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Detection of early apoptosis and cell death in T CD4$^+$ and CD8$^+$ cells from lesions of patients with localized cutaneous leishmaniasis

A.L. Bertho, M.A. Santiago, A.M. Da-Cruz and S.G. Coutinho

Laboratório de Imunidade Celular e Humoral em Protozooses, Departamento de Protozoologia, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brasil

Abstract

Human localized cutaneous leishmaniasis (LCL), induced by *Leishmania braziliensis*, ranges from a clinically mild, self-healing disease with localized cutaneous lesions to severe forms which can present secondary metastatic lesions. The T cell-mediated immune response is extremely important to define the outcome of the disease; however, the underlying mechanisms involved are not fully understood. A flow cytometric analysis of incorporation of 7-amino actinomycin D and CD4$^+$ or CD8$^+$ T cell surface phenotyping was used to determine whether different frequencies of early apoptosis or accidental cell death occur at different stages of LCL lesions. When all cells obtained from a biopsy sample were analyzed, larger numbers of early apoptotic and dead cells were observed in lesions from patients with active disease (mean = 39.5 ± 2.7%) as compared with lesions undergoing spontaneous healing (mean = 17.8 ± 2.2%). Cells displaying normal viability patterns obtained from active LCL lesions showed higher numbers of early apoptotic events among CD8$^+$ than among CD4$^+$ T cells (mean = 28.5 ± 3.8 and 15.3 ± 3.0%, respectively). The higher frequency of cell death events in CD8$^+$ T cells from patients with LCL may be associated with an active form of the disease. In addition, low frequencies of early apoptotic events among the CD8$^+$ T cells were observed in two patients with self-healing lesions. Although the number of patients in the latter group was small, it is possible to speculate that, during the immune response, differences in apoptotic events in CD4$^+$ and CD8$^+$ T cell subsets could be responsible for controlling the CD4/CD8 ratio, thus leading to healing or maintenance of disease.

Correspondence
S.G. Coutinho
Laboratório de Imunidade Celular e Humoral em Protozooses
Departamento de Protozoologia
Instituto Oswaldo Cruz, FIOCRUZ
Av. Brasil, 4365
21045-900 Rio de Janeiro, RJ
Brasil
Fax: +55-21-280-1589
E-mail: coutinho@gene.dbbm.fiocruz.br

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- Flow cytometry
- Human leishmaniasis
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- T cells

Introduction

Human cutaneous leishmaniasis in Rio de Janeiro, Brazil, is caused mainly by the obligate intracellular protozoan *Leishmania braziliensis* (1). The parasite replicates inside the parasitophorous vacuoles of phagocytic cells. Infections in humans range from a single skin ulcer (localized cutaneous leishmaniasis - LCL), which heals spontaneously or after antimony therapy, to severe forms which involve either secondary metastatic lesions on the mucous membranes of the face (mucocutaneous leishmaniasis) or mul-

tiple nodular lesions all over the body (diffuse cutaneous leishmaniasis). Few parasites are detectable in LCL lesions, but tissue destruction and focal points of necrosis suggest a cell-mediated hypersensitivity mechanism of injury.

Several studies utilizing the mouse model suggest that T cell-mediated immunity may be responsible for either a favorable outcome of the disease by activation of macrophages and killing of the parasites or aggravation of the lesions by inhibition of macrophage function (2-4). Cytokines such as IFN-γ and TNF-α and -ß, produced by Th1 $CD4^+$ T lymphocytes, play a pivotal role in this process of macrophage activation and parasite destruction. Alternatively, the mechanisms for aggravating the disease in mice are related to the effects of cytokines such as IL-4 and TGF-ß, which are primarily produced by Th2 $CD4^+$ T lymphocytes (5-8). $CD8^+$ T lymphocytes also appear to play an important role in the immunologic response leading to cure of murine leishmaniasis (9-11); antigen-activated $CD8^+$ T lymphocytes have been shown to produce IFN-γ and may have a cytolytic effect on parasitized macrophages (11,12).

Research in histology, genetics, and molecular biology during the past 25 years has shown that all cells are genetically programmed to die. Under physiological circumstances, damaged and senescent cells sacrifice themselves through a type of cell death termed apoptosis. This form of programmed cell death is characterized by shrinkage, dense chromatin condensation, DNA fragmentation and formation of apoptotic bodies (13,14), and plays a pivotal role in the development and homeostasis of normal tissues (15), as well as in the pathogenesis of different diseases (16).

The hallmark for identifying apoptosis is fragmentation of cell DNA via endonucleases, and demonstrable by gel electrophoresis (17). However, flow cytometry has become a method of choice for analysis of cell death in a variety of cell systems (18-20).

A method using 7-amino actinomycin D (7-AAD) and single laser flow cytometry has been described (21-23) which permits discrimination between early apoptotic and live or dead cells (late apoptosis and accidental cell death, ACD). The 7-AAD method was used in the present study to measure the frequencies of live, early apoptotic or dead $CD4^+$ and $CD8^+$ T cells from lesions of LCL patients with active disease or in the spontaneous healing phase in order to better understand the role of cell death in the regulation of the T cell-mediated immune responses in human leishmaniasis.

Material and Methods

Study subjects

Seventeen adult patients were studied (11 men and 6 women), 15 with active LCL and two with spontaneously healed lesions. After diagnosis, tissue samples were collected for examination and the patients were immediately treated with antimony therapy. Since treatment with antimony compounds is usually started immediately, few "spontaneous healed" lesions were available for inclusion in the study. All patients were from areas in the regions surrounding Rio de Janeiro where the disease is endemic. The following criteria were used for diagnosis: a) clinical picture and epidemiological evidence for LCL, b) positive Montenegro skin test - delayed-type hypersensitivity reaction to leishmanial antigens, and c) isolation of *Leishmania* from lesion biopsy samples after culture in McNeal, Novy and Nicolle medium (24).

Mononuclear cells obtained from lesions and blood

Lesion mononuclear cells (LMC) were obtained as described elsewhere (25). Briefly, ellipsoid lesion biopsy samples were ob-

tained under local anesthesia from LCL patients. Fat tissue was removed, and the remaining fragment was placed in a sieve fitted with a mesh filter on a Petri dish containing RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) with 10% fetal calf serum (FCS; Sigma). The tissue was cut into small pieces with a surgical scalpel and extruded through the mesh with a glass rod. The mononuclear cells were separated by centrifugation over a Ficoll-Hypaque gradient (Sigma), adjusted to 5×10^5-1×10^6 cells per ml and then stained for surface markers and 7-AAD (Sigma) or used in apoptosis-induction experiments.

Blood from healthy adult donors was collected into heparinized tubes and peripheral blood mononuclear cells (PBMC) were separated by centrifugation over a Ficoll-Hypaque (Sigma) gradient.

Induction of apoptosis

For induction of apoptosis in LMC- and PBMC-derived cells, 10 µM staurosporine (Stau, Sigma) was added to 1×10^6 cells of LMC or PBMC cultured in RPMI 1640 supplemented with 1% penicillin/streptomycin (Sigma), 10% human AB+ serum (Sigma), 10 mM HEPES, 1.5 mM L-glutamine (Sigma), and 40 µM 2-mercaptoethanol (Sigma) at 37°C in a humidified atmosphere of 5% CO_2 in air for 3 days (21). Cell cultures without Stau treatment were used as control. ACD was induced by placing LMC or PBMC in a water bath at 60°C for 5 min.

Dual-color cell surface phenotyping, 7-AAD staining and flow cytometry

For surface antigen staining, 5 µl of each anti-CD4-FITC or anti-CD8-PE monoclonal antibody (Coulter Electronics Inc., Miami, FL, USA) diluted in 90 µl of phosphate-buffered saline (PBS) containing 2% FCS and 0.1% sodium azide (Sigma) (PBSAz) was added to 5×10^5-1×10^6 LMC followed by incubation at 4°C for 20 min. After two washes with 1 ml of PBSAz, the supernatant was removed and the cell pellet was resuspended in 1 ml of PBSAz with 20 µg of 7-AAD, and then incubated for 20 min at 4°C protected from light. Samples stained with 7-AAD and surface markers were analyzed with an EPICS 751 flow cytometer (Coulter) equipped with a 488-nm argon-ion laser. Green fluorescence was measured with a 525-nm band pass (BP) filter and orange fluorescence was measured with a 575-nm BP filter. The red fluorescence from 7-AAD was filtered through a 630-nm long pass filter. Electronic compensation among the fluorescence channels was used to remove residual spectral overlap. Fluorescence data were displayed on four-decade log scales. Approximately 30,000 events were analyzed for each sample.

Cell staining with PI and flow cytometry

Cells were stained with hypotonic citrate solution containing propidium iodide (PI; Sigma) as described elsewhere (26). Briefly, 1 ml of staining solution containing 50 µg of PI, 0.1% sodium citrate (Sigma), and 0.1% Triton X-100 (Sigma) in PBS was added to 1×10^6 cells, kept overnight at 4°C, and then analyzed with an EPICS 751 flow cytometer. Red PI fluorescence was measured with a 600-nm BP filter and displayed on a four-decade log scale. A low flow rate was set at approximately 400 events/s to improve the coefficient of variation in the DNA histograms. A minimum of 15,000 events were analyzed per sample.

Statistical analysis

All results are reported as mean ± standard error (SEM). The two-tailed Mann-Whitney U-test was used for statistical analysis, with the level of significance set at $P \leq 0.05$.

Results

Monitoring the accuracy of 7-AAD staining

To determine whether the 7-AAD method effectively distinguished between live, early apoptotic and dead cells, LMC and PBMC were heated for 5 min at 60ºC to induce death, or cultured in the presence of Stau for induction of early apoptosis (see Material and Methods).

Figure 1 shows one representative series of 3 individual experiments in which the fluorescence intensity of 7-AAD *vs* forward scatter (FSC) dot plot was used to define dead cells induced by heating (Figure 1A), and early apoptotic cells induced by treatment with Stau (Figure 1B). Untreated cells were used as controls (Figure 1C). Similar procedures were carried out using mononuclear cells obtained from lesions of a cutaneous leishmaniasis patient (data not shown). Negligible 7-AAD incorporation was seen in live cells (region R1), while 7-AAD^{dim} was observed in early apoptotic cells (R2) and 7-AAD^{bright} in dead cells (R3). For a better definition of the apoptotic cell region, cells were sorted by flow cytometry fol-

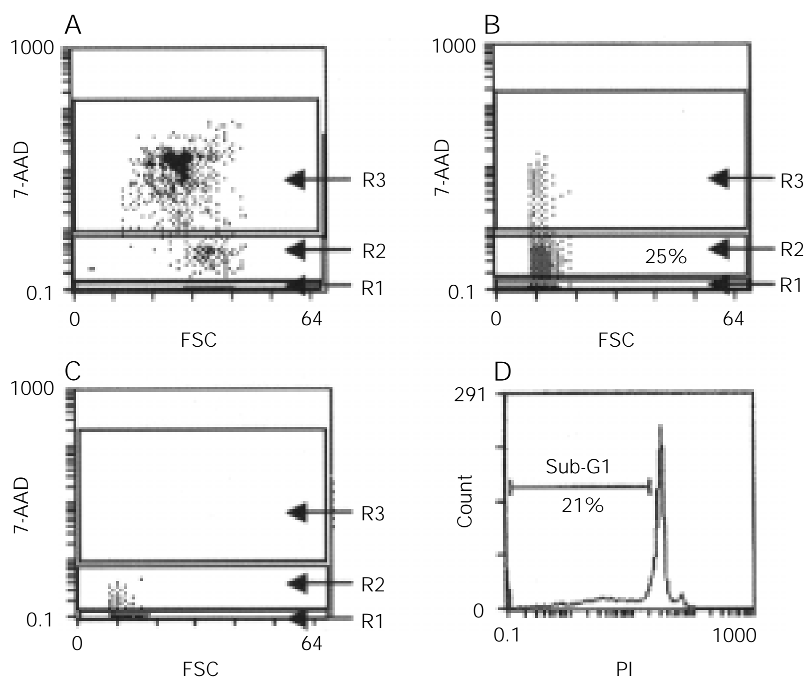

Figure 1 - Two-parameter flow cytometry analysis (forward scatter, FSC vs 7-amino actinomycin D, 7-AAD). PBMC from LCL patients were obtained after centrifugation over a Ficoll-Hypaque gradient and heated for 1 h at 60ºC to induce ACD (A) or cultured in the presence of Stau for 3 days to induce early apoptosis (B). Cell cultures without Stau were used as control (C). R1 - Live cells (7-AAD^{-}); R2 - early apoptotic cells (7-AAD^{dim}); R3 - dead cells (7-AAD^{bright}). D, Analysis of Stau-induced apoptosis by hypotonic propidium iodide (PI) staining was done to confirm the results. The sub-G1 region encompasses the apoptotic cells.

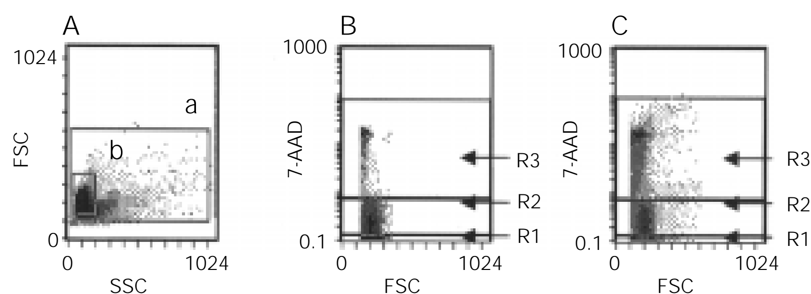

Figure 2 - A representative flow cytometry analysis used to determine 7-amino actinomycin D (7-AAD) incorporation based on two different gates (A) created in side scatter/forward scatter (SSC/FSC) dot plot: a, all cells; b, cells with viable patterns; B, cells within gate b; C, cells within gate a. Region R1 encompasses live cells; R2 - early apoptotic cells; R3 - dead cells (ACD).

lowed by agarose gel electrophoresis where the formation of characteristic DNA ladders was observed (data not shown).

Cells undergoing induced apoptosis or ACD were detectable in side *vs* forward light scatter plots. Treatment with Stau reduced FSC and increased side scatter (SSC), which is a well-established morphological feature of apoptosis; on the other hand, thermal injury at 60°C increased both FSC and SSC, which is a characteristic of cells undergoing ACD (data not shown).

The 7-AAD and hypotonic PI staining techniques were also compared. PBMC or LMC were cultured for 3 days in medium containing Stau, or heated for 5 min at 60°C and then stained in parallel with 7-AAD or with a hypotonic solution containing PI. In the 7-AAD dot plots (R2) we observed 25% of cells within the early apoptosis gate. These data closely matched the percentage of lymphocytes in the "sub-G1" region where the DNA fragmented content was detected in the PI histogram (21%) (Figure 1B and D, respectively).

Cell death analysis of LMC obtained from LCL patients

The 7-AAD method was used to evaluate the cell death events occurring in the lesions of LCL patients with active disease or spontaneous healing. Figure 2 shows a representative experiment in which a flow cytometry protocol was used to quantitatively determine the frequencies of live, early apoptotic or dead cells in these groups of patients. 7-AAD incorporation (Figure 2B and C) was evaluated by two different gates created in the SSC/FSC dot plot: gate a, surrounding all analyzed cells including those supposed to be dead according to the light scatter patterns and deeply stained with PI, and gate b, constructed around cells with apparently normal morphology (unmodified SSC/FSC) and considered as "viable cells" by their ability to exclude vital dyes such as PI (Figure 2A). Analysis within gate a (Figure 3A) showed higher numbers of early apoptotic cells in lesions of patients with active disease (mean = 39.5 ± 2.7%) as compared to patients with spontaneous healing (mean = 17.8 ± 2.2%). Regarding dead cells, no significant difference was observed between the two groups of patients (16.3 ± 2.0 and 12.6 ± 4.0%, respectively, P = 0.49). It is interesting to note that even in gate b, cells supposed to be viable according to their scatter profiles showed important frequencies of early apoptotic events. Patients with active disease showed high numbers of early apoptotic cells (mean = 28.5 ± 3.8%) when compared to patients with spontaneously healing lesions (mean = 15.3 ± 3.0%) (P = 0.0001). As expected, Figure 3B shows small numbers of dead cells within gate b (as described in Figure 2), regardless of the group of patients analyzed (active disease = 5.1 ± 2.0%; healing lesions = 2.0 ± 1.0%).

Analysis of cell death in CD4$^+$ or CD8$^+$ T cells from lesions of LCL patients

Live, early apoptotic or dead CD4$^+$ or CD8$^+$ T cells were evaluated in the LMC obtained from lesions of LCL patients by the 7-AAD method and by dual-color cell surface staining for phenotypic analysis. The

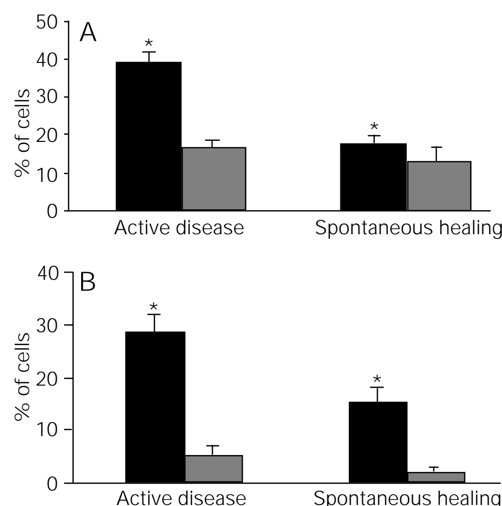

Figure 3 - Quantitative analysis of early apoptotic and dead cells obtained from lesions of LCL patients within gates a and b according to Figure 2. A, Analysis within gate a; B, cells analyzed within gate b. Black bars - Mean frequencies ± SEM of early apoptosis in cells from patients during active disease (N = 15) and during spontaneous healing (N = 2). Gray bars - mean frequencies ± SEM of dead cells in cells from patients during active disease and during spontaneous healing. *P<0.0001 compared to % dead cells (Mann-Whitney U-test).

quantitation of the frequency of cell death events in cells within gate a was difficult because cells in advanced stages of apoptosis and necrosis can lose their surface antigen expression. In this respect, early apoptosis and phenotypic determinations were performed only on gate b, created as described in Figure 2. Two other gates were also constructed around the $CD4^+$ (gate c) or $CD8^+$ (gate d) T cells. Double positive or double negative cells were not considered relevant in this analysis. The 7-AAD incorporation in each subset was measured in two 7-AAD *vs* FSC dot plots which combined definitions for gate b + c or gate b + d. These dot plots provided simultaneous determinations of live (region R1), early apoptotic (region R2) or dead (region R3) $CD4^+$ or $CD8^+$ T lymphocytes (Figure 4).

In patients with active disease, $CD8^+$ and $CD4^+$ T cells exhibited high frequencies of apoptosis, with significantly more apoptosis among $CD8^+$ T cells than $CD4^+$ T cells (means: $CD8^+ = 31.3 \pm 5.9\%$; $CD4^+ = 16.7 \pm 4.7\%$; P = 0.00003). Conversely, patients with spontaneous healing showed a general reduction in the frequency of apoptotic cells. Moreover, the numbers of apoptotic events was significantly lower among $CD8^+$ T cells than $CD4^+$ T cells (mean $CD8^+ = 4.3 \pm 3.6\%$; mean $CD4^+ = 15.2 \pm 4.1\%$, P = 0.0004). Additionally, the numbers of apoptotic $CD4^+$ T cells in spontaneously healed lesions remained similar to those observed in patients with active disease, whereas the number of apoptotic $CD8^+$ T cells decreased in patients showing spontaneous healing (P = 0.016) (Figure 5).

Discussion

Apoptosis has been described as essential for normal organogenesis and tissue development. This phenomenon plays an important role in the immunopathogenesis of several parasitic diseases (27,28). Studies on cell death in protozoonoses have also focused on its possible role in the immunopathology of experimental Chagas' disease (29-31). Infection with the intracellular protozoan *Toxoplasma gondii* induces apoptosis of host $CD4^+$ T lymphocytes, which may involve a cooperative effect of IFN-γ on Fas-mediated cell death (32). In leishmaniasis there is evidence suggesting that the sensitivity of *Leishmania* promastigotes to programmed cell death is induced by heat shock

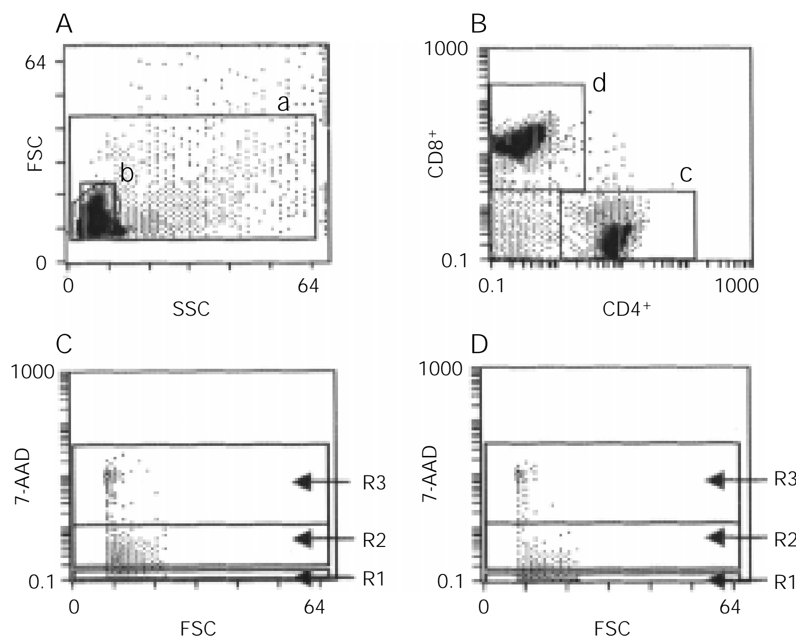

Figure 4 - Representative flow cytometric analysis carried out to determine live (R1), early apoptotic (R2) or dead (ACD) (R3) $CD8^+$ or $CD4^+$ T cells obtained from lesions of an LCL patient. A, Scatter histogram (side/forward, SSC/FSC) in which gates a and b were created following scatter patterns according to Figure 2. B, Dual-color dot plot of $CD8^+$-PE and $CD4^+$-FITC T cells based on gate b according to Figure 2. C, FSC vs 7-amino actinomycin D (7-AAD) gated on $CD8^+$ T cells within gate d; D, FSC vs 7-AAD gated on $CD4^+$ T cells within gate c.

and modulated by calcium (33). It has also been demonstrated that intracellular infection by *Leishmania donovani* inhibited macrophage apoptosis (34).

The importance of apoptotic events in the modulation of the immune response occurring in lesions of LCL patients is still unclear. To quantify cell death events in T cell subsets after they were obtained *ex vivo* from lesions of LCL patients, we utilized the 3-color staining method which permitted us to measure live, early apoptotic or dead T cell subsets.

As expected, by analyzing all LMC from active LCL patients, we detected high numbers of early apoptotic and dead cells. These data are in accordance with some histological findings which show the presence of fibrinoid necrosis in active LCL (35). Expressive numbers of early apoptotic cells were detected in another analysis based on cells which exhibited a normal, unmodified scatter profile. This means that, despite the apparently normal morphological patterns, cells were undergoing early apoptosis.

When cell death events were evaluated in $CD4^+$ or $CD8^+$ T lymphocytes we observed that active LCL patients had larger numbers of apoptotic $CD8^+$ T cells than apoptotic $CD4^+$ T cells. These data suggest that during active disease a large number of $CD8^+$ lymphocytes underwent early apoptosis and thereby had their immunologic functions compromised, leading to low cytokine production (mainly IFN-γ) and probably impairing a favorable effect on the course of the disease.

Regarding patients with a tendency to self-healing, the small number of patients studied here did not allow us to reach consistent conclusions, although some interesting data were obtained. A lower rate of cell death was observed in these patients, a fact probably related to the clearance of infection. In this situation, preserved T cells may maintain their efficacy, producing cytokines and activating macrophages to kill the parasites. In the case of apoptotic $CD4^+$ and $CD8^+$ T cell analysis, these patients with spontaneously healed lesions showed small numbers of apoptotic $CD8^+$ T cells although the frequencies of apoptotic $CD4^+$ T cells were similar to those observed in patients with active disease. Despite the small number of patients studied, we can speculate that the low percentage of apoptosis in $CD8^+$ T cells of spontaneous healing patients may point to a significant role of this cell subpopulation in the mechanism of cure of LCL. This possibility is in accordance with previous results from our group (36-39) showing that $CD8^+$ T cells are associated with cure and protection in LCL.

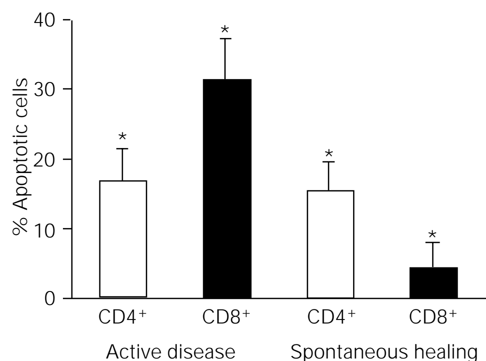

Figure 5 - Early apoptosis in $CD4^+$ or $CD8^+$ T cells obtained from lesions of patients with active disease (N = 15) or spontaneous healing (N = 2) as measured by flow cytometry. Solid bars represent early apoptotic $CD8^+$ T cells, and open bars, early apoptotic $CD4^+$ T cells. Data are reported as mean ± SEM. *$P<0.0004$ - early apoptotic $CD8^+$ T cells compared to early apoptotic $CD4^+$ T cells (Mann-Whitney U-test).

Some hypotheses can be raised in order to explain the role of apoptosis mediating the immune responses in active or healing lesions. One possibility refers to a T cell hypersensitivity to leishmanial antigens, which is apparently the main immunopathological component in this disease, leading to activation-induced cell death (AICD). The phenomenon of AICD is thought to operate in situations in which there is an excess of antigen, and may be a mechanism that prevents immunopathology resulting from overactivation of the immune system (40). This AICD is mediated by interaction of Fas (CD95) with its ligand (FasL), which is transiently expressed on activated T cells. In fact, such affirmation is in agreement with our preliminary observations showing that there was a high expression of CD95 in cells

from lesions of patients with active disease (data not shown). Apoptosis may also be associated with modulation of the immune response leading to a beneficial effect or with cell and tissue destruction leading to aggravation of lesions. In active disease an important number of $CD8^+$ and $CD4^+$ T cells undergo early apoptosis and therefore have their functional characteristics altered.

To our knowledge, this is the first study investigating the occurrence of apoptosis in T lymphocytes obtained from lesions of human cutaneous leishmaniasis using the advantages of flow cytometry and the 7-AAD method. Further investigations are needed to better understand the involvement of apoptotic events in leishmaniasis.

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