

Chemical Definition, Cloning, and Expression of the Major Protein of the Leprosy Bacillus

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The decline in prevalence of leprosy is not necessarily matched by a fall in incidence, emphasizing the need for new antigens to measure disease transmission and reservoirs of infection. *Mycobacterium leprae* obtained from armadillo tissues was disrupted and subjected to differential centrifugation to arrive at preparations of cell wall, cytoplasmic membrane, and cytosol. By committing 0.3 g of *M. leprae* to the task, it was possible to isolate from the cytosol and fully define the major cytosolic protein. Amino-terminus sequencing and chemical and enzymatic cleavage, followed by more sequencing and fast atom bombardment-mass spectrometry of fragments, allowed description of the entire amino acid sequence of a protein of 10,675-Da molecular mass. The sequence derived by chemical means is identical to that deduced previously from DNA analysis of the gene of a 10-kDa protein, a GroES analog. The work represents the first complete chemical definition of an *M. leprae* protein. PCR amplification of the 10-kDa protein gene, when cloned into *Escherichia coli* with a pTRP expression vector, allowed production of the recombinant protein. Chemical analysis of the expressed protein demonstrated that it exactly reflected the native protein. The recombinant major cytosolic protein appears to be a promising reagent for skin testing, still probably the most appropriate and pragmatic means of measuring incidence of leprosy.

The aggressive implementation of multiple drug therapy, in addition to the decision to redefine a case of leprosy, has resulted in a dramatic downward revision of estimated numbers of leprosy cases worldwide from 10 to 12 million to a new estimate of about 5.5 million (40). Consequently, the World Health Assembly has set the goal of leprosy elimination, defined as reducing the prevalence of the disease to less than one case per 10,000 population, by the year 2000 (56). However, as Smith (50) points out, there is a need for considerable caution in interpreting the apparent dramatic fall in cases, since not only is the reduction due in some measure to the redefinition of a case of leprosy but also because reduction in prevalence does not necessarily match incidence. It is clear from years of effort that serology will not provide the tools to identify reservoirs of infection or the cause of disease transmission. Unlike tuberculosis, leprosy is devoid of a good skin test reagent. Accordingly, a major thrust of basic research in leprosy should be the identification of protein antigens capable of gauging exposure to disease, i.e., a "leprosy purified protein derivative." In view of the scarcity of *Mycobacterium leprae*, a noncultivable organism (this laboratory is one of the few remaining sources), several innovative approaches have been developed to generate the major protein antigens. Of greatest impact was the approach, pioneered by Young et al. (61, 62), resulting in the construction of a genomic library of *M. leprae* and, through screening recombinant clones with monoclonal or appropriate polyclonal antibodies or oligonucleotide probes, the identification of 11 to 12 different proteins or their genes. Of these, the 70- and 65-kDa products, the DnaK and GroEL analogs, have received the greatest attention in the light of

their roles in response to various external stresses, in autoimmunity, and perhaps in reactional states in leprosy (15, 18, 45, 46, 48, 52, 60). The 18-kDa protein is clearly a major immunogen (3, 10, 11, 39). Other proteins that have emerged from this exercise, such as the 28-kDa superoxide dismutase (51), another 28-kDa product (6), and a 36-kDa protein (8, 53), have received less attention (59). In this and a previous short communication (24), we have taken a more direct but technically demanding approach to the proteins of *M. leprae* involving the processing of sufficient quantities of the armadillo-derived bacteria to allow isolation and chemical characterization of the major cellular proteins, thereby facilitating an immunological comparison of individual proteins with each other and with those derived by genetic means. From this exercise has emerged the recognition of one dominant protein. The unexpected abundance of this protein allowed the development of a purely chemical approach to structural definition.

MATERIALS AND METHODS

Preparation of *M. leprae*. *M. leprae*-infected armadillo livers and spleens were generated by E. E. Storrs, Florida Institute of Technology, Melbourne, through the medium of the National Institutes of Health (NIAID contract AI 52563). Tissues contained at least 10⁹ acid-fast bacilli per g of wet weight. The absence of *Mycobacterium avium*, a frequent contaminant of *M. leprae*-infected armadillos, was confirmed by the inability to cultivate the bacilli and the absence of chemical evidence for the *M. avium*-specific antigens, the glycopeptidolipids (34). Spleens and livers (150-g lots) were irradiated with ¹²⁶Cs (2.5 Mrads), and *M. leprae* cells were isolated from 50-g lots (32, 56). The yield of bacilli was variable (100 to 200 mg) and depended on several factors besides bacillary load, including

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the presence of a liver pigment, a lipofuscin, which interferes with recovery of bacteria (32).

Disruption and subcellular fractionation of *M. leprae*. Pure bacilli, 150 to 300 mg, were suspended in 2 to 4 ml of phosphate-buffered saline (PBS) containing Tween (0.05%), phenylmethylsulfonyl fluoride (0.8 mg/ml), benzamide (156 µg/ml), pepstatin A (30 µg/ml), iodoacetamide (10 mg/ml), L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride (50 µg/ml), EDTA (3 mg/ml), and 1,10-phenanthroline (0.26 mg/ml). The suspension was separated into 2-ml aliquots and intermittently sonicated with a probe sonicator (W-385 Sonicator, Ultrasonic Liquid Processor; Heat Systems-Ultrasonic, Inc., Farmingdale, N.Y.) for three 15-min periods on ice. No visible intact acid-fast bacilli remained after this treatment when homogenates were examined under the microscope. The sonicate was centrifuged at 27,000 × g for 30 min. The resulting pellet was washed with the breaking buffer and re-centrifuged as before. The new pellet represented the cell wall fraction of *M. leprae*. The combined supernatants from the 27,000 × g centrifugation were further centrifuged at 100,000 × g for 2 h. The 100,000 × g pellet was resuspended in the breaking buffer, and ultracentrifugation was repeated. The washed pellet represented the cytoplasmic membrane fraction of *M. leprae*, and the combined 100,000 × g supernatants made up the cytosolic fraction. The cytosol was immediately applied to a column of Sephacryl S-300 in 50 mM Tris-HCl (pH 7.4) containing 6 M guanidine-HCl.

Chromatographic and electrophoretic systems for protein resolution. Details of the gel filtration systems are provided in the text. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under standard reducing conditions (31). Samples were boiled in SDS-mercaptoethanol buffer for 2 min. Electrophoresis was performed on gel slabs 0.75 mm thick and 16 cm long with a 6% stack over a 15% resolving gel. Each gel was run at a constant current of 10 mA per gel until the tracking dye (bromophenol blue) entered the resolving gel; the current was then increased to 15 mA per gel for the duration of the run. Silver staining was performed according to the method of Morrissey (38) to demonstrate proteins; at times, the periodic acid oxidation step of Tsai and Frasch (54) was applied immediately following fixation to enhance the appearance of lipoarabinomannan and phosphatidylinositol mannosides, persistent contaminants of most native mycobacterial protein preparations (22, 24). Two-dimensional electrophoresis was performed on purified native major cytosolic protein I (MCP-I) and recombinant MCP-I 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. Gels were silver stained or transblotted to nitrocellulose membranes.

BNPS-skatole cleavage of MCP-I and N-group sequencing. Purified MCP-I (200 µg) was dissolved in 200 µl of 50% acetic acid in H₂O. L-Tyrosine (100 µl [200 µg] in 50% CH₃COOH) and BNPS-skatole [2-(2-nitrophenylsulfonyl)-3-methyl-3-bromoindolene] (100 µl [800 µg] in 50% CH₃COOH) were added to the solution, and mixtures were sealed under argon (14). The mixture was shaken in the dark at room temperature for 96 h. After the addition of 1 ml of H₂O, the mixture was dried in a Speed-Vac lyophilizer (Savant, Farmingdale, N.Y.). The hydration and drying were repeated one more time. Peptides generated by this cleavage method were separated on an SDS-PAGE gel (Tricine 16% gel; Novex, Encinitas, Calif.) running at 125 V for 1.5 h. Peptides were then blotted at 45 V for 1.5 h onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, Mass.). After the membrane was

stained with Coomassie brilliant blue (0.1% [wt/vol] in 10% [vol/vol] methanol containing 2% acetic acid) for 10 to 15 min, it was destained with 7% CH₃COOH in 45% CH₃OH followed by 7% acetic acid in 90% CH₃OH until bands were clearly visible. Peptide bands were then excised, and amino acid sequencing was applied directly to the membrane, using the Applied Biosystems (San Jose, Calif.) 470A Gasphase Sequencer equipped for on-line narrow-bore phenylthiohydantoin (PTH)-amino acid analysis. Data were collected on a Waters 730 data module. Direct N-terminal sequencing of the intact protein was also performed in this way.

Endoproteinase Lys-C digestion of MCP-I for amino acid sequencing. Purified protein (MCP-I; 1.5 nmol) was dissolved in 72 µl of H₂O, 18 µl of Tris-HCl (0.5 M; pH 9.0) and 10 µl of a 1.1-pmol/µl solution of Lys-C (27) (Boehringer Mannheim, Indianapolis, Ind.) were added, and the protein was digested at 37°C overnight. The Lys-C digest (100 pmol) was applied directly to a Vydac C₁₈ column (2.1 by 250 mm) eluting at 250 µl/min in 0.1% trifluoroacetic acid in H₂O (28). Peptide fragments were separated by application of a linear gradient of 5 to 60% CH₃CN in 0.1% trifluoroacetic acid. Peaks were monitored at 214 nm and collected. Purified peptides arising from high-performance liquid chromatography (HPLC) fractionation of Lys-C digests were applied to a peptide sequencer equipped with a continuing-flow reactor at City of Hope (Duarte, Calif.), as described previously (20, 49). Samples were also applied to polyvinylidene difluoride membranes and subjected to Edman degradation followed by amino acid on-line, narrow-bore PTH analysis.

MS of purified MCP-I and its peptide fragments. The molecular weight of purified MCP-I was determined by electrospray ionization-mass spectrometry (MS) on a Finnegan TSQ 700 MS with triple quadrupole equipped with an electrospray ionization source at City of Hope (20, 49). Fragments of the Lys-C digest of MCP-I collected from HPLC columns were also analyzed for molecular weight by fast atom bombardment (FAB)-MS on a Jeol two-sector MS, HF100, at City of Hope.

Antibodies and antibody-based assays. Monoclonal antibodies (MAbs) that recognize MCP-I were prepared with purified MCP-I as immunizing antigen (24). Immuno-dot enzyme-linked immunosorbent assay and Western blotting (immunoblotting) were conducted as described previously (22–24).

PCR amplification of *M. leprae* MCP-I DNA. Oligonucleotide primers for MCP-I were synthesized on a MilliGen 7500 DNA synthesizer. The upstream primer was constructed with a *Hind*III restriction site, and the downstream primer was constructed with a *Sal*I restriction site. *M. leprae* chromosomal DNA was purified by a modification of methods described by Whipple et al. (55). Template DNA consisted of ca. 20 ng of *M. leprae* chromosomal DNA in a 100-µl reaction volume. Amplification conditions involved a 1-min 96°C denaturation step and a 30-s 55°C primer annealing and extension step over 35 cycles. PCR products of 320 bp were analyzed on a 1.5% agarose gel.

Cloning of the MCP-I gene. Recombinant DNA cloning procedures were as described by Maniatis et al. (33). PCR-amplified MCP-I DNA was purified using a GeneClean kit (Bio 101, La Jolla, Calif.), digested with restriction enzymes *Hind*III and *Sal*I (Molecular Biology, Boehringer Mannheim), and inserted into the expression vector pTRP obtained from the American Type Culture Collection (ATCC 39946) with the aid of T4 ligase (Promega, Madison, Wis.). DNA was transformed into DH5α competent cells (Gibco BRL, Gaithersburg, Md.), and transformants were selected on LB agar containing ampicillin. A total of 40 recombinant clones were screened by PCR, using a toothpick scraping of individual colonies. Twenty

clones contained the MCP-I gene and were designated BHRP 1 through 20.

Expression and purification of recombinant MCP-I. Five MCP-I recombinant clones (BHRP-9, -12, -16, -19, and -20) and two nonrecombinant clones were grown overnight at 37°C in 2 ml of M9 media containing 0.1% Casamino Acids, 0.2% glucose, 1 mM MgSO₄, 0.1 mM CaCl₂, 2 µg of thiamine per ml, 100 µg of ampicillin per ml, and 20 µg of tryptophan per ml. Expression of the recombinant MCP-I, as described by Hoey (21), was induced by tryptophan starvation in which 1 ml from each culture was centrifuged, and cells were resuspended in 400 ml of the above M9 medium without tryptophan. Cultures were grown to an optical density of 0.5 at 600 nm with shaking at 225 rpm at 37°C. Cell lysates were analyzed by SDS-PAGE, using 15% gels. Proteins were transferred to nitrocellulose and probed with MCP-I-specific MAb CS-01 at a 1:20,000 dilution (24). Reactivity was detected with goat anti-mouse alkaline phosphatase-conjugated antibodies at a 1:6,000 dilution (Sigma, St. Louis, Mo.) and visualized with nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma).

A 20-liter batch of *E. coli* clone BHRP-9 was prepared by inoculating 60 ml of M9 media containing tryptophan with a single colony and incubating overnight at 37°C. To express the recombinant protein, the cells were pelleted at 2,700 × *g* for 5 min at room temperature and resuspended in 20 ml of M9 media without tryptophan. Each 20-liter batch of medium was then inoculated with 1 ml of culture, which was incubated at 37°C, shaking rapidly, until the A₆₀₀ reached 0.5. Indole acrylic acid (a tryptophan analog; Sigma) was added to a final concentration of 10 µg/ml, and cultures were incubated for an additional 2 h with shaking at 37°C.

To extract the total protein and purify the recombinant MCP-I, cells were pelleted at 4,000 × *g* for 10 min at 4°C and washed once with 100 ml of cold PBS. Cells were then suspended in 50 ml of PBS containing 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1% Nonidet P-40 and the following protease inhibitors: 2 mg of aprotinin per ml, 1 mg of leupeptin per ml, 1 mg of pepstatin per ml, 100 mM phenylmethylsulfonyl fluoride, and 100 mM sodium metabisulfide. Lysozyme (Worthington Biochemicals Corp., Freehold, N.J.) was added to a final concentration of 0.5 mg/ml from a stock solution of 50 mg/ml in 0.2 M Tris (pH 8.0), and cells were incubated for 30 min on ice, followed by probe sonication twice for 15 min each on ice. DNase and RNase were added to final concentrations of 1 µg/ml each, and the extract was incubated for 15 min on ice and centrifuged at 27,000 × *g* for 15 min at 4°C. The supernatant containing ca. 4% soluble recombinant protein was removed; the pellet was resuspended in 50 ml of the above-mentioned buffer and recentrifuged, and the supernatants were combined. The remaining pellet containing ca. 96% recombinant protein in the form of inclusion bodies was resuspended in 4 M guanidine hydrochloride (United States Biochemical, Cleveland, Ohio) in buffer and rocked gently at 4°C for 30 min to solubilize inclusion bodies. The suspension was centrifuged as above, and the supernatant was dialyzed in precooled 1 M guanidine hydrochloride buffer for 36 h. All supernatants containing soluble recombinant MCP-I were pooled and dialyzed in PBS. Purification of the recombinant MCP-I was accomplished by affinity chromatography on a protein A-agarose matrix (Scheicher & Schuell, Keene, N.H.) covalently bound to the MCP-I-specific MAb CS-01. Unbound proteins were removed with 6 column volumes of PBS and 4 column volumes of 1 M NaCl. Pure recombinant MCP-I was eluted from the column with 4 column volumes of 0.2 M glycine, pH 2.3.

RESULTS

Resolution and structure of the major cytosolic protein of *M. leprae*. Simplicity of fractionation of harvested *M. leprae* was essential in order to reduce losses from a product which at most can be generated in only 0.1- to 0.2-g lots. Repeated centrifugation of ruptured *M. leprae* at medium speeds yielded the soluble contents of *M. leprae* free of particulate material. SDS-PAGE showed three MCPs of ca. 14,000 (MCP-I), 17,000 (MCP-II), and 28,000 (MCP-III) M_r (Fig. 1, inset). MCP-II was recognized as the 18-kDa small heat shock protein (3, 11, 39) and MCP-III was recognized as superoxide dismutase (51) by reaction with the corresponding antibodies (58). Successful purification of the predominant cytosolic protein, MCP-I, was accomplished by application of the whole 100,000 × *g* supernatant fraction to columns of Sephacryl S-300 in 50 mM Tris-HCl (pH 7.4) containing 6 M guanidine-HCl (Fig. 1). Final purification of MCP-I was accomplished by rechromatography of fractions 55 to 61 in the same column. MCP-I is the major single polypeptide in *M. leprae*, representing about 1% of the mass of the organism. In the present instance, about 1.5 mg of MCP-I was recovered from 150 mg of *M. leprae*. Affinity chromatography as described below for the recombinant MCP-I can also be used for purification of the native protein.

Previously, the first 30 amino acid residues of MCP-I of *M. leprae* were successfully sequenced (24). On this occasion, the sequence was readily extended to the first 51 residues through N-group analysis at the City of Hope facility (Fig. 2). The close relationship of MCP-I to the *M. bovis* BCG-a 10-kDa MPB-57 protein (2, 24, 37, 47, 57) was obvious (Fig. 2) and, with it, the realization that MCP-I should be amenable to selective chemical cleavage. Of the several nonenzymatic cleavage methods tried (14), BNPS-skatole-catalyzed cleavage was the most successful (Fig. 3). Sequencing of isolated fragment A from the BNPS-skatole-catalyzed cleavage reaction showed that it contained a mixture of two peptides, and both peptides were sequenced simultaneously on the sequencer. One fragment (A-1) contained residues 1 to 35 (Fig. 2), and the other fragment (A-2) contained sequences 51 to 84. Fragment B contained sequences 51 to 80. Thus, specific cleavage at Try (W), as well as nonspecific cleavage at Glu (E) and Tyr (T), had occurred. However, residues 50, 56, 71, 72, 75, 76, 79, and 82 of MCP-I could not be categorically identified through conventional sequencing. The identity of these residues was established instead by mass analysis of the Lys-C digestion fragments of MCP-I. The full range of fragments arising from enzymatic cleavage was isolated by HPLC (Fig. 4) and subjected to FAB-MS to derive molecular weights and hence sequence. The results of this exercise are summarized in Table 1. Fragment 1 contained residues 73 to 79 with a determined MH⁺ of *m/z* 767 (theoretical MH⁺, *m/z* 767.39). Fragment 3 contained two subfragments, one corresponding to residues 35 to 56 with an MH⁺ of *m/z* 2,326 (theoretical MH⁺, *m/z* 2,325.15) and the other indicative of residues 37 to 56 with a determined MH⁺ of *m/z* 2,068 (theoretical MH⁺, *m/z* 2,069.24). Fragment 5 contained residues 57 to 72 with a determined MH⁺ of *m/z* 1,789 (theoretical MH⁺, *m/z* 1,789.97). Fragment 6 contained residues 12 to 34 with a determined MH⁺ of *m/z* 2,368 (theoretical MH⁺, *m/z* 2,368.24). FAB-MS analysis of fragment 10 suggested strongly that its sequence was identical to that of residues 80 to 99 of the *M. tuberculosis* product (Fig. 2) on the basis of the following information. First, an MH⁺ ion of *m/z* 2,239 was recognized, in excellent agreement with the theoretical value of *m/z* 2,239.20 for residues 80 to 99 of the *M. tuberculosis* product. Second, the amino acid composition of fragment 10 in

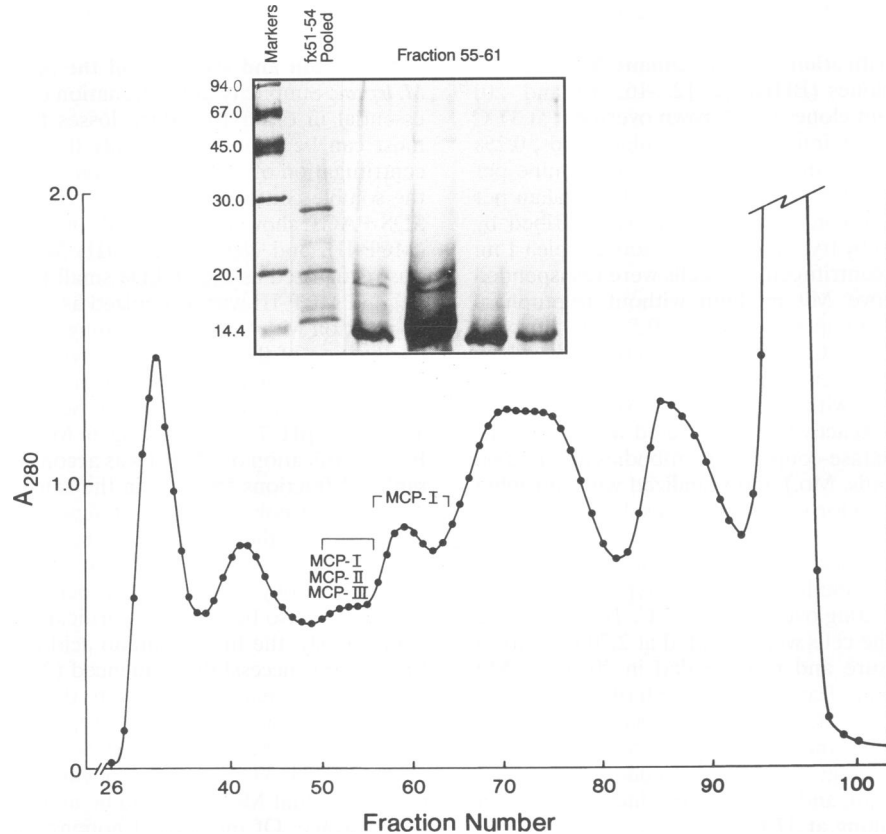


FIG. 1. Sephacryl S-300 column (1 by 100 cm) chromatography of the whole cytosolic proteins derived from 150 mg of *M. leprae* in 50 mM Tris-HCl (pH 7.4) containing 6 M guanidine-HCl. Fractions (fx) (1 ml) were collected, and samples of some were subjected to SDS-PAGE (inset) and stained with silver.

terms of residues per mole of peptide was the same as that predicted for residues 80 to 99 of the *M. tuberculosis* 10-kDa protein. On the other hand, direct N-terminal sequencing of fragment 10 was not successful, except for the first amino acid of the fragment (amino acyl 80), due to α - β -transpeptidation of the succeeding residue, resulting in blockage of Edman degradation at the cyclization stage by the imide of the

β -aspartyl structure (28). Nevertheless, on the basis of the presence of identical FAB-MS MH^+ ions and comparable amino acid analyses, residues 80 to 99 in both the *M. leprae* and *M. tuberculosis* proteins were considered identical. The complete amino acid sequence of MCP-I determined in this fashion is shown in Fig. 2. This sequence is in accord with that



FIG. 2. Comparison of complete amino acid sequences of MCP-I and the *M. tuberculosis* 10-kDa protein. The sequence reported in reference 2 is used. It differs from the product from *M. bovis* BCG at the C-terminal end (57).

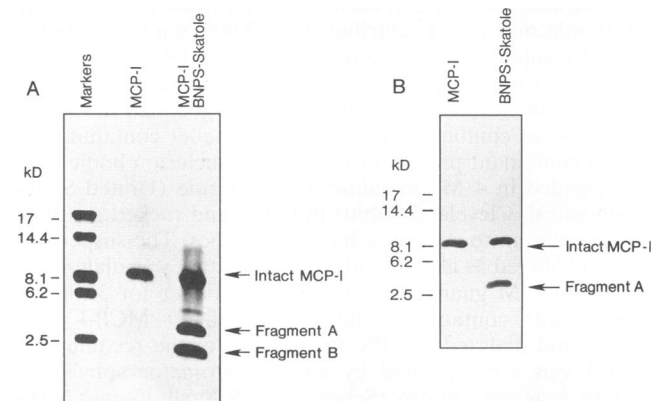


FIG. 3. Results of cleavage of MCP-I with BNPS-skatole. (A) MCP-I (1 mg) was treated with BNPS-skatole as described in the text. The reaction products were run on a 16% Tricine gel and silver stained. (B) A parallel gel was immunoblotted with the anti-MCP-I MAAb CS-01.

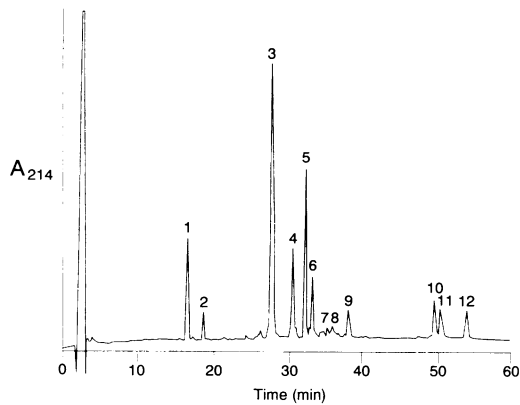


FIG. 4. Peptide fingerprint of Lys-C-digested MCP-I on reverse-phase HPLC. Experimental details are provided in the text.

derived from the gene as established independently by us (36). The calculated average M_r for the protein shown in Fig. 2 is 10,670.21, which is to be compared with the empirical molecular weight determined by electrospray ionization-MS of 10,675.0. This value gave an accuracy of 0.045%, further confirming the sequence shown in Fig. 2 for MCP-I. On SDS-12.5% PAGE in a Tris-glycine buffer system, MCP-I displayed migration properties similar to those of a 14,000-molecular-weight marker. However, on a Tricine-16% PAGE gel, MCP-I migrated much closer to its true molecular weight of ca. 10,700 (Fig. 3A, middle lane).

Antibody-reactive site of MCP-I. Despite the close similarity in amino acid sequence of MCP-I and the 10-kDa protein of *M. tuberculosis*, the prototype antibody (MAb SA12 [37]) to the *M. tuberculosis* product did not react with MCP-I. As demonstrated in Fig. 5, MAb SA12 reacted only with the 10-kDa protein in late culture filtrates of *M. tuberculosis* but did not react with pure MCP-I or any of the soluble proteins from *M. leprae*. Conversely, MAb CS-01 reacted strongly with MCP-I but not with the 10-kDa protein in culture filtrates from *M. tuberculosis* (Fig. 5). MAb ML06, one of the original anti-*M. leprae* MAbs (13) and known to recognize a 12,000- M_r protein in soluble extracts of *M. leprae* (25, 26), also reacts with MCP-I and not with the 10-kDa protein of *M. tuberculosis* (data not shown). Furthermore, when the BNPS-skatole cleavage products were blotted with anti-*M. leprae* MCP-I MAb CS-01 (Fig. 3B), the antibody reacted with MCP-I and fragment A only and not with fragment B, indicating that the antibody-binding region was located in either fragment A-1 or A-2. However, since fragment A-2, which contained amino acyl residues 51 to

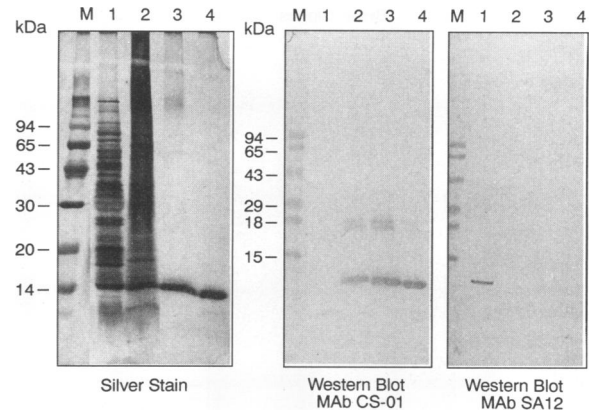


FIG. 5. Demonstration of the specificity of the two 10-kDa proteins from *M. leprae* and *M. tuberculosis* for their corresponding antibodies. Proteins were run on SDS-15% polyacrylamide gels and stained with silver or subjected to immunoblotting. M, molecular weight markers. Lanes: 1, *M. tuberculosis* late culture filtrate, 5 μ g; 2, *M. leprae* soluble proteins, 2.5 μ g; 3, native MCP-I, 0.5 μ g; 4, recombinant MCP-I, 0.5 μ g.

84, is similar to fragment B, which contained amino acids 51 to 80, and CS-01 did not react with fragment B, the epitope for CS-01 must be located in fragment A-1, i.e., within the first 35-amino-acyl segment of MCP-I.

Preparation of recombinant MCP-I. In order to assess the full immunological potential of MCP-I, its expression without a fusion partner was undertaken. Specific oligonucleotide primers were constructed to allow PCR amplification of the MCP-I gene from *M. leprae* chromosomal DNA. The 320-bp product was inserted into the *Hind*III and *Sal*I restriction sites of the plasmid expression vector pTRP, which carries a transcriptional promoter controlled by Try. Transformants were grown on LB agar in the presence of ampicillin. A total of 40 colonies were screened by PCR, and 20 were found to be positive. The amplified PCR product of five positive clones is shown in Fig. 6. Expression of clones containing the MCP-I gene was induced by Try starvation. The recombinant MCP-I was released in the form of insoluble inclusion bodies or aggregates, presumably of improperly folded protein, which were solubilized in the SDS-PAGE buffer, electrophoresed on 15% polyacrylamide gels, and transferred to nitrocellulose for Western blot analysis. All recombinant MCP-I preparations tested were reactive against the specific MAb CS-01, and all showed the same M_r value as the native MCP-I except clone BHRP-21, which produced a product of slightly smaller size (Fig. 7).

TABLE 1. FAB-MS analysis of some of the peptide fragments arising from Lys-C digestion of MCP-I

HPLC fragment ^a	MH ⁺ (m/z)		Deduced sequence ^c	Deduced residues
	Experimental ^b	Theoretical		
1	767	767.39	YGGTEIK	73-79
3	2,068	2,069.24	PQEGTVVAVGPRWDEDGEK	37-56
	2,326	2,325.15	EKPQEGTVVAVGPRWDEDGEK	35-56
5	1,789	1,789.97	RIPLDVAEGDVTIYSK	57-72
6	2,368	2,368.24	ILVQANEAEETTTASGLVIPDTAK	12-34
10	2,239	2,239.20	YNGEELYILSARDVLAVVSK	80-99

^a See Fig. 4.

^b Experimental conditions are described in the text.

^c See Fig. 2.

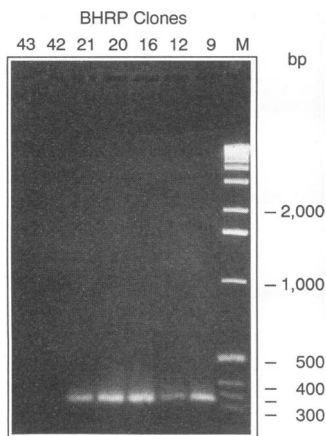


FIG. 6. Results of PCR amplification of the 320-bp insert of MCP-I DNA from five recombinant clones designated BHRP-9, -12, -16, -20, and -21. Two nonrecombinant clones designated no. 42 and 43 were chosen as negative controls. Products were run on a 1.5% agarose gel and stained with ethidium bromide.

To purify and characterize recombinant MCP-I, the protein was expressed from 20 liters of M9 media inoculated with *E. coli* clone BHRP-9. Only about 4% of the released product was soluble MCP-I; the remainder was insoluble. To solubilize the aggregated form, the protein pellet was resuspended in 4 M guanidine hydrochloride and dialyzed slowly into PBS. Recombinant MCP-I was then readily purified from *E. coli* lysate by affinity chromatography, using MAb CS-01 (Fig. 8). The yield of purified recombinant MCP-I from this 20-liter batch was 100 mg. Both the recombinant MCP-I and its native counterpart appeared as a doublet on two-dimensional gel electrophoresis (Fig. 9), and both reacted with MAb CS-01, indicating that they are isoforms. However, whereas the native protein shows equal intensity of both isoforms, the recombinant product contains considerably less of the more acidic isoform, perhaps due to less degradation. N-terminal amino acid sequencing of recombinant MCP-I (up to 17 residues) demonstrated com-

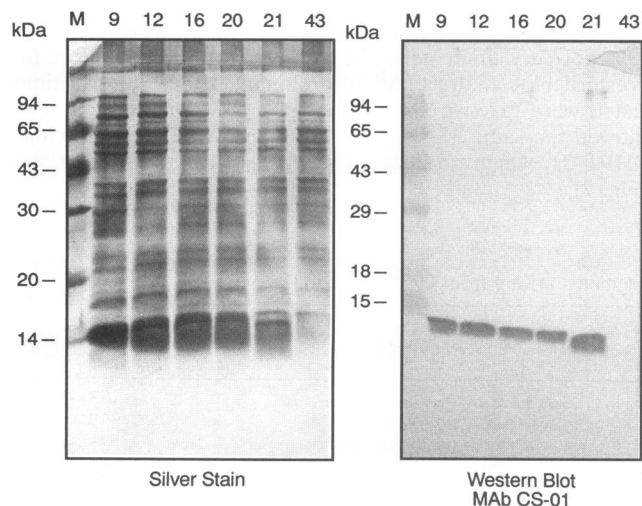


FIG. 7. Analysis of the *E. coli* cell lysates from recombinant MCP-I clones (BHRP-9, -12, -16, -20, and -21) and one nonrecombinant clone (no. 43). Products were analyzed by SDS-15% PAGE and silver staining or Western blotting with the MCP-I-specific MAb CS-01.

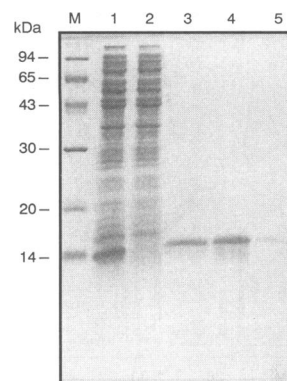


FIG. 8. Purification of recombinant MCP-I by affinity chromatography based on MAb CS-01. Lane 1, *E. coli* clone BHRP-9-solubilized cell lysate; lane 2, unbound lysate; lanes 3 to 5, 0.2 M glycine (pH 2.3) eluant containing the purified recombinant MCP-I.

plete correspondence with the native product (Fig. 2). Again, the first amino acid was Ala. It should be noted that previously we had concluded, on the basis of nucleotide sequence (36), that the protein had an additional Val at the N terminus. This discrepancy is due to the fact that the initial codon for this gene, GUG, encodes Val when internal in the polypeptide, but when it serves as the initiation codon, it is recognized by the tRNA carrying amino acid formylmethionine and becomes part of the initiation signal. Accordingly, the amino acid sequence of the native and the fusionless recombinant products and that deduced from the nucleotide sequence are the same, with Ala as the N-terminal residue.

DISCUSSION

The dramatic downward revision of estimates of global prevalence of leprosy represents a watershed for those in basic biological research on leprosy, necessitating a reexamination of research priorities. Aggressive case finding and multiple drug therapy may soon reduce active leprosy below the level of a pressing public health problem. The sustenance of a vaccine program, long the mainstay of fundamental immunological research in leprosy, can hardly be justified while adhering to such optimistic predictions. Many now argue that resources should be directed to a study of immunopathogenesis in leprosy, in light of the millions still suffering from disabilities, and to development of diagnostic tools to measure incidence of infection and latent disease.

In the light of retrenchment in leprosy research, the arma-

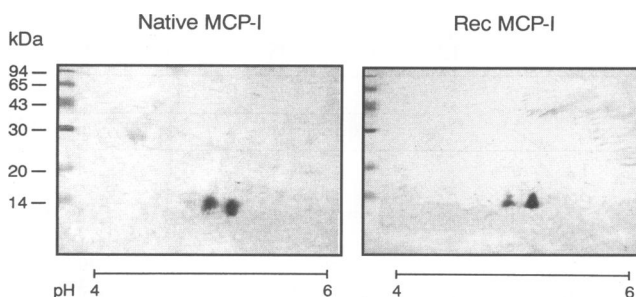


FIG. 9. Comparative analysis of the native and recombinant (Rec) MCP-I (2 μ g each) by two-dimensional gel electrophoresis and silver staining.

dillo-derived bacillus, long the source of key antigens and genomic DNA, may be endangered, lending some urgency to phenotypic and genomic (12) characterization. Thorough biochemical characterization of *M. leprae* may be the only means of acquiring the new epidemiological tools required to measure the true extent of the disease. Already, most of the secondary gene products of *M. leprae* have been described, such as the phthiocerol-containing phenolic glycolipids (16), much of the cell wall core (i.e., the mycolylarabinogalactan-peptidoglycan complex [35]), and the particular type of mannose-capped lipoarabinomannan that distinguishes *M. leprae* (5, 22). Indeed, this approach has been responsible for the few pragmatic tools available for the serodiagnosis of leprosy (16), although a decade of effort has shown that serology merely provides an alternative means for diagnosis of lepromatous leprosy, certainly not a pressing need.

It is also more likely that biochemical fractionation of *M. leprae* will ultimately lead to appropriate skin test antigens. Certainly the approach of defining protein antigens through screening of recombinant libraries with antibodies, a facile but inappropriate approach, has not uncovered promising candidates. In fact, MCP-I, obviously the single most dominant protein of the organism, was not revealed through these means. The heat shock proteins were the primary products revealed through antibody screens. The 65- and 70-kDa heat shock proteins are probably involved in protein folding and translocation in light of considerable sequence identity with *E. coli* Dnak and GroEL, respectively (4, 15, 19, 48). The precise role of these in the immunology of leprosy is in debate, complicated by their roles in autoimmunity and arthritis and as an antigen for $\gamma\delta$ T cells (29). Moreover, the sizable sequence homology with host counterparts compromises their practical use. The lack of appreciable quantitation of the recombinant forms of many of the other proteins of *M. leprae* has thwarted extensive immunological comparisons. A family of proteins of 30 to 31 kDa with extensive sequence homology to the antigen 85 complex of *M. tuberculosis* (42, 43), the well-known 18-kDa protein (39), a new low-molecular-weight protein (30, 44), and now MCP-I-10-kDa protein appear the most promising to date. Of these, the 10-kDa protein is quantitatively the most prominent, representing about 1% of the bacterial mass.

The predominance of MCP-I, a stress protein and obviously the GroES analog, may be a reflection of the intracellular obligate nature of *M. leprae*, or its peculiar life cycle within the host, such that only 5 to 10% of the bacilli are deemed to be viable on harvesting. The GroES and GroEL proteins are encoded by a cistronic *groESL* operon (9) similar to that in other organisms (4). However, the earlier belief that the *groEL* and *groES* genes are on separate loci also seems to be correct in that there is now evidence that *M. leprae* contains a separately arranged second *groEL* gene in addition to the *groESL* operon (9). The chromosomal arrangement of the *groES* and one of the *groEL* genes should allow for coordinated expression. In fact, we (23) and others (17) have reported on the presence of considerable quantities of the 65-kDa protein in *M. leprae*, perhaps in physical association with the 10-kDa protein. The possibility should be seriously considered that the immunoreactivity of the key proteins of *M. leprae* is dependent on a polymeric state rather than a monomeric or, indeed, peptidic state (41). It is known that the native 65-kDa protein of *M. bovis* BCG occurs as a multimer, and it is in this form, which was once designated skin-reactive protein P64 (1, 7), that it demonstrates its greatest potency.

Previously, we concluded that MCP-I-10-kDa protein is the dominant T-cell reactive protein of *M. leprae* in that it was recognized by most *M. leprae*-reactive human T-cell lines and

was more potent in stimulation of proliferation of blood mononuclear cells than other *M. leprae* native and recombinant proteins (36). Yet the same protein, both native and recombinant forms, has shown disappointingly weak delayed-type hypersensitivity responses in sensitized guinea pigs compared with whole *M. leprae* cytosol from which this protein arose (our unpublished results). It now appears that the protein that emerged from extensive fractionation and subsequently tested in guinea pigs was the monomeric denatured form. Efforts are now under way to isolate and test the polymeric form of MCP-I-10 kDa, either the homopolymer or the heteropolymer of the 10- and 65-kDa proteins.

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