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Research Article

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Potential Role for Interleukin-10 in the Immunosuppression Associated with Kala Azar

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Abstract

Patients with acute kala azar are generally nonreactive in a number of immunologic assays, including T cell proliferation and generation of macrophage-activating cytokines, principally IFN-γ, in response to leishmania antigens in vitro. To test for potential immunosuppressive factors, a series of T cell lines and clones were established from patients with acute kala azar, from patients after chemotherapy for kala azar, and from skin test–positive adults from the same endemic region. Although CD4$^+$ T cell lines and clones could be readily established from the skin test–positive adults, lines and clones from acute or treated patients were heavily biased in expression of CD8$^+$. The CD8$^+$ cells from acute patients did not themselves release cytokines in response to leishmania antigens in vitro, but markedly affected the cytokine profile of peripheral blood mononuclear cells isolated 1 yr later after recovery. Addition of the CD8$^+$ cells caused inhibition of lymphoproliferation and IFN-γ release, with augmentation of IL-6 and IL-10 release. The inhibitory effects of the CD8$^+$ cells could be partially abrogated by antibodies to IL-10 but not by antibodies to IL-4. Analysis of four patients with acute kala azar demonstrated release of IL-10 that could not be demonstrated in supernatants from asymptomatic skin test–positive individuals. Generation of IL-10 may contribute to the profound suppression of IFN-γ release that occurs during kala azar due to *Leishmania chagasi*. (*J. Clin. Invest.* 1993. 92:2626–2632.) Key words: leishmania • interleukin-10 • kala azar • interferon-γ • immunosuppression

Introduction

Leishmania infection produces a spectrum of illness that depends both upon the species of the infecting organism and the host immune response. Cellular immunity capable of activating host macrophages, the only cell known to support growth of the intracellular forms of leishmania, is felt to be critical for successful eradication of the parasite. Peripheral blood lymphocytes taken from patients with visceral leishmaniasis, or kala azar, proliferate poorly and release little IFN-γ in response to parasite antigens in vitro (1–3), and do not activate infected macrophages to a leishmanicidal state (2). In contrast, lymphocytes taken from individuals with delayed-type skin reactions to leishmania antigens who remain asymptomatic despite residence in highly endemic areas proliferate and release IFN-γ in vitro (1, 2) and activate their autologous infected macrophages to a leishmanicidal state (2). Such findings have encouraged the successful use of IFN-γ in refractory kala azar (4), although the basis for these underlying immune responses remains obscure.

In murine leishmaniasis produced by *Leishmania major*, counter-regulation between protective T helper type 1 (Th1)[1] CD4$^+$ cells that release IFN-γ and nonprotective Th2 CD4$^+$ cells that release IL-4 and IL-10 has been suggested by several investigators (5, 6). Although IFN-γ release from lymphocytes taken from patients with solid immunity to leishmania has suggested the occurrence of protective Th1 CD4$^+$ cells in humans, it has been more difficult to establish a role for Th2-like cells during acute disease in humans with kala azar (1–3) or mice infected with *L. donovani* (7). In this report, we characterized T cell lines and clones taken from patients with acute kala azar, patients studied after therapy with antimonials, and normal skin test–positive adults in order to test for the presence of potential immunosuppressive factors in human visceral leishmaniasis.

Methods

Human subjects. Six patients representing the spectrum of clinical responses to *L. chagasi* infection were studied. Informed consent was obtained in accordance with institutional guidelines at each of the participating universities. All were from the state of Ceara in northeastern Brazil, where kala azar is endemic (8). Two adults males, 17 and 39 yr old (designated D4 and D5), were skin test positive to *L. chagasi* antigens using reagents and criteria previously described (8). Peripheral blood lymphocytes from both men proliferated and released IFN-γ in response to *L. chagasi* antigens in vitro, and activated autologous infected macrophages to kill intracellular amastigotes in vitro (2). Neither had any signs or symptoms attributable to *L. chagasi* infection at the time of study or had any history of kala azar. Two adult males, 17 and 33 yr old (designated R2 and R8), were treated for kala azar upon presentation with typical signs and symptoms and demonstrable amastigotes in biopsies of bone marrow. Both received 20 d of therapy with pentavalent antimony (20 mg/kg per d) and neither required retreatment. Cells were collected within 6 mo after completion of therapy. One of the men (R8) was completely free of symptoms. The other (R2)

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1. *Abbreviations used in this paper:* LT, lymphotoxin; Th, T helper.

had residual splenomegaly and intermittent fatigue without fever. PBMC from both R2 and R8 failed to secrete IFN-γ in response to *L. chagasi* antigens but their peripheral blood T cells were able to activate autologous macrophages to kill amastigotes (2). Two adult males, 17 and 33 yr old (designated P1 and P2), had cells collected for study 7 and 12 d after the initiation of pentavalent antimony therapy for acute kala azar diagnosed by signs and symptoms and the presence of amastigotes in bone marrow aspirates. PBMC from P1 and P2 failed to secrete IFN-γ in response to *L. chagasi* antigens, and their peripheral blood T cells were unable to activate autologous macrophages to kill amastigotes (2). These same patients were studied again 1 yr later at a time when both were entirely well and free of all signs and symptoms of kala azar.

Isolated PBMC were prepared as described (2) from seven patients with acute kala azar, four patients who had completed therapy with antimonials 2 wk to 6 mo before the study, and two skin test–positive asymptomatic adults from the endemic area. Washed PBMC were suspended in Iscove's modified Dulbecco's medium containing 20% human AB serum and antibiotics with or without 20 μg/ml *L. chagasi* antigens prepared as described (2). In designated experiments, 3×10^6 washed PBMC were mixed with 1 μg/ml murine anti-human CD8 mAb (Caltag Laboratories, South San Francisco, CA) for 30 min at 4°C, washed, and further incubated with 4×10^7/ml goat anti-mouse IgG-coated magnetic beads (Advanced Magnetics, Inc., Cambridge, MA). A magnetic separator (Advanced Magnetics, Inc.) was used to retain antibody-bound cells while collecting unbound cells. The procedure was repeated twice and the numbers of remaining CD8$^+$ cells in the unbound cell population were quantitated by FACS® (Becton Dickinson & Co., Mountain View, CA) analysis after incubation with anti-mouse FITC-conjugated anti-human CD8 mAb (Caltag Laboratories). The CD8-depleted cells were assayed using comparable cell numbers as with total PBMC. Supernatants were collected after 72 h and frozen before assaying for IL-10 (below).

Parasites and parasite antigens. *L. chagasi* promastigotes were cultured in HOSMEM II medium (9) containing 20% FCS and harvested from stationary phase cultures by centrifugation before use.

Establishment of T cell lines and clones. PBMC collected as described (2) were washed twice in HBSS (GIBCO BRL, Gaithersburg, MD) containing 0.25% BSA and cultured at 4×10^6 cells/ml in Iscove's modified Dulbecco's medium (GIBCO BRL) containing 20% human AB-positive serum, penicillin/streptomycin (GIBCO BRL), and 20 μg/ml *L. chagasi* antigens at 37°C in 5% CO_2, air for 2 wk. Subsequent expansion used one of two methods depending upon the availability of frozen autologous cells and the extent of antigen-specific proliferation after the initial 2-wk period.

The first method was used for both skin test–positive patients (D4 and D5) and one of the recovered patients (R2). After expansion in the initial bulk culture, cells were examined by flow cytofluorometric (FACS®) analysis (FACScan®; Becton Dickinson & Co.; or Epics-Profile II; Coulter Electronics Inc., Hialeah, FL) for surface expression of CD4, CD8, and Ig using FITC- or PE-labeled mAbs (Becton Dickinson & Co.). Cells were serially diluted to 0.5 CD4$^+$ cells/well either directly from bulk culture (patient D5) or after one (D4) or two (R2) rounds of stimulation with *L. chagasi* antigens for 2 wk using 5×10^5/ml mononuclear cells with 2×10^6/ml thawed, autologous, gamma-irradiated (3,000 rad) PBMC. Cells from patients D4 and D5 were cultured at 0.5 CD4$^+$ T cells/well and from patient R2 at 0.5 T cells/well.

The second method was used for the acutely ill patients (P1 and P2) and the recovered individual (R8). PBMC from none of these patients proliferated well to *L. chagasi* antigens, and insufficient cells were available to freeze cells for future use. Two initial bulk stimulations were accomplished using 2×10^5/ml antigen-stimulated PBMC with 5 μg/ml PHA (Sigma Chemical Co., St. Louis, MO), 10^6/ml gamma-irradiated (4,000 rad) allogeneic PBMC, 10^5/ml JY cell line (10) that had been treated with 50 μg/ml mitomycin c for 45 min before gamma irradiation (2,000 rad), and 25 U/ml rIL-2 (Cetus Corp., Emoryville, CA). Aliquots of the polyclonal T cell lines established in this manner from the two patients with acute kala azar (designated P1 10/90 and P2 10/90) were frozen for later use. A separate culture of PBMC from each donor was infected with EBV using the conditions described (11) in order to establish transformed B cell lines from each donor. EBV supernatant was generated by culturing B95-8 cells (CRL1612; American Type Culture Collection, Rockville, MD) in RPMI 1640 containing 25 mM Hepes, 10% FCS, 2 mM L-glutamine, 250 U/ml penicillin, and 250 μg/ml streptomycin. The supernatant was centrifuged at 600 *g* for 30 min and passed through a 0.22-μm filter before use. Subsequently, 1 ml of EBV supernatant was incubated with 10^6 PBMC from each donor at 37°C for 1.5 h before washing and resuspending the cells in medium with 125 ng/ml cyclosporin A (Sandoz, East Hanover, NJ). The PBMC were cultured with cyclosporin for 2–3 wk; cyclosporin was omitted from the medium thereafter. The PHA-stimulated bulk cultures were expanded twice at 2-wk intervals using 10^6/ml EBV-transformed, irradiated (5,000 rad), autologous B cells pulsed with 20 μg/ml *L. chagasi* antigens and $1–2 \times 10^5$/ml patient T cells plus rIL-2 with inactivated allogeneic PBMC and JY cells. Aliquots of these T cell lines (designated P1 1/91 and P2 1/91) were also frozen for later use. The P1, P2, and R8 T cell lines were each cloned by limiting dilution at 0.5 T cells/well.

T cell lines and clones were analyzed for CD4, CD8, and α/β or γ/δ T cell receptor expression using FACS® analysis after incubation with the respective mAbs (Becton Dickinson & Co.). CD8$^+$ cells were further analyzed for expression of CD56. P2 10/90 was also assessed for the presence of CD11a, CD11b, and CD57. The P1 and P2 10/90 cell lines were sorted after staining with anti-CD4 and anti-CD8 (Laboratory for Cell Analysis, UCSF) in order to establish cell lines from acute patients that were relatively enriched for CD4$^+$ T cells. CD4$^+$ T cell clones were derived from these lines by serial dilution at 0.5 T cells/well.

Proliferation and lymphokine assays. Freshly isolated PBMC were assayed for IL-10 secretion by culturing at 4×10^6/ml with or without 20 μg/ml *L. chagasi* antigens. The supernatants were collected on day 3. Designated T cells from long-term cultures at 2×10^5/ml were cultured with or without autologous PBMC at 4×10^6/ml in the presence or absence of 20 μg/ml *L. chagasi* antigens in a final volume of 250 μl. Monoclonal anti-IL-4 (12) and anti-IL-10 (13) were added to designated cultures at 100 μg/ml. Supernatants were collected on day 1 for IL-4 assays and on day 3 for IL-6, IL-10, and IFN-γ assays. Cultures were pulsed with 0.5 μCi/well [^{3}H]thymidine (sp act, 6.7 Ci/mmol; New England Nuclear, Boston, MA) on day 5 and collected using a multiple sample harvester on day 6 for scintillation counting. Cultures of T cells alone were incubated with 10 ng/ml PMA and 250 ng/ml ionomycin (Sigma Chemical Co.) in the presence or absence of 25 U/ml rIL-2, and the supernatants were collected on days 1 and 3 for lymphokine assays.

IFN-γ, IL-4, IL-6, and IL-10 were assayed using monoclonal-based immunoenzymatic ELISA as described (2, 13, 14). These assays are sensitive to 40–100 pg/ml. Lymphotoxin (LT) was assayed using the WEHI-164 bioassay as described (15). mAbs specific for TNF-α (6.2×10^5 neutralizing U/mg; 3.3 EU/mg) and LT (5×10^5 neutralizing U/mg; 3.0 EU/mg) were kindly provided by Genentech, Inc. (S. San Francisco, CA).

Macrophage activation and cytotoxicity. These assays were performed as described previously (2). Briefly, PBMC were allowed to adhere to microculture slides for 3 d, after which nonadherent cells were removed by vigorous washing. Washed stationary phase promastigotes were added at 3×10^6/ml to the adherent macrophages. Cloned T cells were washed twice and added to the infected macrophage cultures at 10^5/ml. The supernatants and nonadherent cells were removed after 3 d and the adherent cells were fixed and stained with Wright Giemsa. The slides were scored for the number of macrophages/field and number of intracellular amastigotes/field for each of 10 fields. Assays for T cell cytotoxicity to autologous macrophages presenting nonviable *L. chagasi* antigens were performed in the same way as the assays described above except that the macrophages were not infected and 20 μg/ml of *L. chagasi* antigens was incubated with the macrophages and cloned T cells for 3 d.

Designated T cell clones were evaluated for NK activity using the K562 cytotoxicity assay as described (16). Briefly, 5×10^3 ^{51}Cr-labeled K562 cells were incubated with 5.0, 2.5, or 1.2×10^4 T cells in a volume of 200 μl of medium for 4 h before assaying for chromium release.

Statistics. Analysis of variance was used to determine the significance of differences between means collected from the designated groups.

Results

Analysis of T cell lines and clones from skin test-positive individuals. 70 clones were isolated from subject D4, and 20 were isolated from subject D5. The latter and 20 of the fastest growing D4 clones were analyzed for CD4 and CD8 expression; all of the D5 and 19 (95%) of the D4 clones were α/β TCR$^+$,CD4$^+$,CD8$^-$ (Fig. 1 *A*). The remaining D4 clone was CD8$^+$,CD4$^-$. Polyclonal D4 and D5 T cell lines maintained by PHA stimulation became 100% CD4$^+$,α/β TCR$^+$ after two cycles of stimulation (Fig. 1 *B*).

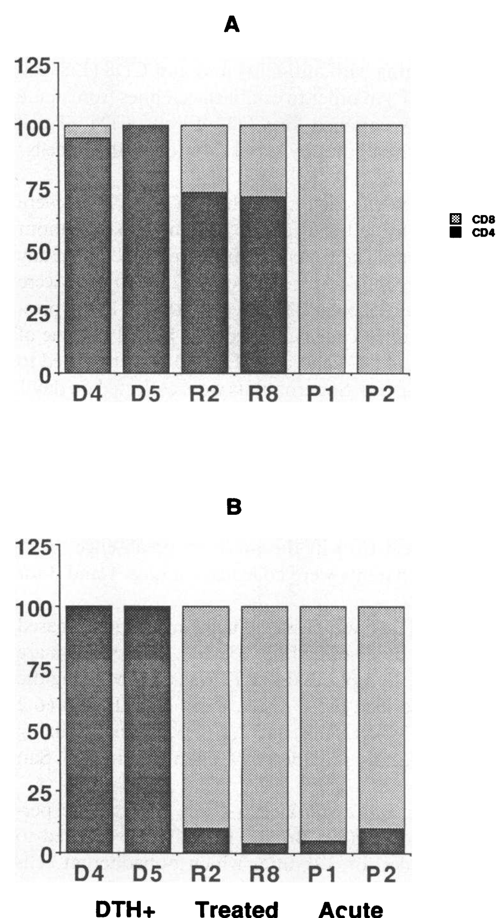

Figure 1. CD4 and CD8 expression on leishmania-specific lines and clones. (*A*) Expression of CD4 (*solid bars*) and CD8 (*stippled bars*) antigens on human T cell clones generated from patients with kala azar (*P1, P2*), recovered patients after antimony therapy (*R2, R8*), and subjects with delayed-type hypersensitivity to parasite antigens with no prior history of kala azar (*D4, D5*). Analysis used flow cytofluorometric study after incubation with the appropriate labeled mAbs and expressed as percent positive cells. (*B*) Expression of CD4 (*solid bars*) and CD8 (*stippled bars*) on polyclonal T cells lines developed from the same patients as studied in *A*.

Table I. Cytokine Release by Clones from Delayed-type Hypersensitivity–positive Subjects

Subject	Clone	IFN-γ	LT
		ng/ml	*U/ml*
D4	25	1.5	480
	46	1.5	92
	59	1.3	0
	66	1.3	0
D5	13	8.0	310
	14	4.2	110
	15	3.0	180
	16	4.0	310
	17	3.9	150
	18	5.1	120
	19	2.4	50

Supernatants from cloned T cells from subjects D4 and D5 were incubated with autologous irradiated PBMC in the presence or absence of 20 μg/ml *L. chagasi* antigens and assayed after 3 d for IFN-γ and LT by ELISA or bioassay. In the absence of antigens, clones secreted 0–0.5 ng/ml IFN-γ and no detectable LT.

The clones displayed a range of leishmania-specific IFN-γ secretion from 0 to 1.5 ng/ml for D4 clones and 0 to 8.0 ng/ml for D5 clones. Similarly, the leishmania-specific LT responses varied from 0 to 480 U/ml for D4 clones and 0 to 310 U/ml for D5 clones. None of the clones secreted IL-4 or IL-10. 11 clones secreted > 1 ng/ml of IFN-γ in response to leishmanial antigens (Table I).

Designated clones from D4 and D5 were assayed for their ability to activate autologous macrophages to kill intracellular amastigotes (Table II). All of the clones that secreted significant quantities of IFN-γ were able to activate macrophages to kill > 99% of intracellular amastigotes as assessed microscopically. Most of the clones also displayed either cytotoxicity or inhibition of adherence by macrophages, since the infected

Table II. Leishmanicidal Activity of Selected CD4$^+$ Clones

Subject	Clone	Amastigotes/field	Macrophages/ field	IFN-γ	LT
				ng/ml	*U/ml*
D4	None	8.4	13.1	0.1	0.0
	25	0	9.5	13.4	125
	59	0	6.7	18.6	150
D5	None	12.7	28.3	0.4	7
	14	0	13.0	12.0	173
	16	0	15.0	29.7	310
	18	0.2	8.6	6.2	63
	19	0.7	14.0	6.3	41

Peripheral blood monocytes were purified by adherence before incubation with stationary phase promastigotes for 3 h. After washing, media were replaced with or without the designated autologous CD4$^+$ T cell clones at a ratio of 1 T cell/10 MF. After an additional 3 d, supernatants were assayed for IFN-γ by ELISA and LT by bioassay. The slides were stained and the numbers of macrophages and intracellular amastigotes quantitated by microscopy.

monolayers became significantly disrupted in the presence of autologous T cells. The D4 clones secreted an average of 7.5 times more and D5 clones secreted 5.4 times more IFN-γ in response to infected macrophages than they did to autologous antigen-presenting cells pulsed with nonviable antigens (data not shown).

Characterization of T cell lines and clones from recently treated patients. 70 clones were isolated from patient R2, of which 11 were analyzed for expression of CD4 and CD8. Eight (73%) of the clones were CD4$^+$,CD8$^-$ and three (27%) were CD4$^-$,CD8$^+$. Seven clones were isolated from patient R8, of which five (71%) were CD4$^+$,CD8$^-$ and two (29%) were CD4$^-$,CD8$^+$. Both polyclonal R2 and R8 T cell lines maintained by PHA stimulation became 90–100% CD8$^+$ after three to four rounds of stimulation and rest (Fig. 1).

32 of the R2 clones were assayed for leishmania antigen–specific proliferation and cytokine secretion. Few CD4$^+$ or CD8$^+$ R2 clones showed significant responses to antigen. None of the clones secreted > 1 ng/ml of IFN-γ. One CD4$^+$ clone and one CD8$^+$ clone secreted large amounts of IL-10 in response to leishmania antigens.

Characterization of T cell lines and clones from patients with acute kala azar. 14 clones were isolated from patient P1, and 21 from patient P2. Polyclonal P1 and P2 T cell lines maintained by PHA stimulation became 90–100% CD8$^+$ after two rounds of stimulation and rest (lines P1 10/90 and P2 10/90) (Fig. 1). The 14 P1 and 21 P2 clones isolated from the polyclonal T cell lines were α/β TCR$^+$,CD8$^+$,CD4$^-$. All of the CD8$^+$,CD4$^-$ clones were CD56$^+$. The polyclonal P2 10/90 T cell line was also CD11a$^+$,CD11b$^-$,CD57$^-$. Both monoclonal and polyclonal CD8$^+$ T cell lines required exogenous IL-2 for growth and survival in vitro.

P2 CD4$^+$ and CD8$^+$ T cells were easier to maintain in culture than P1 T cells, and more assays were done using cells from subject P2. Similar results, however, were obtained using cells from P1. Five of the eight P2 CD4$^+$ clones established by sorting demonstrated small proliferative responses to leishmania antigens or secreted limited amounts of IFN-γ or IL-6. No IL-4 or IL-10 secretion was noted. Both P1 and P2 CD8$^+$ clones and polyclonal lines were assayed for leishmania antigen–specific activation using mitomycin c–inactivated PBMC as antigen-presenting cells. In most of these assays there was little response to antigen-presenting cells with or without antigen. PMA and ionomycin stimulation of both CD4$^+$ and CD8$^+$ T cell clones alone generally resulted in increased proliferation if exogenous IL-2 was present, but little lymphokine secretion with or without exogenous IL-2 present.

Because all P1 and P2 polyclonal cell lines were exposed to allogeneic PBMC and JY before cloning and some were also exposed to autologous EBV-transformed B cells before cloning, P1 and P2 polyclonal cell lines were tested for reactivity with alloantigens or EBV antigens. The cell lines were tested and found to be unresponsive to allogeneic PBMC mixed with JY or with EBV-transformed B cells alone under the conditions used (data not shown). They were also tested for cytotoxicity to autologous EBV-transformed B cells, autologous macrophages presenting nonviable leishmania antigens, or for the NK target cell line, K562. No cytotoxicity was demonstrable using any of these target cell populations (data not shown).

Effect of P1 and P2 CD8$^+$ T cell clones on proliferation and cytokine secretion by autologous PBMC collected after clinical recovery. The P1 and P2 CD8$^+$ T cells were tested for their ability to suppress autologous PBMC collected 1 yr after creation of the cells lines after both patients had completely recovered from clinical leishmaniasis. PBMC collected at this time from both P1 and P2 demonstrated proliferation and IFN-γ secretion in response to *L. chagasi* antigens in vitro (Table III). 14 P1 and 18 P2 clones were assayed, as well as the polyclonal lines 10/90 and 1/91; all were found to inhibit PBMC proliferative responses to parasite antigens. No IL-4 was detected in any of the supernatants. All P2 clones and the polyclonal P2 10/90 line suppressed leishmania antigen–specific IFN-γ secretion and proliferation, and increased IL-6 and IL-10 secretion. P1 clones were generally less active than P2 clones, but also suppressed leishmania antigen–specific proliferation and IFN-γ secretion, and increased IL-6 secretion (data not shown). P2 10/90 had no effect on proliferation to tetanus toxoid or candida antigens (data not shown).

Table III. Effects of CD8$^+$ Clones from Patients with Kala Azar on Antigen-specific Responses by PBMC after Recovery

Cells added	Antigen	Proliferation	IFN-γ	IL-6	IL-10
			ng/ml	*ng/ml*	*pg/ml*
None	–	1,722±194	0	1.0	0
	+	27,560±913	6.9	3.2	200
CD8$^+$ line	–	1,858±588	0.9	7.6	20
	+	8.548±729	3.2	15.9	280
Clone 3	–	3.947±998	0.1	21.0	180
	+	13,642±3,021	3.7	22.4	300
Clone 4	–	3,606±280	0.1	16.0	130
	+	14,546±3,823	3.4	23.2	480
Clone 15	–	4,220±1,027	0.1	7.2	30
	+	8,752±452	3.1	17.0	380
Clone 20	–	3,430±876	0.2	5.7	160
	+	10,547±1,051	4.2	12.5	210

PBMC were collected from patient P2 1 yr after recovery and incubated in vitro with or without *L. chagasi* antigens with or without the designated CD8$^+$ cell line or clones cultured from the patient at the time of acute kala azar. Supernatants were collected for cytokine assays on day 3. Cells were harvested for the proliferation assay on day 6, 18 h after the addition of [^{3}H]thymidine.

Table IV. Effect of Cytokine Neutralization on the Inhibition by CD8$^+$ Cell Lines

Cells	*L. chagasi* antigens	Additions (IFN-γ/IL-10)		
		None	Anti-IL10	Anti-IL4
PBMC	–	0/0	23.4/0	0.1/4.4
	+	40.8/0.2	99.0/0	6.0/6.9
PBMC + CD8	–	0/0	3.4/0	0/6.1
	+	5.0/1.1	92.4/0	4.1/5.7

PBMC were incubated with or without the autologous CD8$^+$ cell line (20:1 ratio) obtained at the time of kala azar in the presence or absence of *L. chagasi* antigens. mAbs that neutralize IL-10 or IL-4 were added to the culture media where indicated. Supernatants were collected after 3 d and assayed for IFN-γ and IL-10 using ELISA. Shown is one of two comparable experiments.

The polyclonal CD8$^+$ T cell line, P2 10/90, was chosen for further study because it was easily maintained in culture and was more active in the previous assays (Table IV). The addition of the P2 10/90 CD8$^+$ cell line from the same patient 1 yr earlier caused a marked reduction in the amount of IFN-γ recovered together with a fivefold increase in IL-10. Addition of anti-IL-10 completely restored the IFN-γ response. Addition of anti-IL-4 had little effect in this assay, although an increase in IL-10 levels with a concomitant decrease in IFN-γ was detected. IL-4 was undetectable in these experiments.

IL-10 secretion by PBMC. The suggestion that IL-10 might be involved in some of the immunomodulation by the CD8$^+$ lines in vitro prompted studies using freshly isolated cells. PBMC from patients in each of the study groups were stimulated in vitro with *L. chagasi* antigens for 72 h and the supernatants quantitated for IL-10 using ELISA (Fig. 2). All four patients with acute kala azar had readily detectable levels of IL-10; one of these patients had detectable levels in cultures maintained in the absence of antigen (1.32 ng/ml). Patients studied during the 6 mo after therapy with glucantime had detectable but lower IL-10 levels, whereas the two skin test–positive adults had no antigen-dependent release of IL-10. The differences between the levels released by patients with acute kala azar and the other groups was significant ($P < 0.05$).

The capacity of CD8$^+$ cells lines isolated during acute kala azar to augment IL-10 production from cells isolated after recovery suggested that CD8$^+$ T cells may be mediating IL-10 production during acute disease. Three patients with biopsy-proven kala azar had PBMC isolated and aliquots were depleted of CD8$^+$ T cells using antibody coated-magnetic beads. Bead-treated cells consisted of 1–6% CD8$^+$ cells, as compared with 14–23% CD8$^+$ cells in the unfractionated PBMC. After stimulation, supernatants were assayed for IL-10. In two cases, IL-10 levels increased after CD8$^+$ depletion (from 0.8 to 1.7 and 0.9 to 1.4 ng/ml for antigen-stimulated release); in the third, IL-10 levels were comparable under both conditions.

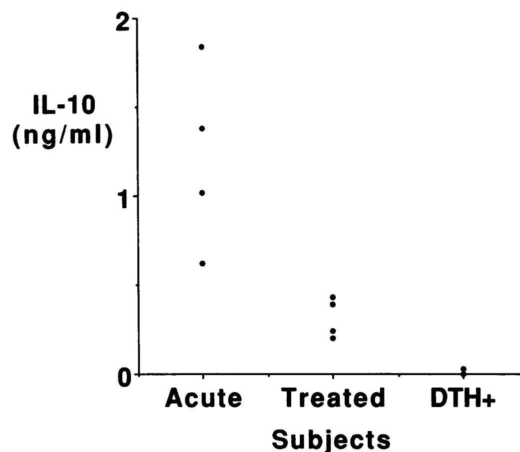

Figure 2. Recovery of IL-10 from patients infected with *L. chagasi.* PBMC from patients in the designated groups were incubated with 20 μg/ml *L. chagasi* antigens for 72 h, and the supernatants were harvested for assay for IL-10 using ELISA.

Discussion

L. chagasi infection constitutes a spectrum from acute kala azar to asymptomatic skin test conversion believed to depend upon the immune response of the host. A number of studies have documented the correlation between the capacity of PBMC to generate macrophage-activating factors, particularly IFN-γ, after stimulation with parasite antigens in vitro and immunity to infection. Conversely, PBMC from patients with acute kala azar are unable to generate IFN-γ in vitro (1–3) or to activate autologous infected macrophages to kill intracellular amastigotes (2). These studies demonstrate the ability to clone CD4$^+$ cells of the Th1-like phenotype from immune individuals. Although IFN-γ was not recovered from stimulation of PBMC taken from patients with kala azar, these supernatants, unlike those from immune skin test–positive individuals, contained IL-10. Establishment of T cell lines from acute patients consistently yielded CD8$^+$ lines and clones that had the capacity to abrogate parasite-specific proliferation and IFN-γ production when added back to autologous cells 1 yr later. The diminished IFN-γ release in the presence of CD8$^+$ cells could be reversed by the addition of neutralizing IL-10 antibody. Together, these data support a possible role for IL-10 in mediating the immunosuppression of acute kala azar and suggest complex interactions between CD8$^+$ and CD4$^+$ cells in determining the ultimate outcome of infection of humans with *L. chagasi.*

The ability to establish Th1-like lines and clones from individuals with skin test positivity to *L. chagasi* antigens was not unexpected. Others have similarly cloned antigen-specific CD4$^+$ Th1-like cells from patients with immunity to *L. chagasi* (17) and *L. donovani* (18). These studies together with a number of investigations of murine *L. major* infection (5, 6) have established the central role for CD4$^+$ Th1 cells in elaboration of macrophage-activating factors implicated in eradication of the parasite (19). As noted by others (17, 18), cloning of human cells by the methods used led to an outgrowth of CD4$^+$ cells that was readily maintained in culture. Although most of the macrophage-activating clones also released IFN-γ and LT, this was not an invariant response, suggesting other mechanisms, including cytotoxicity (20), might underlie host defense against leishmania.

Unexpected was the outgrowth of CD8$^+$ lines and clones from PBMC taken from patients with kala azar after culture in vitro. Similarly, cells taken from patients recently treated for kala azar developed a marked bias for CD8$^+$ with time in culture. Proliferation and cytokine release by the CD8$^+$ lines and clones were not consistently observed, raising concern regarding the antigen specificity of these cells. Despite being generated using allospecific and EBV-transformed cells, no cytotoxicity against alloantigens or EBV-infected targets could be demonstrated. The best evidence suggesting the leishmania reactivity of these lines was generated with lines P1 and P2; each had profound antigen-specific effects on autologous PBMC taken from the donors 1 yr later when Th1-like responses could be demonstrated. The reason underlying the preferential outgrowth of CD8$^+$ cells in the in vitro cultures from acutely infected patients, however, remains unknown. Others have similarly noted the difficulty in culturing long-term CD4$^+$ populations from patients with kala azar (3, 18). As reported here, even sorted CD4$^+$ lines did not release IL-4 or IL-10 after stimulation with PMA and ionomycin in vitro, suggesting that the circulating IL-4 detected in patients with kala azar (2, 21) may arise from non-T cell sources (22) or from T cells out of the circulating compartment.

The effects of the P1 and P2 lines and clones on the subsequent activation of autologous PBMC by leishmania antigens suggest novel counter-regulatory systems in visceral leishmaniasis that might account for the profound immunosuppression during acute disease (23). Addition of CD8$^+$ lines established at the time of disease to competent PBMC caused abrogation of lymphocyte proliferation and IFN-γ release associated with increases in IL-6 and IL-10 release. In the mouse, both IL-6 and IL-10 are released by Th2 cells and various antigen-presenting cell populations, and have been demonstrated to deactivate macrophages (24, 25), the target cell for leishmania. In human studies, IL-10 is produced by Th0, Th1, and Th2 cells (14, 26), as well as keratinocytes (27) and antigen-presenting cells, including macrophages and B cells (28). Increases in IL-6 and IL-10 and a decrease in IFN-γ would be expected to profoundly interfere with the capacity of the infected macrophages to be activated to kill amastigotes. How these CD8$^+$ cells mediate these effects is unclear, although antibodies to IL-10 restored IFN-γ production. The CD8$^+$ cells themselves did not consistently release IL-4 or IL-10, and depletion of CD8$^+$ cells before generation of IL-10 from PBMC in vitro yielded an increased amount of IL-10, rather than the decreased amount that might have been expected if these cells directly mediated IL-10 production. Together, these experiments suggest complex interactions between the regulatory T cells and antigen-presenting cells that determine the ultimate proliferative and cytokine profile of the mixed culture. Similar observations were recently reported in studies of lepromatous and tuberculoid leprosy (29), suggesting that Th2-like regulatory functions may not be unusual features of CD8$^+$ cells that appear during acute progressive infectious diseases. The CD8$^+$ cells analyzed here were consistently α/β TCR$^+$,CD56$^+$,CD57$^-$, suggesting that a particular phenotype may be associated with the functional effects noted.

The capacity for cells from acutely ill patients to suppress immunospecific responses caused us to examine the supernatants from stimulated PBMC from each of the patient groups. In prior experiments we had been unable to recover IL-4 from acute patients' cells, although one-third of these patients, as in a prior report (21), had IL-4 recovered from sera by ELISA (2). In the experiments reported here, PBMC from acute patients released readily detectable IL-10 into the media, whereas cells from treated patients released less IL-10, and cells from skin test–positive individuals released no antigen-specific IL-10. A recent study further documents the capacity to recover IL-10 cytokine transcripts from bone marrow aspirates of patients with kala azar (30). These data suggest that the triggering of IL-4 and IL-10 release during acute infection may contribute significantly to the measured suppression of IFN-γ release, and contribute to the progressive nature of disease. The possibility remains that IL-10 may be released by infected macrophages and may contribute to the abrogation of IL-1 and TNF generation by these cells (31).

The spectrum of response to leishmania has similarities to a number of other infectious diseases. Indeed, CD8$^+$ suppression in the lepromatous type of leprosy has been associated with production of Th2-like cytokines by these cells (29). As additional investigations establish these biased cytokine-mediated responses in a number of infectious diseases, the challenge will remain to determine the mechanisms that lead to the initial maturation of effective or ineffective T cell responses. The data reported here suggest that counter-regulatory mechanisms between both CD4 and CD8 cells may contribute to the marked bias in the immune response associated with leishmaniasis.

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