



## Variations in the serum levels of soluble CD23, nitric oxide and IgE across the spectrum of American cutaneous leishmaniasis

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### Abstract

Serum IgE levels and the expression of low affinity receptor for IgE (FcεRII/CD23) are increased in cutaneous leishmaniasis. The ligation of CD23 receptor by IgE–anti-IgE immune complexes results in nitric oxide (NO) production, which is a critical leishmanicidal factor. Human monocytes/macrophages express FcεRII/CD23 after activation with IFN-γ and IL-4. In the present study, we assessed the relationship between NO, total and *Leishmania*-specific IgE and soluble CD23 across the clinical spectrum of American cutaneous leishmaniasis (ACL). Sixty-nine ACL patients and 22 endemic controls were studied. NO concentration was estimated using the Greiss method. Soluble CD23, total IgE and anti-*Leishmania* IgE levels were measured using ELISA. Soluble CD23 was increased in the four patient groups ( $0.001 < P < 0.05$ ) compared by the Mann–Whitney test to healthy subjects, particularly in mucocutaneous leishmaniasis (MCL) and diffuse cutaneous leishmaniasis (DCL) patients. Total IgE levels were higher in ACL patients ( $P < 0.0001$ ). Anti-*Leishmania* IgE showed a similar tendency, where MCL and DCL patients had greater levels. All patients groups, except intermediate or chronic cutaneous leishmaniasis (ICL) patients, showed elevated levels of  $\text{NO}_2^-/\text{NO}_3^-$  compared to healthy individuals ( $0.001 < P < 0.01$ ). Interestingly, ICL patients did not produce significant levels of  $\text{NO}_2^-/\text{NO}_3^-$ . ACL patients showed a significant positive correlation between soluble CD23 and anti-*Leishmania* IgE. DCL patients showed a positive correlation between both parameters.

Overall results suggest that sCD23, IgE and NO may play different roles across the ACL spectrum.

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### 1. Introduction

American cutaneous leishmaniasis (ACL) is a disease caused by infection with *Leishmania* parasites that invade and grow within macrophages and affect millions of people in the New World. The infection

shows a spectrum of clinical, histopathological and immunological manifestations (Convit, 1974; Convit et al., 1972, 1993). These are: localised cutaneous leishmaniasis (LCL), which presents limited skin lesions that usually self-heal; diffuse cutaneous leishmaniasis (DCL), a rare manifestation characterised by extensive cutaneous involvement by non-ulcerated nodules rich in parasites; and the intermediate forms, which include mucocutaneous leishmaniasis (MCL) as well as the extensive lesions and verrucous cutaneous

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forms (intermediate or chronic cutaneous leishmaniasis, ICL). MCL patients are characterised by destructive lesions of the oral and nasopharyngeal cavities that usually appear long after the healing of the primary skin lesions (Convit, 1974; Convit et al., 1972).

Evidence from experimental models demonstrates that cellular immune mechanisms play a central role in resistance to *Leishmania* infection. Moreover, protection and cure are associated with the generation of CD4<sup>+</sup> Th1 cells, and disease progression with CD4<sup>+</sup> Th2 lymphocytes (Scott et al., 1988; Heinzel et al., 1989). Similar CD4<sup>+</sup> T lymphocytes subsets are associated with pathogenesis and resistance in human leishmaniasis (Pirmez et al., 1990, 1993; Cáceres-Dittmar et al., 1993; Melby et al., 1994, 1996; Castés et al., 1996). Nevertheless, the role of the immune cells and their different mediators in healing or progression of leishmaniasis remains unclear.

Serum IgE levels and the expression of low affinity receptor for IgE (FcεRII/CD23) are increased in cutaneous leishmaniasis (Lynch et al., 1982; O'Neil et al., 1993; Vouldoukis et al., 1994; Castés et al., 1996; Sousa-Atta et al., 2002).

Vouldoukis et al. (1995) demonstrated that the ligation of CD23 on human macrophages was a strong inducer of NO, which restricts the growth of *Leishmania major*. Moreover, the protective role of the CD23/NO pathway has been supported by previous studies in malaria and in *Plasmodium falciparum* and *Ascaris lumbricoides* coinfection (Nacher et al., 2000, 2002).

Human monocytes/macrophages express FcεRII/CD23 after activation with interferon-γ or interleukin-4 (Delespesse et al., 1991). During the activation of macrophages, CD23 is cleaved by proteolysis and released as a soluble form (sCD23). CD23 receptor is also related with antigen capture and antigen presentation. In order to understand the role of IgE, CD23 and proinflammatory factors in the protection or pathogenesis of ACL, we analysed the relation between sCD23, NO, total and specific IgE in ACL patients.

## 2. Materials and methods

### 2.1. Patients

Patients aged  $31.83 \pm 18.64$  with LCL ( $n = 22$ ), ICL ( $n = 9$ ), MCL ( $n = 21$ ) and DCL ( $n = 17$ )

were studied in the Instituto de Biomedicina. They were diagnosed by established clinical, epidemiological and histopathological criteria (Convit, 1974). Parasitological confirmation of the clinical diagnosis was based on Giemsa staining of smears from biopsies, culture of minced biopsy material on blood agar base slants containing 15% defibrinated rabbit blood and 200 U of penicillin/ml, inoculation of hamsters with biopsy macerate, and histopathological examination of Giemsa and hematoxylin–eosin stained sections. LCL patients had less than 4 months of evolution. The patients were not under treatment at the time of study.

### 2.2. Healthy controls

A total of 22 healthy volunteers were studied (aged  $33.72 \pm 18.13$ ). All were from Venezuelan endemic areas, but had no clinical history of leishmaniasis.

### 2.3. Measurement of NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> concentration in serum

The serum concentration of nitrite (NO<sub>2</sub><sup>-</sup>) as determined by the Griess reaction was used as an indicator of NO production (Green et al., 1982). Nitrate was reduced to nitrite by an enzymatic process described in Moshage et al. (1995). Briefly, serum samples were diluted 1:5 with dH<sub>2</sub>O<sub>2</sub> and incubated for 20 min at 37 °C with 5 μM flavin adenine dinucleotide (FAD), 50 μM nicotinamide adenine dinucleotide phosphate reduced form (NADPH) and 200 U/l of reductase nitrate (all purchased from Sigma). Samples were mixed with 0.001 g/ml lactate dehydrogenase plus 10 mM of sodium pyruvate. After incubation for 5 min at 37 °C the samples were diluted 1:5 with distilled water and deproteinized by 1:20 dilution with 0.3 g/ml ZnSO<sub>4</sub>. Finally, samples were centrifuged at  $10,000 \times g$  for 5 min at room temperature. The amount of NO<sub>2</sub><sup>-</sup> was assayed by mixing 50 μl of supernatant with 150 μl Griess reagent (1% sulfanilamide, 0.1% naphthylethylene-diamide-dihydrochloride and 2.5% H<sub>3</sub>PO<sub>4</sub>) and incubation for 10 min at room temperature. The absorbance of the reaction product at 540 nm was determined in an ELISA reader. The NO<sub>2</sub><sup>-</sup> concentration was determined by using sodium nitrite as standard. Data are expressed in nM.

#### 2.4. Total serum IgE levels

These were measured by a capture ELISA developed at the Immunochemistry Laboratory (Instituto de Biomedicina). These were standardized against the commercial Phadebas IgE PRIST (Pharmacia Sweden) test. Briefly, flat bottom 96-well microplates (Immunolon IV Dynatech Laboratories Inc., Virginia, USA) were coated overnight at 4 °C with 1 µg per well of murine monoclonal antibody anti-human IgE (produced in the Institute for Child Research, Perth, Australia) in carbonated buffer (pH 9.6) and blocked with 1% bovine serum albumin (BSA). The plates were washed with phosphate saline buffer containing 0.1% Tween 20 (PBS-T, pH 7.4), then incubated with serum samples (diluted 1:400) for 2 h at 37 °C. After another wash, detection of the captured IgE was performed by addition of biotinylated murine anti-human IgE at 1:500 (Vector Laboratories, California, USA) for 1 h at 37 °C. The plates were washed thoroughly and allowed to react with avidin–biotin–peroxidase complex (Vectastain kit, Vector Laboratories) for 30 min at 37 °C. *o*-Phenylenediamine dihydrochloride (OPD; Sigma) plus H<sub>2</sub>O<sub>2</sub> were added and the optical density (OD) at 490 nm was read with an ELISA reader. A standard titration curved using a pool of sera with high IgE levels was run with each assay. Results were expressed as international units/ml (IU/ml).

#### 2.5. Anti-*Leishmania* IgE levels

The leishmanial antigen ( $2 \times 10^7$  ml<sup>-1</sup> fixed *Leishmania mexicana* MHOM/BZ/82/BEL21 promastigotes) was coated onto 96-well microplates (Immunolon IV Dynatech Laboratories Inc., Virginia, USA) and incubated overnight at 4 °C. Excess antigen was washed off with PBS-T and blocked for 2 h at 37 °C with 1% bovine serum albumin. The undiluted test sera were plated and incubated for 1 h at 37 °C. After further washes with PBS-T, peroxidase-conjugated murine anti-human IgE (Sigma) was added at 1:1000 dilution and incubated for 1 h at 37 °C. The washing process was repeated and OPD plus H<sub>2</sub>O<sub>2</sub> were added. The OD at 490 nm was read with an ELISA reader.

#### 2.6. Soluble CD23 levels

A capture ELISA was used to detect soluble CD23. Briefly, Dynatech Immunolon IV plates were coated

overnight at 4 °C with 6 µg per well murine monoclonal antibody anti-human CD23 (Biosdesign International, NY, USA) in coating buffer (carbonated buffer, pH 9.6) and then blocked with 5% BSA in PBS-T for 2 h at 37 °C. Serum samples (undiluted) were added and incubated for 1 h at 37 °C. After washing, peroxidase-conjugated murine anti-human CD23 (1:1000; Biosdesign International, NY, USA) was added and incubated for 1 h at 37 °C. OPD and H<sub>2</sub>O<sub>2</sub> were added as substrate to develop the reaction. The OD at 490 nm was read with an ELISA reader. Results were expressed as OD.

#### 2.7. Statistical analysis

The Mann–Whitney test was used to test for statistical significance between patient groups. A value of  $P < 0.05$  was considered statistically significant. The Spearman's rank correlation coefficient was used to determine the significance of the different correlations calculated.

Cut-offs were set to define responder status in IgE anti-*Leishmania* ( $\geq 0.134$  OD) and for NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> production ( $\geq 312.2$ ), based on endemic control responses. Mean OD of IgE anti-*Leishmania* or NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> levels plus 2 standard deviations were considered as cut-off values. Fisher's exact test was used to determine whether differences in the proportion of responders:non-responders observed between groups were statistically significant.

### 3. Results

In general, the different groups showed a high variability in the responses evaluated (IgE, sCD23 and NO). Similar heterogeneous responses have been observed in ACL patients in lymphocyte proliferation and cytokine production assays (Cabrera, 1994). Different host genetic make up could account for this observation.

#### 3.1. Nitrate/nitrite in serum from ACL patients and endemic volunteers

Fig. 1 shows the in vivo production of NO in ACL patients and healthy controls. The proportion of responders and the concentration of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> in

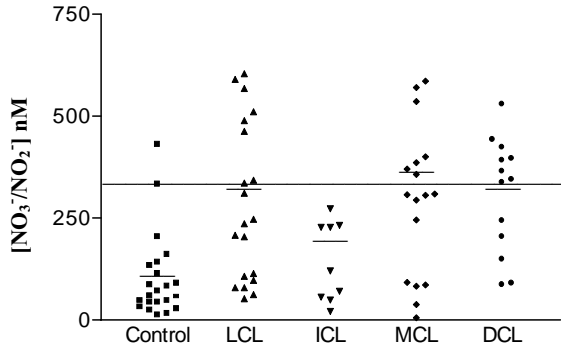


Fig. 1. Concentration of  $\text{NO}_3^-/\text{NO}_2^-$  in serum from American cutaneous leishmaniasis patients. Points represent individual subjects. The horizontal line represents the cut-off value. Crossbars indicate means  $\text{NO}_3^-/\text{NO}_2^-$  levels.  $0.001 < P < 0.01$  for differences between ICL patients and healthy individuals versus LCL, MCL and DCL patients.

serum from LCL, MCL and DCL patients were significantly higher ( $0.05 < P < 0.001$ ) compared to ICL patients and endemic controls. Interestingly, we observed an increased proportion of responders in LCL compared to DCL patients ( $P < 0.05$ ).

### 3.2. Total and anti-Leishmania serum IgE

Total serum IgE (Table 1) was higher in the four patient groups compared to healthy volunteers ( $P < 0.0001$ ). Similar results (Table 1, Fig. 2) were found for anti-Leishmania IgE. This antibody showed signif-

Table 1  
Total and anti-Leishmania IgE levels in American cutaneous leishmaniasis patients and endemic volunteers

Subjects	Total IgE	Specific IgE
Controls	300 (1088) <sup>a</sup>	$0.059 \pm 0.021^b$ (0.0)
LCL	1595 (5656)	$0.207 \pm 0.083$ (73)
ICL	3339 (9539)	$0.206 \pm 0.118$ (67)
MCL	1603 (7267)	$0.260 \pm 0.126$ (81)
DCL	2065 (7166)	$0.282 \pm 0.184$ (88)

Geometric mean (GM) (GM + SD) IU/ml for total IgE or mean  $\pm$  SD OD for specific IgE are shown with percent responders in parentheses.

<sup>a</sup>  $P < 0.0001$  for differences between controls and LCL, MCL and DCL patients.

<sup>b</sup>  $0.0001 < P < 0.01$  for differences between controls and LCL, ICL, MCL and DCL patients.

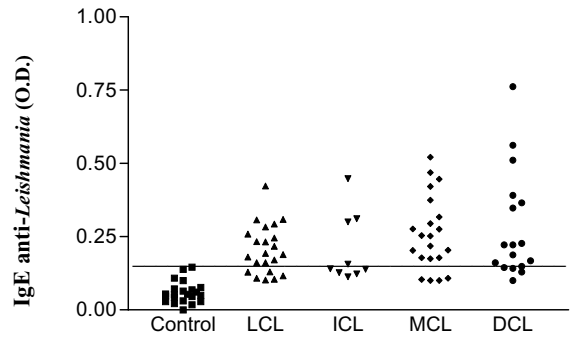


Fig. 2. Serum IgE anti-Leishmania levels in American cutaneous leishmaniasis patients. Points represent individual subjects. The horizontal line represents the cut-off value.  $0.0001 < P < 0.01$  for differences in mean and in the proportion of responders between controls and LCL, ICL, MCL and DCL patients.

icantly increased levels and a higher proportion of responders among patient groups (LCL, ICL, MCL and DCL) versus healthy individuals ( $0.001 < P < 0.01$ ).

### 3.3. Soluble CD23 levels

Serum soluble CD23 levels from all patient groups were higher when compared with endemic controls ( $0.001 < P < 0.05$ ) (Fig. 3). Interestingly, MCL and DCL patients showed higher levels of sCD23 than LCL patients ( $P < 0.01$ ).

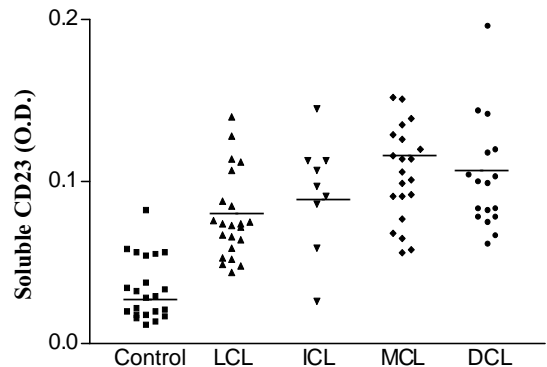


Fig. 3. Soluble CD23 levels in endemic volunteers and American cutaneous leishmaniasis patients. Points represent individual subjects. Crossbars indicate mean sCD23 levels.  $0.0001 < P < 0.001$  for differences between controls versus LCL, ICL, MCL and DCL patients.  $P < 0.01$  for differences in between LCL patients versus MCL and DCL patients.

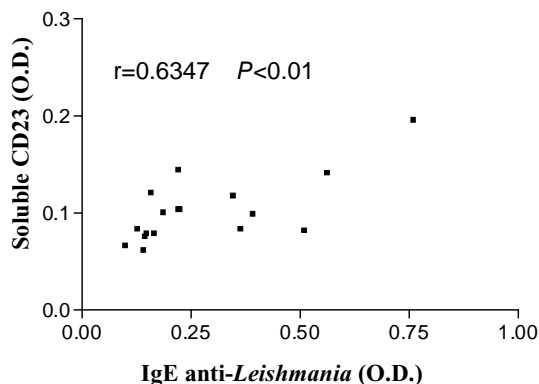


Fig. 4. Correlation analysis between specific IgE and soluble CD23 in DCL patients.

#### 3.4. Correlation between NO, sCD23 and total and specific IgE

In order to determine whether the in vivo production of NO, sCD23 and total and specific IgE show a different relationship among the three clinical manifestations of ACL, we analysed the correlation among these parameters by Spearman's rank correlation coefficient. Interestingly, ACL patients (LCL, ICL, MCL and DCL patients pooled) had a very significant positive correlation between sCD23 and anti-*Leishmania* IgE ( $r = 0.459$ ,  $P < 0.001$ ). Among this group, DCL patients showed a positive correlation between both parameters (Fig. 4). Regarding the specific IgE responses, we found that 22 out of 45 ACL patients were responders by NO production and specific IgE. In contrast, 2 out of 14 non-responders by specific IgE, showed positive NO levels ( $P < 0.01$ ). In addition, a higher percentage of responders by specific IgE were also responders by NO production in LCL (40%) and DCL (62%) patients, compared to non-responder patients by specific IgE ( $P < 0.0001$ ). The total and anti-*Leishmania* IgE did not show any significant correlation.

#### 4. Discussion

FcεRII/CD23, the low affinity IgE receptor, plays a key signaling role in several immunologic processes. Human CD23 has two isoforms: a and b. The latter belongs to the c-type lectin family and is

an activation antigen expressed by human, monkey and rat macrophages. IFN- $\gamma$ , IL-4 and IL-13 induce the expression of CD23b by human macrophages (Mossalayi et al., 1997). During activation, CD23 is cleaved by proteolysis into soluble fragments with cytokine-like activity. Moreover, sCD23 is a potent macrophage stimulator (Armant et al., 1994, 1995). As mentioned before, it has been shown that ligation of membrane associated CD23 or stimulation with recombinant sCD23 elicits monocytes to release nitrite and L-citruline (Paul-Eugene et al., 1995). Therefore, both transmembrane and soluble CD23 regulate NO generation by human macrophages. In cutaneous leishmaniasis lesions (the mild form of the disease), relatively high CD23 gene expression has been detected compared to normal human skin (Vouldoukis et al., 1994). The crosslinking of CD23 by IgE and anti-IgE immune complexes (IgE-IC) or by specific monoclonal antibodies has been shown to induce proinflammatory responses in human and rat macrophages (Mossalayi et al., 1997). In addition, IgE-IC, via CD23 binding, induces intracellular killing of *L. major* in human monocyte-derived macrophages through NO production (Vouldoukis et al., 1995).

The present study shows for the first time the relationship between sCD23, IgE and NO with the more severe forms of ACL: MCL, DCL and ICL, which include atypical forms of the disease.

Previous studies on IgE antibodies in ACL patients are limited to cutaneous localised and mucocutaneous forms of disease or in a pooled group of ACL patients (Lynch et al., 1982; Afchain et al., 1983; O'Neil et al., 1993; Sousa-Atta et al., 2002). These studies had shown contrasting results, which could be due to variation in test antigens. In our study, total serum IgE levels were higher in ACL patients. Specific IgE was increased among ACL patients, particularly in MCL and DCL patients, which may reflect a stimulation of the CD4<sup>+</sup> Th2 response. Non-healing disease in cutaneous leishmaniasis is associated with a CD4<sup>+</sup> Th2 response, which is characterised by the production of IL-4, IL-5, IL-10 and others. These cytokines regulate eosinophilopoiesis and isotype switching to IgE (Scott et al., 1988; Coffman and Corty, 1986; Heinzl et al., 1989). Th2 cytokines had been identified in DCL patients and a mixture of Th1 and Th2 cytokines in MCL and ICL patients (Cáceres-Dittmar et al., 1993;

Pirmez et al., 1993; Castés et al., 1996; Díaz et al., 2002).

The increased serum  $\text{NO}_3^-/\text{NO}_2^-$  concentration observed in LCL, MCL and DCL patients indicates that NO may play a different role across the spectrum of the disease. Interestingly, the chronic forms of ACL: DCL and MCL patients characterised by opposite responsiveness to *Leishmania* spp., showed a similar high NO response. MCL patients may genetically be more susceptible to the detrimental effect mediated by NO. On the other hand, the infecting *Leishmania* strains are different in both groups of patients. Diffuse and mucocutaneous leishmaniasis have been associated with infection with members of *L. mexicana* and *Leishmania braziliensis* complexes, respectively (Convit et al., 1993). Probably, *L. mexicana* is more tolerant to the leishmanicidal mechanisms dependent on NO. Then, the parasite persists and disseminates in DCL patients.

MCL patients showed the highest levels of sCD23, followed by DCL patients. This could be related to local cytokine production. Upon stimulation with various cytokines, notably IL-4 and IFN- $\gamma$ , human monocytes/macrophages express CD23 and release sCD23 fragments. Both cytokines are secreted during the early phases of the immune response and as part of Th1 and Th2 responses, as mentioned before. Alternatively, some parasite enzymes such as the metallo-proteinase GP63, which is the major parasite glycoprotein, could be implicated in cleavage of CD23.

DCL patients had high levels of sCD23, total and specific IgE; specific IgE and sCD23 were positively associated. In addition, we detected high nitrite levels. Nevertheless, these factors were not associated with cure in these patients. In contrast, MCL patients showed a higher production of sCD23 and NO with significant levels of specific IgE. Crosslinking of CD23 leads to induction of iNOS in human monocytes. Since IgE binds CD23, IgE may account for the production of NO. CD23-mediated generation of NO is known to be involved in TNF- $\alpha$  production, which is increased in MCL patients (Castés et al., 1993). In addition, CD23-induced iNOS activation could ultimately result in NO-mediated toxic effects through the inactivation of enzymes with haeme groups or an iron-sulphur cluster, the nitrosilation of proteins, or the interaction with superoxide to yield the highly

toxic peroxynitrite  $\bullet\text{ONOO}$ . Under pathological situations the CD23/NO transduction cascade could be enhanced to provide a non-specific defence mechanism. Although MCL and ICL patients belong to the middle portion of the ACL spectrum, characterized by a similar cytokine profile, both groups had different sCD23, NO and anti-*Leishmania* IgE responses. ICL patients do not show mucosal lesions. Interestingly, ICL patients did not produce significant levels of NO. In addition, similar specific IgE and sCD23 responses were observed between ICL and LCL patients.

Finally, LCL patients had significant levels of sCD23, specific IgE and NO. Moreover, 40% of them were responders by specific IgE and NO, which may contribute to parasite killing. Nevertheless, other leishmanicidal mechanisms such as those mediated via IFN- $\gamma$  receptor should be considered.

Overall results suggest that CD23/NO/IgE could result in *Leishmania* killing or disease chronicity depending on predominant local cytokines and other undefined factors.

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