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Serological Response of Patients with Leprosy to a 28- to 30-Kilodalton Protein Doublet from Early Cultures of *Mycobacterium bovis* BCG

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Analysis by Western immunoblotting of *Mycobacterium bovis* BCG short-term culture filtrates with a pool of serum samples from lepromatous leprosy patients revealed an immunodominant protein doublet with apparent molecular masses of 28 and 30 kilodaltons (kDa). The humoral response to these antigens was also investigated by using individual serum samples from patients representative of the whole spectrum of leprosy and from tuberculosis patients, as well as from contacts of leprosy patients and control groups. The protein doublet was recognized by 92% of the sera from patients with lepromatous leprosy (51 of 56), whereas essentially negative results were obtained with sera from the other groups. Similar immunodominant bands were also detected by Western blotting analysis of sonic extracts of seven other slow- and fast-growing mycobacterial species, suggesting a broad distribution of these antigens within the genus. Analysis of the purified doublet by Western blotting after two-dimensional gel electrophoresis fractionation showed that the 28- and 30-kDa doublet consisted of at least five different components with pIs from 5.2 to 5.7 and molecular masses from 28 to 31 kDa. These results indicate that the protein doublet could be used as a potential marker in the diagnosis of lepromatous leprosy.

Little is known about the role of secreted proteins in host-parasite interactions in mycobacterial infections. Evidence that their recognition by the immune system may be essential for an efficient response comes from in vivo experiments which indicate that vaccination with live mycobacteria evokes a better protection than vaccination with dead bacteria (14; for a review, see reference 22). Indeed, some proteins that induce a cellular immune response have recently been isolated from short-term mycobacterial culture media, suggesting their potential as protective immunogens (4, 6, 9, 18). A series of experiments showing the involvement of mycobacterial-specific cytolytic T lymphocytes in the host immune response in leprosy and tuberculosis (11, 17, 24) support the view that secreted or shed components could be responsible for protection and pathogenic sequelae in these diseases. There is evidence that cytotoxic T cells recognize nonprocessed antigens in association with class I major histocompatibility complex molecules expressed in all nucleated host cells (30). Thus, it is reasonable to assume that components that are secreted or shed by intracellular parasites could reach the host cell surface more easily and hence be recognized by cytolytic T cells.

The secreted proteins from mycobacteria may also contribute to the pathology of tissue lesions observed in patients with leprosy and tuberculosis, as indicated by the fact that protease, mucinase, lipase, and RNase activities were recently detected in early culture media of pathogenic mycobacteria (10). Proteins with fibronectin-binding activity have also been found on the surfaces and in the culture medium of pathogenic mycobacteria (20), but the physiological significance of these receptors in mycobacterial infections is not yet known. Since cultures of *Mycobacterium leprae* have never been successfully implanted, the construction of genomic libraries constitutes an alternative for immunological and biochemical studies involving proteins from this microorganism. This approach, however, has yielded only a limited number of recombinant *M. leprae* proteins, essentially owing to the lack of adequate probes for selection of relevant clones (reviewed in reference 29).

The similarity between the antigenic composition of *M. leprae* and *Mycobacterium bovis* BCG is well known (7). This feature can be exploited in the sense that information on the biochemistry of and immune response to both structural and secreted BCG proteins may be relevant to leprosy. More specifically, the recognition of common major antigens from the two different mycobacteria may lead to the selection of clones coding for proteins directly involved in the pathogenesis of and protective immunity in leprosy.

In the present paper, we describe the humoral immune response of leprosy and tuberculosis patients to immunodominant cross-reactive proteins secreted or shed by *M. bovis* BCG, with the aim of establishing the role of these proteins in disease.

MATERIALS AND METHODS

Bacterial culture. *M. bovis* BCG subspecies Moreau was kindly provided by the Ataulpho de Paiva Foundation, Rio de Janeiro, Brazil. Cultures were grown on the surface of Sauton medium and incubated at 37°C for 7 days. The bacteria were collected by filtration and washed with distilled water. Remaining bacteria in the culture medium were removed by filtration through a membrane (pore size, 0.45 μm).

Mycobacterium kansasii ATCC 12478, *Mycobacterium phlei* ATCC 11758, *Mycobacterium smegmatis* ATCC 19420, *Mycobacterium simiae* ATCC 25275, *Mycobacterium marinum* ATCC 927, *Mycobacterium vaccae* ATCC 15483, *Mycobacterium szulgai* NCTC 10831, and *Mycobacterium*

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tuberculosis H37 Rv were grown in 10-ml roller tubes containing 5 ml of Sauton medium at 37°C, with the exception of *M. marinum*, which was incubated at 31°C. After 7 to 11 days, cells were harvested by centrifugation, and the medium was filtered through a 0.45-μm membrane. Media and cells were stored at −20°C until used.

Preparation of crude fractions. Proteins in short-term BCG culture medium were concentrated by precipitation of culture filtrate with ammonium sulfate, at 50% or 75% saturation. Salts were removed by Sephadex G-25 gel filtration.

To obtain BCG subcellular fractions, the bacteria were suspended in 0.01 M phosphate-buffered saline (PBS) (pH 7.2) and sonicated on ice for 30 min at 150 W in an MSE sonicator. Remaining whole cells were removed by centrifugation at 10,000 × *g* for 30 min. The sonic extract was centrifuged at 100,000 × *g* for 2 h, and the supernatant was collected and precipitated with ammonium sulfate at 50% saturation; this material is referred to as the soluble BCG fraction. The pellet from the centrifugation was suspended in PBS and subjected to the same conditions of sonication and centrifugation as described above for whole-cell suspension. The final 100,000 × *g* pellet was the cell envelope fraction. The protein concentration was estimated by the method of Lowry et al. (15) with a bovine serum albumin standard. The antigen preparation was stored at −20°C until used.

Individuals tested. The humoral response to BCG proteins in 89 leprosy patients, 110 clinically healthy household contacts of leprosy patients, 30 patients with active pulmonary tuberculosis, 27 members of the staff of the leprosy department at the Oswaldo Cruz Foundation, and 123 randomly chosen hospitalized patients with diseases other than leprosy was investigated. The leprosy patients were carefully diagnosed by the Ridley-Jopling method (21). They were classified as 18 full lepromatous leprosy patients, 38 borderline lepromatous leprosy patients, 10 patients with the indeterminate form, and 23 borderline tuberculoid patients. The majority of patients were either untreated or at the onset of chemotherapy (less than 6 months of treatment). All tuberculosis patients were sputum positive at the time of diagnosis and were under treatment.

Western immunoblot and PAGE analysis. BCG subfractions and fractions of the precipitated culture filtrate were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a 10 or 12.5% gel, as described by Laemmli (13). The gels were stained with Coomassie blue or silver stain. They were scanned by using a green filter with a Quick Scan (Helena Laboratories). Two-dimensional PAGE was performed by the method of O'Farrell (19). For serum reactivity tests, the proteins were blotted overnight onto a nitrocellulose membrane (pore size, 0.45 μm; Schleicher & Schuell, Inc.) in Tris-glycine-methanol buffer, as described by Towbin et al. (25). The membranes were washed three times with 0.1% Tween 20 in PBS (pH 7.4) (PBST) and incubated with serum diluted in PBST containing 5% of fat-free milk powder (PBSTM) for 90 min, with constant shaking at room temperature. The membranes were washed with PBSTM and treated with goat anti-human immunoglobulin G labeled with peroxidase diluted 1:5,000 in PBSTM (Protatek). After incubation for 1 h at room temperature and further washing in PBS, the membranes were immersed in 10 ml of PBS containing 3,3-diaminobenzidine tetrahydrochloride (2.5 mg), 70 μl of 1% $CaCl_2$ solution, and 20 μl of 30% H_2O_2. The reaction was stopped by washing the membranes with distilled water.

Purification of the 28- and 30-kilodalton (kDa) doublet. The proteins precipitated from 340 ml of BCG short-term culture medium with ammonium sulfate at 50% saturation were suspended in 0.05 M phosphate buffer (pH 8.7) with 0.5 M NaCl and 2% butanol and fractionated in a column of Sephacryl S-200 (1.5 by 46 cm) at a flow rate of 40 ml/h. Elution was monitored by recording the A_{280}, and fractions containing proteins were analyzed by SDS-PAGE and immunoblotting.

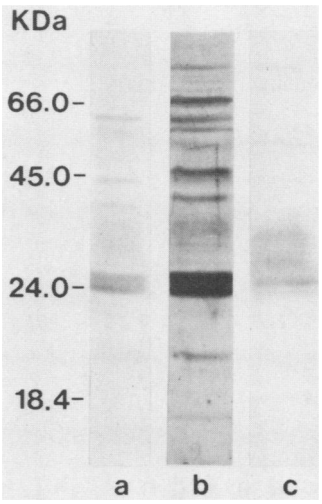

FIG. 1. Immunoblot pattern of culture filtrate (lane a), soluble sonic fraction (lane b), and purified cell envelope fraction (lane c). Blots were developed with a pool of serum samples from lepromatous leprosy patients. In each lane, 12 μg of proteins from each fraction was applied. Molecular weight markers, from top to bottom, are bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000), and lactoglobulin (18,400).

RESULTS

Identification of short-term BCG culture medium components that cross-react with sera from lepromatous leprosy patients. Figure 1, lane a, shows the Western blot pattern obtained after analysis of a BCG culture filtrate precipitated with ammonium sulfate and developed with a pool of serum samples from five lepromatous leprosy and five borderline lepromatous leprosy patients. Clearly, a doublet of apparent molecular mass 28 and 30 kDa appeared as the immunodominant bands, together with other less prominent components of apparent molecular masses 40, 45, and 56 kDa. Comparison of this pattern with those obtained from a BCG soluble fraction (Fig. 1, lane b) and from a purified cell envelope fraction (Fig. 1, lane c) also shows the presence of the 28- and 30-kDa doublet in these subcellular fractions.

Humoral immune response to the 28- and 30-kDa doublet. The humoral reactivity to the 28- and 30-kDa doublet in the BCG soluble fraction was determined by Western blotting, with individual serum samples from patients representing the entire spectrum of leprosy, tuberculosis patients, healthy household contacts of leprosy patients, and control groups. All samples were diluted 1:100, and the reactivity to the test was classified as positive, doubtful, or negative. In all experiments, a strong positive, a weak positive, and a negative serum sample were included as controls. Some representative Western blots are demonstrated in Fig. 2, and all results are summarized in Fig. 3. Of the lepromatous leprosy patient population, 94.5% (17 of 18) were positive; 89.5% (34 of 38) of borderline lepromatous leprosy patients

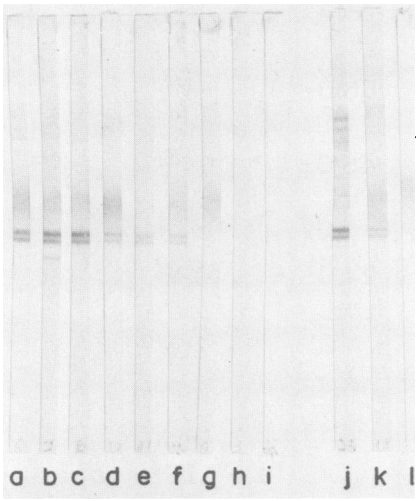

FIG. 2. Reactivity of several sera against the 28- and 30-kDa doublet as revealed by immunoblotting. Sera were grouped as strong positive (lanes a to c), weak positive (lanes d to f), doubtful (lane g), and negative (lanes h and i). Positive, weak-positive, and negative control sera are in lanes j, k, and l, respectively.

were positive. All patients with the indeterminate form were negative, and only 1 of the 23 borderline tuberculoid leprosy patients was positive. Frequencies of positive reactions of 1.8% (2 of 110) and 5.1% (2 of 39) were detected among leprosy contacts and tuberculosis patients, respectively. None of the 150 serum samples from the control groups was positive.

Occurrence of possibly similar antigens in extracts from other *Mycobacterium* species. The occurrence of immunodominant proteins in the molecular weight range of 28,000 to 30,000 in extracts from slow- and fast-growing mycobacteria was suggested after inspection of total sonic extracts of eight other *Mycobacterium* species developed with a serum sample from a lepromatous leprosy patient (diluted 1:100) (Fig. 4A). An immunodominant protein doublet very similar to the one in BCG was identified in *M. marinum*, *M. kansasii*, and *M. tuberculosis*. A single prominent band of similar molecular weight was observed in all of the species except *M. smegmatis*. The broadly reactive area seen in most of these gels is probably due to the presence of the highly antigenic, cross-reactive lipoarabinomannan (8).

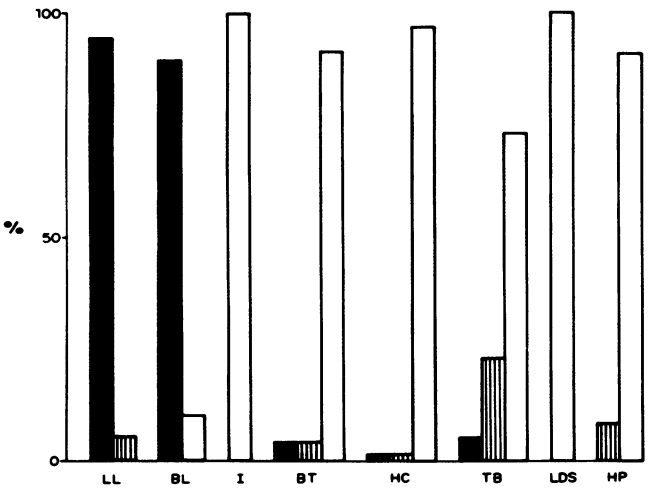

FIG. 3. Serum reactivity spectrum to the 28- and 30-kDa antigens as analyzed by immunoblotting. The frequencies of positive, doubtful, and negative results are expressed as percentages. Abbreviations: LL, polar lepromatous leprosy patients; BL, borderline lepromatous leprosy patients; I, indeterminate leprosy patients; BT, borderline tuberculoid leprosy patients; HC, household leprosy contacts; TB, tuberculosis patients; LDS, Leprosy Department staff; HP, randomly chosen hospitalized patients. Symbols: ■, positive sera; □, negative sera; ▥, doubtful sera.

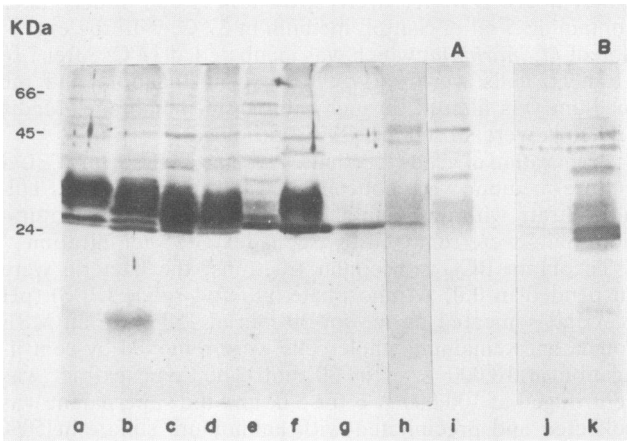

FIG. 4. Immunoblot of total sonic extracts (A) and culture filtrates (B) of mycobacterial species, with a serum sample from a lepromatous leprosy patient (diluted 1:100). Lanes: a, *M. marinum*, 12 μg; b, *M. kansasii*, 12 μg; *M. tuberculosis*, 8 μg; d, *M. bovis* BCG, 8 μg; e, *M. phlei*, 12 μg; f, *M. simiae*, 12 μg; g, *M. vaccae*, 18 μg; h, *M. szulgai*, 8 μg; i, *M. smegmatis*, 12 μg; j, *M. kansasii*, 12 μg; k, *M. tuberculosis*, 12 μg.

The culture media of these mycobacteria were also tested for the presence of these antigens. Starting with 25 ml of culture filtrate, we could detect antigen only in *M. kansasii* and *M. tuberculosis* media (Fig. 4B).

Purification and two-dimensional gel electrophoresis analysis of the 28- and 30-kDa doublet. To carry out a more detailed biochemical and immunological characterization of these immunodominant antigens, we purified the 28- and 30-kDa doublet from the culture medium. The medium filtrate from a 7-day-old BCG culture contained 20 to 30 μg of nondialyzable proteins per ml. Figure 5 shows the components of culture media fractionated by SDS-PAGE and stained with silver, as well as the proteins precipitated with ammonium sulfate. Proteins from *M. tuberculosis* culture filtrate, precipitated with ammonium sulfate, are also shown for comparison. Quantitative analysis of the gel-scanning profile showed that the 28- and 30-kDa peaks correspond to 3.6 and 6.7%, respectively, of the total silver-staining components of the BCG culture filtrate. A third faint band of molecular mass 31 kDa was detected as a shoulder of the 30-kDa peak in the scanning profile, corresponding to 2.9% of the BCG culture medium components. These bands are also major constituents of a short-term *M. tuberculosis* culture filtrate (Fig. 5, lane d) as already shown on a Western blot. These components could be stained with Coomassie blue, but failed to stain with Schiff-periodic acid reagent, suggesting that they have little or no carbohydrate in their structure.

Proteins precipitated from the culture medium with ammonium sulfate at 50% saturation were fractionated by gel filtration in Sephacryl S-200 (Fig. 6). After analysis by SDS-PAGE and Western blotting, fraction 5 showed the presence of the purified doublet (Fig. 6, right-hand side). The

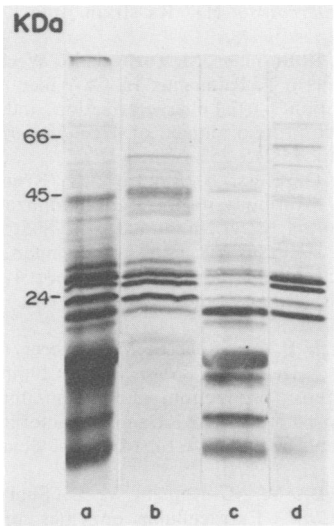

FIG. 5. Analysis of culture filtrate components by SDS-PAGE with silver staining. Lanes: a, lyophilized 7-day-old BCG medium (12 μg); b, components of BCG culture medium precipitated with ammonium sulfate at 50% saturation (9 μg); c, components of BCG culture which did not precipitate with ammonium sulfate at 50% saturation but did precipitate with ammonium sulfate at 75% saturation (12 μg); d, proteins of 9-day-old *M. tuberculosis* culture medium precipitated with ammonium sulfate at 75% saturation (6 μg).

complexity of these antigens was analyzed by two-dimensional gel electrophoresis followed by Western blotting. At least five components with a pI between 5.2 and 5.7 and a molecular mass between 28 and 31 kDa were found; all of these reacted with a pool of sera from lepromatous leprosy patients.

DISCUSSION

In this study, Western blot analysis of BCG short-term culture filtrates revealed the presence of five major bands

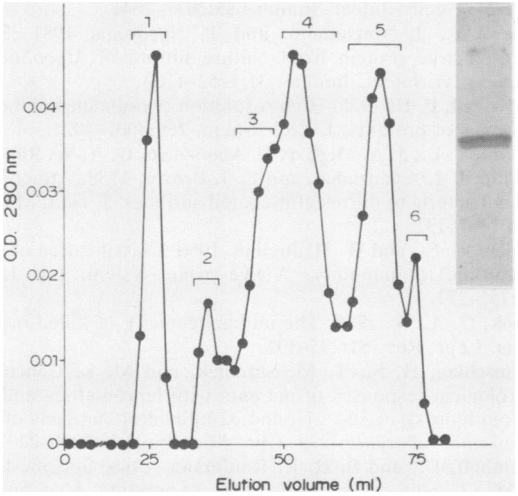

FIG. 6. Gel filtration chromatogram of BCG culture medium, after precipitation with ammonium sulfate at 50% saturation from a Sephacryl S-200 column. On the right-hand side, the immunoblotted pattern of fraction 5 shows the presence of the enriched 28- and 30-kDa antigens. O.D. 280 nm, Optical density at 280 nm.

that reacted with a pool of sera from lepromatous leprosy patients. The most prominent cross-reactive component, a doublet of molecular weight 28,000 and 30,000, was also detected in sonic extracts of the slow-growing *M. marinum*, *M. kansasii*, and *M. tuberculosis* H37 Rv species. All the other *Mycobacterium* species assayed, with the exception of *M. smegmatis*, showed only a single prominent band with a molecular weight similar to that of the BCG doublet. These results suggest a broad distribution of these antigens within the genus. The relatively simple silver-staining SDS-PAGE profile of BCG culture medium and of *M. tuberculosis* medium, in which the 28- and 30-kDa bands form the major components, supports the idea that these proteins are secreted or shed by mycobacteria rather than being autolytic bacillary products. The two-dimensional electrophoresis and blotting analysis showed that the immunodominant bands observed as a doublet in one-dimensional SDS-PAGE and Western blot actually consist of a mixture of five or six antigenic components reactive to a pool of sera from lepromatous leprosy and borderline lepromatous leprosy patients.

To understand the role of the 28- and 30-kDa doublet in leprosy, we investigated the humoral immune response to these prominent antigens by Western blotting, using individual serum samples from patients representing the entire spectrum of leprosy, patients with tuberculosis, and control groups. Relative high immunoglobulin G titers were observed in most patients with the lepromatous pole of leprosy (92%), whereas essentially no reactivity was detected in patients with the tuberculoid pole of leprosy, nor in patients with the indeterminate form or with tuberculosis. The negative results observed for healthy household contacts and members of the Leprosy Department staff might suggest that previous exposure to *M. leprae* or other mycobacteria does not evoke a detectable antibody response to these antigens. Furthermore, previous vaccination with BCG does not seem to affect the test, since 45% of the leprosy contact group were vaccinated individuals. There was no correlation between the bacteriological index as determined at the time of serum collection and the level of binding to the doublet, suggesting that the humoral immune response against these antigens is not just a consequence of host bacterial load (results not shown). An important detail which should also be stressed is that almost all individual serum samples assayed reacted in the same way with both the 28- and 30-kDa band in Western blots. This fact suggests that these bands, and perhaps the five or six different components identified in two-dimensional electrophoresis and blotting, have common epitopes and are probably related proteins with a common origin.

Among recent reports concerning the identification of the antigens eliciting an antibody response in leprosy, the 28- and 30-kDa doublet could be related to a trypsin-sensitive 33-kDa immunodominant doublet detected in ultrasonic extracts of *M. leprae* and BCG (12). Our results are also in agreement with the findings obtained on immunoprecipitation of radiolabeled sonic extracts of *M. leprae* and BCG from sera of patients with leprosy and tuberculosis (2). That report showed that a doublet of molecular weight 31,000 and 32,000, present in both sonic extracts, was recognized only by antibodies in serum samples from lepromatous leprosy patients. Furthermore, in a recent report, a similar reactivity against a triplet of molecular mass 31, 32, and 33 kDa, prepared from *M. tuberculosis* culture medium, was found with serum samples from tuberculosis and leprosy patients (23). Probably these antigens are the same as the ones described in this study, the third (33-kDa) component being

the spot of molecular mass 31 kDa detected in the two-dimensional blotting and the third 31-kDa band in SDS-PAGE with silver staining. Different groups have purified antigens from *M. tuberculosis* and *M. bovis* BCG which are probably related to the 28- and 30-kDa doublet (5, 6, 28), but, more recently, reports have shown that these components belong to a common antigenic structure complex, having common epitopes (3, 26, 27). Also, an antigen belonging to the BCG 85 complex referred to as α antigen, was recently cloned and expressed in *Escherichia coli*, revealing for the first time the structure of a mycobacterial signal peptide (16).

Although there is evidence suggesting that all the antigenic components mentioned above are related proteins, controversial findings about the humoral response to these antigens in tuberculosis patients (2, 4, 9, 23) indicate the need for a more careful biochemical and immunochemical analysis. Monoclonal antibodies available to mycobacterial antigens probably do not react with the 28- and 30-kDa doublet (1, 4). Regarding the biological activity of these proteins, a recent report (1) suggests that they could be the mycobacterial fibronectin-binding proteins (12). The preliminary results of this study suggest a potential use for the easily obtainable 28- and 30-kDa antigens in a serological test for differential diagnosis of lepromatous leprosy patients, both in leprosy control programs and in epidemiological investigations. However, when screening large populations for leprosy, the use of combinations with other antigens will be necessary to cover the whole spectrum of the disease. Regarding the potential use of these antigens to detect multibacillary subclinical infection, the two household contracts that were positive in our analysis are now being followed up to obtain further information.

Finally, the strong humoral immune response evoked by these antigens in lepromatous leprosy patients, in contrast to patients in the remaining leprosy spectrum and patients with tuberculosis, indicates that these antigens may play an important role in the regulation of the immune system in leprosy, leading to an inadequate response in the lepromatous pole.

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LITERATURE CITED

1. **Abou-Zeid, C., I. Smith, J. M. Grange, T. L. Ratliff, J. Steele, and G. A. W. Rook.** 1988. The secreted antigens of *Mycobacterium tuberculosis* and their relationship to those recognized by available antibodies. J. Gen. Microbiol. **134**:531–538.
2. **Britton, W. J., L. Hellqvist, R. J. Garcia, and A. Basten.** 1988. Antigens of *Mycobacterium leprae* identified by immunoprecipitation with sera from leprosy and tuberculosis patients. Clin. Exp. Immunol. **71**:394–398.
3. **Closs, O., M. Harboe, N. H. Axelsen, K. Bonch-Christensen, and M. Magnusson.** 1980. The antigens of *Mycobacterium bovis*, strain BCG, studied by crossed immunoelectrophoresis: a reference system. Scand. J. Immunol. **12**:249–263.
4. **Collins, F. M., J. R. Lamb, and D. B. Young.** 1988. Biological activity of protein antigens isolated from *Mycobacterium tuberculosis* culture filtrate. Infect. Immun. **56**:1260–1266.
5. **Daniel, T. M., and L. E. Ferguson.** 1970. Purification and characterization of two proteins from culture filtrates of *Mycobacterium tuberculosis* H37 Ra strain. Infect. Immun. **1**:164–168.
6. **De Bruyn, J., R. Bosmans, M. Turneer, M. Weckx, J. Nyabenda, J. P. van Vooren, P. Falmagne, H. G. Wiker, and M. Harboe.** 1987. Purification, partial characterization, and identification of a skin-reactive protein antigen of *Mycobacterium bovis* BCG. Infect. Immun. **55**:245–252.
7. **Harboe, M., O. Closs, B. Bjorvatn, G. Kronval, and N. H. Axelsen.** 1977. Antibody response in immunization with *Mycobacterium leprae*. Infect. Immun. **18**:792–805.
8. **Hunter, S. W., H. Gaylord, and P. J. Brennan.** 1986. Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. J. Biol. Chem. **261**:12345–12351.
9. **Huygen, K., J. P. van Vooren, M. Turneer, R. Bosmans, P. Dierckx, and J. De Bruyn.** 1988. Specific lymphoproliferation, gamma interferon production, and serum immunoglobulin G directed against a purified 32 KDa mycobacterial protein antigen (P32) in patients with active tuberculosis. Scand. J. Immunol. **27**:187–194.
10. **Kannan, K. B., V. M. Katoch, V. D. Sharma, and V. D. Bharadwaj.** 1987. Extracellular enzymes of mycobacteria. FEMS Microbiol. Lett. **48**:31–33.
11. **Kaufmann, S. H. E., S. Chiplunkar, I. Flesch, and G. De Libero.** 1986. Possible role of helper and cytolytic T cells in mycobacterial infections. Lepr. Rev. **57**:101–111.
12. **Klatser, P. R., M. M. van Rens, and A. Eggelte.** 1984. Immunochemical characterization of *Mycobacterium leprae* antigens by the SDS-polyacrylamide gel electrophoresis immunoperoxidase technique (SGIP) using patients' sera. Clin. Exp. Immunol. **56**:537–544.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Lovik, M., and O. Closs.** 1984. Induction of immunity against live *Mycobacterium lepraemurium*: a requirement for viable bacilli. Immunology **53**:165–173.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Matsuo, K., R. Yamaguchi, A. Yamazaki, H. Tasaka, and T. Yamada.** 1988. Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular α antigen. J. Bacteriol. **170**: 3847–3854.
17. **Muller, I., S. P. Cobbold, H. Waldmann, and S. H. E. Kaufmann.** 1987. Impaired resistance to *Mycobacterium tuberculosis* infection after selective in vivo depletion of $L3T4^+$ and $Lyt\text{-}2^+$ T cells. Infect. Immun. **55**:2037–2041.
18. **Nagai, S., J. Matsumoto, and T. Nagasuga.** 1981. Specific skin-reactive protein from culture filtrate of *Mycobacterium bovis* BCG. Infect. Immun. **31**:1152–1160.
19. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
20. **Ratliff, T. L., J. A. Mcgarr, C. Abou-Zeid, G. A. W. Rook, J. L. Stanford, J. Aslanzadeh, and E. J. Brown.** 1988. Attachment of mycobacteria to fibronectin-coated surfaces. J. Gen. Microbiol. **134**:1307–1313.
21. **Ridley, D. S., and W. H. Jopling.** 1966. Classification of leprosy according to immunity. A five-group system. Int. J. Lepr. **34**:255–271.
22. **Rook, G. A. W.** 1980. The immunogenicity of killed mycobacteria. Lepr. Rev. **51**:295–301.
23. **Rumschlag, H. S., T. M. Shinnick, and M. L. Cohen.** 1988. Serological responses of patients with lepromatous and tuberculoid leprosy to 30-, 31-, and 32-kilodalton antigens of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **26**:2200–2202.
24. **Steinhoff, U., and S. H. E. Kaufmann.** 1988. Specific lysis by $CD8^+$ T cells of Schwann cells expressing *Mycobacterium leprae* antigens. Eur. J. Immunol. **18**:969–972.
25. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.

26. **Wiker, H. G., M. Harboe, and T. E. Lea.** 1986. Purification and characterization of two protein antigens from the heterogeneous BCG 85 complex in *Mycobacterium bovis* BCG. Int. Arch. Allergy Appl. Immunol. **81**:298–306.
27. **Wiker, H. G., M. Harboe, S. Nagai, M. E. Patarroyo, C. Ramirez, and N. Cruz.** 1986. MPB 59, a widely cross-reacting protein of *Mycobacterium bovis* BCG. Int. Arch. Allergy Appl. Immunol. **81**:307–314.
28. **Yoneda, M., and Y. Fukui.** 1961. Extracellular proteins of tubercle bacilli. I. Zone-electrophoresis purification of the proteins of a virulent strain (H37 Rv) of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. Suppl. **92**:9–18.
29. **Young, D. B.** 1988. Structure of mycobacterial antigens. Br. Med. Bull. **44**:562–583.
30. **Zinkernagel, R. M., and P. C. Doherty.** 1979. MHC-restricted cytotoxic T cells: studies on the biological role of determining T-cell restriction—specificity, function and responsiveness. Adv. Immunol. **27**:51–177.