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Generation and Characterization of Monoclonal Antibodies to the Phenolic Glycolipid of *Mycobacterium leprae*

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Nine cloned cell lines producing antibodies to the unique phenolic glycolipid of *Mycobacterium leprae* have been established as a result of fusions with spleens from mice immunized with the glycolipid complexed with methylated bovine serum albumin. One of the antibodies was relatively nonspecific, binding to a related glycolipid from *Mycobacterium kansasii*, but the remaining antibodies were specific for the *M. leprae* lipid. Some of the antibodies required the intact (trisaccharide) carbohydrate portion for recognition of the glycolipid antigen, whereas others recognized partially hydrolyzed forms lacking one or two sugar residues. Monoclonal antibodies directed at the terminal saccharide of the glycolipid showed the greatest specificity for *M. leprae* in enzyme-linked immunoassays. These antibodies brightly labeled whole mycobacteria in indirect immunofluorescence experiments, demonstrating the surface location of *M. leprae*-specific determinants of the glycolipid antigen. In addition to their use in providing information about the antigenic properties of the phenolic glycolipid, these antibodies have potential applications for elucidating the roles of glycolipid in the pathogenesis of leprosy.

Mycobacterium leprae synthesizes a phenolic glycolipid (PG) which is related to that of *Mycobacterium kansasii* (9) but contains a unique carbohydrate portion consisting of terminal 3,6-di-*O*-methyl glucose linked β1→4 to 2,3-di-*O*-methyl rhamnose, which in turn is linked α1→2 to 3-*O*-methyl rhamnose (11, 12). This lipid is found in large amounts in leprosy-infected tissues of human (18) and armadillo (11) origin, and it has recently gained considerable interest for its antigenic properties (3, 12, 15, 19). Several groups have reported the presence of antibodies to the glycolipid in serum samples from leprosy patients (3, 12, 15, 19), and it is of interest to determine whether similar antibodies might allow early detection of preclinical infection with *M. leprae* (T. M. Buchanan, D. B. Young, R. A. Miller, and S. R. Khanolkar, Int. J. Lepr., in press).

The ease with which the glycolipid can be separated from the bacilli during the mechanical processes involved in isolating *M. leprae* from infected tissues has led to the suggestion that the lipid is present in the form of a surface capsule surrounding the bacilli (11) in a manner analogous to that of the peptidoglycolipids of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex (2). A role for the peptidoglycolipids in protecting pathogenic mycobacteria such as *Mycobacterium lepraemurium* from the host bactericidal activities in phagocytic cells has been proposed (5), and by extension, a similar role can be postulated for the *M. leprae* PG.

Monoclonal antibodies can be used as highly specific probes to investigate the antigenic determinants present on particular molecules and also as diagnostic reagents for the localization and quantitation of antigen in infected material. To further our understanding of the antigenicity and pathogenic role(s) of the *M. leprae* PG, we have generated a set of monoclonal antibodies to this molecule.

MATERIALS AND METHODS

PG. The *M. leprae* PG was purified from supernatant material derived from homogenization of infected armadillo liver (11). Extraction by the Bligh-Dyer monophasic method was followed by elution from a silica gel-Celite column with methanol (2% by volume) in chloroform (11) and preparative thin-layer chromatography (TLC) as described previously (19). The PG from *M. kansasii* was purified in the same way from bacteria grown on Middlebrook 7H11 medium. The identities of the glycolipids were confirmed by combined gas-liquid chromatography and mass spectroscopy of the alditol acetate derivatives (19). For use in the routine enzyme-linked immunosorbent assay (ELISA), PG from *M. leprae* was deacylated by alkaline hydrolysis, and the product was purified by TLC (19). Partially hydrolyzed forms of the lipid that had lost one or two sugar residues were generated by mild acid hydrolysis and were isolated by preparative TLC with a diethyl ether-acetone (9:1) solvent as described by Hunter et al. (12). The concentration of glycolipids was determined by estimation of the carbohydrate content (6), with rhamnose used as the standard.

Immunization and fusion protocol. Partially purified PG from *M. leprae* (i.e., eluate from silica gel-Celite column with 2% methanol in chloroform) was complexed with methylated bovine serum albumin (Sigma Chemical Co.) by Vortex mixing and sonication as described by Barrow and Brennan for complexing peptidoglycolipids (1). The lipid-methylated bovine serum albumin complex contained 20% by weight of the PG as judged by assay for carbohydrate content. Lipid-methylated bovine serum albumin complex suspended in phosphate-buffered saline (PBS; 1 mg/ml) was injected into the peritoneal cavities of BALB/c mice at weekly intervals for 5 weeks (50 μl per injection), and then, 2 days before sacrifice, a final injection was delivered by the intravenous route. Fusion of spleen cells from injected mice with those from the BALB/c myeloma cell line NSI/1 was carried out as described previously (8), except that a thymocyte feeder layer was not used. Supernatant fluid from each well was tested for antibody production by ELISA, and positive reactors were replated at a density of 5 cells per well and then finally as formal clones. Ascites fluid rich in antibody was obtained by injecting the cloned cells into the peritoneum of pristane-primed BALB/c mice.

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TABLE 1. Reactivity patterns of monoclonal antibodies to PG[a]

Antibody	Reactivity pattern			
	M. leprae PG with:			*M. kansasii* PG
	Trisaccharide	Disaccharide	Monosaccharide	
A1E	+	+	+	−
A2A	+	+	+	−
A3H	+	−	−	−
B2G	+	−	−	−
B5B	+	−	−	−
B5H	+	+	+	−
B8F	+	−	−	−
B9D	+	+	+	+
B10H	+	−	−	−

[a] The reactivity pattern for each antibody was determined by ELISA with intact and partially hydrolyzed glycolipid as described in the text.

ELISA. (i) ELISA with deacylated PG. For the routine ELISA, deacylated PG from *M. leprae* was suspended in distilled water at a concentration of 50 μg/ml by Vortex mixing and incubation at 55°C (19). This suspension was diluted 10-fold in distilled water and used to coat polystyrene microtiter plates (0.1 ml per well) by overnight incubation at 37°C. Plates were washed with PBS and blocked by incubation with 5% bovine serum albumin in PBS. Culture supernatants or diluted ascites fluid were then added, and plates were incubated for 2 h at 37°C. After being washed with PBS, plates were incubated with enzyme-conjugated secondary antibody. For initial screening of clones, peroxidase-linked goat anti-mouse immunoglobulin G (IgG; heavy and light chains) (Miles Laboratories, Inc.) and peroxidase-linked goat anti-mouse IgM (mu chain specific) (Cappel Laboratories) were used. Subsequently, with monoclonal antibodies characterized as belonging to the IgM class of immunoglobulin, only the mu chain-specific secondary antibody was used. After being washed with PBS, plates were developed with the peroxidase substrate *o*-phenylenediamine (19).

(ii) ELISA with partially hydrolyzed PG. Native PG and the products of partial acid hydrolysis were dissolved in hexane and coated to polystyrene microtiter plates by adding 10 μl to each well and drying at 37°C for 15 min (12). Subsequent steps in the ELISA were identical to those described for the routine ELISA above.

(iii) ELISA with whole mycobacteria. *M. leprae* was purified from infected armadillo liver by the standard Draper procedure (4), except that benzamidine (1 mM) was added during the initial homogenization and Tween was not used. Other mycobacterial cultures were grown on Middlebrook 7H11 agar (Difco Laboratories). Bacteria were scraped from the plates, washed with normal saline, and stored at 0 to 4°C in saline. The concentration of bacterial suspensions was estimated by determination of protein concentration after boiling with NaOH (14). Whole mycobacteria were coated to polystyrene microtiter plates by preparing a suspension of 5 μg of protein per ml in 10 mM Tris-hydrochloride, pH 8, and adding 50 μl per well. This corresponded to a concentration of approximately 5×10^7 organisms per ml. After overnight incubation at room temperature, cells were fixed with glutaraldehyde (0.25%) for 10 min, and then plates were washed with PBS and blocked with bovine serum albumin (5%, wt/vol) for 2 h. Subsequent screening for antibodies directed against the mycobacteria was carried out as described for the PG ELISA.

(iv) Inhibition ELISA. To determine the ability of whole mycobacteria or purified lipids to competitively inhibit binding of monoclonal antibodies to microtiter plates coated with PG, samples were preincubated with diluted ascites fluid for 30 min at room temperature. The mixture was then added to the coated plates and incubated for 1 h at 37°C. Detection of antibody by means of peroxidase-linked goat anti-mouse IgM was then performed as before.

^{125}I labeling of antibody. Monoclonal antibody was purified from ascites fluid by gel filtration by using a Bio-Gel A5M column (16). The antibody was labeled with ^{125}I by using chloramine T (10). Binding of the radiolabeled antibody to PG was assayed with Immulon I Removawell strips (Dynatech Laboratories, Inc.) coated with deacylated PG.

TLC autoradiography. TLC plates (Si-HPF, J. T. Baker Chemical Co.) on which lipids had been separated were washed with PBS and then incubated for 1 h at room temperature with ^{125}I-labeled monoclonal antibody diluted in PBS containing 5% fetal calf serum. Plates were washed for 3 h with six changes of PBS, allowed to dry, and then exposed to X-ray film overnight. After an autoradiograph was obtained, plates were sprayed with orcinol-sulfuric acid to visualize glycolipids and then were photographed.

Immunofluorescence. Mycobacteria (10^7 per slide) were attached to immunofluorescence slides by heat and Formalin fixation. After washing with PBS, monoclonal antibody was added at a dilution of 1:40 in PBS. After 1 h of incubation at 37°C in a moist chamber, slides were washed with PBS and then incubated for 30 min with fluorescein-conjugated goat anti-mouse IgM (mu chain specific, Cappel Laboratories) diluted 1:50 in PBS. After being washed with PBS and distilled water, slides were mounted with *p*-phenylenediamine to inhibit fluorescence quenching (13). Fluorescence was observed with a Zeiss fluorescence microscope at a magnification of ×1,000.

RESULTS

Nine cloned cell lines producing antibodies to the PG were established as a result of two fusions with cells from immunized mice (Table 1). Ascites fluid derived from the clones

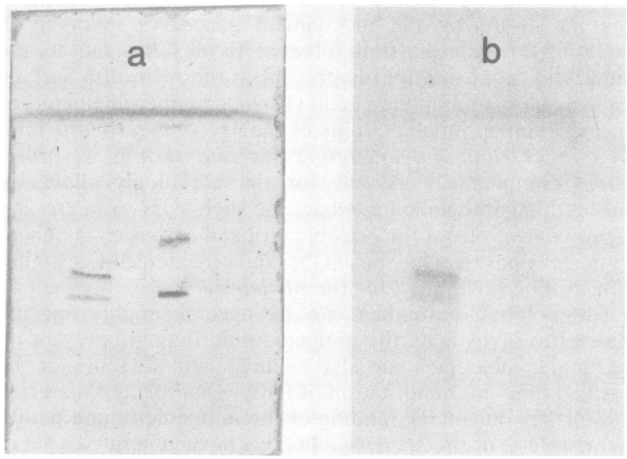

FIG. 1. Specificity of monoclonal antibody for *M. leprae* glycolipid. Native (upper spots) and deacylated (lower spots) PGs from *M. leprae* (left lanes) and *M. kansasii* (right lanes) were separated on a TLC plate developed with chloroform-methanol (15:1). The plate was then reacted with ^{125}I-labeled monoclonal antibody (B2G) and processed as described in the text. (a) TLC plate sprayed for visualization of glycolipids. (b) Autoradiograph of TLC plate.

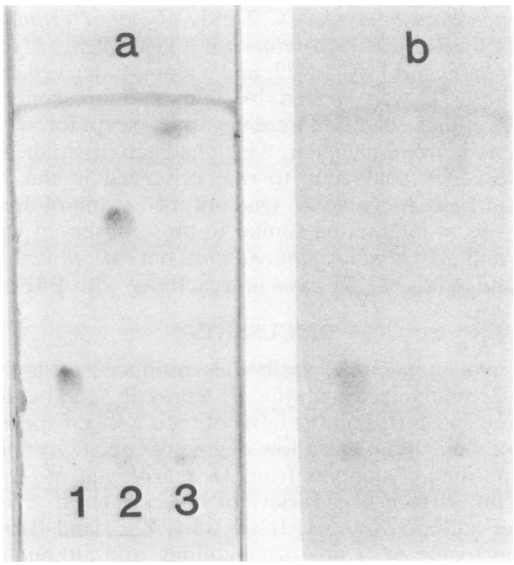

FIG. 2. Specificity of monoclonal antibody for PG with intact carbohydrate portion. PG from *M. leprae* (lane 1) and its partial hydrolysis products lacking one (lane 2) or two (lane 3) sugars were separated on a TLC plate developed with diethyl ether-acetone (9:1). Each lipid was applied in an amount corresponding to 0.5 μg of carbohydrate. The plate was then reacted with ^{125}I-labeled monoclonal antibody (B2G) and processed as described in the text. (a) TLC plate sprayed for visualization of glycolipids. (b) Autoradiograph of TLC plate.

showed a positive reaction in the PG ELISA at dilutions ranging from 1:2,000 (B5B) to 1:10,000 (B2G). All of the monoclonal antibodies were identified as IgM immunoglobulins by immuno-diffusion experiments with class-specific anti-mouse immunoglobulin antibodies.

Antibody from one of the cell lines (B2G) was purified and labeled with ^{125}I. Each of the other antibodies was found to compete with B2G for binding to the PG antigen, suggesting that they all recognize epitopes present on the same region of molecule.

Each antibody was tested for its ability to distinguish between the *M. leprae* and *M. kansasii* PG bound to microtiter plates in their deacylated forms. Only B9D bound to the *M. kansasii* lipid, indicating that, unlike the eight other antibodies, this clone is not specific for the unique trisaccharide portion of the *M. leprae* PG. Figure 1 shows the specificity of antibody B2G as determined by TLC autoradiography. Binding of antibody to the native and deacylated forms of the *M. leprae* PG is clearly seen and contrasts to the absence of recognition in the case of the *M. kansasii* glycolipid.

The effect of sequential removal of the carbohydrate residues on the binding of antibody was investigated by preparing derivatives of the *M. leprae* PG lacking one or two sugar residues, as described by Hunter et al. (12). Figure 2a shows the native PG (trisaccharide) along with the disaccharide and monosaccharide forms separated by their different migration patterns during TLC. Figure 2b shows the interaction of ^{125}I-labeled B2G with the same TLC plate and demonstrates a specificity for antibody binding to the intact trisaccharide form of the lipid with a marked decrease in binding after the removal of a single sugar residue. The eight monoclonal antibodies which recognized the carbohydrate portion of the PG were tested for specificity by using an ELISA system with the trisaccharide, disaccharide, and monosaccharide forms of the lipid as solid-phase antigen. This test allowed the distinction of two separate groups among these antibodies: (i) those which bound strongly to the intact lipid but had no detectable binding to the disaccharide and monosaccharide forms (e.g., B8F, Fig. 3a), and (ii) those which showed a preference for binding to the trisaccharide but also showed significant binding to the disaccharide and monosaccharide forms (e.g., A1E, Fig. 3b). The specificity pattern shown by each monoclonal antibody is recorded in Table 1.

The presence of PG on the surface of *M. leprae* was confirmed by the ability of intact organisms to inhibit binding of specific monoclonal antibodies to deacylated PG bound to

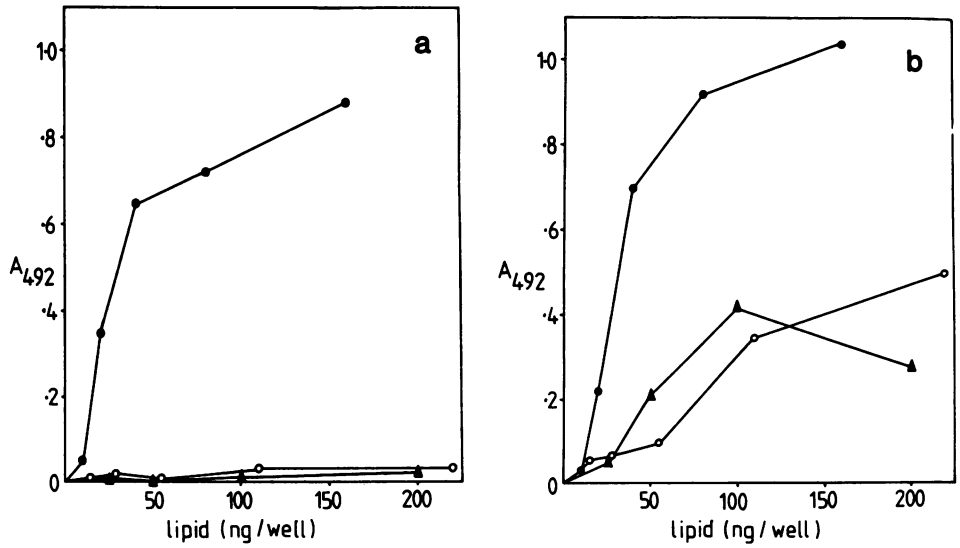

FIG. 3. Specificity of monoclonal antibodies of ELISA. PG from *M. leprae* (●) and its partial hydrolysis products lacking one (○) or two (▲) sugars were coated to microtiter plates, and the reactivity of monoclonal antibodies to the three lipids was determined by ELISA as described in the text. Shown are specificity patterns of antibodies B8F (a) and A1E (b).

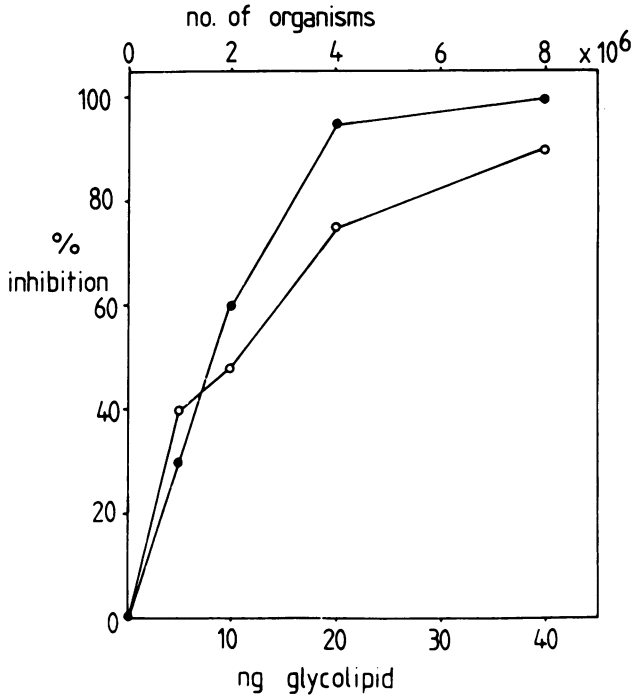

FIG. 4. Competitive inhibition of monoclonal antibody in PG ELISA. The ability of whole *M. leprae* (●) or purified deacylated PG (○) to inhibit binding of monoclonal antibody (B8F) was determined as described in the text.

microtiter plates. Figure 4 shows that preincubation with 1.5×10^6 *M. leprae* resulted in a 50% inhibition of binding of monoclonal antibody B8F used in the ELISA at a dilution of 1:4,000. Approximately the same level of inhibition was obtained with deacylated PG as inhibitor at a concentration equivalent to 10 ng of the native glycolipid. Thus, the amount of surface-exposed PG on purified *M. leprae* approximates 10 ng/1.5×10^6 organisms.

When whole *M. leprae* was used as solid-phase antigen in ELISA, binding of monoclonal antibodies followed a pattern similar to that observed with the purified lipid antigen but with lower maximum absorbance levels (Fig. 5). As a further test of specificity, each monoclonal antibody was screened by ELISA with a panel of 20 mycobacterial strains as antigen (Table 2). The relatively nonspecific antibody B9D bound to all of the strains tested. A1E showed a significant binding to *Mycobacterium terrae*, *Mycobacterium nonchromogenicum*, and *Mycobacterium fortuitum*, although at levels less than half of that seen with *M. leprae*. The remaining antibodies showed marked specificity for *M. leprae* with little or no binding to other mycobacteria. Some degree of interaction with *M. terrae* and *M. nonchromogenicum* was observed with several of the antibodies. This interaction was eliminated by washing these strains with Tween 80 before use in the ELISA, but the same treatment also significantly reduced the binding of monoclonal antibodies to *M. leprae*.

The surface location of the PG was further demonstrated by indirect immunofluorescence with monoclonal antibodies. Figure 6 shows the fluorescence of leprosy bacilli after incubation with specific monoclonal antibody B8F. A fluorescent zone was seen surrounding the bacilli and was particularly marked in bacterial clumps. All of the other mycobacteria shown in Table 2, plus *M. avium-M. intracellulare-M. scrofulaceum* serovars 1, 2, 8, 9, 14, and 16, *Mycobacterium bovis* ATCC 27291, *Mycobacterium ulcerans* ATCC 19423, *M. scrofulaceum* ATCC 19981, *Mycobacterium chelonei* ATCC 14472, and a leprosy-derived corynebacterium (7), were tested by immunofluorescence with B8F. All of these bacteria were negative except for *M. terrae* and *M. nonchromogenicum*, which had activity in the immunofluorescence analogous to that observed in the whole-organism ELISA (Table 2). One untyped strain of *M. bovis* gave levels of interaction similar to those observed with *M. terrae* and *M. nonchromogenicum*, but *M. bovis* ATCC 19015 and ATCC 27291 gave no reactivity with B8F.

DISCUSSION

The nine monoclonal antibodies produced after fusions with mice immunized with the *M. leprae* PG can be divided into three categories on the basis of their antigen specificity. One antibody (B9D) has a low degree of specificity, binding to the purified glycolipids from *M. leprae* and *M. kansasii* and to the surface of a variety of mycobacteria. A second group of antibodies (A3H, B2G, B5B, B8F, and B10H) do not bind to the *M. kansasii* glycolipid, and although their interaction is unaffected by removal of the two long-chain fatty acids from the lipid antigen, removal of a single sugar residue is sufficient to destroy antibody-antigen binding. A third group of antibodies (A1E, A2A, and B5H) are specific for the *M. leprae* lipid, but although they preferentially bind to the lipid with its trisaccharide portion intact, they also show significant binding after the removal of one or two sugar residues. Our unpublished studies with serum samples from leprosy patients suggest that the dominant human immune response to this molecule is IgM immunoglobulin directed specifically at the trisaccharide portion containing 3,6-di-*O*-methyl glucose linked β1→4 to 2,3-di-*O*-methyl rhamnose.

The terminal 3,6-di-*O*-methyl glucose of the *M. leprae* PG is a sugar which has not been previously been found in

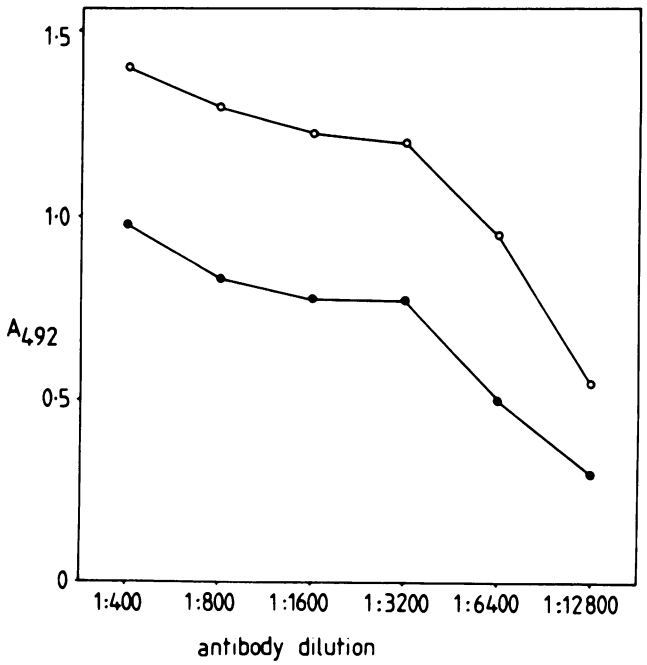

FIG. 5. Activity of monoclonal antibody (B2G) in ELISA tests with deacylated PG (○) or whole *M. leprae* (●) as antigen.

TABLE 2. Reactivity of monoclonal antibodies in the whole organism ELISA

Mycobacterium	Reactivity of antibody[a]								
	A1E	A2A	A3H	B2G	B5B	B5H	B8F	B9D	B10H
M. leprae	4+	2+	3+	4+	+	3+	4+	2+	2+
M. bovis ATCC 19015				−			−		
M. diernhoferi ATCC 19304	+/−	−	+/−	+/−	−	+/−	−	2+	−
M. duvalii	−	−	−	−	−	−	−		−
M. flavescens ATCC 14474	−	−	−	−	−	−	−		−
M. fortuitum	+	−	−	+/−	−	−	−	2+	−
M. gastri ATCC 15754	+/−	−	−	−	−	−	−		−
M. gordonae ATCC 14470	−	−	−	−	−	−	−		−
M. intracellulare TMC 1403	−	−	−	−	−	−	−	2+	−
M. kansasii TMC 1203	−	−	−	−	−	−	−	2+	−
M. marinum ATCC 927	−	−	−	−	−	−	−		−
M. nonchromogenicum ATCC 19530	+	+/−	+/−	+/−	−	+/−	+/−	2+	−
M. peregrinum ATCC 14467	−	−	−	−	−	−	−	+	−
M. phlei ATCC 11758	−	−	−	−	−	−	−		−
M. smegmatis ATCC 19420	+/−	−	−	−	−	−	−		−
M. terrae ATCC 15755	2+	−	+/−	+/−	−	+/−	+/−	+	−
M. thamnopheos ATCC 4445	−	−	−	−	−	−	−		−
M. triviale ATCC 23290	−	−	−	−	−	−	−	+	−
M. tuberculosis ATCC 25177	−	−	−	−	−	−	−		−
M. vaccae	+/−	−	−	−	−	−	−	+	−

[a] Monoclonal antibodies were used at dilutions of 1:500 to 1:2,000 in the whole organism ELISA test as described in the text. Results are expressed in terms of absorbance readings at 492 nm as follows: −, <0.1; +/−, 0.1 to 0.2; +, 0.2 to 0.4; 2+, 0.4 to 0.6; 3+, 0.6 to 0.8; 4+, >0.8.

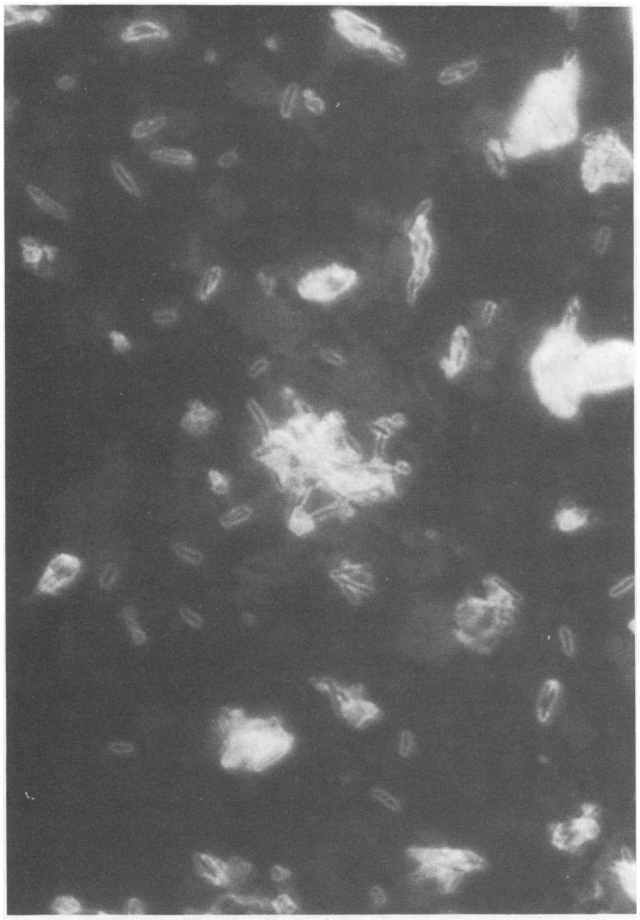

FIG. 6. Immunofluorescence of *M. leprae* after interaction with monoclonal antibody (B8F) and fluorescein-conjugated secondary antibody.

nature (11). It is, therefore, likely that the monoclonal antibodies requiring this sugar for antigen recognition will be highly specific reagents. This is confirmed by their specificity in the whole-organism ELISA and immunofluorescence tests (Table 2). In our experience, IgM antibodies are more susceptible to nonspecific binding effects than are IgG antibodies, and the small degree of interaction with *M. terrae* and *M. nonchromogenicum* may result from some nonspecific effect. However, the possibility that these strains (which are closely related taxonomically; 17) produce an antigen related to the PG of *M. leprae* cannot be excluded at the present time.

The results reported here with immunofluorescence and whole-organism ELISA experiments involving specific monoclonal antibodies strongly support the idea that the PG is a significant component of the surface capsule surrounding *M. leprae*. Further experiments will be necessary to elucidate the role of the PG and the capsule for intracellular survival of leprosy bacilli.

In addition to their use in providing information about the antigenic properties of the PG, the antibodies generated in this study have a wide variety of possible applications. These include the quantitation of antigen levels (the experiment shown in Fig. 4 is an example of this) and localization of the antigen within infected lesions by immunofluorescence or immunocytochemistry. The PG is the first *M. leprae*-specific antigen to be available for detailed immunological analysis, and it is hoped that these antibodies will be useful tools in clarifying the role of this intriguing molecule in the pathogenesis of leprosy.

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LITERATURE CITED

1. **Barrow, W. W., and P. J. Brennan.** 1982. Immunogenicity of the type-specific C-mycoside glycopeptidolipids of mycobacteria. Infect. Immun. **36**:678–684.
2. **Barrow, W. W., B. P. Ullom, and P. J. Brennan.** 1980. Peptidoglycolipid nature of the superficial cell wall sheath of smooth-colony-forming mycobacteria. J. Bacteriol. **144**:814–822.
3. **Brett, S. J., P. Draper, S. N. Payne, and R. J. W. Rees.** 1983. Serological activity of a characteristic phenolic glycolipid from *Mycobacterium leprae* in sera from patients with leprosy and tuberculosis. Clin. Exp. Immunol. **52**:271–279.
4. **Draper, P.** 1979. Annex 1, protocol 1/79. Report on the enlarged supervisory council meeting for research on the immunology of leprosy. World Health Organization, Geneva.
5. **Draper, P., and R. J. W. Rees.** 1970. Electron-transparent zone of mycobacteria may be a defence mechanism. Nature (London) **228**:860–861.
6. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
7. **Freer, J., K. S. Kim, M. R. Krauss, L. Beaman, and L. Barksdale.** 1969. Ultrastructural changes in bacteria isolated from cases of leprosy. J. Bacteriol. **100**:1062–1075.
8. **Gillis, T. P., and T. M. Buchanan.** 1982. Production and partial characterization of monoclonal antibodies to *Mycobacterium leprae*. Infect. Immun. **37**:172–178.
9. **Goren, M. B.** 1972. Mycobacterial lipids: selected topics. Bacteriol. Rev. **36**:33–64.
10. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. **89**:114–123.
11. **Hunter, S. W., and P. J. Brennan.** 1981. A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J. Bacteriol. **147**:728–735.
12. **Hunter, S. W., T. Fujiwara, and P. J. Brennan.** 1982. Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. J. Biol. Chem. **257**:15072–15078.
13. **Johnson, G. D., R. S. Davidson, K. C. McNamee, G. Russel, D. Goodwin, and E. J. Holborow.** 1982. Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. J. Immunol. Methods **55**:231–242.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **192**:265–275.
15. **Payne, S. N., P. Draper, and R. J. W. Rees.** 1982. Serological activity of purified glycolipid from *Mycobacterium leprae*. Int. J. Lepr. **50**:220–221.
16. **Rice, P. A., and D. L. Kasper.** 1982. Characterization of serum resistance of *Neissera gonorrhoeae* that disseminate. J. Clin. Invest. **70**:157–167.
17. **Tsukamura, M.** 1976. Numerical classification of slowly growing mycobacteria. Int. J. Syst. Bacteriol. **26**:409–420.
18. **Young, D. B.** 1981. Detection of mycobacterial lipids in skin biopsies from leprosy patients. Int. J. Lepr. **49**:198–204.
19. **Young, D. B., and T. M. Buchanan.** 1983. A serological test for leprosy using a glycolipid specific for *Mycobacterium leprae*. Science **221**:1057–1059.