IgM and IgG Antibodies to Phenolic Glycolipid I from *Mycobacterium leprae* in Leprosy: Insight into Patient Monitoring, Erythema Nodosum Leprosum, and Bacillary Persistence

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Serum IgM and IgG antibodies against *Mycobacterium leprae*-derived phenolic glycolipid I (PG) were determined in leprosy patients, contacts, and controls by enzyme-linked immunosorbent assay (ELISA). Anti-PG IgM levels increased from the tuberculoid (TT) to the lepromatous (LL) pole of the disease spectrum. There was a positive linear correlation between anti-PG IgM and bacillary index (BI). Patients with erythema nodosum leprosum (ENL) had lower levels of serum anti-PG IgM than non-ENL patients of comparable BI, suggesting that anti-PG IgM is involved in the pathogenesis of ENL. Initial observations indicate that high anti-PG IgM levels in bacillary-negative patients might reflect bacillary persistence. A study of 2 different substrate reagents in the ELISA [2,2′-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS), 0.1 mM H_2O_2, serum diluted 1:20, and *o*-phenylenediamine (OPD), 5 mM H_2O_2, serum diluted 1:300] showed generally good correlation in detection of anti-PG IgM. However the OPD system detected more paucibacillary disease (BT), while the ABTS system detected the significant effect of ENL on the relationship between BI and anti-PG IgM. Anti-PG IgM was clearly dominant over anti-PG IgG. However, certain patients, including several patients who had upgraded from LL and borderline lepromatous leprosy (BL), showed high levels of anti-PG IgG. Since studies have shown that LL patients are selectively deficient in cell-mediated immunity, T-cell products may be required for the IgM to IgG isotype switch. We conclude that anti-PG IgM is useful for monitoring the bacillary load in individual patients and should prove useful for leprosy control strategies. *J Invest Dermatol 86:529–534, 1986*

The most widely used method for following disease activity and effect of treatment in leprosy patients is to assess the load of *Mycobacterium leprae* by measuring the bacillary index (BI) in skin biopsies and slit smears. In recent years, several advances in the serologic detection of leprosy infections have been reported. Hunter et al [1] have described a phenolic glycolipid I (PG) obtained from armadillo-derived *M. leprae* containing an *M. leprae*-specific trisaccharide. Serum antibodies against this glycolipid in leprosy have been detected by immunodiffusion [2] and enzyme-linked immunosorbent assay (ELISA) techniques [1–4]. Serum levels of anti-PG IgM predominating over anti-PG IgG [2,3] have been shown to increase from the tuberculoid (TT) toward the lepromatous (LL) pole of the disease spectrum [1–4]. Long-term treatment of leprosy has been reported to result in a decrease in antibodies to PG [2,3]. These results suggest a relationship between bacterial load and serum anti-PG Ig levels.

In the current study we have developed a reproducible ELISA for detection of IgM and IgG antibodies to PG. In keeping with the work of others [1–4], our study has shown a characteristic pattern of anti-PG IgM in patients grouped according to the Ridley-Jopling classification [5]. In addition, by emphasizing clinical correlations, the current study has shown 4 additional findings that make antibodies against PG especially useful for monitoring leprosy patients.

1. A direct correlation of anti-PG IgM with the BI with some exceptions noted below.
2. BI alone cannot account for elevated anti-PG IgM values in LL and borderline lepromatous leprosy (BL). Significant anti-PG IgM levels elevated out of proportion to BI were noted. Active neuropathies and recent elevated BI values suggested that anti-PG IgM measured occult bacillary activity, bacillary persistence undetected by biopsy, or antigenic persistence of PG.
3. A decrease in the relationship between BI and anti-PG IgM (but not anti-PG IgG) with erythema nodosum leprosum

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Abbreviations:
- ABTS: 2,2′-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid)
- BB: midborderline leprosy
- BI: bacillary index
- BL: borderline lepromatous leprosy
- BSA: bovine serum albumin
- BT: borderline tuberculoid leprosy
- ELISA: enzyme-linked immunosorbent assay
- ENL: erythema nodosum leprosum
- E: extinction
- HC: household contacts
- LL: lepromatous leprosy
- NC: nosocomial contacts
- OPD: *o*-phenylenediamine
- PBS: phosphate-buffered saline
- PG: phenolic glycolipid I
- TT: tuberculoid leprosy

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(ENL), suggested an involvement of anti-PG IgM in the pathogenesis of ENL.

4. Finally, while anti-PG IgM predominates over anti-PG IgG, some patients develop good IgG responses. Initial observations indicate the lack of anti-PG IgG may be related to selective immune defects of lepromatous leprosy.

MATERIALS AND METHODS

Sera Leprosy patients were clinically and histologically classified according to the Ridley-Jopling scale [5]. The BI was measured on a semiquantitative scale (0–6+) approximating that of Ridley [6]. Histology (including BI) was reported from the National Hansen's Disease Center by 2 pathologists (C. K. Job, J. A. Freeman), whose agreement on BI was good ($r = 0.94$). Contacts of leprosy patients consisted of household contacts (HC) and 14 nosocomial contacts (NC). Healthy subjects were used as normal controls. Serum samples were stored in aliquots at −70°C.

ELISA for Anti-PG IgM and IgG ELISA screening of sera for anti-PG Ig was performed with PG incorporated into liposomes, as described for antibodies against glycosphingolipids [7]. PG-liposomes were prepared as described by other authors [2]. PG (armadillo-derived PG, kindly provided by P. J. Brennan), sphingomyelin, cholesterol, and dicetyl phosphate (0.1:2.0:1.5:0.2, molar ratio) were combined in chloroform-methanol 2:1 (v/v), the solvent was removed by rotary evaporation and the lipid mixture was sonicated (Branson Sonifier) in Tris-buffered saline (0.15 M NaCl, 20 mM Tris, pH 8.0) for 3 min (300 μg PG/ml). Control liposomes without PG were prepared as above. Flat-bottomed polystyrene microtiter plates (Dynatech) were coated by incubation with 100 μl of the PG-liposome suspension (diluted to 2.5 μg PG/ml in Tris-buffered saline), or with the control-liposome suspension, for 18 h at 37°C. After washing (3×) with phosphate-buffered saline (PBS) (0.1 M phosphate buffer, pH 7.2, 0.15 M NaCl), plates were incubated for 1.5 h at 37°C with 200 μl of 3% (w/v) bovine serum albumin (BSA, Sigma) in PBS (BSA-PBS). BSA-PBS was replaced by 100 μl of test serum (in duplicate), diluted 1:20 in BSA-PBS. A positive lepromatous serum pool was included as reference on each plate. After incubation with test serum for 1.5 h at 37°C, plates were again washed with PBS (3×) and incubated for 1.5 h at 37°C with 100 μl of peroxidase-conjugated goat antihuman IgM or IgG (Cappel), diluted 1:1000 in BSA-PBS. After washing with PBS (3×) 100 μl of substrate solution, containing 1.8 mM 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Boehringer) and 0.1 mM H_2O_2 in 0.1 M phosphate buffer pH 7.0, was added for 1 h at room temperature. The reaction was stopped with 100 μl of 0.32% (w/v) NaF and the extinction (E) was read at 405 nm using a Titertek microtiter plate reader. The results were expressed as Δ E = E (PG-liposome coat) − E (control liposome coat).

In order to compare our results with those of others [3], anti-PG IgM levels were also determined by ELISA using an *o*-phenylenediamine (OPD) substrate solution. The OPD ELISA procedure was identical to the ABTS ELISA with the following exceptions: (1) Sera were diluted 1:300 in BSA-PBS. (2) The substrate solution contained 2.2 mM OPD (Sigma) and 5 mM H_2O_2 in citrate phosphate buffer pH 5.0. (3) The reaction was stopped after 30 min with 100 μl 2.5 N H_2SO_4 and absorbance read at 492 nm.

Statistical Analysis Analysis was done by computer using BMDP statistical packages [8]. Differences in anti-PG IgM and IgG levels among leprosy patients divided into Ridley-Jopling classes and controls were ascertained using analysis of variance with follow-up *t*-tests. In addition, because of the nonnormal distribution of ΔE values, the data were also analyzed by the nonparametric Kruskal-Wallis test to confirm the analysis of variance results. Differences in anti-PG IgM in Ridley-Jopling groups and controls were also determined by analysis of variance using BI as covariate in order to determine whether factors other than BI contributed to high anti-PG IgM in leprosy patients.

Multiple correlations of IgM, IgG, and ENL were done with BI as an independent variable using data from leprosy patients only to determine effects of ENL on the relationship between anti-PG IgM and BI, and anti-PG IgG and BI.

RESULTS

Experiments comparing techniques described by other authors for coating PG onto microtiter plates are shown in Fig 1. The most effective of these, PG in hexane, was compared with coating with PG incorporated into liposomes (Fig 2). It can be seen that

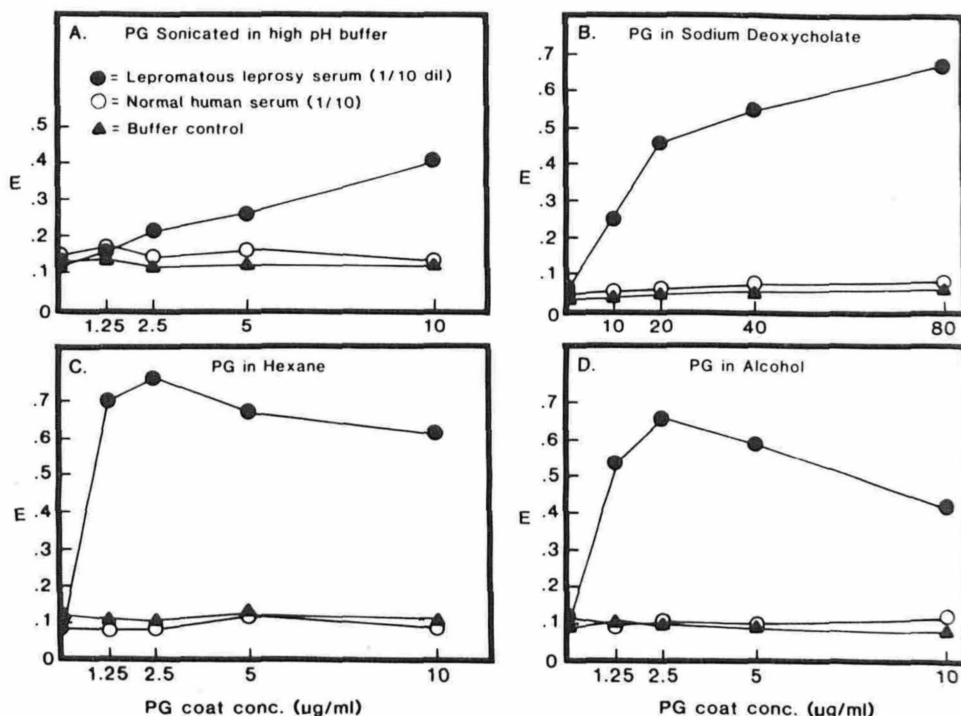

Figure 1. Comparison of 4 techniques for coating PG onto microtiter plates for ELISA. *A*, PG (100 μg/ml) suspended in carbonate-bicarbonate buffer pH 9.6 by sonication, diluted to appropriate concentration, and incubated in microtiter plates overnight at 37°C [3]. *B*, PG (80 μg/ml) dissolved in PBS + 0.1% sodium deoxycholate, diluted to appropriate concentrations, and incubated overnight at 37°C [2]. *C and D*, PG (10 μg/ml) dissolved in hexane (*C*) or ethanol (*D*), diluted to appropriate concentrations, dispensed in microtiter plates and air-dried until solvent evaporated [1].

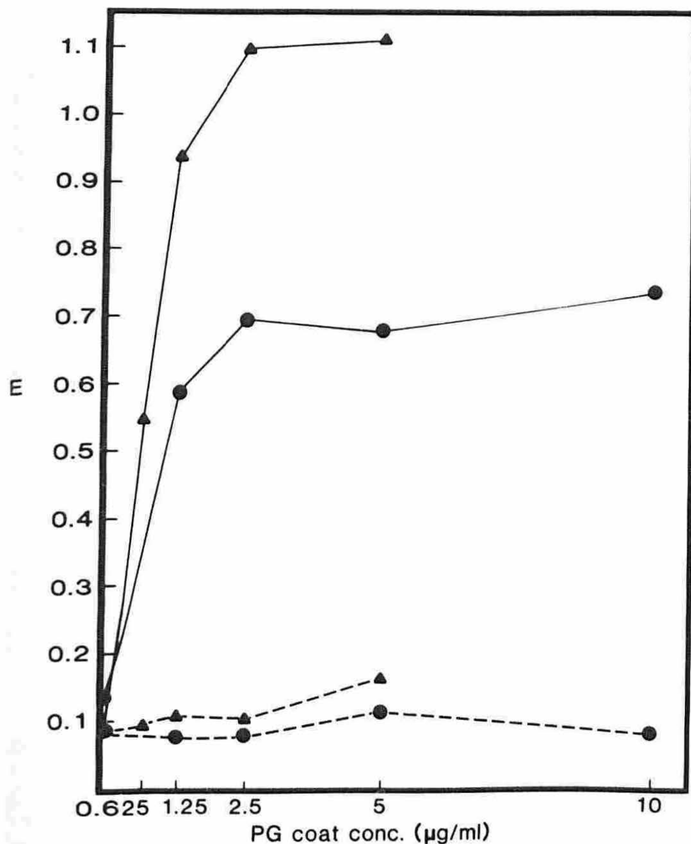

Figure 2. Comparison of coating techniques using PG dissolved in hexane and PG liposomes. ▲ = PG-liposomes; ● = PG in hexane; *broken lines* = control serum (1:10); *solid lines* = lepromatous leprosy serum (1:10).

greater antibody binding was obtained with PG liposomes than with PG in hexane. Optimal coating concentration for PG liposomes was achieved by 2.5 μg PG/ml, resulting in the most efficient utilization of limited PG supplies.

Screening of sera from leprosy patients, contacts, and controls for anti-PG IgM antibodies revealed a characteristic pattern of anti-PG IgM in patients grouped according to the Ridley-Jopling classification. When the ELISA was performed using sera at 1:20 dilution and the ABTS substrate solution, the LL and BL groups had significantly elevated anti-PG IgM levels compared with controls ($p < 0.001$, $p < 0.05$, respectively) by analysis of variance (Fig 3*A*). The nonparametric Kruskal-Wallis test indicated that LL, BL, midborderline leprosy (BB), and borderline tuberculoid leprosy (BT) groups were higher than controls ($p < 0.05$). Testing of HC and controls yielded one serum in each group with highly elevated ΔE values for anti-PG IgM. Another serum sample was taken from the positive control 3 months after the initial bleeding which gave similar results.

When sera were tested for anti-PG IgM at a 1:300 dilution using the OPD substrate solution LL, BL, and BT groups were significantly higher than controls ($p < 0.001$, $p < 0.01$, $p < 0.01$, respectively) (Fig 3*B*). Nonparametric methods again showed LL, BL, BB, and BT groups to be higher than controls ($p < 0.05$). Correlation between ΔE values for the ABTS and OPD assays was good ($r = 0.8038$, $p < 0.01$).

Anti-PG IgM in leprosy patients was found to increase with BI when assayed with the ABTS ($r = 0.5162$, $p < 0.01$) and the OPD ($r = 0.4409$, $p < 0.01$) substrate solutions (Fig 4*A*,*B*). When all leprosy patients were used for analysis it was found that ENL had a significant effect on the relationship between BI and anti-PG IgM, with ENL patients having lower levels of serum anti-PG IgM than non-ENL patients of comparable BI (Fig 4*A*, $p =$ 0.0024). The effect of ENL on the relationship between anti-PG IgM and BI was apparent using the ABTS substrate solution, but not the OPD substrate solution, as the difference in the slopes of the regression lines for ENL and non-ENL patients did not reach

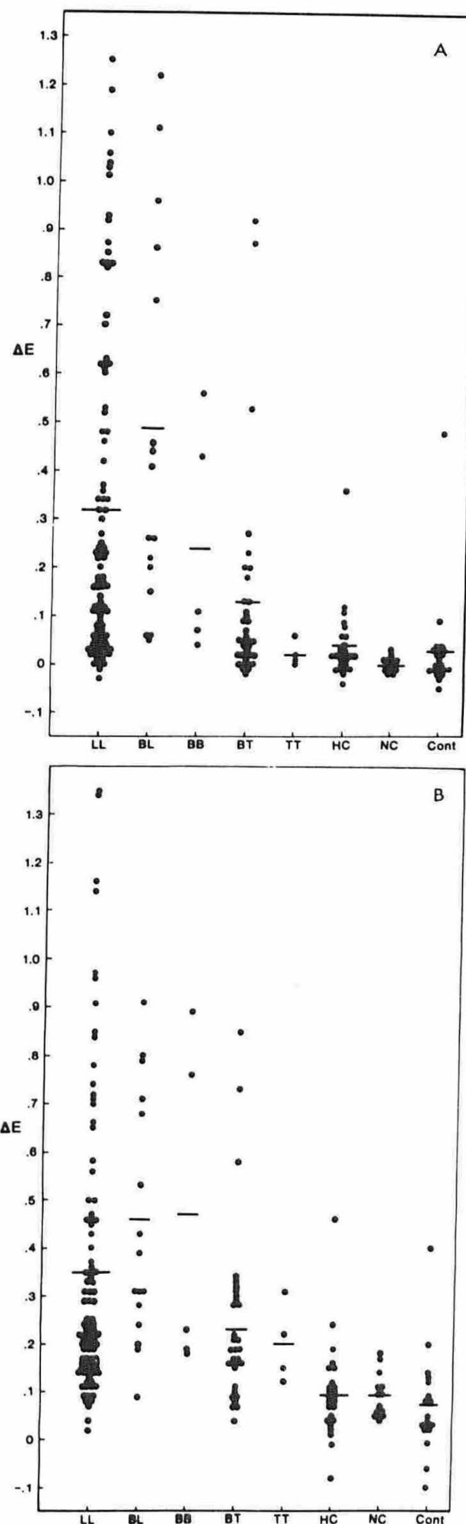

Figure 3. *A*, Anti-PG IgM levels vs Ridley-Jopling classification. Sera diluted 1:20, ABTS substrate solution. *B*, Anti-PG IgM levels vs Ridley-Jopling classification. Sera diluted 1:300, OPD substrate solution. *LL*, lepromatous leprosy; *BL*, borderline lepromatous; *BT*, borderline tuberculoid; *TT*, tuberculoid leprosy; *HC*, household contacts; *NC*, nosocomial contacts; *Cont*, normal controls.

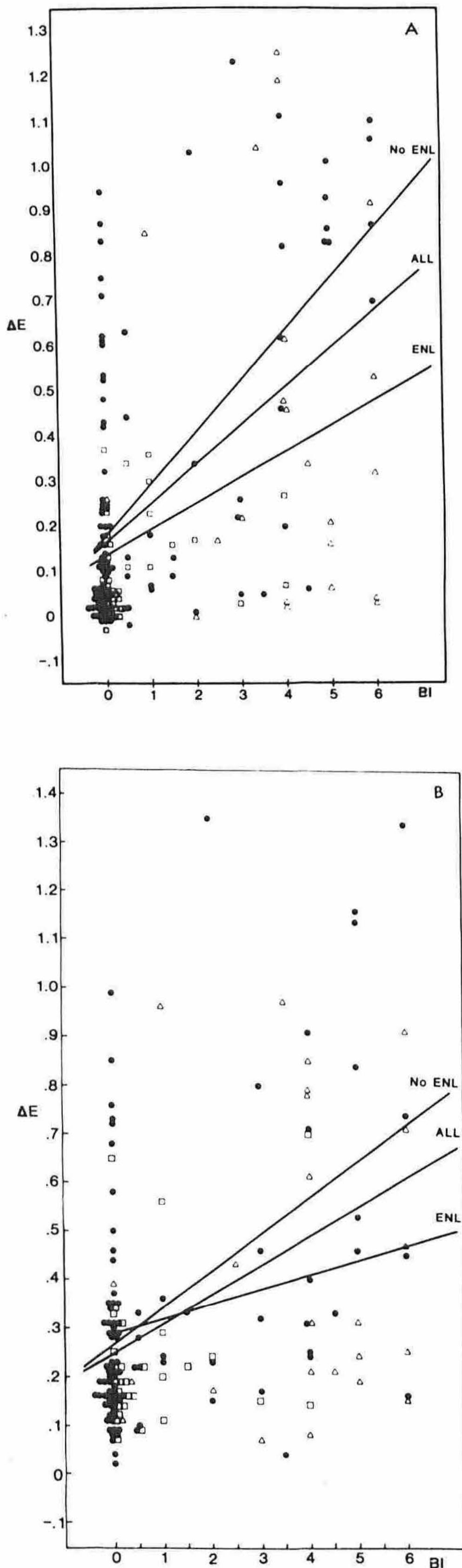

Figure 4. *A*, Effect of ENL on the relationship between BI and anti-PG IgM. Sera diluted 1:20, ABTS substrate solution. ● = non-ENL patients, □ = ENL more than 2 years ago, Δ = ENL within the last 2 years. *B*, Effect of ENL on the relationship between BI and anti-PG IgM. Sera diluted 1:300, OPD substrate solution.

significance ($p = 0.089$) (Fig 4*B*). The effect of ENL on the relationship between BI and anti-PG IgM was also seen when only BL and LL patients were used for analysis. With ABTS substrate solution and sera diluted 1:20, the regression line for BI vs anti-PG IgM was significantly different for ENL patients as opposed to non-ENL patients. Again, with OPD and sera at 1:300 the effect of ENL on BI vs anti-PG IgM was not seen (Table I).

Although anti-PG IgM increased significantly with increased BI, factors other than BI contribute to elevated anti-PG IgM levels in leprosy patients. A number of patients with BI = 0 are seen to have elevated anti-PG IgM (Fig 4*A*,*B*). Statistical analysis was performed to determine whether factors other than BI significantly contribute to anti-PG IgM levels in leprosy patients divided into Ridley-Jopling groups. Covariance analysis, using BI as covariate determined that anti-PG IgM was significantly elevated in leprosy patients due to factors other than BI (Table II). Using ABTS data, LL and BL groups showed significantly elevated anti-PG IgM levels not related to BI. OPD data indicate LL, BL, BB, and BT groups have elevated anti-PG IgM levels not related to BI.

ELISA screening for anti-PG IgG showed LL patients as a group had significantly higher anti-PG IgG levels than controls ($p < 0.01$) (Fig 5). The nonparametric Kruskal-Wallis test showed LL, BL, and BB groups to be significantly higher in anti-PG IgG than controls ($p < 0.05$). Although some BT patients had elevated anti-PG IgG, as a group BT was not significantly higher than control.

There was not a significant correlation between BI and anti-PG IgG as there was for anti-PG IgM when all patients were analyzed. However, when only BL and LL patients were analyzed it appeared that a relationship existed among BI, anti-PG IgG, and ENL in which anti-PG IgG increased with rising BI in ENL patients and remained the same or decreased slightly with rising BI in non-ENL patients (Table III). Thus the effect of ENL on anti-PG IgG levels was opposite to the ENL effect on anti-PG IgM levels.

DISCUSSION

Our study shows that, under appropriate conditions, anti-PG IgM antibodies correlate directly with the BI. This finding means that anti-PG IgM can be used to monitor disease activity in leprosy patients. While exceptions occur (such as ENL and possible bacillary or antigenic persistence) an elevated anti-PG IgM should alert the clinician to bacillary activity and serve as an adjunct to skin biopsies and slit smears. Because bacillary activity varies significantly from one region to another, a negative skin biopsy in one region does not exclude bacillary activity in other regions of the skin, nerves, or even visceral involvement such as the liver, spleen, or testicles. We currently utilize slit smears on patients who are bacillary-negative by skin biopsy, but have elevated anti-PG IgM and are proven hepatitis B antigen-negative. Factors other than BI, ENL, and bacillary persistence, such as genetics [9], are likely to also affect the antibody response to PG. Nevertheless, from the epidemiologic vantage, the finding that anti-PG IgM reflects bacillary load should make it possible to evaluate bacillary activity within a given program or given geographic region. This ability may prove important for evaluating vaccination or other strategies, such as the use of recombinant T-cell products, for leprosy control.

Bacillary persistence is an identified problem in leprosy [10]. The reasons for bacillary persistence are currently poorly understood. Subpopulations of Schwann cells or macrophages may harbor antibiotic-sensitive bacilli although in selected individuals antibiotic distribution patterns, or other mechanisms, may be involved. Existing methods for monitoring bacillary persistence in leprosy are cumbersome. Multiple skin biopsies, slit smears, and even nerve biopsies sample only small regions and do not exclude bacillary activity in other areas. The degree to which an elevated anti-PG IgM reflects bacillary persistence in patients,

Table I. Relationship Among Anti-PG IgM, BI, and ENL in LL and BL Patients

	ENL		No ENL	
	No. of Patients	Regression Line	No. of Patients	Regression Line
ABTS	50	ΔE IgM = 0.140 + 0.051(BI)[a]	55	ΔE IgM = 0.238 + 0.115(BI)
OPD	50	ΔE IgM = 0.242 + 0.041(BI)	55	ΔE IgM = 0.262 + 0.082(BI)

[a]Slope of ENL regression line significantly different than non-ENL regression line ($p < 0.01$).
Abbreviations: *PG*, phenolic glycolipid I; *BI*, bacillary index; *ENL*, erythema nodosum leprosum; *LL*, lepromatous leprosy; *BL*, borderline lepromatous leprosy; *ABTS*, 2,2′-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid); *OPD*, *o*-phenylenediamine.

who by other available clinical criteria are bacillary-negative, is still not known. The slow elimination of the insoluble glycolipid antigen may require introducing the term "antigenic persistence" as well as "bacillary persistence." An elevated anti-PG IgM could indicate both in some instance and only antigenic persistence in others. Whether or not an elevated anti-PG IgM requires more aggressive therapy will be determined by future relapse rates. The duration of treatment with multiple antibiotics vs changing to a single "maintenance" regime is still undecided in leprosy [11,12]. Multibacillary leprosy patients treated with antibiotics probably never become totally bacillary-negative. This is supported by the recidivistic nature of multibacillary leprosy when antibiotic therapy is discontinued as well as by the phenomenon of bacillary persistence [10]. The persistence of *M. leprae* and mycobacterial antigens has been demonstrated in nerve biopsies from leprosy patients considered inactive by other criteria [13]. Thus our study suggests that the determination of serum anti-PG IgM levels, annually performed, as an adjunct to skin biopsies and skin scrapings, should assist the clinician in the decision on duration as well as regime of antibiotic treatment. Further study and sequential monitoring of leprosy patients is clearly indicated. Furthermore, sequential monitoring of contacts with good clinical evaluation and correlation should elucidate the usefulness of PG antibodies as a serologic screening test for leprosy, e.g., we already have detected a contact with an elevated anti-PG IgM. The contact (wife of a BL patient) had a mononeuropathy by clinical examination and nerve conduction velocity tests. A skin biopsy was read as nonspecific dermatitis and slit smears were negative. This symptomatic contact has been placed on Dapsone, 50 mg q.d. Her neuropathy will be followed clinically and by electrophysiologic monitoring. Only by further study of contacts with clinical correlation will we be able to determine at what stage antibodies to PG will be able to diagnose leprosy.

ENL, a major complication of multibacillary leprosy, has been attributed to a possible Arthus hypersensitivity reaction, induced by immune complexes [14]. The demonstration of IgM, but not IgG in ENL skin biopsies [15] as well as the inhibition of IgM synthesis by thalidomide [16], an effective treatment for ENL, suggest that IgM plays a major role in the pathogenesis of ENL. In keeping with the studies of Cho et al [17] and Andreoli et al [18], our study demonstrates significantly lower anti-PG IgM levels in patients with ENL reaction as compared with those without ENL, especially in patients with very high BI. The differences between results achieved with OPD and ABTS are most probably at the level of sensitivity and serum dilution. The 2 detection systems are generally in agreement, but the slightly increased background with OPD did not allow the ENL effect to achieve statistical significance. Additional comparison of our results with other laboratories is also in keeping with this interpretation (unpublished observations). Conversely, anti-PG IgG for ENL patients was somewhat higher than for non-ENL patients of comparable BI (also BB and BL higher than LL). LL and BL together and BL individually all showed significant differences in anti-PG IgG levels of ENL vs non-ENL patients of comparable BI. In addition, Modlin et al [19,20] have shown an increase in putative interleukin 2 producer cells during ENL by immunocytochemistry. These findings suggest that T-cell function and probably T-cell products are involved in the isotype switch from IgM to IgG and that T-cell function as well as immune complexes may be involved in ENL. The finding of opposite effects (i.e., decreasing anti-PG IgM and increasing anti-PG IgG) in ENL may reflect immune complex formation of IgM antibodies and an increase in T-cell products required for IgG production. Further studies, including sequential studies are indicated to further investigate these findings.

Overall, anti-PG IgG was significantly less pronounced than anti-PG IgM, in keeping with the findings of others [2,3]. How-

Table II. Anti-PG IgM Levels for Ridley-Jopling (R-J) Groups Adjusted for Effects of BI

		ABTS		OPD	
R-J Group	No. of Patients	Mean ΔE	Adjusted ΔE	Mean ΔE	Adjusted ΔE
LL	95	0.33	0.27[a]	0.35	0.31[a]
BL	15	0.49	0.42[a]	0.45	0.41[a]
BB	5	0.24	0.31	0.47	0.52[a]
BT	33	0.13	0.19	0.24	0.29[a]
TT	4	0.02	0.11	0.20	0.26
HC	8	0.03	0.11	0.08	0.14
NC	14	0.00	0.09	0.09	0.16
Cont	18	0.04	0.12	0.07	0.13

[a]Significantly different than controls ($p < 0.05$).
Abbreviations: *PG*, phenolic glycolipid I; *BI*, bacillary index; *ABTS*, 2,2′-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid); *OPD*, *o*-phenylenediamine; *E*, extinction; *LL*, lepromatous leprosy; *BL*, borderline lepromatous leprosy; *BB*, midborderline leprosy; *BT*, borderline tuberculoid leprosy; *TT*, tuberculoid leprosy; *HC*, household contacts; *NC*, nosocomial contacts; *Cont*, controls.

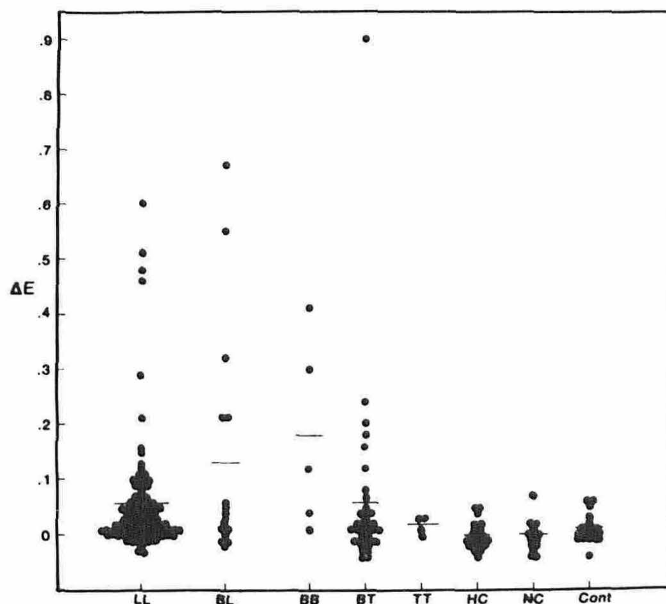

Figure 5. Anti-PG IgG vs Ridley-Jopling classification, ABTS substrate solution, sera diluted 1:20.

Table III. Relationship Among Anti-PG IgG, BI, and ENL in LL and BL Patients

Group	ENL		No ENL	
	No. of Patients	Regression Line	No. of Patients	Regression Line
BL and LL	50	ΔE IgG = 0.023(BI) + 0.014[a]	55	ΔE IgG = −0.005(BI) + 0.091
BL	4	ΔE IgG = 0.143(BI) + 0.010[b]	11	ΔE IgG = −0.016(BI) + 0.065
LL	46	ΔE IgG = 0.016(BI) + 0.008	44	ΔE IgG = −0.003(BI) + 0.083

[a]Slope of ENL regression line significantly different than non-ENL regression line ($p = 0.02$).
[b]Slope of ENL regression line significantly different than non-ENL regression line ($p = 0.012$).
Abbreviations: *PG*, phenolic glycolipid I; *BI*, bacillary index; *ENL*, erythema nodosum leprosum; *LL*, lepromatous leprosy; *BL*, borderline lepromatous leprosy; *E*, extinction.

ever, since several of the BB and BL (and one BT) patients had very high levels of anti-PG IgG it seems less likely that the inability to mount an IgG response to PG is an inherent property of PG. More likely it has to do with patient characteristics and requirements for isotype class switching. T-cell products, possibly including γ-interferon and other factors involved in B-cell maturation have been identified [21–23]. T-cell products, including interleukin 2 and γ-interferon, have been found deficient in some leprosy patients, particularly toward the lepromatous end of the spectrum [24,25]. Cells active in suppressing mitogenic [26] and specific antigen [27] responses have been described in LL and subclinical leprosy infections. At this time, little is known on the nature and diversity of B-cell differentiation factors in humans. Studies in the mouse already indicate a variety of different B-cell differentiation factors [21]. Thus, the inability of many LL patients to mount an anti-PG IgG response may be related to deficiencies of T-cell products required for isotype switching. Alternatively, suppressor mechanisms and active inhibition of the isotype switch may be involved in the pathogenesis of leprosy infections.

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