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Atypical cutaneous leishmaniasis cases display elevated antigen-induced interleukin-10

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SUMMARY

In humans, Leishmania chagasi *parasites can produce subclinical infections, atypical cutaneous leishmaniasis (ACL) and visceral leshmaniasis that is potentially fatal if not treated in a timely fashion.* L. chagasi *parasites that cause both ACL and visceral disease appear to be genetically similar, which suggests that host factors such as the immune response play an important role in controlling infection. We evaluated the immunologic response in ACL using peripheral blood mononuclear cells (PBMCs) of 37 subjects divided into three groups: (i) active ACL cases, (ii) asymptomatic cases and (iii) persons with no history of* Leishmania *infection. The supernatants of stimulated PBMCs were analysed for production of IL-10, IFN-γ and IL-2. Robust production of IL-10 in response to* Leishmania *stimulation was observed in active ACL cases, compared to low levels in asymptomatic cases and negative controls. Serum IgE levels, measured by ELISA, were not significantly different among the three groups. In addition, ACL cases displayed depressed levels of all cytokines in response to mitogen. Thus, this first characterization of the immune response in ACL suggests a role for IL-10 as well as partial immunosuppression.*

Keywords *immune response,* L. chagasi*, Leishmaniasis, Nicaragua*

INTRODUCTION

Leishmania chagasi can cause a range of diseases, from subclinical infections to fatal visceral leishmaniasis (VL). A nonulcerating cutaneous infection with *L. chagasi* termed atypical cutaneous leishmaniasis (ACL) has been described in several countries in Central America (1,2) but may be present anywhere *L. chagasi* is endemic. The parasites that cause both VL and ACL appear to be genetically similar (2,3) implying that host factors such as the immune response play an important role in controlling infection. However, the immune response in ACL had never been characterized, prior to this study.

T helper cells can be divided into two functionally distinct subsets, Th1 cells and Th2 cells, based on their cytokine production (4). Mouse models of *Leishmania* infection have demonstrated that the ability to control infection is a result of activating Th1 cytokines, especially IFN-γ, and inhibiting the production of Th2 cytokines, such as IL-4 (5). Thus, a major host immune defence mechanism against *Leishmania* is the activation of macrophages by IFN-γ produced by T cells (6). The IFN-γ-activated *Leishmania*-infected macrophages produce nitric oxide in order to kill the intracellular replicating amastigotes. With respect to the range of clinical manifestations in human leishmaniasis, healing or asymptomatic disease is correlated with IFN-γ/Th1 cytokine production, as in the mouse studies, whereas VL and diffuse cutaneous leishmaniasis appear to result from an inability to produce sufficient Th1 cytokines to control the infection (7,8). However, recent evidence suggests that the immune response to *Leishmania* is multifactorial and not solely the result of a Th1/Th2 cytokine bias (9–11). IL-10 was originally considered a Th2 cytokine that could play a role in the pathology of leishmaniasis (12), but more recently has also been implicated as a regulatory cytokine (13). Overall, the role of immune responses to *Leishmania* antigens in determining clinical outcomes of *L. chagasi* infection, particularly in ACL, remains poorly understood.

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We sought to characterize immune responses in persons with active ACL disease compared to persons with asymptomatic or healed *Leishmania* infections and persons with no history of *Leishmania* infection. Peripheral blood mononuclear cells (PBMCs) were obtained from all subjects and stimulated *in vitro* with *Leishmania* antigens and mitogens. The levels of IL-2, IL-10 and IFN-γ in the cellular supernatants were analysed by ELISA. The plasma from these subjects was tested for total IgE antibody levels and for *Leishmania*-specific IgE. Overall, the ACL subjects expressed reduced cytokine production in response to mitogen. In response to *Leishmania* antigen, IL-10 was increased only in the ACL subjects, and much higher levels were expressed than by the other groups of subjects. In addition, subsets of both asymptomatic individuals and ACL cases expressed IFN-γ in response to soluble *Leishmania* antigen (SLA). In this first report of the immune response in ACL cases, our results demonstrate high IL-10 expression during active disease.

MATERIALS AND METHODS

Study population

All study participants were recruited from March 2000 to March 2002 in the region of León, Nicaragua, where there is a high incidence of ACL and few cases of VL caused by *L. chagasi* (1,2). Active cases of ACL were defined by the identification of nodular skin lesions in which *L. chagasi* infection was confirmed by PCR analysis of dermal lesion scrapings. All subjects were tested by Montenegro Skin Test (MST). Asymptomatic cases were MST-positive in the absence of lesions, whereas control subjects had no lesions and were negative by MST (Table 1). The study was approved by the Committee for the Protection of Human Subjects at UC Berkeley (#99-6-39) and the Ethical Review Committee of the Centro Nacional de Diagnóstico y Referencia of the Nicaraguan Ministry of Health.

Diagnostic methods

Montenegro skin test

Leishmania chagasi promastigotes (1×10^6) were lysed in 1 mL of sterile saline solution with 0·01% thimerosal. A 100 µL aliquot of this *L. chagasi* antigen was injected intradermally, and the induration at the site of injection was measured 48 h later. If the diameter of the induration was equal to or greater than 5 mm, the skin test was considered positive.

Dermal scraping

Dermal scrapings of lesions were obtained with a scalpel. Dermal cells were placed on slides, fixed with methanol and stained with Giemsa for analysis by microscopy for *Leishmania* amastigotes.

Leishmania-specific PCR

After the removal of the upper layers of lesions using a scalpel, dermal cells were collected with a sterile wooden toothpick, placed into a microcentrifuge tube containing 100 µL of 5% Chelex-100 (Bio-Rad Laboratories, Hercules, CA) in water, boiled for 10 min and stored at –20°C until testing. Amplification of *Leishmania* minicircle DNA by PCR (14) was followed by confirmation of *L. chagasi* infection via amplification of the mini-exon region (15).

Leishmania antigens

Soluble *Leishmania* antigen (SLA) was prepared using promastigotes of *L. chagasi* (strain HN029). Parasites were grown in RPMI-1640 medium with 2 mM L-glutamine for

Table 1 Characteristics of study population

	ACL cases	Asymptomatic individuals	Negative controls
n	18	7	10
Age in years mean (range)	25·9 (15–40)[a]	26·7 (14–45)[a]	24·5 (15–42)[a]
Sex	7M/11F[b]	2M/5F[b]	4M/6F[b]
Montenegro skin test # (%) positive	17 (94%)	7 (100%)	0 (0%)
Dermal scraping # (%) positive	6 (33%)	ND[c]	ND[c]
PCR # (%) positive	18 (100%)	ND[c]	ND[c]

[a]χ^2-square for all three groups *P*-value = 0·87.
[b]ANOVA for all three groups *P*-value = 0·88.
[c]Not determined.

14 days. After harvest, parasites were washed three times with phosphate-buffered saline (PBS) followed by sonication. After centrifugation at 16 000 ***g*** for 20 min, the supernatant was collected and protein concentration was determined by the Lowry assay.

Peripheral blood mononuclear cell stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated using a ficoll–histopaque gradient from 10 mL of heparinized blood. PBMCs were diluted to a concentration of $2{\cdot}3 \times 10^6$ cells/mL and were cultured in RPMI supplemented with 20 U/mL penicillin, 20 μg/mL streptomycin and 2·5% normal human AB serum. PBMCs were incubated with 20 μg/mL of soluble *Leishmania* antigen (SLA) or 10 μg/mL of Phytohemagglutinin (PHA) (Sigma Chemical Co., St. Louis, MO) or no antigen as a negative control. Supernatants were collected 3 days after stimulation for PHA-treated wells or after 7 days for SLA-stimulated and negative control wells.

Cytokine ELISAs

IFN-γ, IL-2 and IL-10 were measured by antigen capture ELISAs using antibodies purchased from Endogen (Pierce Biotechnology, Rockford, IL). In brief, polystyrene plates (Maxisorp™ Nalgene Nunc International, Rochester, NY) were coated with polyclonal antibodies for each cytokine (IL-2 (6 μg/mL), IL-10 (2 μg/mL) and IFN-γ (1 μg/mL)) overnight in carbonate buffer, pH 9·6. A 50 μL aliquot of supernatant from stimulated PBMCs was added to each well and incubated with biotinylated secondary antibodies to IL-2 (0·2 μg/mL), IL-10 (0·25 μg/mL) or IFN-γ (0·25 μg/mL). Purified recombinant cytokine standards (Pierce Biotechnology) were prepared (16–2000 pg/mL) and added to each plate. Streptavidin-conjugated alkaline phosphatase was added for 1 h, followed by *p*-nitrophenyl phosphate (Sigma). Plates were read at 405 nm on an El×808 Microplate Reader (Bio-Tec Instruments Inc., Winooski, VT).

IgE ELISAs

Total human IgE was measured using a kit from Bethyl Laboratories (Montgomery, TX). In brief, polystyrene plates were coated overnight with rabbit antihuman IgE polyclonal antibodies (1 μg in sodium carbonate buffer, pH 9·6) and blocked with 3% normal goat serum (NGS). A 50 μL aliquot of subjects' serum or positive control was added to wells and incubated overnight. Plates were washed with PBS-T, followed by the addition of horseradish peroxidase (HRP)-conjugated monoclonal mouse antihuman IgE. After washing, *o*-phenylenediamine (OPD) substrate was added, and results were analysed at 492 nm using a microplate reader. To determine the levels of *Leishmania*-specific IgE, plates were coated with SLA antigen (10 μg/mL), blocked with 3% NGS, incubated with 50 μL of human serum sample overnight, and detected with 100 μL of HRP-conjugated antihuman IgE and OPD substrate as above.

RESULTS

Cytokine measurements

Thirty-seven subjects were recruited from the region surrounding León, Nicaragua, and classified into three groups. As shown in Table 1, 18 subjects had active ACL disease, seven subjects were asymptomatic and 10 subjects presented no immune response to *Leishmania* antigens (negative controls). PBMCs were collected from all subjects and cultured in medium alone or in the presence of SLA or PHA. Cytokine levels were determined in the supernatants by ELISA, and the average amounts of IL-2, IL-10 and IFN-γ are shown in Table 2. Interestingly, 10 of the 18 ACL cases produced IL-10 in response to SLA stimulation; in contrast, IL-10 was not detected in the supernatants of the asymptomatic or negative control subjects (Figure 1). The average amount of IL-10 generated by SLA-stimulated PBMCs from all ACL subjects was 127 pg/mL (Figure 1 and Table 2).

Table 2 Average cytokine levels in PBMC supernatants after SLA or PHA stimulation

	IFN-γ SLA	IL-10 SLA	IL-2 SLA	IFN-γ PHA	IL-10 PHA	IL-2 PHA
Active ACL (18)	375[a]	127	59	875	695	351
Asymptomatic (7)	486	0	0	1577	1467	628
Negative (10)	190	0	0	1639	1370	786
P-value[b]	0·74	0·02[c]	–[d]	0·03[c]	0·01[c]	0·44

[a]Amounts shown are in pg/mL.
[b]*P*-values were calculated using the Kruskal–Wallis test.
[c]$P < 0{\cdot}05$.
[d]No *P*-value could be calculated since only 2 of 18 samples from ACL had values above 0.

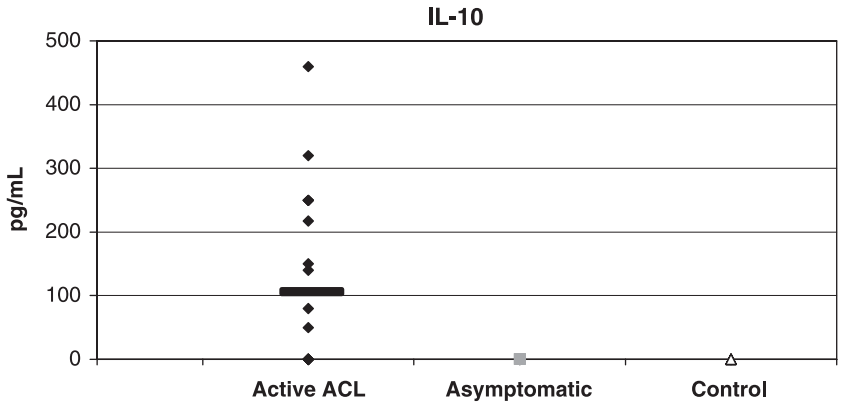

Figure 1 Average levels of IL-10 produced by peripheral blood mononuclear cells (PBMCs) incubated with soluble *Leishmania* antigen (SLA). PBMCs were incubated with 20 μg/mL of SLA for 3 days before supernatant collection. The amount of IL-10 in the supernatants was measured by ELISA using an antigen capture ELISA with a standard curve consisting of recombinant cytokine. Results for all three subject groups are reported in pg/mL. In all three groups, there were PBMC supernatants after SLA stimulation with undetectable levels of IL-10, which were graphed as 0 pg/mL. The numbers of PBMC supernatants with no detectable IL-10 were 8 of 18 ACL cases, 7 of 7 asymptomatic subjects and 10 of 10 control subjects. The average amount of IL-10 was significantly higher in ACL cases than in controls and asymptomatic subjects ($P < 0{\cdot}02$, Kruskal–Wallis).

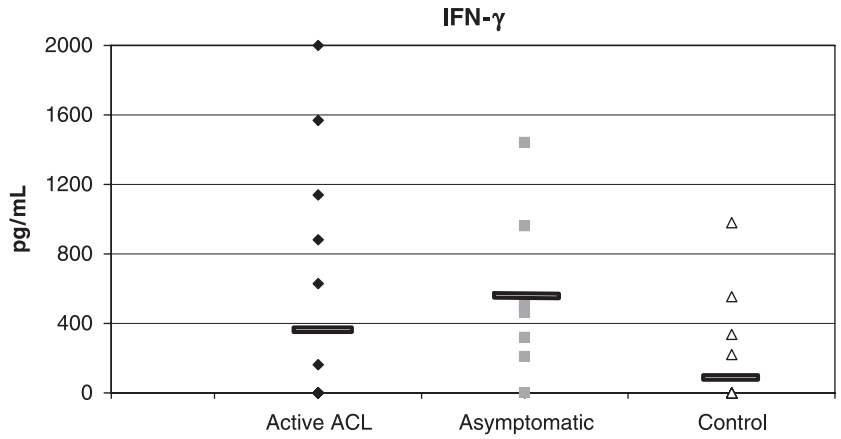

Figure 2 Average levels of IFN-γ produced by peripheral blood mononuclear cells (PBMCs) incubated with soluble *Leishmania* antigen (SLA). PBMCs were incubated with 20 μg/mL of SLA for 3 days before supernatant collection. The amount of IFN-γ in the supernatants was measured using an antigen capture ELISA with a standard curve consisting of recombinant cytokine. Results for all three subject groups are reported in pg/mL. In all three groups, there were PBMC supernatants after SLA stimulation with undetectable levels of IFN-γ, which were graphed as 0 pg/mL. The numbers of PBMC supernatants with undetectable IFN-γ levels were 11 of 18 ACL cases, 2 of 7 asymptomatic subjects and 5 of 10 control subjects. The average amount of IFN-γ was lower in controls than in ACL cases and asymptomatic subjects, but the difference was not significant by Kruskal–Wallis analysis.

IFN-γ was detected in five of seven (71%) asymptomatic subjects, and the average level of IFN-γ was higher than in ACL subjects (Figure 2). Seven of the 18 (39%) ACL cases produced detectable levels of IFN-γ, and three of these had greater than 1000 pg/mL of IFN-γ in response to SLA. Overall, the PBMCs from those ACL cases that produced IFN-γ generated low or undetectable levels of IL-10. Unexpectedly, 5 of 10 control subjects did produce IFN-γ in response to *Leishmania* antigen stimulation, but the average production of IFN-γ in ACL and asymptomatic subjects was greater.

IL-2 was detected in response to SLA in only two ACL subjects, and none of the asymptomatic or negative control subjects produced IL-2 in response to SLA antigen stimulation. The low level of IL-2 detected in cultures after 7 days may be a result of consumption rather than lack of production. In response to PHA stimulation, the average IL-2 levels produced were reduced in the ACL subjects compared to the

other two groups (Table 2), and only 11 of 18 ACL subjects produced any IL-2 under these conditions. With respect to nonspecific mitogen stimulation, the average production of IFN-γ, IL-2 and IL-10 by PBMCs from ACL individuals when stimulated with PHA were all less than 50% of the levels obtained from asymptomatic subjects or control subjects (Table 2).

IgE levels in plasma

Total IgE in plasma was measured as an indication of potential cytokine bias towards a Th2 phenotype. In all three groups, total IgE ranged from < 15 up to 615 ng/mL, and all but 1 of 37 subjects had detectable levels of IgE (> 15 ng/mL). The average IgE in 18 ACL subjects was 233 ng/mL, 212 ng/mL in 7 asymptomatic subjects and 280 ng/mL in the control subjects; thus, there were no significant differences between the average amount of IgE produced in the three groups of subjects. We further tested the plasma for SLA-specific IgE. Low levels of SLA-specific IgE (< 50 ng/mL) were detected in only two of the ACL subjects and none of the asymptomatic subjects or negative control subjects (data not shown).

DISCUSSION

We hypothesized that immune factors may play a role in limiting disease in ACL because it presents a less severe clinical manifestation of *L. chagasi* infection than VL. We found that the production of IL-10 by PBMCs was increased in response to *Leishmania* antigen stimulation by the majority of ACL subjects, while high levels of IFN-γ were observed in some patients. However, IL-2 production stimulated by *Leishmania* antigen was minimal, with low levels found in only 2 of 18 subjects. In addition, ACL subjects displayed reduced production of IFN-γ, IL-2 and IL-10 in response to PHA mitogen stimulation. The primary immunological difference between ACL cases and asymptomatic subjects appears to be increased IL-10 production in response to SLA stimulation.

The role of IL-10 as part of the Th1/Th2 paradigm in *Leishmania* infections is controversial (11,16); IL-10 may help generate immune activation that results in resolution of infection or may inhibit immune responses to *Leishmania* antigens. We detected IL-10 in response to SLA stimulation in the majority of subjects with ACL, whereas asymptomatic subjects, presumably having resolved past *Leishmania* infection, were all negative for IL-10 production in response to SLA stimulation. This suggests that IL-10 production may be associated with chronic ACL disease, preventing parasite elimination or resolution of infection. This hypothesis is consistent with previous studies in which increased IL-10 mRNA was detected after *Leishmania* antigen stimulation of PBMCs obtained from patients with acute VL (12). Interestingly, elevated levels of both IFN-γ and IL-10 mRNA have been reported at the site of infection in VL cases (17,18), and human T cells from patients cured of VL have been found to produce both IFN-γ and IL-10 in response to *Leishmania* antigens (17–19). However, we found that PBMCs from only very few ACL cases (4/17) produced both cytokines.

In *Leishmania* infections, the ability of host macrophages to effectively kill the intracellular parasite limits the disease. This usually requires the production of adequate IFN-γ by Th1 cells to activate infected macrophages (20). We detected IFN-γ production in response to *L. chagasi* SLA stimulation by our PBMCs in only 7 of 18 ACL cases; this is reminiscent of the immunologic response in VL, where the absence of IFN-γ production was also noted (8). For the most part, PBMCs from ACL cases that produced high levels of IFN-γ generated little or no IL-10, suggesting that antigen-induced production of IFN-γ may be indicative of an immune response towards disease resolution among the ACL subjects. The surprising finding that 5 of the 10 control subjects produced antigen-induced IFN-γ, albeit at a lower level than asymptomatic subjects, may suggest false-negative skin tests due to an inherent low ability to respond to *Leishmania* antigens. Therefore, we cannot be completely certain that these control subjects were never exposed to *Leishmania*.

The average levels of IFN-γ, IL-10 and IL-2 made in response to PHA stimulation were lower in ACL subjects than in either the asymptomatic group or the negative controls. This suggests that the ACL subjects may be experiencing partial immune suppression rather than Th1- or Th2-specific immune suppression. This is consistent with immunosuppression in response to mitogen stimulation found in American VL (21). In addition, our finding that total serum IgE levels were comparable in all three subject groups provides no evidence of a specific Th2 bias. Anti-*Leishmania*-specific IgE has been associated with cutaneous and visceral leishmaniasis (22,23) status; however, we were only able to detect anti-*Leishmania*-specific IgE in 2 of 18 ACL subjects and none in asymptomatic cases.

This study is the first characterization of the immune responses of individuals with ACL. We determined that ACL cases produced more IL-10 in response to *Leishmania* antigen stimulation in comparison to asymptomatic subjects and healthy controls. Though there is no clear Th2 cytokine bias, the increased IL-10 may be part of the general immune regulation limiting the elimination of parasites in these subjects. In addition, ACL cases displayed suppressed cytokine responses to mitogen. Together, these data are consistent with findings reported for VL patients; thus, similar immune mechanisms appear to be at work in mild and severe manifestations of *L. chagasi* infection.

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