Brief Definitive Report

PHENOLIC GLYCOLIPID-I OF *MYCOBACTERIUM LEPRAE* INDUCES GENERAL SUPPRESSION OF IN VITRO CONCANAVALIN A RESPONSES UNRELATED TO LEPROSY TYPE

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The varied forms of leprosy form a spectrum (1), with the paucibacillary tuberculoid leprosy at one pole manifesting good cell-mediated immunity (CMI). The multibacillary lepromatous leprosy, at the other extreme, is associated with poor T cell responses. Studies on the role of Ts cells in the understanding of the selective *Mycobacterium leprae*-related T cell anergy in lepromatous leprosy have led to conflicting results. Whereas Mehra et al. (2), using a costimulant assay, indicated that 84% of lepromatous leprosy patients had lepromin-induced OKT8+ Ts cells that inhibited in vitro Con A responses, repeated studies from our laboratory (3, 4) as well as others (5–8) using similar assays and modified protocols failed to confirm these findings. Recent evidence from Mehra et al. (9) indicated that the unique antigen phenolic glycolipid-I (PGL-I) may be a major suppressor molecule on *M. leprae* capable of inducing suppressor cells in lepromatous patients. Furthermore, vaccinated patients were shown to have reversal of suppression, implicating thereby a pathogenetic association to this in vitro phenomenon (9). In the present investigation, we provide the first evidence that PGL-I induces a general suppressive effect on mitogenic responses across the leprosy spectrum and is not unique to the lepromatous leprosy type. Moreover, this antigen used in identical concentrations has both a stimulatory and suppressive role in some healthy and lepromatous subjects.

Materials and Methods

Patients. We included 42 leprosy patients in this study, including 15 just diagnosed as having bacilliferous lepromatous (2BL, 13LL), 9 treated for bacilli negative lepromatous, 18 tuberculoid patients (15 BT, 3TT), and 11 healthy contacts (6 of whom were exposed to leprosy patients for >5 yr) attending the leprosy clinics in Delhi (Safdarjung Hospital). The leprosy patients were classified on the basis of the Ridley and Jopling scale (1) after clinical and histopathological examination. The bacillary load (BI) was evaluated by slit smear examination and Ziehl-Neelsen staining of three to six sites in the body (10). The treated patients had been mainly on dapsone (4,4'-diamino diphenyl sulfone; DDS) monotherapy for >5 yr. The other patients were either just diagnosed or on <1 yr of dapsone therapy.

Stimulants. Integral *M. leprae* bacilli were extracted from human lepromas, heat killed, and used at an optimal concentration of 5 × 10^6 bacilli/ml in lymphoproliferative and suppressor assays as described earlier (3).

PGL-I was kindly donated by Dr. P. J. Brennan (Dept. of Microbiology, Colorado State University, Fort Collins, CO) and Mrs. Sheila Payne, National Institute for Medical Research, London, United Kingdom. Because of its insolubility in aqueous media, it was incorporated into liposomes (11). Briefly, a mixture of 1 mg PGL-I, 8.67 mg sphingomyelin, 3.16 mg cholesterol, and 0.28 mg dicetyl phosphate (Sigma Chemical Co., St. Louis, MO) were dissolved in chloroform and methanol (2:1) and dried in an air stream at 37°C. 1 ml of RPMI 1640 (Gibco Laboratories, Irvine, United Kingdom) was added and sonicated in a Branson sonifier (Branson Cleaning Equipment Co., Shelton, CT) cold water bath for 1 h. They were stored at 4°C and resonicated before use. The incorporation of PGL-I in liposomes was checked by (1) immunodiffusion in 1% agar using varying dilutions of mAb to PGL-I (12), kindly donated by Dr. D. B. Young (MRC Unit in Tuberculosis and Related Mycobacterial Diseases, Hammersmith Hospital, London, United Kingdom), and (2) agglutination in 96-well microtiter plates using checkerboard titration with one-fourth, one-eighth, and one-sixteenth dilution of stock PGL-I incorporated and control liposomes with doubling dilutions of (*a*) anti-PGL mAb and (*b*) pooled lepromatous sera containing anti-PGL antibodies.

Control liposomes devoid of PGL-I were prepared in a similar manner and used in similar concentrations in parallel replicate cultures.

Con A (Pharmacia Fine Chemicals, Uppsala, Sweden) was found to optimally stimulate PBMC of normal subjects at a concentration of 10 μg/ml.

Lymphoproliferation. PBMC were separated by Ficoll-Hypaque (13) density gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) from sterile heparinized blood (10 units of heparin per ml of blood; The Upjohn Co., Kalamazoo, MI) and put up for culture in RPMI 1640 + 10% AB serum as described earlier (3). In brief, 10^5 cells in 100 μl medium/well were dispensed in 96-well round-bottomed microtiter plates (Nunc Intermed, Kamstrup, Denmark). Four replicates each were set up with (*a*) cells only and cells with 25 μl of (*b*) *M. leprae*, (*c*) varying concentrations of PGL-I liposomes, and (*d*) similar concentrations of control liposomes devoid of PGL-I. The cultures were incubated at 37°C with 5% CO$_2$ and air. They were terminated on day 6, except where stated after 18 h of pulsing with 1 μCi [^{3}H]thymidine (2 Ci/mmol, Bhabha Atomic Research Centre, Trombay, India). Cultures were harvested on glass fiber discs using a semiautomatic cell harvester (PHD, Cambridge, MA) and processed for liquid scintillation counting in an LKB Rackbeta 1712, Turku, Finland. Stimulation index was expressed as: (*a*) (mean cpm of cells with *M. leprae*)/(mean cpm of cells only), (*b*) (mean cpm of cells with PGL-I liposomes)/(mean cpm of cells with control liposomes).

Suppressor Cell Activity. Antigen-induced suppressor cell activity on mitogen responses was undertaken as described earlier (3). Cultures were set up as above with (*a*) medium only and 25 μl of the stimulants (*b*) Con A, (*c*) *M. leprae*, (*d*) PGL-I liposomes (1 μg/ml, except where stated), (*e*) control liposomes of appropriate dilution, (*f*) Con A + *M. leprae*, (*g*) Con A + PGL-I liposomes, (*h*) Con A + control liposomes. The cultures were incubated as above and terminated on day 3 after 18 h of pulsing with [^{3}H]thymidine. Δ cpm of replicate cultures were calculated as (*a*) [(mean cpm of cells with Con A or *M. leprae*) − (mean cpm of cells alone)], (*b*) [(mean cpm of cells with PGL-I liposomes) − (mean cpm of cells with control liposomes)]. Percent suppression induced by antigens *M. leprae* and PGL-I liposomes was expressed as: 100 × [1 − (mean Δ cpm of cultures with antigens + Con A)/(mean Δ cpm of cultures with Con A)]. 10% inhibition/enhancement was found to be statistically significant (p <0.05).

Statistical Analysis. Mann-Whitney U test and Kruskal Wallis tests were used for calculating p values (14).

Results

Since PGL-I is insoluble in aqueous medium it was presented to PBMC in the liposomal form. The definitive presence of PGL-I in liposomes was established by screening with pooled lepromatous sera containing anti-PGL antibodies and

TABLE I

Stimulation Index* and Effect of M. leprae and Phenolic Glycolipid-I (PGL-I) on
Con A Responses

Leprosy type	Stimulation index (range)		Mean cpm (range) of Con A cultures		
	M. leprae	PGL-I	−	+ M. leprae	+ PGL-I
Tuberculoid (18)[‡]	2.3–8.0	2.3–6.3 (9)[‡]	10,560–82,983	2,337–74,128	1,464–81,827
Healthy (11)	2.0–4.2	1.5–2.4 (11)	25.664–50,523	4,818–34,406	3,572–59,327
Treated Lepromatous (9)	0.8–1.6	1.3–1.7 (7)	47,520–89,669	44,966–142,080	50,776–73,783
Untreated Lepromatous (15)	0.6–1.4	0.8–1.9 (13)	1,486–33,720	1,424–40,561	1,180–48,447

* Stimulation index, (Mean cpm of cells with M. leprae or PGL-I liposomes)/(Mean cpm of cells alone or cells with control liposomes).
‡ Figures in parentheses indicate number of patients.

an mAb to PGL (12). At 1/8 dilution of stock liposomes prepared from 1 mg/ml of PGL-I, agglutination was observed at 1/32 and 1/320 dilution of pooled lepromatous sera and the mAb, respectively. Furthermore, immunodiffusion in agar using the mAb gave a strong precipitin line.

PGL-I-Induced Lymphoproliferation. We evaluated the ability of PGL-I liposomes to stimulate PBMC from leprosy patients and healthy lepromin responsive contacts (Table I). Over a concentration range of 0.25–10 µg/ml PGL-I, no stimulation was observed in 20 lepromatous leprosy patients. In contrast, 6 of 9 tuberculoid patients and 5 of 11 healthy subjects had significant lymphoproliferation over a 1–10 µg/ml range, with a stimulation index range of 2.3–8.0. The time kinetics of PGL-I-induced lymphoproliferation was similar to that of integral M. leprae, with maximal [³H]thymidine incorporation seen on day 6.

Suppression of Con A Responses in Vitro. Over a concentration range of 0.25–10 mg/ml of PGL-I, optimal in vitro suppression of Con A responses was seen at 1 mg/ml in six responder subjects and subsequent studies were undertaken at this concentration.

Significantly increased (p <0.05 to <0.01) levels of suppression of lymphoproliferation to Con A was induced by PGL-I liposomes as compared with M. leprae in many leprosy patients. The percent suppression ranged from 13–64% and 12–79% in untreated lepromatous and tuberculoid patients, respectively. Both the number of individuals showing suppression as well as the level of suppression was increased in the presence of PGL-I. This feature was observable across the leprosy spectrum; 9 of 15 untreated lepromatous (p <0.05) and 10 of 18 tuberculoid patients (p <0.01) showed significantly higher levels of suppression of mitogenic responses by PGL-I as compared with M. leprae. The level of inducible suppression was unrelated to the background Con A responses (Table I). To evaluate the temporal nature of the suppression we studied nine treated lepromatous patients who were devoid of bacilli in the skin. None of them showed PGL-I-induced suppression.

Moreover, at the same concentration, PGL-I showed both stimulatory and inhibitory effects on Con A responses of lepromatous and healthy subjects but not in any of the tuberculoid patients.

In agreement with our earlier studies (3), we also found that M. leprae-induced suppression was infrequent in untreated lepromatous (13%) and more common in the tuberculoid (67%) and healthy responder (54%) individuals. Interestingly,
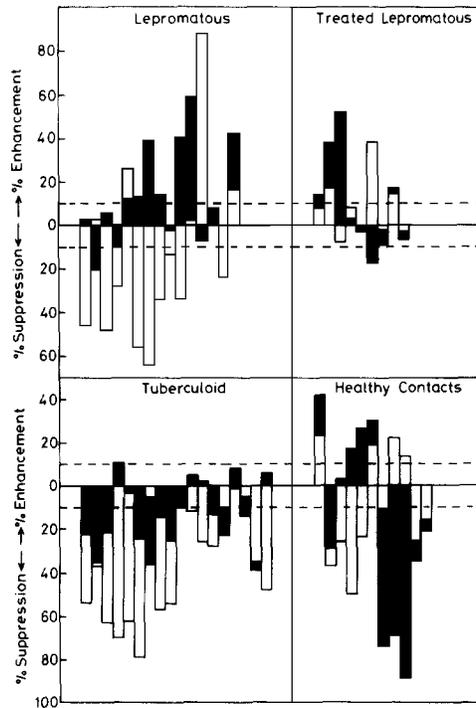
FIGURE 1. The effect of PGL-incorporated liposomes and integral M. leprae on Con A–induced responses of PBMC. 15 untreated and 9 treated lepromatous, 18 tuberculoid and 11 healthy subjects were studied. Each bar indicates an individual subject tested concurrently with PGL ($\square$) and M. leprae ($\blacksquare$). Using a 3-d costimulant assay (3), four replicates of 10^5 cells/well of Ficoll-Hypaque-purified PBMC were cultured in 96-well microtiter plates with Con A alone and with Con A + antigens. Cells alone and cells with liposomes devoid of PGL were used as controls for the appropriate experiments. % suppression = 100 × 1 − [(mean cpm of PBMC + Antigen + Con A)/(mean cpm of PBMC + Con A)]. Δ cpm = (a) (mean cpm of PBMC with ML or Con A) − (mean cpm of PBMC alone) and (b) (mean cpm of PBMC with PGL-I liposomes) − (mean cpm of PBMC with control liposomes). Dashed lines indicate ± 10% used as cutoff point based on p <0.05 as evaluated by two-way analysis of variance. The p values for significance of suppression (Kruskall-Wallis test) are: M. leprae antigen, <0.01 for tuberculoid vs. untreated lepromatous and <0.05 for vs. treated lepromatous patients; PGL antigen, <0.05 for tuberculoid vs. untreated lepromatous and <0.001 vs. treated lepromatous patients; PGL vs. M. leprae, <0.05 for untreated patients, and <0.01 for tuberculoid patients.

4 of 6 healthy laboratory staff members showing in vitro suppression in the presence of M. leprae had been exposed to leprosy patients for more than 5 yr. Except for one, all the others showing lack of suppression had been in contact with leprosy patients for ≤2 yr.

Discussion

The present study indicates that PGL-I liposomes are potent inducers of suppression of Con A responses across the leprosy spectrum. The suppression induced by the glycolipid is seen in 60% of lepromatous patients and parallels that seen in tuberculoid leprosy (67%). Though the number of tuberculoid patients showing significant suppression to both antigens was the same, the level of suppression exerted by PGL-I was significantly higher (p <0.05 to <0.01, Fig.

1). Interestingly, lepromatous patients with undetectable bacillary load subsequent to prolonged chemotherapy showed reversal of suppression.

This antigen also stimulated lymphoproliferation in responder patients and healthy population with similar time kinetics as integral *M. leprae* antigens. Paradoxically, at the same concentration PGL-I had both a stimulatory and suppressive role in some lepromatous and healthy subjects.

In confirmation of our earlier studies (3), integral *M. leprae*–induced suppression of Con A responses was lower in lepromatous as compared with tuberculoid leprosy and healthy lepromin responder subjects. Moreover, in agreement with studies of Stoner et al. (15), the healthy laboratory staff members showing in vitro induced suppression had been in contact with leprosy patients for 5 yr, whereas all except one showing no evidence of suppression had recent exposure of 1–2 yr. We have interpreted these results to indicate that a well-regulated T cell–mediated immune response resulting from natural exposure to *M. leprae* as in the healthy staff members or limited infection as in tuberculoid patients may induce a parallel Ts cell function as part of a physiological response.

The proposed role of PGL-I as a unique suppressor epitope (9) appears unlikely to be the central mechanism responsible for the antigen-specific anergy peculiar to lepromatous leprosy. Several points appear to disfavor its singular role in the biology of the disease: (*a*) the same molecule at the same concentration has both a stimulatory and inhibitory effect in some subjects, (*b*) in ~60% of cases it shows a general in vitro suppressive effect in both the localized and disseminated forms of leprosy, (*c*) moreover, the reversal of in vitro suppression induced by PGL-I in long term–treated bacilli-negative lepromatous patients is not accompanied by improvement in their antigen-specific anergic state or a return to skin test reactivity. Nevertheless, it is possible that the observed accumulation of PGL-I in the tissues of bacilliferous lepromatous leprosy patients may help to protect the *M. leprae* organism within the host macrophages or play a role in the secondary depression of general T cell responses seen in some lepromatous patients or contribute to the immunopathology of the lesions.

Other suppressive mechanisms need to be explored to explain the wide spectrum of clinicopathological features seen in leprosy and the antigen-specific anergy associated with lepromatous leprosy. Our earlier data suggested that monocytes of lepromatous patients released suppressive factors on contact with antigen (16) that inhibited IL-2 production (17).

Summary

Using a costimulant assay, in vitro Con A responses of patients across the leprosy spectrum were found to be markedly suppressed by phenolic glycolipid-I (PGL-I), a unique antigen of *M. leprae*. The degree of inducible suppression as well as the number of leprosy patients showing suppression of mitogenic responses was higher with PGL-I as compared with integral *M. leprae* ($p <0.05$ to <0.01). Both untreated lepromatous (60%) as well as tuberculoid leprosy (67%) patients showed significant suppression ranging from 13 to 64% and 12 to 79%, respectively. Thus, PGL-I appears to have a universal suppressive effect on Con A responses and is unlikely to play a central role in determining the leprosy spectrum.

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