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Cellular Immune Responses of Leprosy Contacts to Fractionated *Mycobacterium leprae* Antigens

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Antigens of armadillo-derived *Mycobacterium leprae* sonic extract were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane, and the unstained blot was converted into 20 fractions of antigen-bearing particles. These were tested in cellular proliferation assays, and reproducible results were obtained between batches of fractions. Peripheral blood mononuclear cells from healthy contacts of leprosy patients (presumed to have protective immunity) were tested with the fractions to investigate which antigens they recognized. A small group of tuberculoid leprosy patients were also tested. Both groups showed a wide range of responses. Almost every fraction stimulated proliferation with at least one donor, yet none was clearly immunodominant or inhibitory in either group. Thus, protective immunity did not appear to be associated with proliferation caused by any single fraction.

The spectrum of clinical manifestations found in leprosy indicates the type of vaccine that is required for protection. Patients at the lepromatous pole of this spectrum possess large numbers of *Mycobacterium leprae* in their skin despite increased levels of antibody. However, they lack cellular immunity to the bacillus. At the tuberculoid pole, clearance of *M. leprae* from the tissues is associated with a strong cellular response to the bacillus (3). These observations demonstrate that the ability to restrict the growth of *M. leprae* is mediated by cellular rather than humoral immunity. Therefore, to produce a rational vaccine against leprosy we need to identify the antigens involved in this immunity.

Although tuberculoid patients display a strong cellular response to the bacillus, this often leads to neuropathy (3). Thus, rather than develop a vaccine that might induce such damage, we have studied the cellular responses of long-term contacts of leprosy patients. Contacts appear to be infected with *M. leprae* (1, 10) but presumably mount a protective and nonpathogenic immune response such that the infection remains subclinical.

M. leprae antigens recognized by antibodies have been identified by Western (immuno-) blotting (using sera to probe nitrocellulose-bound antigens separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (6, 9, 15, 23). Recently, a technique was described that again uses the high resolving power of sodium dodecyl sulfate-polyacrylamide gel electrophoresis to separate antigens but probes the T-cell response (2, 25). We have assessed this technique and have applied it to identify *M. leprae* antigens recognized by T cells from leprosy contacts and tuberculoid leprosy patients.

MATERIALS AND METHODS

Preparation of fractionated antigen bound to nitrocellulose particles. An armadillo-derived *M. leprae* sonic extract (batch CD99) (prepared as described in the Report on the Fifth Meeting of the Scientific Working Group on the Immunology of Leprosy [Immlep], World Health Organization document TDR/IMM-LEP-SWG [5] 80.3 annex 4, p. 23, 1980) was kindly provided by R. J. W. Rees (Clinical Research Centre, Harrow, United Kingdom). A 300-μg sample of this sonic extract was boiled for 5 min in the presence of sodium dodecyl sulfate and separated on a 1.5-mm-thick 16% preparative mini-gel (monomer-dimer ratio, 44:0.8) (Hoeffer Scientific Instruments, San Francisco, Calif.) (17). Electrophoresis was carried out at a constant current of 10 mA until the dye front had travelled 4.5 cm into the separating gel. Molecular mass markers were run alongside the antigen. Proteins were electroblotted onto nitrocellulose membranes (0.45-μm pore size; Schleicher & Schuell GmbH, Dassel, Federal Republic of Germany) (21) with a constant voltage of 50 V for 1 h (batches V and VI) or 15 V for 16 h (batches VII and IX).

The membrane was cut with a clean scalpel as indicated in Fig. 1. The distribution of the *M. leprae* proteins and the positions of the molecular mass markers were identified by briefly staining the edges with 0.1% amido black and then washing in distilled water. A 1-mm-wide strip cut down the center of the blot was also stained to give an indication of the degree of "smiling" that had occurred during electrophoresis. By using two vertical strips (2 mm wide) the positions of the six cloned *M. leprae* protein antigens (of molecular masses 70, 65, 36, 28, 18, and 12 kilodaltons [kDa]) were identified with murine monoclonal antibodies (L7, L12, F47-9, SA-1 B11H, L5, and MLO6, respectively) (5, 11, 16, 24). Another vertical strip (3 mm by 4 cm) was cut from the center of the blot to be used as a positive control. The remaining nitrocellulose onto which *M. leprae* antigens had been blotted was divided into 20 horizontal strips (2 mm by 6 cm). Two fractions of nitrocellulose (2 mm by 6 cm) taken from a region of the blot devoid of protein acted as negative controls. (Note that all test and control fractions contained the same amount of nitrocellulose). The molecular masses of proteins contained within each fraction were estimated from the positions of the molecular mass markers (taking into account the curvature of the protein bands), and the positions of the cloned proteins were identified by the monoclonal antibodies (Fig. 1).

The 20 test fractions, the positive control, and both

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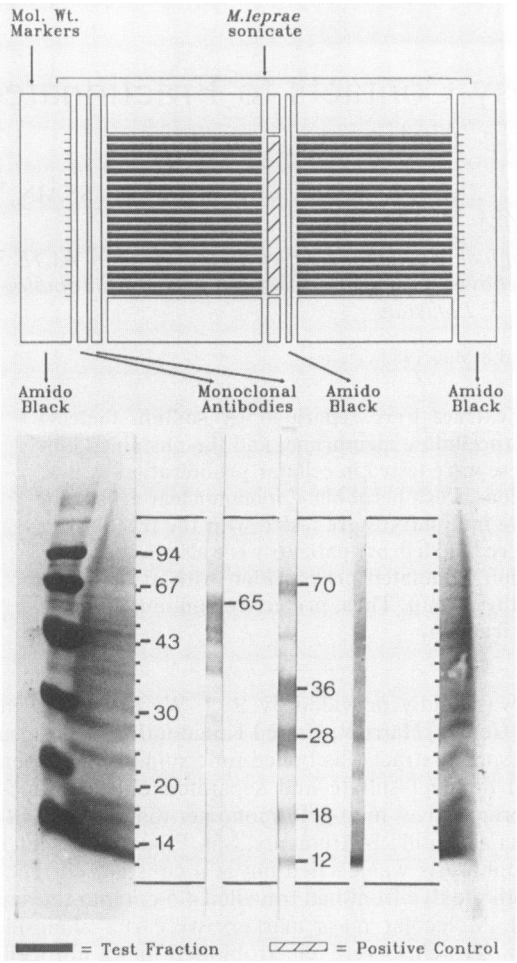

FIG. 1. *M. leprae* sonic extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a preparative gel and blotted onto a nitrocellulose membrane, and the membrane was divided up as shown. The test fractions and a positive control were converted into antigen-bearing particles, as described in the text. Staining (with 0.1% amido black) of strips taken from the center and both the right- and left-hand sides of the membrane revealed the distribution of *M. leprae* antigens and the positions of the molecular mass markers (in kilodaltons) relative to the test fractions. The positions of the cloned *M. leprae* proteins were identified with monoclonal antibodies. The photographs are of batch IX.

negative controls were converted into fine antigen-bearing particles (2) by dissolving them in 3.5 ml of dimethyl sulfoxide (Sigma Chemical Co., Poole, England) and reprecipitating them with an equal volume of carbonate-bicarbonate buffer (50 mM, pH 9.6). The buffer was added dropwise at a constant rate (0.8 ml/min), using a peristaltic pump, while the fraction dissolved in dimethyl sulfoxide was continually mixed with a magnetic glass stirrer.

The particles were washed three times with RPMI 1640 (GIBCO Ltd., Paisley, Scotland) containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer–100 IU of penicillin per ml–100 μg of streptomycin per ml by centrifuging at 9,000 × *g* for 10 min. The pellet was resuspended by carefully aspirating it through a 26-gauge needle. The particles were finally suspended in 1 ml of RPMI 1640 with penicillin and streptomycin, divided into aliquots, and stored at −70°C.

Proliferation assays. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood on Ficoll Histopaque 1077 (Sigma) and suspended in growth medium (RPMI 1640 containing 10% autologous plasma, 2 mM L-glutamine, 25 mM HEPES buffer, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml). A total of 2×10^5 cells per well were cultured with antigen in 96-well round-bottom microdilution plates (Nunc, Roskilde, Denmark) for 5 to 6 days at 37°C in humidified air with 5% CO_2. The cells were then pulsed (1 μCi per well) with [^{3}H]methyl thymidine (Amersham International plc, Amersham, United Kingdom) (specific activity, 5 Ci/mmol). Approximately 16 h later, they were harvested onto glass fiber filter strips, and [^{3}H]thymidine incorporation was measured by liquid scintillation spectroscopy. PBMC from donors C18, C22, C69, and LP71 were stored frozen in liquid nitrogen before being tested.

As a test of cellular viability, all donors were tested in proliferation assays with phytohemagglutinin (PHA-P; Wellcome Diagnostics Ltd., Beckenham, Kent, United Kingdom) at 1 μg/ml. They were also tested with tuberculin-purified protein derivative (Statens Seruminstitut, Copenhagen, Denmark) at 10 μg/ml.

Proliferative results are expressed as kilocounts per minute in Fig. 2, with background responses to nitrocellulose alone shown. To compare profiles of different donors, the results are expressed as stimulation indices (see Fig. 3, 4, and 5). A stimulation index of 2.0 to 3.9 was considered a weak response, 4.0 to 5.9 was considered a moderate response, and >6.0 was considered a strong response. The mean background response of contacts and patients to nitrocellulose alone was 4,740 cpm (frozen cells gave lower backgrounds than fresh cells).

Donors. To assess the use of the fractionated antigens in proliferation assays, blood was taken from a healthy *Mycobacterium bovis* BCG-vaccinated donor in the United Kingdom and a leprosy contact from India, now resident in the United Kingdom.

(i) Leprosy contacts. The leprosy contacts were 13 staff members (10 male, 3 female; mean age, 30 years) at the Marie Adelaide Leprosy Centre, Karachi, Pakistan, who had been in regular contact with patients for between 1 and 21 years (mean, 9 years). The only contacts who had received BCG vaccination were C38, C62, C18, and C57.

(ii) Leprosy patients. The leprosy patients were 5 tuberculoid patients (two male, three female; mean age, 52 years) who attended the same center in Karachi. All were untreated except for LP71, who had received dapsone for the previous 10 months. LP71 and LP52 had not received BCG vaccination. The BCG vaccination status of the other three patients was not known.

Statistics. Results were compared by using the Student *t* test and were considered significant when *P* was less than or equal to 0.01.

RESULTS

To assess the reproducibility of results obtained with this technique, a healthy BCG-vaccinated donor in the United Kingdom was tested on separate occasions with three different batches of fractions (Fig. 2). The profiles of responses obtained indicate that results are reproducible between batches. This reproducibility was also found when using the same batch with cells from the same donor tested repeatedly (correlation coefficient, 0.92) (data not shown).

The fractions were tested at a range of dilutions with PBMC from a leprosy contact now resident in the United

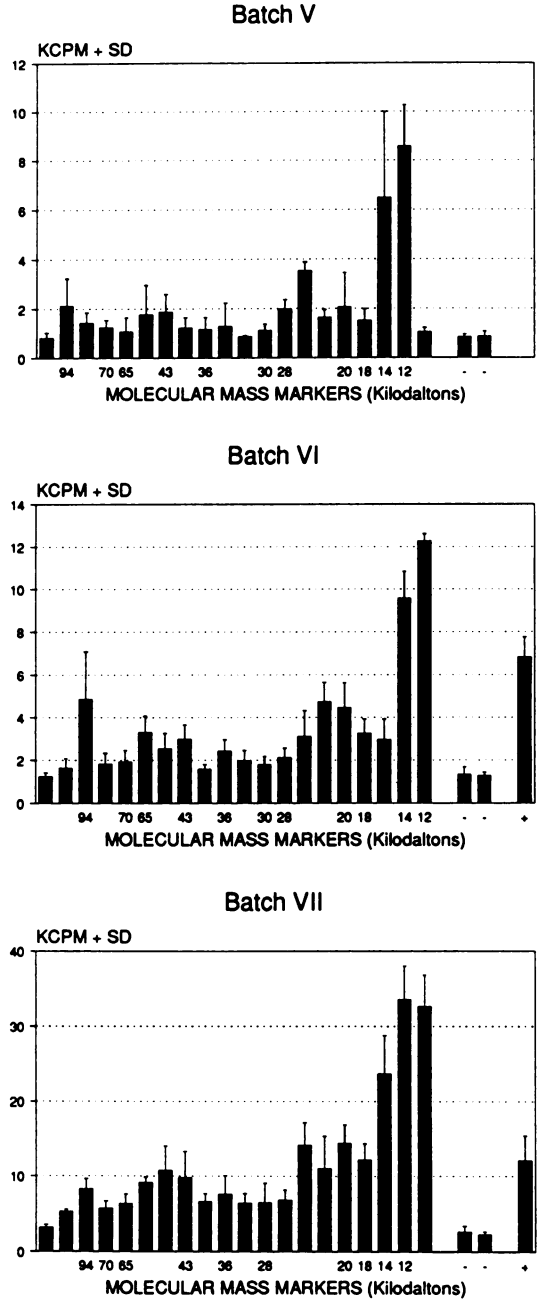

FIG. 2. Proliferative responses of PBMC from a healthy BCG-vaccinated donor in the United Kingdom to three different batches of fractionated *M. leprae* antigens. The antigen-bearing nitrocellulose particles were used at a 1/100 dilution. Values represent the mean kilocounts per minute (kcpm) plus standard deviation (SD) of triplicate cultures. Negative controls (−) (background response to nitrocellulose alone) and positive controls (+) (response to unfractionated *M. leprae* bound to nitrocellulose; batches VI and VII only) are indicated.

Kingdom (Fig. 3). As expected, diluting the fractions reduced the cellular response, but the shape of the profile remained largely unaltered. Repeating this experiment with PBMC from the BCG-vaccinated United Kingdom donor mentioned above yielded the same results (data not shown). A 1/100 dilution appeared to be optimal, yet responses were found even to some fractions diluted 1/10,000. The nitrocel-

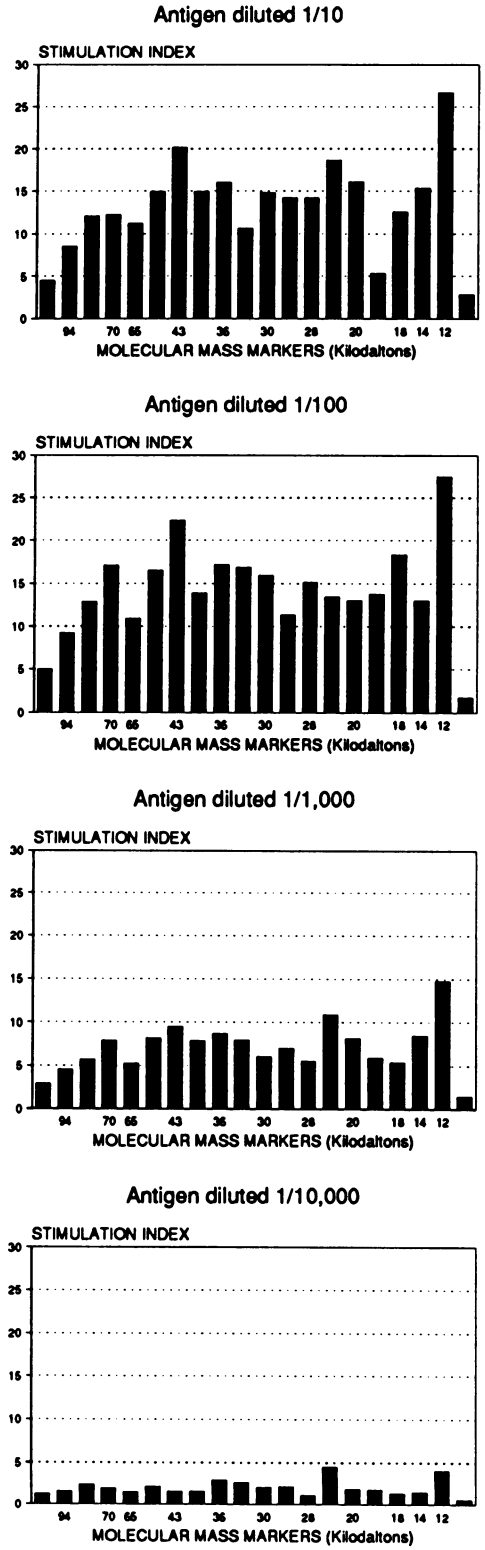

FIG. 3. Proliferative responses of PBMC from a leprosy contact to fractionated *M. leprae* antigens (batch V) tested over a range of dilutions. Stimulation index = (mean response to test fraction [n = 3])/(mean response to negative controls [n = 6]).

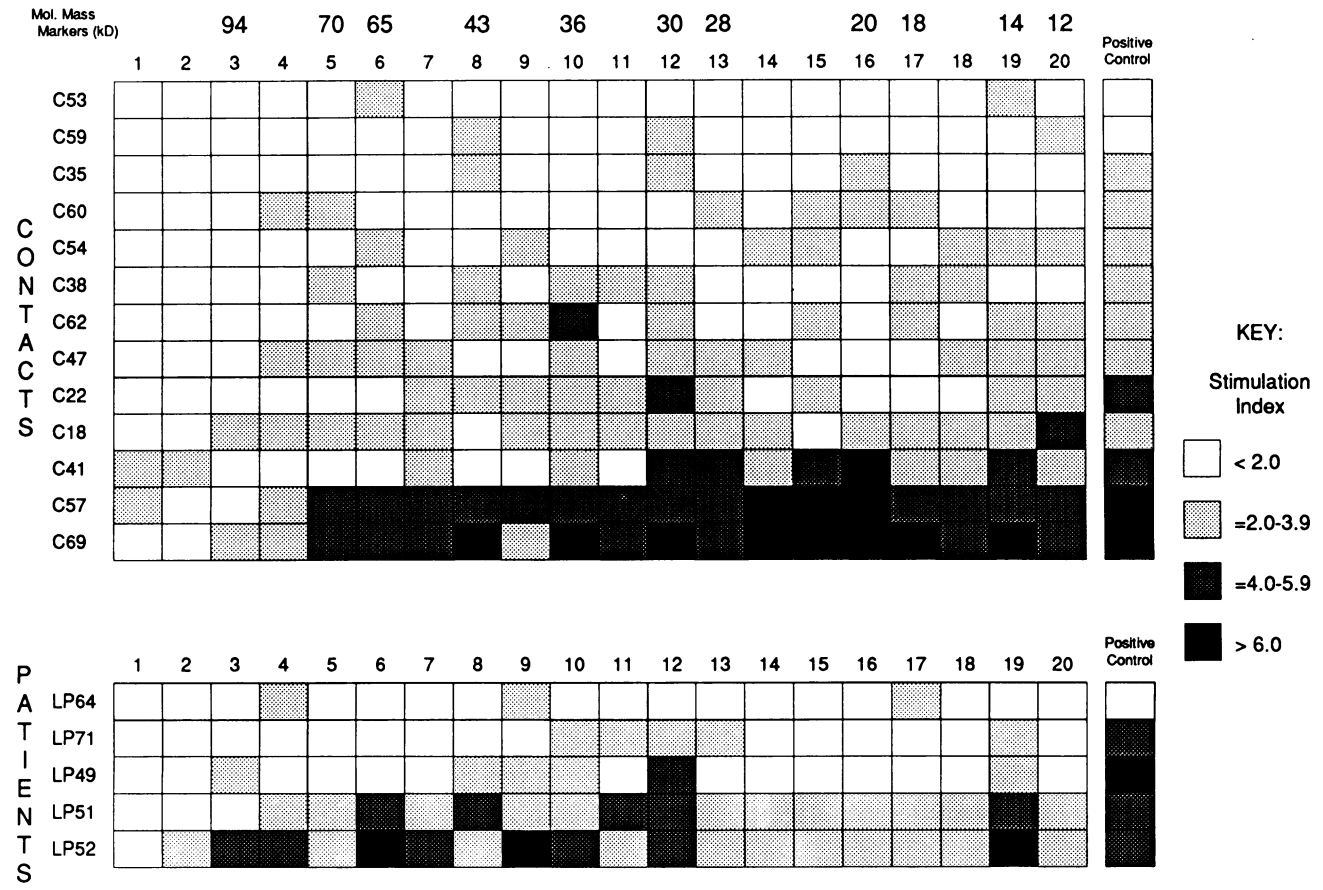

FIG. 4. Proliferative responses of PBMC from leprosy contacts and tuberculoid leprosy patients to fractionated *M. leprae* antigens (batch VI). The positive control was unfractionated *M. leprae* bound to nitrocellulose. The stimulation index was calculated as described in the legend to Fig. 3.

lulose particles themselves had no mitogenic effect on the cells, but when used at a 1/10 dilution, they reduced the response to below background levels. With some donors, this inhibition was also found at a 1/100 dilution.

The resolution of individual proteins was confirmed by this technique, using *M. leprae*-reactive human T-cell lines, one of which produced two clear peaks of response to antigens of approximately 20 and 14 kDa (S. P. Lee, H. M. Dockrell, N. G. Stoker, and K. P. W. J. McAdam, manuscript in preparation), and another two lines peaked at approximately 18 kDa (8).

The cellular responses of 13 contacts and 5 patients were analyzed by using these fractions (batch VI), and the results are summarized in Fig. 4. A wide variety of profiles was seen, with every fraction causing proliferation with at least one donor. Some donors were capable of responding to virtually all the fractions (e.g., C57 and LP52), whereas others had a more selective response (e.g., LP49). No test fraction caused significant inhibition of the response below background levels ($P > 0.05$). The duration of contact, age, or sex of the donor did not appear to correlate with the pattern or intensity of the profile. All donors showed a strong response (stimulation index >6.0) to tuberculin-purified protein derivative. The mean stimulation index of PBMC from contacts and patients to each of the fractions is shown in Fig. 5. With contacts, the relatively flat profile and large standard deviations indicated that no fraction was immunodominant. With patients, there was a suggestion of immunodominance with fractions 12 and 19 (which contained proteins of approximately 30 and 14 kDa), although again the standard deviations were high.

DISCUSSION

Presenting nitrocellulose-bound antigens to T cells appears to be more efficient when the nitrocellulose is converted to particles small enough to allow uptake by macrophages (2). The technique used to reprecipitate the nitrocellulose dissolved in dimethyl sulfoxide is therefore important. Using a peristaltic pump and magnetic stirrer means that the carbonate-bicarbonate buffer is added at a slow, controlled rate and is immediately mixed with the dimethyl sulfoxide, thus producing a fine suspension of particles. It is crucial that the production of each fraction is consistent, and such automation will ensure this. This consistency no doubt contributed to the reproducibility of results obtained with different batches of fractionated antigen. When producing these batches, we also avoided the need to stain the blot, thus eliminating any possible effects that staining may have on the cellular response to the antigens.

Testing the fractions at a range of dilutions showed that cells proliferated even when the particle suspensions were diluted 1/10,000. This confirms the increased efficiency of presenting antigen bound to fine nitrocellulose particles. To give a rough estimate of the protein levels present at this

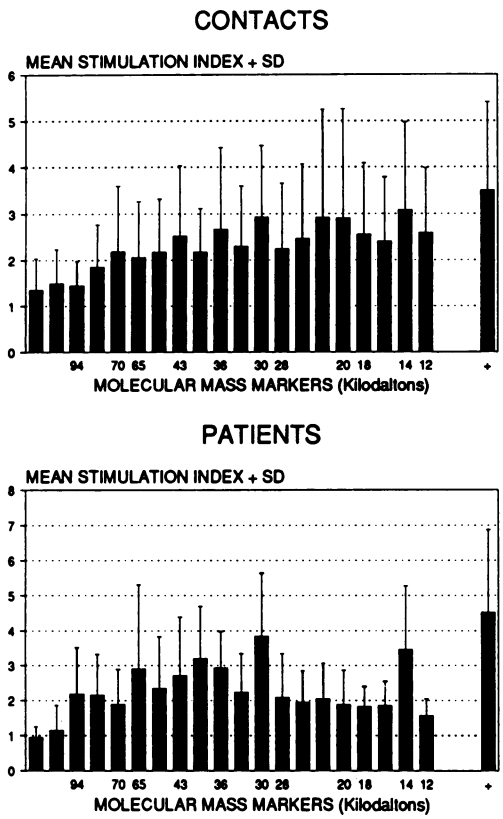

FIG. 5. The mean stimulation index (plus standard deviation [SD]) of PBMC from leprosy contacts (n = 13) and tuberculoid leprosy patients (n = 5) to fractionated *M. leprae* antigens. The stimulation index was calculated as described in the legend to Fig. 3. The positive control (response to unfractionated *M. leprae* antigen bound to nitrocellulose particles) is indicated (+).

dilution, if all of the *M. leprae* sonic extract loaded on the gel was evenly distributed among the 20 test fractions, at this dilution cells are responding to approximately 1.5 ng of total protein. The optimum dilution was considered to be 1/100, and thus from a single batch of these fractions, which requires 300 μg of protein, it is possible to test over 100 donors.

Studying the cellular responses of leprosy contacts and patients to these fractionated antigens produced a wide variety of profiles, with no reproducible patterns of response within either group. Comparing the mean stimulation index to each fraction also revealed no significant difference ($P > 0.05$) between these groups (although the number of donors was limited). Among contacts, the strongest responses were found with fractions 12, 15, 16, and 19. Fraction 12 induced a stimulation index of 2 or above with 10 of 13 contacts. Fractions 19 and 20 caused such stimulation with 9 of 13 contacts. However, from the mean stimulation indices to each fraction (Fig. 5), it is clear that there was no immunodominant fraction. The stronger responses to fraction 19 may well reflect the presence of breakdown products from various proteins of higher molecular masses that have accumulated at the bottom of the gel. In a similar study with leprosy contacts in Ethiopia, the strongest cellular responses were to regions of 11 to 16 and 22 to 26 kDa of *M. leprae* proteins (7). However, this apparent immunodominance may be due to the low numbers of contacts tested (n = 5).

Healthy, BCG-vaccinated donors in the United Kingdom, when tested with the fractionated antigens, gave either specific responses to certain fractions (as seen in Fig. 2) or a broad response to nearly all the fractions (data not shown). These donors were presumably responding to antigens in *M. leprae* which cross-react with those of such closely related organisms as *M. bovis* BCG. We are currently investigating the responses of non-BCG-vaccinated individuals. *M. leprae* also possesses proteins which display high levels of amino acid sequence homology with proteins in *Escherichia coli*, to which such donors are likely to have been exposed (26). The leprosy contacts and patients are also likely to have responded to cross-reactive antigens, since all of them responded to tuberculin-purified protein derivative and some of them had been BCG vaccinated.

The 65-kDa protein of *M. leprae* and also the 65-kDa proteins of *Mycobacterium tuberculosis* and *M. bovis* BCG (which are closely homologous to the *M. leprae* protein [20]) appear to be immunodominant for T-cell responses (13, 22). However, such immunodominance was not observed here. This may well be explained by the breakdown of this protein in the sonic extract of *M. leprae*, as revealed by Western blotting (Fig. 1).

Although these findings may discourage attempts to identify individual protective antigens in *M. leprae*, the possibility remains that such antigens exist. For example, not all antigens may be present on the nitrocellulose at an optimal concentration to stimulate T cells, and others may have been destroyed by the fractionating process. The response to a single protein within a fraction may have been masked by the response to other proteins within the same fraction, and donors who responded to the same fraction may not have been responding to the same antigen. Improving the resolution of proteins by using two-dimensional gel electrophoresis may help to overcome some of these problems. It is also possible that cellular proliferation is not the best marker of protection and that studying lymphokine production (e.g., interleukin-2 and gamma interferon) may reveal a different answer.

There was no evidence of inhibition of the T-cell response to below background levels with any of the test fractions. It has been reported that lipoarabinomannan, a component of mycobacteria that migrates on the gel at the 30- to 40-kDa range, acts as a nonspecific inhibitor of T-cell proliferation (12). However, in this system, no such inhibition was observed.

Since a lambda gt11 DNA library of *M. leprae* was constructed (27), a great deal of leprosy research has concentrated on the six proteins subsequently identified. These proteins were detected by using murine monoclonal antibodies and therefore were not necessarily of importance to human cell-mediated immunity. T-cell responses to some of them have now been reported (4, 8, 14, 18, 19), but the results described here, using the fractionated sonic extract, indicate that there are T-cell epitopes distributed throughout the molecular mass range. This highlights the need to study the many other as yet uncloned *M. leprae* antigens.

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