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Phenolic Glycolipid 1 of *Mycobacterium leprae* Causes Nonspecific Inflammation but Has No Effect on Cell-Mediated Responses in Mice

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The involvement of the phenolic glycolipid from *Mycobacterium leprae* in cell-mediated immunity has been investigated in this study. The phenolic glycolipid itself does not appear to stimulate cell-mediated immunity directly, as shown by its failure to elicit a classical delayed-type hypersensitivity response in mice immunized with *M. leprae* or to stimulate *M. leprae*-immune lymph node cells in a lymphoproliferative assay. Intradermal vaccination with the phenolic glycolipid failed to influence the growth of *M. leprae* in mouse footpads. A nonspecific inflammatory response to the sonicated glycolipid was observed in mice vaccinated with whole *M. leprae* and in control animals. No evidence was obtained for any adjuvant or suppressive effect on cell-mediated immunity by the phenolic glycolipid either to *M. leprae* or an unrelated antigen (sheep erythrocytes); neither sensitization nor elicitation to either antigen was affected.

Mycobacterium leprae synthesizes a phenolic glycolipid (PG) which is found in large amounts in leprosy-infected tissues of humans (28) and armadillos (10). This lipid contains a unique trisaccharide consisting of terminal 3,6-di-*O*-methyl glucose-linked β1→4 to 2,3-di-*O*-methyl rhamnose-linked α1→2 to 3-*O*-methyl rhamnose (6, 11). The trisaccharide appears to be the major antigenic determinant of the PG to which antibodies in the serum of leprosy patients are directed (3, 6, 30). It also appears to be immunologically specific since antibodies to the PG are not found in patients with other mycobacterial infections, including tuberculosis (2, 4, 29).

The presence of determinants on the PG specific for *M. leprae*, together with the large amounts of PG found in tissues of heavily infected (lepromatous) patients, may be one factor associated with the specific immunological unresponsiveness seen in these patients. It has recently been demonstrated that PG is capable of inducing suppression of mitogenic responses in vitro in lymphocytes of lepromatous but not tuberculoid leprosy patients (16). Also, certain mycobacterial lipids and glycolipids have been shown to modulate both humoral and cell-mediated responses in vivo and in vitro (23).

The present study was a preliminary investigation into the influence of *M. leprae* PG on cell-mediated immunity to *M. leprae* and to an unrelated antigen, sheep erythrocytes (SRBC), in mice. Initially, it was necessary to analyze the cellular response to the PG alone in relation to dose, kinetics, and histology.

MATERIALS AND METHODS

Animals. Specific pathogen-free, female CBA mice, 6 to 9 weeks of age and bred at the National Institute for Medical Research, were used in these experiments.

Antigens. A whole-cell antigen preparation of *M. leprae* cells was prepared from livers and spleens of experimentally infected armadillos (27) after killing the bacilli with 2.5 Mrad of γ irradiation. A soluble antigenic extract (MLS) from the purified *M. leprae* suspensions was prepared by ultrasonication as previously described (22). The protein concentration was measured by the method of Lowry et al. (13).

M. leprae PG was obtained from supernatants generated during the preparation of *M. leprae* suspensions from armadillos. It was purified by column and thin-layer chromatographic procedures, with chloroform-methanol (9:1 [vol/vol]) as the solvent in the final thin-layer chromatography stages as previously described (10, 11). A single band was seen on the chromatograms. Under these conditions, it is extremely unlikely that antigenically active mycobacterial protein or particular debris could migrate on the chromatograms and contaminate the purified product. Phthiocerol dimycocerosate (PDIM) prepared in a similar way was donated by D. Minnikin (Newcastle University).

Emulsions of PG or PDIM were prepared in normal saline by ultrasonication for 8 to 10 min at 80 kHz with a Megason ultrasonic cleaner. In some experiments, PG was incorporated into liposomes (18).

A glycosidic aldehyde of the terminal disaccharide of the PG was chemically synthesized (7) and conjugated to bovine serum albumin (BSA) with reductive amination by standard procedures (19).

SRBC were obtained from Gibco Biocult (Paisley, Scotland) in Alsevers solution. Before they were used, SRBC were washed three times and suspended in saline at the required concentration.

Immunization. Mice were immunized with 10^9 *M. leprae* cells (ca. 100 μg [freeze-dried weight]) either intradermally (i.d.) in 0.02 ml of saline in the right flank or intravenously (i.v.) in 0.2 ml of saline via the tail vein. In some experiments, they were injected i.v. with 100 μg of sonicated PG. Mice were immunized subcutaneously with 10^8 washed SRBC into the right hind footpad.

Footpad tests. The animals were tested with 10^8 autoclaved *M. leprae* cells, 5 μg of MLS, various concentrations of sonicated PG, 50 and 5 μg of the synthetic disaccharide-BSA conjugate, BSA alone, or 10^7 SRBC in 0.02 ml of saline injected subcutaneously in the left hind footpad.

The thickness of both footpads was measured with a screw gauge micrometer (Moore and Wright, Sheffield, United Kingdom) at 0, 24, 48, and 72 h. The increase in footpad thickness was expressed as a percentage of the thickness of

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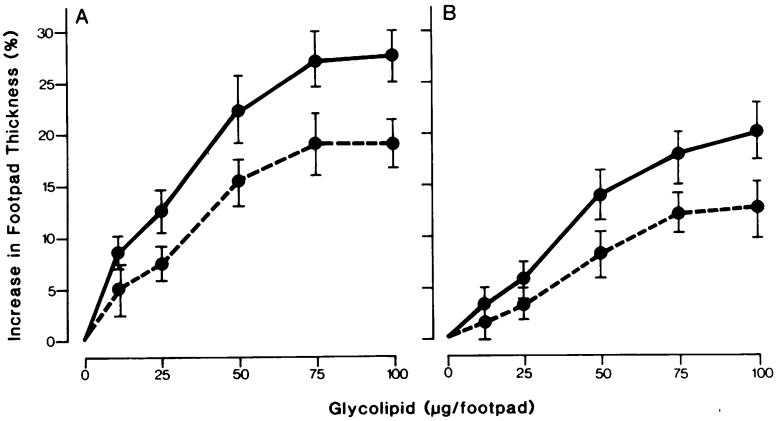

FIG. 1. Increase in footpad thickness after skin testing with PG 4 weeks after i.d. immunization (●———●) with 10^9 *M. leprae* organisms at 24 h (A) and 48 h (B) or in nonimmunized mice (●– – –●).

the unchallenged footpad, and the increase in footpad size when challenged with saline was subtracted from this value.

Challenge infections. A total of 10^4 *M. leprae* cells that had been maintained by passage in mice or armadillos was injected in 0.03 ml of saline into the left hind footpad of immunized and control mice. After 6 and 9 months, the footpads were harvested and homogenized in 2 ml of 0.1% albumin-distilled water. The acid-fast bacilli were stained by the Ziehl-Neelsen technique and counted by the method described by Hart and Rees (9).

In vitro stimulation of draining lymph node cells. Pooled cell suspensions were made from the inguinal lymph nodes from 8 to 10 CBA mice by gently pushing the cells through a nylon sieve. After two washes, cells were suspended in RPMI 1640 supplemented with 2 mM glutamine (Gibco Biocult), 100 IU of penicillin per ml (Glaxo, Middlesex, United Kingdom), 100 μg of streptomycin per ml (Evans, Middlesex, United Kingdom), 5×10^{-5} M 2-mercaptoethanol, and 5% fetal calf serum (heat inactivated [Gibco Biocult]), at a cell density of 5×10^5 cells per 0.2 ml in microtiter plates (Nunc Paisley, United Kingdom). The cells were cultured in the presence or absence of various doses of MLS or sonicated PG for 5 days; 18 h before harvest, 1 μCi of [^{3}H]thymidine was added. Cells were harvested with a Skatron cell harvester (Flow Laboratories, Inc., McLean, Va.). Results were expressed as the mean counts per minute, plus or minus standard deviation, of triplicate cultures.

Histology. Mouse footpads were fixed in Bouins solution, sectioned, and stained with hematoxylin and eosin.

Statistics. Results are expressed at all times as the mean plus or minus standard error. Means of skin test responses were compared by the Student *t* test. Counts of bacilli in mouse footpads were compared by the Wilcoxon test.

RESULTS

Elicitation of footpad swelling with PG. Animals were immunized i.d. with 10^9 *M. leprae* cells, and footpads were tested after 4 weeks with various doses of sonicated PG. Footpad readings were taken at 24 and 48 h (Fig. 1A and B, respectively). A small but significant ($P > 0.01$) enhancement in footpad swelling was found in immunized animals compared with nonimmunized animals, but a strong nonspecific inflammatory response was also seen in the nonimmunized controls. The dose-response curve was similar in immunized and control animals.

The kinetics of the footpad test response to sonicated PG (50 μg) was compared with the response to *M. leprae* cells (10^8) or MLS (5 μg) in animals immunized with *M. leprae* (10^9) and in control animals (Fig. 2). When PG was used as the eliciting agent, the maximal response occurred at 24 h and had declined by 72 h. When *M. Leprae* and MLS were used, the kinetics of the response were characteristic of classical delayed-type hypersensitivity (DTH), with the maximal response occurring at 48 h and being maintained for at least 72 h. Again, nonspecific footpad swelling elicited with PG was evident in nonimmunized mice.

Further experiments were carried out to determine whether the inflammatory response to PG was attributable to the lipid PDIM part of the molecule or to the carbohydrate region. It was found that the terminal disaccharide conjugated to BSA did not produce a significant response when either

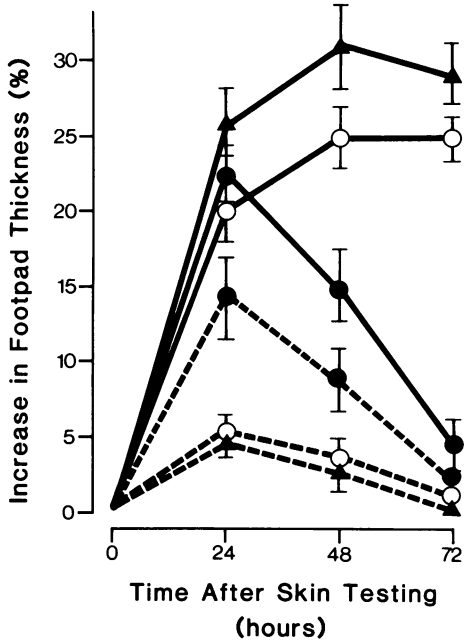

FIG. 2. Kinetics of increase in footpad thickness after skin testing in mice 4 weeks after i.d. immunization with 10^9 *M. leprae* cells (———) or in unvaccinated mice (– – –). Mice were skin tested with 50 μg of PG (●), 10^8 *M. leprae* cells (○), or 5 μg of MLS (▲).

50 or 5 μg was injected. However, the lipid PDIM, which contains no sugar residues, also did not elicit an inflammatory response when 50 μg was injected (Fig. 3).

The preparations which elicited a strong inflammatory response in uninfected control mice were also found to give a slightly enhanced response in mice immunized with 10^9 *M. leprae* cells. This included the nonspecific inflammation elicited with a high dose of a totally unrelated antigen (SRBC) to which the mice had not been previously primed.

Effect of PG on growth of *M. leprae* in mouse footpads. Experiments were also carried out to investigate whether pretreatment of mice with purified PG influenced the multiplication of *M. leprae* in mouse footpads (Table 1). Immunization of CBA mice with 10^9 *M. leprae* cells (100 μg [freeze-dried weight]) i.d. invariably resulted in significant protection against challenge with 10^4 *M. leprae* cells ($P < 0.001$). No significant difference from untreated controls given the same challenge infection was observed when mice were pretreated i.d. with doses of between 1 and 100 μg of sonicated PG or 40 to 80 μg of PG incorporated into liposomes instead of whole bacteria. Incorporation into liposomes has been shown to enhance the immunogenic capacity of many substances. It probably orientates the PG such that the more polar trisaccharide regions to which antibodies are directed are exposed to the aqueous environment and the PDIM part of the molecule is buried in the lipid bilayer.

Histology of footpad test sites. Figure 4 shows the histology of representative footpad test sites of nonimmunised mice and animals immunized with *M. leprae* i.d. and then skin tested after 4 weeks with 5 μg of MLS or 50 μg of sonicated PG. The overall cellular reaction, at low magnification, shows a much more intense focal pattern in response to PG compared with the more dispersed pattern observed with MLS (Fig. 4A and D). This may be because the glycolipid does not readily diffuse through the tissue. Similar patterns were found at 24 and 48 h.

Immunized mice challenged with MLS showed a histological picture consistent with DTH reactions. At 24 h, there was marked cellular infiltration of mononuclear cells and polymorphs, but by 48 h, mononuclear cells were predominant (Fig. 4E and F). Immunized mice challenged with PG showed an infiltrate consisting predominantly of polymorphonuclear leukocytes at 24 h (Fig. 4B); this was also seen in nonimmunized animals (Fig. 4H). At 48 h, the cellular infiltrate in both immunized and control mice consisted of both polymorphs and mononuclear cells (Fig. 4C and I).

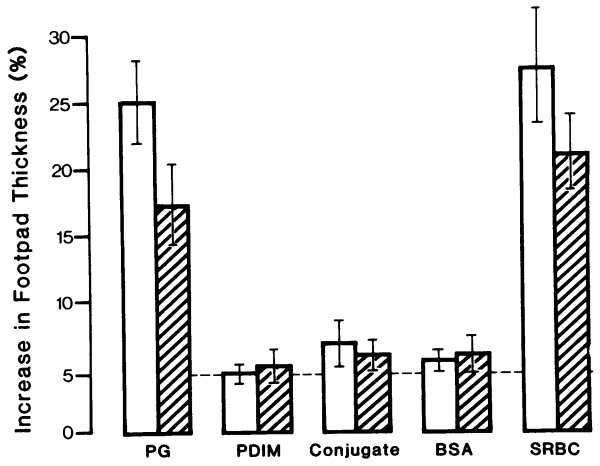

FIG. 3. Increase in footpad thickness after skin testing in mice 4 weeks after i.d. immunization with 10^9 *M. leprae* cells (open bar) or in nonimmunized mice (shaded bar). Mice were skin tested with 50 μg of sonicated PG, 50 μg of PDIM, 50 μg of disaccharide conjugate, 50 μg of BSA, and 10^8 SRBC. The response at 24 h is shown. The dotted line indicates the response to saline alone.

TABLE 1. Effect of i.d. injection with PG on growth of *M. leprae* in mouse footpads[a]

No. of expt	Vaccination with:	*M. leprae*/footpad at 6 months ([mean ± SE] [10^5])	
1	10^9 *M. leprae* cells	0.3 ± 0.2	$P < 0.001$
	—	4.5 ± 2.4	
	1 μg of PG	4.9 ± 1.4	$P > 0.05$
	10 μg of PG	4.3 ± 3.8	
	50 μg of PG	5.6 ± 3.1	
	100 μg of PG	4.3 ± 2.4	
2	10^9 *M. leprae* cells	0.13 ± 0.1	$P < 0.001$
	—	8.7 ± 5.3	
	80 μg of PG-L	12.3 ± 5.8	$P > 0.05$
	80 μg of L	8.4 ± 3.9	
	80 μg of PG	3.6 ± 1.5	

[a] In experiments 1 and 2, results are expressed as the mean plus or minus standard error ($n = 6$ and $n = 8$, respectively). —, Nonimmunized mice. L, Liposomes.

In vitro assay of antigen responsiveness. Four weeks after i.d. immunization of CBA mice with 10^9 *M. leprae* cells, cells from the draining lymph node were assayed for their ability to proliferate in vitro to MLS or PG. Strong proliferative responses were obtained by stimulating cells of immunized but not control mice with MLS (Table 2). No proliferation was detectable with sonicated PG in either the immunized or control mice.

Investigation of immunomodulatory properties of PG. Experiments were carried out to investigate whether the PG influences either sensitization or elicitation of DTH responses. When MLS (5 μg) and high concentrations of PG (75 μg) were used in combination as eliciting agents, a synergistic enhancement of footpad response was seen (Fig. 5A). At lower concentrations, which did not produce a nonspecific inflammatory response, the PG did not interfere with the elicitation of a DTH response to MLS in *M. leprae*-immune mice when both PG and MLS were given together (Fig. 5B). Similarly, at low concentrations, PG did not interfere with the elicitation of DTH to SRBC in mice previously immunized with SRBC (data not shown). As found with MLS, high concentrations of PG in combination with SRBC resulted in an enhanced response, probably due to the summation of DTH responses to SRBC and the nonspecific inflammatory response to PG.

To investigate whether PG could act as a nonspecific adjuvant, the effect of PG on the response to SRBC was investigated. Mice were immunized in the left hind footpad with 100 μg of sonicated PG. At the same time or after 35 days, the same mice were immunized with 10^8 SRBC at the site of PG inoculation. Tests for DTH to SRBC were made after an additional 6 days by injecting 10^7 SRBC in the right hind footpad, and swelling was measured at 24 and 48 h (Table 3). In either of the two experiments did previous inoculation with PG significantly influence the DTH response to SRBC ($P > 0.05$), indicating that PG did not exert any adjuvant effects or interfere with sensitization to an unrelated antigen.

Further experiments were also carried out to investigate

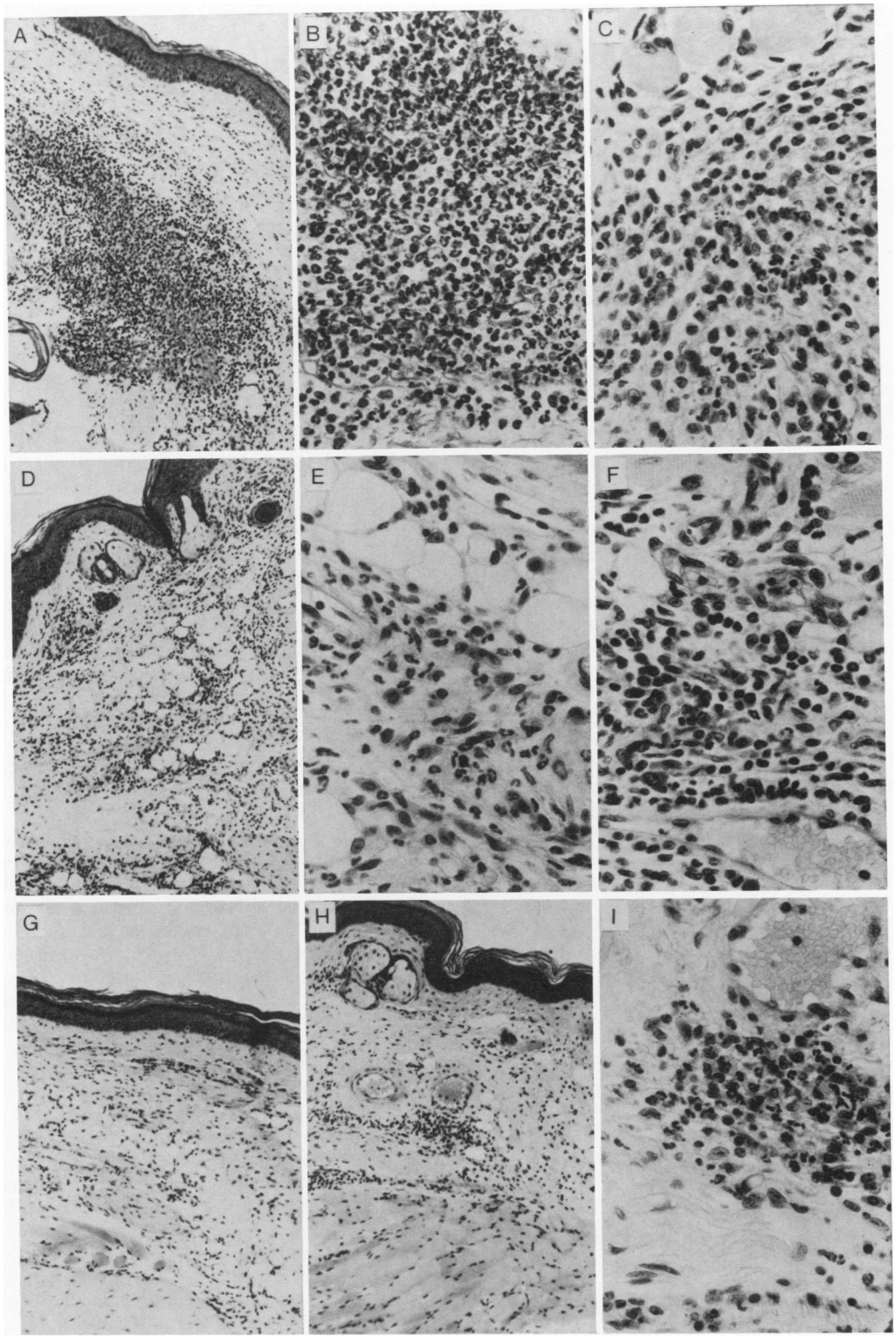

FIG. 4. Histological appearance of skin test sites of mice immunized previously with 10^9 *M. leprae* cells after elicitation response at 24 h (A and B) and 48 h (C) with 50 μg of PG and at 24 h (E) and 48 h (F) with 5 μg of MLS. The response at 24 h in nonimmunized mice to MLS (G) and PG (H and I) are also shown. Magnification: ×100 (A, D, G, and H); ×400 (B, C, E, F, and I).

TABLE 2. Stimulation of draining lymph node cells in vitro[a]

Dose of antigen (μg/ml)	[^{3}H]thymidine incorporation (cpm) (mean ± SD) in mouse cells			
	MLS treated		PG treated	
	Immune cells	Normal cells	Immune cells	Normal cells
0	1,935 ± 180	1,699 ± 109	1,726 ± 292	1,831 ± 97
0.1	1,673 ± 212	1,809 ± 215	1,978 ± 195	1,767 ± 150
1	2,342 ± 450	2,157 ± 242	1,940 ± 143	1,915 ± 204
10	5,329 ± 989	1,732 ± 133	2,155 ± 272	1,970 ± 238
50	11,925 ± 2124	2,990 ± 291	760 ± 151	990 ± 338
100	9,034 ± 1005	3,256 ± 193	844 ± 218	507 ± 149

[a] Mean plus or minus standard deviation of [^{3}H]thymidine incorporation (cpm) was obtained from lymph nodes pooled from 5 mice. Cultures were set up in triplicate.

whether the PG could modulate the DTH response induced in mice by i.d. vaccination with whole *M. leprae*. Injection of 10^9 *M. leprae* cells i.v., followed by subsequent i.d. immunization and footpad testing, is associated with an abrogation of the DTH response (Table 4).

Injection of PG or PG-liposomes (100 μg) i.v. instead of *M. leprae* resulted in only a slight reduction of the DTH response (Table 4).

DISCUSSION

The effect of PG on cell-mediated immunity in mice has been investigated in this study; cell-mediated immunity appears to mediate resistance to infection with *M. leprae* (15, 17, 25). The PG itself does not appear to stimulate cell-mediated immunity directly. It fails to elicit a classical DTH response, as assessed by dose response, kinetics, and histology, in mice immunized with *M. leprae* or to stimulate *M. leprae*-immune lymph node cells in lymphoproliferative assays. It was also found that i.d. vaccination with PG failed to inhibit the growth of *M. leprae* in mouse footpads. Many other workers have shown that lipid or carbohydrate antigens do not appear to stimulate T helper cell-dependent, cell-mediated responses (1), although antibody (12) and cell-mediated (20) responses to some carbohydrates may be regulated by T cells.

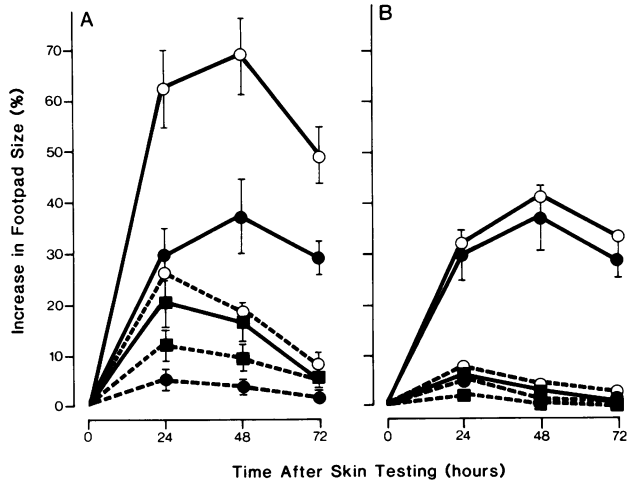

FIG. 5. Increase in footpad thickness 4 weeks after i.d. immunization (———) with 10^9 *M. leprae* cells or in nonimmunized mice (– – –) after skin test challenge with (A) 75 μg of PG alone (■), 5 μg of MLS alone (●), or 75 μg of PG plus 5 μg of MLS (○) or (B) 7.5 μg of PG alone (■), 5 μg of MLS alone (●), or 7.5 μg of PG plus 5 μg of MLS (○).

TABLE 3. Investigation into adjuvant properties of PG

No. of expt and immunization[a]	Challenge[b]	% Increase (mean ± SE) after:	
		24 h	48 h
1			
Day 0	Day 6		
PG-SRBC	SRBC	24 ± 9	13 ± 4
SRBC only	SRBC	22 ± 7	14 ± 3
PG only	SRBC	4 ± 1	2 ± 1
Nonimmunized	SRBC	3 ± 1	2 ± 0.5
2			
Day 0–day 35	Day 42		
PG–SRBC	SRBC	21 ± 3	10 ± 2
Nonimmunized–SRBC	SRBC	23 ± 8	14 ± 5
PG–nonimmunized	SRBC	5 ± 2	1 ± 0.5
Nonimmunized–nonimmunized	SRBC	3 ± 2	2 ± 1

[a] Mice were immunized subcutaneously in the left hind footpad with 100 μg of sonicated PG, 10^8 SRBC, or both.
[b] Mice were challenged in the right hind footpad with 10^7 SRBC.

Although the PG did not elicit a classical DTH response, such as that found to MLS or *M. leprae*, it did produce a strong inflammatory response when doses of more than 20 μg were injected into mouse footpads. This response was slightly enhanced in mice immunized with *M. leprae* compared with control mice. However, the results suggest that this is due to an enhanced ability of vaccinated animals to produce a nonspecific inflammatory response rather than to specific immunological recognition. The response to PG peaked at 24 h, when it consisted predominantly of an infiltrate of polymorphonuclear cells. By 48 h, a mixture of polymorphonuclear and mononuclear cells could be seen. This appears to be similar to the response observed when various inflammatory irritants such as starch, thioglycolate, mineral oil, and lipids from other mycobacteria are injected into mice (26).

The inflammatory response to PG at the doses investigated appeared to require the intact molecule, as neither PDIM nor the disaccharide conjugated to BSA elicited a response. The reason that PDIM did not elicit an inflammatory response at this dose may be because it is a much more apolar molecule than the PG. Several workers have shown that physical properties such as solubility may influence the intensity of the response (26). Mycocerosic acids which are esterified in the PG have been shown to cause a nonspecific inflammatory response; however, in these studies, much larger quantities were injected into guinea pigs (26).

There has been evidence accumulating in scientific literature that certain carbohydrate determinants play an important role in cellular interactions of the immune system. For example, interaction between natural killer cells (5), cytotoxic cells (14), suppressor cells (24), and migration inhibition factor (8) with their targets may be inhibited by certain sugars. Mycobacterial cell wall components are known to exert strong immunomodulating effects (23). The presence of large amounts of glycolipid in the tissues of lepromatous patients may therefore have diverse effects on the functioning of the immune system. In this study, however, the glycolipid did not interfere with sensitization of mice to SRBC; neither did it exert an adjuvant effect when injected at the same time or 5 weeks later. Whole *M. leprae*, however, exerts a strong adjuvant effect on the cell-mediated responses to SRBC, which is maximal 5 weeks after vaccination with *M. leprae* (17). Elicitation of DTH with a mixture

TABLE 4. Influence of PG on DTH response induced by vaccination with *M. leprae*

Group	Antigen given at day (type of immunization):[a]			% Increase (mean ± SE) in footpad size[b]	% Tolerance[c]	Significance (*P*) vs:[d]	
	−14 (i.v.)	Antigen 0 (i.d.)	28 (s.c.)			A	E
A (*n* = 7)	None	10^9 ML	10^8 ML	20.3 ± 2.8 (24 h)			$P < 0.001$
				24.7 ± 2.7 (48 h)			$P < 0.001$
				24.1 ± 6.9 (72 h)			$P < 0.001$
B (*n* = 10)	10^9 ML	10^9 ML	10^8 ML	5.44 ± 1.69 (24 h)	90	$P < 0.001$	NS
				2.65 ± 1.97 (48 h)	91.5	$P < 0.001$	NS
				1.01 ± 1.2 (72 h)	99	$P < 0.001$	NS
C (*n* = 8)	100 μg of PG	10^9 ML	10^8 ML	15.4 ± 4.32 (24 h)	30	$P < 0.01$	$P < 0.001$
				16.9 ± 5.8 (48 h)	29	$P < 0.001$	$P < 0.001$
				14.5 ± 5.13 (72 h)	46	$P < 0.001$	$P < 0.001$
D (*n* = 6)	100 μg of PG	None	10^8 ML	6.86 ± 2.7 (24 h)	ND	ND	$P > 0.05$
				3.7 ± 3.2 (48 h)	ND	ND	NS
				2.3 ± 1.3 (72 h)	ND	ND	NS
E (*n* = 6)	None	None	10^8 ML	3.8 ± 0.82 (24 h)	ND		
				1.14 ± 0.9 (48 h)	ND		
				1.68 ± 1.8 (72 h)	ND		

[a] ML, *M. leprae* cells; s.c., subcutaneous.
[b] Times in parentheses indicate when during the experiment that responses were measured.
[c] Tolerance expressed as B or C ÷ A × 100.
[d] ND, Not done.

of MLS and PG in mice vaccinated with *M. leprae* resulted in a summation of the response of the two products injected separately. A similar result was found with SRBC as the antigen, suggesting that PG does not interfere with the effector phase of a DTH response.

It has previously been shown that if mice are preinjected with *M. leprae* i.v., a subsequent i.d. vaccination with *M. leprae* does not induce a DTH response (21). We were able to confirm these results in this study. It was also observed, however, that if mice are pretreated with PG i.v. instead of *M. leprae*, the DTH response is only slightly reduced. This suggests, therefore, that the PG did not interfere with sensitization of mice to *M. leprae*.

In this study, therefore, no suppressive or adjuvant activities could be attributed to the PG from *M. leprae*. Suppression of in vitro mitogenic responses by the PG has, however, been observed in lepromatous leprosy patients (16). Our results do not necessarily contradict those of the latter study, as the suppressive effects of the PG were only observed in patients with untreated lepromatous leprosy. Normal, immunologically intact mice are able to limit multiplication of *M. leprae* in the footpad and therefore their response resembles the immune response in tuberculoid patients or healthy contacts rather than that of patients with lepromatous leprosy. The results of the study by Mehra et al. (16) suggest that specific T cells are generated to the PG in lepromatous leprosy patients which nonspecifically suppress the response to concanavalin A in vitro. It is clear from our study that the injection of large amounts of PG into mice infected with *M. leprae* does not exacerbate the infection or influence the cell-mediated response to *M. leprae* or SRBC. It is therefore unlikely that these protocols induced either specific or nonspecific T suppressor cells to the PG.

ACKNOWLEDGMENTS

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