Dichotomy of the T cell response to *Leishmania* antigens in patients suffering from cutaneous leishmaniasis; absence or scarcity of Th1 activity is associated with severe infections

A. GAAFAR*,†, A. KHARAZMI†, A. ISMAIL‡, M. KEMP†, A. HEY†, C. B. V. CHRISTENSEN†, M. DAFALLA§, A. Y. EL KADARO‡, A. M. EL HASSAN§ & T. G. THEANDER† *Institute of Tropical Medicine, MRC, Khartoum, Sudan, †Centre for Medical Parasitology, Institute for Medical Microbiology and Immunology, University of Copenhagen and Department of Infectious Diseases and Clinical Microbiology, National University Hospital of Denmark (Rigshospitalet), Copenhagen, Denmark, Departments of ‡Medicine and §Pathology, Faculty of Medicine, University of Khartoum, and ¶Faculty of Medicine, Wadi El Niel University, Khartoum, Sudan

(Accepted for publication 10 January 1994)

SUMMARY

The T cell response was studied in 25 patients suffering from cutaneous leishmaniasis caused by *Leishmania major* with severe (*n* = 10) and mild (*n* = 15) disease manifestations. Peripheral blood mononuclear cells (PBMC) from the patients were activated by sonicates of *Leishmania* promastigotes (LMP) and amastigotes (LDA), and the surface protease gp63. The proliferative responses to *Leishmania* antigens were lower in patients with severe disease than in patients with mild disease (*P* = 0.01–0.05), and such a difference was not observed in the response to purified protein derivative of tuberculin (PPD) or tetanus toxoid (TT). LMP-induced interferon-gamma (IFN-γ) production was lower in patients with severe than in patients with mild disease (*P* < 0.05). When the IL-4 and IFN-γ responses of each patient were considered, two response patterns were observed in the cultures activated by the *Leishmania* sonicates. One response pattern was characterized by high production of IFN-γ without production of IL-4 (a Th1-like pattern), the other was characterized by low IFN-γ levels which in most cases were associated with IL-4 production (not a Th1-like pattern). These patterns could not be distinguished when the cells from the same donors were stimulated by TT and PPD. The percentages of patients with a Th1-like response pattern after stimulation by LMP in patients with severe and mild disease manifestations were 30% and 80%, respectively. This difference was statistically significant (*P* = 0.034).

Keywords cutaneous leishmaniasis human T cell subsets

INTRODUCTION

Members of the genus *Leishmania* are protozoan parasites infecting and multiplying inside the phagolysosomes of macrophages in the mammalian host. Diseases caused by *Leishmania* parasites, the leishmaniases, constitute serious health problems, which affect millions of people in the tropical and subtropical parts of the world [1]. The clinical manifestations of leishmaniasis range from self-healing cutaneous ulcers (cutaneous leishmaniasis) to fatal systemic disease (visceral leishmaniasis, kala-azar) depending on the infecting parasite species and the immune response of the host [2].

The most common clinical manifestation of *L. major* is simple cutaneous leishmaniasis (CL) characterized by an ulcerated lesion(s) which heals spontaneously leaving depressed scar(s) and long-lasting immunity [3]. However, in some patients complications may occur as the parasite metastasizes through the lymphatics to the lymph node, leading to the formation of subcutaneous nodules and/or enlargement of the regional lymph nodes [4,5]. Severe disfigurement might result from large ulcers. Recently, an epidemic of CL hit the central Sudan along the Nile River in Greater Khartoum and spread to the North [6,7]. The disease was caused by *L. major* zymodeme LON-1 [8,9].

In experimental murine models the outcome of *L. major* infection depends on the activation of one of the two subsets of T helper/inducer cells, Th1 and Th2 [10]. Th1 cells produce interferon-gamma (IFN-γ), IL-2, and lymphotoxin, whereas Th2 cells secrete IL-4, IL-5, IL-6, and IL-10. Functionally, Th1 cells have mainly been associated with DTH reaction, whereas Th2 cells have been related to B cell help. When *L. major*
infection in mice results in activation of Th1 cells the mice recover and are resistant to reinfection, whereas activation of Th2 cells leads to progressive disease and death of the animals [11,12].

We have previously shown that both Th1- and Th2-like T cell reactivity exist in individuals who have been drug cured from visceral leishmaniasis caused by L. donovani [13,14]. Th2-like cytokine production profiles have been demonstrated in T cells from peripheral blood [15] and bone marrow [16] of patients suffering from kala azar. We have also shown that the Leishmania-specific T cells of individuals recovered from uncomplicated CL were of the Th1-like subset [17]. In this study we describe the cytokine production pattern in patients suffering from mild and severe CL, and report that the presence in peripheral blood of Leishmania-specific T cells with Th1-like cytokine production profile is associated with mild disease, whereas the absence or scarcity of Th1 activity and the presence of Th2 activity is associated with severe disease.

PATIENTS AND METHODS

Donors

After giving informed consent 25 patients with CL presenting to the Tropical Diseases Hospital, Omdurman, Sudan, were included in the study. From each patient a clinical history was obtained and a clinical examination was performed. The diagnosis of CL was made on the demonstration of amastigotes on slit smears, impression smears of biopsies and/or histological sections. On the basis of the clinical history and the clinical picture on admission the patients were divided into severe and mild cases. Patients were categorized as severe if: (i) they presented with a lesion of more than 40 mm in diameter, and had a clinical history of more than 6 months; and/or (ii) had subcutaneous nodules at admission. Nine normal Danish volunteers were included as controls not exposed to Leishmania antigens.

Treatment

Patients with single small lesions were not treated, as these usually healed spontaneously. Patients with multiple lesions or subcutaneous nodules were treated with iv sodium stibogluconate (Pentostam) at a dose of 10 mg/kg per day for 3 weeks, although some patients with severe disease needed several courses of Pentostam before healing was achieved.

Blood sampling and peripheral blood mononuclear cell preparation

Blood was collected by venipuncture into heparinized vacutainers (Becton Dickinson Ltd., Rutherford, NJ) at the day the patients presented to the Clinic. Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Nyegaard, Oslo, Norway) density centrifugation, washed twice in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), and frozen at −196°C in RPMI 1640 supplemented with 10% dimethylsulphoxide and 20% FBS. The cells were frozen by a manually operated version of a recently described device for gradient freezing of cells under field conditions [18] and stored and transported in liquid nitrogen. Before use, the cells were rapidly thawed and washed. The viability of the cells was confirmed by trypan blue exclusion.

Antigens

A sonicate preparation from L. major (Baringo strain; Kenya—MHOM/KE/87/NLB 455) was made as described previously [19]. The crude L. major preparation was used in a final concentration of 70 µg/ml.

Crude soluble amastigote antigen was prepared from L. donovani amastigotes grown in the human monocyte-like cell line, U937 (Batch no. F-8918; American Type Culture Collection, Rockville, MD) as described [20]. Briefly, U937 cells were grown. Before infection the cells were transferred to tissue culture plates (Nunc, Roskilde, Denmark) at 1 x 10⁶ cells/ml and stimulated by phorbol 12-myristate 13-acetate (PMA), causing cells to differentiate and grow as a monolayer. U937 cells were infected for 24 h at a parasitacell ratio of 10:1 followed by washing to remove extracellular parasites. After 6 days of incubation at 37°C, the U937 cells were harvested. Amastigote purification was performed as described [21] by homogenization of the cells, followed by differential filtration through polycarbonate filters of decreasing pore size (Isopore; Millipore, Bedford, MA). Purified amastigotes were washed three times before sonication for 5 x 45 s (MSE-150; Soniprep, Crawley, UK). Soluble antigens were collected after centrifugation (30 min, 30 000g, 4°C), and stored at −80°C until used at a final protein concentration of 1-3 µg/ml.

The abundant surface protein gp63 was purified from L. major promastigotes as described [22]. The protease activity of gp63 was inactivated by heating to 70°C for 20 min and the protein was used at a final concentration of 10 µg/ml.

Purified protein derivative of tuberculin (PPD) and tetanus toxoid (TT) were purchased from Statens Seruminstitut (Copenhagen, Denmark) and used at 12 µg/ml and 3 µg/ml, respectively.

Lymphoproliferation and cytokine production

Cells were cultured in RPMI 1640 with 10 mM HEPES, 20 U penicillin and 20 µg streptomycin per ml supplemented by 15% heat-inactivated, pooled, normal human serum (NHS). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. PBMC were incubated with antigen at 6.6 x 10⁵ cells/ml in volumes of 170 µl in round-bottomed microculture plates (Nunc). The cultures were incubated for 7 days and pulsed with 20 µl/well of ³H-thymidine (New England Nuclear, Boston, MA) (1-85 MBq/ml) for the last 24 h of incubation. The culture supernatants were recovered and stored at −20°C for later determination of IFN-γ. The cells were harvested onto glassfibre filters and the incorporation of ³H-thymidine into DNA was determined by a Matrix β-counter (Packard, Greve, Denmark). All proliferation assays were performed in triplicate. For each set of samples the median ct/min was recorded. The antigen response was expressed as the difference between antigen-stimulated cultures and cultures incubated without antigen. A response was considered measurable if the stimulation index (SI; ct/min in antigen-stimulated culture/ct/min in unstimulated culture) was ≥ 2.5.

For the measurement of IL-4 release by antigen-stimulated cultures of PBMC, parallel cultures were carried out for 6 days and then pulsed with 1 µM ionomycin and 50 ng/ml PMA (both from Sigma, St Louis, MO) for 24 h before the culture supernatant from triplicate wells was harvested [23].
**Cytokine measurements**

IFN-γ and IL-4 in the culture supernatants pooled from the triplicate cultures were measured by ELISA as described elsewhere [19,23]. The detection ranges of the ELISAs were: 1–66 U/ml for IFN-γ (specific activity of the reference protein: 2.5 × 10^8 U/mg) and 30–2000 pg/ml for IL-4. Antigen-induced cytokine production was calculated as the difference between antigen-stimulated and unstimulated cultures. A response was considered measurable if the SI (cytokine level in supernatant from antigen-stimulated culture/cytokine level in supernatant from cultures without antigen) was ≥ 2.0.

**Statistical analysis**

The magnitude of the responses in patients with mild and severe disease were compared by the Mann–Whitney two-sample rank sum test. The Fisher exact test was used to compare the frequency of Th1 and non-Th1 responders in patients with severe and mild disease. P < 0.05 was considered significant.

**RESULTS**

**Clinical features**

Most patients were from villages of Khartoum region or from the outskirts of Omdurman City. The patients were divided into a group with mild and a group with severe disease according to the size of the lesion, duration of the disease and the presence of subcutaneous nodules. Table 1 summarizes the clinical information of the two groups. The groups were comparable with regard to age and number of lesions. The lesions were typically nodular or noduloucerative, and usually involved the upper or lower limb. In 32% of the patients subcutaneous nodules, which usually followed the lymphatics draining into the regional lymph nodes, were present.

**Proliferative responses**

The proliferative responses of PBMC isolated from patients with severe and mild disease and of Danish controls presumably not previously exposed to *L. major* are summarized in Table 2. The responses to both the purified *Leishmania* antigen, gp63, and the crude *Leishmania* antigen preparations were significantly lower in the group with severe disease than in the group with mild disease. PBMC from Danish donors did not proliferate in response to gp63 and amastigote sonicate (LDA), whereas a response to the promastigote sonicate (LMP) was found in six of nine PBMC isolates.

No differences were found between the groups in the response to PPD. Some of the Sudanese patients responded to TT, and the responders were mainly women (data not shown), probably due to tetanus toxoid vaccination during pregnancy.

**IL-4 and IFN-γ assays**

The IFN-γ response to the *Leishmania* antigens was lower in the group with severe than in the group with mild disease (Table 3), but only the difference in the response to promastigote sonicate reached statistical significance (P < 0.05). The PBMC from Danish donors did not produce IFN-γ in response to gp63 or LDA. Some Danes produced IFN-γ in response to LMP, and the IFN-γ response to LMP in Danes and patients with severe disease was similar.

The IFN-γ response to PPD was comparable in patients and controls. The TT response was higher in the patients with mild disease than in the two other groups, but the difference was not statistically significant. The difference between the

**Table 1. Clinical features of cutaneous leishmaniasis patients at diagnosis**

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Severe (n = 10)</th>
<th>Mild (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age* (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (F/M)‡</td>
<td>3/7</td>
<td>7/8</td>
</tr>
<tr>
<td>Lesion features</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration (weeks)†</td>
<td>25 ± 5</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>Number of lesions</td>
<td>4 ± 5</td>
<td>6 ± 5</td>
</tr>
<tr>
<td>Size of lesions (mm)</td>
<td>41 ± 1</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>Subcutaneous nodules§</td>
<td>8 (80)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lymphadenopathy§</td>
<td>3 (30)</td>
<td>1 (67)</td>
</tr>
</tbody>
</table>

* Median (range).
† Female/male.
‡ Mean ± s.d.
§ Number of patients and (percentages).

**Table 2. Antigen-induced proliferation (median kct/min (95% confidence interval)) in peripheral blood mononuclear cells (PBMC) from patients with severe and mild cutaneous leishmaniasis (CL) and non-exposed Danish controls**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Severe CL (n = 10)</th>
<th>Mild CL (n = 15)</th>
<th>Controls (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp63</td>
<td>0.1 (0.06)**</td>
<td>1.9 (0.6, 5.1)</td>
<td>0.2 (0.1, 0.3)</td>
</tr>
<tr>
<td>LDA†</td>
<td>4.3 (2.3, 6.3)**</td>
<td>7.2 (4.6, 11.4)</td>
<td>2.0 (0.7)</td>
</tr>
<tr>
<td>LMP‡</td>
<td>7.7 (4.5, 11.4)*</td>
<td>12.4 (9.4, 15.9)</td>
<td>2.1 (0.5)</td>
</tr>
<tr>
<td>PPD</td>
<td>5.6 (1.9, 9.3)</td>
<td>6.4 (3.1, 11.0)</td>
<td>4.4 (2.4, 7.29)</td>
</tr>
<tr>
<td>TT</td>
<td>0.2 (0, 6.4)</td>
<td>0.8 (0.3, 2.9)</td>
<td>2.4 (0.9, 3.8)</td>
</tr>
</tbody>
</table>

* **Response significantly lower than in patients with mild disease (*P < 0.05; **P < 0.01).† ‡ Sonicates of Leishmania promastigote (†) or amastigote (‡).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Severe CL (n = 10)</th>
<th>Mild CL (n = 15)</th>
<th>Danes (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp63</td>
<td>0.0 (0, 3.5)</td>
<td>4.4 (0.6, 8.4)</td>
<td>0 (0)†</td>
</tr>
<tr>
<td>LDA</td>
<td>2.0 (0, 8.2)</td>
<td>8.7 (4.6, 13.5)</td>
<td>0 (0)**</td>
</tr>
<tr>
<td>LMP</td>
<td>10.0 (4.6, 19.6)*</td>
<td>26.0 (13.5, 38.7)</td>
<td>12.5 (0, 26.5)**</td>
</tr>
<tr>
<td>PPD</td>
<td>25.0 (1.4, 40.4)</td>
<td>24.6 (11.2, 42.8)</td>
<td>18.0 (36.5)</td>
</tr>
<tr>
<td>TT</td>
<td>1.3 (0.4, 10.6)</td>
<td>5.57 (1.9, 9.9)</td>
<td>2.2 (0.27)</td>
</tr>
</tbody>
</table>

* **Production statistically significantly lower than in patients with mild disease (*P < 0.05; **P < 0.01).† n = 6.**
‡ PPD, Purified protein derivative; TT, tetanus toxoid.
response (Table 3) probably reflected a difference in the sex ratio between those with mild and those with severe disease.

The IL-4 (Table 4) responses were generally low, but PBMC from some donors, mainly among the patients with severe disease, produced IL-4 in response to the Leishmania antigens. The differences in Leishmania antigen-induced IL-4 production between patients with mild and severe disease were not statistically significant. PPD induced only sporadic IL-4 production. A few of the Sudanese donors and approximately half of the Danes had a measurable IL-4 response to TT.

Figures 1 and 2 show the corresponding IL-4 and IFN-γ responses of each patient. Most responses to Leishmania antigens could be divided into two response patterns (Fig. 1): (i) those with a considerable IFN-γ production and no IL-4 (Th1-like); and (ii) those with lacking or moderate IFN-γ production often associated with some IL-4 production (not Th1-like). Most of the patients with mild disease (open circles) showed a Th1-like responsiveness, as opposed to the patients with severe disease (closed circles), in whom a non-Th1-like response pattern was most frequent. When the response of the patients was defined as Th1-like and non-Th1-like on the basis of IFN-γ production as indicated in Fig. 1 (dotted lines), the fraction of patients with Th1-like response pattern to Leishmania antigens was higher among those with mild disease compared with those with severe symptoms (Table 4). The responses to PPD and TT could not be divided into distinct response patterns, and no correlation between the responsiveness and disease severity was evident (Fig. 2).

**DISCUSSION**

Leishmania parasites cause human disease ranging from self-healing cutaneous ulcers to fatal systemic infections. In addition many individuals become infected without developing disease [24–27]. CL caused by L. major is often an uncomplicated disease, characterized by a localized skin lesion which heals spontaneously. However, in some cases complications might occur as the infection results in large ulcers or spreads through the lymphatics to the lymph nodes. To correlate Th1- and Th2-like immune responses to the clinical disease, we attempted to identify patients who had difficulties in controlling inflammation and parasite multiplication. Hence patients with large lesions and a long clinical history and/or apparent spread of the parasite as evidenced by swelling of subcutaneous nodules were defined as suffering from severe disease. This classification of the patients is probably not accurate. For instance, a patient with mild disease might later develop severe symptoms, and signs of severity could be caused by complications due to secondary infections. There was a higher proportion of men in the group with severe CL than in the group with mild disease. Furthermore, the duration of disease was shorter among the women than among the men (data not shown). These differences might be due to a tendency for women to seek treatment earlier than men.

The proliferative response to Leishmania antigens was lower in PBMC from patients with severe disease than in PBMC from patients with mild disease. This finding is in agreement with the findings by Jaffe et al. [28] and Frankenburg et al. [29], who reported on Leishmania antigen-specific proliferative response in patients with mild CL caused by L. major, and the findings of Castes et al., and Caceres-Dittmar et al., who found that Leishmania antigen-induced PBMC proliferative response correlated with disease severity in South American patients suffering from CL [30–32]. The Danish controls did not respond to LDA and gp63 antigen, whereas LMP as reported previously [33,34] activated the PBMC of some non-exposed donors.

Leishmania antigen-induced IFN-γ responses were higher in patients with mild than in patients with severe disease, and the
Human T cell response to Leishmania antigens

![Graph showing cytokine production](image)

**Table 5. Number of individuals with a Th1-like peripheral blood mononuclear cell (PBMC) response to the Leishmania antigens, gp63, LDA, and LMA in patients suffering from severe and mild cutaneous leishmaniasis, respectively**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Disease severity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe (n = 10)</td>
</tr>
<tr>
<td>gp63</td>
<td>2 (20)</td>
</tr>
<tr>
<td>LDA</td>
<td>3 (30)</td>
</tr>
<tr>
<td>LMA</td>
<td>3 (30)</td>
</tr>
</tbody>
</table>

*Th1-like responsiveness was defined according to the production of IFN-γ (Fig. 1).

*, **Number statistically significantly higher than in patients with severe disease (*P = 0.049; **P = 0.034, Fisher's exact test).
ACKNOWLEDGMENTS

Dr M. Hag-Ali, Dr Suad Sulaiman and Professor James Jensen are acknowledged for making our work possible at the MRC-NIH laboratory in the Sudan. The excellent technical assistance of Jette Dalsten is greatly appreciated. This work was supported by grant 104.Dan.8.L/401 from the Danish International Development Agency (DANIDA) and the Danish Biotechnology program and NIH grant AI-16312.

REFERENCES


13 Kemp M, Kurtzhals JAL, Bentzen K et al. Leishmania donovani-reactive Th1- and Th2-like T-cell clones from individuals who have recovered from visceral leishmaniasis. Infect Immun 1993; 61:1069–73.


Human T cell response to Leishmania antigens