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Cellular and Humoral Immune Response to a Phenolic Glycolipid Antigen (PhenGL-I) in Patients with Leprosy

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The ability of phenolic glycolipid I (PhenGL-I) of *Mycobacterium leprae* to stimulate in vitro lymphocyte proliferation (LP) was tested in cultures of peripheral blood cells from 42 patients with leprosy in Chicago and Thailand, 9 individuals with household contact in Thailand, and 10 unexposed North American controls. Only 10 responders (24%) were found among the patients, and the degree of LP was small. Responders were found among patients with lepromatous (18%) or tuberculoid (30%) leprosy without relation to age, complications, duration of treatment, or lepromin responsiveness. The specificity of the response was supported by a lack of response to two other glycolipids, by responses by T cells but not B cells, and by the observation that three of four responders tested maintained their responses to PhenGL-I for at least 1 year. Serum immunoglobulin M (IgM) and IgG antibodies were measured in the same patients by using PhenGL-I or its terminal monosaccharide conjugated to a bovine serum albumin carrier in an enzyme-linked immunosorbent assay. The presence of IgM antibody correlated negatively with LP to lepromin and to PhenGL-I in patients with tuberculoid leprosy. We conclude that circulating T cells from some leprosy patients proliferate in the presence of PhenGL-I in vitro, but the response is weak, possibly due to concomitant suppression or inhibition. The predominance of IgM antibody to PhenGL-I may be related to a lack of a T-helper-cell-mediated switch to IgG antibody response.

The antigen-specific unresponsiveness of circulating lymphocytes in the lepromatous form of leprosy, in contrast to the responsiveness in the tuberculoid form, has been recognized for many years (1, 10), but the origin of this unresponsiveness remains obscure. Studies of lymphocyte responsiveness have been hampered by the lack of a *Mycobacterium leprae*-specific antigen. Immunoelectrophoretic techniques have revealed that the majority of the major protein and polysaccharide antigens of *M. leprae* are cross-reactive with antigens of Bacillus Calmette-Guérin and other mycobacterial species (12). New evidence indicates that some of these antigens contain specific epitopes among cross-reactive determinants (9).

In addition, *M. leprae* synthesizes three specific triglycosylphenolic diacylphthiocerols, termed phenolic glycolipid I (PhenGL-I), PhenGL-II, and PhenGL-III (13, 14). In the trisaccharide portion of PhenGL-I, the terminal sugar, a 3,6-di-*O*-methyl-β-D-glucopyranosyl substituent, is the primary antigenic determinant recognized by the specific antibody in patients infected with *M. leprae* (4, 6, 7, 25) and by monoclonal antibodies (26). More than 95% of patients with lepromatous leprosy have circulating antibody to PhenGL-I, as do smaller percentages of patients with tuberculoid leprosy (7, 8, 24).

However, the role of PhenGL-I in cellular immune activity has not been fully explored. A role in immunoregulation was suggested by a study of nonspecific T suppressor cells from patients with lepromatous leprosy (18). The present study was undertaken to demonstrate whether T lymphocytes from patients with leprosy proliferate in response to stimulation in vitro with PhenGL-I. Since PhenGL-I is a single-epitope antigen, it was also of interest to compare the T-cell reactivity of each patient with specific antibody production, since this may provide clues toward understanding disordered immunoregulation in leprosy.

MATERIALS AND METHODS

Patients. A total of 42 patients with leprosy, 9 individuals with household contact, and 10 unexposed controls were studied. Eighteen of the patients were from the Hansen's Disease Clinic of the University of Illinois, Chicago; five of these patients had polar lepromatous leprosy, five had borderline lepromatous leprosy, and eight had borderline tuberculoid leprosy. Three of the patients with polar lepromatous leprosy and two of the patients with borderline lepromatous leprosy were experiencing repeated episodes of erythema nodosum leprosum. The countries of origin of these patients were as follows: four patients from the Phillippines, six patients from Mexico, two patients from India, and one patient each from Thailand, the United States, Cambodia, Korea, Brazil, and Pakistan. All of the patients were receiving 50 to 100 mg of dapsone daily, and nine were receiving rifampin; none received corticosteroids at the time of the study. The patients had been treated for 2 weeks to 7 years prior to the study; six patients were studied within 1 year of the onset of treatment.

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TABLE 1. Peripheral blood LP stimulated by PhenGL-I

	Chicago			Thailand		
	Reactive patients	Nonreactive patients	Unexposed controls	Reactive patients	Nonreactive patients	Individuals with household contact
No.	6	12	10	4	20	9
No. of males/no. of females	3/3	10/2	7/3	3/1	15/5	7/2
Age (yr)	34 ± 7[a]	33 ± 13	35 ± 5	54 ± 11	39 ± 13	28 ± 3
Histological class[b]						
No. with LL or BL	3	8		3	11	
No. with BT or TT	3[c]	4		3	9	
LP to PhenGL-I						
PBMC S.I.	3.1 ± 0.9[a]	1.2 ± 0.4	1.0 ± 0.1	3.4 ± 1.0	1.3 ± 0.3	1.4 ± 0.8
PBMC Δcpm (cpm)	1,395 ± 489[a]	179 ± 209	−159 ± 129	1,169 ± 222	295 ± 130	186 ± 293
T-cell S.I.	2.3, 2.1, 1.6, 8.3[d]	1.1 ± 0.4 (n = 6)	0.9 ± 0.2 (n = 6)	ND[e]	ND	ND
T-cell Δcpm (cpm)	3,334, 2,350, 1,231, 2,187[d]	60 ± 416	−180 ± 293	ND	ND	ND

[a] Mean ± standard deviation.
[b] LL, Polar lepromatous leprosy; BL, borderline lepromatous leprosy; BT, borderline tuberculoid leprosy; TT, tuberculoid leprosy.
[c] One woman with borderline tuberculoid leprosy was found to be a reactor in a study of her T cells in culture; since her PBMC studies were not completed, data for this woman do not appear in Fig. 2.
[d] No PBMC study was performed.
[e] ND, Not determined.

A total of 24 patients were from the Chiang Mai/Illinois Leprosy Research Project in Chiang Mai, Thailand; 4 of these patients had polar lepromatous leprosy, 7 had borderline lepromatous leprosy, 10 had borderline tuberculoid leprosy, 2 had tuberculoid leprosy, and 1 had borderline leprosy. Nine individuals with household contact were also studied; these individuals were determined not to have clinical leprosy by examination by a physician. The sexes and ages of the patients are shown in Table 1. Controls in United States studies included 10 healthy adults living in the United States. All patients were graded on the basis of the Ridley-Jopling histopathological classification of skin biopsies (19), in addition to slit skin smears stained by Fite stain for acid-fast organisms and careful clinical examination.

Antigens. Details of the isolation and structure of PhenGL-I, which consists of 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1 - 4)-2,3-di-*O*-methyl-α-L-rhamnopyranosyl-(1 - 2)-α-L-3-*O*-methylrhamnopyranosyl-para-29-phenol-9,11-dimycocerosyl-3-methoxyl-4-methyl-nonacosene, have been described previously (9, 10). In addition, synthesis of the terminal monosaccharide as the 8-methoxy-carboynyloctyl-3,6-di-*O*-methyl-β-D-glucopyranoside, its conjugation to bovine gamma globulin (BGG) to yield monosaccharide-linked BGG (MS-BGG), and its serological testing have been described elsewhere (5a). PhenGL-I and two control glycolipid antigens, mycoside A derived from *Mycobacterium kansasii* (13) and galactocerebroside (Sigma Chemial Co., St. Louis, Mo.), were diluted in methanol and dried onto the wells of polystyrene microtiter plates for in vitro cell culture. In some experiments PhenGL-I was incorporated into liposomes as described previously (14). The lepromin used in the studies in Thailand was obtained through the courtesy of R. J. W. Rees (IMMLEP Program, World Health Organization). Phytohemagglutinin (PHA) was used in 3-day cultures (1:500 dilution; Sigma).

Serological assay. Serum antibody titers were determined for 16 of the 18 Chicago patients on two samples 1 year apart and for all Thai patients on only one blood sample. In a standard enzyme-linked immunosorbent assay, 96-well Immulon-1 U-bottom plates (Dynatech Laboratories, Inc., Alexandria, Va.) were first coated with 50 μl of PhenGL-I (2 μg/ml) or MS-BGG conjugate at a 1:5,000 dilution (stock solution, 320 μg of monosaccharide in a solution containing 2,800 μg of BGG per ml). Serum samples were diluted 1:300 and incubated for 120 min at 37°C. Horseradish peroxidase-labeled antibody to human immunoglobulin M (IgM) or IgG (Cappel Laboratories, Cochranville, Pa.) was added at the dilution recommended by the manufacturer, and the preparation was incubated for 30 min. Results are reported as optical density at 490 nm. A significant antibody titer was indicated by an optical density reading that was 2 standard deviations above the mean for 19 unexposed United States controls. A strong response was arbitrarily defined as a titer that was 20 standard deviations above the control mean.

Preparation of PBMC. For studies on Chicago patients and unexposed controls, heparinized blood was drawn 12 to 18 h before culture and was shipped overnight at ambient temperature to Albuquerque, N.M.; in Thailand, cultures were established on the same day that blood was obtained. In the studies of both groups of patients peripheral blood was sedimented by centrifugation at 400 × g on Ficoll-Hypaque cushions to isolate peripheral blood mononuclear cells (PBMC) containing 5 to 15% monocytes, as determined by using nonspecific esterase stain. The cells were washed three times in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 1,000 U of penicillin G per ml, and 250 μg of streptomycin sulfate per ml. Cultures were also grown with 10% autologous serum in place of fetal calf serum; in each case the result was the same. The results obtained with autologous serum are reported below for four cases in which background stimulation with fetal calf serum was unacceptably high (more than 2,000 cpm).

Separation of T- and B-cell fractions. T-cell fractions were obtained by incubating PBMC with aminoethylisothiouronium bromide-treated sheep erythrocytes for spontaneous rosetting (15), followed by separation on Ficoll-Hypaque cushions. This resulted in populations of 75 to 84% T cells, as determined by staining with the pan-T-cell monoclonal antibody OKT3 (23) in fluorescence-activated flow cytometry. T-cell preparations contained 3 to 5% monocytes. The nonrosetting fraction was incubated on plastic dishes coated with mouse anti-human immunoglobulin, and the resulting

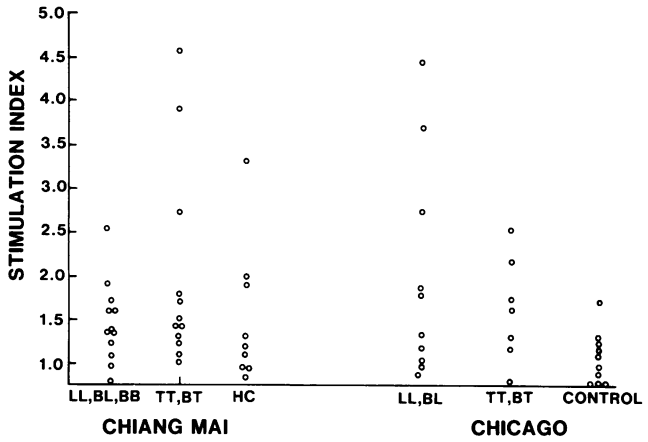

FIG. 1. [^{3}H]thymidine incorporation by PBMC after a 5-day incubation in the presence of 20 or 200 ng of PhenGL-I. There were 22 patients with lepromatous leprosy (LL, BL, BB) and 19 patients with tuberculoid leprosy (TT, BT); 17 of the patients were from Chicago, and 24 were from Thailand. In Thailand, 9 individuals with household contacts (HC) were studied; in the United States, 10 unexposed controls were studied. Positive LP was defined as an S.I. of >2.0. LL, Polar lepromatous leprosy; BL, borderline lepromatous leprosy; BB, borderline leprosy; TT, tuberculoid leprosy; BT, borderline tuberculoid leprosy.

adherent cells were cultured as a B-cell-enriched population which contained 50 to 80% immunoglobulin-positive B cells. This preparation contained less than 5% OKT3$^+$ cells and failed to respond to PHA.

Culture conditions. PBMC and T- and B-cell-enriched fractions were cultured in 96-well microtiter plates (Nunclon; Nunc A/S, Roskilde, Denmark) at a cell density of 0.5×10^6 cells per ml (final volume, 0.2 ml/well) at 37°C in a 5% CO_2 atmosphere. The blastogenic response was determined on day 5 of culture by pulsing the cells with [^{3}H]thymidine (6.7 Ci/mmol; 0.5 μCi/well; New England Nuclear Corp., Boston, Mass.). The amount of thymidine taken up by the cells was determined 8 h later by harvesting the cells on glass fiber filter paper (MASH II) and using standard scintillation counting. Results are reported below as the difference between the radioactivity of stimulated cultures and the radioactivity of unstimulated cultures (Δcpm) and as the stimulation index (S.I.), which was calculated by dividing the mean counts per minute for antigen-stimulated cells by the mean counts per minute for unstimulated cells. Group means for unstimulated cultures (background) ranged from 363 to 1,070 cpm.

Statistical analysis. Differences between groups were tested by a two-tailed t test and a chi-square test; a P value of <0.05 was considered significant. To test the relationship between lymphocyte reactivity and antibody titers, correlation coefficients and single-variable regression equations were calculated by using a microcomputer (Statpak; Northwest Associates, Portland, Oreg.).

RESULTS

LP response to PhenGL-I. A positive lymphocyte proliferation (LP) response was defined as an S.I. of >1.7. Among all patients, PBMC from 9 of 41 patients (22%) responded in vitro to PhenGL-I (Fig. 1). Of 22 patients with lepromatous disease, 4 (18%) responded, compared with 6 of 20 patients (30%) with tuberculoid leprosy; this included one borderline tuberculoid leprosy responder for whom only T-cell data were available (Table 1). Of the nine individuals with household contact in Thailand, one was a responder. The magnitude of all responses was small, as indicated by both the S.I. and the mean counts per minute (Table 1). There was no reactivity to the control glycolipids galactocerebroside (S.I., 1.06 ± 0.21 [mean ± standard deviation]) and mycoside A with both responder and nonresponder PBMC.

The absence of a proliferation response to PhenGL-I did not appear to be explained by general unresponsiveness, since proliferation in PBMC nonresponders stimulated by a suboptimal concentration of PHA in a 3-day culture was maintained (mean ± standard deviation, 44,725 ± 9,411 cpm). Among the Thai patients PhenGL-I reactors had a mean response (S.I.) to lepromin of 4.4 ± 5.1, compared with 3.3 ± 6.1 for nonreactors (not significant as determined by the t test).

Among the patients from Thailand and Chicago, responders to PhenGL-I did not differ from nonresponders with respect to sex, age, or histological classification. Of the three PhenGL-I responders with lepromatous leprosy from Chicago, two had histories of chronic erythema nodosum leprosum, compared with five patients with erythema nodosum leprosum among the seven PhenGL-I nonresponders. The bacillary index ranged from 1.0 to 2.0 for the PhenGL-I responders and from 1.0 to 6.0 for the PhenGL-I nonresponders. For the six PhenGL-I responders, the interval between time of diagnosis and time of lymphocyte studies ranged from 12 months to 7 years; the range for nonresponders was 2 weeks to 7 years. All four patients tested within 4 months of diagnosis and onset of treatment were PhenGL-I nonresponders.

When liposome-packaged PhenGL-I antigen was used, nine experiments performed by using PBMC from Chicago patients showed no significant difference (<50% change) in eight cases and a 200% increase in the response of one polar lepromatous leprosy responder (data not shown).

PBMC responding to PhenGL-I in vitro demonstrated a broad dose-response profile. Of the nine responders, five showed a maximum response at 200 ng/well, and four showed a maximum response at 20 ng/well. In the presence of 2,000 ng of PhenGL-I per well, a marked decrease in cell proliferation occurred in both controls and responders. To examine whether PhenGL-I impaired the proliferation of lymphocytes, lymphocytes were stimulated by a suboptimal dose of PHA in the presence of different doses of PhenGL-I

TABLE 2. Inhibition of PHA response by PhenGL-I

Group	No.	PHA Δcpm (cpm)	% inhibition by PhenGL-I at the following concn:		
			1.0 μg/ml	0.1 μg/ml	0.01 μg/ml
Patients	11	30,055 ± 6,736	21.5 ± 3.9[a]	10.1 ± 4.4	1.8 ± 5.3
Controls	5	46,373 ± 9,928	35.9 ± 15.0	21.0 ± 3.8	ND[b]

[a] Mean ± standard deviation.
[b] ND, Not determined.

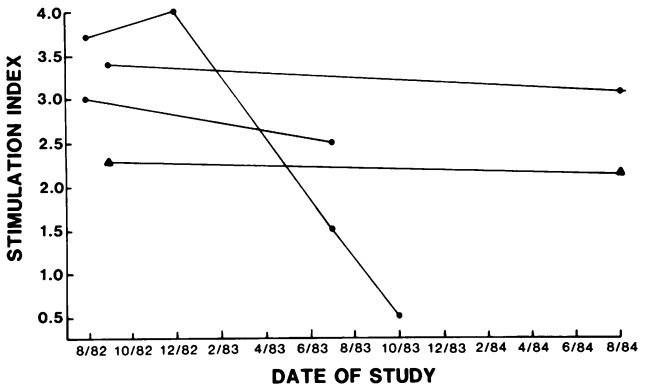

FIG. 2. Longitudinal study of [^{3}H]thymidine incorporation of PBMC from four PhenGL-I responders in Chicago who were studied a second time at least 1 year after the initial study. Symbols: ●, patients with polar lepromatous leprosy or borderline lepromatous leprosy; ▲, patient with tuberculoid leprosy or borderline tuberculoid leprosy.

(Table 2). A dose-dependent decrease in thymidine incorporation was seen in both patients with leprosy and controls.

T-cell and B-cell responsiveness to PhenGL-I. Using cells from 12 Chicago patients, we cultured sheep erythrocyte-rosetting T cells in the same way that we cultured PBMC. Of three patients with PhenGL-I-responsive PBMC, two (one with polar lepromatous leprosy and one with borderline tuberculoid leprosy) were responsive (S.I., 2.3 and 2.1, respectively). T cells from the third PBMC-responsive patient had an S.I. of 1.6 and a Δcpm value of 1,232 cpm to PhenGL-I, but a Δcpm value to PHA of only 8,108 cpm; the level of PHA stimulation for all other T-cell studies was 53,990 ± 12,806 cpm.

B-cell LP stimulated by PhenGL-I was studied in eight patients, including three PBMC responders. All showed no specific response, with a mean S.I. of 1.15 and a mean Δcpm of 65 cpm. Unexposed controls also showed no response (mean S.I., 1.06; mean Δcpm, −84 cpm).

Longitudinal study of cellular responsiveness to PhenGL-I. Four Chicago responder patients were tested a second time 1 to 2 years later, and three remained responsive (Fig. 2). A woman with polar lepromatous leprosy and repeated bouts of erythema nodosum leprosum showed a LP response to PhenGL-I 24 and 28 months after the beginning of therapy. Responsiveness decreased during continued episodes of erythema nodosum leprosum while she was receiving steroids at intermittent low doses. Thus, reactivity to PhenGL-I appeared to be commonly, but not always, stable over time, at least in this small sample of patients.

Serum antibody to PhenGL-I. MS-BGG conjugate was more sensitive than PhenGL-I for detecting antibody by an enzyme-linked immunoassay. The MS-BGG conjugate detected IgM antibody in 34 of 40 cases (85%), compared with 24 of 41 cases (58%) for PhenGL-I ($P < 0.05$; chi-square test). The MS-BGG conjugate detected IgG antibody in 12 of 40 cases (30%), compared with 6 of 32 cases (19%) for PhenGL-I. When the IgM antibody detected by the MS-BGG conjugate was examined, patients with lepromatous disease were more likely than patients with tuberculoid leprosy to have high titers (optimal density, >0.75; $P = 0.05$; Fisher exact test). There was no difference between patients from Thailand and patients from Chicago with respect to the antibody detected by either antigen.

Correlation between PBMC proliferation and antibody optical density. LP responsiveness to lepromin was negatively correlated with IgM binding to the MS-BGG conjugate ($r = -0.60$; $P < 0.001$) (Fig. 3). The LP response to PhenGL-I correlated negatively with IgM antibody binding to the MS-BGG conjugate ($r = -0.33$; $P < 0.05$). IgG binding to MS-BGG tended to be positively correlated with LP ($r = 0.34$; $P < 0.05$; one-tailed test) among Chicago patients.

DISCUSSION

In this study we sought evidence for a specific T-cell proliferative response to the glycolipid PhenGL-I, which bears a unique carbohydrate epitope (14). Specificity was indicated by (i) a lack of reactivity among unexposed controls; (ii) a lack of reactivity to galactocerebroside, another common glycolipid of *M. leprae*; (iii) consistent reactivity when a repeat study was performed months later; and (iv) the response of T cells but not B cells in a few of the responder patients studied. However, The magnitude of the proliferative response was small, and the frequency of

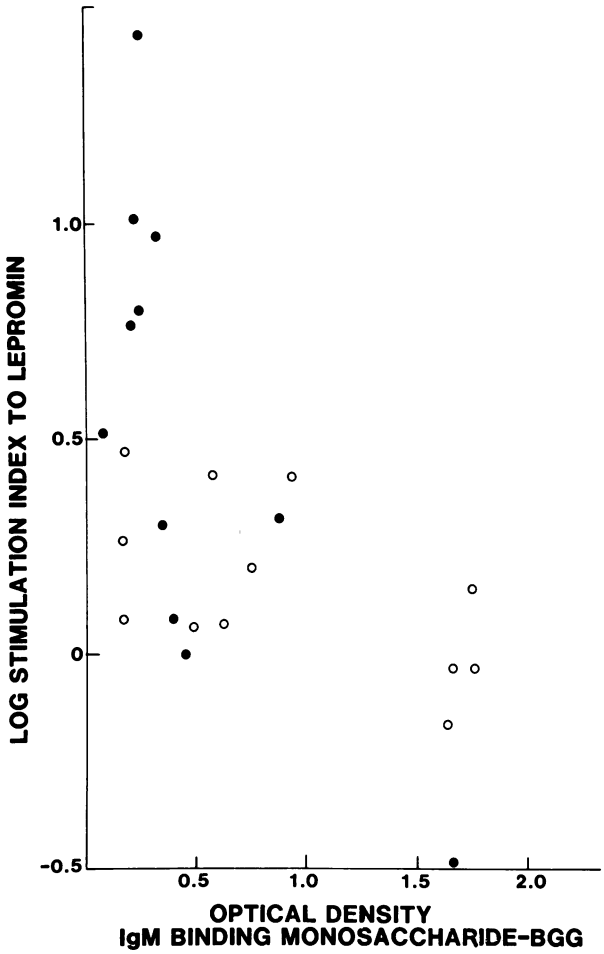

FIG. 3. Relationship between titer of IgM anti-PhenGL-I antibody binding to MS-BGG complex in an enzyme-linked immunosorbent assay and log S.I. of PBMC response to lepromin in 23 patients in Thailand. Symbols: ○, patients with polar lepromatous leprosy, borderline lepromatous leprosy, or borderline leprosy; ●, patients with tuberculoid leprosy or borderline tuberculoid leprosy. $r = -0.602$; $P < 0.001$.

responsiveness (24%) was also small. Several explanations can be proposed.

First, cellular responsiveness could be a function of duration or intensity of exposure to PhenGL-I. The titer of IgM antibody to PhenGL-I appears to be related to antigen load (7, 8). In this study cellular responsiveness was negatively correlated with specific IgM antibody, suggesting that low bacillary loads permitted responsiveness. However, there was no correlation between LP and bacillary index, nor were there significant differences in the number of responders among patients with lepromatous leprosy (18%) compared with patients with tuberculoid leprosy (30%).

Second, it is possible that pure carbohydrate antigens may not elicit proliferative responses by T cells. T cells have been clearly documented to recognize capsular polysaccharides of *Bacteroides fragilis* (22). Specific T cells mediate the shift from IgM to IgG antibody responses to many polysaccharides (2, 5). T cells from infected calves (20) and vaccinated humans (17) have been shown to proliferate specifically in response to the O-polysaccharide antigen derived from the lipopolysaccharide of the homologous (infecting or vaccinating) *Salmonella* species. In these studies the optimum concentration of oligosaccharide antigen was 200 to 1,000 ng/well, and the degree of responsiveness was small (S.I., <3.0; Δcpm, <3,000 cpm). However, specificity was assured by a lack of response to a slightly different control *Salmonella* O-polysaccharide and, in the study of young calves, by concordance with developing a positive skin test response to the same antigen (21). Finally, in lepromin-sensitive infected armadillos, PhenGL-I elicited a delayed-type hypersensitivity dermal reaction, with granulomas demonstrated in skin biopsies (C. K. Job, submitted for publication). Thus, a variety of carbohydrate antigen epitopes do stimulate T-cell-directed immunity and proliferative T-cell responses in vitro, and it is likely that PhenGL-I stimulates proliferative T cells if it can also stimulate delayed-type hypersensitivity. The relationship between the operational subclass of T cells which proliferate in vitro in the presence of specific antigen and the functional subclasses of T cells, such as helper and suppressor cells, is unclear. In the case of several protein antigens, different T-cell subclasses recognize completely different epitopes (11). Thus, in the case of single-epitope antigens, such as PhenGL-I, only a restricted number of T-cell subclasses may respond to the antigen.

Third, carbohydrate antigens may elicit concomitant suppression, particularly if higher doses of antigen prime the immune response (2). In patients with untreated lepromatous leprosy, PhenGL-I specifically activated T suppressor cells to nonspecifically suppress concanavalin A-activated T-cell proliferation (18). Thus, PhenGL-I may selectively stimulate suppression, obscuring responses by other T-cell subclasses. Our observation that PhenGL-I decreases thymidine incorporation in PHA-stimulated lymphocytes may be explained by active cell-mediated suppression (18) or by more direct effects on proliferating cells or thymidine incorporation itself. PhenGL-I did not appear to inhibit the development or expression of delayed-type hypersensitivity reactions to *M. leprae* in vivo in CBA mice (3), but this does not necessarily exclude an immunoregulatory role for PhenGL-I.

We confirmed that patients with polar lepromatous leprosy and borderline lepromatous leprosy were more likely to have high titers of antibody to PhenGL-I than patients with tuberculoid leprosy or borderline tuberculoid leprosy and that IgM antibody predominated over IgG antibody (7, 8, 25). Cho et al. (7) proposed that the persistence of IgM antibody to PhenGL-I may be due to a lack of a T-helper-cell-mediated switch to IgG antibody. In our patients, LP to PhenGL-I correlated negatively with IgM anti-PhenGL-I and positively with IgG anti-PhenGL-I. In a study of *M. leprae*-immunized inbred mouse strains (16), LP responses to PhenGL-I tended to correlate ($r = 0.43$; $P < 0.1$) with IgG anti-PhenGL-I titers. Our results support the notion that a lack of T cells mediates the switch from IgM to IgG antibody, although the small proliferative responses encountered necessitate caution in drawing any conclusions.

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