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Mycobacterium leprae Produces Extracellular Homologs of the Antigen 85 Complex

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The antigen 85 complex is a set of at least three closely related secreted proteins (85A, 85B, and 85C) of 30 to 32 kDa produced by *Mycobacterium tuberculosis* and other mycobacteria. Their prominence in *Mycobacterium leprae*, the one obligate intracellular pathogen of the genus, had been assumed on the basis of immunological evidence and proof of the existence of the gene encoding the 85B protein of the complex. We have now observed the production of this family of proteins by *M. leprae* through analysis of various fractions by Western blotting (immunoblotting) with monospecific rabbit antisera raised against the individual *Mycobacterium bovis* BCG 85A, 85B, and 85C proteins. A predominant cross-reactive band with an apparent molecular mass of 30 kDa was detected in extracts of nondisrupted whole *M. leprae* and in soluble fractions prepared from the tissues of *M. leprae*-infected armadillos. Further studies of the subcellular distribution of this protein within the bacterium confirmed that it is secreted by the organism, an observation that explains past difficulties in detecting the antigen 85 complex in *M. leprae*. Confirmation that the *M. leprae* product is a member of the antigen 85 complex was obtained by comparison of peptide fingerprints with those from the BCG product. The pattern of reactivity of the *M. leprae* antigen 85 complex with anti-*M. bovis* BCG 85B serum, as well as two-dimensional electrophoresis, established that the 85B component was the predominant member of the complex in *M. leprae*. The fibronectin-binding capacity of the *M. leprae* and BCG 85 complexes was reinvestigated by new approaches and is questioned. Nevertheless, the results obtained with the native proteins reinforce previous reports, derived primarily from the use of homologous proteins, that the antigen 85 complex is one of the dominant protein immunogens of the leprosy bacillus.

Leprosy and tuberculosis, caused by *Mycobacterium leprae* and *Mycobacterium tuberculosis*, respectively, remain major diseases, affecting more than 40 million people worldwide (24, 25). Considerable current attention is also being devoted to the "atypical" mycobacteria, such as members of the *Mycobacterium avium* complex, since they are often encountered in patients with AIDS (3). Despite much effort in recent years, little is known of the antigenic composition, host-parasite interactions, pathogenesis, and physiology of mycobacteria. Extension of our knowledge in these areas will certainly contribute to efforts in disease eradication through vaccination, early diagnosis, and efficient chemotherapy.

M. leprae is an obligate intracellular parasite and remains one of the few pathogenic bacteria unable to grow outside the host. Studies of the bacillus itself are restricted to isolates from highly infected armadillos, *Dasypus novemcinctus*. In this sense, *M. leprae* constitutes a model for in vivo-grown mycobacteria in which bacterial components expressed inside the host and probably necessary for survival and virulence can be compared with those produced by other cultured, pathogenic mycobacteria. A major protein expressed by *M. tuberculosis* and *Mycobacterium bovis* BCG in cultures is the so-called antigen 85 complex (3, 7, 34–36). It constitutes a family of structurally related secreted proteins found in all mycobacterial species but not in closely related genera (5). Proteins of the antigen 85 complex react with antibodies and peripheral blood mononuclear cells from individuals with leprosy or tuberculosis (8, 10, 16, 18, 28) and thus may hold promise as constituents of subunit vaccines, as well as diagnostic reagents. A hallmark of the antigen 85 complex is the ability to bind to fibronectin (1), a feature that plays a role in the virulence of some bacterial infections (11). At least three members of the antigen 85 complex of proteins, designated 85A (also called P32), 85B (also called alpha antigen, MPB 59, or antigen 6), and 85C, have been characterized. Independent genes that code for the 85A, 85B, and 85C proteins have been cloned from different species of mycobacteria (4, 6, 9, 20, 21, 23, 32), and a high degree of homology in the amino acid sequences of the individual mature proteins of the complex within a species, as well as between species, has been observed.

The immunological data already accumulated strongly suggest that antigens of the 85 complex are expressed by *M. leprae* and *M. tuberculosis* during the course of an active human infection. However, tangible evidence for the expression of the individual components of the antigen 85 complex in *M. leprae* has not emerged, largely for want of sufficient bacilli. The present study was designed specifically to search for these proteins in *M. leprae* and surrounding tissue as part of a larger effort to identify the protein, peptide, and carbohydrate antigens of tissue-derived *M. leprae* (14).

MATERIALS AND METHODS

Fractionation of *M. leprae*. *M. leprae* was purified from irradiated armadillo spleens and livers by the Draper protocol as described previously (13). Bacilli (300 mg, dry weight), stored at −70°C in 1 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 7.0) (buffered water), were thawed and recovered by centrifugation at 10,000 × *g* for 10 min. The supernatant was recentrifuged, dialyzed overnight against water, and lyophilized to produce a buffered-water

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extract of whole *M. leprae*. One-half of the resulting bacterial preparation was resuspended in 10 mM phosphate-buffered saline (PBS) (pH 7.2) (5 ml) containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine and extracted for 2 h on ice with gentle agitation. The suspension was centrifuged at 10,000 × *g*. The supernatant was recentrifuged, dialyzed against water, and lyophilized to provide a second extract of undisrupted *M. leprae*, the PBS extract. The remainder of the bacterial preparation was resuspended in 3 ml of PBS containing 0.05% Tween 80 and the same protease inhibitors as those listed above and disrupted by probe sonication (W-385 sonicator, an ultrasonic liquid processor; Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) for 15 min (on 50% of the time) three to five times on ice. Breakage of at least 95% of the cells was obtained by this procedure. The sonicate was centrifuged at 27,000 × *g* for 30 min. The new pellet represented the cell wall fraction of *M. leprae*. The supernatant was recentrifuged at 100,000 × *g* for 2 h; the resulting pellet represented the membrane fraction of *M. leprae*, and the resulting supernatant was considered the soluble cytosolic fraction (14). Soluble extracts of both uninfected and *M. leprae*-infected armadillo spleens were prepared by suspending 300 mg of tissue in 2 ml of homogenizing buffer (1 mM EDTA, 1 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride in 10 mM Tris-HCl buffer [pH 7.4]). Samples were probe sonicated for 5 min (on 50% of the time) on ice and centrifuged for 15 min at 27,000 × *g*. The supernatants were further centrifuged at 100,000 × *g* for 2 h. The protein content of the fractions was estimated as described previously (31).

Analysis of *M. leprae* subfractions and soluble tissue extracts. The PBS extract of whole *M. leprae* was fractionated on a Superose 12 fast-performance liquid chromatography (FPLC) gel filtration column (Pharmacia, Uppsala, Sweden) equilibrated with PBS at a flow rate of 0.3 ml/min. Fractions were monitored for UV A_{280} and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 6% stacking and 15% separating gels as described previously (17) or on 10% Tricine gels (Novex, Encita, Calif.). Proteins were also transferred from gels to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Calif.) for Western blotting (immunoblotting) as described previously (28, 33). Rabbit polyclonal antibodies against the BCG 85A, 85B, and 85C proteins were gifts from Morten Harboe (Institute of Immunology and Rheumatology, University of Oslo, Rikshospitalet, Oslo, Norway). These sera were used separately at a 1:2,000 dilution or were combined together in a pool. A conjugate of goat anti-rabbit immunoglobulin G–alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.), diluted 1:9,000, was used as the secondary antibody. Color development was accomplished with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate at 0.2 mg/ml (Sigma). Purified BCG 85A and 85B proteins and proteins from a 4-week-old BCG culture filtrate were used as positive controls. The relative concentrations of the proteins of the antigen 85 complex in each *M. leprae* subfraction were determined by quantitative densitometry of the immunoblots with a MicroScan 1000 two-dimensional gel analysis system (Technology Resources, Inc., Nashville, Tenn.). The volumes of the peaks were normalized in terms of micrograms of total protein applied to each lane. Fractions of *M. leprae* were also analyzed by two-dimensional electrophoresis, initially by isoelectric focusing in tube gels containing 4% ampholytes in the pH range of 4 to 6 and 1% ampholytes in the pH range of 3 to 10 (Bio-Lyte; Bio-Rad) and then by SDS-PAGE in 15% separating gels (26). Gels were silver stained or transblotted to nitrocellulose membranes.

Peptide fingerprint analysis of the *M. bovis* BCG and *M. leprae* antigen 85 complex proteins. Purification of the *M. bovis* BCG antigen 85 complex and its individual 85A and 85B proteins has been described (28). A comparison of the partial proteolytic cleavage patterns of the pure BCG 85A protein and the PBS extract of whole *M. leprae* was made through the use of a commercially available protein fingerprinting system (Promega, Madison, Wis.). Digestions were performed in accordance with the recommendations of the manufacturer with the following modifications. The pure BCG 85A component (1 μg) and the *M. leprae* PBS extract (18 μg) were heat denatured by incubation at 95°C for 5 min and then cooled. Five microliters of endoproteinase Glu-C (*Staphylococcus aureus* V8 protease) or alkaline protease was added to each sample. Glu-C was used at protease/protein ratios of 2% (wt/wt) for the BCG 85A protein and 10% (wt/wt) for the *M. leprae* PBS extract. Alkaline protease was added at concentrations of 0.05% (wt/wt) for the BCG 85A protein and 0.2% (wt/wt) for the *M. leprae* PBS extract. The mixtures were incubated for 2 h at 37°C, and the resulting peptides were resolved by SDS-PAGE on 16% Tricine gels. The gels were run at a constant voltage of 50 V until the tracing dye entered the resolving gel; the voltage was then increased to 125 V, and the rest of the run was performed in the cold. Peptides were electrotransferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, Mass.) in CAPS buffer [3-(cyclohexylamino)-1-propanesulfonic acid] (19) at a constant voltage of 50 V for 45 min and detected by immunostaining with the anti-BCG 85 sera.

Fibronectin binding assays. The fibronectin-binding capacity of the *M. leprae* antigen 85 complex was assayed by Western blotting as described by Abou-Zeid et al. (1). Gelatin, BCG culture filtrate proteins, and bovine serum albumin (BSA) were included as controls. Membranes were incubated in pure human fibronectin (Boehringer Mannheim Co., Indianapolis, Ind.) for 2 h at room temperature. Fibronectin was detected with monoclonal antibodies 333, HFN 7.1, and HFN 36.3, kindly provided by Daniel R. Salomon and Geraldo M. B. Pereira (Laboratory of Immunology, National Institutes of Health, Bethesda, Md.). Antibodies HFN 7.1 and 333 recognize the cell-binding domain of fibronectin, and antibody HFN 36.3 recognizes a different epitope outside this domain (2, 2a). Supernatants of these clones were used at a 1:2 dilution. Alkaline phosphate conjugates of goat anti-mouse immunoglobulin G and anti-rat immunoglobulin G (Sigma), diluted 1:6,000 and 1:2,000, respectively, were used as secondary antibodies. The binding of fibronectin was also assayed by probing the nitrocellulose membranes directly with radiolabeled fibronectin. For this purpose, human fibronectin (Boehringer) was labeled with ^{125}I by the chloramine-T method (15). The specific activity was estimated to be 2×10^6 cpm/μg of protein. Nitrocellulose membranes were incubated for 3 h in blocking solution containing 5% BSA and 0.1% polyvinylpyrrolidone in Tris buffer-saline-Tween (TBST) (0.14 M NaCl, 50 mM Tris-HCl buffer [pH 7.4], 0.05% Tween 20), probed overnight at room temperature with ^{125}I-fibronectin (5×10^4 cpm/ml) in TBST, and washed extensively with TBST. Labeled fibronectin was visualized by autoradiography of the nitrocellulose membranes with X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

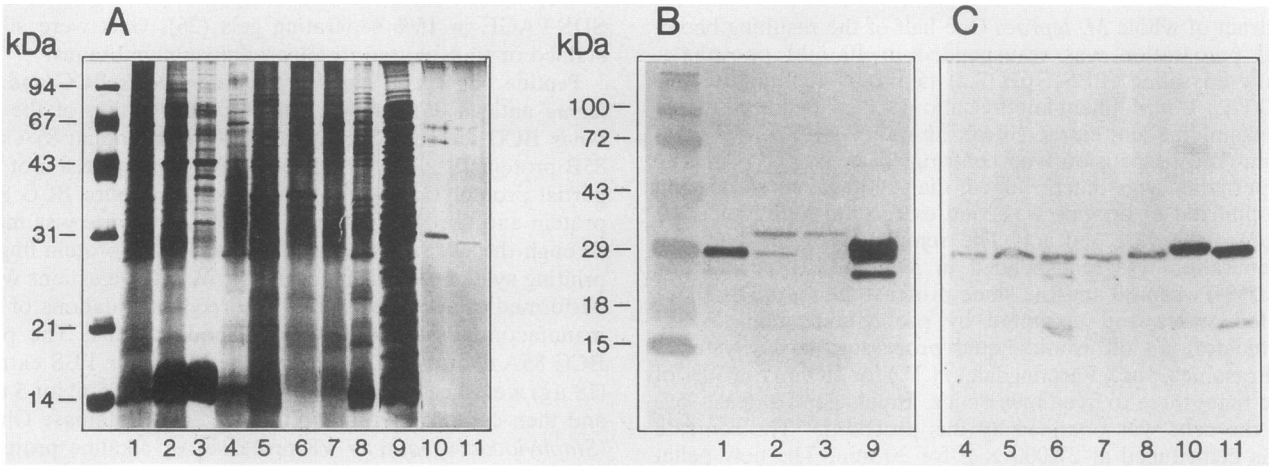

FIG. 1. Distribution of the antigen 85 complex in subcellular fractions of *M. leprae*. (A) All fractions were subjected to SDS-PAGE on 15% acrylamide gels and stained with $AgNO_3$. (B and C) The same samples were fractionated on 10% Tricine gels and probed with pooled monospecific sera raised against individual members of the BCG antigen 85 complex. Lanes: 1, whole-cell lysate (21 μg); 2, soluble fraction from an infected armadillo spleen (40 μg); 3, soluble fraction from an uninfected armadillo spleen (50 μg); 4, buffered-water extract of whole *M. leprae* (10 μg); 5, PBS extract of whole *M. leprae* (12 μg); 6, 100,000 × *g* membrane fraction of *M. leprae* (50 μg); 7, cell wall fraction of *M. leprae* (50 μg); 8, soluble fraction of *M. leprae* (50 μg); 9, culture filtrate proteins of BCG (8 μg); 10, purified BCG antigen 85A (0.5 μg); 11, purified BCG antigen 85B (0.5 μg) (only about one-quarter of these amounts were used in chemically stained gels). Molecular mass markers (nonprestained and prestained; GIBCO, Grand Island, N.Y.) are shown to the left of panels A and B.

RESULTS

Recognition of the antigen 85 complex in *M. leprae*. A search was conducted for the presence of the antigen 85 complex in extracts of nondisrupted *M. leprae*, whole bacterial lysates, subcellular fractions, and soluble extracts from *M. leprae*-infected and uninfected armadillo tissue (Fig. 1). SDS-PAGE revealed a complex mixture of proteins and mycobacterial lipopolysaccharides (lipoarabinomannan, lipomannan, and phosphatidylinositol mannosides) in all fractions (14). A major protein with an electrophoretic migration identical to that of the BCG 85B protein was identified through the use of pooled anti-BCG 85A, 85B, and 85C sera in bacterial lysates and extracts from the organs of *M. leprae*-infected armadillos but not uninfected animals (Fig. 1). From these experiments, we concluded that *M. leprae* produced the antigen 85 complex or parts of it. The expected three-component complex was not observed; rather, only one protein, apparently the 85B protein, predominated.

Information on the subcellular location of the homologous antigen 85 complex in *M. leprae* was obtained by Western blot analysis of various fractions (Fig. 1C). The 85B protein was detected in all subfractions studied. However, densitometry scans demonstrated that the buffered-water and PBS extracts of whole bacteria contained, respectively, 2.5 and 4.0 times more of the protein than the soluble bacterial extract. In two other batches of pure *M. leprae* similarly analyzed, there was virtually no antigen 85 complex in the soluble cytosolic fractions, further indicating that these proteins were actively secreted by *M. leprae*.

Other characteristics of the *M. leprae* antigen 85 complex. Gel filtration chromatography of the PBS extract of *M. leprae* on a calibrated Superose 12 FPLC column established a molecular mass of 27.8 kDa (Fig. 2). The *M. leprae* product and the BCG antigen 85B component always showed identical migrations in SDS-PAGE, and all of the evidence indicated that the *M. leprae* complex was primarily composed of the 85B antigen. To confirm this point for the *M. leprae* complex, we reacted a whole *M. leprae* lysate and BCG culture filtrate proteins against individual anti-BCG 85A, 85B, and 85C sera and against normal rabbit serum (Fig. 3). The anti-BCG 85A serum allowed the detection of a single band of ca. 31 kDa in the BCG culture filtrate and a weak band in the *M. leprae* lysate with the same molecular mass. The anti-BCG 85C serum also reacted with a single band of ca. 31 kDa in the BCG culture filtrate but did not recognize a corresponding product in the *M. leprae* lysate. The anti-BCG 85B serum reacted with the three proteins of the antigen 85 complex in the BCG culture filtrate, demonstrating considerable intracomplex cross-reactivity. However, the same antiserum demonstrated the presence of only the 30-kDa band in the *M. leprae* lysate, reinforcing the conclusion that the 85B component is the major, if not the only, member of the antigen 85 complex in *M. leprae*. When *M. leprae* fractions were analyzed by two-dimensional electrophoresis and immunoblotting and the profiles of components reacting with pooled anti-BCG 85A, 85B, and 85C sera were compared with our published profiles for the BCG antigen 85 complex (28), three major acidic spots of 30 kDa with pIs of 5.1, 5.2, and 5.3, constituting isoforms of the 85B component, predominated (28) (Fig. 4). However, a minor basic spot of 31 kDa representing the 85A component was also present.

In the absence of sufficient material for N-terminal analysis but in an effort to obtain more direct evidence of homology between the BCG antigen 85 complex and the equivalent material from *M. leprae*, we performed a comparative peptide fingerprint analysis. The gel electrophoresis and immunoblotting patterns of fragments generated by the action of both endoproteinase Glu-C and alkaline protease were similar, consisting of several peptides all still recognized by pooled anti-BCG 85A, 85B, and 85C sera (Fig. 5).

Binding of fibronectin to the antigen 85 complex. For exploration of the capacity of the homologous *M. leprae* antigen 85 complex proteins to bind to fibronectin, proteins from a whole *M. leprae* cell lysate and a BCG culture filtrate

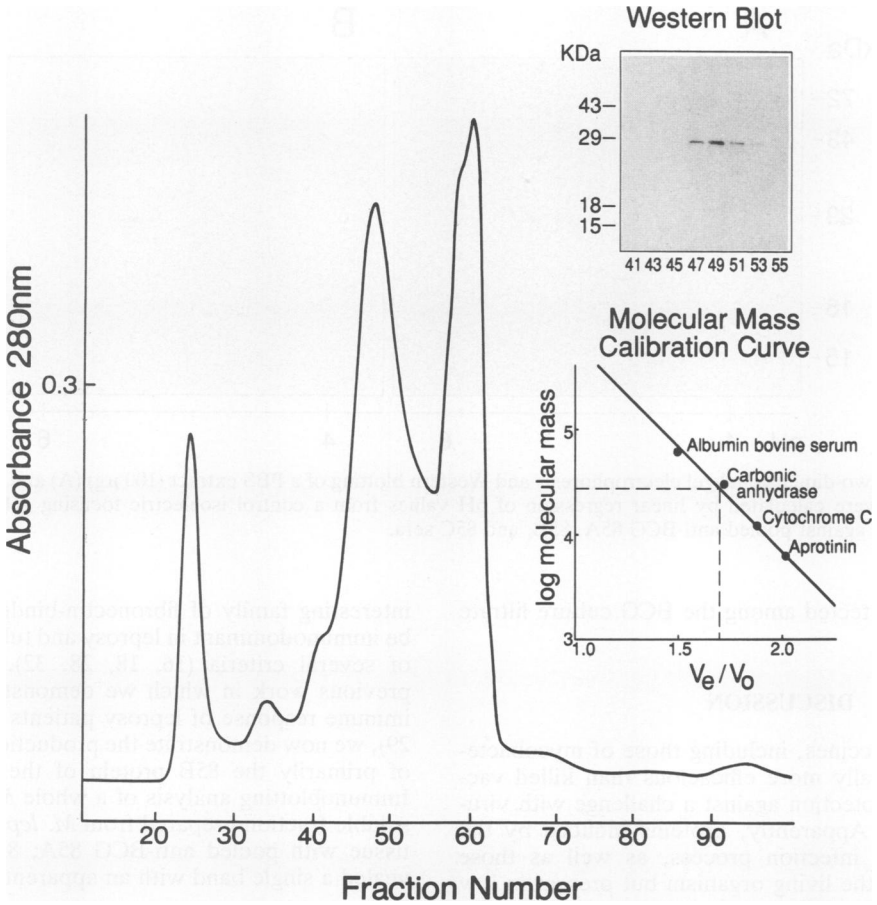

FIG. 2. Determination of the molecular mass of the *M. leprae* antigen 85 complex by gel filtration chromatography. The PBS extract of whole *M. leprae* was applied to a Superose 12 FPLC gel filtration column. Fractions (0.3 ml) were collected, and samples (10 μl) were subjected to Western blot analysis with the pooled anti-BCG antigen 85 complex serum (upper inset). The lower inset shows the molecular mass calibration curve obtained by testing a mixture of molecular mass standards under identical gel filtration conditions. The molecular mass of the *M. leprae* 85 complex was extrapolated (broken line) from its elution volume/void volume ratio (V_e/V_0).

were separated by SDS-PAGE, transferred to nitrocellulose, probed with fibronectin, and analyzed by immunostaining. Membranes submitted to all steps except for incubation with fibronectin and membranes tested against pooled anti-BCG

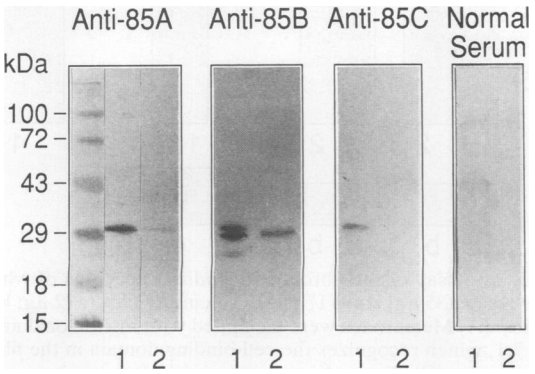

FIG. 3. Reactivity of the *M. leprae* antigen 85 complex with sera raised against BCG 85A, 85B, and 85C components and with normal rabbit serum. Lanes: 1, BCG culture filtrate proteins (2 μg); 2, whole-cell lysate (35 μg). Antisera were used at a 1:2,000 dilution. Prestained standards (GIBCO) are shown to the left.

85 sera were included as controls. Figure 6 shows the results obtained when monoclonal antibody HFN 7.1 was used as the detecting reagent. There was no evidence of binding between either the *M. leprae* or the BCG antigen 85 complex and fibronectin. Some binding activity, seen as a diffuse reaction in the high-molecular-mass range in the case of the whole *M. leprae* lysate, was observed but was apparently not related to the antigen 85 complex. Application of rat monoclonal antibody 333 and mouse monoclonal antibody HFN 36.3 produced the same negative reactions. To determine whether the general denaturing conditions to which the antigen 85 complex had been subjected had a bearing on the absence of fibronectin-binding activity, we performed a series of dot blot analyses on the BCG culture filtrate proteins, pure BCG antigen 85A and antigen 85B, and the *M. leprae* products. Regardless of whether the fibronectin or the antigen 85 complex was adsorbed to the membranes, the results were negative. Another effort to examine the fibronectin-binding capacity of the antigen 85 complex involved incubation of the individual products with ^{125}I-fibronectin and assay for binding by autoradiography (Fig. 6). For the whole *M. leprae* lysate, reactive bands with apparent molecular masses of 25, 35, 43, 55, 80, and 100 kDa were observed, results similar to those obtained with the monoclonal antibodies. A reactive band of 55 kDa and a faint

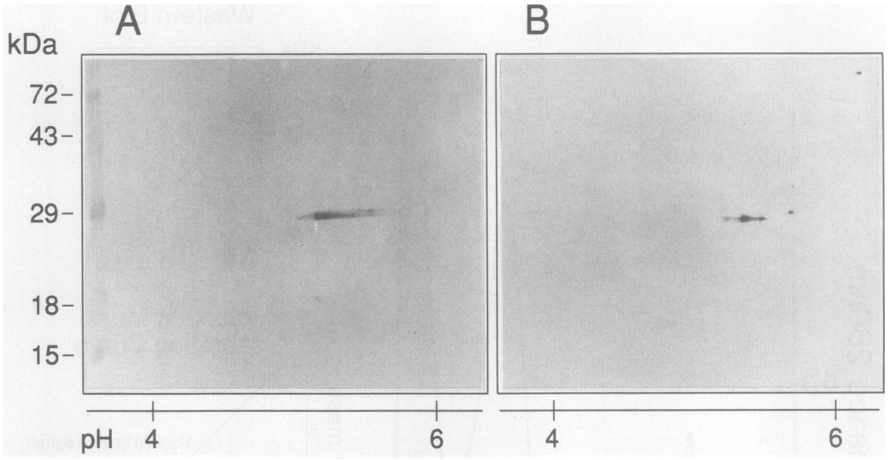

FIG. 4. Analysis by two-dimensional gel electrophoresis and Western blotting of a PBS extract (100 μg) (A) and a whole *M. leprae* lysate (200 μg) (B). The pIs were calculated by linear regression of pH values from a control isoelectric focusing gel cut into 1-cm sections. Membranes were tested against pooled anti-BCG 85A, 85B, and 85C sera.

31-kDa band were detected among the BCG culture filtrate proteins.

DISCUSSION

Live attenuated vaccines, including those of mycobacterial origin, are generally more efficacious than killed vaccines in providing protection against a challenge with virulent organisms (12). Apparently, proteins induced by the pathogen during the infection process, as well as those actively secreted by the living organism but present in low amounts in dead cells, constitute important protective immunogens (27). One approach to the identification of such key antigens in the case of leprosy is to compare the protein composition, particularly of secreted proteins, of in vivo-grown *M. leprae* with that of actively metabolizing, cultured, pathogenic mycobacteria. An outcome of such efforts in the past was the recognition of the antigen 85 complex, an interesting family of fibronectin-binding proteins shown to be immunodominant in leprosy and tuberculosis on the basis of several criteria (16, 18, 28, 32). As an extension of previous work in which we demonstrated a high humoral immune response of leprosy patients to these proteins (28, 29), we now demonstrate the production by *M. leprae* in situ of primarily the 85B protein of the antigen 85 complex. Immunoblotting analysis of a whole *M. leprae* lysate and a soluble fraction prepared from *M. leprae*-infected armadillo tissue with pooled anti-BCG 85A, 85B, and 85C sera revealed a single band with an apparent molecular mass of 30

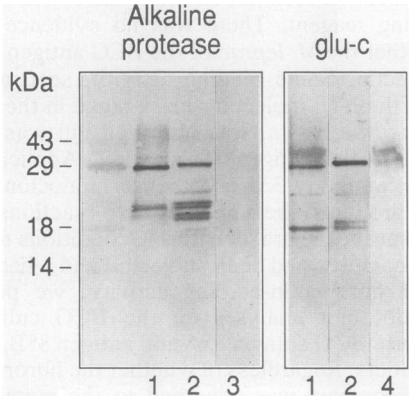

FIG. 5. Comparative peptide fingerprint analysis of the *M. leprae* antigen 85 complex and the BCG 85A antigen. Proteins were digested with alkaline protease and endoproteinase Glu-C by use of a commercial fingerprinting system (Promega). The resulting fragments were transferred onto a polyvinylidene difluoride membrane and probed with pooled anti-BCG 85A, 85B, and 85C sera. Lanes: 1, PBS extract (18 μg); 2, BCG antigen 85A (1 μg); 3, alkaline protease alone; 4, endoproteinase Glu-C alone. Prestained low-molecular-mass standards (GIBCO) are shown to the left.

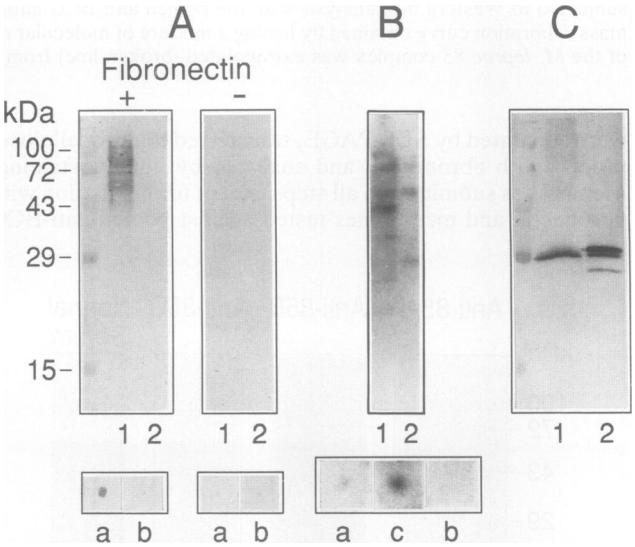

FIG. 6. Assay of the fibronectin-binding capacities of whole *M. leprae* lysate (35 μg; lanes 1) and BCG culture filtrate (2 μg; lanes 2) proteins. (A) Membranes were incubated with monoclonal antibody HFN 7.1, which recognizes the cell-binding domain in the fibronectin molecule. (B) Autoradiogram obtained after incubation of the membranes with ^{125}I-fibronectin. (C) Members of the antigen 85 complex were detected with pooled anti-BCG 85A, 85B, and 85C sera. Dot blots with gelatin (a), BSA (b), and BCG culture filtrate (c) were produced to serve as further controls. Prestained standards (GIBCO) are shown to the left.

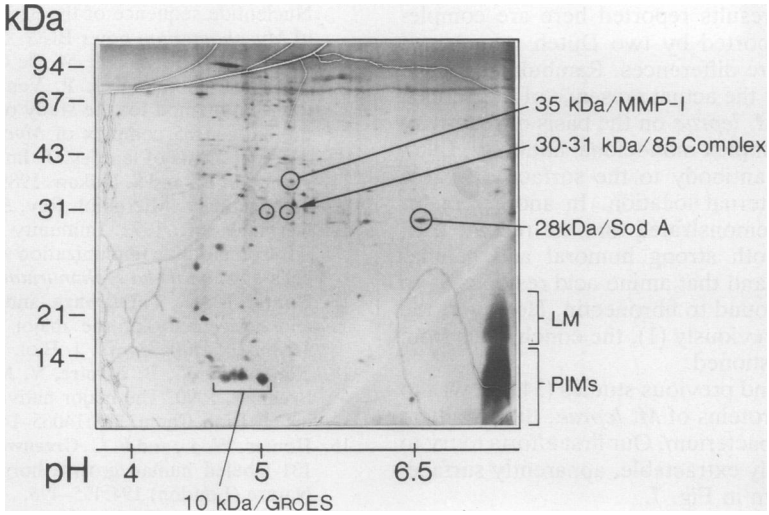

FIG. 7. Two-dimensional map of the PBS-extractable proteins of whole *M. leprae*. Experimental details are provided in the text. Trivial names for the identifiable proteins are provided; corresponding agreed-upon systematic names have been published (37).

kDa. The same single component was revealed by SDS-PAGE and immunoblotting analysis of all *M. leprae* subcellular fractions studied. Extracts from whole *M. leprae* cells contained relatively higher levels of the antigen 85 complex than somatic cellular fractions, indicating that *M. leprae* secrets the antigen 85 complex within the host cell. Previously, immunofluorescence staining of whole *M. leprae* with a monoclonal antibody that recognized both the antigen 85A and the antigen 85B components had also indicated a surface location for these proteins (30), and the gene that encodes the *M. leprae* antigen 85B protein showed evidence of a signal peptide (23, 32). Two lines of evidence strongly indicate that the *M. leprae* antigen 85 complex is composed primarily of the 85B protein. Firstly, the *M. leprae* protein selectively reacts with the anti-BCG 85B serum and not with the anti-BCG 85A or the anti-BCG 85C serum. Secondly, the profile of the *M. leprae* antigen 85 complex revealed by two-dimensional electrophoresis followed by immunoblotting showed three acidic proteins of ca. 30 kDa similar to those already described for the BCG antigen 85 complex (28). A recent analysis of the levels of antibodies to the individual members of the antigen 85 complex in lepromatous leprosy patients suggested that the response occurs predominantly against the 85B protein (28), an observation that tallies with the current evidence of the predominance of the 85B protein in *M. leprae*-derived materials.

In a recent report, Thole et al. (32) presented hybridization data indicating that *M. leprae* has for the antigen 85 complex only a single gene, which codes for a protein homologous to the 85B protein and, as discussed above, our results suggest a preferential expression by the bacteria of the gene encoding this protein. However, by using an anti-BCG 85A serum and two-dimensional electrophoresis, we also detected trace amounts of a 31-kDa protein, corresponding to the 85A homolog. On the other hand, we were unable to detect the 85C protein in *M. leprae* preparations, a not-unexpected result in view of the low level of production of this antigen in other mycobacterial species (34). Thus, neither the present study nor earlier studies have provided an unequivocal answer to the question of the number of genes for the individual antigen 85 proteins present in *M. leprae*.

A series of experiments was performed to examine the fibronectin-binding capacity of the *M. leprae* antigen 85 complex. Using the procedures described in the literature (1), we were unable to ascribe convincing fibronectin-binding activity to the BCG or the *M. leprae* antigen 85 complex. The generally low binding affinity of proteins of the antigen 85 complex for fibronectin, as demonstrated by the current experiments, was unexpected in light of the long-standing attribution of this feature to this protein complex (1). An alternative, but seemingly improbable, explanation for these negative results is that the same sites that are normally occupied by the antigen 85 complex proteins on fibronectin coincide with those recognized by the three monoclonal antibodies used. On the other hand, the BCG antigen 85A component as well as a band with an apparent molecular mass of 55 kDa in BCG culture filtrates did bind to ^{125}I-fibronectin in assays that measured direct binding capacity. Indeed, the literature had already indicated the presence of a second major fibronectin-binding molecule of 55 kDa in mycobacteria (1) and the higher binding affinity of the antigen 85A component for fibronectin (1). The current observation that the *M. leprae* product is composed primarily of the antigen 85B component and contains little of the antigen 85A component may explain the absence of appreciable fibronectin-binding capacity. Moreover, two other observations suggest that the binding of fibronectin to the antigen 85 complex proteins is not specific. When membranes were preincubated with an excess of unlabeled fibronectin, no inhibition of the binding of iodinated proteins was observed. In addition, in several immunostaining assays, the 55- and 30-kDa proteins were detected in control membranes submitted to all steps except for incubation with fibronectin. These experiments did permit the identification in lysates of whole *M. leprae* of other components, apparently unrelated to the antigen 85 complex, that bind fibronectin, and the fact that a pool of lepromatous leprosy patient sera, but not of normal sera, inhibited the binding of fibronectin to these entities indicates that they are bacterial, not host, in origin. Our results clearly indicate the need for a detailed study of the interaction between mycobacterial components and fibronectin in terms of specificity, affinity constant, and binding sites to better evaluate the potential role of these molecules in the pathogenesis of leprosy.

In most respects, the results reported here are complementary to those just reported by two Dutch groups and collaborators, but there are differences. Rambukkana et al. (30) provided evidence for the actual presence of the antigen 85 complex in sonicated *M. leprae* on the basis of reactivity with an anti-antigen 85 complex monoclonal antibody. Likewise, the binding of the antibody to the surface of whole bacteria suggested an external location. In another major study, Thole et al. (32) demonstrated that the recombinant 85B protein displayed both strong humoral and cellular immunological responses and that amino acid residues 55 to 266 and 265 to 377 both bound to fibronectin. However, the assays were those used previously (1), the conclusions from which are now being questioned.

One goal of this study and previous studies (14, 22) was to develop a profile of the proteins of *M. leprae*, the paradigm of an in vivo-grown mycobacterium. Our first efforts to try to identify the range of readily extractable, apparently surface-located proteins are shown in Fig. 7.

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