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Serological Specificity of Phenolic Glycolipid I from *Mycobacterium leprae* and Use in Serodiagnosis of Leprosy

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The serological activities of the specific phenolic glycolipid I from *Mycobacterium leprae*, its dissected parts, and related glycolipids from other mycobacteria were examined by enzyme-linked immunosorbent assay against hyperimmune anti-*M. leprae* rabbit antiserum and sera from patients with leprosy and other mycobacterial diseases. High anti-phenolic glycolipid I immunoglobulin M antibodies were found in 23 of 24 (96%) of lepromatous leprosy patients on short term chemotherapy and in 8 of 13 tuberculoid leprosy patients (62%). Sera from patients with tuberculosis or atypical mycobacterial infections were devoid of anti-phenolic glycolipid I activity. The structurally related phenolic glycolipids from *Mycobacterium kansasii* and *Mycobacterium bovis* and the aglycone segments of the *M. leprae* product showed no significant activity. Thus, the trisaccharide determinant of phenolic glycolipid I is specific in its structure, serological activity, and, to a lesser extent, the antibody class it evokes.

The recent isolation of a number of unique phthiocerol-containing lipids from the surface of *Mycobacterium leprae* (9, 10) affords the unprecedented opportunity to relate specific, fully defined, leprosy bacillary moieties to the immunological responses of leprosy patients. Since these lipids comprise the bulk of the copious outer bacillary extensions (9), they may represent the primary functional interface between the organism and the host's defense. The principal lipid is a diacylphthiocerol (9), but the more interesting are three triglycosylphenolic diacylphthiocerols, two of which have been fully characterized and shown to contain the previously unrecognized trisaccharide appendages: 3,6-di-*O*-methyl-β-D-glucopyranosyl(1→4)2,3-di-*O*-methyl-α-L-rhamnopyranosyl(1→2)3-*O*-methyl-α-L-rhamnopyranose (in phenolic glycolipid I [Phen GL-I]) and 6-*O*-methyl-β-D-glucopyranosyl(1→4)2,3-di-*O*-methyl-α-L-rhamnopyranosyl(1→2)3-*O*-methyl-α-L-rhamnopyranose (in phenolic glycolipid III) (9, 10). Since the trisaccharides hold the promise of the long-sought *M. leprae*-specific antigen determinants, immediate interest concerns the potential of these trisaccharides for the specific serodiagnosis of leprosy. To this end, the activities of the native phenolic glycolipid I and its dissected components were examined against anti-*M. leprae* rabbit antiserum and sera from leprosy patients across the clinicohistopathological spectrum of the disease. In addition, the activity of a closely related triglycosylphenolic diacylphthiocerol from *Mycobacterium kansasii* and that of sera from patients with mycobacterial infections other than leprosy were examined to better delineate the specificity of the glycolipid antigen for serological assessment of leprosy.

MATERIALS AND METHODS

Source of lipids. Lipids were obtained by extraction of *M. leprae*-infected armadillo tissues and fractionated on silicic acid columns (10). Dimycocerosyl phthiocerol was eluted with $CHCl_3$ and purified by preparative thin-layer chromatography (TLC) in $CHCl_3$ (9). Phthiocerol was derived from it by alkalinolysis and fully purified by preparative TLC (9). Phen GL-I was obtained by further irrigation of silicic acid columns with 2% CH_3OH in $CHCl_3$, followed by preparative TLC in ether-acetone (8:2) (10). Deacylated Phen GL-I and its fatty acids were derived from the native glycolipid by alkalinolysis followed by separation of the component parts on silicic acid (10). The deglycosylated phenolic phthiocerol core was derived from the deacylated Phen GL-I by methanolysis (10). Preparation and characterization of the triglycosylphenolic diacylphthiocerol (mycoside A) from *M. kansasii* has been described (8). The monoglycosylphenolic diacylphthiocerol (mycoside B) of *M. bovis* was extracted from lyophilized BCG with $CHCl_3$–CH_3OH (2:1), and partially purified by elution from silicic acid columns with $CHCl_3$–1% methanol in $CHCl_3$. Final purification was accomplished by preparative TLC in diethyl ether-acetone (9:1). The structure was examined by procedures described previously (9, 10), and that reported by Demarteau-Ginsburg and Lederer (5) was confirmed and amplified. It is a mixture of 27-[p(2-O-methyl-α-L-rhamnopyranosyloxy)phenyl]-9,11-dimycocerosyl-3-methoxyl-4-methyl-heptacosane, and 29-[p(2-O-methyl-α-L-rhamnopyranosyloxyphenyl]-9,11-dimycocerosyl-3-methoxyl-4-methyl-nonacosane (C. V. Knisley and P. J. Brennan, unpublished data).

Sera. Hyperimmune antiserum was obtained from rabbits immunized with *M. leprae* purified from armadillo liver and emulsified in incomplete Freund adjuvant. Animals were inoculated intramuscularly and bled 7 weeks after the final boost. Human sera were obtained from individual leprosy patients attending the clinic of the U.S. Public Health Service Hansen's Disease Program, Seton Medical Center, Dale City, Calif. Patients were classified clinically and pathologically according to the Ridley and Jopling scale (14). A pool of serum was obtained by mixing equal volumes of sera from several patients (of R. H. Gelber) with lepromatous leprosy. Sera were also obtained from patients with mycobacterial infections other than *M. leprae* attending National Jewish Hospital and Research Center, Denver, Col. In all cases, clinical symptoms of disease were evident, acid-fast bacilli were detected in sputa or other body fluids or organs, and mycobacteria had been cultured and, in most cases, identified by biochemical means, serology, and TLC of the specific lipid antigens (4). The atypical mycobacteria isolated were *M. kansasii*, *M. fortuitum*, or one of the various *M. intracellulare* serotypes. Normal sera were obtained from students and graduating Doctors of Veterinary Medicine at Colorado State University.

ELISA conditions. For enzyme-linked immunosorbent assay (ELISA) analysis of human sera, lipids (100 μg/ml) were suspended in carbonate-bicarbonate coating buffer (pH 9.6; reference 16) by direct sonication for 20 to 30 s with a 3-mm probe. The suspension was diluted to the required concentration with the same buffer; 50 μl was added to wells of polystyrene microtiter plates which were incubated at 37°C for 14 to 16 h in a moist chamber. Wells were washed with phosphate-buffered saline (PBS), blocked by the addition of 100 μl of PBS containing 5% bovine serum albumin (BSA), and incubated at 37°C for 1 h in a moist chamber. The contents were aspirated, and 50 μl of human serum diluted with PBS containing 20% normal goat serum (PBS-NGS) was added. Plates were incubated at 37°C for 1 h, washed with PBS, followed by the addition of goat anti-human immunoglobulin M (IgM)- or immunoglobulin G (IgG)-peroxidase conjugate reagent (Cappel Laboratories, Downington, Pa.) diluted 1/1,000 in PBS-NGS. After a 1-h incubation and five further washings, 50 μl of H_2O_2-O-phenylenediamine substrate-dye reagent in citrate phosphate buffer (16) were added and incubated at 37°C for 30 min in the case of the IgM conjugate and 2 h for the IgG conjugate. Reactions were terminated with 2.5 N H_2SO_4 after a 30-min incubation and the absorbance was read at 488 nm (A_{488}). ELISA was also conducted with whole *M. leprae* suspended in coating buffer (50 μg/ml). ELISA on hyperimmune rabbit serum was conducted as described (10). To abolish IgM activity, reduction with 2-mercaptoethanol was performed as described by Melsom and Duncan (12). IgG was separated from IgM with a commercial IgM isolation kit (Isolab Inc., Akron, Ohio) based on the procedure described by Johnson and Libby (11).

Statistics. Statistical analysis was performed by using the group comparison Student *t* test.

RESULTS

Development of ELISA. The ELISA conditions described previously using hyperimmune anti-*M. leprae* antiserum were suitable for demonstrating the inherent serological activity of native Phen GL-I, but the few human sera tested were marked by abnormally high background absorbances (10). In another set of conditions, in which ethanol was employed as the coating medium and PBS-Tween was used for nonspecific blocking and as the diluent, intact Phen GL-I reacted poorly, and deacylated Phen GL-I did not react at all; presumably, it did not adsorb and was lost as the fluid phase.

Variation of the conditions basically designed for protein antigens (7, 16) proved to be the most reproducible for Phen GL-I. The important features of the procedure seem to be thorough sonication of the glycolipid in the coating buffer, deletion of detergents from all subsequent steps, use of a double block with heterologous BSA and homologous NGS, and also use of the latter as a diluent. Figure 1 shows the activity observed with the pooled human lepromatous leprosy sera and a range of glycolipid concentrations with either the IgM- or IgG-conjugated reagent. IgM responses were uniformly much higher than those of IgG. When pooled sera from lepromatous leprosy patients were reduced with 2-mercaptoethanol all anti-Phen GL-I IgM activity was abolished, and only the low residual IgG activity remained. When the majority of IgG subclasses were removed from IgM by aminoethyl–Sephedex A-50 ion-exchange chromatography (11), there was little diminution in activity; the correlation coefficient between the A_{488} values for purified IgM and for whole serum IgM was 0.992. Thus, the reactive immunoglobulin class is mostly IgM. The lower limit of antigen reactivity was about 250 ng/ml, i.e., 12.5 ng or 6 pmol per well. Antibody titration of the pooled active sera showed an approximate linear relationship between absorption and dilution of serum pool in the range 1:100 to 1:6,400. Based on several trial experiments with pooled active and negative sera, a dilution of 1:300 was chosen, and plates were routinely coated with 2 μg of Phen GL-I per ml of buffer (100 ng per well) at 37°C for 14 to 16 h. Under these conditions, an absorbance value of 0.972 ± 0.100 (triplicate) for the pooled active sera was obtained compared with a value of 0.048 ± 0.016 (triplicate) for pooled sera from healthy controls.

Specificity of Phen GL-I. To compare the serological activity of the native glycolipid with its various entities, Phen GL-I and the dimycocerosyl phthiocerols were subjected to alkaline

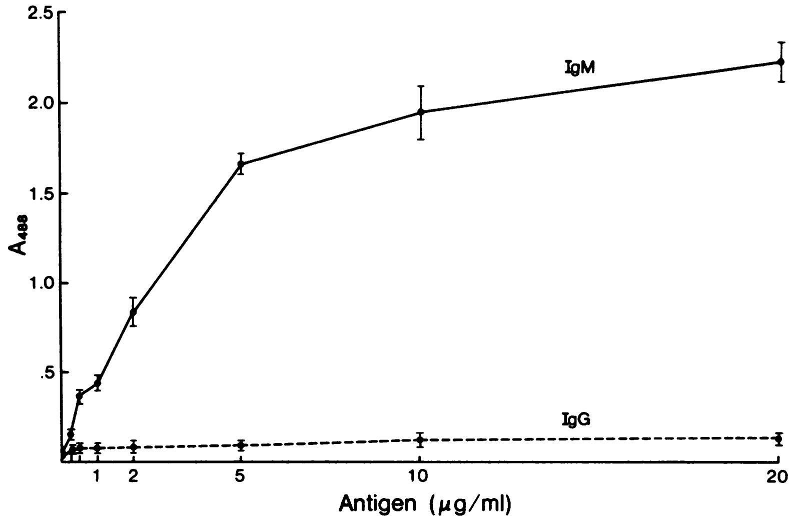

FIG. 1. Comparison of IgG and IgM antibody activities against Phen GL-I (2 μg/ml) in pooled sera from human lepromatous leprosy patients (diluted 1:300).

and acidic degradations (9, 10) (Fig. 2). Removal of the fatty acyl functions from Phen GL-I did little to enhance activity (Fig. 3). Little serological activity was found in the aglycone segments of the phthiocerol-containing lipids, such as the phenolic phthiocerol core, phthiocerol itself, the dimycocerosylphthiocerol or the free mycocerosic acids. Moreover, the monoglycosylphenolic dimycocerosylphthiocerol from *M. bovis* and the triglycosylphenolic diacylphthiocerol from *M. kansasii* were devoid of significant activity against the anti-*M. leprae* rabbit antibodies or those from human lepromatous leprosy patients.

To further examine the serological specificity of the triglycosyl entity, anti-*M. leprae* rabbit antiserum was reacted against the full range of 31 mycoside C glycopeptidolipid antigens from members of the *M. avium-M. intracellulare-M. scrofulaceum* serocomplex (2, 4). These glycolipids are endowed with short type-specific tetra- or trisaccharide antigen determinants, are also rich in *O*-methyl-6-deoxyhexoses and *O*-

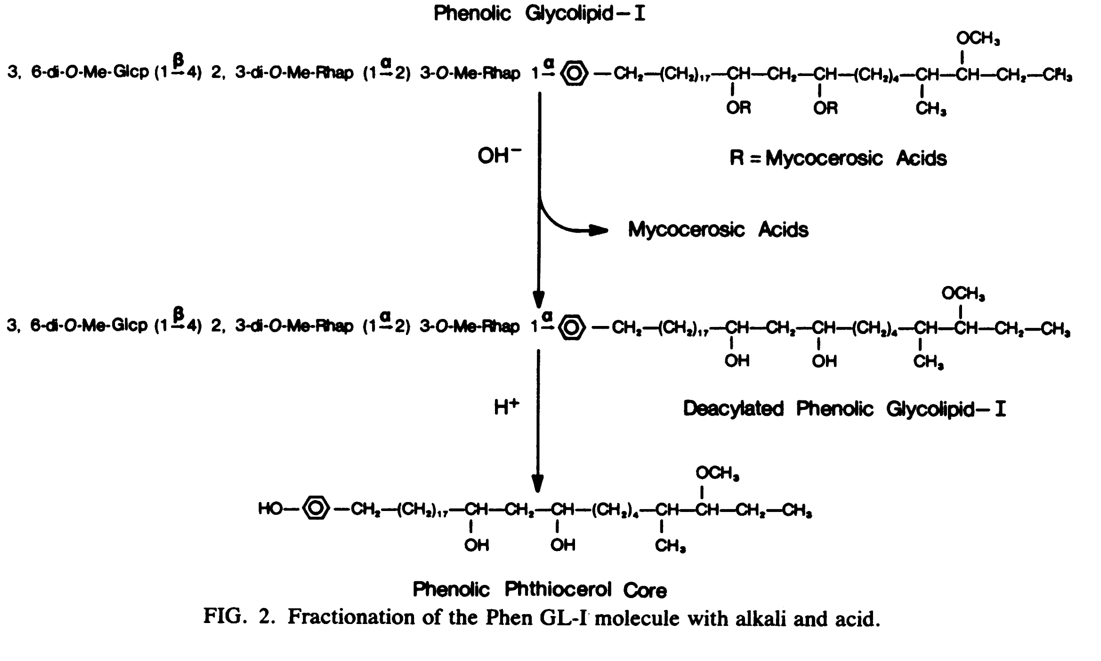

FIG. 2. Fractionation of the Phen GL-I molecule with alkali and acid.

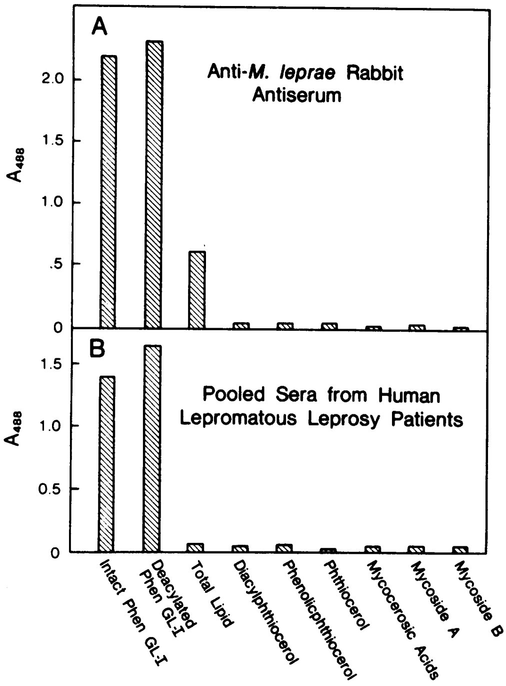

FIG. 3. (A) Rabbit IgG antibody activity against Phen GL-I, dimycocerosyl phthiocerol, and various degradation products. The concentration of the lipids was 5 μg/ml of coating buffer. Anti-*M. leprae* rabbit antiserum was diluted 1:90 in PBS-Tween (10). (B) Human IgM antibody activity against the various lipids. The concentration of lipids was 10 μg/ml of coating buffer and pooled sera from human lepromatous patients was diluted 1:100 in PBS.

methylglucoses (2), and are highly reactive in ELISA against hyperimmune rabbit antisera raised against the homologous strains (D. L. Yanagihara, V. L. Barr, and P. J. Brennan, unpublished data). Despite a plethora of 31 potentially reactive oligosaccharides, there were surprisingly few cases of cross-reaction; only the glycopeptidolipids from serovars 5, 12, 18, 20, 23, 24, and 28 showed significant activity (>0.10) with absorbances of 0.57 ± 0.4, 0.11 ± 0.07, 0.25 ± 0.17, 0.31 ± 0.22, 0.29 ± 0.21, 2.53 ± 1.79, and 0.38 ± 0.27, respectively, compared with an absorbance of 1.33 ± 0.94 for the Phen GL-I anti-*M. leprae* antiserum combination (one set of values from three such experiments, all qualitatively similar). Of these, the glycopeptidolipid from serovar 24 was the most reactive. However, when the reverse reactions were conducted (i.e., Phen GL-I against each of the hyperimmune rabbit antisera to the individual serovars), there was not one incidence of a positive reaction. Moreover, when the individual glycopeptidolipids were reacted with pooled sera from lepromatous leprosy patients there was no cross-reactivity.

Analysis of human sera. Sera from individual leprosy patients, patients with other mycobacterial infections, and healthy individuals were analyzed using both the IgM- and IgG-conjugated reagents (Table 1). With the IgM conjugate, the sera of 29 of 33 lepromatous patients (88%) demonstrated anti-glycolipid antibodies, and the mean value (A_{488}, 0.580 ± 0.514) was significantly higher ($P < 0.01$; Student t test) than that of healthy subjects. Treatment of lepromatous leprosy patients for two or more years resulted in significantly lower IgM values ($P < 0.01$). Of those treated for less than two years, 23 of 24 (96%) were judged positive (mean A_{488}, 0.737 ± 0.519), whereas for those treated for more than two years, 6 of 9 (67%) were positive (mean A_{488}, 0.162 ± 0.107) (Table 1 and Fig. 4). Even in this latter group the mean A_{488} value was significantly higher ($P < 0.01$) than in the healthy subjects.

One of the two borderline patients studied had elevated IgM antibodies (A_{488}, 0.315), but the other did not (A_{488}, 0.065) (Table 1 and Fig. 5). Of the 13 tuberculoid patients studied, 8 (62%) had significant elevations of IgM antibody to Phen GL-I (mean A_{488}, 0.186 ± 0.251); the mean value for tuberculoid patients was significantly

TABLE 1. Human IgM seroreactivity to phenolic glycolipid I

Infection	No. positive[a]/negative	Mean response (A_{488}) ± SD for		IgM/IgG ratio
		IgM	IgG	
Healthy controls	0/23	0.048 ± 0.016	0.046 ± 0.016	
Tuberculosis	0/12	0.059 ± 0.022	0.069 ± 0.022	
Atypical mycobacterial infections	0/15	0.038 ± 0.016	0.048 ± 0.020	
Tuberculoid leprosy (TT, BT)[b]	8/5	0.186 ± 0.251	0.048 ± 0.140	3.9
Lepromatous leprosy (LL_p, LL_s, BL)[c]	29/4	0.580 ± 0.514	0.084 ± 0.068	6.9
With a <2-yr therapy	23/1	0.737 ± 0.519	0.098 ± 0.075	7.5
With a >2-yr therapy	6/3	0.162 ± 0.107	0.045 ± 0.021	3.6

[a] Positive, >3 SD from the mean of healthy controls.
[b] TT, Tuberculoid; BT, borderline tuberculoid.
[c] LL_p, Polar lepromatous; LL_s, subpolar lepromatous; BL, borderline lepromatous.

higher ($P < 0.05$) than that of the healthy controls, and significantly lower ($P < 0.01$) than that of the lepromatous patients. Five of the leprosy patients who had initially demonstrated positive sera were restudied 1 month to 1 year later; all remained positive, antibody levels either staying essentially the same (two patients) or decreasing (three patients). For all patients throughout the disease spectrum, the IgM-to-IgG ratio was variable, in the range of 3.6 to 7.5, again indicating that anti-glycolipid IgG activity is of questionable significance. However, an inverse relationship of IgM to IgG applied to anti-whole *M. leprae* antibodies; when whole suspended *M. leprae* was the coating antigen, the ratio was about 0.5. No patients with tuberculosis ($n = 12$; mean A_{488}, 0.059 ± 0.022) or patients with various atypical mycobacterial infections ($n = 15$; mean A_{488}, 0.038 ± 0.016) demonstrated IgG or IgM seroreactivity to Phen GL-I. Nevertheless, all of these sera showed a high IgG response in ELISA to whole *M. leprae* coating antigen, indicating that the sera had antibodies to the common mycobacterial antigens.

DISCUSSION

Since their isolation and recognition of their structural uniqueness, the trisaccharide appendages of the phenolic glycolipids had promised to be specific *M. leprae* haptens, especially when considered in light of the mycoside C polar glycopeptidolipids (2, 4) and the trehalose-containing lipooligosaccharides (S. W. Hunter, R. C. Murphy, K. Clay, M. B. Goren, and P. J. Brennan, J. Biol. Chem., in press), all highly antigenic and endowed with short oligosaccharides of exquisite specificity. However, unlike these others, the phenolic glycolipids are highly apolar and not readily amenable to serological manipulations. Nevertheless, their potential as tools for the serodiagnosis of leprosy was inferred from initial recognition of them as the likely serologically active components in crude bacillary extracts (3), and, subsequently, Payne et al. (13) incorporated pure Phen GL-I into liposomes and demonstrated reactivity with sera from lepromatous leprosy patients by gel diffusion. Previously, using hyperimmune anti-*M. leprae* antiserum, we recognized the felicity of ELISA and assumed that the orientation of the lipid on the polystyrene substratum was such as to render the diacylphthiocerol cryptic and preferentially expose the trisaccharide moiety (10). As described here, ELISA combined with the simple ploy of thorough sonication of the glycolipid in an aqueous medium allows ready reaction between glycolipid and antibodies in sera from leprosy patients. The most gratifying aspect of the present study is the absence of

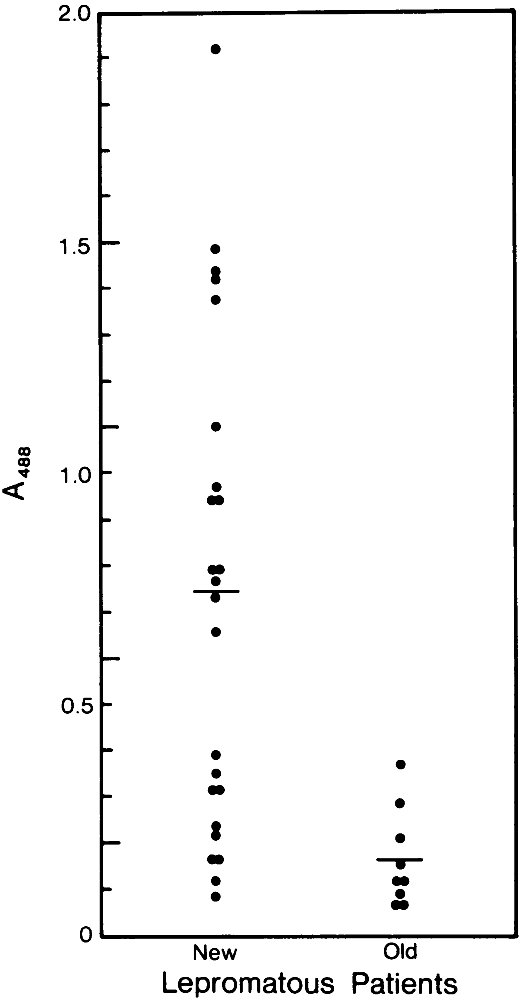

FIG. 4. Anti-Phen GL-I activity in lepromatous patients treated with dapsone for less than two years (new patients) and more than two years (old patients).

significant binding between the so-called mycoside A of *M. kansasii* and anti-Phen GL-I antibodies. Previously, we had used mycoside A as an aid to establishing the triglycosylated nature of the product from *M. leprae* (8). Although the sugar composition of mycoside A is known (5), the sequence has not been fully established. Nevertheless, there are striking similarities in the two products; both contain trisaccharides, the nonreducing terminus of both is di-*O*-methylated, there is a preponderance of *O*-methyl-6-deoxyhexoses, and there are similarities in the configurations of the anomeric protons. Accordingly, future emphasis must be on the [3,6-di-*O*-methyl-β-D-glucopyranosyl(1→4)2,3-di-*O*-methyl-α-L-rhamnopyranosyl(1→2)3-*O*-methyl-α-L-rhamnopyranosyl] antigen determinant, on its chemical synthesis, its conjugation

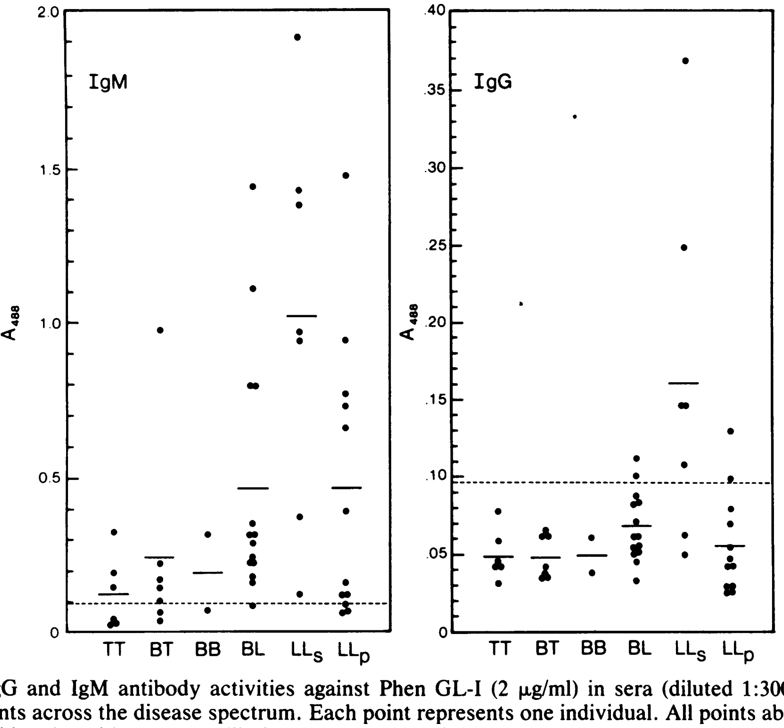

FIG. 5. IgG and IgM antibody activities against Phen GL-I (2 μg/ml) in sera (diluted 1:300) from human leprosy patients across the disease spectrum. Each point represents one individual. All points above the dashed line are considered positive (serum dilution 1:300). The solid lines represent median values. TT, Tuberculoid; BT, borderline tuberculoid; BB, borderline; BL, borderline lepromatous; LL_s, subpolar lepromatous; LL_p, polar lepromatous.

to peptide carriers, and its use as a tool for serodiagnosis and as a modulator of cell mediated immunity.

Anti-whole *M. leprae* IgG and IgM have been amply demonstrated in lepromatous leprosy patients, and the anti-*M. leprae* IgG was about twice as high as IgM (12, 15). Thus, the anti-*M. leprae* IgM antibodies may be mostly in response to the specific phenolic glycolipids, whereas IgG may be directed primarily against the more common mycobacterial cell wall antigens, such as the arabinogalactan-peptidoglycan complex (1). Indeed, sera from patients with other mycobacterial infections, although devoid of anti-Phen GL-I antibodies, showed high anti-whole *M. leprae* IgG. Anti-*M. leprae* IgM has been attributed to persistent infection (15). However, it is also possible that IgM might be a bystander response to the glycolipid, driven by nonspecifically acting lymphokines produced by a specifically activated T cell (6). Thus, the absence of a direct cell-to-cell signal delivered by the T cell to the responding B cell could be the reason for the lack of a substantial IgM-to IgG switch.

Chemotherapy of leprosy has been associated with decreasing antibody titers, whereas relapsed tuberculoid patients generate higher antibody levels (17). Touw et al. have indicated that at the tuberculoid end of the spectrum, solid-phase antibodies to whole *M. leprae* correlated with severity of disease (15). A specific assay for IgM activity to the specific *M. leprae* glycolipid, particularly in the preclinical state of the lepromatous form of the disease, may enable earlier chemotherapy and thereby prevent deformity and eliminate the infectious reservoir.

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