In Vitro Modulation of Proliferation and Cytokine Production by Human Peripheral Blood Mononuclear Cells from Subjects with Various Forms of Coccidioidomycosis

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Using peripheral blood mononuclear cells (PBMC) from individuals with or without coccidioidal delayed-type hypersensitivity (DTH), we examined and attempted to modulate the in vitro responses of PBMC from various donors to the coccidioidal antigen toluene spherule lysate (TSL). Among healthy DTH-positive donors, 100 ng of human recombinant interleukin-10 (IL-10) per ml suppressed both PBMC proliferation ($P = 0.01$) and gamma interferon (IFN-$\gamma$) and IL-12 production (for both, $P < 0.05$). In vitro proliferation and production of IFN-$\gamma$ and IL-12 by PBMC were significantly higher in DTH-positive donors with active coccidioidomycosis than in healthy, nonimmune controls ($P < 0.05$) but not in active DTH-negative donors with or without human immunodeficiency virus infection (for both, $P > 0.05$). Human recombinant IL-12 increased IFN-$\gamma$ production by PBMC from active, DTH-positive donors ($P = 0.01$) but not by PBMC from DTH-negative groups. For healthy DTH-positive donors, the median antigen-reactive cell frequency per $10^5$ PBMC was 3.7, compared to 1.7 in DTH-negative donors with active coccidioidomycosis ($P = 0.03$). These data indicate that the in vitro TSL response is highly dependent on coccidioidal DTH. Not only do PBMC from individuals with DTH appear to respond to TSL, but their response can be modulated in vitro with either IL-10 or IL-12. On the other hand, PBMC from DTH-negative individuals do not respond in vitro to TSL and their response is not modulable, suggesting a lack of antigen response.

The clinical expression of human coccidioidomycosis extends over a wide range. On one end are the majority of individuals, in whom infection is completely asymptomatic except for the expression delayed-type hypersensitivity (DTH). At the other extreme are individuals who develop widely disseminated infection without DTH and who have elevated serum anticoccidioidal antibodies (16). This pattern is reminiscent of a murine model first described by Mosmann and colleagues in which T-helper lymphocytes are categorized based on their cytokine response to antigen (33). In this model, a T-helper lymphocyte type 1 (Th1) response is characterized by the production of interleukin-2 (IL-2) and gamma interferon (IFN-$\gamma$) and a strong cellular immune response. On the other hand, a Th2 response is characterized by production of IL-4, IL-5, and IL-10 and the lack of cellular immune response (34).

More recent data have suggested that IL-10 has a counter-regulatory effect on the Th1 response (19, 20). IL-10 inhibits macrophage production of IL-12, which itself stimulates Th1 cells to produce IFN-$\gamma$ (15). However, the roles of the Th2 response and of IL-10 in human infectious diseases are far from established (29, 39). We recently showed that peripheral blood mononuclear cells (PBMC) obtained from patients with disseminated coccidioidomycosis and stimulated in vitro with the coccidioidal antigen toluene spherule lysate (TSL) produced significantly less IFN-$\gamma$ than did PBMC from healthy, immune donors. However, PBMC production and mRNA expression of IL-10 were low and not significantly different between these two groups (9).

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However, that study left unanswered several questions, including the timing of cytokine production, the relation of in vitro PBMC proliferation and cytokine production to clinical expression of DTH, and whether in vitro PBMC proliferation and cytokine production could be modulated by the addition of cytokines or antibodies against cytokines. To answer these questions, we examined the in vitro responses to TSL among donors with different forms of coccidioidomycosis. We hypothesized that there would be significant differences in the in vitro TSL responses to TSL based on the DTH responses of the subjects and that these responses would be modulable in vitro.

MATERIALS AND METHODS

Subjects. The use of human subjects in this study was performed under the guidelines and approval of the Human Subjects Committee of the University of Arizona. In total, five different groups of subjects were recruited. Healthy adults without active coccidioidomycosis with coccidioidal DTH made up the healthy, immune control group. Healthy adults without active coccidioidomycosis and without coccidioidal DTH made up the healthy, nonimmune control group. Individuals with chronically active coccidioidomycosis requiring antifungal therapy were recruited consecutively from the Infectious Diseases Clinic of the Tucson Veterans Affairs Medical Center and comprised the active group. All these subjects were clinically stable at the time of study. These individuals were divided into those with coccidioidal DTH and those without. The final group of subjects consisted of individuals with known infection with human immunodeficiency virus type 1 (HIV) and concomitant coccidioidomycosis requiring antifungal therapy. For the nine patients with HIV infection, the median peripheral blood CD4 lymphocyte count was 135/µl (range, 2 to 291/µl).

Assessment of DTH. Coccidioidal DTH was determined by injecting 0.1 ml of usual-strength Spherulin (ALK Laboratories, Berkeley, Calif.) intradermally by a trained individual. Induration of the skin over the injection site of ≥5 mm after 24 or 48 h was considered indicative of DTH. To exclude any possibility that DTH testing might affect the results of in vitro assays, DTH testing was done immediately after blood was drawn for study in subjects with active coccidioidomycosis and was done ≥3 months prior to the study in the healthy, control subjects.

Clinical scoring. The clinical scoring of subjects with active coccidioidomycosis at the time of the study was performed by using a previously established system (25). A point was awarded for each symptom related to coccidioidomycosis, and...
points were awarded for each coccidioidal lesion and its severity based on physical and radiological examination. In addition, points were awarded for whether an involved site was culture positive and for the serum titer of complement fixation antibody. The final clinical score was the sum of all points awarded.

**LT assay.** The lymphocyte transformation (LT) assay was performed as previously described (1). Briefly, PBMC were isolated from heparinized blood by using Ficoll-Hypaque (Pharmacia, Piscataway, N.J.). Subsequently, 5 × 10^5 PBMC in RPMI 1640 (GIBCO) and 10% heat-inactivated pooled AB human serum (GIBCO) were added to flat-bottom wells of 96-well plates (Corning Glass Works, Corning, N.Y.). Either a final concentration of 100 μg of TSL per ml or RPMI 1640 alone was added to the wells in replicates of four. PBMC plate was incubated at 37°C in 95% air–5% CO_2_ for 5 days, after which time [3H]thymidine (0.5 Ci/ml; NEN, Boston, Mass.) was added. After an additional 18 h of incubation, the wells were harvested onto glass filter paper and counted by scintillation spectrometry. Results were expressed in counts per minute.

**Cytokine assay of cell supernatants.** Cell supernatants were prepared by placing 5 × 10^5 viable PBMC into flat-bottom wells in the same manner as in the proliferation assay. Cell supernatants were harvested after 4, 24, 48, and 72 h of incubation. Supernatants were immediately frozen and stored at −70°C until assayed. Concentrations of IFN-γ and IL-10 in PBMC supernatants were determined by enzyme-linked immunosorbent assay (ELISA; Genzyme, Cambridge, Mass., and Biosource International, Camarillo, Calif.). Preliminary experiments revealed that the median superantigen concentration of IL-12, using an assay which measured only the p70 heterodimer (R&D Systems, Minneapolis, Minn.), among six healthy donors with coccidioidal DTH was 10.4 pg/ml (range, 4.9 to 54.5 pg/ml), compared to 456.4 pg/ml (range, 17.2 to 472.9 pg/ml) in supernatants from five other healthy DTH-positive donors when measured by an ELISA which detected both p70 and p40 IL-12 (Biosource International). In subsequent experiments, supernatant IL-12 was determined by the ELISA which measured both p70 and p40 (Biosource International).

**Modulation of cytokine response of PBMC from healthy, immune donors.** We initially chose to examine the response in vitro responses among donors with active coccidioidomycosis. The clinical assessment scores between the two groups were not significantly different. For the DTH-positive subjects, the median score was 1.0 (range, 0 to 9), compared with a median of 1.5 (range, 0 to 4) for DTH-negative subjects (P = 0.54).

The proliferative response of PBMC from HIV-negative, DTH-positive donors was significantly greater than the re-
in any of the three groups. The addition of Levels of IL-10 were not significantly higher than in nonimmune controls among active DTH-positive subjects but with or without HIV infection. IL-10 occurred after 72 h of incubation. As shown in Table 1, g IFN-γ, IL-12, and IL-10 among four groups of donors had significantly increased proliferation as well as increased production of IFN-γ and IL-12 in response to TSL, while hu rIL-12 significantly and markedly increased levels of IFN-γ in samples from DTH-positive donors but not in other groups of subjects. In addition, production of IFN-γ by PBMC from five healthy, immune donors also significantly increased from a median of 125 pg/ml (range, 69 to 407 pg/ml) with TSL alone to 1,323 pg/ml (range, 348 to 4514 pg/ml) with the addition of hu rIL-12 (P = 0.04).

Comparison of ARC frequency. Five healthy DTH-positive subjects and five DTH-negative subjects with active coccidioidomycosis were assayed to determine the frequency of cells reactive to TSL in vitro. As shown in Fig. 3, the median ARC frequency per 10^6 PBMC stimulated with TSL was 3.7 (range, 2.6 to 4.1) for the healthy, immune subjects compared to 1.7 (range, 1.1 to 3.3) for the subjects with active coccidioidomycosis and negative DTH (P = 0.03).

**DISCUSSION**

It has long been recognized that patients with disseminated coccidioidomycosis have a diminished in vitro response to a variety of coccidioidal antigens and that this lack of response is associated with a failure to manifest cutaneous DTH (3, 11, 14). This lack of response has led to speculation that cytokine imbalances may play a role in the failure of patients with disseminated coccidioidomycosis to control their disease. This concept is bolstered by animal data showing that genetically susceptible mice produce IL-4 in lung extracts after coccidoidal infection, whereas genetically resistant mice produce IFN-γ (30). Moreover, systemic administration of IL-12 to susceptible mice results in significant amelioration of disease and shifts the cytokine message expression from a Th2 to a Th1 pattern (31). However, no data supporting this model have been obtained in human studies.

The most striking finding of the present study was that there was a profound difference in antigen responsiveness in vitro based on whether the donors with active coccidioidomycosis manifested coccidoidal DTH. PBMC from donors with DTH had significantly increased proliferation as well as increased production of IFN-γ and IL-12 in response to TSL, while PBMC from donors without DTH were not different from PBMC from nonimmune controls. All subjects with active coccidioidomycosis were stable and receiving antifungal therapy at the time of study. There was no difference in clinical score between the subjects with active coccidioidomycosis who expressed DTH and those who did not. These data suggest that

**TABLE 1. Supernatant concentrations of IFN-γ, IL-12, and IL-10 among four groups of donors**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Supplement</th>
<th>DTH+</th>
<th>DTH−</th>
<th>HIV</th>
<th>Nonimmune</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>TSL</td>
<td>195 (40–862)*</td>
<td>33 (25–54)</td>
<td>46 (27–68)</td>
<td>32 (25–49)</td>
</tr>
<tr>
<td></td>
<td>+ IL-12</td>
<td>3,798 (902–11,357)†</td>
<td>86 (37–267)</td>
<td>47 (28–919)</td>
<td>59 (36–80)</td>
</tr>
<tr>
<td></td>
<td>+ αIL-10</td>
<td>186 (42–438)*</td>
<td>36 (30–68)</td>
<td>38 (25–68)</td>
<td>33 (30–56)</td>
</tr>
<tr>
<td>IL-12</td>
<td>TSL</td>
<td>54 (31–81)*</td>
<td>21 (15–31)</td>
<td>24 (17–137)</td>
<td>15 (14–30)</td>
</tr>
<tr>
<td></td>
<td>+ αIL-10</td>
<td>52 (15–86)*</td>
<td>22 (13–30)</td>
<td>25 (16–98)</td>
<td>15 (14–25)</td>
</tr>
<tr>
<td>IL-10</td>
<td>TSL</td>
<td>16 (12–23)</td>
<td>15 (12–16)</td>
<td>16 (11–32)</td>
<td>17 (13–18)</td>
</tr>
<tr>
<td></td>
<td>+ IL-12</td>
<td>30 (15–68)*</td>
<td>15 (13–19)</td>
<td>16 (12–39)</td>
<td>14 (11–19)</td>
</tr>
<tr>
<td></td>
<td>+ αIL-10</td>
<td>14 (12–22)</td>
<td>16 (15–17)</td>
<td>16 (12–41)</td>
<td>15 (15–16)</td>
</tr>
</tbody>
</table>

*Samples were incubated for 72 h with either 100 µg of TSL per ml (TSL), TSL plus 10 ng of hu rIL-12 per ml (+ IL-12), or TSL plus 10 µg of anti-IL-10 per ml (+ αIL-10).

† Ranges are given in parentheses. Donor groups: DTH+, DTH-positive subjects with active coccidioidomycosis (n = 8); DTH−, DTH-negative subjects with active coccidioidomycosis (n = 4); HIV, subjects with active coccidioidomycosis and HIV infection (n = 9); nonimmune, DTH-negative healthy subjects without coccidiodal infection (n = 3). * P < 0.05 compared to results for nonimmune donors in the same row. † P < 0.05 compared to results with TSL alone for the same cytokine.
even under therapy, there are distinct groups of subjects with active coccidioidomycosis based on immunological responsiveness. Although this study has no data regarding clinical response, others have found that expression of DTH and in vitro proliferation are associated with improved outcome in coccidioidomycosis (3).

The in vitro response was modulable in donors who expressed coccidioidal DTH. Among healthy, immune donors, the addition of IL-10 resulted in virtually complete suppression of IL-12 and partial suppression of proliferation and IFN-γ. These findings do not directly implicate IL-10 as an in vivo modulator in human coccidioidomycosis, however. In fact, the lack of complete suppression of proliferation and IFN-γ production by relatively high concentrations of IL-10 (15) indicates that these responses are, at most, only under partial control of IL-10.

The assay used to measure IL-12 in PBMC supernatants detected both the p40 monomer and the p70 heterodimer of the cytokine. Preliminary data indicated that the levels of IL-12 were approximately 45 times higher than the levels seen when only p70 was measured. This difference is in keeping with observations of a marked overproduction of the p40 subunit of IL-12 when the entire heterodimer is produced (37). While recombinant p40 has been reported to inhibit IL-12 activity in mice (7, 22, 23, 32), there is no evidence that native p40 is inhibitory in humans either in vitro or in vivo (37). Moreover, others have used the measurement of either p40 mRNA (21) or p40 itself (8) as indicators of total IL-12 production in humans. The data from our study support the concept that measurement of both p40 and p70 is a sensitive way to detect in vitro IL-12 production by human PBMC.

Among both DTH-positive donors with active coccidioidomycosis and healthy immune donors, the addition of IL-12 resulted in striking increases in IFN-γ production. While proliferation was not enhanced by IL-12 in these subjects, it is likely that the PBMC were already maximally stimulated in the lymphocyte transformation assay. Unlike the results among DTH-positive subjects, addition of IL-12 failed to significantly enhance IFN-γ production among DTH-negative donors with active coccidioidomycosis.

Anti-IL-10 did not significantly alter the response among any of the subject groups. Although it is possible that an inadequate concentration was used, this concentration was as high as or higher than those reported in other studies (5, 24, 28). A more likely possibility is that IL-10 is not playing a significant role in suppressing antigen responsiveness in DTH-negative subjects with active coccidioidomycosis. This finding, combined with the evidence that IL-12 does not enhance responsiveness and that there is a diminished frequency of cells reactive to TSL in DTH-negative subjects with active coccidioidomycosis, suggests that lack of antigen responsiveness rather than imbalance of cytokine production accounts for the pattern of in vitro responsiveness observed in this group of subjects. This lack of in vitro modulation among DTH-negative subjects to IL-12 and anti-IL-10 appears to differentiate coccidioidomycosis from other human infectious diseases, including visceral leishmaniasis (2, 5), tuberculosis (24), and leprosy (35).

The diminished proliferative response of TSL-stimulated PBMC from subjects with active coccidioidomycosis who were coccidioidal DTH negative could be due to a decrease in the number of cells responding to TSL antigen, to a decrease in antigen responsiveness of individual cells, or to a combination of these factors. Using limiting-dilution analysis, we found a significant decrease in the number of cells responsive to TSL from subjects who were DTH negative compared to PBMC from DTH-positive subjects. These data indicate that at least part of the diminished responsiveness of PBMC from DTH-negative subjects is due to a lower frequency of antigen-reactive cells. However, the etiology for this lack of responsiveness is not identified. Immune suppressive factors in coccidioidomycosis have been hypothesized by others (4, 6, 10, 12, 27) and could account for these findings. Of these, a serum suppressor (13, 27) seems unlikely since pooled human sera were used to perform these experiments. A suppressive cytokine is a possibility, but results of this and other work (9) indicate that it is unlikely to be either IL-10 or IL-4. The presence of suppressor cells (6) or direct suppression by antigen (4, 10, 12) merits further study. A final possibility is that immune-reactive cells from DTH-negative subjects with active coccidioidomycosis are unable to recognize, process, or respond to the appropriate antigens within TSL to generate a Th1 type response.

Data are conflicting regarding the effect of IL-12 on in vitro responsiveness among persons infected with HIV. Landay and colleagues found that IL-12 and anti-IL-10 increased in vitro proliferation to HIV and influenza virus antigens, but only in those subjects whose peripheral blood CD4 lymphocyte count was ≥200/μl (28). Similar findings were reported by Uherova and colleagues for studies using other antigens (38). Harrison and Levitz (26) found that subjects with HIV infection had impaired proliferation to the fungi Cryptococcus neoformans, Candida albicans, and Pneumocystis carinii. The responses were not dependent on peripheral blood CD4 lymphocyte count and were not enhanced by the addition of IL-12. In our study, the median peripheral blood CD4 count of the nine subjects was well below 200/μl. Despite having active coccidioidomycosis, these subjects did not significantly respond to coccidioidal antigen. While there was a significant increase in proliferation with the addition of IL-12, this increase still remained significantly below the response of HIV-negative, DTH-positive donors. Moreover, no significant increase in production of IFN-γ was observed when cells were incubated with TSL plus IL-12 compared to TSL alone. Hence, IL-12 does not appear to profoundly affect the in vitro responses to coccidioidal antigens in persons with HIV infection.

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