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Leishmania donovani-Reactive Th1- and Th2-Like T-Cell Clones from Individuals Who Have Recovered from Visceral Leishmaniasis

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Infections in humans by *Leishmania donovani* parasites can result in a fatal disease, visceral leishmaniasis (VL), or in a self-limiting asymptomatic infection. In murine models of the infection employing *Leishmania major*, the course of the disease can be directed into a VL-like syndrome by interleukin-4 (IL-4)-producing Th2 cells, or cure may result by Th1 cells secreting gamma interferon (IFN-γ). The present study examined the potential of human T cells to generate Th1 or Th2 responses to *L. donovani*. The profiles of IFN-γ, IL-4, and lymphotoxin secretion after antigen stimulation were analyzed in a panel of *L. donovani*-reactive CD4$^+$ human T-cell clones generated from individuals who had recovered from VL after antimonial treatment. Two of the T-cell clones produced large amounts of IL-4 without production of IFN-γ, seven clones produced both IFN-γ and IL-4, and eight produced only IFN-γ. This is the first report of a Th1- and Th2-type response in human leishmaniasis. These results suggest that in analogy with murine models, there is a dichotomy in the human T-cell response to *L. donovani* infections. Preferential activation of IL-4-producing Th2-like cells may be involved in the exacerbation of human VL, whereas activation of IFN-γ-producing Th1 cells may protect the host from severe disease. Identification of leishmanial antigens activating one or the other type of T cells will be important in the development of vaccines against leishmaniasis.

Human infection with the intracellular parasite *Leishmania donovani* can take one of two courses. In some individuals, it remains a subclinical infection with no or few, mild symptoms, detectable only by development of specific immune responses to *Leishmania* antigens (3, 18, 26). In other cases, the infection results in a systemic fatal disease, visceral leishmaniasis (VL), or kala azar, characterized by parasite invasion in macrophages in all lymphoid tissues in the body. Host factors, especially T lymphocytes, are essential for defense against the parasite. Thus, patients with AIDS and other functional T-cell defects are highly susceptible to *L. donovani* infections (4, 7, 25).

Experimental infection in mice with *Leishmania major* can result either in a fatal visceral disease or in a spontaneous cure, in a manner that is somewhat similar to that of *L. donovani* infection in humans. The course of the infection appears to be determined by the pattern of the lymphokines produced by *Leishmania*-reactive CD4$^+$ T cells (6). On the basis of the pattern of their lymphokine production, murine CD4$^+$ T-helper cells can be divided into at least two subsets (16, 22). Susceptibility to *L. major* is associated with the activation of Th2 cells secreting interleukin-4 (IL-4), IL-5, IL-6, and IL-10. In contrast, when the T-cell response to *L. major* is dominated by the IL-2-, gamma interferon (IFN-γ)-, and lymphotoxin (LT)-producing Th1 subset, infected animals are capable of controlling the infection and subsequently eliminating the parasites. Murine *Leishmania*-reactive T-cell clones expressing distinct Th1 or Th2 lymphokine patterns have been established and have been shown to control the outcome of the infection in adoptive transfer experiments (10). Functionally, Th1 cells have mainly been associated with delayed-type-hypersensitivity reactions, whereas Th2 cells have been related to B-cell help.

The idea that lymphokines play a critical role in regulating *L. donovani* infections in humans is supported by findings of elevated levels of IL-4 in the plasma of kala azar patients (32) and by promising results of including IFN-γ in the treatment of this disease (2). Furthermore, in vitro studies have shown that IFN-γ can stimulate macrophages to kill intracellular *Leishmania* parasites (11, 23), an effect which is reported to be antagonized by IL-4 (19). To examine the potential of *L. donovani*-reactive human T cells to generate IFN-γ- or IL-4-type responses, we established T-cell clones from two individuals from Kenya who were cured of VL by antimonial treatment. The *L. donovani*-reactive T-cell clones were analyzed for expression of surface markers and secretion of IFN-γ, IL-4, and LT after activation by antigen.

MATERIALS AND METHODS

Donors and isolation of mononuclear cells. Heparinized blood was collected from two Kenyan donors (k72 and k75) who had suffered from kala azar 3 and 5 years, respectively, prior to bleeding. The donors had received antimonial treatment after the diagnosis was verified by splenic aspiration. The donors showed no signs of ongoing disease at the time of sampling. Peripheral blood mononuclear cells (PBMC) from the donors proliferated and produced IFN-γ in response to *Leishmania* antigens (18). PBMC were isolated by Lymphoprep (Nyegaard, Oslo, Norway) density centrifugation. The cells were frozen by a manually operated version of a

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recently described device for gradient freezing of cells under field conditions (12) and stored and transported in liquid nitrogen. Before use, the cells were rapidly thawed and washed. The viability of the cells was ascertained by trypan blue exclusion.

Antigens and mitogens. Insoluble and soluble antigen preparations of *L. donovani* promastigotes (Kenya strain MHOM/84/KE/NLB 274) were prepared as described previously (15). The protein concentrations of the antigen preparations were determined by a protein assay kit from Bio-Rad (Brussels, Belgium). For stimulation of T cells, the insoluble material was used at 20 μg/ml and the soluble protein preparation was used at 10 μg/ml. Phytohemagglutinin (PHA) was from Wellcome (Detroit, Mich.).

Generation of *L. donovani*-reactive human T-cell clones. Antigen-reactive T-cell clones were generated according to the method described by Sinigaglia et al. (29). PBMC were incubated at 10^6 cells per ml in RPMI 1640 with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) supplemented with penicillin (20 IU/ml), streptomycin (20 μl/ml), and 10% heat-inactivated, pooled normal human serum (complete medium) in the presence of soluble or insoluble *L. donovani* antigen preparation. After 6 days of incubation at 37°C in a humidified atmosphere containing 5% CO_2, 50 U of recombinant IL-2 (rIL-2) per ml was added to the cultures. The cell lines were further expanded for 1 week, and single cells were then isolated by the limiting dilution technique. The cells were seeded at a calculated concentration of 0.5 cells per well in Terasaki plates (Nunc, Roskilde, Denmark) in a volume of 20 μl in the presence of 50 U of rIL-2 per ml, 50 μg of PHA per ml, and 0.5×10^6 irradiated (2,400 rads) allogeneic PBMC per ml. After 1 week, growing cultures were transferred to 96-well microplates and restimulated with PHA, irradiated allogeneic PBMC, and rIL-2 as described above. The T-cell clones were further expanded in rIL-2-containing medium by weekly restimulation. The probability of clonality in growing cultures was >95% as determined by Poisson analysis. For screening of reactivity to *Leishmania* antigen, 1.25×10^4 cloned T cells were incubated in 96-well round-bottom microplates (Nunc) with 2.5×10^4 irradiated autologous PBMC in 170 μl of complete medium. Triplicate cultures were incubated with or without the relevant antigen preparation for 3 days and pulsed with 20 μl of [^{3}H]thymidine (1.85 MBq/ml) per well for the last 20 h. The cultures were harvested onto glass fiber filters, and incorporation of [^{3}H]thymidine was assessed by liquid scintillation counting. T-cell clones showing more than a fivefold increase in thymidine incorporation after incubation with antigen compared with unstimulated cultures were termed *L. donovani* reactive and were selected for further studies.

Flow cytometry. *L. donovani*-reactive T-cell clones were stained with fluorochromic antibodies by standard procedures (13). Monoclonal mouse antibodies against CD4 and CD8 were from Dako (Glostrup, Denmark). Monoclonal antibodies recognizing the alpha chain of the human T-cell receptor (TcR) were from Becton Dickinson (Mountain View, Calif.), and the anti-delta chain TcR antibody was from the T-Cell Sciences (Cambridge, Mass.). The analyses were done with a FacsStar cell sorter (Becton Dickinson).

Antigen activation of *L. donovani*-reactive human T-cell clones. For the generation of culture supernatants, T cells were incubated with antigen and irradiated autologous PBMC as described above. *L. donovani* antigen preparation, PHA (as positive controls), or medium was added to quadruplicate cultures. After incubation for 2 days, all cultures were pulsed with [^{3}H]thymidine. Twenty hours later, 150 μl of culture supernatant was recovered from each well and the [^{3}H]thymidine incorporation was measured in a scintillation counter. Supernatants from quadruplicate wells were pooled and stored at −20°C until they were analyzed. Cultures of antigen-presenting cells without additional T cells were included as negative controls.

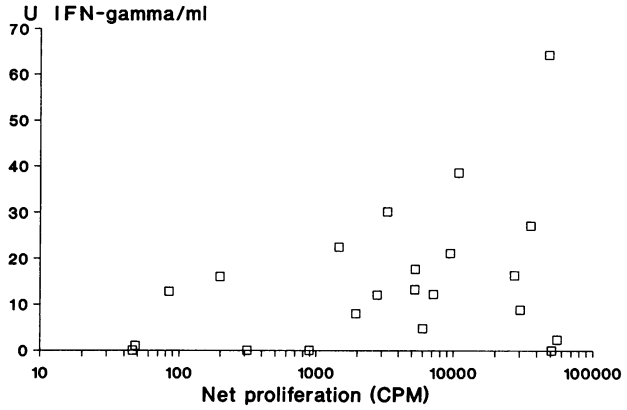

FIG. 1. Proliferation and IFN-γ production by human T-cell clones after stimulation with *Leishmania* antigen preparations, with irradiated PBMC used as antigen-presenting cells.

Activation of T-cell clones by immobilized anti-CD3 antibodies. The bottoms of 96-well round-bottom culture plates were coated with rabbit anti-mouse immunoglobulin antibodies (Dako) by incubation overnight at 4°C with a 40-μl sample of antibodies diluted 1:100 in phosphate-buffered saline (PBS) per well (14). After being washed three times with PBS, 40 μl of monoclonal mouse anti-CD3 antibody (anti-T3; Dako) diluted 1:500 in PBS was added to half of the wells in each plate and 40 μl of PBS was added to the rest of the wells; these served as controls for unstimulated cultures. After incubation for 45 min at room temperature, the plates were washed three times and cloned T cells were added in complete medium at 1.25×10^4 per well in a volume of 150 μl. The cultures were incubated overnight at 37°C, and culture supernatant was recovered and pooled from six replicate wells.

Analysis of cytokine production. The contents of IFN-γ and IL-4 in the culture supernatant were measured by previously described enzyme-linked immunosorbent assays (ELISAs) (15, 17) with recombinant human lymphokines for calibration. The detection ranges of the ELISAs were 1 to 64 U/ml for IFN-γ (specific activity of the reference protein, 2.5×10^8 U/mg) and 63 to 10,000 pg/ml for IL-4. Low background values (in unstimulated cultures) were observed for a few cultures and were subtracted from values for stimulated cultures. LT was also measured by specific ELISA. The detection range of the LT ELISA was 8 to 2,000 pg/ml.

RESULTS

Surface marker expression. All of the T-cell clones expressed CD4 and the α/β chain TcR. None of the clones expressed CD8 or the γ/δ chain TcR.

Proliferation and IFN-γ production by *Leishmania* antigen-activated T-cell clones. Twenty-two *L. donovani*-reactive T-cell clones were tested for proliferation and production of IFN-γ, IL-4, and LT after stimulation with *Leishmania* antigen with irradiated autologous mononuclear cells as

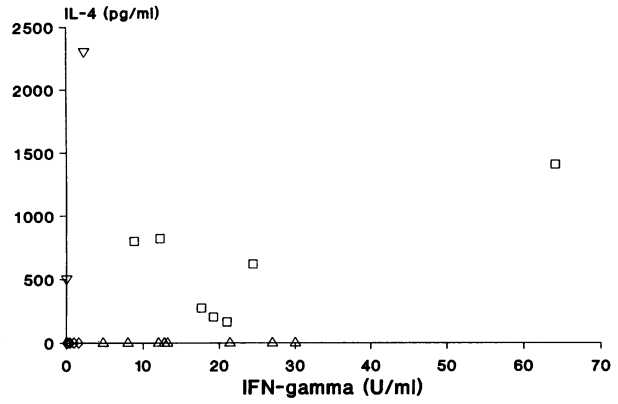

FIG. 2. IFN-γ and IL-4 production by human T-cell clones after stimulation with *Leishmania* antigens. ▽, T-cell clones secreting more than 500 pg of IL-4 per ml and less than 4 U of IFN-γ per ml; △, clones secreting more than 4 U of IFN-γ per ml and less than 78 pg of IL-4 per ml; □, clones secreting more than 4 U of IFN-γ per ml and more than 78 pg of IL-4 per ml; ◇, clones secreting less than 4 U of IFN-γ per ml and no detectable IL-4.

antigen-presenting cells. Various amounts of IFN-γ were produced by proliferating cells (Fig. 1).

Lymphokine pattern by *Leishmania* antigen-activated T-cell clones. Two of the *L. donovani*-reactive T-cell clones produced more than 500 pg of IL-4 per ml and less than 4 U of IFN-γ per ml (Fig. 2). Eight clones produced more than 4 U of IFN-γ per ml without detectable IL-4. Seven T-cell clones produced both IFN-γ and IL-4, resembling murine Th0 cells, the putative precursors of Th1 and Th2 cells (8). The remaining five T-cell clones produced neither IFN-γ nor IL-4. Six of the eight T-cell clones producing IFN-γ but not IL-4 also produced the Th1-associated lymphokine LT (Fig. 3).

Production of IFN-γ and IL-4 after stimulation by immobilized anti-CD3 antibody. To evaluate the consistency of the lymphokine pattern of the T-cell clones, eight clones were restimulated two to three times by PHA and then activated by immobilized anti-CD3 antibody. The results given in Table 1 show that even after repeated restimulation cycles and under totally different activation and cultivation conditions, at least the clones k72.p.2 and k72.p.18 had a clear

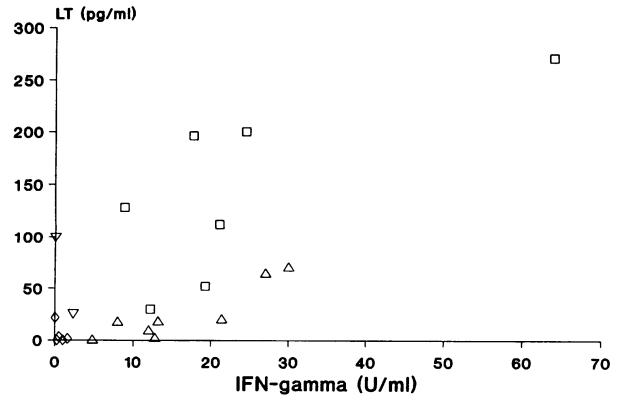

FIG. 3. IFN-γ and LT production by human T-cell clones after stimulation with *Leishmania* antigens. Symbols are as described in the legend to Fig. 2.

TABLE 1. Production of IFN-γ and IL-4 by *Leishmania*-reactive human T-cell clones after antigen activation and after activation by immobilized anti-CD3

Donor	Clone	Amt after antigen stimulation		Amt after anti-CD3 activation[a]	
		IFN-γ (U/ml)	IL-4 (pg/ml)	IFN-γ (U/ml)	IL-4 (pg/ml)
k72	p.2	22.4	0	5.2	0
k72	p.18	30.0	0	38	82
k75	p.5	<1	500	1.0	68
k75	p.14	2.3	2,300	1.2	310
k75	p.2	19.2	200	4.6	98
k75	p.9	>64	1,400	22	63
k75	p.7	12.2	820	1.6	<63
k75	p.6	27.0	0	1.5	320

[a] After antigen-induced lymphokine production by the T-cell clones had been investigated, the cells were expanded for several weeks before lymphokine production after stimulation by immobilized anti-CD3 antibody was measured.

Th1-like pattern as in the initial testing, whereas the clones k75.p.5 and k75.p.14 continued to produce relatively more IL-4 than IFN-γ. Two T-cell clones producing both IL-4 and IFN-γ after antigen activation (clones k75.p.2 and k75.p.9) still produced both lymphokines after stimulation by immobilized anti-CD3. The production of both IFN-γ and IL-4 by the T-cell clone k72.p.7 after antibody stimulation was too low to judge the lymphokine pattern in this clone. Only the T-cell clone k75.p.6 changed the pattern of lymphokine production, since it produced predominantly IL-4 after stimulation by anti-CD3 and only IFN-γ after antigen activation.

DISCUSSION

In this study, we have analyzed proliferation and production of lymphokines by $CD4^+$ T-cell clones isolated from two individuals who had previously had VL. The cells responded to *L. donovani* antigens. T-cell clones secreting predominantly IFN-γ (Th1-like cells) or IL-4 (Th2-like cells) after *Leishmania* antigen stimulation, as well as clones secreting both lymphokines, were identified.

The existence of human analog to the murine Th1 and Th2 subsets has been disputed. The reason for the reservation in accepting human Th1 and Th2 is probably that the dichotomy in the human system is not as clear as in murine cells. However, human Th1- and Th2-like cell clones recognizing epitopes on different microbial antigens and Th2-like cell clones specific for allergens have recently been described elsewhere (9, 21, 24, 27, 31). The results presented in the present paper show that *Leishmania*-reactive T-cells with Th1- and Th2-type lymphokine patterns coexist in humans after *Leishmania* infections, as they do in mice (20). To our knowledge, this is the first report of Th1- and Th2-like human T-cell clones recognizing antigens of intracellular protozoa of the genus *Leishmania*. The demonstration of such cells suggests that there is a potential in humans to generate the IFN-γ- or IL-4-type response to *Leishmania* infections. Work is in progress in our laboratory to evaluate whether VL patients develop lymphokine responses to *Leishmania* infections that are qualitatively different from the responses in individuals with subclinical *L. donovani* infections. The *Leishmania*-reactive Th1- and Th2-like clones were present in the blood years after recovery,

indicating that cure from the disease did not eliminate Th2-like cells.

In order to examine the stability of the lymphokine pattern in the T-cell clones, we expanded eight *L. donovani*-reactive human T-cell clones for several weeks before retesting the lymphokine production under activation and culture conditions completely different from those used in the initial analysis. Activation of cells by immobilized anti-CD3 antibody in the absence of accessory cells showed that six of the eight T-cell clones produced the same pattern of IL-4 and IFN-γ as they did when activated by *L. donovani* antigen, although the absolute amounts of the lymphokines differed. This suggests that at least some *Leishmania*-reactive human T cells express a lymphokine pattern which is consistent and independent of the way of activation.

In murine leishmaniasis, injection of IFN-γ or neutralizing anti-IL-4 antibodies at the time of infection directs the T-cell responses to *L. major* into Th1 type, and the animal becomes resistant to the parasite. When the balance between IL-4 and IFN-γ is changed at the time of infection by anti-IFN-γ antibodies, the response is of the Th2 type and the animal becomes susceptible (5, 6, 28). If similar mechanisms operate in humans, the outcome of *L. donovani* infections may depend on the lymphokine environment in which early activation of *Leishmania*-specific T cells takes place. This could depend on the pattern of lymphokines produced by the T cells which are first activated by *Leishmania* parasites invading a previously unexposed individual. Such cells could be cross-reactive memory T cells previously activated and expanded by the influence of other environmental antigens (1, 15, 30).

Human Th1- and Th2-type cells may have differential antigen recognition (9, 21, 24, 27, 31). *Leishmania* antigens eliciting Th1-like responses in humans can be identified by use of human T-cell clones. Such antigens might be included in a future vaccine against human leishmaniasis.

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