

## Role of PGL-I of *M. leprae* in TNF- $\alpha$ production by *In vitro* whole blood assay

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### ABSTRACT

Phenolic glycolipid-I (PGL-I) is known to be a major antigen of *Mycobacterium leprae*. We have studied the influence of PGL-I on the production of Tumour Necrosis Factor alpha (TNF- $\alpha$ ) using the *in vitro* whole blood assay. Armadillo-derived *M. leprae* (ADML) are thought to be depleted of PGL-I during the purification process. *M. leprae* obtained from mouse foot pad material (MFPML) has been subjected to a less rigorous purification process; their PGL-I coating is therefore believed to be more intact than that of ADML. PGL-I or ADML alone induced the secretion of minimal levels of TNF- $\alpha$  in whole blood assay; when added in combination, higher levels of this cytokine were observed. The highest TNF- $\alpha$  response was seen following stimulation with MFPML. MFP material not infected with ML did not elicit any response. The difference in TNF- $\alpha$  response shown by ADML and MFPML was postulated to be largely due to the presence of higher levels of PGL-I in MFPML. This increase in TNF- $\alpha$  production suggests that PGL-I may play a significant role in the induction of TNF- $\alpha$  during natural infection.

**Keywords:** Phenolic glycolipid-I, armadillo-derived *M. leprae*, MFP *M. leprae*, TNF- $\alpha$ , whole blood assay.

### INTRODUCTION

Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae*, affecting the peripheral nerves, mucosa of the upper respiratory tract and also the eyes. In severe manifestations, other organs may also be involved. Immunity towards *M. leprae* is a complex process which is mainly cell mediated. Mycobacterial antigens are widely distributed in tissues of patients with leprosy.<sup>1</sup> Among the mycobacterial antigens, phenolic glycolipid-I (PGL-I) is specific for *M. leprae*, forming a loose extra-cellular capsule around the bacillus.<sup>2</sup> Antibody responses to this antigen have been shown to be diagnostic of exposure to *M. leprae*.<sup>3</sup>

PGL-I may play a role in the intracellular persistence of *M. leprae* in the human macrophage by scavenging toxic oxygen metabolites of the antimicrobial systems of the host cell.<sup>4</sup> This molecule also induces suppressor T-cells in lepromatous patients and in high concentration may non-specifically suppress T-cell responses.<sup>5</sup> PGL-I also appears to be important in the uptake of *M. leprae* by the macrophage.<sup>6</sup> In mycobacterial infection, the cytokine tumour necrosis factor alpha (TNF- $\alpha$ ) is involved in both host protective responses and disease pathology.<sup>7</sup> Little is known about the factor which triggers TNF- $\alpha$  release during the course of leprosy disease, but there is some evidence that PGL-I is a modulatory factor in the production of this cytokine.<sup>8</sup>

The present study aimed to determine the effect of PGL-I on TNF- $\alpha$  production in the whole blood assay following stimulation with PGL-I, armadillo derived

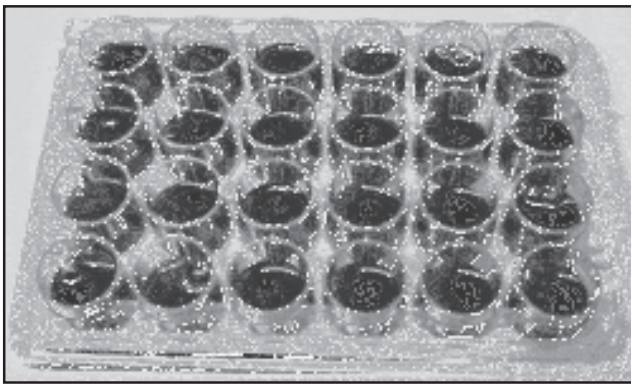
whole *M. leprae* (ADML), PGL-I plus ADML and mouse footpad derived *M. leprae* (MFPML).

ADML are largely depleted of PGL-I as a result of the purification process which is required following culture in armadillo liver<sup>2</sup> while extraction of *M. leprae* from the mouse footpad is less harsh; thus MFPML is likely to have a more intact PGL-I capsule. These preparations were used to stimulate human blood cells in the whole blood assay (WBA) under the same conditions to investigate whether any difference in TNF- $\alpha$  response was observed.

### MATERIALS AND METHODS

**Study subjects:** This study was carried out at Anandaban Hospital after taking informed consent from twenty subjects. Following consent, 10ml of blood was drawn from each participant on one occasion for immediate use in the Whole Blood Assay (WBA). All participants were males between the ages of 18 and 40 years. Five individuals from each of the following categories were recruited: professionally exposed, healthy controls and leprosy patients of borderline tuberculoid (BT) and borderline lepromatous (BL) types.

**Stimulants:** PGL-I and ADML were kindly provided by Dr. P.J Brennan of Colorado State University, USA. Mouse footpad derived *M. leprae* (MFPML) was prepared in our laboratory by differential centrifugation of homogenized *M. leprae* infected mouse footpad materials. Footpads were minced with fine scissors and homogenized in 1ml phosphate buffer saline (PBS), then



**Fig. 1.** *In vitro* whole blood assay stimulation with test antigens for 12 hour and 24 hour time points

centrifuged at 600g for 5 minutes to pellet mouse material. The supernatant obtained was further centrifuged at 2200g for 30 minutes.<sup>9</sup> The number of acid fast bacilli was estimated microscopically at each stage to ensure enrichment for *M. leprae*. A suspension containing  $2 \times 10^6$  organisms/ml was prepared in PBS and used to stimulate the blood cells as described below. Phytohaemagglutinin (PHA) (Sigma, USA) was used as the positive stimulant.

**Whole blood assay and stimulation:** One millilitre of whole blood from each participant was stimulated in appropriate wells of a 24-well plate<sup>10</sup> (Greiner, Germany) as shown in Fig. 1, with either PGL-I (10 $\mu$ g), ADML ( $2 \times 10^5$  organisms), PGL-I + ADML (10 $\mu$ g +  $2 \times 10^5$  organisms), MFPML ( $2 \times 10^5$  organisms) or PHA (10 $\mu$ g). After 12 and 24 hours incubation at 5.0% CO<sub>2</sub> at 37°C in a humidified CO<sub>2</sub> incubator (Labline, UK), approximately 200 $\mu$ l of the supernatant was carefully removed for ELISA. PGL-I stock solutions in ethanol were added to culture wells and dried under sterile conditions before addition of blood. The same concentration of ethanol was added to control wells, but had no detectable effect on TNF- $\alpha$  secretion.<sup>8</sup>

**ELISA for TNF- $\alpha$  detection:** TNF- $\alpha$  was measured in culture supernatants by ELISA using paired monoclonal murine antibodies (Pharmingen, USA). Mouse anti-human TNF- $\alpha$  monoclonal antibody was coated onto the wells of ELISA plates (Immulon 2, Dynatech, USA) at 2 $\mu$ g/ml in coating buffer (0.1M Na<sub>2</sub>HPO<sub>4</sub>, pH 9.0), 50 $\mu$ l per well. Plates were covered and incubated overnight at 4°C. The wells were blocked with 3% bovine serum albumin (BSA, Sigma, USA) in PBS, 200 $\mu$ l per well, for 2 hours at room temperature. Recombinant human TNF- $\alpha$  standard (Pharmingen, USA) was serially diluted in RPMI (Sigma, USA) with 5.0% heat inactivated pooled human serum and was applied in duplicate in a concentration range of 15pg/ml to 2000pg/ml in 100 $\mu$ l in the first two rows of each ELISA plate. A 1:100 dilution in 100 $\mu$ l in RPMI of each

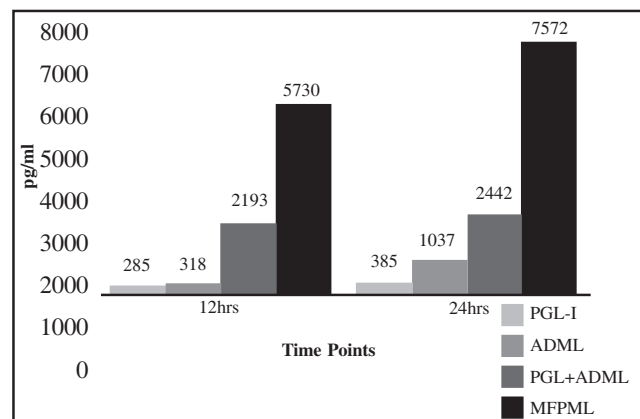
culture supernatant in RPMI was added to six wells and incubated overnight at 4°C. After washing, 100 $\mu$ l of 1 $\mu$ g/ml biotin-labeled anti-human TNF- $\alpha$  detecting antibody (Pharmingen, USA) was added to each well of the plate and incubated for 1 hour at room temperature. After washing, 100 $\mu$ l of 1.25 $\mu$ g/ml of avidin peroxidase (Sigma, USA) was added to each well of the plate and incubated for 1 hour at room temperature. A colour reaction was developed by adding 100 $\mu$ l of 0.4mg/ml O-phenylenediamine dihydrochloride (OPD, Sigma, USA) in citrate phosphate buffer (pH 5.0) and 0.006% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The reaction was stopped with 100 $\mu$ l of 2.5N Sulphuric acid. The plate was read at a wavelength of 490 nm using a Dynatech MRX plate reader. TNF- $\alpha$  results were expressed as means of TNF- $\alpha$  (pg/ml) of six wells after subtraction of any non-specific TNF- $\alpha$  production in non-stimulated cultures. The minimum and maximum detection limits of the TNF- $\alpha$  ELISA standard were 15pg/ml and 2000pg/ml respectively.

**Statistical analysis:** Statistical analysis was done using Student's t-test.

## RESULTS

Optimal incubation time for TNF- $\alpha$  production was initially determined by stimulating the blood cells with different antigens for 2, 4, 12, 18, 24 and 36 hours. Peak level of TNF- $\alpha$  response was observed between 12-24 hours time period (Data not shown).

We compared the TNF- $\alpha$  produced by blood cells from participants incubated with PGL-I, PGL-I + ADML, ADML and MFPML at 12 and 24 hours (Fig). Stimulation of blood cells with PGL-I or ADML alone induced minimal TNF- $\alpha$  response while PGL-I added in combination with ADML showed increased TNF- $\alpha$  response ( $p = 0.077$ ). The highest TNF- $\alpha$  response was observed following stimulation with MFPML. Student t-test showed significant difference in TNF- $\alpha$  response



**Fig. 2.** Mean TNF- $\alpha$  response of all subjects in 12 hours and 24 hours induced by indicated antigens

between ADML and MFPML ( $p=0.027$ ,  $p=0.04$  at 12 hrs and 24 hrs respectively). Uninfected MFP material induced no detectable TNF- $\alpha$  response in the whole blood assay (Data not shown).

## DISCUSSION

PGL-I is present in large amounts during *M. leprae* infection. In view of its abundance and extra cellular location<sup>11</sup> PGL-I is recognized by the immune system and thus is likely to play a significant role in interaction between the pathogen and its environment. This study sought to investigate the influence of PGL-I on the production of TNF- $\alpha$  in a whole blood assay in leprosy patients and their contacts.

In this study, ADML or PGL-I alone induced only baseline levels of TNF- $\alpha$  secretion. ADML and PGL-I in combination elicited an increased TNF- $\alpha$  response. MFPML, which is believed to have intact PGL-I, showed the highest TNF- $\alpha$  response (Fig. 2). These results are consistent with the hypothesis that PGL-I plays a role in the production of TNF- $\alpha$  *in vivo*. A similar study was performed by Charlab *et al*<sup>8</sup> using peripheral blood mononuclear cells (PBMCs) and the human myelomonocytic cell line THP-1. In the latter study cells exposed to armadillo derived *M. leprae* did not secrete detectable levels of TNF- $\alpha$  unless PGL-I was simultaneously added to the culture, suggesting that PGL-I plays a role in the induction of TNF- $\alpha$  during natural infection. Our results extend this finding as we have compared the antigens in whole blood from individuals exposed to *M. leprae* and have also compared responses induced by ADML (known to be depleted of PGL-I) to those induced by MFPML (thought to have a more intact complement of PGL-I).

MFPML is a crude preparation which is contaminated with mouse footpad material, which may play a part in stimulating the production of TNF- $\alpha$ . To investigate this, we compared the TNF- $\alpha$  response induced by uninfected mouse footpad material only (prepared in an identical manner to MFPML) and MFPML. Mouse footpad material alone did not elicit any detectable level of TNF- $\alpha$  in WBA (Data not shown). Thus, the high response shown by MFPML does not appear to be due to contaminating mouse footpad material.

These experiments indicate that PGL-I alone will not induce the secretion of TNF- $\alpha$  by T cells but when added along with ADML, PGL-I induces the cells to release this cytokine. The difference in the response between MFPML and ADML is postulated to be mainly due to the PGL-I factor, which is thought to be present in much lower concentrations in ADML. Researchers throughout the world are using armadillo derived whole *M. leprae* in *in vitro* studies to understand the role of *M. leprae* in

*vivo*. Our study indicates that cell mediated responses shown towards ADML and MFPML differ significantly in production of TNF- $\alpha$ . This is postulated to be due to structural difference in these two preparations of *M. leprae*. Further investigation is required but our results suggest that, where available, using MFPML in *in vitro* culture may elicit more appropriate immunological responses as compared to armadillo derived *M. leprae*. However, while MFPML can be isolated by simple 'in-house' purification, the numbers of *M. leprae* obtained compared to ADML would be small. Batch to batch consistency with MFP material may also be a problem.

## ACKNOWLEDGMENTS

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