

Serology and Leprosy: Immunoassays Comparing Immunoglobulin G Antibody Responses to 28- and 30-Kilodalton Proteins Purified from *Mycobacterium bovis* BCG

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Two major proteins from *Mycobacterium bovis* BCG culture filtrates with molecular masses of 28 kDa (P₂₈) and 30 kDa (P₃₀), identified as components of the BCG 85 complex, were purified and used in enzyme-linked immunosorbent assays (ELISAs) for the determination of specific immunoglobulin G (IgG) levels in patients with leprosy or tuberculosis or with exposure to these diseases. High reactivity to both antigens was observed with sera from lepromatous leprosy patients, whereas antibody levels in sera from paucibacillary leprosy patients were not significantly different from those in sera from healthy individuals from an area in which leprosy is endemic. High IgG responses were also found in some contacts of lepromatous leprosy patients. A comparison of the levels of anti-P₂₈ and anti-P₃₀ within the multibacillary leprosy patient group showed much higher IgG reactivity to P₂₈ than to P₃₀, suggesting that the antibody response of lepromatous patients is directed predominantly against the 28-kDa protein. A high degree of correlation in values of ELISAs based on P₂₈ and on the phenolic glycolipid of *Mycobacterium leprae* was observed in all groups analyzed. The potential use of an assay based on the 28-kDa protein to selectively distinguish individuals destined to develop multibacillary leprosy is discussed, as also is the likelihood that the 28-kDa–30-kDa complex, part of the fibronectin-binding family, is an important component of *M. leprae*.

Infection with mycobacteria induces a complex immune response in the host which involves both cellular immune reactions and antibody production. It is also known that immunological resistance to infection is primarily dependent on mechanisms of cell-mediated immunity (2), while the role played by the humoral immune response remains ill defined.

Evidence now suggests that antibodies and activated T cells can interact and influence each other, affecting immunity and pathogenetic mechanisms in mycobacterial infections. In tuberculosis, immunosuppression has been associated with increased levels of antibodies against mycobacteria (9). Similarly, there is a negative correlation in leprosy between specific cell-mediated immunity and the levels of circulating antibodies. Generally, lepromatous patients have abnormally high levels of antibodies and weak cell-mediated immunity, whereas tuberculoid patients have low antibody levels and strong cell-mediated immunity (2).

In recent years, several mycobacterial components that constitute major targets of antibody response in leprosy patients have been defined and characterized. Dominant carbohydrate-containing epitopes of *Mycobacterium leprae* are found in the specific phenolic I (PGL-I) and the cross-reactive lipoarabinomannan (4). The reactivity frequencies against these antigens in sera from lepromatous patients are relatively high in comparison with those in tuberculoid sera. In addition, some dominant antibody-reactive epitopes have been identified in proteins with apparent molecular masses of 28, 35, and 36 kDa (5, 13, 22).

In a recent report, we were able to identify components in the short-term culture filtrates of *Mycobacterium bovis* that were markers of an appreciable humoral immune response in leprosy (19). The most reactive fractions were composed of a 28- to 30-kDa protein doublet, according to polyacrylamide gel electrophoresis, indicating that these proteins may act as markers in diagnosing this form of the disease.

In the present study, the individual 28- and 30-kDa components of the 28- to 30-kDa doublet were resolved and identified as components of the BCG 85 complex (7, 25, 26), the mycobacterial family of fibronectin-binding proteins (1, 20). The antibody levels against these purified antigens were evaluated in sera from leprosy patients, household leprosy contacts, and control groups, suggesting a differential response to the two antigens.

MATERIALS AND METHODS

Sera. The test group consisted of 109 serum samples from leprosy patients registered for treatment at the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. All patients were clinically and histologically classified by the Ridley and Jopling scale and, on the basis of these results, divided into a polar lepromatous (LL) group consisting of 22 patients, a borderline lepromatous (BL) group (47 patients), an indeterminate leprosy group (8 patients), and a borderline tuberculoid leprosy group (32 patients). The majority of the patients had either been untreated or commenced chemotherapy; none of the patients had been treated for more than 2 years. Sera from 115 clinically healthy household contacts (HC) of leprosy patients were also studied; the majority of these were contacts of multibacillary patients.

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Sera representing the control groups were obtained from (i) healthy subjects, 20 of whom were members of the Leprosy Department staff at the Oswaldo Cruz Foundation and 42 of whom were individuals living in areas in which leprosy is endemic, and (ii) patients (35 in number) with active pulmonary tuberculosis (TB) who were under treatment.

Antigens. Culture filtrates were obtained from *M. bovis* BCG substrain Moreau as previously described (19). Proteins were concentrated by precipitation with ammonium sulfate at 50% saturation. The precipitate was then used to prepare a partially purified fraction containing the 28- to 30-kDa doublet (called P₂₈-P₃₀), the purified 28-kDa protein (P₂₈), and the purified 30-kDa protein (P₃₀). The P₂₈-P₃₀ fraction was obtained as follows. The ammonium sulfate precipitate was dissolved in 50 mM phosphate buffer, pH 7.0, with 2% butanol and 0.5 M NaCl and applied to a column (1.5 by 130 cm) of Sephadex G-75 superfine (Pharmacia Fine Chemicals, Uppsala, Sweden) which was equilibrated and eluted with the same buffer at a flow of 5 ml/h. The second major peak with an A₂₈₀ consisted of a mixture of P₂₈ and P₃₀.

The P₂₈ and P₃₀ antigens were purified by a modification of the procedures described elsewhere (25, 26). Briefly, the ammonium sulfate precipitate was suspended in 10 mM phosphate buffer, pH 6.8, and applied to a column (2.3 by 4 cm) of phenyl-Sepharose (Pharmacia Fine Chemicals) previously equilibrated with the same buffer. Proteins were eluted first with 10 mM Tris-glycine buffer, pH 8.9, and then with a linear concentration (0 to 50%) gradient of ethylene glycol in the same buffer. All fractions of the eluate were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a 12.5% acrylamide gel (14). Further purification of P₂₈ and P₃₀ was accomplished on Sephadex G-50 or DEAE-Sephacel (Pharmacia Fine Chemicals). Sephadex G-50 chromatography employed a column (0.8 by 85 cm) equilibrated with 50 mM phosphate buffer, pH 7.0, with 0.5 M NaCl and 2% butanol and a flow rate of 12 ml/h. The DEAE-Sephacel column (1.5 by 4 cm) was equilibrated with 30 mM Tris-HCl buffer, pH 8.9, and eluted with a linear gradient of NaCl (70 to 150 mM).

Purified antigens were analyzed by two-dimensional electrophoresis with separation in the first dimension by isoelectric focusing and in the second dimension by SDS-PAGE as described by O'Farrel (18). Gels were silver stained or transblotted to nitrocellulose membranes by the method of Towbin et al. (24). Blots were developed with a pool of serum samples from multibacillary leprosy patients as previously described (19). The protein concentration was estimated by the method of Lowry et al. (16). Purified antigens were diluted 1:2 in buffered glycerol and stored in aliquots at -20°C.

Identification of the purified proteins through immunoassay. The identification of P₂₈ and P₃₀ as components of the BCG 85 complex was performed by dot enzyme-linked immunosorbent assay (ELISA) using specific rabbit antisera against the BCG 85A and BCG 85B antigens (kindly provided by Morten Harboe, Institute of Immunology and Rheumatology, University of Oslo). P₂₈ and P₃₀ were spotted onto nitrocellulose paper (Bio-Rad Laboratories, Richmond, Calif.). After blocking with 1% bovine serum albumin (BSA) and 0.01% Tween 80 in 0.01 M phosphate-buffered saline, pH 7.2 (PBS), the nitrocellulose was incubated in antisera previously diluted 1:2,000 in PBS-Tween. Peroxidase-conjugated goat anti-rabbit immunoglobulins (Cappel, Cooper Biomedical, Inc., Malvern, Pa.) diluted 1:4,000 was

used as the secondary antibody. Color development was performed with a 3,3',5,5'-tetramethylbenzidine substrate mixture (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.).

Enzyme immunoassays. The ELISA for antibody determination was based on the method of Engvall and Perlmann (10). The antigens P₂₈-P₃₀, P₂₈, and P₃₀ dissolved in 50 mM carbonate buffer, pH 9.6, at concentrations of 0.5, 0.5, and 1.0 µg/ml, respectively, were applied to flat-bottom polyvinyl chloride microtiter plates (Dynatech Laboratories, Chantilly, Va.) in amounts of 100 µl per well and incubated for 1 h at 37°C. Each well was washed three times with 200 µl of PBS containing 0.3% Tween 20 (PBST), and 100 µl of serum diluted 1:100 in PBST containing 10% bovine serum was added. After incubation for 30 min at 37°C, the wells were washed, and then 100 µl of peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (Bio-Manguinhos, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil) diluted 1:100,000 in PBST containing 10% bovine serum was added. After 1 h at 37°C the washes were repeated, and then 100 µl of a freshly prepared solution of 3,3',5,5'-tetramethylbenzidine in 0.15 M citrate-phosphate buffer, pH 5.0, containing 0.3% hydrogen peroxide was added. After 30 min, the reaction was stopped with 50 µl of 4 N H₂SO₄ and the A₄₅₀ was measured in a Titertek Multiskan Plus ELISA reader (Flow Laboratories, Helsinki, Finland). Wells without sera and without antigen and sera were used as controls for nonspecific conjugate binding. In each experiment, there was always one negative and one positive reference serum sample. The mean value of the positive reference serum in independent experiments was taken as a constant reference value and used to correct variations for plate-to-plate and day-to-day assays.

The anti-PGL-I antibody assay was conducted with the disaccharide-containing neoantigen described previously (6) and flat-bottom polystyrene microtiter plates (Immulon II Dynatech); the A₄₉₂ was measured.

Differences between groups in each ELISA and between assays were assessed for significance by using a multivariate analysis of variance model, procedure GLM from the SAS statistical package (SAS Institute Inc., Cary, N.C.).

RESULTS

Analysis of the isolated 28-kDa, 30-kDa, and 28- to 30-kDa combination antigens. The three antigen preparations that were the basis of the present study were subjected to two-dimensional electrophoresis and examined by chemical stain and by the extent of reaction against a pool of sera from LL and BL patients (Fig. 1). The partially purified P₂₈-P₃₀ doublet fraction showed considerable heterology in the 28- to 30-kDa region; all components reacted with the pool of LL-BL sera. In addition, other components (spots 7, 8, and 9) were identified in the silver-stained gel. Two proteins of the same apparent molecular mass, 28 kDa, were observed in the purified P₂₈ fraction on two-dimensional analysis, and both were serologically reactive. The purified protein fraction of 30 kDa showed the presence of three components, all of which reacted with the pooled LL-BL sera. Immunoassay of the purified antigens with monospecific sera showed that they correspond to components of the BCG 85 complex (Fig. 2). Furthermore, the more intensive reactivity observed between P₂₈ and anti-BCG 85B and between P₃₀ and anti-BCG 85A suggests that P₂₈ and P₃₀ correspond to BCG 85B and BCG 85A, respectively.

Measurement of antibody levels to the 28- to 30-kDa doublet

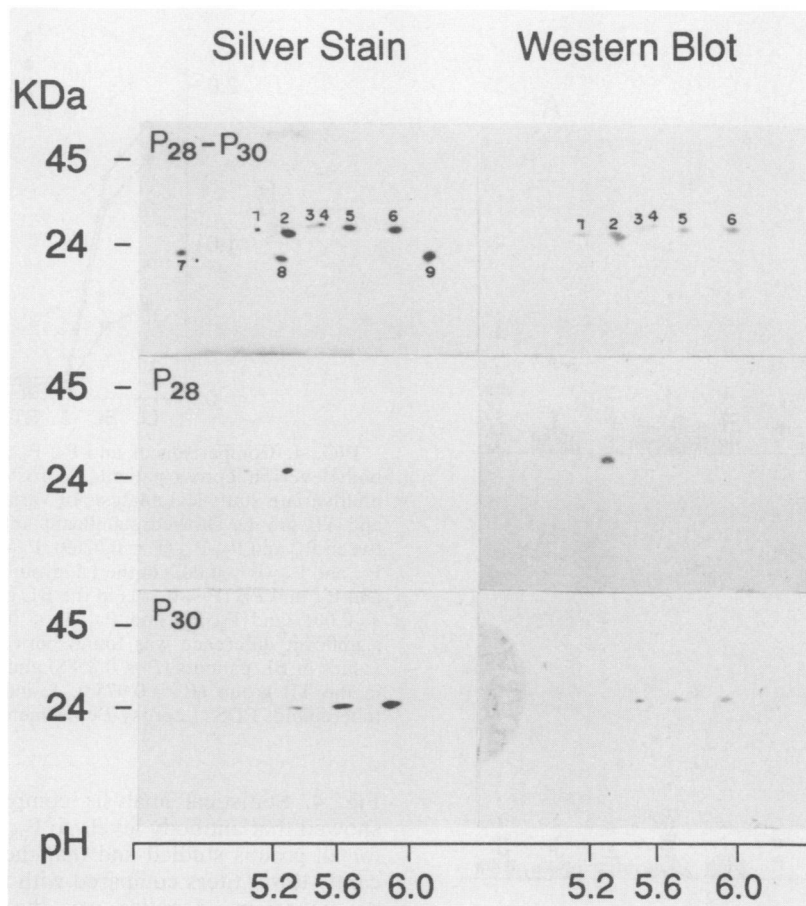


FIG. 1. Two-dimensional fractionation of antigens purified from BCG culture medium. First dimension, isoelectric focusing; second dimension, SDS-PAGE. Blots were revealed with a pool of sera from LL-BL patients. P₂₈-P₃₀ antigen, 8 µg; purified P₂₈, 4 µg; and purified P₃₀, 4 µg. Approximate molecular masses of standard proteins are indicated along the left edge.

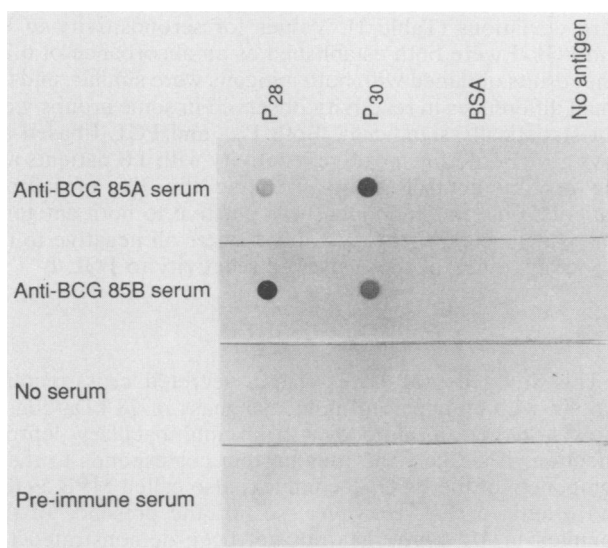


FIG. 2. Immunoassay identification of P₂₈ and P₃₀ proteins. Dot ELISA was conducted using 0.5 µg of purified proteins. BSA was spotted as a negative control. Control incubations were performed with PBS-Tween and a rabbit preimmune serum.

fractions. The levels of IgG antibodies to the P₂₈-P₃₀, P₂₈, and P₃₀ antigens were estimated in sera from leprosy patients, healthy HC, and control groups (Fig. 3). The sera from the control groups contained low IgG titers against the three antigens assayed. On the other hand, much higher antibody titers were observed in sera from multibacillary leprosy patients; both LL and BL patients showed a mean antibody activity significantly higher than that of the healthy control groups ($P < 0.001$). Differences in the mean values between the two multibacillary groups were statistically significant in all assays, with a lower titer in the BL group. There was a wide variation in antibody content in individual sera from patients with multibacillary leprosy. No significant differences were observed among patients with indeterminate and borderline tuberculoid leprosy and the healthy control groups in all three assays. The mean value of the HC group was also low in all assays and not significantly different from that of the healthy control groups. Some contacts, however, showed considerably high titers, especially to the P₂₈-P₃₀ and P₂₈ fractions. One contact showed high IgG titers to the three antigens. Two contacts demonstrated high antibody activity to both the P₂₈-P₃₀ and P₂₈ fractions, and three others had high IgG levels only to the P₂₈-P₃₀ fraction or to the P₂₈ fraction.

Patients with active pulmonary TB showed low mean values against the three antigen preparations, and they were not significantly different compared with values from the

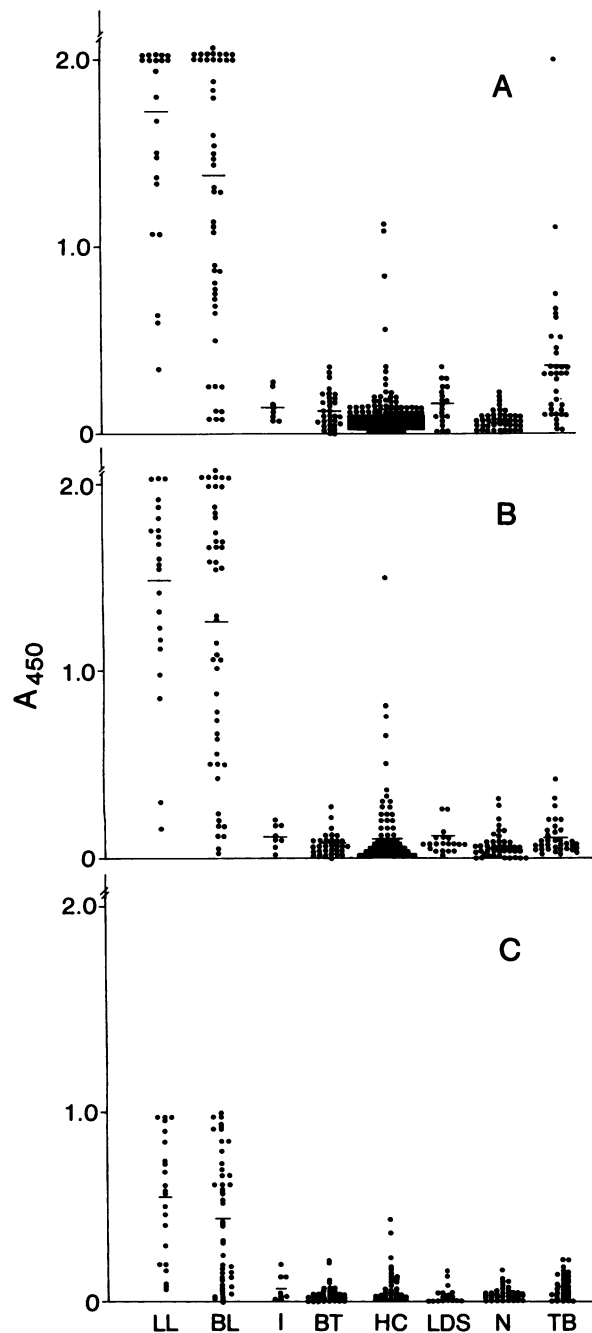


FIG. 3. IgG anti- P_{28} - P_{30} (A), anti- P_{28} (B), and anti- P_{30} (C) antibody levels determined by ELISA in sera from leprosy patients, HC of leprosy patients, and control groups. Individuals were tested in duplicate; each point represents one subject. Leprosy patients were grouped as follows: 22 LL, 47 BL, 8 indeterminate (I), and 32 borderline tuberculoid (BT). One hundred fifteen leprosy HC were assayed. Control individuals were 42 healthy subjects (N), 20 members of the Leprosy Department staff (LDS), and 35 active pulmonary TB patients. The horizontal bars represent mean values.

healthy control groups. On the other hand, a number of individual serum samples showed high IgG anti- P_{28} - P_{30} antibody titers.

The profile of the mean IgG levels obtained throughout the groups as assayed with the three antigens is summarized in

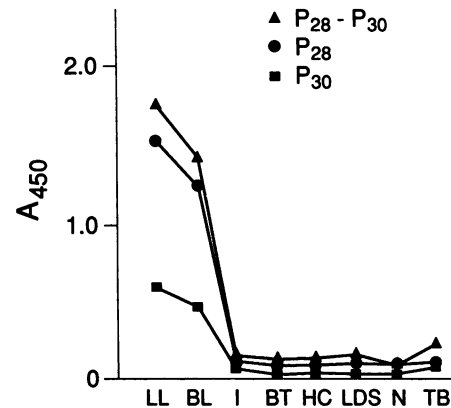


FIG. 4. Comparison of anti- P_{28} - P_{30} , anti- P_{28} , and anti- P_{30} antibody levels in leprosy patients, leprosy HC, and control groups. A multivariate statistical analysis of variance of the critical LL, BL, and TB groups showed significant antibody level differences between P_{28} and P_{28} - P_{30} ($P = 0.0269$), P_{28} - P_{30} and P_{30} ($P < 0.001$), and P_{28} and P_{30} ($P < 0.001$) in the LL group; P_{28} - P_{30} and P_{30} ($P < 0.001$) and P_{28} and P_{30} ($P < 0.001$) in the BL group; and P_{28} - P_{30} and P_{28} ($P < 0.001$) and P_{28} - P_{30} and P_{30} ($P < 0.001$) in the TB group. No significant difference was found between P_{28} - P_{30} and P_{28} ELISA values in BL patients ($P = 0.0588$) and between P_{28} and P_{30} values in the TB group ($P = 0.0758$). I, indeterminate; BT, borderline tuberculoid; LDS, Leprosy Department staff; N, healthy control.

Fig. 4. Statistical analysis comparing the three ELISAs showed that antibody levels to P_{28} - P_{30} and P_{28} were similar for all groups studied and that the P_{30} test showed significantly lower titers compared with titers in the other assays. An important exception was the TB group, for which a significantly higher titer was shown in the P_{28} - P_{30} test compared with titers in the P_{28} ($P = 0.002$) and the P_{30} tests.

Comparison of levels of antibody to the P_{28} protein and PGL-I. Sera from a group of individuals were assayed for both IgM anti-PGL-I and IgG anti- P_{28} , and the ELISA values were compared. In both tests, a serum sample was considered positive when the absorbance exceeded the mean of the results obtained from 42 healthy controls by 3 standard deviations (Table 1). Values for seropositivity to P_{28} and PGL-I were both established as an absorbance of 0.28. The results obtained with both antigens were similar, and the small differences in reactivity observed in some groups were not statistically significant. Both P_{28} - and PGL-I-based assays gave borderline positive reactivity with TB patients and the healthy control groups. Among the HC of leprosy patients, only one individual was positive to both antigens. The paucibacillary leprosy patients were all negative to the P_{28} assay; a few of these showed reactivity to PGL-I.

DISCUSSION

This study demonstrates that a secreted cross-reactive protein with an apparent molecular mass of 28 kDa constitutes a target of antibodies from multibacillary leprosy infection. It is likely that this protein corresponds to the B component of the BCG 85 complex, also called MPB 59 (26) and α -antigen (17). However, so far, the presence of this complex in *M. leprae* has not yet been demonstrated (1). Nevertheless, the results described to date indicate that these proteins are expressed during *M. leprae* infection of humans and may play an important role in immunity, if not in pathogenic mechanisms in leprosy. The biological role of

TABLE 1. Comparison between anti-P₂₈ and anti-PGL-I antibody levels in sera from leprosy patients, HC of leprosy patients, and control groups

Group ^a	No. of serum samples	ELISA result				No. of positive serum samples in both assays
		P ₂₈		PGL-I		
		Mean value ± SD	No. of positive serum samples	Mean value ± SD	No. of positive serum samples	
LL	22	1.45 ± 0.52	21	1.30 ± 0.72	22	21
BL	40	1.17 ± 0.71	35	1.31 ± 0.66	38	34
I	8	0.12 ± 0.06	0	0.15 ± 0.15	1	0
BT	29	0.08 ± 0.06	0	0.11 ± 0.12	3	0
HC	110	0.11 ± 0.19	9	0.10 ± 0.24	7	1
LDS	20	0.10 ± 0.07	0	0.06 ± 0.07	0	0
N	42	0.07 ± 0.07	1	0.04 ± 0.08	2	0
TB	19	0.11 ± 0.10	2	0.09 ± 0.14	2	1

^a I, indeterminate; BT, borderline tuberculoid; LDS, Leprosy Department staff; N, healthy control.

this protein remains unknown. However, there is an intriguing recent report ascribing fibronectin-binding properties to the BCG 85 antigens and showing that these proteins are able to mediate interaction of whole bacteria with fibronectin (20). Fibronectin-binding proteins play a role in virulence of some pathogenic microorganisms (for a review, see reference 11). The significance of this fibronectin-binding activity of mycobacteria is not known, although some potential roles in host-parasite interactions have been discussed previously (1, 20).

Multibacillary leprosy patients showed significantly higher IgG antibody levels against all antigens assayed compared with levels in the healthy control groups. On the other hand, low antibody levels were detected in paucibacillary patients, not significantly different from levels in the healthy control groups. These results are in accordance with those described in other reports showing that there is an increase from BT to LL in the incidence of serum antibodies which react with mycobacterial antigens (15, 23). However, an important observation in this context is the selective reactivity of sera from lepromatous patients in contrast to sera from tuberculoid patients. Healthy controls, as well as healthy HC of leprosy cases, also showed low antibody activities to all of the three antigens assayed, suggesting that previous exposure to *M. leprae* or other mycobacteria did not evoke a detectable antibody response to these cross-reactive antigens. Furthermore, tuberculin testing and BCG vaccination seem to have no influence on antibody response against these proteins, since some of the subjects from the groups studied were BCG vaccinated and reactive to purified protein derivative. Comparison between anti-P₂₈-P₃₀ and anti-P₂₈ IgG antibody levels in multibacillary leprosy patients (LL and BL) indicates similar values in both assays (Fig. 4). On the other hand, significantly lower anti-P₃₀ IgG antibody levels were observed in these patients, suggesting that the antibody response is predominantly directed against the P₂₈ component of the 28- to 30-kDa doublet. The lower anti-P₃₀ antibody titer could also result from a poor adherence of P₃₀ to the test wells. However, the high structural homology between P₂₈ and P₃₀ (3) indicates that the binding capacity of these antigens should be equivalent. Moreover, these results suggest that P₂₈-specific epitopes, and not those shared with P₃₀, are the targets of antibody response in multibacillary patients.

A high humoral immune response against these BCG components was also detected in some HC of multibacillary patients. However, only one individual showed positive

results to both P₂₈ ELISA and PGL-I ELISA. This person could be considered as having a typical subclinical multibacillary infection, since he maintained a positive bacteriological index without any clinical manifestation of leprosy during 2 years of follow-up. Quite recently this individual developed a multibacillary form of the disease. Such individuals with preclinical lepromatous infection are considered the most important link in the transmission of the disease (2), and the development of a specific test for their detection has been one of the main goals in leprosy research. These preliminary results indicate that an assay based on P₂₈ protein in combination with the PGL-I ELISA may be useful in detecting more efficiently individuals prone to develop lepromatous forms of the disease in areas in which leprosy is endemic. A larger sample of individuals at risk tested with both P₂₈ and PGL-I ELISAs over a period of years will give more information regarding the utility of these tests.

All of these latest results confirm and extend our previous data (19) and are in agreement with information recently published by others (8, 21). On the other hand, a 32-kDa protein, isolated from BCG culture medium and identified as the A component of the BCG 85 complex, was shown to be immunodominant when antibodies were evaluated in active pulmonary TB (12). It is now our considered opinion, based on the above and other evidence, that the P₃₀ component of the 28- to 30-kDa doublet corresponds to the BCG 85A and therefore to the P₃₂ protein. Accordingly, the discrepancy in these two sets of results may lie in the different assays, in the BCG strain used for antigen purification, or in the status of the TB patients that are the basis of the assays.

In conclusion, the results presented in this study indicate the potential for an assay based on a secreted protein with an apparent molecular weight of 28 kDa to selectively identify individuals with lepromatous leprosy infection. This protein likely corresponds to the α BCG antigen recently cloned and expressed in *Escherichia coli* (17) and is therefore available in substantial amounts for extended serological studies. In addition, the evidence that this protein is equivalent to the fibronectin-binding protein demands further definition of physiological roles in host-parasite interactions.

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