INFECTION AND IMMUNITY, Mar. 1990, p. 703–710
0019-9567/90/030703-08$02.00/0

Vol. 58, No. 3

Identification of an Immunostimulating Protein from *Mycobacterium leprae*

NAHID MOHAGHEGHPOUR,[1,2]* MALCOLM W. MUNN,[1] ROBERT H. GELBER,[2,3] AND EDGAR G. ENGLEMAN[1]

Department of Pathology, Stanford University, Stanford, California 94305,[1] and Kuzell Institute for Arthritis and Infectious Diseases[2] and Hansen's Disease Program,[3] San Francisco, California 94115

Received 1 September 1989/Accepted 6 December 1989

Despite the recent identification of a number of *Mycobacterium leprae* proteins, the major immunogenic determinants of this organism remain obscure. We isolated from *M. leprae* a potent immunostimulatory preparation, designated the MLP fraction, which contains a major protein of 35 kilodaltons (kDa). This protein was precipitated by monoclonal antibody ML03-A_1, which recognizes a 35-kDa protein of *M. leprae*, and by sera obtained from patients with lepromatous leprosy. Neither sera from healthy controls nor sera from patients with pulmonary tuberculosis recognized the 35-kDa protein, and only one of four serum samples from patients with borderline tuberculoid leprosy reacted with this protein. The MLP fraction stimulated T-cell proliferation in patients with leprosy whose T cells proliferate in response to whole *M. leprae* cells. Apparently, the T-cell epitope associated with MLP is also expressed on *M. tuberculosis* and *M. bovis* BCG, since patients with pulmonary tuberculosis and BCG-vaccinated individuals demonstrated significant responses to the MLP fraction. The 35-kDa *M. leprae* protein, purified to homogeneity in the laboratory of P. J. Brennan, stimulated T-cell proliferative responses in all MLP-responsive subjects. These findings suggest that the 35-kDa protein present in MLP is an immunostimulatory component of *M. leprae*. In addition to serving as a useful probe for study of the T-cell anergy associated with lepromatous disease, this protein may ultimately be useful as a component of a vaccine designed to provide protection against infection with *M. leprae*.

Mycobacterium leprae is responsible for a wide spectrum of diseases in humans (32). At one end of the spectrum, patients with tuberculoid leprosy exhibit strong cell-mediated immune responses to *M. leprae* and their lesions contain low levels of bacteria. By contrast, most patients with lepromatous leprosy (LL) have high-titered antibody responses but fail to display specific cell-mediated responsiveness to *M. leprae* antigens and their lesions contain abundant numbers of bacilli (2, 11, 26, 29, 30). Although some patients with LL have been described with nonspecific anergy (4), many lepromatous patients exhibit vigorous cell-mediated immune responses to antigens other than *M. leprae* (25, 26, 29).

Purified *M. leprae* antigens are essential for dissecting the cellular and humoral immune response to this bacterium. Use of murine monoclonal antibodies (MAbs) (3, 10, 15, 20; H. D. Engers, M. Abe, B. R. Bloom, V. Mehra, W. Britton, M. Buchanan, S. K. Khanolkar, D. B. Young, O. Closs, T. Gillis, M. Harboe, J. Ivanyi, A. H. J. Kolk, and C. C. Shepard, Letter, Infect. Immun. **48**:603–605, 1985), recombinant DNA expression libraries (40), and extraction procedures (13, 23) has led to the identification and initial characterization of a number of *M. leprae* proteins. However, the major immunogenic determinants of *M. leprae* remain obscure. To identify such determinants, we tried to isolate native proteins from *M. leprae*. The 35-kilodalton (kDa) *M. leprae* protein described in this report appears to be a particularly potent immunogen which not only reacts with circulating antibodies from patients with leprosy but, more importantly, elicits T-lymphocyte responses from patients with leprosy that parallel their responses to whole *M. leprae* organisms.

* Corresponding author.

MATERIALS AND METHODS

Patients. Heparinized blood was obtained from 11 patients with LL and 12 patients with borderline tuberculoid leprosy (BTL). The disease stage was classified by T. H. Rea of the Division of Dermatology, University of Southern California, as described by Ridley and Jopling (32). These patients were under treatment with dapsone or a combination of dapsone, rifampin, and clofazimine. None were receiving corticosteroids or thalidomide or had erythema nodosum leprosum when blood samples were taken. All of these patients had been previously studied for the ability of their T cells to proliferate in response to *M. leprae* in vitro (Mohagheghpour et al., unpublished data). Five of the patients with LL were included in this study because their T-cell responses to *M. leprae* were normal while the remaining patients with LL were nonresponders to *M. leprae*. Blood samples were also obtained from 7 patients with pulmonary tuberculosis (PT) from the Curicica Sanitarium, Rio de Janeiro, Brazil; 10 healthy volunteers who had received *M. bovis* BCG vaccinations; and 8 healthy individuals who had not been vaccinated with BCG.

Antigens. Armadillo-derived whole *M. leprae* cells, lyophilized uninfected armadillo liver, and a 35-kDa *M. leprae* protein purified to homogeneity (single silver-stained band by sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis [PAGE]) were kindly provided by P. J. Brennan, Department of Microbiology, Colorado State University, Fort Collins. Recombinant *M. leprae* proteins of 12, 18, and 65 kDa expressed in *Escherichia coli* were kindly provided by Richard A. Young, Whitehead Institute for Biomedical Research, Cambridge, Mass.

Anti-*M. leprae* antibodies. Sera were obtained from four patients with LL and four with BTL known to have antibodies against *M. leprae*. Sera were obtained from the patients with PT described above. In addition, sera from two untreated, bacteriologically smear-positive patients with PT

were kindly provided by Luiz M. Bermudez, Medical Research Institute, San Francisco, Calif. Control serum was obtained from a healthy individual never exposed to leprosy bacilli and known to be a nonresponder to *M. leprae*. The rabbit polyclonal antibodies used were serum pools from two animals immunized with the *M. leprae* protein fraction (MLP) recovered from the pellet fraction of sonicated *M. leprae* as described below. Rabbit anti-*M. bovis* BCG polyclonal antibodies were kindly provided by P. J. Brennan. Murine MAb ML03-A_1, which identifies a 35-kDa *M. leprae* protein, and MAb F47-CL9, which binds to a 36-kDa *M. leprae* protein (15; Engers et al., Letter), were kindly provided by Thomas Shinnick, Centers for Disease Control, Atlanta, Ga.

Surface phenotype analysis of T cells. Murine MAbs to CD3 (anti-Leu-4), CD8 (anti-Leu-2), and CD4 (anti-Leu-3) were generated in our laboratory and purified from ascites. Fluorescein-conjugated antibodies were prepared as described earlier (34). Analysis of surface phenotypes with these antibodies was performed on an Ortho System 50H Cytofluorograf (34).

Preparation of MLP. The MLP fraction containing the 35-kDa protein was recovered from the pellet fraction of sonicated *M. leprae*. Briefly, lyophilized bacilli were rehydrated with phosphate-buffered saline (PBS; pH 7.0) and washed once by centrifugation. Bacilli (33 mg/ml of PBS) were sonicated on ice for a total of 3 min, with 20-s bursts followed by 10-s rests, in a W-10 probe sonicator (Heat System-Ultrasonics, Inc., Plainview, N.Y.). The sonic extract was then centrifuged at 25,000 × *g*. The supernatant containing soluble sonicated proteins (SS) was removed, and the pellet was washed twice with PBS. To remove the lipid moiety, the pellet was suspended in PBS and cold acetone was added dropwise to a concentration of 60% (vol/vol). After 2 h of incubation at 4°C, this suspension was centrifuged at 25,000 × *g* and the resultant pellet was dried under vacuum for 4 h. Subsequently, the pellet was suspended in 1.0 ml of PBS and the soluble fraction designated the MLP fraction was collected by centrifugation at 25,000 × *g*. The protein concentration of the MLP fraction was measured by the Folin reagent. Thereafter, the material was divided into aliquots and stored at −70°C until used. Lyophilized, uninfected armadillo liver was subjected to an identical fractionation procedure, and the final supernatant was used as a control (control protein) in the studies described below.

Protein analysis by SDS-PAGE. *M. leprae* SS, the MLP fraction, and the purified 35-kDa protein (10 μg of protein per lane) were reduced in 5% β-mercaptoethanol and run on SDS–10% PAGE gels as described by Laemmli (21). The protein bands were silver stained under basic pH conditions (38). The molecular size standards ranged from 29 to 205 kDa (Sigma Chemical Co., St. Louis, Mo.).

Immunoprecipitation. The MLP fraction and the purified 35-kDa protein (15 μg) were radiolabeled with ^{125}I (Dupont, NEN Research Products, Boston, Mass.) by using Iodogen (Pierce Chemical Co., Rockford, Ill.) as described by Fraker and Speck (8). Free ^{125}I was removed by gel filtration through Sephadex G-25. The radiolabeled proteins were subsequently preabsorbed for 6 h with fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring, La Jolla, Calif.). The Pansorbin was removed by centrifugation, and ^{125}I-labeled proteins were rocked for 6 h with protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) precoated with various antibodies. Resultant resins were then washed five times with binding buffer (Bio-Rad Laboratories, Richmond, Calif.) The proteins bound were eluted by boiling of the washed resin pellet in 80 μl of sample buffer (0.125 M Tris hydrochloride containing 6% SDS and 10% glycerol [pH 6.8]) for 2 min. The eluates were then analyzed by SDS-PAGE under reducing conditions as described above. Following electrophoresis, the gel was fixed in 50% methanol, dried, and autoradiographed for various lengths of time.

Preparation of PBL. Peripheral blood lymphocytes (PBL) were isolated by Ficoll-Hypaque gradient centrifugation of fresh heparinized blood and suspended in RPMI 1640 supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer–2mM L-glutamine–100 U of penicillin per ml–100 μg of streptomycin per ml–20% heat-inactivated pooled human serum (complete medium).

Proliferation assays. The proliferative responses of PBL to mycobacterial antigens were assayed as previously described (26). Prior studies indicated that 10 μg of whole *M. leprae* cells per ml produces maximal responses in most individuals (26). Thus, 2×10^5 PBL were incubated in 200 μl of complete medium with (i) no additives, (ii) 10 μg of *M. leprae* per ml, or (iii) various concentrations of the MLP fraction, the purified 35-kDa protein, or control protein. Quadruplicate microwells were pulsed with [^{3}H]thymidine (6.7 Ci/mM, 1 μCi per well; Dupont, NEN Research Products) on day 5, and the cells were harvested 16 h later. The results are expressed as mean counts per minute or the stimulation index (SI), calculated by the following equation: SI = mean counts per minute in cultures with antigen/mean counts per minute in cultures without antigen.

Generation of $CD4^+$ T-cell clones. PBL from two patients with BTL (patients 1 and 2) and one patient with LL (patient 3) were separated into T and non-T cells with a sheep erythrocyte rosette-forming technique (16). $CD4^+$ ($CD8^-$) T cells were negatively selected by panning (7) with MAb anti-Leu-2. Fresh $CD4^+$ T cells from the patients with BTL were stimulated with 10 μg of sonicated *M. leprae* per ml of Iscove modified Dulbecco medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum in the presence of irradiated (3,000 rad) autologous PBL as antigen-presenting cells (APC). $CD4^+$ T cells from the patient with LL (patient 3) were stimulated with *M. leprae* after preculture in medium alone for 48 h to allow for immunologic recovery (25). Six days after stimulation with *M. leprae*, the resultant lymphoblasts were isolated on Ficoll-Hypaque gradients and cloned at a density of one cell per well in round-bottom microtiter wells in 200 μl of the above-described medium supplemented with interleukin 2-containing supernatant and 10^5 irradiated APC obtained from the human lymphocyte antigen-identical brother of the patient. An interleukin 2-containing supernatant was generated from activated PBL as previously described (24). Approximately 5 U of interleukin 2 was added per ml every 3 to 4 days, alternating with irradiated APC and 2 μg of antigen per ml. Proliferative responses were assayed by incubating 2×10^4 of each $CD4^+$ T-cell clone isolated on Ficoll-Hypaque gradients in 200-μl triplicate cultures with 5×10^4 irradiated APC plus 10 μg of *M. leprae* per ml. After 72 h of incubation, cultures were labeled with 1 μCi of [^{3}H]thymidine and harvested 8 h later. Phenotype analysis of the cloned cells was performed as described above before their use in proliferation assays, and cells were not assayed until 7 days after the last addition of APC and antigen. A total of 11 *M. leprae*-reactive clones were generated, each of which was $CD3^+$ $CD4^+$ $CD8^-$.

Isolation and immunologic studies of SDS-PAGE-separated proteins from the MLP fraction. MLP proteins were recov-

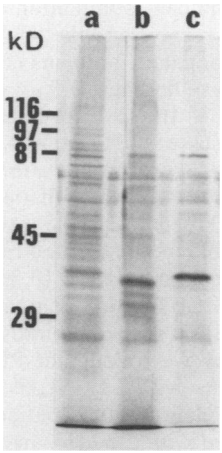

FIG. 1. SDS-PAGE analysis of *M. leprae*. *M. leprae* protein MLP and SS were prepared as described in Materials and Methods. Protein samples (10 μg) were subjected to SDS–10% PAGE under reducing conditions followed by silver staining of protein bands. kD, Molecular size markers (β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; transferrin, 81 kDa; ovalbumin, 45 kDa; carbonic anhydrase 29 kDa). Lanes: a, SS; b, control protein from liver cells obtained from uninfected armadillos (see Materials and Methods); c, MLP fraction.

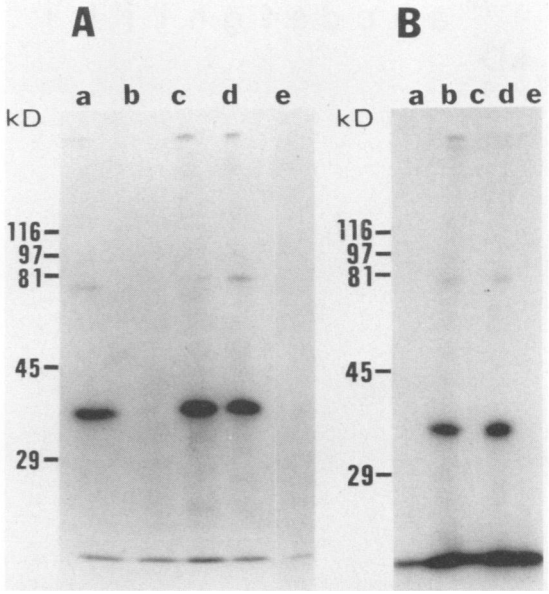

FIG. 2. Analysis of MLP preparation by immunoabsorption. ^{125}I-labeled MLP was mixed with protein A-Sepharose CL-4B which had been precoated with various antibodies. Bound proteins were eluted, analyzed on SDS–10% PAGE, and prepared for autoradiography as described in Materials and Methods. Antibodies used for immunoprecipitation of ^{125}I-labeled MLP in panel A were as follows. Lanes: a, MAb ML03-A_1; b, rabbit anti-*M. bovis* serum; c, rabbit anti-MLP serum; d, pooled sera from patients with LL; e, serum from a healthy control. Those in panel B were as follows. Lanes: a, serum from a healthy control; b, pooled sera from patients with LL; c, serum from a patient with PT; d, MAb ML03-A_1; e, MAb F47-CL9. The molecular size markers used are described in the legend to Fig. 1.

ered from SDS-PAGE gels by the method described by Young and Lamb (39). Briefly, 15 μg of the MLP fraction was fractionated by SDS–10% PAGE. Proteins were then transferred from the gel to nitrocellulose by electroblotting with a Trans-Blot cell apparatus (Bio-Rad). To allow for accurate localization of protein, prestained molecular size markers ranging from 15 to 206 kDa (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used. Each nitrocellulose strip (6 by 35 mm) was divided into 16 equal sections which were tested separately for the ability to stimulate T-cell proliferation. Sections of nitrocellulose strips which contained no protein were added to the control cultures.

RESULTS

Preparation and characterization of MLP. The MLP fraction and SS were prepared from sonicated leprosy bacilli as described in Materials and Methods. From 100 mg of *M. leprae*, approximately 150 μg of the MLP fraction and 5.5 mg of SS were obtained. SDS-PAGE analysis of the MLP fraction (10 μg) under reducing conditions, followed by silver staining, revealed a major band of 35 kDa and several minor bands in the 70- and 12- to 18-kDa ranges (Fig. 1, lane c). These minor bands appeared more prominently in SDS-PAGE of SS together with multiple additional bands dispersed throughout the gel (Fig. 1, lane a). SDS-PAGE of an identically treated sonic extract of armadillo liver cells obtained from uninfected animals (control protein) revealed several bands in the 70-kDa and 18- to 30-kDa ranges; however, it did not reveal a band in the region of 35 kDa (Fig. 1, lane b).

The dominant (35-kDa) protein in the MLP fraction was recognized by MAb ML03-A_1, which is known to react with a 35-kDa protein of *M. leprae* (7, 12, 14) (Fig. 2A, lane a). In addition to the 35-kDa protein, MAb ML03-A_1 reacted with a minor band of 70 kDa. The same 35- and 70-kDa bands were precipitated by rabbit polyclonal antibodies to the MLP fraction and by pooled sera from four patients with LL (Fig. 2A, lanes c and d). By contrast, MAb F47-CL9, which reacts with a 36-kDa molecule of *M. leprae* (12; Engers et al., Letter), did not precipitate the 35-kDa protein (Fig. 2B, lane e). Similarly, neither rabbit anti-*M. bovis* polyclonal antibodies nor serum from a healthy control reacted with the 35-kDa protein (Fig. 2A, lanes b and e).

Figure 3 shows the results of immunoabsorption of the ^{125}I-labeled MLP fraction with individual sera from a panel of patients. Only one of four sera from patients with BTL (lanes d to g) reacted weakly with the 35-kDa protein (lane f). In addition, serum from this patient revealed a minor band of 70 kDa. By contrast, sera from all four patients with LL reacted with the major 35-kDa protein of the MLP fraction, as well as the antigen corresponding to the 70-kDa protein (Fig. 3, lanes h to k). Sera from the nine patients with PT tested, however, did not react with the MLP fraction. Representative data are shown in Fig. 2B (lane c) and 3 (lane 1).

Responses of T cells to the MLP fraction. To test for the presence of T-cell-stimulating epitopes, the MLP fraction was used in proliferation assays. Initially, we compared the abilities of MLP and whole *M. leprae* cells to stimulate PBL from patients with BTL over a wide dose range. Data from these experiments are shown in Fig. 4. The MLP fraction at concentrations of 0.5 to 1 μg/ml induced proliferation comparable to that elicited by 10 μg of whole *M. leprae* cells per ml in PBL from patients with BTL. Increasing the concentration of MLP to 10 μg/ml resulted in profound reduction of proliferation in two of the three PBL samples tested (Fig. 4A and E). Control protein from armadillo liver cells, however,

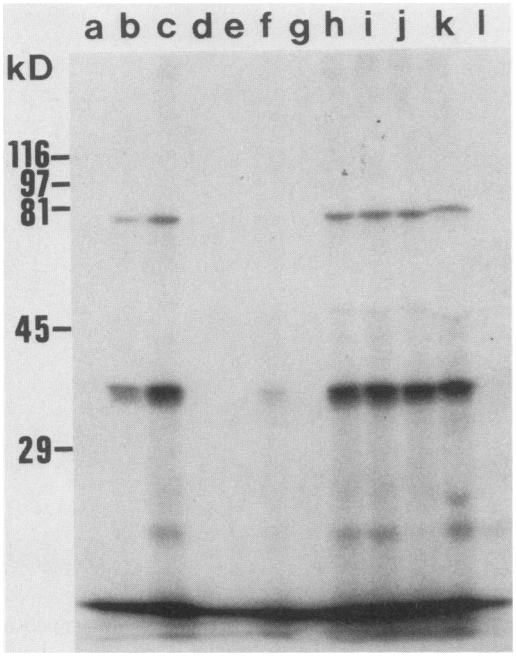

FIG. 3. Ability of sera from patients with leprosy to recognize the MLP fraction. Immunoabsorption of SDS-PAGE analysis of proteins were performed as described in the legend to Fig. 2. The antibodies used for immunoprecipitation of ^{125}I-labeled MLP were as follows. Lanes: a, serum from a healthy control; b, MAb ML03-A_1; c, rabbit anti-MLP serum; d to g, sera from four patients with BTL; h to k, sera from four patients with LL; l, serum from a patient with PT. The molecular size markers used are described in the legend to Fig. 1.

did not produce significant stimulation (SI, ≤2), indicating that the proliferative response to the MLP fraction is not due to contaminating xenogeneic antigens (Fig. 4A, B, and D).

On the basis of these findings, 0.5 μg of the MLP fraction per ml was selected as the test dose for assessment of the proliferative responses of PBL from a larger panel of patients with leprosy. Marked proliferation in response to the MLP fraction was observed in all patients with BTL. The intensity of the T-cell response to 0.5 μg of the MLP fraction per ml was comparable to that elicited by 10 μg of whole *M. leprae* cells per ml (mean SI, 24.8 ± 10.2 versus 19.4 ± 11.3), suggesting that the MLP fraction contains a T-cell immunogen. In a further analysis of the immunostimulatory capacity of the MLP fraction, we found that PBL from *M. leprae* nonresponder patients with LL (SI, ≤2), in contrast to T cells from responder lepromatous patients, failed to proliferate when stimulated with the MLP fraction (Fig. 5). Thus, the profiles of the responses of nonresponder patients with LL to the MLP fraction and intact leprosy bacilli were identical.

To determine whether the MLP fraction possesses T-cell epitopes common to other mycobacteria, the ability of T cells from patients with PT and BCG-vaccinated healthy individuals to proliferate upon stimulation with the MLP fraction was compared with that of T cells from *M. leprae* responder patients. The proliferative responses of patients with PT and BCG-vaccinated individuals to the MLP fraction were similar to those of patients with BTL (Fig. 5). PBL from 5 of 6 patients with PT and 8 of 10 BCG-vaccinated individuals demonstrated significant responses to the MLP fraction (SI range, 4 to 62). In contrast, PBL from six of eight healthy individuals who had no known exposure to *M.*

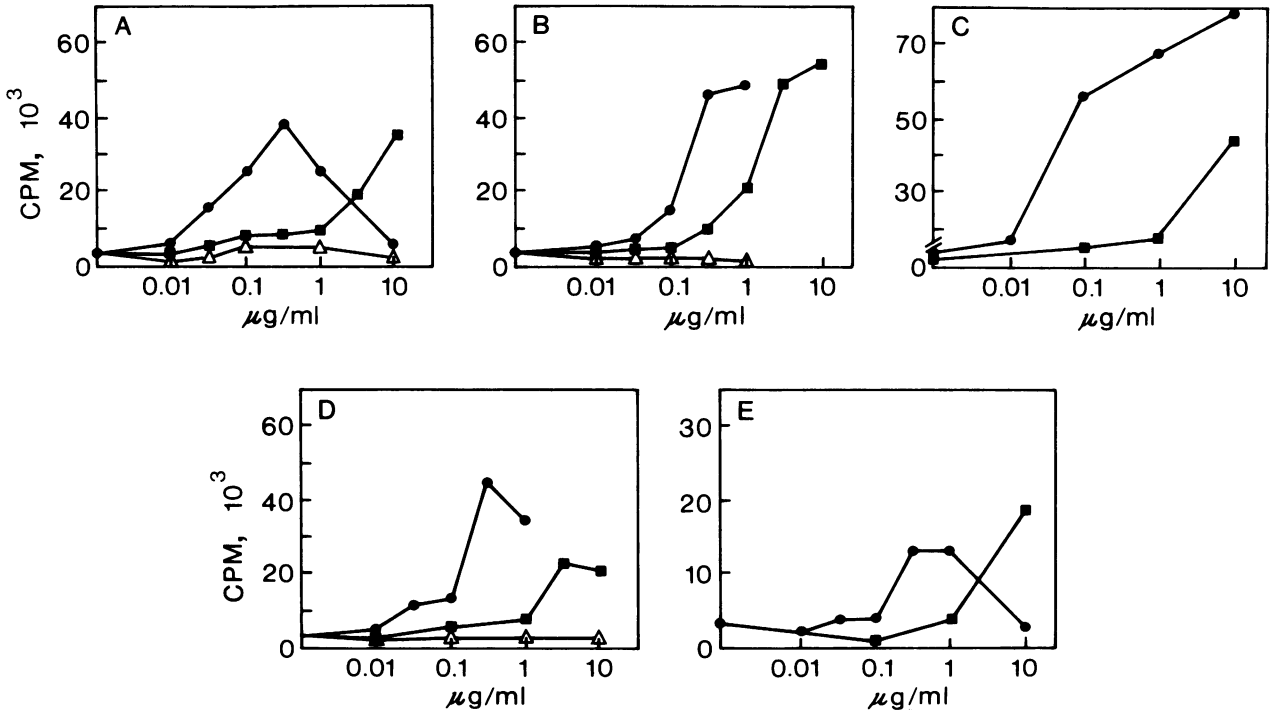

FIG. 4. Ability of the MLP fraction to stimulate PBL from patients with BTL. PBL were isolated from five different patients with BTL (A to E) and cultured for 6 days with various concentrations of the MLP fraction (●), control protein (△), or whole *M. leprae* cells (■). Proliferation was measured by [^{3}H]thymidine incorporation on day 6 of culture. Each point represents the mean counts per minute of four replicate cultures. The standard error of the mean was <10% in all cultures.

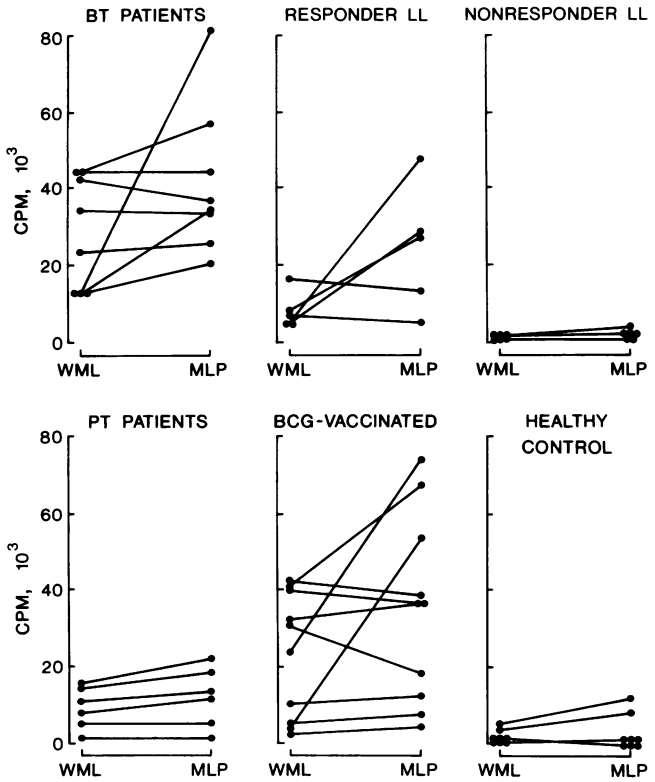

FIG. 5. Stimulation of PBL with the MLP fraction. PBL from 9 patients with BTL, 11 with LL (including 6 *M. leprae* nonresponders and 5 responders), 6 with PT, 10 healthy BCG-vaccinated individuals, and 8 unvaccinated healthy individuals with no known exposure to mycobacteria were stimulated with 0.5 μg of the MLP fraction per ml or 10 μg of whole *M. leprae* cells (WML) per ml. [^{3}H]thymidine incorporation was measured 6 days after stimulation. Results are expressed as mean counts per minute of antigen-stimulated cultures minus the mean counts per minute of equivalent control cultures without antigen. The standard error of the mean was <10% in all cultures.

leprae or BCG did not respond to the MLP fraction or *M. leprae* by proliferation (SI, ≤2) (Fig. 5). PBL from the two healthy blood donors that responded to the MLP fraction and whole *M. leprae* cells by proliferation (Fig. 5) were also responsive to *M. tuberculosis* (data not shown). Taken together, these findings indicate that the MLP fraction is devoid of nonspecific mitogenic activity and an epitope(s) associated with a protein(s) contained in the MLP fraction is recognized by T cells of *M. leprae* responder patients with leprosy, patients with PT, and healthy individuals who respond to mycobacterial antigens.

Next, studies were performed to determine whether a panel of *M. leprae*-reactive CD4$^+$ T-cell clones were capable of responding to the MLP fraction. These clones were generated by stimulating CD4$^+$ T cells of two patients with BTL (patients 1 and 2) and one patient with LL (patient 3) with sonicated *M. leprae* in the presence of autologous or human lymphocyte antigen-identical APC. Of 11 *M. leprae*-reactive clones, 5 responded to the MLP fraction (SI range, 7 to 22), and in all 5 cases, the responses to the MLP fraction were stronger than the responses of the same clones to whole *M. leprae* cells (Table 1). When these 11 *M. leprae*-reactive clones were tested against recombinant *M. leprae* proteins of 12, 18, and 65 kDa, only one clone (1C) was

TABLE 1. Proliferative responses of *M. leprae*-reactive CD4$^+$ T-cell clones to the MLP fraction[a]

Clone[b]	Mean cpm of [^{3}H]thymidine incorporated by CD4$^+$ T-cell clones		
	No stimulation	Stimulated with the MLP fraction	Stimulated with whole *M. leprae* cells
BTL			
1A	2,164	24,969 (12[c])	20,827 (10)
1B	3,084	6,179	8,906 (3)
1C	2,251	5,002	9,087 (4)
1D	1,273	14,892 (12)	12,798 (10)
1E	2,151	3,107	6,474 (3)
BTL			
2A	1,410	18,750 (13)	6,871 (5)
2B	1,751	2,300	23,752 (14)
2C	4,248	3,084	13,569 (3)
LL			
3A	828	5,603 (7)	3,160 (4)
3B	1,382	29,889 (22)	6,092 (4)
3C	1,105	2,296	6,535 (6)

[a] [^{3}H]thymidine incorporation was measured 72 h after stimulation with 1 μg of the MLP fraction per ml or 10 μg of whole *M. leprae* cells per ml. The results shown are for triplicate cultures. The standard error of the mean was <10% for all cultures.
[b] Clones from two patients with BTL and one with LL were tested.
[c] The stimulation index is shown in parentheses.

stimulated with the 18-kDa protein (SI, 3.4) and one clone (3C) was stimulated with the 65-kDa protein (SI, 7) (data not shown). However, none of the clones which responded to the MLP fraction showed reactivity to these *M. leprae* recombinant peptides.

The possibility existed that a protein(s) other than the 35-kDa polypeptide present in the MLP preparation was responsible for the observed T-cell stimulation. To address this question, MLP fraction proteins separated from one another by size with SDS-PAGE were examined individually for the ability to stimulate T-cell responses with *M. leprae*-reactive subjects. While the overall responses of PBL to whole *M. leprae* antigens in cultures containing nitrocellulose membranes was reduced, the highest responses (SI, 18.6 and 6.3) and the only response with an SI of >3 were observed in the wells containing the 35-kDa protein (Fig. 6). The possibility exists that additional proteins present in the MLP preparation are capable of eliciting a T-cell response but failed to do so because they could not be recovered in sufficient quantity from SDS-PAGE. Even if such proteins were present in our preparation, however, the analysis of T-cell-stimulating activity recoverable from SDS-PAGE suggests that the 35-kDa protein present in the MLP fraction is a potent T-cell immunogen.

To address this question further, the 35-kDa protein purified to homogeneity from *M. leprae* bacilli by P. J. Brennan was tested for its ability to react with MAb ML03-A_1 and activate T cells from patients with BTL. MAb ML03-A_1 precipitated the purified 35-kDa protein (Fig. 7, lane b) and the 35-kDa protein contained in the MLP fraction (lane d). Similarly, rabbit anti-MLP polyclonal antibodies reacted with the purified 35-kDa protein (data not shown). Subsequently, the abilities of T cells from MLP-responsive individuals to proliferate upon stimulation with the purified 35-kDa protein were assessed. PBL from eight patients with BTL and healthy BCG-vaccinated individuals demonstrated significant responses to the 35-kDa protein (SI range, 3 to 38)

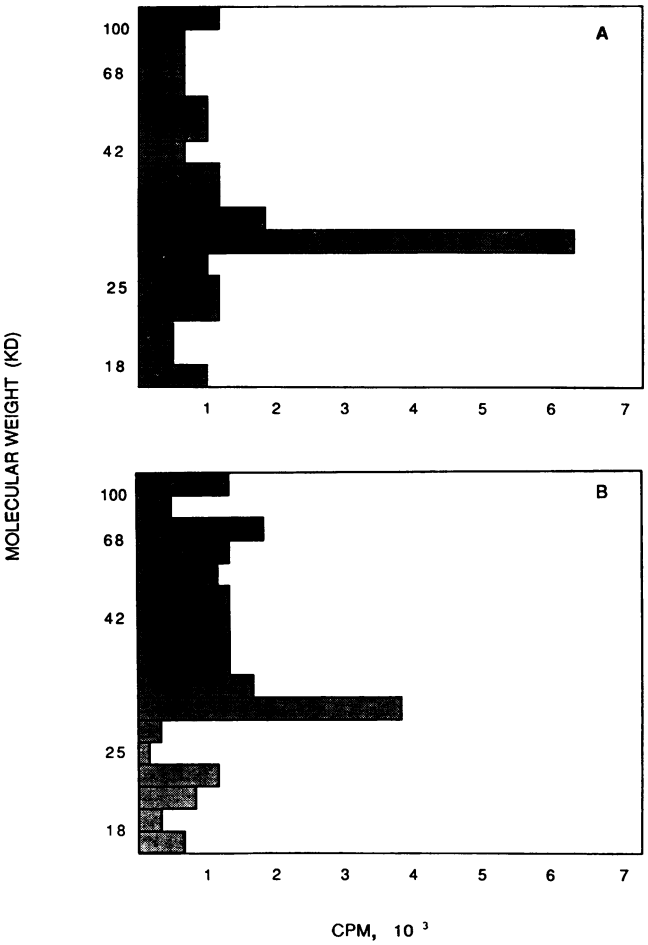

FIG. 6. Stimulation of PBL with proteins recovered from MLP preparation by SDS-PAGE. PBL from two patients with BTL were cultured for 6 days with SDS-PAGE-separated, nitrocellulose-bound fractions (16 fractions) of the MLP preparation. Proliferation was measured as described in Materials and Methods. Localization of proteins was determined by using prestained molecular size markers. The response of PBL from patient A to 10 μg whole *M. leprae* cells per ml in the presence of nitrocellulose was 7,363 ± 1,088 cpm, and the response of PBL from patient B was 22,995 ± 793 cpm; the background uptakes were 1,150 and 600 cpm, respectively.

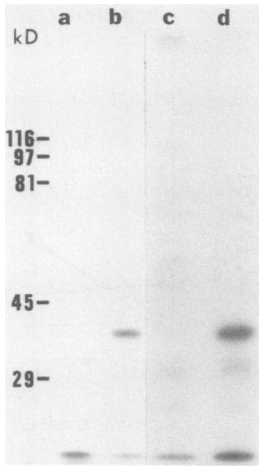

FIG. 7. Ability of the purified 35-kDa protein to react with MAb ML03-A_1. Immunoabsorption and SDS-PAGE analysis of proteins were performed as described in the legend to Fig. 2. The proteins used were the purified 35-kDa protein (lanes a and b) and the MLP fraction (lanes c and d). The antibodies used for immunoprecipitation of ^{125}I-labeled proteins were MAb ML03-A_1 (lanes b and d) and serum from a healthy control (lanes a and c). The molecular size markers used are described in the legend to Fig. 1.

(Fig. 8). The difference in the magnitudes of the responses to the purified 35-kDa protein and the MLP fraction might be due to the presence of other proteins in the MLP fraction. Alternatively, subjecting the 35-kDa protein to purification procedures might have resulted in partial denaturation and reduced T-cell-stimulating activity. Indeed, responses of PBL to a wide dose range (0.2 to 2 μg/ml) of the purified 35-kDa protein did not differ significantly (SI range, 8 to 14). Reduced response to the 35-kDa protein was not due to cell death as assessed by acridine orange-ethidium bromide.

DISCUSSION

Protective immunity to mycobacteria is believed to be mediated by antigen-specific T cells rather than antibodies (1). Therefore, *M. leprae* proteins are essential probes for dissecting the T-cell responses of patients with leprosy. Using a relatively gentle extraction procedure, we isolated the native proteins from the pellet fraction of sonicated leprosy bacilli. This preparation, designated MLP, contains a major protein with a molecular mass of 35 kDa on the basis of SDS-PAGE analysis (Fig. 1). In studies of sera from patients with LL, who characteristically have high levels of circulating anti-*M. leprae* antibodies, the 35-kDa protein was

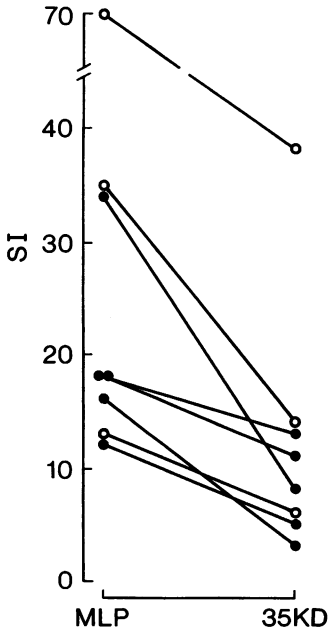

FIG. 8. Ability of the purified 35-kDa protein to stimulate PBL from MLP-responsive individuals. PBL from five patients with BTL (●) and three BCG-vaccinated individuals (○) were stimulated with 1.0 μg of the 35-kDa protein or the MLP preparation. [^{3}H]thymidine incorporation was measured on day 6 of culture. Results are expressed as the SI. The mean counts per minute of cultures without antigen ranged from 477 to 2,401.

found to be a strong B-cell immunogen in the *M. leprae* MLP preparation. PBL and cloned *M. leprae*-reactive $CD4^+$ T cells from patients with leprosy responded vigorously to MLP, even at concentrations as low as 0.1 μg/ml, indicating that the MLP preparation contains one or more strong T-cell immunogens. The MLP fraction also elicited a strong proliferative response in lymphocytes from patients with PT and healthy individuals vaccinated with BCG, indicating that common or cross-reactive mycobacterial determinants are present in MLP.

We considered the possibility that a protein other than the 35-kDa polypeptide present in the MLP preparation was responsible for the observed T-cell stimulation. Initial studies designed to address this question showed that within the MLP preparation, a protein(s) of approximately 35 kDa was the only detectable T-cell stimulus (Fig. 6). In a final series of experiments, the 35-kDa protein purified to homogeneity from leprosy bacilli was examined for its ability to stimulate T-cell responses in MLP-reactive subjects. The results showed that the purified protein was recognized by MAb ML03-A_1 (Fig. 7), which is known to react with a 35-kDa protein of *M. leprae* (7, 12, 14) and the MLP fraction and that this protein stimulates T-cell responses (Fig. 8). We have not ruled out the possibility that lipopolysaccharide comigrating with the 35-kDa protein contributes to the observed T-cell response to the MLP fraction. However, other investigators (5, 6, 17, 33), as well as our group (Mohagheghpour, unpublished data), have failed to detect any T-cell responses to mycobacterium-derived lipopolysaccharides, including lipoarabinomannan B of *M. leprae*. Moreover, previous studies have demonstrated that lipoarabinomannan B nonspecifically inhibits T-cell reactions (6, 17).

Antibody competition studies by Mwatha et al. (28) confirm our findings that the 35-kDa protein is a strong B-cell immunogen. Using MAb ML04, which binds to an epitope expressed by the 35-kDa protein of *M. leprae* and has paratope specificities similar to those of ML03 (15), those investigators found that essentially all patients with LL but no healthy subjects from the leprosy-endemic area produced high levels of antibodies to the 35-kDa protein. Although the 35-kDa protein in MLP is a potent T-cell immunostimulant, other *M. leprae*-derived proteins have been reported to stimulate T cells. For example, Mustafa and co-workers demonstrated that *M. leprae*-reactive T-cell clones derived from volunteers vaccinated with *M. leprae* responded to the recombinant *M. leprae* 18-kDa protein expressed in *E. coli* (27). Ottenhoff et al. (31) have reported that 12- and 36-kDa proteins prepared from *M. leprae* bacilli by fast liquid chromatography stimulated *M. leprae*-reactive T-cell clones derived from the PBL of a single patient with BTL. Klatser et al. (19) reported that 5 of 12 *M. leprae*-reactive clones derived from the PBL of an untreated patient with BTL responded by proliferation to a 36-kDa protein isolated from sonicated *M. leprae*. However, most of the clones reactive with the 36-kDa protein also responded to the 64-kDa protein of *M. leprae*. Recently, Mehra et al. (22) have demonstrated that a number of T-cell lines generated against the cell wall preparations of leprosy bacilli responded to *M. leprae* 7-, 16-, and 28-kDa proteins separated from one another by SDS-PAGE.

The failure of T cells from most patients with LL to respond to whole *M. leprae* cells (2, 11, 26), is thought to be a major contributing factor both to the massive accumulation of *M. leprae* organisms in the skin of patients with LL and to the aggressive clinical course which characterizes these patients. Therefore, our finding that the same patients whose T cells failed to respond to whole *M. leprae* cells also failed to respond to the MLP fraction or the purified 35-kDa protein (data not shown) is of particular interest, since this suggests not only that the 35-kDa protein is an immunostimulatory component of *M. leprae* but also that it is one of potential clinical relevance. Although the precise explanation for the T-cell defect in LL is unknown, we have shown previously that culture of *M. leprae*-unresponsive T cells from patients with LL for 24 to 48 h in medium lacking *M. leprae* antigens reverses the T-cell defect in a number of patients with LL (25). Furthermore, reversal of the defect was prevented by addition of *M. leprae* during the first 48 h of culture, suggesting that *M. leprae* antigens play a direct role in either induction or maintenance of T-cell unresponsiveness in LL. Kingston and Colston (18) have demonstrated that *M. leprae* produces concentration-dependent bimodal effects in cultures of *M. leprae*-responsive T cells. Proliferation by *M. leprae*-responsive T-helper cell clones and interleukin 2 production by *M. leprae*-immune T-cell hybridomas were reduced at high concentrations of *M. leprae* antigens. It will be of interest, therefore, to determine whether the *M. leprae* 35-kDa protein prevents recovery of T-cell responses to whole *M. leprae* cells in vitro.

In addition to serving as a probe in studies of the mechanism of *M. leprae*-specific T-cell anergy, the immunogenicity of the 35-kDa protein observed in convalescent patients with BTL suggests that this protein may be a useful component in a vaccine designed to provide protection against infection with leprosy bacilli. The fact that this antigen is recognized by the T cells of BCG-vaccinated individuals, as well as patients with leprosy, does not necessarily detract from its potential efficacy as a leprosy vaccine. Indeed, recent evidence suggests that the protective antigens of mycobacteria are those which are shared by other mycobacterial species rather than species-specific antigens (35–37). Studies with a murine model of leprosy support this possibility, as immunization of mice with the MLP fraction protected them from subsequent infection with leprosy bacilli (9).

ACKNOWLEDGMENTS

We thank Sher Pask and Carl Grumet of the Stanford Medical School Blood Center, Palo Alto, Calif., for performing tissue typing.

This work was supported by Public Health Service grant AI22653 from the National Institute of Allergy and Infectious Diseases, and a contract from the Gillis W. Long Hansen's Disease Center. Purification of the 35-kDa protein of *M. leprae* was supported by Public Health Service contract N0 1-AI-52582 (to Colorado State University) from the National Institute of Allergy and Infectious Diseases.

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