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The T-cell anergy induced by *Leishmania amazonensis* antigens is related with defective antigen presentation and apoptosis

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ABSTRACT

Leishmania amazonensis is the main agent of diffuse cutaneous leishmaniasis, a disease associated with anergic immune responses. In this study we show that the crude antigen of *Leishmania amazonensis* (LaAg) but not *L. braziliensis* promastigotes (LbAg) contains substances that suppress mitogenic and spontaneous proliferative responses of T cells. The suppressive substances in LaAg are thermoresistant (100°C/1h) and partially dependent on protease activity. T cell anergy was not due to a decreased production of growth factors as it was not reverted by addition of exogenous IL-2, IL-4, IFN-γ or IL-12. LaAg did not inhibit anti-CD3-induced T cell activation, suggesting that anergy was due to a defect in antigen presentation. It was also not due to cell necrosis, but was accompanied by expressive DNA fragmentation in lymph node cells, indicative of apoptosis. Although pre-incubation of macrophages with LaAg prevented their capacity to present antigens, this effect was not due to apoptosis of the former. These results suggest that the T cell anergy found in diffuse leishmaniasis may be the result of parasite antigen-driven apoptosis of those cells following defective antigen presentation.

Key words: *Leishmania*, anergy, apoptosis.

INTRODUCTION

Leishmaniasis is a complex of diseases that affects over 12 million people in 88 countries, and may be caused by over 17 species of the protozoan parasite *Leishmania* (WHO 2002). The disease is transmitted by sandflies (Soares and Turco 2003), which inoculate the promastigote forms of the parasite into the skin during bloodmeal and these will in turn infect local and incoming macrophages. After transformation into amastigote forms inside the macrophage parasitophorus vacuoles, dermotropic parasite species will keep on infecting skin macrophages at the site of the bite whereas viscerotropic species will migrate and infect liver, spleen and bone marrow macrophages. Cutaneous leishmaniasis is generally a benign and common form of the disease, but in some cases of *L. braziliensis* and *L. amazonensis* infections, the skin lesion may evolve into the dreadful mucosal or diffuse forms, respectively. Diffuse cutaneous leishmaniasis which is predominantly caused by *L. amazonensis* resembles lepromatous leprae, is characterized by absence of T cells and delayed-type hypersensitivity responses and may persist for a lifetime (Convit et al. 1972, Castes and Tapia 1998).

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A great effort has been devoted to understanding the pathogenic mechanisms related to dysfunction of the immune system in leishmaniasis. In mice, it is widely accepted that the greater susceptibility of BALB/c mice to *L. major* infection is associated with TH2-type T cells which produce IL-4 and IL-10 whereas resistance by other mouse strains is associated with TH1-type cells which produce IFN-gamma (reviewed by Sacks and Noben-Trauth 2002). However, in *L. amazonensis* infection such dichotomy may not be so clear-cut. While C57Bl/10 mice are able to heal following infection with *L. major*, they fail to heal following infection with *L. amazonensis* due to the absence of a TH1 response (Afonso and Scott 1993). Little is known about the anergic mechanisms which determines the development of the diffuse form of cutaneous leishmaniasis in *L. amazonensis* infection, but TGF-β may be a key cytokine since it is greatly increased in mice immunized with *L. amazonensis* but not with *L. braziliensis* antigens (Pinheiro et al. submitted). The anergic nature of *L. amazonensis* may be of relevance not only in the genesis of the diffuse form of the disease but also in the formulation of one of the most studied human vaccines (Abath 2000). Leishvacin® was previously composed of killed promastigotes of various parasite species (*L. mexicana, L. amazonensis, L. guyanensis*, and two *L. major*-like) but for standardization reasons *L. amazonensis* is now used as a sole component (Marzochi et al. 1998). Although the *L. amazonensis* vaccine may be immunogenic (De Luca et al. 2001), some individuals, possibly those who would develop the diffuse form of infection, are totally unresponsive.

Apoptosis of host T cells appears to be an important immunopathological feature of *Trypanosoma cruzi* (Lopes et al. 1995), *Toxoplasma gondii* (Liesenfeld et al. 1997), and *Leishmania* sp (Das et al. 1999, Bertho et al. 2000) infections. In this work we evaluate whether irrespective of an active infection *L. amazonensis* possess apoptosis-related anergenic properties that would have implications in Leishvacin® inefficiency and in the diffuse form of the disease.

MATERIALS AND METHODS

Animals: BALB/c mice originally obtained from Jackson Laboratory (Bar Harbor, Maine) were bred and maintained at our own facilities using sterilized bedding, filtered water and pelleted food. Animals were used at 6 to 8 weeks of age in all experiments.

Parasites and antigens: For the preparation of antigens, *L. amazonensis* (IFLA/BR/67/PH8), and *L. braziliensis* (MHOM/BR/96/H3456) promastigotes were grown at 28°C in M199 medium (Cultilab, Brazil) containing 10% heat-inactivated fetal bovine serum (HIFCS, GIBCO Laboratories, Grand Island, N.Y). Stationary growth phase promastigotes were washed three times in phosphate buffered saline (PBS) in sterile conditions and concentration adjusted to 2×10^8 promastigotes/ml in PBS. Parasite suspensions were submitted to five cycles of freezing and thawing and microscopically inspected for the absence of intact parasites. The antigens prepared as above (herein named LaAg and LbAg, respectively) were lyophilized, weighted, stored at −20°C and reconstituted with PBS immediately before use. When appropriate, protein-enriched LaAg was prepared using protease inhibitors. Washed promastigotes were suspended in lysis buffer (20 mM Tris, pH8,0; 50 mM NaCl, 5 mM DTT, 5 μm ZnCl$_2$, 1 mM EDTA) containing 1 mM PMSF, 30 μM TLCK, 30 μM TPCK, 10 μg/ml aprotinin, 10 μg/ml pepstatin A and 10 μg/ml leupeptin and ultrasonicated. The lysate was centrifuged first at 15.000 × g/10 min, the supernatant was recentrifuged at 120.000 × g/80 min/4°C and the soluble part precipitated under agitation at 4°C with ammonium sulfate at 60% of saturation (361g/liter) in pH 7.0. After centrifugation, the supernatant was extensively dialyzed against distilled water and stored at −20°C. Lysis buffer with protease inhibitors, but without parasites, was treated in the same way as controls.

Infection: BALB/c mice were infected in the right hind footpad with 2×10^6 stationary phase *L. amazonensis* promastigotes.

Proliferation assay: Cells from inguinal nodes from normal or 7 day-infected BALB/c mice were stimulated *in vitro* with 5 μg/ml Concanavalin A (Sigma) or 0.5 μg/ml anti-CD3 (R&D Systems) in the presence of different concentrations of LaAg for 48h. Alternatively, cells from infected mice were incubated for 2 hours in the presence of 50 U/ml IL-2, 100 U/ml IL-4, 5 ng/ml IL-12 or 50 U/ml IFN-γ (Pharmingen) prior to addition of 40 μg/ml LaAg. The proliferative response was estimated by ^{3}H-thymidine (Sigma, 0,5 μCi/well) incorporation in the last 6 hours of culture. Cells were collected using a Dot-Blot apparatus (Rossi-Bergmann and Noleto 1994), and the radioactivity expressed as cpm.

Antigen presentation assay: Peritoneal cells from normal BALB/c mice were cultured at 37°C and 4% CO_2 in Dulbecco-modified Minimum Essential Medium (D-MEM) at 4×10^5 cells/well a 96-well culture plate for adherence. The non-adherent cells were removed and LaAg was added at different concentrations in D-MEM with 10% HIFCS. After incubation for 17 hours, the cells were washed two times with PBS to remove free LaAg. Nylon wool-purified T cells from 7 day-infected BALB/c were added at 4×10^5 cells/well (200 μl) to the adherent cells and the cultures incubated for a further 72 hours at 37°C. The cell proliferation was assessed as described above.

Apoptosis: Lymph node cells (2×10^6) from infected BALB/c mice were incubated for 17h in the presence of LaAg (40 and 400 μg/ml) or 1 μM dexamethasone. Cells were pelleted and lysed with 0.5% Triton X-100, 5 mM Tris and 5 mM EDTA pH 8,0. After centrifugation (30000 rpm/17 min/ 10°C), the DNA in the supernatants were extracted with phenol/chloroform and precipitated with iso-propanol at -20°C/18h. Samples were treated with RNase and the DNA was air dried and reconstituted with TE buffer. The samples were submitted to electrophoresis in 1.8% agarose gels and the DNA was visualized under ultraviolet light after staining with ethidium bromide. Alternatively, adherent mouse peritoneal cells were incubated in triplicate in 24-well culture plates for 6h with 200 μg/ml LaAg or 1 μM dexamethasone. Cells were then washed and incubated with AnnexinV – FITC (R&D Systems) for 15 min at 37°C and the percentage of fluorescent cells counted under UV inverted microscopy at a minimum of 300 cells/well.

Statistical analysis: All experiments were reproduced at least twice and a representative experiment is shown. The statistical differences between two groups were determined by the Student's *t* test, and were considered significant when p $\leq$ 0.05.

RESULTS

LaAg Inhibits the Spontaneous and Mitogenic Proliferation of Immune Cells

The course of cutaneous leishmaniasis is determined by the immune response in the earlier days of infection (Scott 1996). To investigate the T cell response to LaAg during the course of infection, cells from inguinal nodes were taken at days 0, 7, 14, 21 and 28 of infection with *L. amazonensis* and incubated for 48h with varying concentrations of LaAg in the absence or the presence of the T cell mitogen Concanavalin A. The Fig. 1A shows that as disease progresses, the lymph node cells are increasingly activated and proliferate spontaneously *in vitro*. However, in the presence of LaAg (40 μg/ml) the spontaneous proliferation is significantly abrogated in all time points. The observation that cells from uninfected mice (day 0 of infection) were also suppressed indicates the presence of unspecific suppressor substances in LaAg. Suppression was also observed in Concanavalin A-stimulated cells (Fig. 1B), where LaAg managed to decrease the mitogenic responses in a dose-dependent manner. T cell unresponsiveness was not a post-activation event, since kinetics studies starting at 9 hours and ending at 84 hours demonstrated lack of proliferation in response to LaAg at any time point (data not shown). The incapacity of LaAg to totally abrogate proliferation on day 21 of infection (Fig. 1B) was probably due to the higher state of intrinsic cell activation. Since antigen-specific unresponsiveness was seen throughout infection (Fig. 1A), and the earlier stages are normally critical to the outcome
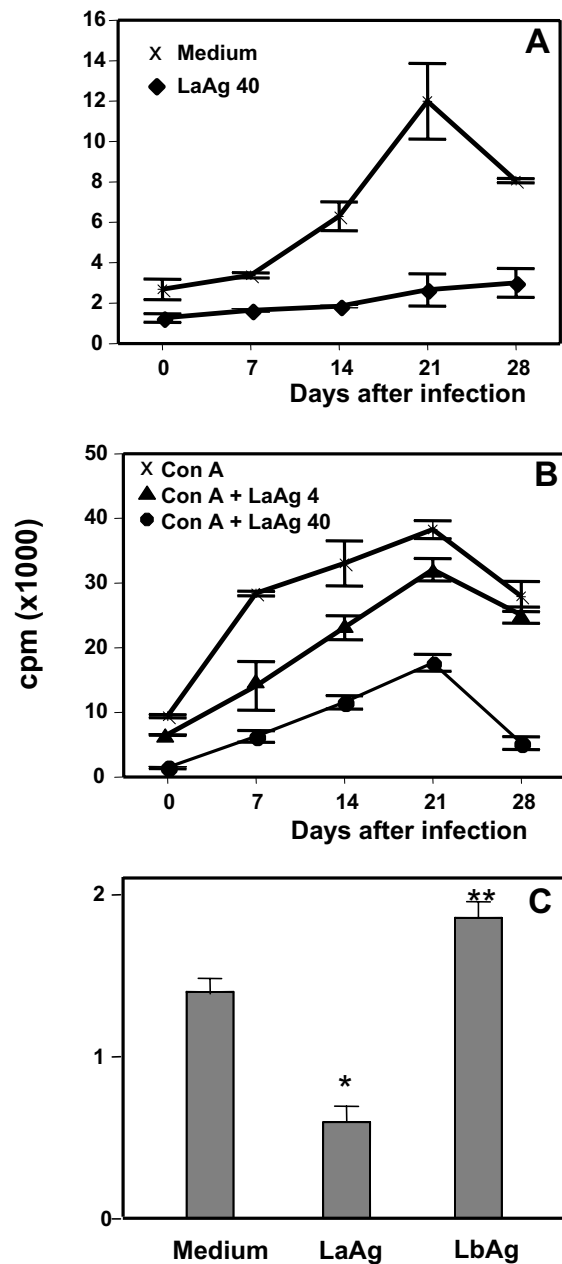
Fig. 1 – LaAg- induced suppression of cell proliferation during the course of infection. Groups of BALB/c mice at different times of infection with *L. amazonensis* were sacrificed and their lesion-draining lymph node cells obtained. The spontaneous (A) or Con A-induced proliferation (B) was measured in the presence of the indicated concentrations of LaAg. In (C), cells from 7-day infected mice were re-stimulated with 40 μg/ml of LaAg or LbAg. Mean $\pm$ SD (n=3). Asterisks indicate (*) $p \leq 0.01$ and (**) $p \leq 0.05$ in relation to control.
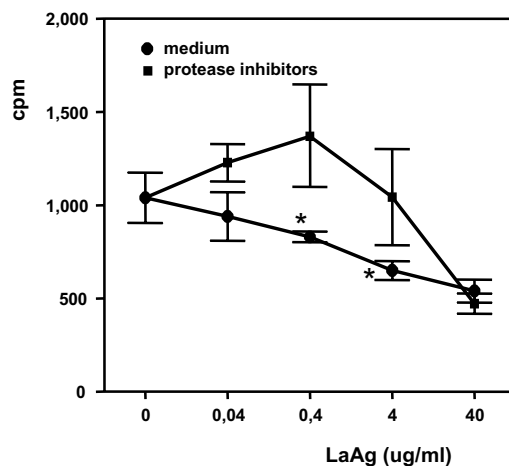
Fig. 2 – Effect of LaAg prepared with protease inhibitors on lymphocyte cell proliferation. Cells from 7 day-infected mice were incubated with different concentrations of LaAg prepared in the presence or absence of protease inhibitors for 72 hours as described in Materials and Methods. Mean ± SD (n=3). Asterisks indicate $p \leq 0,01$ in relation to the same concentration point.

of the disease, we used cells at day 7 as sources of "immune" cells. We found that suppression is a *L. amazonensis* feature, since *L. braziliensis* antigens prepared in the same way were stimulatory (Fig. 1C). There is evidence that *L. amazonensis* has a greater amount of cytolytic proteins than other species, such as *L. guyanensis* (Almeida-Campos and Horta 2000). To determine if the inhibitory effect of LaAg was due to protease activity, the parasites were treated with a cocktail of protease inhibitors (PMSF, TLCK, TPCK, aprotinin, pepstatin and leupeptin) before and during antigen preparation. Figure 2 shows that the resulting protein-rich fraction of LaAg was also T-cell suppressive at concentrations of LaAg above 4 μg/ml, but was devoid of activity at lower concentrations, indicating that the inhibitory effect of LaAg is partially dependent on protease activity.

The Suppression Induced by the LaAg is not Due to the Lack of Key Cytokines

To evaluate if the T-cell suppression induced by LaAg could be reverted by the addition of exo-

genous cytokines, lymph node cells from infected BALB/c mice were pre-incubated for 2 hours in the presence of IL-2, IL-4, IL-12 and IFN-γ alone or in combination before the addition of LaAg. The results expressed in Figure 3 show that in none of the conditions tested there was a reversion of the inhibitory effect, indicating that the anergy was not due to insufficient levels of lymphocyte growth/differentiation factors (IL-2, IL-4 or IL-12) or macrophage-activating cytokines (IFN-γ).
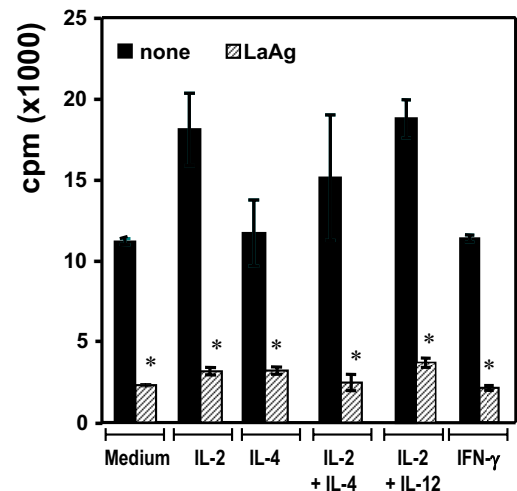

Fig. 3 – Inhibitory effect of LaAg in the presence of exogenous cytokines. Draining lymph node cells from 7-day-infected mice were cultured for 2 hours in the presence of IL-2 (100 U/ml), IL-4 (100 U/ml), IL-12 (5 ng/ml) and/or IFN-γ alone or in combination as indicated. 40 μg/ml LaAg was then added and cells were cultured at 37°C/4% CO$_2$ for a further 46 hours. The proliferative response was expressed in cpm, as described in Materials and Methods. Mean ± SD (n=3). Asterisks indicate $p \leq 0,01$.

LaAg does not Inhibit the Cell Proliferation Induced by Anti-CD3

The observation that the suppression induced by LaAg *in vitro* occurs in cells from both normal and infected animals (Fig. 1) suggested a lack of involvement of the TCR-CD3 complex. Figure 4 shows that unlike Concanavalin A-stimulation, LaAg does not inhibit the lymphocyte proliferation mediated by anti-CD3. Actually, this was increased

in concentrations of LaAg between 0.4 and 4 μg/ml. Stimulation with Concanavalin A requires antigen presentation by accessory cells such as macrophages (Holt et al. 1985). Thus, macrophages were pulsed with LaAg for 2 hours and after antigen removal, they were co-cultured with immune T cells. Figure 5 confirms that LaAg significantly affected the capacity of macrophages to process/present parasite antigens in a dose-dependent fashion.
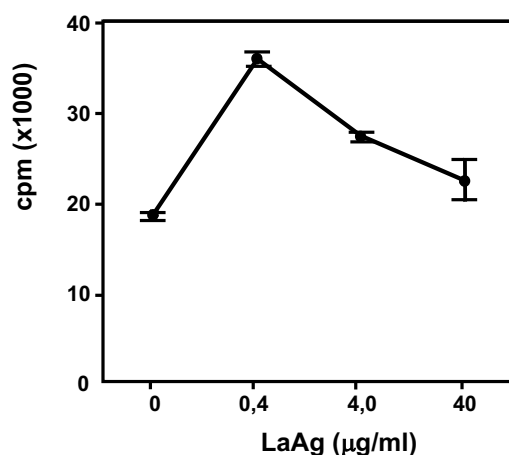

Fig. 4 – Effect of LaAg on anti-CD3-induced proliferation. Lymph node cells from 7-day infected mice were cultured in the presence of different LaAg concentrations for 1h. After this time, cells were transferred with LaAg to a 96-well plate adsorbed with anti-CD3. The proliferative response was measured 48 hours later. Mean $\pm$ SD (n=3).

LaAg Induces Apoptosis in Lymph Node Cells

To investigate if antigen unresponsiveness was due to LaAg toxicity, we first evaluated cell death due to necrosis. However, significant release of lactate dehydrogenase or internalization of Trypan Blue dye used as indicators of cell lysis was achieved only with LaAg concentrations above 400 μg/ml. This is well above the necessary to induce cell anergy (data not shown), indicating that necrosis was not the main mechanism. Electrophoresis of the DNA of cells pre-cultured with LaAg showed an increased DNA fragmentation (indicator of apoptosis), in relation to cells cultured without LaAg (Fig. 6A). DNA
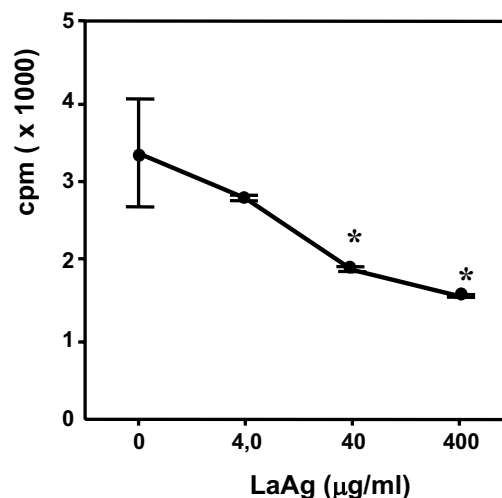

Fig. 5 – Effect of LaAg on antigen presentation. Peritoneal cells from normal BALB/c mice were cultured at 4×10^5 with LaAg at the indicated concentrations. Adherent cells were washed twice and then co-cultured for a further 72h with 2×10^5 immune T cells. The cell proliferation was expressed as cpm. Controls were T cells incubated without adherent cells for the same time period: 301 ± 85 cpm. Mean $\pm$ SD (n=3). Asterisks indicate p $\leq 0,01$ in relation to 0 μg/ml.

fragmentation was confirmed by the more quantitative TUNEL assay, where the percentage of cells with spontaneously fragmented DNA (20%) was significantly increased to 48% when the cells were cultured in the presence of 40 μg/ml LaAg (data not shown). The results above showed that the suppressive effect of LaAg was associated with apoptosis but not necrosis. The cell type undergoing apoptosis is not determined yet, but it is unlikely macrophages since incubation of adherent peritoneal cells with a supraoptimal concentration of LaAg (200 μg/mL) for 8h did not increase the percentage of cells expressing phosphatidylserine (Fig. 6B).

DISCUSSION

The immune regulation during cutaneous leishmaniasis is well described in the murine model of infection with *L. major* (Sacks and Noben-Trauth 2002). Less is known about the host-parasite relationship
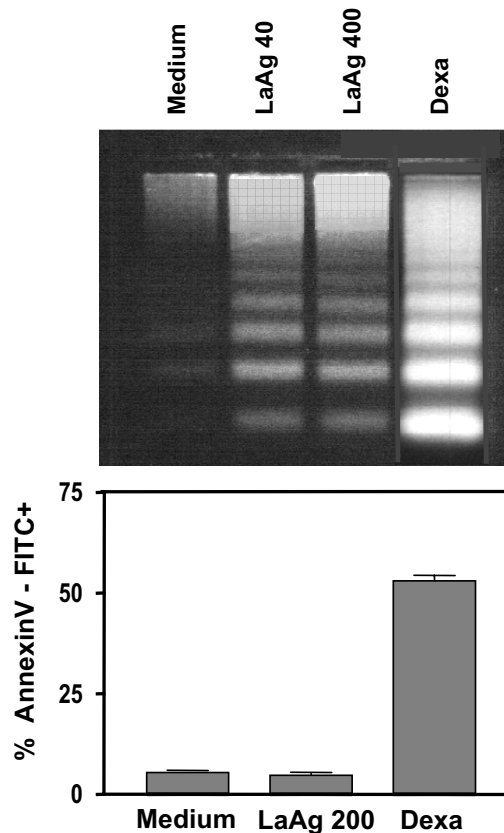
Fig. 6 – LaAg induces apoptosis in BALB/c mice infected cells. (A) Lymph node cells from *L. amazonensis* infected mice (day 7) were cultured for 17 hours in the presence of LaAg at 40 and 400 μg/ml or 1 μM dexamethasone. The total cell DNA was extracted and submitted to electrophoresis in agarose gel as described. (B) Macrophages from normal BALB/c mice were incubated with LaAg at 200 μg/ml for 6 hours. Cells were incubated with AnnexinV-FITC for 15min/37°C.

during infection with *L. amazonensis*. In the search for immunogenic molecules for vaccine development, various *L. major* antigens have been identified, such as the glycoprotein gp63, Hsp80, LACK, 1G6 or 4H6 (WHO 2001). Earlier studies showed that the glycoprotein gp10/20 from *L. amazonensis* promastigotes stimulated the proliferative responses *in vitro*, but induced lesion exacerbation when used to immunize mice by s.c. or i.p. routes (Rodrigues et al. 1987). Recently, we showed that i.m.

immunization with whole *L. amazonensis* antigens (LaAg), as used in the formulation of the vaccine Leishvacin® also promoted disease progression in BALB/c mice infected with *L. amazonensis* in a manner associated with TGF-β induction (Pinheiro et al. submitted). In the present study we demonstrate that LaAg not only is incapable to re-stimulate lymphocytes from infected animals *in vitro* but also renders murine T cells unable to proliferate in response to the mitogen Concanavalin A.

We found no evidence that T cell anergy was due to activation-induced death (Critchfield et al. 1994), as cells cultured with LaAg for shorter times (9 or 34 hours) showed no sign of proliferation (data not shown). Likewise, anergy does not seem to be due to lack of growth factors (Fig. 3) or to production of NO (Bingisser et al. 1998), as verified by the ineffectiveness of the NO synthase inhibitor N-G-methyl-L-arginine added to the culture to revert anergy (data not shown).

It was previously shown that intracellular infection with *L. amazonensis* leads to increased production of TGF-β by macrophages (Barral-Netto et al. 1992). We found that an active infection is not mandatory since culture with LaAg also greatly increases TGF-β production (manuscript in preparation). TGF-β is a cytokine with immunosuppressive and apoptotic properties. The nature of the TGF-β-inducing substance(s) in LaAg is unknown, but it is probably not a cystein protease such as the *L. chagasi* cathepsin B-like proteases (Gantt et al. 2003) since it was demonstrated to be thermoresistant (100°C/1h, data not shown) and T-suppression was only partially affected by a cocktail of protease inhibitors (Fig. 2). We have observed that neutralization with anti-TGF-β antibodies restored the T cell responsiveness to LaAg (Pinheiro et al. submitted). Thus, it is likely that TGF-β production by macrophages is a primary key event in T cell apoptosis/anergy induced by LaAg but the contribution of TGF-β released by the apoptotic T cells (Chen et al. 2001), membrane-bound TGF-β in CD4+CD25+ regulatory T cells (Nakamura et al. 2001) or the unbalance between CTLA-4 and CD28 expression

on CD4+ cells (Gomes and DosReis 2001, Sung et al. 2003) should also be considered. Isolated immune T cells were suppressed when co-cultured with LaAg-pulsed macrophages (Fig. 5). Once defective antigen presentation was extended to unrelated antigens, as observed by the impaired capacity of macrophages pre-pulsed with 40 μg/mL LaAg to present OVA to a TCR-specific DO-11.10 hybridoma (data not shown), it seems likely that the anergic effect of LaAg is primarily on APCs rather than on the induction of regulatory T cells during leishmanial infection (Belkaid et al. 2002).

The cell type(s) in the mixed lymph node cells undergoing apoptosis (Fig. 6, top) were not phenotypically identified here, but they are unlikely adherent APCs as isolated macrophages remained annexin V negative when cultured with supraoptimal concentrations of LaAg (Fig. 6, bottom). The finding that LaAg did not suppress and actually transiently increased T cell responses to anti-CD3 – a TCR-driven APC-independent stimulus (Fig 4) – further corroborates that LaAg T suppression is due to impaired antigen processing/presentation.

In summary, this study suggests that *L. amazonensis* antigens induce unresponsiveness to both related and unrelated antigens by a mechanism possibly initiated by TGF-β production by macrophages and culminated with T cell apoptosis. These findings may contribute to a better understanding of the anergic mechanisms associated with diffuse cutaneous leishmaniasis and propose that the use of LaAg as the sole antigen in Leishvacin$^{\circledR}$ should be reconsidered.

RESUMO

A *Leishmania amazonensis* é o principal agente etiológico da leishmaniose cutânea difusa, uma doença associada a respostas imunes anérgicas. Neste estudo nós mostramos que o extrato bruto de promastigotas de *Leishmania amazonensis* (LaAg), mas não de *L. braziliensis* (LbAg), contém substâncias que suprimem respostas proliferativas, espontâneas e mitogênicas, de células T. As substâncias supressoras no LaAg são termo-resistentes (100°C/1h) e parcialmente dependentes da atividade de proteases. A anergia de células T não foi devida à diminuição na produção de fatores de crescimento, uma vez que não foi revertida pela adição de: IL-2, IL-4, IFN-γ ou IL-12. O LaAg não inibiu a ativação de células T induzida por anti-CD3, sugerindo que a anergia é devida a um defeito na apresentação de antígenos. A anergia não foi devida à necrose celular, mas foi acompanhada de uma expressiva fragmentação de DNA nas células de linfonodos, indicativo de apoptose. Apesar da pré-incubação de macrófagos com LaAg ter inibido sua capacidade de apresentação de antigenos, este efeito não foi devido à apoptose dos primeiros. Estes resultados sugerem que a anergia de células T encontrada na leishmaniose difusa deve ser devida à apoptose dessas células que se segue à apresentação defeituosa de antígenos pelo antígeno do parasito.

Palavras-chave: *Leishmania*, anergia, apoptose.

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