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Effect of unique *Mycobacterium leprae* phenolic glycolipid-I (PGL-I) on tumour necrosis factor production by human mononuclear cells

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Summary *Mycobacterium leprae* cell wall-associated components are found in large amounts in the tissues of leprosy patients, particularly those at the lepromatous pole. Among these molecules, the phenolic glycolipid-I (PGL-I), unique to *M. leprae*, has been involved in the selective anergy observed in the lepromatous patients. Armadillo-derived *M. leprae* retains only a small proportion of the total PGL-I found in infected tissues. Therefore, the addition of PGL-I to *M. leprae in vitro* is important for a better understanding of *M. leprae* effects *in vivo.* We have studied the influence of PGL-I on TNF production by normal human peripheral blood mononuclear cells (PBMC) and by a human monocytic leukaemia cell line (THP-1) following stimulation with killed *M. leprae*. PGL-I alone did not induce TNF secretion by PBMC, but when associated with a sub-optimal dose of armadillo-derived *M. leprae* increased the release of this cytokine. In agreement with these results, *M. leprae*-exposed THP-1 cells did not secrete detectable levels of TNF unless PGL-I was simultaneously added to the culture. This increase in TNF production suggests that PGL-I plays a role in the induction of TNF during the natural infection. In addition, the modulatory effect of PGL-I on TNF release by THP-1 cells reinforces that monocytes are one of the possible targets of this molecule.

Introduction

Glycolipids from the cell wall of *Mycobacterium leprae* are widely distributed in the tissues of patients with leprosy.[1,2] Among these components, the phenolic glycolipid 1 (PGL-I),

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unique to *M. leprae*,[3,4] has been implicated in the selective anergy observed in lepromatous leprosy patients.[5] *In vitro* findings indicate that PGL-I interferes with the cellular immune response at the level of both macrophage function (measured by the release of inflammatory cytokines such as TNF and by generation of oxygen radicals) and lymphocyte proliferation.[5–9] Moreover, PGL-I fixes selectively the complement component C3 and thus helps to mediate *M. leprae* phagocytosis via complement receptors on the surface of mononuclear phagocytes.[10,11]

In mycobacterial infections TNF is involved in both host protective response and disease pathology.[12] Whole mycobacteria and/or cell wall associated components stimulate TNF release by murine macrophages and by mononuclear cells from normal individuals or leprosy patients *in vitro*.[13–16] However, little is known of the factors which trigger TNF release during the course of the disease. We have studied the influence of PGL-I on TNF production by normal human peripheral blood mononuclear cells (PBMC) following stimulation with *M. leprae*. Alternatively, THP-1 cells, a human myelomonocytic cell line that lately has been extensively employed in studies of mechanisms of maturation/differentiation from monocytes to macrophages,[17] were used as host cells.

M. leprae PGL-I is an extracellular product forming a loose capsule around the bacillus which is partially lost during the purification procedure from infected tissues.[4] It therefore seems necessary to add PGL-I to the armadillo-derived *M. leprae* for a better understanding of *M. leprae* effects *in vivo*. In addition, this approach helps to clarify PGL-I contribution to these effects. Our results suggest that PGL-I acts as a co-signal for TNF production by *M. leprae*-exposed mononuclear cells, but has no detectable effect when added alone to unstimulated cultures. The possible relevance of these *in vitro* results to the leprosy pathogenesis is discussed.

Materials and methods

SOURCE OF REAGENTS

RPMI 1640 medium, penicillin/streptomycin, L-glutamine were obtained from Gibco BRL (Gaithersburg, MD, USA), heat-inactivated fetal calf serum was purchased from Fazenda Pigue (Rio de Janeiro, RJ, Brazil), Ficoll was obtained from Sigma Chemical Co. (St Louis, MO, USA).

PBMC CULTURE AND STIMULATION

Peripheral blood mononuclear cells (PBMC) were isolated from six healthy individuals, five of them living in Rio de Janeiro and one being a visitor from the UK. PBMC were separated by Ficoll-Hypaque density gradient from sterile heparinized blood, washed three times in PBS and resuspended in RPMI-1640 medium supplemented with 5% AB human serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes and 2 mM L-glutamine. Two culture conditions were used, as follows: (i) 3×10^5 cells in 0·2 ml medium/well were dispensed in 96-well flat-bottomed microtitre plates or (ii) 2×10^6 cells in 1 ml medium/well were dispensed in 24-well microtitre plates and cultured with or without stimulants (as below) for 24 h at 37°C in a humidified atmosphere of 5% CO_2.

THP-1 CULTURE AND STIMULATION

Cells from a human myelomonocytic cell line, THP-1 (American Type Culture Collection, Rockville, MD, USA), were maintained in suspension cultures in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes and 2 mM glutamine, at 37°C in a humidified atmosphere of 5% CO_2 and used between three and 14 passages. For the experiments, 2×10^5 cells in 1 ml of the same medium/well were dispensed in 24-well microtitre plates and cultured with or without stimulants up to 48 h at 37°C in a humidified atmosphere of 5% CO_2. After the incubation period, the culture supernatants of PBMC or THP-1 cells were collected for TNF quantitation.

STIMULANTS

Irradiated armadillo-derived *M. leprae* and PGL-I were obtained as described.[4] Evaluation of PGL-I for the presence of Gram-negative bacterial endotoxin (LPS) was done with the amebocyte assay (QCL-1000 kit, BioWhittaker, Inc., Walkersville, MD, USA). Two lots of PGL-I obtained between 1994 and 1997 contained <0.5 pg of LPS per μg of PGL-I per batch. Because of its insolubility in aqueous medium, PGL-I was presented to PBMC or THP-1 cells immobilised onto the microplate well. Briefly, PGL-I stock solutions in ethanol were added to culture wells and dried under sterile conditions before cell addition. The same concentration of ethanol was added to control wells, although ethanol alone had no detectable effect in our conditions. PGL-I final concentration in cultures was 1 or 25 μg/ml; *M. leprae* was presented to PBMC or THP-1 cells over a concentration range of 1.25–40 μg/ml (approximately 3×10^6–10^8 organisms/ml).

TNF DETECTION

Supernatants were adequately diluted in PBS and assayed in duplicates. TNF levels were determined by ELISA using specific pairs of monoclonal antibodies and human recombinant TNF as standard, according to the manufacturer recommendations (Pharmingen, San Diego, CA, USA). The limit of sensitivity was 250 pg/ml of recombinant TNF.

STATISTICAL ANALYSIS

The Friedman test and Wilcoxon signed ranks test were performed with data shown in Figure 1. Data related to Figure 2 are reported as means ± SEM.

Results

EFFECT OF PGL-I ON TNF PRODUCTION BY PBMC STIMULATED WITH *M. LEPRAE*

We compared TNF levels produced by PBMC simultaneously treated with PGL-I and *M. leprae* to the levels produced by mononuclear cells exposed to *M. leprae* alone (Figure 1). Optimal incubation time for TNF secretion was initially determined by stimulating the cells with *M. leprae* for 4, 12, 24 and 48 h, with a peak level observed at 12–24 h (not shown). PBMC did not release detectable amounts of TNF into cell medium when cultivated for the same periods of time in the presence of PGL-I or medium alone. The stimulation of PBMC cultures with *M. leprae* induced TNF release by cells of all tested subjects as previously

shown by others.[15] However, the profile of TNF release in response to *M. leprae* exposure varied substantially among the different individuals over the concentration range used. In the presence of the *M. leprae* dose that induced the lowest amount of TNF for each subject (sub-optimal *M. leprae* dose), PGL-I enhanced TNF levels in all subjects (Figure 1, $P < 0{\cdot}009$). When other *M. leprae* doses were used, PGL-I had complex effects, either increasing or decreasing the TNF release in the same individual (not shown). Both PGL-I doses (1 and 25 μg/ml) induced comparable levels of TNF when added to *M. leprae*-stimulated PBMC ($P < 0{\cdot}65$). Subjects a, b, c, d and f were previously exposed to *M. leprae*, differently from subject e, who lives in a non-endemic area. Subject e-derived PBMC, compared to the other subjects, produced lower levels of TNFα in response to all tested doses of *M. leprae*. Removing the values referring to this subject from the statistical analysis, the difference in TNF production between PBMC challenged with *M. leprae* and PBMC challenged with *M. leprae* and PGL-I is still significant ($P < 0{\cdot}023$).

THP-1 cells are functionally very similar to peripheral blood monocytes.[18,19] We thus tested the effect of added PGL-I in *M. leprae* induced TNF. PGL-I qualitatively modified the TNF response pattern of THP-1 cells to *M. leprae*. Differently from PBMC (Figure 1), THP-1 cells did not secrete measurable levels of TNF when stimulated with *M. leprae* unless PGL-I was added to the culture. TNF secretion was detected after 48 h of simultaneous exposure to PGL-I and *M. leprae* (Figure 2), but not at early time points. THP-1 cells also showed similar response kinetics to PMA, followed by morphological criteria[19] and measurement of TNF levels in culture supernatants (M. M. Oliveira, R. Charlab and M. C. V. Pessolani, submitted). As observed before, PGL-I alone did not stimulate the production of measurable levels of TNF (Figure 2).

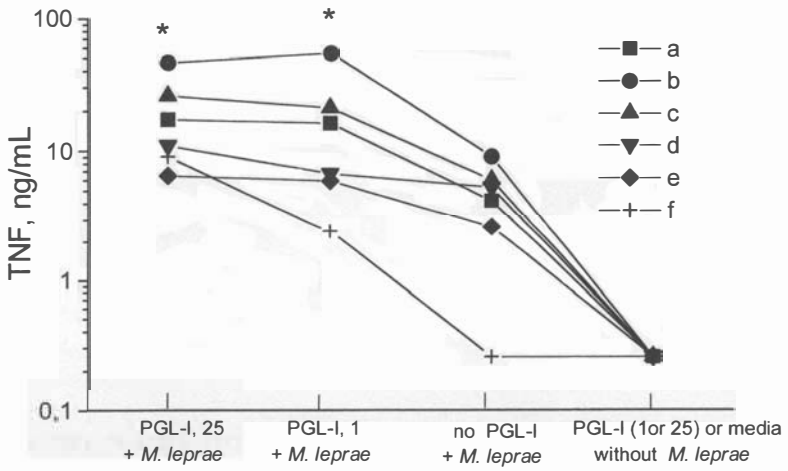

Figure 1. Effect of PGL-I on TNF production by *M. leprae*-stimulated PBMC. PBMC of six healthy subjects (*a*, *b*, *c*, *d*, *e*, *f*; 2×10^6/ml) were incubated with a sub-optimal dose of *M. leprae* for TNF release (over a range of 1.25–40 μg/ml), PGL-I immobilized in the culture microplates (1 and 25 μg/ml) or both for 24 h. TNF concentration in culture supernatants was determined by ELISA. PBMC cultivated without *M. leprae* in the presence of PGL-I or medium alone secreted undetectable levels of TNF. *M. leprae* doses which induced the lowest amount of TNFα for each subject were, respectively: *a* 20 μg/ml; *b* 2 μg/ml; *c* 20 μg/ml; *d* 5 μg/ml; *e* 10 μg/ml; *f* 40 μg/ml. Cultures exposed to PGL-I and *M. leprae* were statistically different from cultures exposed to *M. leprae* alone ($P < 0{\cdot}009$). TNF values of cultures exposed to *M. leprae* and 1 μg/ml of PGL-I were not statistically different from the values of cultures exposed to *M. leprae* and 25 μg/ml of PGL-I ($P < 0{\cdot}65$).

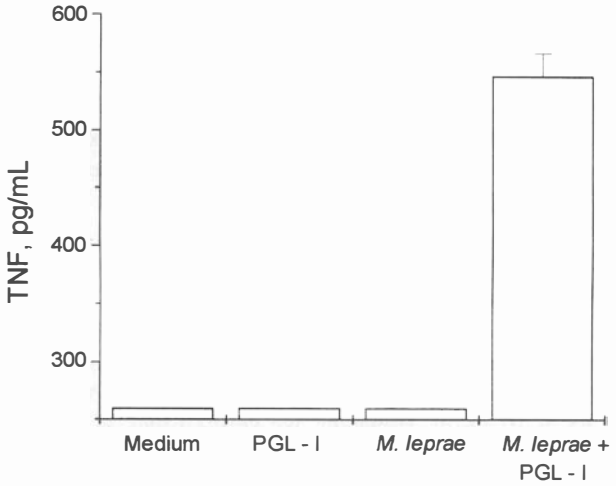

Figure 2. Effect of PGL-I on TNF production by *M. leprae*-stimulated THP-1 monocytic cells. THP-1 cells (2×10^5/ml) were incubated with *M. leprae* (40 μg/ml), PGL-I immobilized in the culture microplates (1 μg/ml) or both for 48 h. TNF concentration in culture supernatants was determined by ELISA. Results are expressed as means ± SE of three independent experiments.

Discussion

It is now well accepted that PGL-I is produced in large amounts during *M. leprae* infection, being found in serum, urine and in the skin lesions of leprosy patients.[1,2,20] This feature allied to its capacity to affect both lymphocyte and macrophage functions (reviewed by Brennan *et al.*[3]) and to elicit a strong antibody response in leprosy patients,[21] points to an involvement of PGL-I in the pathogenesis of *M. leprae*.

To examine whether the addition of PGL-I affects *M. leprae* action *in vitro*, we evaluated TNF production by PBMC of normal individuals or by THP-1 monocytic cells exposed to *M. leprae* in the presence or absence of free PGL-I. In this study, PGL-I alone did not induce TNF secretion by PBMC of healthy individuals or by THP-1 cells, but when associated with *M. leprae* modulated the release of this cytokine. PGL-I increased TNF production by PBMC stimulated with sub-optimal doses of *M. leprae*. However, positive or negative modulatory effects of PGL-I were observed in the different individuals and in the same individual when other concentrations of *M. leprae* were used. These variations may be related to differences in the genetic background (e.g. high or low type TNF producers), functional status of the PBMC donors, heterogeneity of the PBMC population and of the monocyte subset by itself. It is also worthy to mention at this point that we have not adjusted the number of PBMC to turn equal the counts of monocytes to all individuals tested. This should certainly be done if we were working with purified monocytes and not with such a heterogeneous population of cells as PBMC, in which other cell types such as NK and T-cells could also contribute to TNF secretion. Instead, we preferred to use a wide range of *M. leprae* doses for each individual, determining a sub-optimal dose for each one and then use this dose to investigate the effect of PGL-I.

The increase in TNF production observed when PGL-I was added to *M. leprae*-stimulated cultures suggests that this component plays a role in TNF induction during the natural

infection. This observation was reinforced when THP-1 cells were co-cultured with PGL-I and *M. leprae*. We have previously shown that *M. bovis* BCG but not *M. leprae* induced TNFα secretion in THP-1 cells, while both mycobacteria stimulated the release of this cytokine by human PBMC (M. M. Oliveira, R. Charlab and M. C. V. Pessolani, submitted). This result emphasises the potential of THP-1 cells as a model to explore the role of PGL-I on TNF secretion. In the present work, the addition of PGL-I was essential for detection of TNF in the culture supernatants. *M. leprae*-exposed THP-1 cells only secreted detectable levels of TNF when PGL-I was simultaneously present in the culture.

The modulatory effect of PGL-I on TNF production by THP-1 monocytic cells suggests that monocytes are one of the possible targets of this molecule. In this respect it has been shown that PGL-I incorporated in liposomes inhibited the secretion of TNF, IL-1 and IL-6 by LPS-stimulated peripheral blood-derived monocytes from healthy individuals,[22] but, in agreement with our results, had no effect when added alone to non-stimulated controls. The authors used LPS at 10 μg/ml. However, we verified that, at lower LPS doses, PGL-I, either incorporated in liposomes or immobilized in culture microplates was also able to increase TNF production by PBMC of normal subjects. It is worth to mention that undifferentiated THP-1 cells did not secrete detectable levels of TNFα in response to LPS from 100 ng to 10 μg/ml, a behaviour already observed by others.[23] The production of TNF by LPS-stimulated THP-1 cells was not detected even when PGL-I was simultaneously added to the cultures (R. Charlab, unpublished results).

In this study, we showed that PGL-I did not induce the secretion of TNF by PBMC of healthy individuals, but positively or negatively modulated the release of this cytokine induced by *M. leprae*. In this context, one can speculate that PGL-I may have different roles on the pathogenicity along the clinical spectrum of leprosy and in reactional and non-reactional stages of the disease. Depending on the infection status, PGL-I may either increase or suppress the inflammatory response in the infected tissue, contributing, respectively, to sustain a low specific response to *M. leprae* or to enhance tissue damage.

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