In Situ Type 1 Cytokine Gene Expression and Mechanisms Associated with Early Leprosy Progression

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We explored the prognostic value of in situ cytokine patterns in 39 patients with single-skin-lesion paucibacillary leprosy before single-dose therapy, with 3 years of follow-up. Interferon (IFN)–γ, interleukin (IL)–12, IL-10, IL-4, tumor necrosis factor (TNF)–α, and macrophage inflammatory protein (MIP)–1α mRNA was quantified in skin biopsy samples at diagnosis, and Mycobacterium leprae DNA was detected in 51.4% of cases. Type 1 immunity predominance with measurable IFN-γ and undetectable IL-4, which is indicative of effective cell-mediated immunity, is compatible with both the reversal reactions (33.3%) and the resolution of lesions (64.1%) observed. A positive correlation between IL-12 and IFN-γ indicated type 1 polarization via IL-12. The TNF-α/MIP-1α correlation implied the TNF-α induction of chemokines, which is important for granuloma formation. Positive correlations between key regulatory cytokines—IL-10 and IFN-γ, IL-10 and IL-12, and IL-10 and TNF-α—suggests that there may be some level of an intraleosomal pro- or anti-inflammatory mechanism essential in avoiding immunopathology.

Cytokines produced by innate and adaptative immunity have been demonstrated to play an active role in protection against intracellular pathogens that regulate the interaction of immunocompetent cells and act as effectors of antimicrobial immunity. The classically described Th1 and Th2 cells and the cytokines they produce have been demonstrated, in murine models of bacterial and parasitic diseases, to be pivotal for protection or immunopathology [1]. Type 1 cells produce interleukin (IL)–2 and interferon (IFN)–γ and are generally associated with resistance to intracellular infection. Type 2 cells release IL-4 and are related to progressing disease. This polarized pattern of cytokine production, which has been clearly defined in animal models that used T cell clones [2], also has been demonstrated in human infectious diseases and allergic manifestations [1, 3, 4].

The leprosy spectrum, which is composed of well-established chronic polar and borderline forms, has been extensively studied as a model in which clinical manifestations correlate with local cytokine patterns and the nature of T cell responses. At one extreme, tuberculoid (TT) patients mount strong cell-mediated immunity (CMI) to Mycobacterium leprae that effectively restricts bacterial growth via the local production of type 1 cytokines. At the other extreme of the immunological spectrum, lepromatous patients are characterized by multibacillary disseminated disease and a lack of specific T cell responses to M. leprae, and their lesions express a type 2 cytokine profile that promotes a humoral immune response and progressive disease [5–7]. During the chronic course of the disease, acute inflammatory episodes may occur and are classified as
reversal reaction and erythema nodosum leprosum. These episodes are considered to be immunologically mediated phenomena, and they cause motor and sensory loss and, ultimately, nerve-function impairment and disability [7–9].

As leprosy control improves with the extensive use of multidrug therapy, patients with early stage disease, with 1 or few lesions, may represent the majority of new patients during the elimination era [10, 11]. Single-skin-lesion paucibacillary (SSL-PB) leprosy is considered to be the earliest clinical manifestation of the disease and is generally associated with a good prognosis. The diagnosis of early PB leprosy may be difficult, and polymerase chain reaction (PCR) to discover \textit{M. leprae} can enhance the ascertainment of new cases [12, 13]. New short-course drug regimens, such as a single dose of rifampin (600 mg), ofloxacin (400 mg), and minocycline (100 mg) (ROM), which was recommended by the World Health Organization in the late 1990s, have gained support for the treatment of SSL-PB leprosy [14]. In this context, studies concerning the local immune response among incipient clinical leprosy such as SSL-PB are needed.

We determined in situ cytokine patterns in patients with SSL-PB leprosy as defined by IFN-\(\gamma\), IL-12, IL-10, IL-4, tumor necrosis factor (TNF)–\(\alpha\), and macrophage inflammatory protein (MIP)–1\(\alpha\) mRNA expression, quantified by real-time, reverse transcription (RT)–PCR, and assessed their correlation to disease prognosis. PCR for \textit{M. leprae} DNA was used to investigate the presence of the etiological agent in early leprosy lesions. Our original goal was to assess whether the local dermal concentrations of cytokines in early lesions, detected once at baseline, could be informative about the ultimate clinical outcome observed in extended follow-up after single-dose ROM therapy.

**SUBJECTS AND METHODS**

**Study subjects.** Patients with SSL-PB leprosy from the Brazilian Multicentric Cohort \((n = 259)\) were enrolled, treated with 1 dose of ROM therapy, and clinically monitored between October 1997 and December 2001. The study design and the epidemiological, immunological, and histopathological baseline profiles of this cohort have been reported elsewhere [15, 16]. In brief, the inclusion criteria consisted of newly detected, untreated patients with 1 skin lesion without peripheral nerve involvement and smear-negative test results (PB leprosy). Before drug intake, all patients had dermatological and neurological examinations, and a blood sample was obtained for anti-phenolic glycolipid (PGL)–I serological testing. Smear bacilloscopy and the Mitsuda test were done, and 2 skin-punch biopsy samples (4 mm) were obtained from each patient for molecular tests and conventional histopathological testing. The Mitsuda preparation \((40,000,000 \textit{M. leprae} \text{cells/mL; Centro de Produção e Pesquisa de Imunobiológicos, Instituto da Saúde do Paraná, Brazil})\) was injected intradermally \((0.1 \text{mL})\), and the reaction at 3 weeks was measured.

A subset of patients \((n = 39)\) from 3 regions of Brazil where leprosy is endemic (Amazonas, Rondonia, and Goias) was selected for the present study and fell into distinct histopathological categories: 13 TT, 12 borderline tuberculoid (BT), and 14 indeterminate (I). Skin biopsy samples collected before the initiation of treatment were used for quantitating cytokine mRNA by real-time RT-PCR and for assessing the presence of \textit{M. leprae} DNA by \textit{M. leprae}–specific PCR. Informed consent was obtained from all study subjects. The research conformed to all ethical guidelines of the authors’ institutions and was approved by the Brazilian Ethical Research Committee.

**Clinical monitoring.** After 1 dose of ROM therapy, the entire cohort was actively monitored by at least 1 dermatological examination every 6 months during a 3-year follow-up period. Complete disappearance or a reduction in the size of skin lesions without any sign of reactional episodes was judged to be a resolution of lesions and a favorable clinical outcome. An unfavorable outcome was defined as a shift to multibacillary disease, confirmed by histopathological testing, the appearance of new lesion(s), peripheral nerve trunk involvement, or a first episode of reversal reaction with or without neuritis. Reversal reactions were diagnosed if any of the following criteria were met: increased swelling and redness of a preexisting skin lesion or the appearance of new erythematous skin lesion(s), with or without signs of neuritis (pain and/or tenderness of involved peripheral nerves), as evaluated by trained physicians. Because reversal reactions can potentially lead to immunopathological conditions that require intervention to avoid nerve damage, they were considered to be unfavorable outcomes. A standard course of prednisolone \((1 \text{mg/kg body weight for 12 weeks})\) was used to treat reversal reactions. For cases that shifted to multibacillary leprosy, a multidrug regimen was implemented according the official guidelines of the Brazilian Leprosy Control Programme.

**Quantitative PCR for cytokines.** Relative concentrations of IFN-\(\gamma\), IL-12, IL-10, IL-4, TNF-\(\alpha\), MIP-1\(\alpha\), and 18S were determined by quantitative, real-time RT-PCR using TaqMan Gene Expression Quantification assays (PE Applied Biosystems). For the cytokine study, skin biopsy samples were placed in denaturing solution (Promega) immediately after collection, snap frozen, and stored in liquid nitrogen at the coordinating center in Brazil. Selected samples were shipped on dry ice and processed within a median of 2 years \((\text{range, 14–27 months})\) of collection at the National Hansen’s Disease Programs Laboratory (Louisiana State University, Baton Rouge). Total RNA was isolated by guanidine isothiocyanate lysis and 2 cycles of phenol-chloroform-isomyl alcohol extraction and isopropanol-ethanol precipitation (Total RNA Isolation System; Promega). Genomic DNA was removed using RNase-free DNase \((1 \text{U/\muL; Life Technologies Life Science})\). The final RNA concentration used for preparing cDNA from each biopsy sample
was 336 ng RNA/reaction. First-strand cDNA was synthesized using Moloney Murine Leukemia Virus Reverse Transcriptase (200 U/μL; Clontech) and random hexamer primers (RT PCR Kit; Clontech). Amplification of cDNA was done on an ABI 7700 Prism Sequence Detection System with specific gene-targeted predeveloped TaqMan assay reagents (PE Applied Biosystems). A reference reagent was used to establish standard curves for all cytokines and 18S rRNA and was derived from a cDNA library made from concanavalin A–stimulated human peripheral blood mononuclear cells. Slopes for each cytokine and 18S standard curves based on the reference reagent ranged from −3.4 to −3.6. One hundred percent efficiency of amplification in these systems is equal to a slope of −3.3. Amplicons for all TaqMan reactions were between 75 and 200 bp. The correlation coefficient for the IL-10 standard curve was 0.91, and all other cytokine and 18S standard curves yielded correlation coefficients between 0.96 and 0.99. All cytokine and 18S rRNA values from patient biopsy samples fell on the linear portion of the appropriate standard curve. Relative concentrations of 18S rRNA, as cDNA, from each specimen were determined using the reference reagent, to aid in normalizing each specimen cytokine concentration. The normalization of data for each cytokine was accomplished by dividing the relative cytokine concentration for a sample by the relative concentration of 18S rRNA for that same sample. This allowed for comparisons between patient samples for a particular cytokine.

**M. leprae DNA PCR.** Fresh skin biopsy samples (*n* = 37) were snap frozen, stored in liquid nitrogen at the coordinating center in Brazil, shipped in dry ice, and processed at the National Hansen’s Disease Programs Laboratory (Louisiana State University, Baton Rouge). Skin biopsy samples were thawed at room temperature, minced, and lysed through 3 cycles of freezing (at −180°C) and thawing (at 95°C). Samples were digested for 18 h at 60°C with proteinase K (2.5 mg/mL) in 100 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 10 mmol/L EDTA (pH 8.0). Proteinase K was heat inactivated at 95°C for 10 min, and homogenates were extracted with phenol-chloroform-isooamyl alcohol. DNA was extracted with chloroform-isooamyl alcohol and precipitated overnight with ethanol at −20°C at an ionic strength of 100 mmol/L NaCl. The precipitated DNA was washed in cold 70% ethanol, dried at room temperature, and resuspended in Tris-EDTA (TE) buffer. Each specimen was amplified by PCR that used pairs of primers to produce a 360-bp *M. leprae*-specific DNA fragment from the 18-kD protein gene [17]. PCR products were detected by agarose gel electrophoresis with ethidium bromide staining and confirmed by slot-blot hybridization using a digoxigenin-labeled, 212-bp DNA probe that binds to the 360-bp PCR fragment. Each sample was tested undiluted and at 1:5 dilution together with positive (*M. leprae* DNA) and negative (TE buffer) controls.

**Serological testing.** The presence of IgM anti–PGL I antibodies was tested by ELISA, as described elsewhere [18]. PGL I synthetic disaccharide, covalently coupled to bovine serum albumin (gift from M. J. Colston, National Institute for Medical Research, London), was used at a concentration of 0.25 μg/mL, and serum samples were diluted at 1:300. Goat anti–human IgM peroxidase conjugate (μ-chain–specific; Sigma) was used, and the reaction was developed with o-phenylenediamine dye reagent (0.4 mg/mL; Sigma).

**Histopathological testing.** Paraffin sections (5 μm) were stained with hematoxylin and eosin and Fite-Faraco for acid-fast bacilli. Histopathological readings were classified according to Ridley-Jopling criteria [6]. Histopathological and ELISA tests were done at the coordinating center in Brazil.

**Statistical analysis.** mRNA cytokine results were expressed as a raw data ratio relative to 18S RNA quantities detected in each biopsy. To normalize the distribution, log10 transformation of cytokines values was done by adding 1 to 0 values. The correlations between transformed values of cytokines were calculated using Spearman’s correlation coefficients (2-tailed test of significance). The correlation between log cytokine values and outcomes was also examined. To transform cytokine data back to the original units, their values were reported as geometric mean (GMs) and 95% confidence intervals (CIs). Cytokine data were stratified into histopathological groups (TT, BT, and I), and comparisons among groups were done using the Kruskal-Wallis test. Fisher’s exact test was used to assess the difference in proportions of positivity to *M. leprae* PCR according to histopathological categories. All statistical calculations were done with the SPSS software program (SPSS).

**RESULTS**

**Baseline and follow-up data from patients with SSL-PB leprosy.** At the onset of clinical disease, the patients were 11–60 years old (mean, 33.4; SD, 15.3), and the male:female ratio was 20:19. The median size of the single lesions was 4.5 cm, 75% of lesions measured 2.5–6.5 cm (interquartile range, 4.0 cm), and 46.2% of the patients had a bacille Calmette-Guérin vaccination scar. Most patients (76.9%) had positive (≥5 mm) Mitsuda test, and 82.1% had a negative IgM anti–PGL I serological test result. During the 3-year clinical follow-up after ROM intervention, 33.3% (13/39) of patients presented mild reversal reactions, and all responded satisfactorily to steroid therapy. Only 1 patient (0.3%) had recurring multibacillary leprosy, as confirmed by histopathological testing, and was treated appropriately. The majority of adverse events (*n* = 13) occurred after 2 years of follow-up. The remaining 64.1% (25/39) patients had a favorable clinical evolution after treatment, and 11 (44%) of 25 had a complete disappearance of the lesion.

**PCR for *M. leprae.** Nineteen patients tested positive for
Figure 1. The relationship among histopathological groups and interferon (IFN)–γ, interleukin (IL)–10, IL-12, tumor necrosis factor (TNF)–α, and macrophage inflammatory protein (MIP)–1α log₁₀ values is shown. The Y-axis corresponds to the ratio of cytokine:18S RNA as obtained by reverse-transcription polymerase chain reaction. The top and bottom of the box are the 25th and 75th percentiles; thus, the length of the box is the interquartile range (IQR). The line through the middle of the box is the median. The upper error bar is the largest observation: 75th percentile plus 1.5 × IQR. The lower error bar is the smallest observation: 25th percentile minus 1.5 × IQR. Circles, outlier values; asterisks, extreme values. BT ( ), borderline tuberculoid; I, indeterminate ( ); TT, tuberculoid.

In situ cytokine profiles among patients with SSL-PB leprosy. For each cytokine detected, the GM (95% CI) was: IFN-γ, 12.30 (5.49–27.50); IL-10, 3.98 (3.55–4.46); IL-12, 1.07 (1.05–1.10); TNF-α, 6.46 (4.90–8.51); and MIP-1α, 1.35 (1.23–1.50). IFN-γ mRNA was detectable in 37 samples within a wide range of values and was undetectable in only 2 indeterminate cases. The expression of IL-4 mRNA was below the detection limit in all SSL-PB leprosy lesions, whereas TNF-α mRNA was detectable in all samples.

Figure 1 shows the box-plot distributions of mRNA for IFN-
Figure 2. Correlations between cytokines are shown. A, Interferon (IFN)–γ and interleukin (IL)–12; B, macrophage inflammatory protein (MIP)–1α and tumor necrosis factor (TNF)–α; C, IFN–γ and MIP–1α; D, IFN–γ and IL–10; E, TNF–α and IL–10; and F, IL–10 and IL–12.

g–IL–10, IL–12, TNF–α, and MIP–1α, stratified by histopathological groups TT, BT, and I. The highest values of IFN–γ were in the TT group (median, 1.77), followed by BT (median, 1.08), and I (median, 0.02). mRNA median values for IL–10 were similar in the TT and BT groups (0.79 and 0.72, respectively), with the lowest values detected in the indeterminate category. Within distinct histopathological categories, analogous distribution patterns were observed for TNF–α, MIP–1α, and IL–12, the latter at low levels. There was no statistical difference among median values of 18S rRNA stratified into different histopathological groups (TT, BT, and I; Kruskall Wallis test, 5.63; P = .06).

Cytokine correlations and intralesional immunomodulation. Figure 2 illustrates the cytokine correlations observed in lesions. Concerning type 1 polarization and granuloma formation, significant positive correlations of IFN–γ versus IL–12 (r = 0.7; P < .01) (figure 2A) and of MIP1–α versus TNF–α (r = 0.8; P = .01) (figure 2B) were seen. Considering that type 1 and 2 cytokine cross-regulations may occur in vivo, data from key regulatory cytokines involved in proinflammatory (IFN–γ, MIP–1α, and IL–12) and anti-inflammatory (IL–10) responses were analyzed. Significant positive correlations were seen between mRNA levels from IFN–γ and MIP–1α (r = 0.7; P < .01) (figure 2C), IFN–γ and IL–10 (r = 0.7; P < .01) (figure 2D),
TNF-α and IL-10 ($r = 0.5; P < .01$) (figure 2E), and IL-10 and IL-12 ($r = 0.8; P < .01$) (figure 2F). Predicting healing or reversal reaction outcomes on the basis of cytokine correlations was not possible (data not shown).

DISCUSSION

The most notable in situ mRNA cytokine gene-expression feature in early SSL-PB leprosy lesions was the predominance of type 1 immunity. The intraleSIONAL cytokine profile, determined by quantitative real-time RT-PCR, was characterized by the consistent detection of IFN-γ mRNA and the absence of detectable IL-4 mRNA. A major CMI effector mechanism in mycobacterial infections relates to the activation of T cells and the production of type 1 cytokines, such as IFN-γ, IL-12, and TNF-α, which results in macrophage activation and the recruitment of circulating monocytes to initiate long-term granulomatous formation and contain the infection [1].

During 3 years of follow-up, 64.1% of the patients with SSL-PB leprosy had a favorable response to treatment, characterized by resolving dermatological lesions and the restoration of sensory deficits, and 33.3% had mild reversal reactions, a percentage comparable to that seen in other reports [19]. Both reversal reactions and favorable clinical outcome observed after treatment in monolesion leprosy are compatible with the type 1 immunological profile of patients with SSL-PB leprosy [4, 8, 9].

IFN-γ is a critical cytokine that has been implicated in both protective immune responses and immunopathological processes [20]. In the cases of monolesion leprosy that we studied, IFN-γ was readily detected in 94.8% of the lesions. The role of IFN-γ in the protection against mycobacteria has been indicated by the description of distinct inherited disorders of IFN-γ-mediated immunity, which renders patients vulnerable to infections even by weakly pathogenic mycobacteria [21–23]. The greater susceptibility of IFN-γ knockout mouse models to mycobacterial infections by Mycobacterium tuberculosis, Mycobacterium avium, and M. leprae corroborates its protective role [24–27]. The absence of detectable IL-4 in the SSL-PB lesions that we studied supports the view that a type 2 immune response is minimal in these lesions, which is different than in the case of multibacillary lesions [4].

Even though in situ IFN-γ, IL-10, TNF-α, MIP-1α, and IL-12 cytokine gene mRNA expression varied widely, the levels correlated with the histomorphological organization in the lesions. Higher levels of each cytokine were always observed among TT patients, whose biopsy samples showed well-formed granulomas, followed by BT and by indeterminate lesions, each of which had decreasing cellular organization. No statistical difference was observed among median values of the housekeeping gene (18S rRNA) when they were stratified into the 3 different histopathological groups. Therefore, potential differences in the cellularity of lesions among groups did not explain the mRNA cytokine differences observed. Positivity for M. leprae DNA was higher in BT lesions than in TT and indeterminate ones, which suggests less effective antimicrobial activity in less-circumscribed BT granulomas. The detection of M. leprae DNA in only one-half of the SSL-PB lesions, a result that is compatible with the results of other studies of PB leprosy [28], underscores the relatively low sensitivity of M. leprae PCR for detecting the etiological agent at the TT end of the spectrum. This finding argues against the implementation of M. leprae PCR for general diagnostic purposes, except as an adjunct test to histopathological testing.

An important immunological factor for type 1 polarization is the cytokine milieu, particularly IL-12 secretion, at the site of infection [1]. T cells from patients with tuberculoid leprosy have been shown to be responsive to IL-12, and the IL-12 receptor β2 chain has been demonstrated to be highly expressed in tuberculoid lesions, compared with lepromatous lesions [29]. Our observation of a positive correlation between IL-12 and IFN-γ suggests a type 1 polarization in SSL-PB leprosy lesions. Resistance to experimental M. leprae infection has been correlated with the early production of IL-12 at the site of infection [30]. Furthermore, it has been suggested that, once Th1 cells have developed in experimental leishmaniasis, their effector function in vivo is independent of IL-12 [31]. These later points could explain the relatively low levels of IL-12 mRNA observed in the SSL-PB lesions.

TNF-α, a cytokine that plays a role both in antimycobacterial defense and in leprosy immunopathology [32], was detectable in monolesions from all histomorphological categories. TNF-α and other cytokines (e.g., MIP-1α) [33] are essential for the formation and maintenance of granulomas. We found that the highest levels of TNF-α and MIP-1α mRNA were in patients categorized as having TT and BT leprosy and were lowest in indeterminate cases. The absence of defined granuloma in indeterminate cases, coupled with the low levels of TNF-α and MIP-1α, suggests an active role for these 2 cytokines in the development and organization of granulomas as seen in the BT and TT SSLPB leprosy lesions and in other mycobacterial infections [34].

Correlations involving the key regulatory cytokines IL-10 and IL-12 were evaluated, to gain insight into possible intraleSIONAL immune modulation operating in vivo in early leprosy. Positive correlations between IL-10 and IFN-γ and between IL-10 and IL-12 were observed in SSL-PB. Earlier studies have shown that Th1 and Th2 cells can cross-regulate one another—IFN-γ, which is secreted by Th1 cells, directly suppresses IL-4 secretion and Th2 polarization. Conversely, IL-4 and IL-10 have been demonstrated to inhibit the secretion of IL-12 and IFN-γ, thereby blocking Th1 polarization [1]. The absence of mRNA for IL-4 in SSL-PB lesions, coupled with the presence of IL-10...
and the Th1 cytokines IL-12 and IFN-γ, suggests that there may be some degree of intralesional coordination of inflammation that is important in avoiding immunopathology.

It was not possible to determine those patients with SSL-PB leprosy who had favorable outcomes or reversal reactions during follow-up on the basis of the cytokine profile at diagnosis. Similar positive correlation coefficients between cytokines were observed independently of the clinical outcome. Noticeable differences in cytokine profiles would be more likely if healing lesions were compared with new cases of multibacillary leprosy, which, among this SSL-PB group, included only 1 patient. Data analysis of cytokine networks should be interpreted with caution, because cytokine production is highly correlated, which makes it difficult to sort out the interleukins associated with disease outcome. In our study, measurement of the cytokine milieu was done at a single point in time, and validation of the translation of mRNA to proteins by immunohistochemistry could add information to our results. Serial cytokine analyses on biopsy samples taken during and after clinical events may be necessary to distinguish the cytokine gene-expression patterns that are associated with favorable or unfavorable clinical outcomes. The challenge remains to identify a prognostic marker of disease progression among patients with early PB leprosy who are receiving minimal treatment.

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