IN VITRO PRODUCTION OF TYPE 1 AND TYPE 2 CYTOKINES BY PERIPHERAL BLOOD MONONUCLEAR CELLS FROM SUBJECTS COINFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS AND LEISHMANIA INFANTUM

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Abstract. To explore the type 1 and type 2 cytokine profile in cases coinfected with human immunodeficiency virus (HIV) and Leishmania infantum, production of interleukin-4 (IL-4), interleukin-10 (IL-10), interferon-gamma (IFN-γ), and interleukin-2 receptor (IL-2R) was investigated in mitogen-stimulated and unstimulated peripheral blood mononuclear cell cultures from eight HIV/Leishmania coinfected subjects matched with eight anti-HIV-positive subjects with no evidence of Leishmania coinfection. Levels of IL-4 and IL-2R increased significantly from the baseline levels in the peripheral blood mononuclear cell supernatants of HIV/Leishmania coinfected subjects following stimulation with phytohemagglutinin, whereas the postchallenge concentration of IFN-γ was significantly increased in the HIV-infected group. The levels of IL-4 and IL-10 were significantly higher in the HIV/Leishmania group throughout evaluation. Post-stimulation IFN-γ production was significantly higher in the HIV-positive group in comparison with that of the HIV-Leishmania coinfected subjects. These observations support the notion that a Th2 cytokine response is present during a Leishmania infection, even among HIV-coinfected individuals.

In Mediterranean countries, more than 70% of cases of visceral leishmaniasis (VL) in adults are coinfected with human immunodeficiency virus (HIV). An epidemiologic study by the World Health Organization showed that Italy has the second highest number of cases of HIV and Leishmania infantum coinfection in Europe after Spain. In Italy, Sicily has the highest incidence of cases and the highest variety of zymodemes.

It has already been established that the outcome of leishmanial infection is not influenced by humoral immunity but appears to be regulated by CD4+ T helper cells with different patterns of lymphokine activity. Protective immunity can be attributed to Th1 cells, which produce interferon-γ (IFN-γ) and interleukin-2 (IL-2), whereas Th2 cells, which produce IL-4 and IL-10. This may facilitate the intracellular survival of the parasite and influence the unfavorable outcome of the disease.

As far as HIV is concerned, recent in vitro experiments have reported a dominant type 1 cytokine profile as being more protective than a dominant type 2 cytokine profile. A shift from a Th1 to a Th2 cytokine pattern has been previously hypothesized to parallel or even precede the progressive CD4+ impairment that accompanies the transition towards the final stage of acquired immunodeficiency syndrome (AIDS).

To explore the type 1 and type 2 cytokine pattern in cases of HIV/Leishmania coinfection, ex vivo production of IFN-γ, IL-4, IL-10, and interleukin-2 receptor (IL-2R) by the peripheral blood mononuclear cells (PBMC) of patients coinfected by the two pathogens was investigated.

Patients and methods

Subjects. We studied 16 HIV-positive subjects attending the outpatient clinic of the University Institute of Infectious Diseases in Catania. This study was approved by the Ethics Committee of Azienda Ospedaliera Garibaldi, Ascoli Tommaselli S. Luigi (Catania). Informed consent was obtained from all patients. Infection with HIV was diagnosed in all 16 subjects by ELISA detection of anti-HIV antibodies and a Western blot testing according to criteria of the Centers for Disease Control and Prevention (CDC) (Atlanta, GA). Eight of these subjects had VL. The diagnosis of Leishmania infection was performed with antibody detection by indirect immunofluorescent assay (Leishmania-Spot IF; BioMerieux, Marcy l’Etoile, France) and confirmed by bone marrow microscopy and culture (cultures were performed at the Laboratorio di Parassitologia dell’Istituto Superiore di Sanità, Rome, Italy). None of the subjects had anti-leishmanial treatment around the time of sample collection.

The other eight patients had no evidence of L. infantum infection. There was no significant difference between the two groups with regard to sex, age, time since HIV was first diagnosed, CDC stage, proportion of patients treated with zidovudine and months of treatment, CD4+ and CD8+ lymphocyte cell counts (FACS auto-analyzer: Becton-Dickinson, Heidelberg, Germany), HIV-1 P24 serum antigenemia (HIV-1 antigen monoclonal ELISA; Abbott Laboratories, Abbott Park, IL), β2-microglobulin serum levels (competitive ELISA; Behring-Werke, Marburg, Germany), HIV-1 reverse transcriptase as determined by a commercially available quantitative ELISA (Boehringer Mannheim, Mannheim, Germany), plasma levels of HIV RNA as determined by a nucleic-acid-sequence-based amplification method (NASBA; Organon Teknika, Boxtel, The Netherlands) (Table 1).

Cell separation and culture. Whole blood was collected in vacutainer tubes containing preservative-free heparin (Becton Dickinson, Franklin Lakes, NJ). Peripheral blood mononuclear cells were separated by sedimentation on a percoll gradient (Seromed, Berlin, Germany), washed three times in Hanks’ balanced salt solution, and resuspended in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were cultured in 24-well plates at a concentration of 1 × 10⁶ cells/ml, either unstimulated or stimulated with 5 μg/ml of phytohemagglutinin (PHA; Gibco-BRL, Gaithersburg, MD), and incubated at 37°C in a 7% CO₂ humidified atmosphere. After incubation for 48 hr, su-
TABLE 1
Characteristics of the study subjects*

<table>
<thead>
<tr>
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<th>Anti-HIV positive (n = 8)</th>
<th>HIV/Leishmania coinfected (n = 8)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>26.9 ± 5</td>
<td>26.2 ± 7</td>
</tr>
<tr>
<td>Male/female</td>
<td>6/2</td>
<td>7/1</td>
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<tr>
<td>CD4+ cell count/mm³</td>
<td>188 ± 103</td>
<td>180 ± 110</td>
</tr>
<tr>
<td>CD8+ cell count/mm³</td>
<td>689 ± 156</td>
<td>702 ± 164</td>
</tr>
<tr>
<td>β2-microglobulin (mg/L)</td>
<td>6.4 ± 1.4</td>
<td>6.2 ± 1.4</td>
</tr>
<tr>
<td>P24 antigen (pg/ml)</td>
<td>12.2 ± 4</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>HIV-1 reverse transcriptase (ng/ml)</td>
<td>1.22 ± 0.37</td>
<td>1.52 ± 0.38</td>
</tr>
<tr>
<td>HIV-1 RNA (NASBA)</td>
<td>36,000 ± 8,900</td>
<td>31,000 ± 7,800</td>
</tr>
<tr>
<td>CDC HIV stage: I, II, III, IVa, IVb, IVc</td>
<td>0, 2, 0, 0, 2, 4</td>
<td>0, 2, 0, 0, 2, 4</td>
</tr>
<tr>
<td>Time since first diagnosis (months)</td>
<td>44.4 ± 18</td>
<td>42.6 ± 19</td>
</tr>
<tr>
<td>Number of subjects treated with AZT</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Duration of AZT treatment (months)</td>
<td>16 ± 4</td>
<td>16 ± 2</td>
</tr>
</tbody>
</table>

* Values are the mean ± SD where indicated. HIV = human immunodeficiency virus; NASBA = nucleic acid sequence-based amplification method; CDC = Centers for Disease Control and Prevention; AZT = azidothymidine.

permatants were harvested by filtration with a 0.45-μm filter and stored frozen at −70°C until the cytokine levels were measured.

Cytokine measurement. Supernatants, either unstimulated or 48 hr after PHA stimulation, were tested for IL-4, IL-10, IFN-γ and IL-2R levels using a commercially available enzyme immunoassays (Genzyme; Cambridge, MA). Tests were performed according to the manufacturer’s instructions. The sensitivities of the assays for IFN-γ, IL-4, IL-10, and IL-2R were 3, 6, 5, and 100 pg/ml, respectively.

Statistical analysis. Statistical evaluation was performed using paired and unpaired Student’s t-tests; P values < 0.05 were considered statistically significant. Results are given as the mean ± SD.

RESULTS

The overall cytokine profile in the examined groups is shown in Table 2.

Type 1 response. Stimulation with PHA induced a significant increase in IFN-γ and IL-2R production (P < 0.05) from the baseline value in the HIV-positive group. In the HIV/Leishmania coinfected subjects, stimulation with PHA induced a significant increase in the concentration of IL-2R (P < 0.05), but production of IFN-γ was not modified. Basal IFN-γ levels did not differ in HIV-positive compared with HIV/Leishmania coinfected subjects. Secretion of IFN-γ by stimulated PBMC was significantly lower in the HIV/Leishmania coinfected group (P < 0.05). Production of IL-2R was significantly higher (P < 0.001) among coinfected patients both before and after stimulation with PHA.

Type 2 response. Stimulation with PHA did not induce a significant increase in the production of IL-4 by cultured PBMC from HIV-positive subjects, whereas levels of IL-10 increased significantly from the baseline value (P < 0.05). Stimulation with PHA induced a significant increase in IL-4 levels (P < 0.001) in the HIV/Leishmania coinfected group, whereas post-stimulation IL-10 production did not differ significantly from the basal value. The concentration of IL-4 was significantly (P < 0.001) higher in HIV/Leishmania coinfected patients than in those infected with HIV alone both before and after stimulation with PHA. Levels of IL-10 were also higher in the coinfected group both in the baseline sample (P < 0.001) and in the post-stimulation one (P < 0.05).

DISCUSSION

Immune dysregulation observed in individuals infected with HIV during progression to AIDS could be accounted for by a shift from a Th1 to a Th2 type cell-associated cytokine profile. In fact, the reduced production of IL-2, together with the increased production of IL-4 and IL-10, by PBMC has been associated with the progressive depletion of CD4+ cells in HIV-infected patients. The Th1-Th2 shift during the natural course of HIV infection has been recently confirmed on a single-cell level by Klein and others, who reported a decreased percentage of lymphocytes expressing IL-2 and IFN-γ in conjunction with an increased proportion of IL-4- and IL-10-producing cells. The reason for this change in the cytokine profile is due to impaired production of IL-12 (a potent Th1 inducer).

On the other hand, susceptibility or resistance to L. infantum infection has also been demonstrated to be regulated by the two different Th lymphocyte subsets. The most potent cytokine for the induction of leishmanicidal activity in macrophages is IFN-γ. Visceral leishmaniasis is associated with transient T cell immune depression as assessed by deficient IL-2 production. Conversely, an over-expansion of Th2 subsets may take place. Karp and others demonstrated significantly elevated levels of IL-10 mRNA in bone marrow aspirates from patients with VL.

Visceral leishmaniasis in HIV-infected individuals is often the consequence of a reactivation of a latent infection. Amastigotes that multiply slowly in cutaneous macrophages

TABLE 2
Cytokine profiles in HIV-positive and HIV/Leishmania coinfected patients*

<table>
<thead>
<tr>
<th>Subjects</th>
<th>IL-4 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-2R (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV+</td>
<td></td>
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<tr>
<td>Basal</td>
<td>53 ± 10</td>
<td>75 ± 24</td>
<td>280 ± 94</td>
<td>382 ± 152</td>
</tr>
<tr>
<td>PHA stimulated</td>
<td>68 ± 26</td>
<td>124 ± 29</td>
<td>392 ± 100</td>
<td>528 ± 133</td>
</tr>
<tr>
<td>HIV/Leishmania</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>149 ± 19</td>
<td>157 ± 45</td>
<td>296 ± 77</td>
<td>1,228 ± 392</td>
</tr>
<tr>
<td>PHA stimulated</td>
<td>317 ± 92</td>
<td>186 ± 66</td>
<td>287 ± 54</td>
<td>1,646 ± 413</td>
</tr>
</tbody>
</table>

* Values are the mean ± SD. HIV = human immunodeficiency virus; IL-4 = interleukin-4; IFN-γ = interferon-γ; IL-2R = interleukin-2 receptor; PHA = phytohemagglutinin.
and in dendritic cells of the lymph nodes accelerate multiplication and start to invade multiple visceral sites due to progressive T cell immunosuppression. This event has already been demonstrated in patients treated with steroids or other immunosuppressive agents. Previous studies have indicated that Leishmania may be a putative cofactor in the progression of HIV-related disease.

Our goal was to investigate in vitro the mutual immunologic relationship between HIV and Leishmania. It can be speculated that during coinfection, both pathogens may interact while residing inside the same cell type (monocyte-macrophage cell lineage) and synergically interfere with cytokine production.

The present study confirmed the hypothesis that Th2 cytokine production is enhanced by the HIV/Leishmania coinfection. Cultured PBMC of HIV/Leishmania coinfect subjects released greater quantities of IL-4 and IL-10 in comparison with HIV-positive patients not coinfected with Leishmania. Specifically, release of IL-4 was higher in the coinfected group.

According to the data of Preiser and others, HIV-infected patients with VL showed a greater amount of circulating IL-4 and IL-10 than HIV-seropositive individuals without parasitic coinfection. Cacopardo and others have shown that in HIV-positive patients with acute VL, serum levels of Th2 cytokines are higher than those of VL patients without HIV infection. In the first group, both the IL-4 and IL-10 concentrations did not return to normal values after anti-leishmanial treatment. In HIV/Leishmania coinfected patients, an irreversible switch from a Th1 to a Th2 cell-associated cytokine pattern seems to occur.

Our data indicate that IL-4 plays a more prominent role in HIV/Leishmania coinfection than IL-10. This is not unusual since the Th2 response in parasitic infections is largely dominated by IL-4. In VL without HIV coinfection, the primary role of IL-4 production both in vivo and in vitro has been proposed by other investigators. During the natural course of HIV infection, IL-4 also appears to play a primary role.

We found significantly higher production of IL-2R by PBMC in the coinfect group. Other investigators (Bonnet E and others, Hopital Rangueil, Toulouse, France, unpublished data) also found significantly higher levels of serum IL-2R in HIV-infected patients with VL compared with HIV-seropositive without VL.

Interleukin-2 receptor is normally expressed on the T cell surface. Its solubilization accompanies a loss of sensitivity to IL-2 by T cells or a reduction of IL-2 synthesis. Furthermore, high levels of IL-4 are known to affect IL-2R expression on T cells. In HIV-infected patients, increased plasma levels of IL-2R have been reported during the progression phase towards AIDS. Thus, our findings could represent an indirect feature of Th2 cell expansion among HIV/Leishmania coinfect subjects.

Production of IFN-γ by PHA-stimulated PBMC was unmodified when compared with the baseline value in the HIV/Leishmania coinfect group, but it was significantly increased among the patients infected with HIV alone. Production of this cytokine by PBMC of HIV-infected patients has been demonstrated as being low by Fakoya and others. During parasitic coinfection, release of IFN-γ is probably down-regulated further by the suppressive action of IL-4.

It has been proposed that prolonged anti-leishmanial therapy in an immunocompetent host may favor a reverse switch from a Th2 to a Th1 response, thus promoting recovery. Furthermore, sodium stilbogluconate seems to require endogenous production of IFN-γ to function optimally. The simultaneous, irreversible production of low levels of IFN-γ and large amounts of IL-4 may be responsible for drug resistance to Leishmania, as well as for relapsing visceral infection, which are both typical clinical features of VL in HIV-seropositive individuals.

More recent availability of highly active antiretroviral drug combinations could favor an immunologic response with a reversal from type 2 to type 1 cytokines in HIV-infected individuals and probably enhance the efficacy of anti-leishmanial drugs in those who are coinfected with HIV and Leishmania.

Unfortunately, this study is limited by the lack of a Leishmania-infected population without HIV infection, which is becoming a rare finding in the geographic area studied. Nevertheless, the results of this study lead us to hypothesize the existence of a two-way relationship between Leishmania and HIV. The former may induce an irreversible defect in the T cell immune response by triggering Th2 cytokine overexpansion, thereby favoring a progression to AIDS in anti-HIV-positive subjects. The latter may induce a greater susceptibility to leishmanial infection and determine its progression to a chronic stage, either by reducing monocyte-macrophage leishmanicidal activity or by establishing an unfavorable environment for an effective response to treatment.

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REFERENCES


