Up-Regulation of Th1-Type Responses in Mucosal Leishmaniasis Patients

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Received 26 April 2002/Returned for modification 20 June 2002/Accepted 24 August 2002

The cytokine profile produced by peripheral blood mononuclear cells (PBMC) in response to leishmania antigens and the ability of interleukin-10 (IL-10) and transforming growth factor β (TGF-β) to modulate the immune response were evaluated in 21 mucosal leishmaniasis patients. Patients with mucosal disease exhibited increased gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) secretion and decreased IL-10 secretion compared to patients with classical cutaneous leishmaniasis. CD4⁺ Th1 cells were the main source of IFN-γ and TNF-α production in mucosal leishmaniasis patients. Evaluation of cytokine gene expression in PBMC of these patients showed that there was strong up-regulation of IFN-γ transcripts upon stimulation with leishmania antigen, in contrast to the baseline levels of IL-10 mRNA. IL-10 suppressed IFN-γ production by 48% in cell cultures from cutaneous leishmaniasis patients and by 86% in cell cultures from healthy subjects stimulated with purified protein derivative, whereas in similar conditions IL-10 suppressed IFN-γ production by 19% in cell cultures from mucosal leishmaniasis patients stimulated with leishmania antigen. TGF-β suppressed IFN-γ levels to a greater extent in healthy subjects than in mucosal leishmaniasis and cutaneous leishmaniasis patients. These data indicate that a poorly modulated T-cell response in mucosal leishmaniasis patients leads to production of high levels of proinflammatory cytokines, such as IFN-γ and TNF-α, as well as a decreased ability of IL-10 and TGF-β to modulate this response. These abnormalities may be the basis for the pathological findings observed in this disease.

Leishmania spp. are obligately intracellular parasites of macrophages that cause a spectrum of human diseases, including self-healing skin lesions, diffuse cutaneous leishmaniasis, localized cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), and visceral leishmaniasis (VL) (24). In all clinical forms, recovery and resistance to disease are dependent on T-cell responses (11, 14). For example, VL and diffuse cutaneous leishmaniasis are characterized by selective anergy of the specific cell-mediated immunity, which results in extensive involvement of the spleen and liver in the former disease and the skin in the latter disease, and abundant parasites are observed inside macrophages (12, 14).

CL is the most common form of tegumentary leishmaniasis. The hallmark of this illness is the development of single or multiple ulcerated dermal lesions, which usually promptly respond to antimonial therapy. A fraction (3%) of CL patients develop mucosal disease either at the time of ulceration or several months thereafter. ML is severely disfiguring and is developed mucosal disease either at the time of ulceration or several months thereafter. ML is severely disfiguring and is often unresponsive to standard treatment and present strong intra-dermal skin tests and lymphocyte proliferative responses to leishmania antigens (12, 16). On the other hand, microscopic analysis of lesion biopsies reveals an intense inflammatory reaction, in which lymphocytes and macrophages predominate and few or no parasites can be found (6).

The exact pathogenic mechanism of ML has not been established. While it has been suggested that ML may represent a polar hypersensitivity reaction to leishmania infection (1), other studies have revealed a mixture of type 1 and type 2 cytokines in lesions with up-regulation of mRNA for gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), and IL-10 (8, 27). Additionally, it has been shown that lymphocytes from ML patients produce high levels of IFN-γ and TNF-α in response to antigenic stimulation (12, 29). These data raise the question of whether immunological hyperactivity plays a part in ML. If it does, an exacerbated and unmodulated immunological response may ultimately lead to damage of host tissues. TNF-α is a proinflammatory cytokine secreted by macrophages and lymphocytes, whose production is up-regulated by IFN-γ (37). A role for TNF-α in the pathogenesis of this and other chronic inflammatory parasitic and bacterial diseases is strongly suggested by (i) increased levels of TNF-α in sera and supernatants of lymphocyte cultures (14, 29), (ii) high levels of TNF-α expression in mucosal tissue (27), (iii) decreased TNF-α levels after therapy for ML (17), and (iv) an association of TNF-α levels with cerebral malaria (19) and tissue damage following erythema nodosum leprosum (32).

In the present study, we demonstrated that an exaggerated immune response to leishmania antigens plays a role in ML, which is chiefly mediated by Th1-type CD4⁺ T lymphocytes and which is poorly modulated by the regulatory cytokines transforming growth factor β (TGF-β) and IL-10.
Patients. Twenty-one ML patients recruited at the health post of Corte de Pedra, Bahia, Brazil, were enrolled in this study. The criteria for diagnosis were a clinical picture compatible with ML, a positive delayed-type hypersensitivity response to leishmania antigens, and parasite isolation or histopathological findings characteristic of ML. All patients had a previous diagnosis of CL and were evaluated before therapy for mucosal disease. Six patients were evaluated before and 120 days after antimonial therapy (Glucantime; Rhodia, São Paulo, Brazil), at which time they were clinically cured of disease symptoms and the lesions had become scars. In order to evaluate the immunological responses of the 21 patients with ML, we matched these individuals by age and sex with 21 patients with cutaneous disease. Immunomodulation of antigen T-cell responses by IL-10 and TGF-β was evaluated in ML patients and compared to immunomodulation in CL patients. We also evaluated the role of these cytokines in modulating the immune response to a different antigen. To do this, we added IL-10 and TGF-β to cultures of cells stimulated with purified protein derivative (PPD) in PPD-skintest-positive individuals. This research was conducted with approval of the Ethical Committee of the Hospital Universitário Prof. Edgard Santos, and informed consent was obtained from each participant.

Soluble leishmania antigen. The leishmania lysate (crude antigen) used for cytokine production was prepared from a Leishmania amazonensis strain (MHOM-BR-86 BA-125) as previously described (12). L. amazonensis was chosen for antigen preparation because it is easy to grow and maintain and because the L. amazonensis antigen produces an immune response remarkably similar to that of Leishmania brasiliensis soluble antigen. This antigen preparation was negative for endotoxin, and it did not stimulate cells from healthy subjects who were not exposed to leishmania infection.

Evaluation of cytokine protein and mRNA expression. Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood over a Ficoll-Hypaque gradient (Organon Teknika, Durham, N.C.), washed, and resuspended in RPMI 1640 medium (Life Technologies Gibco BRL, Grand Island, N.Y.) supplemented with 2 mM l-glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 10% heat-inactivated pooled human AB serum (complete medium) at a concentration of 3 × 10^6 cells/ml. These cells were plated in 24-well plates (catalog no. 3047; Falcon, Becton Dickinson, Lincoln Park, N.J.) and either maintained unstimulated or stimulated with L. amazonensis antigen (10 µg/ml) or PPD (2 µg/ml). After incubation for 72 h at 37°C in the presence of 5% CO2, the supernatants were collected and stored frozen until cytokine determination. To evaluate the roles of IL-10 (DNAX Institute, Palo Alto, Calif.) and TGF-β (R&D Systems, Minneapolis, Minn.), these cytokines were added to the cultures at concentrations ranging from 0.5 to 20 ng/ml. To measure IFN-γ, TNF-α, and IL-5 levels, an enzyme-linked immunosorbent assay (ELISA) sandwich technique was used (Duoset; Genzyme, Cambridge, Mass., and Pharmingen, San Diego, Calif.). The IL-10 levels were measured by using a kit obtained from R&D Systems. In order to evaluate IL-10 and IFN-γ gene expression, PBMC from three ML patients were cultured in triplicate as described above and stimulated with leishmania antigen, medium, or phytohemagglutinin (PHA) for 72 h. Cells were harvested, mRNA was extracted, and reverse transcription (RT)-PCR were performed with the following primer pairs: for IL-10, AGG GCA CCC AGT CTG AGA ACA and CGG CCT TGC TCT AGT TTT CAC; and for IFN-γ, AGT CTC GTC ACC GTC TCT CAG and CGG CCT TGC TCG TGC TCG ACC TCG CAA CAG CAT. Amplicons were resolved by 1% agarose gel electrophoresis and were photographed by using a UVP apparatus. TGC TCT TCG ACCG TCAAA CAG CAT. Amplicons were resolved by 1% agarose gel electrophoresis and were photographed by using a UVP apparatus.

RESULTS

Profiles of cytokines secreted by PBMC from ML and CL patients. The levels of IFN-γ, TNF-α, IL-10, and IL-5 secreted in vitro by PBMC from ML and CL patients revealed disease-specific patterns (Fig. 1). The mean levels of IFN-γ (3,287 ± 2,442 pg/ml) and TNF-α (1,136 ± 1,023 pg/ml) for the 21 ML patients evaluated were higher than the mean levels of IFN-γ and TNF-α observed in CL patients (1,639 ± 1,245 pg/ml [P < 0.01] and 474 ± 526 pg/ml [P = 0.01], respectively). The IL-5 levels in ML patients were also higher (165 ± 193 pg/ml) than those observed in CL patients (11 ± 18 pg/ml) [P < 0.05]. At the same time, the levels of IL-10 in ML patients were significantly lower than those observed in CL patients (20 ± 14 and 59 ± 65 pg/ml, respectively [P < 0.05]).

A comparison of the IFN-γ and TNF-α levels measured before and after therapy for 6 of the 21 ML patients showed that there were unique responses for each cytokine. Whereas there was no significant difference between the levels of IFN-γ before and after antimonial treatment (2,429 ± 653 and 2,091 ± 1,086 pg/ml, respectively [P = 0.5]), the TNF-α levels fell by approximately 60%, from 1,092 ± 442 pg/ml before therapy to 442 ± 296 pg/ml after therapy (P = 0.01).

Expression of IL-10 and IFN-γ genes was also evaluated in PBMC cultures of three ML patients by RT-PCR (Fig. 2). All individuals showed similar patterns of strong up-regulation of IFN-γ mRNA after stimulation with leishmania antigen, comparable to the pattern obtained with PHA, as confirmed by densitometry of gels. However, these patterns were not accompanied by corresponding increases in IL-10 gene transcription. Densitometry showed that IL-10 mRNA kept baseline unstimulated levels after exposure to SLA except for patient 2,
who was able to produce the transcript, albeit at levels that were less than one-third (29%) of those achieved with mitogen. Furthermore, IL-10 up-regulation following PHA stimulation was similar to up-regulation of IFN-γ, confirming the ability of the PBMC tested to express this cytokine and suggesting that the SLA antigen preparation was a poor inducer of IL-10 in these patients.

**Cell population responsible for IFN-γ production in ML patients.** To determine the cellular source of IFN-γ synthesis in ML patients, CD4⁺ T cells, CD8⁺ T cells, and NK cells were removed from PBMC from six patients (Fig. 3). The IFN-γ levels in supernatants of leishmania antigen-stimulated PBMC were 1,773 ± 777 pg/ml. Removal of CD4⁺ T cells resulted in lower IFN-γ levels (395 ± 480 pg/ml) (P < 0.005). In contrast, the IFN-γ levels were not affected by removal of CD8⁺ T cells (1,858 ± 813 pg/ml) and NK cells (1,547 ± 790 pg/ml). Flow cytometric analysis of the PBMC of four ML patients confirmed that CD4⁺ T cells are responsible for more than 80% of the IFN-γ-producing cells following SLA stimulation (data not shown).

**Flow cytometric and cellular removal analyses to determine the sources of TNF-α in ML patients.** In order to determine the cellular sources of TNF-α in ML patients, macrophages and CD4⁺ T cells were removed from PBMC from three patients (Fig. 4). The TNF-α levels in the supernatants of PBMC stimulated with leishmania antigen were 1,450 ± 89 pg/ml. Removal of macrophages and CD4⁺ T cells resulted in lower TNF-α levels (426 ± 376 and 182 ± 117 pg/ml, respectively), suggesting that both of these cell populations are important for production of this cytokine.

Due to the possibility that removal of macrophages could result in decreased TNF-α production caused by inefficient antigen presentation rather than direct removal of a cytokine-producing cell population, the cellular sources of TNF-α were...
directly determined with a flow cytometer (Fig. 5). Representative contour plots for two ML patients demonstrate that lymphocytes accounted for the majority (range for the four patients analyzed, 60 to 90%) of the TNF-α/H9251-producing cells (Fig. 5B and C), followed by CD14/H11001 monocytes (10 to 40%). The lymphocyte-produced TNF-α came primarily from CD4/H11001 T cells (average, 65%) (Fig. 5D), followed by CD8/H11001 T cells (average, 35%) (Fig. 5E). CD14/H11001 monocytes produced variable amounts of TNF-α in the four patients analyzed by flow cytometry, as shown by the comparison of the profiles for low CD14+ TNF-α expression (Fig. 5B) and high (Fig. 5C) CD14+ TNF-α expression. However, in all cases, the frequency of TNF-α-producing lymphocytes was higher than the frequency of TNF-α-producing CD14+ monocytes, as mentioned above.

**IL-10 regulation of IFN-γ production in ML patients.** The role of IL-10 in the regulation of IFN-γ production was evaluated by using seven ML patients, seven CL patients, and three healthy subjects. IL-10 was used at concentrations ranging from 0.5 to 20 ng/ml. At low concentrations (0.5 to 2 ng/ml), IL-10 significantly suppressed IFN-γ production in cell cultures prepared from healthy subjects stimulated with PPD, but...
it had little effect on cell cultures prepared from ML and CL patients stimulated with Leishmania antigen (data not shown).

The ability of IL-10 to down-regulate IFN-γ production at a concentration of 5 ng/ml is shown in Fig. 6. The mean IFN-γ levels in cell culture supernatants stimulated with leishmania antigen (10 μg/ml) were 1,641 ± 580, and addition of IL-10 (5 ng/ml) resulted in levels of 1,330 ± 583 pg/ml (19% suppression). These results contrast with those obtained by using PBMC from CL patients. In CL patients, the mean IFN-γ levels in cell culture supernatants stimulated with leishmania antigen were 918 ± 175 pg/ml. Addition of IL-10 at a concentration of 5 ng/ml resulted in IFN-γ levels of 428 ± 24 pg/ml (48% suppression) (P < 0.005). In cell culture supernatants from healthy controls stimulated with PPD (2 μg/ml), the mean IFN-γ levels were 1,529 ± 825 pg/ml, while addition of IL-10 at a concentration of 2 ng/ml resulted in lower IFN-γ levels (214 ± 283 pg/ml; 86% suppression). The ability of IL-10 to down-regulate IFN-γ production in ML patient PBMC stimulated with leishmania antigen was tested by using 0.5 to 20 ng of this cytokine per ml. The maximal suppression observed was 22%. In three ML patients, IL-10 (2 ng/ml) down-regulated IFN-γ production by 42% in cultures stimulated with PPD, compared to the 86% down-regulation in the controls (data not shown).

**FIG. 5.** Cell population responsible for TNF-α production following antigenic stimulation. The cellular sources of TNF-α production are shown for two ML patients, as determined by using single-cell cytoplasmic staining and analysis with a flow cytometer. (A) TNF-α production without stimulus. (B and C) Frequency of CD14⁺ monocytes which produce TNF-α in two independent individuals, one exhibiting a low frequency of TNF-α-producing monocytes (B) and the other exhibiting a higher frequency of TNF-α-producing monocytes (C) following 20 h of culture with SLA. (D and E) TNF-α-producing CD4⁺ and CD8⁺ T cells, respectively, from another ML patient following short-term in vitro stimulation with SLA as described in Materials and Methods. TNF-A-PE, TNF-α-phosphatidylethanolamine; CD4-CYCHROM, CD4-Cychrome.

**FIG. 6.** Regulation of IFN-γ production by IL-10 in ML and CL patients. As described in Materials and Methods, PBMC from ML or CL patients were stimulated with leishmania antigen (La Ag) in the presence or absence of IL-10. IL-10 was used at a concentration of 5 ng/ml. The data are means and standard deviations for seven ML patients and seven CL patients. The percentage of suppression is indicated next to the single asterisk. Two asterisks indicate that the P value is <0.005.
Modulation of IFN-γ production by TGF-β. Another cytokine known to inhibit IFN-γ production is TGF-β. Therefore, we also tested the inhibitory effects of TGF-β on IFN-γ production in PBMC from ML patients and CL patients stimulated with leishmania antigen. Addition of TGF-β (20 ng/ml) to PBMC cultures from ML and CL patients resulted in a 15% decrease (from 1,427 ± 434 to 1,210 ± 456 pg/ml) and in no decrease (from 928 ± 204 to 931 ± 202 pg/ml), respectively, in IFN-γ production (P > 0.1), while it suppressed IFN-γ levels up to 47% in cell cultures from healthy subjects stimulated with PPD (from 1,490 ± 896 to 780 ± 343 pg/ml).

DISCUSSION

The present study showed that ML is characterized by increased IFN-γ and TNF-α production and decreased secretion of IL-10 in response to leishmania antigens and that the changes are accompanied by a reduced ability of IL-10 and TGF-β to down-regulate these immune responses. IFN-γ, TNF-α, and NO are products that are necessary to kill leishmania (22), but they are also implicated in the inflammation leading to tissue damage (19, 32). Although IFN-γ and TNF-α represent important defense mechanisms against this parasite, previous studies have shown that disease control and cure are not ultimately followed by sterilization (33). This conclusion is supported by the fact that exogenous addition of IFN-γ or of T-cell products from ML patients to human macrophages infected with leishmania does not eliminate the microorganism (12, 28). Thus, overproduction of these proinflammatory cytokines does not necessarily lead to parasite clearance and may be harmful to the host, supporting our hypothesis that ML results from an exacerbated and improperly modulated Th1 immune response to leishmania antigens. In accordance with this reasoning, we have recently shown that the combination of antimony with pentoxifylline, a potent inhibitor of TNF-α production (18), was able to cure ML patients who were refractory to standard antimonial therapy (21). In light of the present results, we suggest that the major contribution of pentoxifylline was to decrease the inflammatory reaction responsible for the tissue damage.

IL-10 is a major cytokine modulating immune responses in leishmaniasis patients. We previously showed that the inability of VL and early CL patients to produce IFN-γ can be restored in vitro by neutralization of IL-10 (10, 30). Conversely, exogenously added IL-10 down-regulates T-cell responses in subjects cured of VL (2). Our demonstration that cells of ML patients have a decreased ability to produce and to respond to IL-10 after stimulation with leishmania antigen suggests that the response to this major immunoregulatory cytokine is impaired in ML patients. This could represent a major mechanism whereby immune responses are deregulated, leading to this severely disfiguring form of human disease.

The pathways underlying the improper production of and response to IL-10 during ML remain elusive. One possibility is that the concentrations of IFN-γ in ML patients down-regulate the expression of IL-10R in lymphocytes and consequently prevent IL-10 modulatory effects. IL-10 acts via a specific ligand (IL-10R) (23), which is structurally related to interferon receptors (20) and might be negatively influenced by high levels of this Th1-type cytokine. Another possibility is that the increased secretion of other proinflammatory cytokines observed in ML patients could also down-regulate IL-10R. In this respect Michel et al. demonstrated that there is decreased expression of the IL-10 receptor in acute exanthematic psoriatic epidermis influenced by the proinflammatory cytokine IL-8 (25).

TGF-β is another cytokine with the ability to modulate immune responses in experimental leishmaniasis, as well as in other conditions (3, 5). For example, TGF-β reduces the antigen-induced cytotoxicity against K562 tumor cells by PBMC of CL patients (4). It has also been reported that addition of TGF-β to PBMC cultures from PPD-skin-test-positive individuals stimulated with PPD results in suppression of IFN-γ production by 70% (26). The data presented here suggest that along with IL-10, TGF-β may play an important regulatory role in ML patients, in which a lack of TGF-β may result in the high damaging levels of IFN-γ and TNF-α observed.

We showed that the major source of leishmania antigen-induced IFN-γ and TNF-α in ML patients is CD4+ Th1 cells. These helper T lymphocytes play a central role in antigen-specific adaptive immune responses, in contrast to the non-antigen-specific innate NK cell response. We propose that improper control of inflammatory reactions mediated by CD4+ cells in ML patients may occur via anergy to and/or lack of adequate levels of IL-10 and TGF-β. A compensatory response leading to down-regulation of the type 1 response might occur through overproduction of Th2-type cytokines, as shown here by the increased IL-5 observed in these patients.

We are tempted to speculate that the biased proinflammatory response described here may be induced in part by selective overexpansion of T-cell clones specific for certain leishmania antigens. Our previous data, as well as the data of other workers, have shown that there are distinct immune responses to recombinant leishmania antigens, as follows: (i) gp63 induces high IFN-γ levels and low IL-4 levels in ML patients (31); (ii) L. braziliensis HSP83 strongly induces mRNA for both Th1-type and Th2-type cytokines in vitro (34); (iii) rLACK generates weak Th1-type responses but apparently activates monocytes (7); and (iv) the elongation initiation factor (LeIF) induces predominantly IL-12 and TNF-α from PBMC of CL and ML patients (35). In keeping with this reasoning, we hypothesize that both host and parasite factors influence the development of ML.

ML is a disease that is associated with a strong Th1-type immune response to leishmania antigen, and parasites are often absent or rarely found in lesions (6). We offer evidence that CD4+ Th1-type cells are responsible for the exaggerated leishmania antigen-specific TNF-α and IFN-γ production in ML patients. This response is poorly regulated by IL-10 and TGF-β. Although Th1-type immunity protects against most forms of leishmaniasis, our data provide evidence that inadequate modulation of a type 1 response may lead to an exaggerated reaction and may constitute the core mechanism driving the immunopathogenesis of ML.

ACKNOWLEDGMENTS

This work was supported by an International Research Scholars grant from the Howard Hughes Medical Institute and by National Institutes of Health grant AI-30639. Edgar M. Carvalho is senior investigator of the Brazilian Council of Research (CNPq).
We thank Elbe M. Silva and Jackson L. Moreira for manuscript preparation.

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