed as described previously (42). Briefly, 1.5 μg of protein in a 3-μl volume was spotted onto nitrocellulose paper and air dried. Nonspecific binding was blocked by 2% bovine serum albumin (BSA) with 0.2% Tween 20 and 1% gelatin in PBS, and nitrocellulose papers were incubated with preevaluated dilutions of MAbs (1:500) overnight at room temperature. After the washing steps, nitrocellulose papers were incubated with horseradish peroxidase-labeled sheep anti-mouse immunoglobulin G (IgG) $F(ab)'_2$ (1:2,000) and further processed to visualize the reaction by using standard diaminobenzidine tetrahydrochloride as an indicator system.

Mapping of epitopes on *M. leprae* antigen 85 complex. Mapping of MAb-reactive epitopes on the *M. leprae* antigen 85 complex was performed by immunoblotting analysis with

TABLE 1. Summary of MAb reactivity with native and denatured 30-kDa-region and 26- or 27-kDa secreted proteins compared with that with total culture fluid and cytosolic proteins of *M. tuberculosis*

MAb	MAb reactivity[a] with:									
	Native proteins[b]			Denatured proteins[b]			Total culture fluid[c]		Cytosol fraction[c]	
	MPT59 (component 85B)	MPT51 (27 kDa)	MPT64 (26 kDa)	MPT59 (component 85B)	MPT51 (27 kDa)	MPT64 (26 kDa)	30 kDa	26 or 27 kDa	30 kDa	26 or 27 kDa
A3c12 (IgG2a)	3+	−	−	−	−	−	−	3+	+[d]	3+
A4e5 (IgG2a)	2+	−	−	−	−	−	−	3+	+[d]	3+
A4d6 (Ig2a)	−	−	−	−	−	−	+	−	3+	−
A4g4 (IgG1)	2+	−	−	−	−	−	−	−	3+	−
A3e2 (IgM)	3+	−	−	3+	−	−	3+[d]	−	3+[d]	+/−
B5e10 (IgG1)	−	−	−	−	−	−	−	−	3+	−
3A8 (IgG1)	3+	−	−	3+	−	−	3+[d]	−	3+[d]	−
5F9 (IgG1)[e]	2+	−	−	−	−	−	−	−	3+	−
5D2 (IgG1)	2+	−	−	−	−	−	−	−	3+	−
5D5 (IgG1)	2+	−	−	−	−	−	−	−	3+	−
Rb2 (IgG1)[f]	3+	−	−	3+	−	−	3+[d]	−	3+[d]	−
Pe12 (IgG1)[f]	3+	−	−	+/−	−	−	+[d]	−	+/−[d]	−
A2h11 (IgG1)[f]	3+	−	−	+	−	−	+[d]	−	+/−[d]	−
HYT27 (IgG)	3+	3+	+	3+	2+	−	3+[d]	−	3+[d]	−

[a] +/− to 3+, increasing intensity of the reaction; −, no reaction.
[b] As analyzed by dot blot ELISA or by SDS-PAGE and immunoblotting with purified native and denatured proteins.
[c] As analyzed by SDS-PAGE and immunoblotting with total culture fluid or cytosol fraction of *M. tuberculosis*. (Note that the MAb-reactive proteins are denatured in these preparations.)
[d] Reactivity with a doublet (30/31-kDa or 29/33-kDa) band. Other MAbs bound to a single band in the 30-kDa region.
[e] Reacted with a weak 93-kDa band from the cytosol fraction.
[f] MAbs that are reacted with a 65-kDa protein from cytosol fraction alone; they also recognized the mycobacterial hsp65 (23).

protein extracts from *E. coli* POP2136 carrying recombinant *M. leprae* fusion proteins as reported earlier (34).

Immunohistochemistry of skin lesions of leprosy patients. A well-defined collection of lesional skin biopsies frozen at −90°C was obtained from untreated lepromatous leprosy patients, and the biopsies were classified by the method of Ridley and Jopling (27). Details of these leprosy biopsies have been described in our previous report (25).

For immunoperoxidase staining, frozen 6-μm tissue sections from leprosy lesions were cut and fixed in acetone. MAbs specific for the antigen 85 complex were used in a two-step MAb-horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin staining system with 3-amino-9-ethyl-carbazol as the staining reagent as described previously (26). Specificity of the staining was performed by preabsorbing the optimally diluted MAbs with purified antigen 85B. Absorption was carried out for 2 h at 37°C prior to application of the MAbs to frozen sections. Staining of absorbed MAbs was compared with that of unabsorbed MAbs treated with 1% BSA in place of antigen 85B.

RESULTS

MAbs. A total of 13 MAbs directed to mycobacterial 30-kDa-region proteins and to the secreted antigen 85 complex were characterized. Eleven of these clones (A3c12, A4e5, A4d6, A4g4, A3e2, B5e10, 3A8, 5F9, 5D2, 5D5, A2h11) were obtained from fusion experiments after immunization of BALB/c mice with SDS-polyacrylamide gel-purified 30-kDa-region antigen fraction from the total *M. tuberculosis* sonicate. Two other clones (Pe12, Rb2) included in this study were isolated from a single fusion experiment after immunization with a gel-purified 65-kDa antigen fraction because they reacted with both the hsp65 and 30-kDa proteins as well as the antigen 85 complex (23). Six of the MAbs (A3c12, A4e5, A4d6, A4g4, A3e2, B5e10) included in this study have not been reported previously, whereas the initial characterization of the other MAbs has been described elsewhere (23, 24). In this study, we further characterize these MAbs by using highly purified native and denatured secreted components of the antigen 85 complex of *M. tuberculosis*. These MAbs appeared to react with different epitopes of the 30-kDa-region proteins on the basis of their different reactivity patterns and also the results of inhibition ELISA (data not shown) (24). The reactivity of all MAbs with the antigen 85 complex or corresponding 30-kDa-region proteins and their isotypes are summarized in Table 1.

MAb reactivity with secreted proteins: identification of different epitopes on proteins of the antigen 85 complex. The major proteins of the culture fluid of *M. tuberculosis* and *M. bovis* BCG are the 30-kDa-region proteins which correspond to the actively secreted components of the antigen 85 complex. Figure 1 illustrates the binding of MAbs to the purified native components of the antigen 85 complex in a dot blot assay as compared with their immunoblot reactivities with total *M. tuberculosis* culture fluid from which antigen 85 complex proteins were derived. It can be seen in Fig. 1A that all MAbs except B5e10 and A4d6 bound strongly to the native components of the antigen 85 complex with various specificities. None of the MAbs reacted with MPT51 and MPT64 despite their extensive cross-reactivity within the antigen 85 complex (42). MAbs A4g4 and A4e5 reacted only with 85B, while MAbs A3c12 and A2h11 reacted with both 85A and 85B. The other MAbs bound strongly to 85A and 85B, while they showed a moderate to weak reaction with 85C. Interestingly, MAbs B5e10 and A4d6 did not (or did very weakly) react with the proteins of the antigen 85 complex, although they bound strongly to a cytosolic 30-kDa protein in immunoblotting (described below). In contrast, the previously characterized MAb HYT27 reacted with all three components of the antigen 85 complex as well as the MPT51 and MPT64 proteins in both native and denatured

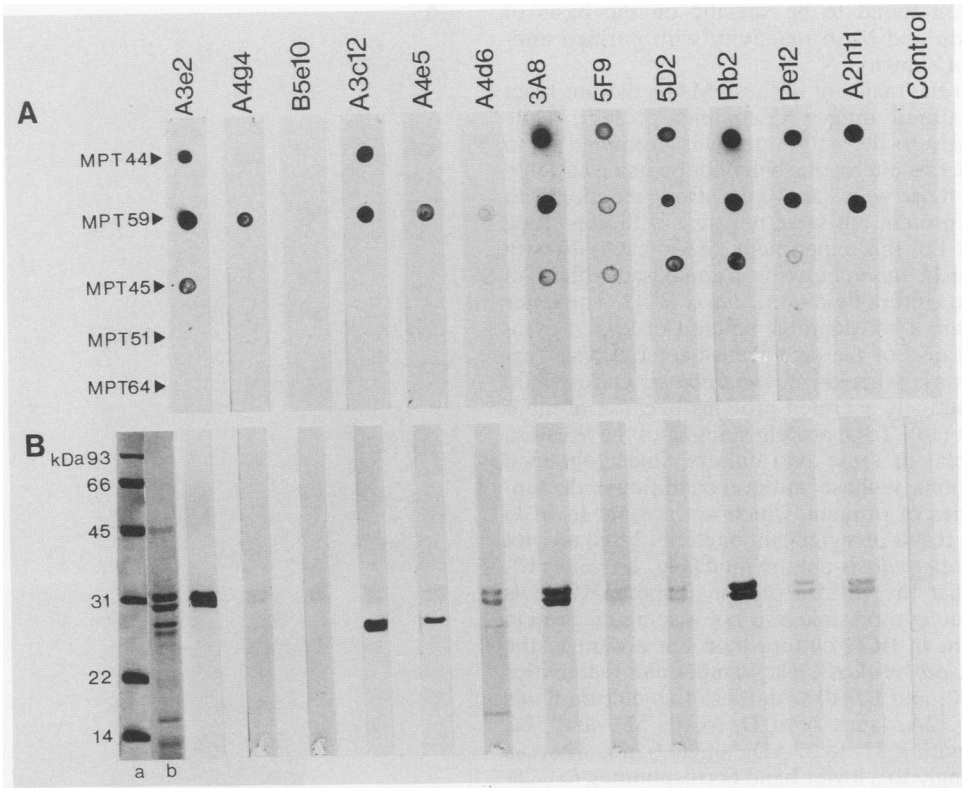

FIG. 1. MAbs recognizing 30-kDa-region secreted proteins of *M. tuberculosis*. (A) Dot blot ELISA showing the reactivity of MAbs with purified native MPT proteins. Note that the MAbs recognize proteins belonging to the antigen 85 complex (MPT44 [85A], MPT59 [85B], and MPT45 [85C]) with different specificities. (B) Immunoblot analysis of MAbs recognizing 30-kDa-region proteins from *M. tuberculosis* culture fluid from which all MPT proteins were derived. Note the nonreactivity of most of the MAbs with the denatured 30-kDa proteins corresponding to the antigen 85 complex, and also note the identification of a distinct 27-kDa protein by two MAbs (lanes A3c12 and A4e5). Reactive MAbs and the control with PBS for both dot blot and immunoblotting are indicated at the top. Amido-black-stained Western blots of molecular mass markers (lane a) and the total culture fluid of *M. tuberculosis* (lane b) are shown on the extreme left.

forms (Table 1), indicating that HYT27 represents a common epitope among proteins of the antigen 85 complex and other related secreted proteins which is quite different from the presently described MAbs. Furthermore, the binding of each of these MAbs to purified individual components of the antigen 85 complex was found to be specific, because these MAbs showed a consistent positive reaction without background even in a high concentration.

Although 11 of 13 MAbs bound to the native proteins of the antigen 85 complex with different specificities and intensities, their reactivities with denatured proteins of the antigen 85 complex and the total culture fluid of *M. tuberculosis* in immunoblotting presented a different picture (Table 1 and Fig. 1B). Only MAbs 3A8, A3e2, and Rb2 showed strong immunoblot reactivities with a doublet band at the 30-kDa region in the culture fluid which corresponds to three components of the antigen 85 complex. These three MAbs also reacted strongly with purified antigen 85 complex in immunoblotting or in a dot blot with denatured proteins (Table 1). A similar reactivity pattern was also observed with the previously characterized MAb HYT27 (Table 1). All other MAbs showed background reactivities (in comparison with the control experiment with PBS) except for A4d6, A2h11, 5D2/5D5, and Pe12, which bound to a 30-kDa doublet band with weak to moderate intensities (Fig. 1B). The reactivity of MAb A4d6 with a 30-kDa doublet protein from the total culture fluid is of particular interest since this MAb did not show positive reactions with purified antigen complex in either native or denatured conditions, indicating that A4d6 may recognize another related 30-kDa protein(s) from the culture fluid. On the basis of MAb reactivity with native and denatured proteins of the antigen 85 complex, it appears that the majority of these MAb-defined epitopes on the antigen 85 complex are present as conformational epitopes.

Interestingly, MAbs A3c12 and A4e5, which recognize native 85A/85B and 85B, respectively, reacted with a distinct 27-kDa band from the total culture fluid in immunoblotting. These two MAbs showed no reaction with either the denatured 30-kDa doublet protein from *M. tuberculosis* culture fluid (Fig. 1B) or the purified denatured antigen 85 complex (Table 1). Since these MAbs did not react with MPT51 and MPT64 proteins in both native and denatured forms, we conclude that MAbs A3c12 and A4e5 recognize a new 27-kDa protein(s) in *M. tuberculosis* culture fluid.

Comparison of MAb reactivity with secreted and cytosolic proteins in the 30-kDa region. A summary of the MAb reactivity with secreted 30-kDa-region proteins and the relationship of these secreted proteins with the cytosolic proteins of *M. tuberculosis* is also given in Table 1. Interestingly, all MAbs reacted with denatured cytosolic proteins in the 30-kDa region with various intensities as demonstrated by immunoblotting. Some of these MAb-reactive epitopes on cytosolic 30-kDa protein(s) are also shared with 26- or 27-kDa, 65-kDa, and 93-kDa cytosolic proteins. The last

reactivity has been found to be specific on the basis of relevant absorption and MAb reactivity with purified antigens (23) (data not shown).

The finding that the majority of these MAbs did not react with purified denatured antigen 85 complex proteins while they bound strongly to the native proteins prompted us to further investigate this interesting behavior by using selected MAbs, especially those which showed a strong reaction with 30-kDa cytosolic protein but were negative with denatured secreted proteins. For this experiment, MAb reactivity with the culture fluid of *M. tuberculosis* was compared with MAb reactivity with the culture fluid of *M. bovis* BCG. The latter is known to contain a considerable amount of leaked cytosolic proteins because of the significant bacterial lysis that occurs during culture, whereas *M. tuberculosis* culture fluid contains mostly actively secreted proteins because of minimal bacterial lysis (14). Total protein staining of the Western blots (immunoblots) of these two culture fluids (obtained from similar stationary-phase culture conditions) demonstrated that a series of proteins which are present in BCG culture fluid (as a result of significant bacterial lysis) are not detectable in *M. tuberculosis* culture fluid (Fig. 2A, lanes E). It can be seen that the 65-kDa protein (hsp65), which is known to be a true cytoplasmic and not a secreted protein (14, 39), is present in BCG culture fluid but absent in the culture fluid of *M. tuberculosis*. The immunoblot reactivities of MAbs 5F9, 5D2, and B5e10 with these two culture fluids are shown in Fig. 2A, lanes A to D. MAbs 5F9 and 5D2 reacted strongly with a 31- to 33-kDa single band, whereas B5e10 bound strongly to a lower band corresponding to a 28- to 29-kDa protein from BCG culture fluid, but none of them showed reactivity with a 30-kDa protein or other proteins from *M. tuberculosis* culture fluid (the weak reactivity corresponds to background staining as shown in Fig. 2A, lanes D). Binding patterns of these MAbs with BCG culture fluid were found to be identical with those of the cytosol fraction of *M. tuberculosis* (Fig. 2B). A similar MAb reactivity pattern was also observed for the *M. bovis* BCG cytosol fraction (data not shown). It should be noted that 5F9 and 5D2 reacted only with native 85 complex whereas B5e10 reacted only with cytosolic 30-kDa proteins and not with secreted antigen 85 complex. Interestingly, MAb 5F9 also showed a weak but specific band at 93 kDa in the cytosol fraction, while it gave a strong reaction with a protein of similar molecular size only in BCG culture fluid. Since proteins of these two mycobacteria are highly related, the differences in MAb reactivity seen in BCG culture fluid may be due to the recognition of some of the leaked cytosolic 30-kDa protein(s). These results suggest that BCG, in comparison with *M. tuberculosis*, releases the cytosolic form of a 30-kDa protein(s) during culture.

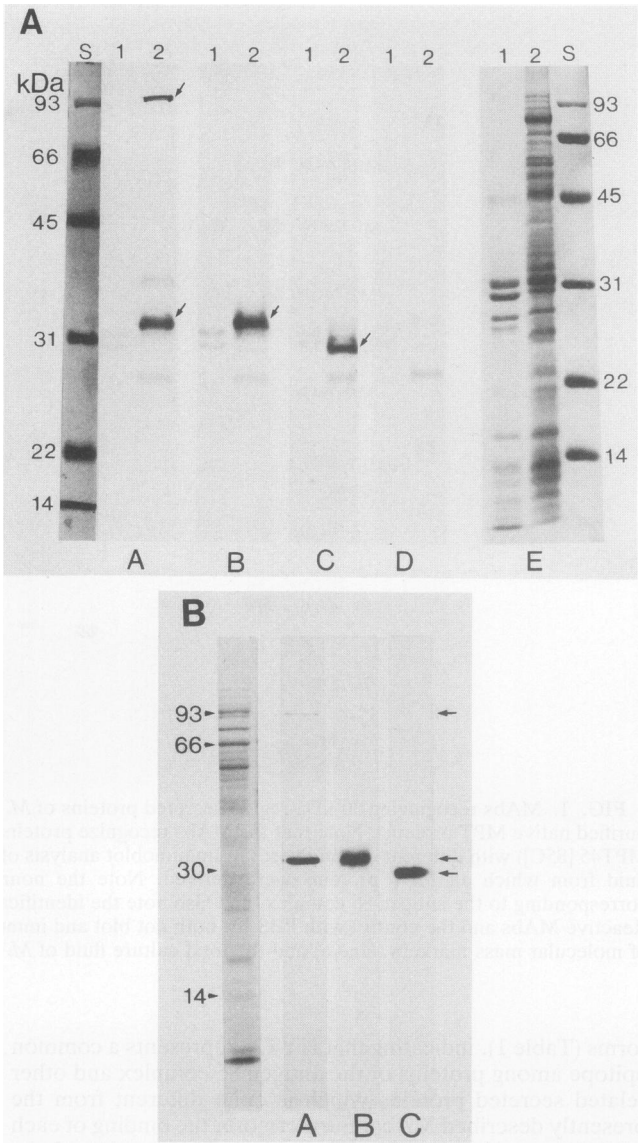

FIG. 2. Comparative immunoblot analysis of MAbs reacting with the culture fluids of *M. tuberculosis* and *M. bovis* BCG and with the cytosol fraction of *M. tuberculosis*. (A) Reactivity of MAbs 5F9 (lanes A), 5D2 (lanes B), and B5e10 (lanes C) with the Western blots of culture fluids of *M. tuberculosis* (lane 1) and *M. bovis* BCG (lane 2). A control strip incubated with PBS is shown in lanes D), and the amido-black-stained Western blots of culture fluids together with molecular mass markers are shown in lanes E. (B) Reactivity of the MAbs shown in panel A (lanes A to C) with the Western blots of the cytosol fraction of *M. tuberculosis*. Amido black staining of the cytosol fraction is shown to the left of lane A. Arrowheads indicate the specific bands recognized by the MAbs. A similar reactivity pattern was also observed with the cytosol fraction of *M. bovis* BCG (data not shown).

Occurrence of cross-reactive epitopes of the antigen 85 complex in various mycobacterial species. Figure 3 shows the different distribution patterns of cross-reactive epitopes of the antigen 85 complex in similar-molecular-sized proteins of 19 different sonicated mycobacterial species as analyzed by immunoblotting with six novel MAbs. The distribution of epitopes defined by seven other MAbs in the same mycobacterial species has been described elsewhere (23, 24). None of these MAbs reacted with *E. coli*. The overall results indicate that each of these MAbs showed a distinct reactivity pattern with these mycobacterial species, indicating the heterogeneity and complexity of these proteins in relation to epitope distribution. The results also indicate the apparent conservation of individual components of the antigen 85 complex within the genus mycobacteria. Since cytosolic proteins contribute to the major constituents of the mycobacterial sonicate, it is likely that these MAb-reactive 30-kDa proteins in different mycobacterial sonicates are cytosolic in origin. Interestingly, only MAbs A3c12and A4e5 showed strong reactivity with a 26- or 27-kDa protein(s) in addition to 30-kDa-region proteins. This MAb-reactive 27-kDa protein(s) was found to be present specifically in myco-

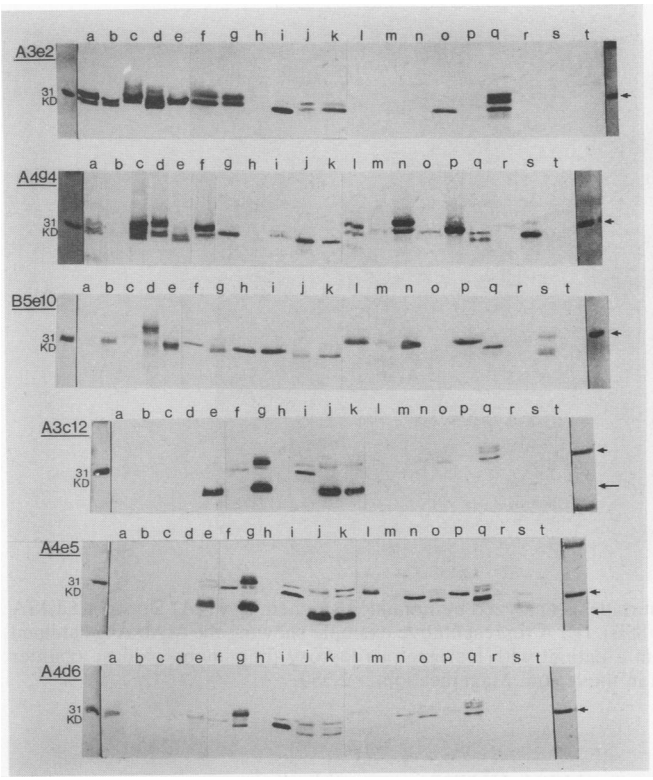

FIG. 3. Heterogeneous distribution of MAb-reactive epitopes on a 30-kDa-region protein(s) in various mycobacterial species. The reactivity of MAbs with Western blots containing 19 different mycobacterial sonicates is shown. Lanes: a, *M. marinum*; b, *M. gastri*; c, *M. scrofulaceum*; d, *M. intracellulare*; e, *M. africanum*; f, *M. avium*; g, *M. microti*; h, *M. nonchromogenicum*; i, *M. kansasii*; j, *M. bovis* BCG; k, *M. tuberculosis* H37Rv; l, *M. fortuitum*; m, *M. terrae*; n, *M. duvalii*; o, *M. gordonae*; p, *M. smegmatis*; q, *M. xenopi*; r, *M. vaccae*; s, *M. flavescens*; t, *E. coli* as a nonmycobacterial sonicate. Reactive MAbs are indicated at the upper left corner of each panel of lanes. In both extreme left and right lanes are shown amido-black-stained molecular mass markers of 31 kDa. Short and long arrows on the extreme right indicate the molecular masses of the 30-kDa region and the 26- to 27-kDa region, respectively.

bacterial species belonging to the *M. tuberculosis* complex (Fig. 3). Therefore, these two epitopes are *M. tuberculosis* complex-specific epitopes in relation to their distribution on 27-kDa protein alone. Such heterogeneous epitope distribution further indicates that these MAbs are directed to different epitopes of 30-kDa-region proteins, some of which are shared with other related proteins.

Epitope mapping on recombinant *M. leprae* 30/31-kDa antigen 85 complex. To ascertain the epitope-containing region of the antigen 85 complex, MAbs were tested with fusion proteins of *M. leprae* antigen 85 complex. Table 2 depicts the immunoblotting reactivity pattern of MAbs with two fusion proteins that span an amino-terminal and a carboxy-terminal region of the molecule. Specific binding of six MAbs only to the fusion protein carrying pIHB1004 plasmid enabled us to locate at least six different epitopes within the amino acid sequence of residues 55 to 266 of the *M. leprae* 85 complex. The location of other MAb-defined epitopes was uncertain because they bound strongly to the fusion protein derived from pIHB1004 and weakly to protein derived from pIHB1006. None of these MAbs reacted with the control derived from pEX2 plasmid, indicating the specificity of these MAbs to recombinant *M. leprae* antigen 85 complex.

In situ demonstration of antigen 85B in *M. leprae*. To evaluate the in situ presence of the proteins of antigen 85 complex in *M. leprae*, we performed immunoperoxidase staining of bacilliferous lepromatous leprosy lesions by using MAbs specific for the antigen 85 complex. We found that a novel MAb, A4g4, which specifically recognizes the 85B component of the antigen 85 complex, stained *M. leprae* bacilli within macrophages in highly bacilliferous lepromatous leprosy lesions (Fig. 4). The number of bacteria detected by this MAb varied by the bacillary index of the lesions. The in situ reactivity of MAb A4g4 appeared as clear rod-shaped bacterial staining, indicating that the A4g4 epitope is present in high density on the cell wall surface of *M. leprae*. However, in all bacilliferous lepromatous leprosy lesions tested, A4g4 detected only a few bacilli in comparison with conventional Fite-Faraco-Wade (FFW) bacterial staining. Completely negative staining was observed in paucibacillary tuberculoid leprosy lesions and in other control skin lesions from patients with sarcoidosis and psoriasis (data not shown). Preincubation of MAb A4g4 [including the $F(ab')_2$ fragment] with purified antigen 85B completely abolished the *M. leprae* staining in the tissues. This ensured the specificity of the immunohistochemical reaction with this MAb. On the other hand, MAb A4e5, which also specifically recognized the native antigen 85B (Fig. 1A), did not show any in situ reactivity with *M. leprae* bacilli in lepromatous lesions, indicating the heterogeneity of the in situ expression of specific 85B epitopes on *M. leprae*.

DISCUSSION

The abundant secretion of the proteins of the antigen 85 complex from live mycobacteria may be relevant to their immunodominant character at both the T-cell and B-cell levels. In the present study, by using a panel of 13 MAbs directed to 30-kDa-region proteins, we have further characterized the antigen 85 complex proteins at the epitope level. We have identified at least 10 different MAb-reactive

TABLE 2. Reactivity of MAbs with fusion proteins of *M. leprae* 30/31-kDa antigen 85 complex

Fusion protein[a]	Amino acid sequence expressed	MAb reactivity[b]											
		A3c12	A4e5	A4d6	A4g4	A3e2	B5e10	3A8	5F9	5D2	A2h11	Pe12	Rb2
pIHB1004	55–266	+	+	+	+	+	+	+	+	+	+	+	+
pIHB1006	265–327	−	−	+/−	+/−	+/−	−	+/−	+/−	+	−	−	−
pEX2	None	−	−	−	−	−	−	−	−	−	−	−	−

[a] Expressed by recombinant plasmids containing two different parts of the 30/31-kDa antigen 85 complex gene of *M. leprae*.
[b] Determined by immunoblotting analysis by using protein extracts from *E. coli* POP2136 carrying recombinant plasmids (34). +/− to +, increasing intensity of the reaction; −, no reaction.

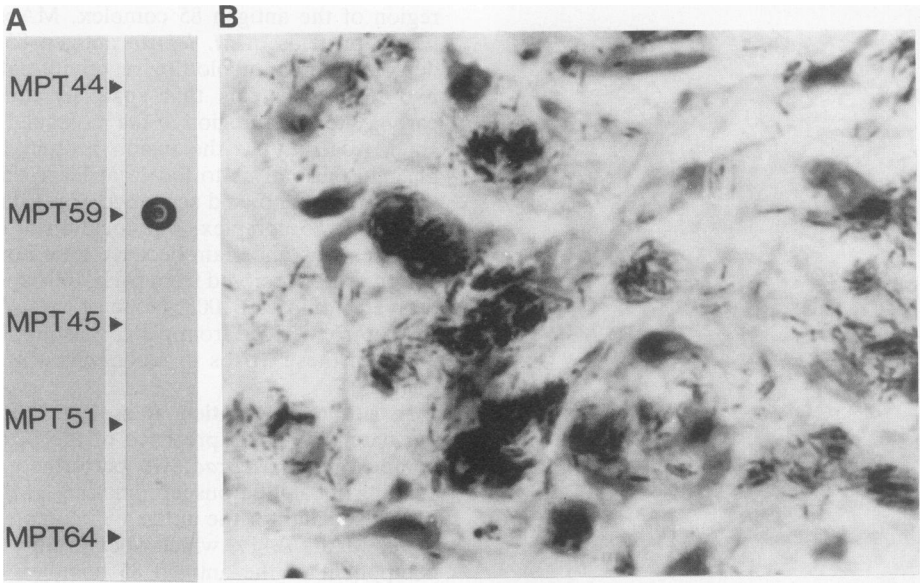

FIG. 4. In situ demonstration of antigen 85B on *M. leprae* cell wall surface as analyzed by immunohistochemistry. (A) Dot blot ELISA showing the specific reactivity of MAb A4g4 with native MPT59 (antigen 85B). (B) A representative example showing the MAb A4g4 stained *M. leprae* bacilli in a bacilliferous skin lesion (bacillary index, 6+) from a patient with lepromatous leprosy (immunoperoxidase counter stained with hematoxylin). Note the rod-shaped staining of many bacilli in the lesion. Magnification, ×1,180.

epitopes on the components of the antigen 85 complex. The differences among these epitopes were evaluated on the basis of heterogeneous distribution of each of these epitopes among different mycobacterial species (Fig. 3) (24), inhibition ELISA (data not shown) (23, 24), shared characteristics of some of the epitopes with different proteins (Table 1) (23), and the variation of their presence in individual purified proteins of the antigen 85 complex (Table 1; Fig. 1). At least two distinct epitopes specific for antigen 85B have been identified by MAbs A4g4 and A4e5. Two common epitopes restricted to components 85A and 85B were recognized by MAbs A3c12 and A2h11, whereas another two epitopes shared among all three components of the antigen 85 complex were identified by MAbs 5F9, 5D2, and 5D5. We have previously shown that MAbs 5D2 and 5D5 are directed to the same epitope and thus are denoted 5D2/5D5 (24). The remaining MAbs (A3e2, 3A8, Rb2, and Pe12) reacted strongly with 85A and 85B but showed moderate to weak reactivity with 85C, and they were found to be directed to four different epitopes of the antigen 85 complex (Fig. 1 and 3; Table 1) (23). None of these MAb-defined epitopes were specific for 85C, and they were not shared with the related MPT51 and MPT64 secreted proteins in spite of their extensive cross-reactivity within the antigen 85 complex (14, 21, 42). On the basis of MAb reactivity with secreted proteins, both purified and culture fluid antigens of *M. tuberculosis*, it was found that most of the MAb-defined epitopes are specific for the proteins of the antigen 85 complex and in the 30-kDa region alone.

Although MAbs that are reacting with the proteins in the 30-kDa region have been previously described (2, 9, 17, 29, 30, 38, 44), only a very few have been shown to recognize the well-characterized proteins of the antigen 85 complex of *M. tuberculosis* and *M. bovis* BCG (29, 40). These MAbs are not specific for a single component of the antigen 85 complex. Moreover, none of them has been shown to recognize the *M. leprae* antigen 85 complex. The previously characterized MAb HYT27 reacts with all three components of the antigen 85 complex of *M. tuberculosis* and *M. bovis* BCG as well as the related MPT51 and MPT64 (1, 21, 25, 42) (Table 1). A recent study has shown that six MAbs of the TB-C series also recognize the antigen 85 complex, and all six MAbs are directed to a single epitope that is shared among the three components of this complex (29). Therefore, to our knowledge, only two epitopes on the antigen 85 complex proteins have been identified so far by known MAbs reported by other investigators. Direct comparison of the latter two epitopes of the antigen 85 complex revealed that the MAbs described in this study recognize new epitopes of the antigen 85 complex and they are different from HYT27 and TB-C epitopes (29, 40, 42) (Table 1).

Yoneda et al. and other independent investigators have shown convincing evidence that antigens related to the antigen 85 complex contain both species-specific and cross-reactive epitopes (2, 31, 32, 44–46). Our results of species specificity of MAbs provide additional evidence for the presence of widely cross-reactive epitopes of the antigen 85 complex. However, we were unable to demonstrate any species-specific epitopes on the antigen 85 complex by using these MAbs. Interestingly, two of these MAb-defined epitopes, A3c12 and A4e5, which react only with native 85B and 85A/85B, respectively, are shared with a 27-kDa protein specifically present in mycobacterial species belonging to the *M. tuberculosis* complex. It is remarkable that these epitopes on 30-kDa-region proteins (corresponding to the antigen complex) are widely cross-reactive among many mycobacterial species, whereas the same epitopes on 27-kDa protein are specific for the *M. tuberculosis* complex. These data may provide indirect evidence for the existence of species-specific or *M. tuberculosis*-complex-specific epitopes on the antigen 85 complex. Moreover, the MAb-reactive 27-kDa protein was identified as a new protein in *M. tuberculosis* culture fluid since MAbs A3c12 and A4e5 did not recognize the previously characterized similar-molecu-

lar-sized MPT51 and MPT64 proteins purified from the same culture fluid (21). Since the *M. tuberculosis* culture fluid presently used contains predominantly secreted proteins (14, 21), this MAb-reactive 27-kDa protein could be a new secreted protein of mycobacteria.

Another interesting finding of the present study is the different behaviors of these MAbs with native and denatured proteins. It seems that most of the MAb-reactive epitopes on the proteins of the antigen 85 complex exist as conformational epitopes as evidenced by the strong reactivity of the MAbs to purified native proteins and their lack of binding to denatured proteins. Those MAbs which do not bind to the denatured antigen 85 complex proteins fail to recognize the corresponding 30-kDa-region proteins in the culture fluid of *M. tuberculosis* from which the antigen 85 complex was derived. In contrast, when the cytosol fraction of *M. tuberculosis* was used in place of *M. tuberculosis* culture fluid, we found that the majority of the MAbs recognized a related protein(s) in the 30-kDa region even when they were denatured by SDS-PAGE. Therefore, it is likely that these MAb-reactive epitopes may be present in a linear form on a 30-kDa protein within the cytosolic compartment of the bacterium. It is also probable that these MAbs recognize the same or different 30-kDa intracellular and secreted protein(s). If the former assumption is true, one can speculate that when 30-kDa-region proteins are actively secreted from the bacterium, certain molecular changes occur in such a way that some of the linear epitopes on cytosolic 30-kDa protein acquire conformational configuration after secretion. The second possibility is that this cytosolic 30-kDa protein differs from the actively secreted antigen 85 complex proteins although they show clear epitope similarities. Further studies are needed to resolve this complexity and to identify the nature of this cytosolic 30-kDa protein(s) and its relationship with the actively secreted antigen 85 complex.

Of particular interest is MAb B5e10, which seems to recognize a nonsecreted 30-kDa protein in the cytosolic fraction, because this MAb did not react with both native and denatured antigen 85 complex and with a 30-kDa-region protein in *M. tuberculosis* culture fluid. However, B5e10 reacted strongly with a 30-kDa-region protein in BCG culture fluid. On the other hand, MAbs 5F9 and 5D2/5D5, which bind only to native antigen 85 complex and not to denatured 30-kDa protein in *M. tuberculosis* culture fluid, also reacted strongly with a denatured 30-kDa protein in BCG culture fluid. The former culture fluid is known to consist mainly of actively secreted proteins, while the latter is contaminated with leaked cytosolic proteins (14, 21). Therefore, it is probable that these MAbs recognize leaked cytosolic protein but not the secreted 30-kDa protein(s) in BCG culture fluid because MAbs B5e10, 5F9, and 5D2/5D5 do not recognize denatured secreted 85 complex proteins. These findings suggest that BCG releases the cytosolic form of 30-kDa protein.

The observed epitope heterogeneity of the antigen 85 complex in the present study is in accord with the findings of Matsuo et al., who showed by analyzing the hydropathy profile of α antigen (85B) that certain amino acid sequences of this antigen might account for the antigenic diversity of the 85B antigen (19, 20). These structural analyses have provided important clues concerning the location of epitopes of the 85B component which may also be valid for the *M. leprae* antigen 85 complex since the amino acid sequences of components 85A and 85B of *M. tuberculosis*, *M. bovis* BCG, and *M. kansasii* display a strong similarity to those of the corresponding *M. leprae* antigen 85 complex (5, 8, 19, 20, 34). On the basis of specific binding of MAb to the fusion protein pIHB1004 of *M. leprae* 30/31-kDa antigen 85 complex (34), we have identified at least six different epitopes within the *M. leprae* protein fragment containing the amino acid sequence of residues 55 to 266. Other MAb-defined epitopes could not be precisely mapped on antigen 85 complex protein because they reacted weakly with fusion protein expressed by pIHB1006 but bound strongly to the fusion protein pIHB1004. A possible explanation for this reactivity is that these MAb-defined epitopes may be shared between the C-terminal region of the amino acid sequence of residues 55 to 266 expressed by fusion proteins pIHB1004 and N-terminal regions of the amino acid sequence of residues 265 to 237 expressed by pIHB1006. Further studies with more fusion proteins or truncated proteins or synthetic peptides of the antigen 85 complex are needed to clarify the precise mapping of these epitopes. Nevertheless, the nonreactivity of these MAbs with the control expressed by pEX2 clearly indicates that all of these MAb-defined epitopes are specifically present in the *M. leprae* antigen 85 complex.

Interestingly, the amino acid sequence of residues 55 to 266 of *M. leprae* antigen 85 complex to which six of the MAb-defined epitopes have been precisely mapped was also shown to be one of the fibronectin-binding sites of *M. leprae* (34). The fibronectin-binding antigen 85 complex released by live mycobacteria may play an important role in the pathogenesis of mycobacterial diseases (1, 34). These fibronectin-binding proteins exposed on the cell surface have been suggested to play a role in the adherence and uptake of *M. leprae* by phagocytes and nonprofessional phagocytic cells and thus may be relevant in the pathogenesis of leprosy (1, 34). Although there are reports providing indirect evidence for the association of the antigen 85 complex with the *M. leprae* surface (1, 4, 14, 22, 39, 40), so far none of these studies have directly demonstrated components of the antigen 85 complex on the *M. leprae* surface. However, by using MAbs recognizing both the antigen 85 complex and hsp65, we have previously described the in situ expression of these MAb-defined shared epitopes on the *M. leprae* surface (23). Since these MAbs are not specific for the antigen 85 complex alone, we were unable to confirm the presence of the antigen 85 complex on the *M. leprae* surface. Moreover, in a separate study, we have also demonstrated the presence of the antigen 85 complex within the lesions of both tuberculoid and lepromatous leprosies by a MAb (3A8) specific for this antigen, but we failed to demonstrate its in situ reactivity with intact *M. leprae* bacilli (25). In the present study, by using the novel MAb A4g4, which reacts only with native 85B, we provide direct evidence for the presence of antigen 85B on the cell surface of *M. leprae* within the lepromatous leprosy lesions. The binding of this MAb to *M. leprae* in tissue sections appears to be related to a high bacillary load (bacillary index, >6+). The sensitivity of detection of bacilli by MAb A4g4 is rather low in comparison to that by Fite-Faraco-Wade staining. It is well known that acid-fast cell wall remnants in lepromatous lesions exist even long after the death of the *M. leprae* organisms. While certain antigenic determinants can be rapidly lost after bacterial death, the continuous secretion of proteins can only occur with live bacilli. Although these assumptions are speculative, the detection of comparatively fewer bacilli by MAb A4g4 may be indicative of the presence of viable bacilli in lepromatous leprosy lesions.

Our results clearly indicate that the antigen 85 complex, or at least the 85B component, is not only present as soluble secreted antigen but also simultaneously associated with the

mycobacterial surface. Identification of antigen 85B on the *M. leprae* surface is in agreement with the findings of Harboe et al. and Wiker et al., who showed that the localization index for antigen 85A has been consistently higher than that of 85B, indicating that antigen 85B may be more closely associated with the bacterial surface (14, 39). The association of antigen 85B with the cell wall may be relevant to the marked immunogenicity of this component relative to that of the other members of the antigen 85 complex, because cell wall proteins of *M. leprae* and *M. tuberculosis* are known to contain powerful immunologic activity (4, 15).

In conclusion, our study provides the first evidence for the identification of different MAb-reactive epitopes on individual secreted proteins of the antigen 85 complex. At least six different epitopes were localized within amino acid residues 55 to 266 of the fibronectin-binding *M. leprae* 30/31-kDa antigen 85 complex. One of the antigen 85B-specific epitopes was localized on the *M. leprae* cell wall surface. Therefore, these MAbs may be useful probes for the elucidation of each epitope of the antigen 85 complex and for the identification of the exact location of the epitopes within the molecule. It is likely that some of the MAb-reactive epitope regions, especially those which are associated with the *M. leprae* cell wall or within the leprosy lesions, have potential use in the future study of the pathogenesis of leprosy.

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