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A 35-Kilodalton Protein Is a Major Target of the Human Immune Response to *Mycobacterium leprae*

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The control of leprosy will be facilitated by the identification of major *Mycobacterium leprae*-specific antigens which mirror the immune response to the organism across the leprosy spectrum. We have investigated the host response to a 35-kDa protein of *M. leprae*. Recombinant 35-kDa protein purified from *Mycobacterium smegmatis* resembled the native antigen in the formation of multimeric complexes and binding by monoclonal antibodies and sera from leprosy patients. These properties were not shared by two forms of 35-kDa protein purified from *Escherichia coli*. The *M. smegmatis*-derived 35-kDa protein stimulated a gamma interferon-secreting T-cell proliferative response in the majority of paucibacillary leprosy patients and healthy contacts of leprosy patients tested. Cellular responses to the protein in patients with multibacillary leprosy were weak or absent, consistent with hyporesponsiveness to *M. leprae* characteristic of this form of the disease. Almost all leprosy patients and contacts recognized the 35-kDa protein by either a T-cell proliferative or an immunoglobulin G antibody response, whereas few tuberculosis patients recognized the antigen. This specificity was confirmed in guinea pigs, with the 35-kDa protein eliciting strong delayed-type hypersensitivity in *M. leprae*-sensitized animals but not in those sensitized with *Mycobacterium tuberculosis* or *Mycobacterium bovis* BCG. Therefore, the *M. leprae* 35-kDa protein appears to be a major and relatively specific target of the human immune response to *M. leprae* and is a potential component of a diagnostic test to detect exposure to leprosy or a vaccine to combat the disease.

Leprosy is a chronic intracellular infection affecting predominantly the skin and peripheral nerves. The clinicopathological spectrum of the disease reflects the host immune response to *Mycobacterium leprae*, the causative agent (4). At the tuberculoid pole of the spectrum, patients mount a strong cell-mediated immune response against antigens of *M. leprae*. This is characterized by a strong type 1-like cytokine pattern, resulting in a reduction of the bacterial load in patient tissues (36). Individuals exposed to leprosy who control the infection show strong cellular reactivity but without immune pathology. At the lepromatous end of the spectrum, patients display disseminated infection, with large numbers of mycobacteria in tissues, high levels of anti-*M. leprae* antibodies, and weak or absent cellular immunity to *M. leprae* (4). Although the introduction of multidrug therapy for the treatment of leprosy has been associated with a reduction in the number of registered patients, an effective leprosy vaccine would complement chemotherapy and help eradicate the disease. Monitoring of the effectiveness of leprosy control will rely on the development of specific diagnostic tests for the early detection of infection with *M. leprae*, as the currently used lepromin and leprosin skin test reagents lack specificity. Such tests will need to distinguish between exposure to leprosy and other antigenically similar mycobacteria, such as *Mycobacterium tuberculosis*. Furthermore, candidate antigens should be recognized by cellular or antibody responses across the leprosy spectrum in order to detect all infected individuals. Although a number of *M. leprae* antigens have been recognized as major targets of the host immune response (15–18), all have closely related homologs in *M. tuberculosis*, making them unlikely candidates as leprosy-specific diagnostic reagents.

The 35-kDa protein of *M. leprae* was initially identified through the binding of *M. leprae*-specific monoclonal antibodies (MAbs) (12, 13). Independently, major membrane protein I of *M. leprae* was purified by biochemical methods and identified as the same 35-kDa protein (11). Serological assays based on a MAb inhibition enzyme-linked immunosorbent assay (ELISA) detected antibodies to the 35-kDa protein in almost all lepromatous leprosy patients (25, 31). The levels of these antibodies correlate strongly with the antigenic load and decline with effective chemotherapy (20, 26). A crude membrane fraction of *M. leprae*, containing predominantly the 35-kDa protein, elicited strong T-cell proliferative responses in tuberculoid leprosy patients, suggesting that the protein was also recognized by the human cellular immune response (19).

Recently, the gene encoding the *M. leprae* 35-kDa protein has been isolated and characterized (35). The limited distribution of the protein (13) was confirmed at the genetic level, as the gene is absent from *M. tuberculosis* and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), the current vaccine used against tuberculosis (TB) and leprosy. In order to explore the cellular reactivity of the protein across the leprosy spectrum, we have purified recombinant 35-kDa proteins from mycobacterial and *Escherichia coli* expression systems and compared the recombinant products. The *Mycobacterium smegmatis*-derived recombinant 35-kDa protein was recognized by the immune response of over 90% of leprosy patients and healthy leprosy contacts. Moreover, the 35-kDa protein elicited specific delayed-type hypersensitivity (DTH) responses in *M. leprae*-sensitized animals, highlighting its potential use in leprosy diagnosis and/or vaccination.

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MATERIALS AND METHODS

Patients. The study population consisted of 63 subjects (47 males and 16 females) aged between 17 and 61. The 31 Nepali leprosy patients included in the study were diagnosed according to the classification of Ridley and Jopling (24). Three tuberculoid leprosy and 15 borderline tuberculoid leprosy patients constituted the paucibacillary (PB) group. Five polar lepromatous, one borderline, and seven borderline lepromatous leprosy patients made up the multibacillary (MB) group. All leprosy patients were either undergoing multidrug therapy or had recently completed treatment. The contact group was made up of three Nepali individuals with household exposure to leprosy and nine health workers from Anandaban Leprosy Hospital. None of the contacts had any clinical signs of leprosy. Of the 10 Nepali TB patients included, 7 had pulmonary and 3 had active nonpulmonary TB. All TB patients were seronegative for the human immunodeficiency virus and were undergoing multidrug therapy. The BCG vaccinee group consisted of 10 Caucasians not exposed to leprosy infection.

Antigens and antibodies. Whole *M. leprae* (batch CD140) cells were kindly provided by R. J. W. Rees through the Immunology of Mycobacteria program of the World Health Organization. *M. leprae* sonicate (MLS) and BCG sonicate were prepared as described previously (6). *M. tuberculosis* purified protein derivative (PPD) was purchased from the Statens Serum Institute, Copenhagen, Denmark. MAbs ML-03 and CS-38 were kind gifts of J. Ivanyi (MRC Unit for Tuberculosis and Related Infections, Hammersmith Hospital, London, United Kingdom) and P. J. Brennan (Department of Microbiology, Colorado State University, Fort Collins), respectively.

Purification of the recombinant *M. leprae* 35-kDa protein from *M. smegmatis* and *E. coli*. Construction of plasmid pWL19, expressing the gene encoding the *M. leprae* 35-kDa protein, has been described previously (35). In order to purify recombinant 35-kDa protein, single *M. smegmatis*/pWL19 colonies were inoculated into 1 liter of Middlebrook 7H9 broth plus ADC (Difco Laboratories, Detroit, Mich.) and incubated for 3 days at 37°C with shaking. Cells were pelleted and resuspended in phosphate-buffered saline (PBS) containing 1% Triton X-100, 10% glycerol, and 1 M NaCl, and the suspension was sonicated four times for 4 min each. Anti-35-kDa-protein MAb ML-03 immunoglobulin G (IgG) was purified from ML-03 ascites by ammonium sulfate precipitation and protein A-Sepharose chromatography and coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). The sonicate was applied to the affinity column at a rate of 0.7 ml/min and recirculated for 3 h. The column was washed with 5 volumes of PBS containing 0.5% Triton X-100, 10% glycerol, and 1 M NaCl, 5 volumes of the initial wash buffer without Triton X-100, and 5 volumes of PBS containing 0.5 M NaCl. Bound protein was eluted with 0.1 M diethylamine (pH 11.0), dialyzed against PBS, and freeze-dried.

Primers JN8 (5′ TAGCTGCAGGGATCCATGACGTCGGCT) and JN9 (5′ CTAGAATTCGAGCTCAAGCTTTCACTTGTACTC) were used to amplify the 35-kDa-protein-encoding gene from plasmid pLJ3 (35). The *Bam*HI-*Pst*I-digested product was cloned into the pQE9 vector (Qiagen Inc., Chatsworth, Calif.) for the generation of histidine-tagged 35-kDa protein. Protein was purified from recombinant *E. coli* MC1061 under denaturing conditions with the use of nickel-nitrilotriacetic acid resin (Qiagen). The protein was resolubilized by dialysis against PBS. In addition, the above PCR product was inserted into the pMAL-c2 vector (New England Biolabs, Beverly, Mass.) for the purification of maltose-binding protein (MBP)-*M. leprae* 35-kDa fusion protein. Soluble MBP–35-kDa protein was purified by use of amylose resin (New England Biolabs). Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with MAb CS-38 performed as described previously (27). The presence of other *M. smegmatis* antigens in the *M. smegmatis*-derived 35-kDa protein was examined in immunoblots with anti-lipoarabinomannan MAb L9 (6), anti-HSP65 MAb L22 (7), and polyclonal antisera to antigen 85B (27).

Molecular mass estimation of the 35-kDa protein was performed by gel filtration using a Superose 6 fast-performance liquid chromatography (FPLC) column (Pharmacia). The column was calibrated with six standard proteins: chymotrypsinogen A (25 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) (Pharmacia).

Lymphocyte proliferation and cytokine assays. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a 1077 Ficoll-Hypaque gradient (Sigma, Sydney, Australia), and 2×10^5 cells were cultured in triplicate in RPMI medium containing 10% heat-inactivated group A serum with antigen at 37°C in 5% CO_2. MLS and the *M. leprae* 35-kDa protein were added at final concentrations of 10 to 0.1 μg/ml. *M. tuberculosis* PPD was used at 10 and 1 μg/ml, and concanavalin A was used at 10 μg/ml. After 5 days, 0.5 μCi of [^{3}H]thymidine (DuPont NEN, North Ryde, Australia) was added to each well, and the cultures were harvested after 16 h. Specific [^{3}H]thymidine incorporation, expressed as counts per minute, was calculated by subtracting the mean counts per minute in control wells (no antigen) from the counts per minute of the test samples. The stimulation index (SI) was the ratio of the mean counts per minute in the test wells divided by the mean counts per minute in the control wells. A response to a particular antigen was considered positive if the net [^{3}H]thymidine incorporation (Δcpm) was >2,000 cpm and the SI was >4.

Gamma interferon (IFN-γ) in the supernatant of 5-day cultures was detected by the *Quanti*FERON MAb ELISA (CSL Limited, Parkville, Australia). The control group consisted of 10 non-leprosy-exposed individuals. An individual with an IFN-γ level of >10.1 IU/ml, the mean of the control group plus 2 standard deviations (SD), was considered positive.

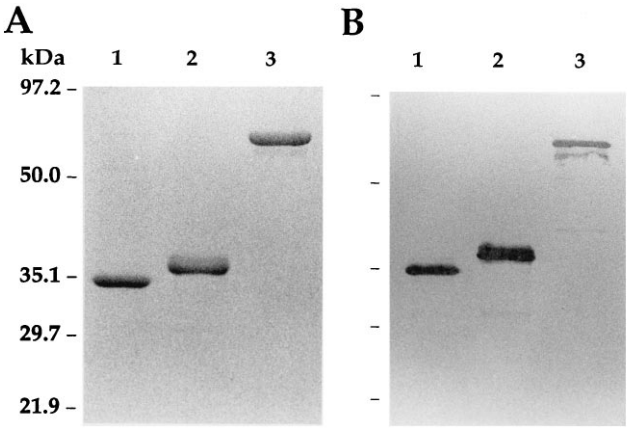

FIG. 1. SDS-PAGE analysis of the *M. leprae* 35-kDa protein purified from *M. smegmatis* and *E. coli*. (A) Coomassie blue-stained SDS-polyacrylamide gel of the three forms of the recombinant 35-kDa protein. (B) Immunoblotting with MAb CS-38. Lanes 1, *M. smegmatis*-derived 35-kDa protein; lanes 2, *E. coli* histidine-tagged 35-kDa protein; lanes 3, *E. coli* MBP–35-kDa protein.

Antibody binding to the 35-kDa protein. Antibodies to the 35-kDa protein were detected by indirect ELISA. Microtiter plate wells were coated with antigen at a concentration of 10 μg/ml overnight at room temperature. The plates were washed and blocked with 3% bovine serum albumin, patient's sera (diluted 1:100) were added, and the plates were incubated for 90 min at 37°C. The plates were washed, alkaline phosphatase-conjugated anti-human IgG was added, and the plates were incubated for 60 min at 37°C. Binding was visualized by the addition of *n*-nitrophenyl phosphate (1 mg/ml), and the A_{405} was measured. Individuals who had an absorbance of greater than 0.337, which was the mean of 40 endemic control serum samples plus 2 SD, were considered positive. The binding of MAbs and pooled lepromatous leprosy sera to the recombinant 35-kDa proteins was measured by ELISA with protein concentrations of 100 pg/ml to 100 μg/ml. Denaturation of the *M. smegmatis*-derived 35-kDa protein for use in ELISA was performed by heating at 100°C for 5 min.

Measurement of DTH. Outbred female guinea pigs (10 to 12 weeks old) were sensitized intradermally with 0.5 mg (wet weight) of heat-killed *M. leprae*, heat-killed *M. bovis* BCG, or gamma-irradiated *M. tuberculosis*. Controls received 400 μl of PBS. The guinea pigs were challenged 4 weeks later by intradermal injection of 10 μg of MLS or the *M. smegmatis*-derived *M. leprae* 35-kDa protein. The area of induration was measured 24 h later.

RESULTS

Characterization of the *M. leprae* 35-kDa protein purified from *M. smegmatis* and *E. coli*. In plasmid pWL19 (35), the gene encoding the *M. leprae* 35-kDa protein was placed under the control of the promoter region from the β-lactamase gene of a highly amoxicillin-resistant mutant of *Mycobacterium fortuitum* (33). This resulted in high-level expression of the gene in *M. smegmatis*. *M. leprae* 35-kDa protein was purified by affinity chromatography from the sonicate of recombinant heat-killed *M. smegmatis* containing pWL19. The preparation contained a single 35-kDa species as assessed by SDS-PAGE (Fig. 1A). Typical yields of protein ranged from 1 to 2 mg of protein per liter of culture. Immunoblotting for lipoarabinomannan, HSP65, and antigen 85B showed that the protein preparation was free of these major immunogenic mycobacterial components (data not shown). Two forms of *E. coli*-derived *M. leprae* 35-kDa protein were also produced, one as an MBP–35-kDa fusion protein and the second as a histidine-tagged 35-kDa protein (Fig. 1A). All three forms of the recombinant protein reacted in an immunoblot with anti-*M. leprae* 35-kDa protein MAb CS-38 (Fig. 1B).

Since the native *M. leprae* 35-kDa protein, purified from the sonicate of *M. leprae*, exists as a multimer of 955 kDa (35), the

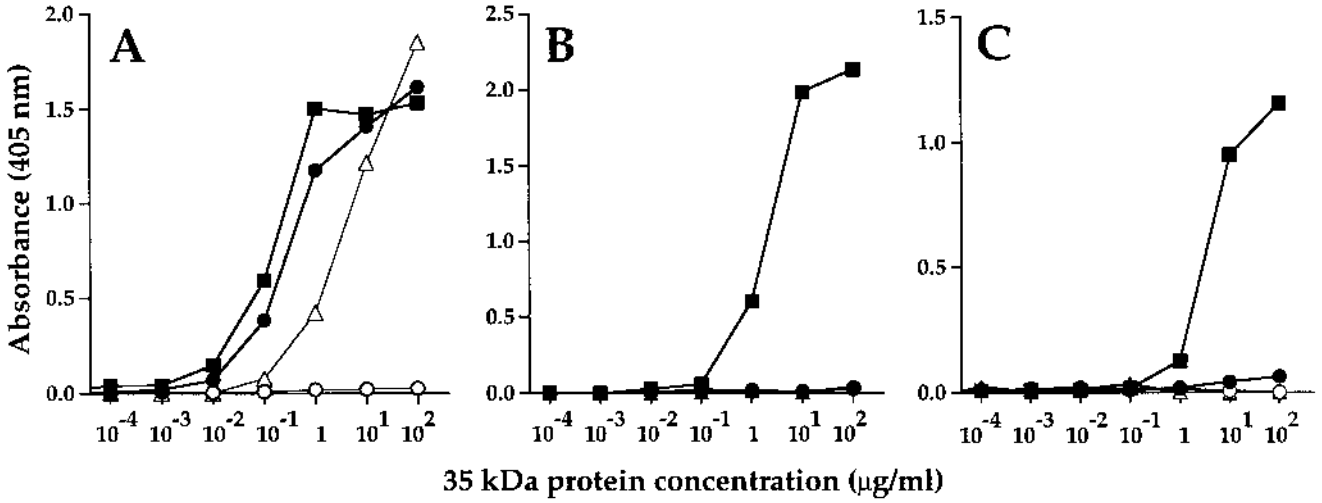

FIG. 2. Binding of MAbs and lepromatous leprosy sera to the *M. leprae* 35-kDa protein. The binding of the three forms of the recombinant 35-kDa protein and MBP by MAb CS-38 (A), MAb ML-03 (B), and pooled lepromatous leprosy sera (C) was determined by ELISA. ■, *M. smegmatis*-derived 35-kDa protein; △, histidine-tagged 35-kDa protein; ●, MBP–35-kDa protein; ○, MBP.

ability of the recombinant preparations of the 35-kDa protein to form such multimeric complexes was assessed. FPLC analysis indicated that the *M. smegmatis*-derived *M. leprae* 35-kDa protein existed as a multimeric complex with an estimated size of 1,010 ± 40 kDa (mean ± SD of three experiments). By contrast, the sizes of the two *E. coli* forms of the protein were in agreement with their predicted monomeric size. Histidine-tagged 35-kDa protein eluted at 34.6 ± 9.9 kDa, similar to the 37 kDa estimated by SDS-PAGE (Fig. 1A). The size of the MBP–35-kDa fusion protein was 87.3 ± 10.7 kDa, compared with 80 kDa as determined by SDS-PAGE.

The conformational state of the recombinant 35-kDa protein was investigated by using MAbs recognizing both linear and conformational determinants on the native *M. leprae* 35-kDa protein. MAb CS-38, generated against the purified native *M. leprae* 35-kDa protein (11), recognizes a linear determinant on both native and denatured protein. CS-38 bound all forms of the recombinant 35-kDa protein, with slightly reduced recognition of the histidine-tagged 35-kDa protein (Fig. 2A). MAb ML-03, which was generated against MLS, reacts only with the protein in its nondenatured state (8, 13). This MAb bound only the *M. smegmatis*, not the *E. coli*, forms of the protein (Fig. 2B). Sera from lepromatous leprosy patients reacted exclusively with the *M. smegmatis*-derived 35-kDa protein (Fig. 2C). Denaturation of the *M. smegmatis* recombinant 35-kDa protein by heating abolished the reactivity of MAb ML-03 and the leprosy serum pool but did not affect the binding of MAb CS-38 (data not shown). These data indicate that the recombinant 35-kDa protein purified from *M. smegmatis* retains conformational determinants present on the native antigen but absent from the *E. coli* products.

The 35-kDa protein induces strong and specific lymphoproliferative responses. The proliferation of PBMCs from leprosy patients and their healthy contacts and TB patients was tested to determine the magnitude and specificity of the cellular immune response recognized by the *M. smegmatis*-derived *M. leprae* 35-kDa protein. None of the groups showed any defect in proliferative responses to *M. tuberculosis* PPD (Fig. 3A) or the mitogen concanavalin A (data not shown). The majority of contacts and PB leprosy patients showed strong proliferative responses to MLS, with similar proportions of individuals responding to the 35-kDa protein (Fig. 3B and C). A total of 26 of 28 individuals in these two groups who mounted a response to MLS were responsive to the 35-kDa protein. Significantly reduced proliferative responses to the 35-kDa protein were observed in MB leprosy patients, consistent with the defective T-lymphocyte proliferative response to *M. leprae* observed in these patients. A few MB leprosy patients (3 of 13) who failed to respond to MLS did respond to the 35-kDa protein (Fig. 3B and C). Only 2 of 10 TB patients exhibited positive PBMC proliferation in response to the 35-kDa protein, and both had strong proliferative responses to MLS, indicative of exposure to leprosy (Fig. 3B and C). No significant responses were seen in the PBMCs of the 10 BCG vaccinees tested, in response to either MLS (mean Δcpm ± SD, 1,035 ± 160) or the 35-kDa protein (mean Δcpm ± SD, 1,335 ± 271).

IFN-γ production in response to the 35-kDa protein. IFN-γ is a key cytokine in the resistance to mycobacterial infection (9, 10). We examined whether the stimulation of PBMCs by the 35-kDa protein is associated with enhanced production of this cytokine. All four groups produced similar amounts of IFN-γ in response to *M. tuberculosis* PPD (Fig. 4A). The 35-kDa protein induced high levels of IFN-γ from the PBMCs of healthy leprosy contacts and PB leprosy patients, with 83% of contacts and 72% of PB leprosy patients exhibiting positive responses to the protein (Fig. 4C). IFN-γ responses comparable to that elicited by the recombinant 35-kDa protein were produced in response to MLS by these two groups (Fig. 4B and C). Lower levels of IFN-γ were produced by MB leprosy patients in response to the recombinant 35-kDa protein and MLS (Fig. 4B and C). As observed in the proliferative responses, some MB patients produced IFN-γ in response to the 35-kDa protein in the absence of such responsiveness to MLS. PBMCs from the majority of TB patients did not produce appreciable amounts of IFN-γ upon stimulation with the 35-kDa protein, while most responded to MLS (Fig. 4B and C).

Responses to the 35-kDa protein correlate with exposure to the leprosy bacillus and not *M. tuberculosis*. As the patterns of proliferative and IFN-γ responsiveness to the 35-kDa protein appeared to resemble the corresponding responses to MLS and not PPD of *M. tuberculosis*, the statistical significance of these responses was compared by using Spearman's rank correlation. There was a strong correlation between proliferative responses to the *M. leprae* 35-kDa protein and MLS (r_s =

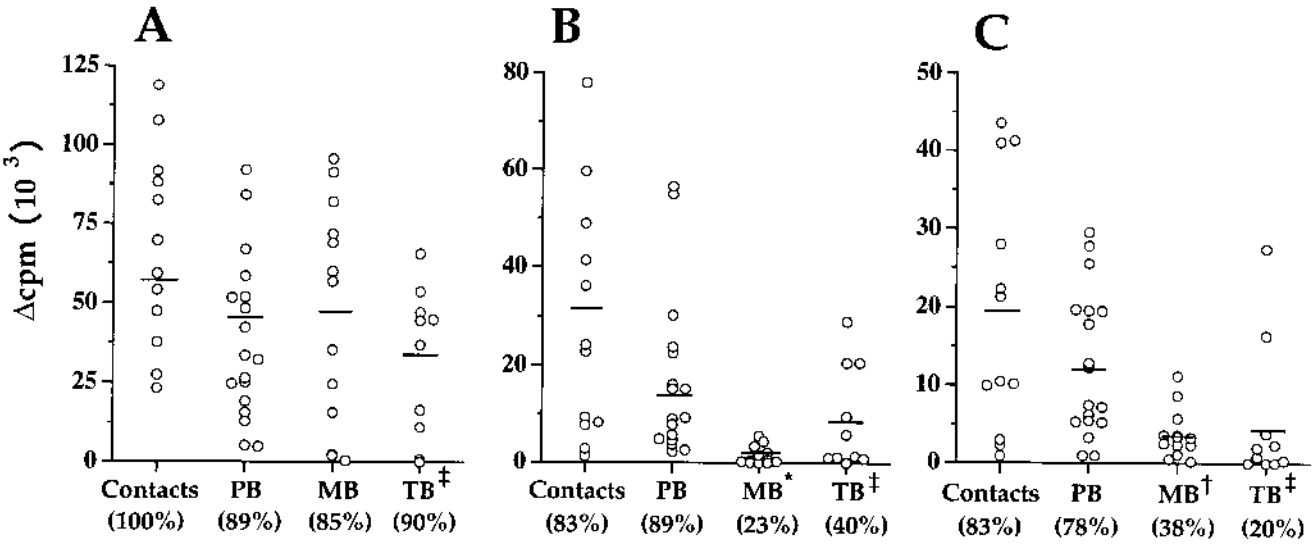

FIG. 3. Proliferative response of PBMCs from leprosy and TB patients and healthy leprosy contacts in response to the 35-kDa protein. PBMCs from subjects were stimulated with 10 μg of *M. tuberculosis* PPD (A), MLS (B), or *M. leprae* 35-kDa protein (C) per ml. The percentage of positive responders (Δcpm > 2,000; stimulation index > 4) is shown in parentheses beneath each patient group. The mean Δcpm for each group is represented by a horizontal bar. Significant differences in the proliferative responses of patient groups compared with that of leprosy contacts were determined by Student's *t* test (*, $P < 0.001$; †, $P < 0.01$; ‡, $P < 0.05$). The mean cpm ± standard error of the mean for control cultures was 1,531 ± 162.

0.760; $P < 0.001$). The correlation was the strongest in the contact group ($r_s = 0.881$; $P < 0.001$) and weakest in the MB leprosy group ($r_s = 0.198$; $P = 0.49$). This highly significant relationship was also observed for the IFN-γ responses to these two antigens ($r_s = 0.667$; $P < 0.001$). As with the proliferative responses, the strongest correlation was seen in the contact group ($r_s = 0.923$; $P < 0.001$) and the weakest was seen in the MB leprosy group ($r_s = 0.629$; $P < 0.05$). By contrast, weaker correlation was seen for both the proliferative ($r_s = 0.409$; $P < 0.05$) and the IFN-γ ($r_s = 0.318$; $P < 0.05$) responses between the 35-kDa protein and *M. tuberculosis* PPD.

Humoral immune responses to the *M. leprae* 35-kDa protein. Although a high proportion of PB leprosy patients and healthy leprosy contacts showed strong T-cell proliferative responses to the 35-kDa protein, this was not reflected in the MB leprosy group, who characteristically have weak T-cell responses and high levels of antibodies to *M. leprae* antigens (4). The level of antibodies to the purified *M. smegmatis*-derived *M. leprae* 35-kDa protein in all subjects was examined by ELISA (Fig. 5). MB leprosy patients had the highest levels of IgG antibodies recognizing the 35-kDa protein, although the proportion of seropositive individuals in this group (55%) was lower than previously observed for untreated MB leprosy patients (25). This was due to the inclusion of patients who either were

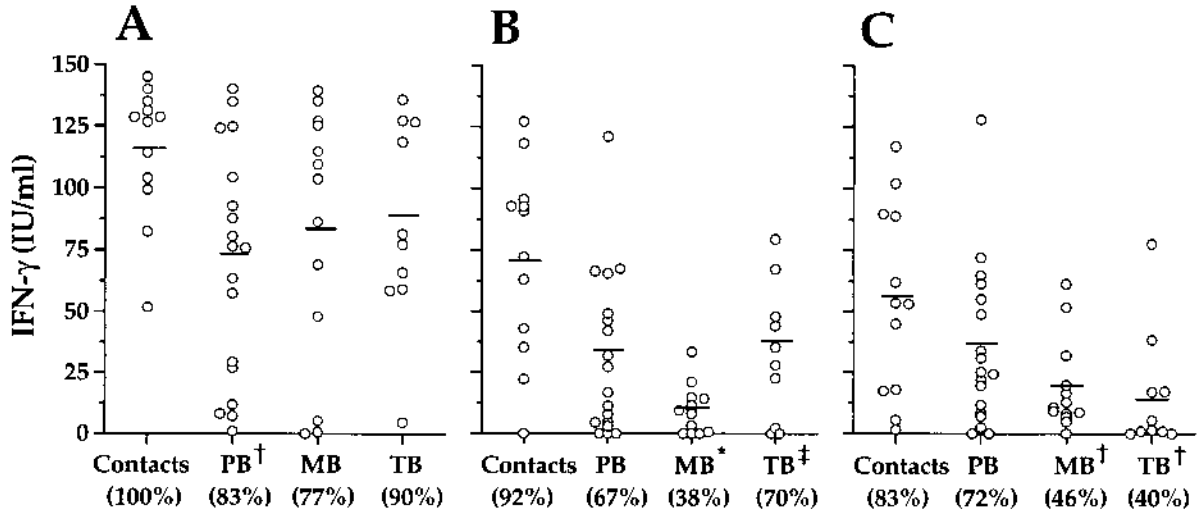

FIG. 4. IFN-γ production by PBMCs of leprosy and TB patients and healthy leprosy contacts in response to the 35-kDa protein. Supernatants of 5-day proliferative cultures of PBMCs stimulated with 10 μg of *M. tuberculosis* PPD (A), MLS (B), or the *M. leprae* 35-kDa protein (C) per ml were collected, and IFN-γ levels were measured by ELISA. The percentage of positive responders (IFN-γ, >10.1 IU/ml) is shown in parentheses beneath each patient group. The mean IFN-γ level for each group is represented by a horizontal bar. Significant differences in the IFN-γ levels of patient groups compared with that of leprosy contacts were determined by Student's *t* test (*, $P < 0.001$; †, $P < 0.01$; ‡, $P < 0.05$).

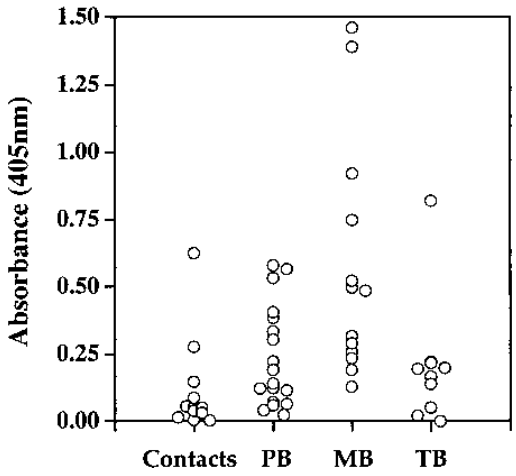

FIG. 5. Antibody levels to the 35-kDa protein in sera of leprosy and TB patients and healthy leprosy contacts. Sera from all subjects were diluted 1/100, and the levels of anti-35-kDa-protein IgG antibodies were determined by ELISA. A level greater than the mean plus 2 SD of control subjects ($A_{405} > 0.337$) was considered positive.

undergoing or had completed multidrug therapy, as the levels of anti-35-kDa-protein antibodies decline with effective chemotherapy (20, 26). A smaller proportion of PB leprosy patients (28%) had anti-35-kDa-protein antibodies, and only one individual in each of the leprosy contact and TB groups was seropositive. Serum specimens from 15 TB patients from an area in which leprosy is not endemic were all seronegative for the 35-kDa protein (data not shown).

When both T-cell proliferative and antibody responses to the 35-kDa protein were considered, over 90% of leprosy patients and contacts recognized this protein (Fig. 6). By contrast, only 2 of 10 TB patients responded to the protein. Most individuals mounted exclusively either a T-cell proliferative response or an antibody response to the 35-kDa protein, not both. No overlap in responses was observed in the MB and contact groups, and only a small degree of overlap occurred in the PB (2 of 14) and TB (1 of 10) groups. This apparent dichotomy in the response to the 35-kDa protein was not exhibited in response to *M. leprae* itself, since a number of patients exhibited both proliferative and antibody responses to MLS (data not shown).

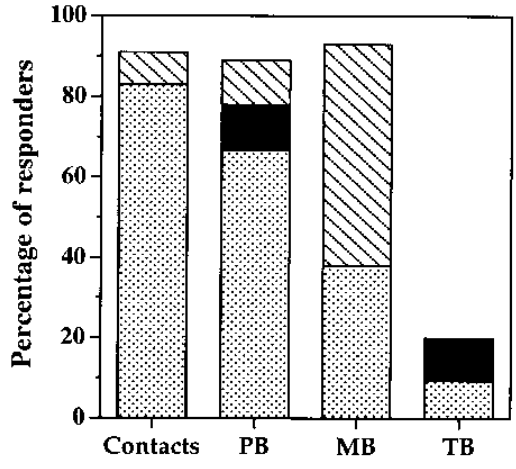

FIG. 6. Comparison of proliferative responses and levels of antibody to the 35-kDa protein. The percentage of individuals in each group with a positive PBMC proliferative response (Δcpm > 2,000; stimulation index > 4) (dotted bars), the percentage of individuals with a positive antibody response ($A_{405} > 0.337$) (striped bars), and the percentage of individuals with both positive proliferative and antibody responses (black bars) are indicated.

The *M. leprae* 35-kDa protein elicits specific DTH. The suitability of the *M. leprae* 35-kDa protein as a skin test reagent for the detection of leprosy exposure was assessed. The *M. smegmatis* recombinant 35-kDa protein elicited strong DTH in all *M. leprae*-sensitized animals, similar to the response elicited by MLS (Table 1). When guinea pigs were sensitized with *M. tuberculosis*, the 35-kDa protein elicited weak DTH in only one of eight animals. A similar result was obtained with animals sensitized with the vaccine strain BCG. By contrast, MLS lacked this specificity, eliciting DTH in all *M. tuberculosis*- and BCG-sensitized animals.

DISCUSSION

As an effective T-lymphocyte response is essential for protection against mycobacterial infection, the identification of antigens involved in such responses is of prime importance. This study indicates that the *M. leprae* 35-kDa protein is one such antigen. Almost all subjects (26 of 28) who demonstrated a cellular response to *M. leprae* showed a T-cell proliferative response to the 35-kDa protein with the release of IFN-γ. Immune responses to the antigen parallel those to *M. leprae* across the leprosy spectrum, with cellular immune responses dominant in PB leprosy patients and healthy subjects exposed to leprosy and antibody responses maximal in MB leprosy patients (Fig. 6). The previously documented discrimination between leprosy and TB patients in antibody responses to the protein (23, 32) was evident in cellular reactivity to the antigen. The small proportion of TB patients with proliferative responses to the 35-kDa protein also demonstrated strong responses to MLS, suggesting that the reactivity of these patients in an area in which leprosy is endemic may have been due to their exposure to leprosy. This is consistent with the observation that proliferative and IFN-γ responses to the 35-kDa protein were more strongly correlated with responses to MLS than to *M. tuberculosis* PPD.

Although many of the MB leprosy patients showed no response to the 35-kDa protein, consistent with their specific unresponsiveness to *M. leprae*, a proportion (3 of 13) who failed to respond to *M. leprae* did respond to the protein. It was not considered that the reactivity was due to contamination of the 35-kDa protein with other *M. smegmatis* antigens, as other proteins were not apparent upon SDS-PAGE fractionation (Fig. 1A) and immunoblots for three other major mycobacterial antigens were negative. Furthermore, TB patients who were PPD positive but unresponsive to the 35-kDa protein would have recognized any cross-reactive *M. smegmatis* contaminating proteins if present. Previous reports have described a level of T-cell reactivity to single *M. leprae* antigens in *M. leprae*-unresponsive MB leprosy patients (21, 22, 30). This has been attributed to the presence of suppressive components in unfractionated *M. leprae* preparations, but not recombinant proteins, which inhibit T-cell responses to the whole organism. This possible explanation for lepromatous unresponsiveness is different from that proposed by Kaleab et al. (14), who suggested that the reactivity of MB leprosy patients to *M. tuberculosis* PPD is due to recognition of specific determinants on *M. tuberculosis* proteins.

The inverse relationship between cell-mediated and IgG antibody responses to the 35-kDa protein is a striking example of the immune deviation present in leprosy. On the basis of our

TABLE 1. DTH elicited by the *M. leprae* 35-kDa protein in mycobacterium-sensitized guinea pigs

Sensitizing organism[a]	Result with the indicated recall antigen[b]			
	MLS		35-kDa protein	
	No. positive/ total	Mean induration (mm) ± SEM	No. positive/ total	Mean induration (mm) ± SEM
None (control)	0/6	0	0/6	0
M. leprae	6/6	10.8 ± 0.9	6/6	10.8 ± 1.6
M. tuberculosis	8/8	11.9 ± 0.9	1/8	0.6 ± 0.6
M. bovis BCG	5/5	11.0 ± 1.5	1/5	1.8 ± 1.8

[a] Guinea pigs were sensitized by intradermal injection of 0.5 mg of each mycobacterial species.
[b] Intradermal injection of 10 μg of each antigen was used for recall.

criteria, 37 of the 43 leprosy contacts or patients tested made exclusively a proliferative or an antibody response to the protein, irrespective of the clinical classification of the patients. A variety of immunoregulatory mechanisms may contribute to this dichotomous response, including age, route of inoculation and bacterial load at the time of infection (3), genetic contribution from the host (2), and the cytokine milieu at the site of T-cell activation (29, 36). Determining the relative importance of these mechanisms will require the availability of *M. leprae* antigens, such as the 35-kDa protein, which mirror the response to the whole organism.

The utility of recombinant mycobacterial antigens may be enhanced if the recombinant form more closely resembles the native protein. Our approach was to purify recombinant protein from the rapidly growing, nonpathogenic *M. smegmatis*. The 35-kDa protein of *M. leprae* purified from this mycobacterial host folded into the correct conformation, as assessed by its recognition by conformation-dependent MAbs (Fig. 2) and its formation of multimeric complexes. By contrast, the recombinant 35-kDa protein appeared to be incorrectly folded in *E. coli*. The *M. smegmatis* form of the protein was preferentially bound by sera from leprosy patients, indicating that nonlinear determinants were dominant in the human antibody response to this antigen. The lack of recognition of denatured *M. smegmatis* recombinant 35-kDa protein suggested that the inability of the *E. coli* recombinant 35-kDa protein to bind leprosy sera was most likely due to differences in the conformation of the proteins, rather than posttranslational modification. Previous studies have also shown enhanced recognition of mycobacterium-derived proteins by the cellular immune response. For example, the MPT64 protein of *M. tuberculosis* when purified from *M. smegmatis* was more potent in eliciting DTH than the *E. coli* recombinant MPT64 and preferentially stimulated lymphocytes from *M. tuberculosis*-infected individuals to secrete IFN-γ (28). The *M. smegmatis*-derived recombinant 35-kDa protein elicited strong and specific DTH in *M. leprae*-sensitized guinea pigs (Table 1). Although the basis for the enhanced immunogenicity of recombinant proteins purified from mycobacteria in DTH and T-cell assays is unclear, presentation of the protein in the correct conformation may lead to more-efficient antigen processing and T-cell activation.

Monitoring of the effectiveness of leprosy control programs is hindered by the lack of specific and sensitive diagnostic reagents with which to detect subclinical infection with *M. leprae* and measure transmission within a community. For a single mycobacterial protein to be useful as a diagnostic reagent, it should be limited in distribution, must be widely recognized by the desired population, and must stimulate the correct pattern of immune responses. The *M. leprae* 35-kDa protein fulfills all these criteria; it is absent from *M. tuberculosis* and *M. bovis* BCG, over 90% of leprosy patients and leprosy contacts respond to the protein, and it stimulates IFN-γ production and DTH. Only a few mycobacterial proteins have been evaluated for each of these criteria. For example, the *M. leprae* 10-kDa, or GroES, protein elicited DTH in *M. leprae*-sensitized guinea pigs and is widely recognized by leprosy patients but lacks specificity, as it shares 90% identity with its *M. tuberculosis* counterpart (18). The immunodominant *M. bovis* BCG 70-kDa heat shock protein elicits DTH in tuberculin-positive subjects (5), but the dominant T-cell epitopes are shared with the *M. leprae* homolog (1). Similarly, the 85B antigen of *M. tuberculosis* also elicits DTH, but the protein is common to all mycobacteria (34). Our finding that the 35-kDa protein elicits no or only weak DTH in *M. tuberculosis*- or BCG-sensitized animals further highlights the potential use of this protein as a component of a diagnostic test for leprosy exposure.

In addition, the *M. leprae* 35-kDa protein is a strong candidate as a component of an *M. leprae* vaccine. As the current vaccine strain BCG lacks the antigen, the introduction of this protein to extend the antigenic repertoire of BCG may enhance the protective efficacy of BCG vaccination against leprosy infection. We are currently evaluating the immunogenicity of BCG overexpressing the gene encoding the *M. leprae* 35-kDa protein, with a view to developing a more effective recombinant vaccine for immunoprophylaxis against leprosy.

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REFERENCES

1. **Adams, E., W. J. Britton, A. Morgan, S. Sergeantson, and A. Basten.** 1994. Individuals from different populations identify multiple and diverse T-cell determinants on mycobacterial HSP70. Scand. J. Immunol. **39:**588–596.
2. **Bloom, B. R., R. L. Modlin, and P. Salgame.** 1992. Stigma variations: observations on suppressor T cells and leprosy. Annu. Rev. Immunol. **10:**453–488.
3. **Bretscher, P.** 1992. A strategy to improve the efficacy of vaccination against tuberculosis and leprosy. Immunol. Today **13:**342–345.
4. **Britton, W. J.** 1993. The immunology of leprosy. Trans. R. Soc. Trop. Med. Hyg. **46:**508–514.
5. **Britton, W. J., L. Hellqvist, A. Basten, and S. Inglis.** 1986. Immunoreactivity of a 70 kD protein purified from *Mycobacterium bovis* BCG by monoclonal antibody affinity chromatography. J. Exp. Med. **164:**695–708.
6. **Britton, W. J., L. Hellqvist, A. Basten, and R. L. Raison.** 1985. *Mycobacterium leprae* antigens involved in human immune responses. I. Identification of four antigens by monoclonal antibodies. J. Immunol. **135:**4171–4177.
7. **Britton, W. J., L. Hellqvist, R. Garsia, and A. Basten.** 1987. Dominant cell wall proteins of *M. leprae* recognised by monoclonal antibodies. Clin. Exp. Immunol. **67:**31–42.
8. **Britton, W. J., L. Hellqvist, J. Ivanyi, and A. Basten.** 1987. Immunopurification of radiolabelled antigens of *Mycobacterium leprae* and *Mycobacterium bovis* (Bacillus Calmette-Guérin) with monoclonal antibodies. Scand. J. Immunol. **26:**149–159.
9. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffen, J. P. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon-γ gene-disrupted mice. J. Exp. Med. **178:**2243–2247.
10. **Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom.** 1993. An essential role for interferon-γ in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med. **178:**2249–2254.
11. **Hunter, S. W., B. Rivoire, V. Mehra, B. R. Bloom, and P. J. Brennan.** 1990. The major native proteins of the leprosy bacillus. J. Biol. Chem. **265:**14065–14068.
12. **Ivanyi, J., J. A. Morris, and M. Keen.** 1985. Studies with monoclonal antibodies to mycobacteria, p. 51. *In* A. J. L. Macario and E. G. Macario (ed.),

Monoclonal antibodies to bacteria. Academic Press, London.
13. **Ivanyi, J., S. Sinha, R. Aston, D. Cussel, M. Kenn, and U. Sengupta.** 1983. Definition of species specific and cross-reactive antigenic determinants of *M. leprae* using monoclonal antibodies. Clin. Exp. Immunol. **52:**528–536.
14. **Kaleab, B., A. Wondimu, R. Likassa, N. Woldehawariats, and J. Ivanyi.** 1995. Sustained T-cell reactivity to *Mycobacterium tuberculosis* specific antigens in 'split-anergic' leprosy. Lepr. Rev. **66:**19–25.
15. **Launois, P., M. N'Diaye, J. L. Cartel, I. Mane, A. Drowart, J. P. van Vooren, J. L. Sarthou, and K. Huygen.** 1995. Fibronectin-binding antigen 85 and the 10-kilodalton GroES-related heat shock protein are the predominant Th1 response inducers in leprosy contacts. Infect. Immun. **63:**88–93.
16. **Launois, P., M. N. Niang, J. L. Sarthou, F. Rivier, A. Drowart, J. P. van Vooren, J. Millan, and K. Huygen.** 1993. T-cell stimulation with purified mycobacterial antigens in patients and healthy subjects infected with *Mycobacterium leprae*: secreted antigen 85 is another immunodominant antigen. Scand. J. Immunol. **38:**167–176.
17. **McKenzie, K. R., E. Adams, W. J. Britton, R. J. Garsia, and A. Basten.** 1991. Sequence and immunogenicity of the 70 kDa heat shock protein of *Mycobacterium leprae*. J. Immunol. **147:**312–319.
18. **Mehra, V., B. R. Bloom, A. C. Bajardi, C. L. Grisso, P. A. Sieling, D. Alland, J. Convit, X. Fan, S. W. Hunter, P. J. Brennan, T. H. Rea, and R. L. Modlin.** 1992. A major T cell antigen of *Mycobacterium leprae* is a 10-kD heat-shock cognate protein. J. Exp. Med. **175:**275–284.
19. **Mohagheghpour, N., M. W. Munn, R. H. Gelber, and E. G. Engleman.** 1990. Identification of an immunostimulating protein from *Mycobacterium leprae*. Infect. Immun. **58:**703–710.
20. **Mwatha, J., C. Moreno, U. Sengupta, S. Sinha, and J. Ivanyi.** 1988. A comparative evaluation of serological assays for lepromatous leprosy. Lepr. Rev. **59:**195–199.
21. **Ottenhoff, T. H. M., P. J. Converse, N. Gebre, A. Wondimu, J. P. Ehrenberg, and R. Kiessling.** 1988. T-cell responses to fractionated *Mycobacterium leprae* antigens in leprosy. The lepromatous nonresponder defect can be overcome *in vitro* by stimulation with fractionated *M. leprae* components. Eur. J. Immunol. **19:**707–713.
22. **Ottenhoff, T. H. M., A. Wondimu, and N. N. B. Reddy.** 1990. A comparative study on the effects of rIL-4, rIL-2, rIFN-γ, and rTNF-α on specific T-cell non-responsiveness to mycobacterial antigens in lepromatous leprosy *in vitro*. Scand. J. Immunol. **31:**553–565.
23. **Parkash, O., V. Chaturvedi, B. K. Girdhar, and U. Sengupta.** 1995. A study on performance of two serological assays for diagnosis of leprosy patients. Lepr. Rev. **66:**26–30.
24. **Ridley, D. S., and W. H. Jopling.** 1966. Classification of leprosy according to immunity. A five group system. Int. J. Lepr. **34:**255–273.
25. **Roche, P. W., W. J. Britton, S. S. Failbus, D. Williams, H. M. Pradhan, and W. J. Theuvenet.** 1990. Operational value of serological measurements in multibacillary leprosy patients: clinical and bacteriological correlates of antibody responses. Int. J. Lepr. **58:**480–490.
26. **Roche, P. W., S. S. Failbus, K. D. Neupane, W. J. Theuvenet, and W. J. Britton.** 1993. Serological monitoring of the response to chemotherapy in leprosy patients. Int. J. Lepr. **61:**35–43.
27. **Roche, P. W., P. W. Peake, H. Billman-Jacobe, T. Doran, and W. J. Britton.** 1994. T-cell determinants and antibody binding sites on the major mycobacterial secretory protein MPB59 of *Mycobacterium bovis*. Infect. Immun. **62:**5319–5326.
28. **Roche, P. W., N. Winter, J. A. Triccas, C. Feng, and W. J. Britton.** 1996. Expression of *Mycobacterium tuberculosis* MPT64 in recombinant *M. smegmatis*: purification, immunogenicity and application to skin tests for tuberculosis. Clin. Exp. Immunol. **103:**226–232.
29. **Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom.** 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T-cell clones. Science **254:**279–282.
30. **Sela, S., J. E. R. Thole, T. H. M. Ottenhoff, and J. E. Clark-Curtiss.** 1991. Identification of *Mycobacterium leprae* antigens from a cosmid library: characterization of a 15-kilodalton antigen that is recognized by both humoral and cellular immune systems in leprosy patients. Infect. Immun. **59:**4117–4124.
31. **Sinha, S., U. Sengupta, G. Ramu, and J. Ivanyi.** 1983. A serological test for leprosy based on competitive inhibition of monoclonal antibody binding to the MY2a determinant of *Mycobacterium leprae*. Trans. R. Soc. Trop. Med. Hyg. **77:**869–871.
32. **Sinha, S., U. Sengupta, G. Ramu, and J. Ivanyi.** 1985. Serological survey of leprosy and control subjects by a monoclonal antibody-based immunoassay. Int. J. Lepr. **53:**33–38.
33. **Timm, J., M. G. Perilli, C. Duez, J. Trias, G. Orefici, L. Fattorini, G. Amicosante, A. Oratore, B. Joris, J. M. Frere, A. P. Pugsley, and B. Gicquel.** 1994. Transcription and expression analysis, using *lacZ* and *phoA* gene fusions, of *Mycobacterium fortuitum* β-lactamase genes cloned from a natural isolate and a high-level β-lactamase producer. Mol. Microbiol. **12:**491–504.
34. **Wiker, H. G., and M. Harboe.** 1992. The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. Microbiol. Rev. **56:**648–661.
35. **Winter, N., J. A. Triccas, B. Rivoire, M. C. V. Pessolani, K. Eiglmeier, S. W. Hunter, P. J. Brennan, and W. J. Britton.** 1995. Characterization of the gene encoding the immunodominant 35 kDa protein of *Mycobacterium leprae*. Mol. Microbiol. **16:**865–876.
36. **Yamamura, M., K. Uyemura, R. J. Deans, K. Weinberg, T. H. Rea, B. R. Bloom, and R. L. Modlin.** 1991. Defining protective immune responses to pathogens: cytokine profiles in leprosy lesions. Science **254:**277–279.

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