In vitro and skin lesion cytokine profile in Brazilian patients with borderline tuberculoid and borderline lepromatous leprosy

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Summary

Objective: We investigated the in vitro and skin lesions production of cytokines in non-treated borderline tuberculoid (BT) and borderline lepromatous (BL) patients.

Patients and Methods: Seven untreated, non-reactional BT patients and 12 untreated, non-reactional BL patients were studied. Levels of the cytokines IFN-γ, IL-10, TGF-β1 and TNF-α were measured in supernatant of peripheral blood mononuclear cells (PBMC) cultures, stimulated with specific M. leprae antigen (sonicated and whole). The cytokines iNOS, IL-10 and TGF-β1 were detected by immunohistochemistry in skin biopsies.

Results: BT patients produced higher levels of IFN-γ than BL patients; iNOS expression in skin lesions was also higher in BT patients. TGF-β1 was detected in
more cells in BL patients; IL-10 expression was similar in both groups. There was a negative correlation between iNOS and TGF-β1 expression in skin biopsies, positive correlation between TGF-β1 in skin lesions and bacillary index, as well as positive correlation between iNOS detected in skin biopsies and PBMC IFN-γ production.

Conclusions: The BT patients had a mainly a Th1-profile of cytokines in their skin lesions and BL patients had a Th2 profile.

Introduction

Leprosy is characterised by a patient developing clinical, histopathologic, bacteriologic and immunologic manifestations on a spectrum related to the host response to *M. leprae*. The polar forms of the disease are associated with the production of cytokines by T-cell subsets. Tuberculoid patients (TT) have Th1 profile cytokines production such as interleukin-2 (IL-2) and IFN-γ; whereas lepromatous patients (LL) produce Th2 type cytokines, IL-4, IL-5 and IL-10.

However, the data on cytokine production are scarce in the borderline group, with small studies, and researchers have grouped the results of BT with TT patients, BL patients with BB or with LL patients when using peripheral blood mononuclear cells (PBMC) cultures. Misra et al. verified Th1 and Th0 profiles in BT patients, while BL patients had Th2 and Th0 profiles in PBMC culture supernatant obtained from seven patients.

Studies on cytokine detection in cutaneous lesions are also scarce and conflicting. McClean et al. described lesions from BT patients showing more CD4+ cells and BL patients more CD8+ cells, but they did not show significant differences in IFN-γ, TNF-α, IL-2, IL-4 and IL-6 production. Schon et al. verified the presence of iNOS in lesions of BT and BL patients and described that only lesions of BT patients had more than 20% of immunostained cells. In contrast, Khanolkar-Young et al. did not find this enzyme present in cutaneous lesions of BT patients. The research on TGF-β also has showed divergent results. Gouhart et al. found intense immunostaining of TGF-β in the subgroup BL and no immunostained cells were observed in the subgroup BT, whereas Khanolkar-Young et al. found positivity for TGF-β in BT patients too. Recently, Andersson et al. found this cytokine in 10–30% of cells present on granulomas from 10 borderline patients; the patients, however, were grouped as BT/BL.

These mediators have been evaluated in either cell culture supernatants or in cutaneous lesions, and there is no study where both aspects were concomitantly evaluated, and that BT or BL patients are frequently grouped with patients with polar forms, the objective of this study was to evaluate the role of macrophage and lymphocyte-derived cytokines (TNF-α, TGF-β1, IL-10 and IFN-γ) in the immune response in non-treated and non-reactional borderline patients. Furthermore, this study looked for correlations between the skin lesions cytokines production and the presence of acid-fast bacilli (AFB) in lesions. The correlation between the levels of cytokines produced in the PBMC supernatant and in the skin lesions was evaluated.

Materials and Methods

**Patients**

Nineteen non-treated leprosy patients were refereed and diagnosed at Instituto Lauro de Souza Lima, Bauru, Brazil and classified according to Ridley & Jopling. Seven of these
patients were classified as BT (skin smear bacterial index –BI: 0–1+), three of which were male and four female, with mean age 38.3 years. Twelve patients were classified as BL (skin smear BI: 2–4+), 10 were male and two female, the mean age of this group was 49.1 years. These patients did not have Type 1 or Type 2 reaction at diagnosis. The study was approved by the Ethics Committee of Instituto Lauro de Souza Lima. All patients were verbally informed of the purpose of the study and signed an informed written consent.

**CUTANEOUS LESION SAMPLE**

A skin biopsy was taken with a 5 mm punch for classification of patients and immunohistochemical analysis. The specimen was fixed in 10% formalin for 24 hours and processed using routine procedures for embedding in paraffin. Histological sections (4 μm) were stained with Hematoxylin-Eosin (HE) and Faraco-Fite. The AFB were evaluated using a scale of 0 to 6+, according to Ridley.

**PBMC CULTURE**

Twenty milliliters of venous blood were collected from each patient in Vacutainer® tubes (BD Becton Dickinson, Franklin Lakes, NJ, USA) containing heparin anticoagulant. Mononuclear cells were separated by Ficoll-Hypaque19 (Sigma, St Louis, MO, USA) density gradient and suspended in 1 ml RPMI-1640 medium containing L-glutamine and 25 mM HEPES buffer (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco), penicillin (100 UI/ml) and streptomycin (100 μg/ml) (Gibco). The total number of mononuclear cells was determined in a Neubauer chamber by 1:2 dilution of the cell suspension with 5% Turk dye in 4% glacial acetic acid. The final concentration was adjusted to 2 × 10⁶ mononuclear cells/ml. The viability of the cell suspensions was determined with 0.1% Trypan blue in buffered saline solution, pH 7.2. The cell suspension (100 μl/well) was distributed in 96-well, flat-bottom tissue culture plates (Corning, New York, NJ, USA) in the presence of 8 μg/ml PHA-M (Gibco), 10 μg/ml E. coli lipopolysaccharide (Sigma, St Louis, MO, USA), 10 μg/ml of M. leprae sonicated antigen (MLSON) and 4 μg/ml of whole M. leprae antigen (8 bacilli:1 mononuclear cell – WML). The antigens were provided by Dr. Patrick Brennan, Department of Microbiology, Immunology and Pathology, Colorado State University, USA. Control cultures (CC) were not stimulated. Plates were incubated at 37°C in an atmosphere with 5% CO₂ for 48 hours. After incubation, the cell suspensions were transferred to tubes and centrifuged at 1500 rpm for 10 minutes. The supernatants were collected, aliquoted and stored at −70°C for cytokine determination.

**QUANTIFICATION OF CYTOKINES IN SUPERNATANTS OF PBMC**

The cytokines IL-10, TGF-β1, IFN-γ and TNF-α present in the mononuclear cell culture supernatant were quantified by ELISA using Cytokine Duo-Set Kit (R&D Systems, Minneapolis, USA). The results are reported as pg/ml based on the standard curve established for each assay.
DETECTION OF IL-10 AND TGF-β1 CYTOKINES AND iNOS ENZYME IN CUTANEOUS LESIONS

The IL-10, TGF-β1 and iNOS were detected by immunohistochemistry. Serial 4 μm thick sections were mounted on silane slides (Dako, Carpinteria, CA, USA). The primary antibodies used were: mouse anti-human IL-10 antibody (R&D Systems), goat anti-human TGF-β1 antibody (R&D Systems) and rabbit anti-human iNOS antibody (Santa Cruz, San Diego, CA, USA), diluted respectively at 1:10, 1:50 and 1:250. After endogenous peroxidases blocking with 3% hydrogen peroxide in phosphate-buffered saline (PBS) pH 7-4 for 30 minutes, the slides were incubated with trypsin (Sigma) 0·25% in PBS or with 10 mM citrate buffer, pH 6·0. The slides were incubated with the primary antibodies overnight at 4 °C. For IL-10 and iNOS detection samples were incubated for 30 minutes with the mouse and rabbit Dako EnVision™ System, respectively. For TGF-β1 marker, the slides were incubated with goat anti-IgG rabbit biotinylated antibody (Dako) for 60 minutes followed by incubation with the ABC complex (avidin-biotyne-peroxidade) (Vector Laboratories, Burlingame, CA, USA) for 45 minutes. After, 1 mg/ml DAB solution (3·3′ tetrahydrochloride dianinobenzidine; Sigma, USA) in PBS pH 7·4, plus 1% hydrogen peroxide (Merck) for 5 minutes. The slides were counterstained with Harris’s hematoxylin and mounted in Permount resin. Between each step slides were washed with PBS. Previously characterised microscopic sections from cutaneous lesions of patients with Jorge Lobo’s disease²⁰ were used as positive controls, since the lesions have granulomas with cells expressing TGF-β and IL-10.

The negative controls used were: 1) samples in which the primary antibody was omitted; 2) sections incubated with normal goat and rabbit serum replacing the primary antibodies; 3) microscopic sections of normal mammary skin taken from patients undergoing plastic surgery. Positive labeling was identified as a brown staining on the cell cytoplasm. The histological sections immunohistochemistry were evaluated by three independent observers, using a semi-quantitative scale ranging from 0 to 4+. The score 0 was considered negative; 1+, minimum; 2+, discrete; 3+, moderate and 4+, intense.²⁰–²²

STATISTIC ANALYSIS

To determine whether there was a significant difference in the cytokine levels of cell culture supernatants between BT and BL patients, the data were analysed by Student’s ‘t’ test. The non-parametric Mann-Whitney test was used to determine whether there was a difference of cytokines and iNOS expression in lesions from BT and BL patients. Spearman correlation coefficient was calculated to verify the correlation between cytokines in culture cell supernatants and cutaneous lesions and the same test was used to determine whether there was correlation of cytokines in cutaneous lesions and the number of AFB. In all analyses, the level of significance was set at P < 0·05.²³

Results

The histopathological sections of BT patients had epithelioid cells granulomas in the centre with peripheral lymphohistiocytic halos. The epithelioid cells appeared in a loose tuberculoid arrangement scattered by small numbers of lymphocytes. The peripheral halo comprised
Figure 1. Histological sections of cutaneous lesions obtained from BT and BL patients (objective 40×, 100×). A. BT patient (HE). Tuberculoid granuloma with multinucleated giant cells and epithelioid cells, permeated with lymphocytes. B. BL patient (HE). Loose non-tuberculoid granuloma, composed by non-modified histiocytes interposed by lymphocytes. C-D. IL-10 immunolabeling in microscopic sections of BT (C) and BL patients (D). IL-10+ cells at the periphery of granulomas. E-F. Immunolabeling of TGF-β1 in microscopic sections of BT (E) and BL patients (F). (E) Focal expression in histiocytes. (F) Diffuse expression in histiocytes cytoplasm. G-H. Immunolabeling of iNOS in microscopic sections of BT (G) and BL patients (H). (G) Diffuse cytoplasmatic immunolabeling in histiocytes and epithelioid cells. (H) Cytoplasmatic immunolabeling in some histiocytes.
mainly lymphocytes, and a few histiocytes and plasma cells. Some of the granulomas showed foreign body and Langhans giant cells (Figure 1A).

In lesions from BL patients, the granulomas were formed by non-epithelioid cells and scattered lymphocytes. Lymphocytes appeared in clusters or bundles around the inflammatory infiltrate (Figure 1B).

The BI of cutaneous lesions ranged from 0 to 1+ (median 0) in BT patients and from 2 to 6+ (median 4.5+) \( (P = 0.001) \) in BL patients (Figure 2A).

IL-10 was detected in mononuclear cells, showing granular pattern, especially on the periphery of granulomas (Figures 1C and 1D).

The presence of this cytokine in lesions was higher in BL patients (median 3+) than in BT patients (median 2+) (Figure 2B).

The TGF-β1 was predominantly detected in histiocytes in a homogeneous and diffuse pattern, mainly in lesions of BL patients (median 3+), significantly more than in BT patients \( (P = 0.033) \) (median 1+) (Figures 1E, 1F and 2C).

iNOS expression was significantly higher in BT patients (median 3+) than in BL patients (median 2+) \( (P = 0.043) \) (Figure 2D).

The iNOS positive cells were histiocytes, epithelioid cells (Figures 1G and 1H) and multinuclear giant cells present on granulomas.

IL-10 and TGF-β1 production in BL patients skin lesions was negatively correlated \( (r = -0.68; P = 0.03) \). There was no other correlation between evaluated parameters.

Independently of the clinical forms, we found a positive correlation between TGF-β1 and BI \( (r = 0.563; P = 0.029) \) and negative correlation between TGF-β1 and iNOS \( (r = -0.705; P = 0.002) \).

![Figure 2](image-url)

**Figure 2.** Semi-quantitative analysis of bacterial index (BI) (A) and detection of IL-10 (B), TGF-β1 (C) and iNOS (D) in BT and BL skin lesions.
Table 1. Quantification of cytokines in the supernatants of peripheral blood mononuclear cells from BT and BL patients. Values are reported as mean ± standard deviation, in pg/ml.

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>TGF-β1 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BT</td>
<td>BL</td>
<td>BT</td>
<td>BL</td>
</tr>
<tr>
<td>PHA</td>
<td>1677.1 ± 1006.1*</td>
<td>1658.2 ± 866.1*</td>
<td>2494.0 ± 960.2*</td>
<td>1760.8 ± 805.8*</td>
</tr>
<tr>
<td>LPS</td>
<td>9.8 ± 15.1*</td>
<td>9.1 ± 19.7*</td>
<td>1829.8 ± 1184.7*</td>
<td>1898.7 ± 614.9*</td>
</tr>
<tr>
<td>WML</td>
<td>10.0 ± 6.4†</td>
<td>1.1 ± 1.9†</td>
<td>1150.4 ± 830.8*</td>
<td>1127.6 ± 517.3*</td>
</tr>
<tr>
<td>MLSON</td>
<td>42.0 ± 39.8†</td>
<td>9.6 ± 12.2†</td>
<td>1695.2 ± 925.1*</td>
<td>1778.9 ± 602.9*</td>
</tr>
<tr>
<td>CC</td>
<td>8.3 ± 22.0</td>
<td>3.8 ± 7.3</td>
<td>929.2 ± 781.2</td>
<td>419.6 ± 364.1</td>
</tr>
</tbody>
</table>

*P value <0.05 versus control cultures (CC).
†P value <0.05 between BT and BL patient.
PHA: phytohemagglutinin; LPS: lipopolysaccharide; WML: whole *M. leprae* antigen; MLSON: *M. leprae* sonicated antigen; CC: control cultures (spontaneous release).
The analysis of cytokines in PBMC supernatants showed higher levels of IFN-\( \gamma \) in BT patients compared to BL patients in \( M. \text{leprae} \)-stimulated cultures (\( P < 0.05 \)) (Table 1). The production of IL-10, TGF-\( \beta 1 \) and TNF-\( \alpha \) was similar between the BT and BL patients. The production of cytokines was higher in culture cells supernatants stimulated by \( M. \text{leprae} \)-specific (MLSON and WML) and non-specific stimulus when compared to non-stimulated cultures (\( P < 0.05 \)) (Table 1).

The levels of IFN-\( \gamma \), IL-10 and TGF-\( \beta 1 \) in PBMC supernatants and the iNOS, IL-10 and TGF-\( \beta 1 \) tissue expression in lesions from patients was positively correlated between the IFN-\( \gamma \) production of MLSON-stimulated culture cells supernatants and the iNOS tissue expression (\( r = 0.55; \ P = 0.027 \)) but not for the other parameters.

**Discussion**

The immunological paradigm of tuberculoid and lepromatous leprosy clinical forms is well established; however, in the borderline group patients show immunological instability. In this group of patients, approximately 40% develop type I or type II reactions, before, during or after the treatment, that may lead to neural damage and physical disabilities.

We found higher iNOS expression in cutaneous lesions from BT patients, and higher IFN-\( \gamma \) levels in PBMC supernatants. iNOS may be used as a marker of CMI response and indicator of IFN-\( \gamma \) production. Therefore, BT patients have a more efficient \( M. \text{leprae} \)-specific CMI response than BL patients, with consequent killing of bacilli. The finding of BI ranging from 0 to 1+ in BT patients supports this premise.

We found only three studies in which cutaneous iNOS expression was analysed in borderline patients. In one of them, the researchers evaluated only BT patients and did not find immunostained cells in cutaneous lesions; in the other studies, however, iNOS was present in lesions of BT patients, corroborating our findings. Misra \textit{et al.} has identified Th1 and Th0 profiles in BT patients and Th0 and Th2 in BL patients.

We found higher TGF-\( \beta 1 \) expression in lesions from BL patients; however we did not find significant difference in cytokine production PBMC supernatants between the BT and BL groups. The cutaneous lesion studies demonstrate different results, i.e. TGF-\( \beta 1 \) cells found only in lesions from BL patients or both BT and BL groups. Thus, in the borderline group there does not seem to be agreement about the presence of this cytokine in skin lesions. Goulart \textit{et al.} studying adhered PBMC cells in cell culture supernatants found differences in TGF-\( \beta 1 \) production in the polar forms of the disease; nevertheless, in BT and BL patients, these researchers did not find differences in PGL-I-stimulated or not-stimulated cultures. We have used mixed blood cells populations (lymphocytes and monocytes) in our study and found similar production of TGF-\( \beta 1 \) in the borderline group.

We found production of IL-10 in lesions of BT and BL patients and high levels of this cytokine in PBMC supernatants in both subgroups. The presence of IL-10 together with TGF-\( \beta 1 \) in lesions of BL patients may favour the maintenance of bacilli in granulomas, since both IL-10 and TGF-\( \beta 1 \) could suppress CMI response to \( M. \text{leprae} \). In the BT group, the high iNOS and low TGF-\( \beta 1 \) tissue expression, associated with to the presence of IL-10+ cells in the periphery of the granuloma and in the connective tissue suggests an immunoregulatory role of IL-10.
The immunoregulatory role of IL-10 in leprosy was also suggested by Stefani et al. who studied paucibacillary patients with single lesions, found positive correlation between the mRNA expression for IL-10 and IFN-γ, IL-12 and TNF-α. Belone reported consistent IL-10 immunostaining in reactional and non-reactional lesions of TT and BT patients, however, only in reactional lesions there was a significant increase of IL-10 cells, suggesting the activation of Th3 cells in the attempt to deactivating the strong damaging tissue reaction. Thus, these results, together with our findings, reinforce the role of IL-10 as an immunoregulatory cytokine.

We found similar levels of TNF-α in PBMC supernatants from BT and BL patients. Considering that TNF-α is a cytokine that acts in synergy with IFN-γ increasing the phagocytes microbicidal capacity and favouring the maintenance of the granulomatous process, it is possible that in BT patients it restricts the infectious process decreasing the bacillary load, in BL patients, however, the presence of high TGF-β1 and low IFN-γ levels allows bacillary proliferation.

The results obtained on this study reflect only one moment in the clinical course of disease and these patients may develop reactions. Thus the presence of IFN-γ and iNOS in BT patients points to a Th1 profile and the presence of high levels of TGF-β1 in BL patients suggests the participation of suppressor mechanisms. We also verified the increased IL-10 levels in BL patients suggesting a Th2 profile. It is also possible to infer the participation of T regulatory cells, such as Th3 lymphocyte and/or Treg cells (CD4+CD25+Foxp3+), in the production of IL-10 and TGF-β1. In granulomatous infectious diseases, such as paracoccidioidomycosis and leishmaniasis, an increased expression and function of Treg cells in lesions has been demonstrated. It has been suggested that these cells could contribute with persistence of the parasite and induction of chronicity of the disease. It is possible that similar cell changes occur in the borderline leprosy group, especially in BL patients, as numerous bacilli are present in lesions of these patients.

We found a positive correlation between iNOS tissue expression and IFN-γ production by PBMC stimulated with M. leprae antigens. The production of IL-10 in skin lesions was similar to that found in PBMC supernatant, with no differences between BT and BL patients. We suggested that the PBMC culture supernatant is a good parameter for evaluation of the immunological changes that occur in cutaneous lesions and can be used on determination of the immune response profile, especially when skin lesion analysis is not possible. However, analysing the profile of cytokine production in skin lesions shows subtle differences that are not detected in the PBMC system.

Our results suggest that borderline patients have different cytokines profiles in their skin lesions, mainly a Th1 profile in BT patients and Th2 profile in BL patients.

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Authors’ Contributions

James Venturini performed the laboratory assays and wrote the manuscript. Cleverson Teixeira Soares evaluated the immunohistochemistry and photographed the slides for the manuscript. Andréa de Faria Fernandes Belone participated on the immunohistochemical evaluation. Jaison Antonio Barreto and Somei Ura selected and classified the patients. José Roberto Pereira Lauris statistically analysed the data. Fátima Regina Vilani Moreno designed the study, supervised data analysis and checked the manuscript. All authors participated on the interpretation of data, read and approved the final manuscript.

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Ethical approval: The study was approved by the ethics committee of Instituto Lauro de Souza Lima, Bauru, SP, Brazil.

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Cytokines profile in borderline leprosy


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