Intestinal helminthiasis as a relevant factor on immunological markers status of cutaneous leishmaniasis patients

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Abstract

**Background:** The intestinal helminths and the tegumentary leishmaniasis are frequent in rain forest area of Bolivia by absence of basic sanitation services and the natural presence of sand fly, vector of Leishmania parasites. Each one of these infections triggers a specific immune response by the host, nevertheless there is scant information regarding the behaviour of immune response during simultaneous Leishmania and intestinal helminths infections. The purpose of this study was contributing to the knowledge on this matter.

**Results:** Forty-four cutaneous leishmaniasis patients and thirty controls entered in the study. The percentage of eosinophils from peripheral blood and plasma concentration of IgE and TNF-α were increased at after antimony treatment, respect the values presented before treatment in Albendazole and Non Albendazole groups, those increases were not statically significant. A decrease between before and after antimony treatment was observed in the ratio CD4/CD8 in both groups, but only in the receiving Albendazole group it was presented differences statistically significant.

**Conclusions:** Several changes in the status of immunological markers including the number of eosinophils were found after the antimony treatment of patients. No additional clinical, neither immunological benefit of Albendazole treatment could be documented probably because the contact of individuals with helminths is long-standing.

**Background**
The intestinal helminths are the most widely disseminated infectious agents in the human population [1], likewise, the tegumentary leishmaniasis a zoonotic infection [2, 3], is a serious public health problem in the Americas region and also at worldwide [4]. Both infections are frequent in Bolivian’s rain forest area characterized by absence of basic sanitation services [5, 6], and because it is the natural ecosystem of *Lutzomyia* species, vector of Leishmania parasites [7, 8].

The human infection by intestinal helminths triggers a strong inflammatory response in the host with production of NO [9], IgE, IL-2, IL-4, IL-10 [10, 11], and eosinophilia [12]. Likewise, the American tegumentary leishmaniasis also stimulates complex immune and inflammatory responses at local level in the skin with production of IL-4, IL-6 and IL-22 by cells Th17 [13, 14]. However, the chronic
mucous form of the infection is characterized by an immune response oriented towards Th2 favouring a persistent inflammation at local and/or systemic level [15, 16, 17].

Previous evidences demonstrated as well that during leishmania infection the changes in the proportion of CD4 and CD8 T-cells with the consequent modification of CD4/CD8 ratio with values as great as 1.8 or more were frequent [18, 19], with the decrease of this value after therapy for leishmaniasis [20, 21].

Although it is well known the behaviour of immune response triggered by intestinal helminths and Leishmania parasite in the host, nevertheless there is scant information regarding of the behaviour of immune system during a concomitant infection of both, Leishmania and intestinal helminths. One report indicates an increased plasma concentration of IgE and IL-5 in subjects with both infections before the anti leishmanial therapy [22]. The purpose of this study was contributing to the knowledge regarding the behaviour of immune response in a concomitant intestinal helminths and leishmania infections before and after antileishmanial therapy in Bolivian cutaneous leishmaniasis patients.

**Results**

Forty-four cutaneous leishmaniasis patients and thirty controls entered in the study. The demographic data of patient and control groups is presented in the table 1. The number of male is greater than female in both groups.

*Clinical Observations*

The healing process of cutaneous lesions, in the patient groups (treated or not with Albendazole) did not present statistical significant changes between before (T0) and after (T1) antimony treatment (Chi square test, \( p > 0.05 \)) (Figure 1). However, there were significant differences in the reduction of area of lesion between T0 and T1 in both Albendazole and non Albendazole groups (Wilcoxon test, \( p = 0.001 \) in both cases) and also between groups (Mann-Whitney test, \( p = 0.003 \)) (Figure 2), even when a thirty percent of subjects in both groups did not complete the healing process after intervention (figure 1).

*Changes in immunological markers*

The number of eosinophil from peripheral blood (expressed as fraction of white blood cells), were
increased at T1 respect the number presented in T0 in both (Albendazole and Non Albendazole groups) (Table 2), those increases were not statically significant (Wilcoxon test, \( p > 0.05 \) in both groups). Similar behaviours were presented by the IgE and TNF-\( \alpha \) plasma concentration (Wilcoxon test, \( p > 0.05 \)), and a small decrease of IL-17 plasma concentration but also without statistical significance (Table 2). A decrease between T0 and T1 was observed in the ratio CD4/CD8 in both groups, but only in the receiving Albendazole group it was presented differences statistically significant (Wilcoxon test, \( p = 0.034 \); Table 2).

The comparison of these immunological markers between patients and controls (Table 3) evidenced a tendency of increased values of number of eosinophil’s and plasma concentration of IgE and IL-17 in the patients group previous to start the intervention and a small decrease in the values ten days after intervention for CD4/CD8 ratio and IL-17. Nevertheless, those continued increased in comparison with the values presented by the subjects control (Mann-Whitney test \( p < 0.05 \) in all the cases). However, it is more remarkable the great increase of number of eosinophil and IgE plasma concentration in the patient group ten days after the intervention.

Discussion
The effect of persisting intestinal helminths in clinical changes on lesions and the behaviour of immunological markers representative of Th2 or Th1 orientation of immune response of cutaneous leishmaniasis Bolivian patients who received pharmacological treatment with antimony were explored in this study.

The healing process in 70% of the patients was completed after the treatment with antimony, but there were persistent lesions in the 30% of the patients (Fig. 1), at similar manner reported by others [23, 24]. In this study, the healing process of lesions was independent of the administration of Albendazole. Probably because of the administration of this drug (400 mg as single dose) was not efficient in the eradication of some intestinal helminth [25, 26]. Although was previously indicated that the coexistence of helminth infection and cutaneous leishmaniasis produced a weak response to therapy [22], also was demonstrated that the early antihelminthic treatment did not improve the cure rates of leishmaniasis [27].
Regarding the behaviour of immunological markers, the findings were an increased number of eosinophil’s and the IgE plasma concentration in the patient groups after intervention (T1) (Table 2), and also in comparison with the values presented by the controls (Table 3). This behaviour responds probably to a pre-existing type Th2 immune response triggered by intestinal helminth infection [28] exacerbated by the antimony compounds treatment due the toxicity of the antimony [29] or the additives used in the formulation of the drug [30], although the report of allergic reactions by treatment of leishmaniasis with antimony is reported in few occasions [23, 31, 32, 33]. It was also an interesting finding the decrease of plasma concentration of IL-17 after antimony therapy, although there was not a statistical significance between before and after intervention (Table 2), the comparison with the controls showed a persistent high concentration of this molecule ($p = 0.001$, Table 3). That finding is in line of a previous study in animal model that indicate the role of IL-17 in the activation and recruitment of neutrophils in the progression of cutaneous leishmaniasis [34]. Nevertheless, the decreasing of the level of IL-17 plasma concentration associated with the absence of changes in plasma concentration of TNF-α after antimony treatment reflect the control of leishmania infection by the therapy with a Th1 orientation of immune response [21, 35, 36]. Concurrently with this results was also observed a reduction of CD4/CD8 index after intervention in both groups although continued increased respect to the control group (Tables 2 and 3 respectively), at similar manner of other studies in which the value of the index was around 1.5 and evidence of healing of cutaneous lesions [18, 20, 21]. However, the observed changes in the behaviour of these markers were not influenced by the Albendazole therapy, probably because individuals with intestinal helminths are long-standing and therefore that chronic contact conditions a tendency for a Th2 response.

Conclusions
This study is the first one that explores the effect of intestinal helminthiasis on the status of immunological markers of Bolivian cutaneous Leishmaniasis patients. Several changes in the status of immunological markers including the number of eosinophils were found after the antimony treatment of patients. No additional clinical neither immunological benefit of Albendazole treatment could be
documented probably because the long-standing contact of individuals with helminths.

Methods

**Study design and ethical consideration**

The patients and controls were recruited in the municipal hospital of Villa Tunari, a tropical town of Bolivia endemic for intestinal helminthiasis, because did not have a basic sanitation services, in the period 2013 to 2015.

A total of 54 patients with confirmed cutaneous leishmaniasis and 40 control subjects, both permanent residents in the tropical area were invited to participate in the study. But it just started with 44 patients and 30 control subjects. The patients and controls were age-sex matched.

For the patients were considered the following exclusion criteria: pregnancy, chronic non-communicable diseases, concomitant infectious diseases such as Tuberculosis and HIV/AIDS, superinfection by bacteria of the cutaneous ulcerative lesions and previous therapeutic failure to antimony compounds treatment. The control subjects were clinically no evidences of any diseases at time of recruitment.

Ethic permission for all procedures involving human volunteers was obtained from the Bolivian Ethic Committee of the Medical Faculty, Universidad Mayor de San Simón.

The patients and controls signed a write consent accepting to participate in the study.

**Intestinal helminths and cutaneous Leishmaniasis treatment**

The patients were randomly assigned to groups: “A” which received the Albendazole to treat the intestinal helminths and posteriorly the antimony compounds as treatment of cutaneous Leishmaniasis and the group “B” which received only the antimony compounds for the treatment of cutaneous Leishmaniasis.

The Albendazole was administered orally (Dose 400mg) once according to WHO recommendations [37].

The antimony compounds were administered intramuscularly (Dose/Kg/weight) daily by 20 days according the norms of Bolivian Health ministry [38].
**Materials**

Venous blood tubes of the Vacutainer® systems (cat no 366430 and 367587) were obtained from Becton Dickinson AB (New Jersey, USA). The Panotic fast staining system (cat no 620529) was obtained from LB (Laborclin, Saõ Paulo, Brazil). The IgE ELISA kit (cat no 2525-300A) was obtained from Monobind Inc. (Lake Forest, California, United States of America). The TNF-α (cat no DTA00C) and IL-17 (cat no D1700) ELISA kits were obtained from R&D systems (Minneapolis, United States of America). The CD4, CD8 kit (cat no 340167) was obtained from Becton Dickinson FACSCount™ (United States of America).

**Blood samples collection**

The patients were blood sampled twice, once before therapy intervention (T0) and then ten days after ending the treatment with antimony compounds (T1). The control subjects were blood-sampled once, at the beginning of the study.

The blood samples were collected by venepuncture after 12 h of fasting in all of subjects in two tubes. The samples for haematology were processed immediately and one aliquot was sent to Medical Faculty laboratory (LABIMED) for the assessment of CD4/CD8 T cells ratio. The samples collected in tubes without additives were centrifuged, fractioned in aliquots of 500 µL and preserved at -80°C until processing by lab tests.

**Laboratory test for diagnostic of cutaneous leishmaniasis**

The leishmania parasite (amastigote forms) was detected in stained smears of scrapings of border of cutaneous ulcerative lesions according with the procedures of Bolivian Health ministry [39] in the laboratory of Villa Tunari Hospital.

**Laboratory test in blood samples**

The haematological test was performed in the laboratories of Villa Tunari Hospital. The following parameters were measured: Total number of white blood cells, neutrophils, eosinophil and
lymphocytes (as fraction of white blood cells), the Total number of red blood cells, haemoglobin and haematocrit in an autohaematology analyser BC-3000 Plus, Mindray™ (Nanshan, Shenzhen, P. R. China).

The plasma concentration of IgE, IL-17 and TNF-α were determined in the laboratories of CUMETROP by ELISA test kit according with the assay instructions. The detection limit was 0.125 IU/mL for IgE and 3pg/mL for IL-17 and TNF-α (Data supplied by the manufacturers).

The CD4/CD8 T cells ratio was performed in LABIMED laboratory by count of CD4 and CD8 T cells in the BD FACSCount™ and using a kit BD for determination of CD markers according with the assay instructions. Briefly, the procedure consisted in the petrification of the cells with formaldehyde solution, subsequently fluorescein-labelled anti-CD4, CD8 and CD3 monoclonal antibodies were added and the intensity of the fluorescence emitted by the complexes formed was measured in a FACSCount™. The results were expressed in number and percentage of CD4, CD8 cells and also expressed as CD4 / CD8 ratio.

**Lesion healing assessment**

The cutaneous lesions of patients were measured twice; once at the beginning of treatment with antimony compounds and ten days after complete the last dose of treatment. The following measurements were made: Area of ulcerative lesion (mm²), presence of raised edge of lesion and presence of humidity. The healing lesions were evaluated as reduction of the area expressed in mm², absence of raised edge and humidity.

**Statistical analysis**

The SPSS software v. 22 was used. The Chi square test was used for comparison of individual characteristics of the lesions of the groups “A” (Albendazole) and “B” (Non Albendazole). Wilcoxon signed rank test was used to compare the same variable of patients in T0 and T1 for the two groups. The Mann-Whitney test was used for testing the significance of differences between two non-normally distributed continuous variables such as area of lesion (groups A and B), plasma concentration of
immunological markers and Number of eosinophil and CD4/CD8T cell ratio between patients and controls. It was considered in all of cases as level of statistical significance p<0.05

Declarations

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Author´s contributions

AV-O and MG-R, designed the study, carried out the immunological measurement, performed the statistical analysis of the data and drafted the manuscript. ER, performed the clinical diagnostic of cutaneous leishmaniasis, performed the discrimination of the patient according inclusion-exclusion criteria and contributed in the drafting of the manuscript MA and FB, recruited the study participant, performed collection of data and samples and participated to the sample analysis. AV-O, ER, MA and MG-R, revised the critical content of the manuscript. All the authors read and approved the final version of manuscript.

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Availability of data and materials

All data used and analysed during study are included within this article and supporting files.

Consent for publication

Not applicable

Competing interest

The authors declare no competing interest

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Abbreviations

IgE: Immunoglobulin E; IL-2: interleukin -2; IL-4: interleukin - 4; IL-5: interleukin - 5; IL-6: interleukin - 6; IL-10: interleukin - 10; IL-17: interleukin - 17, IL-22: interleukin - 22; TNF-α: Tumour necrosis factor -α; Th-1: T helper -1; Th-2: T helper - 2; Th-17: T helper - 17; CD: Cluster of differentiation of T lymphocytes ; CD: Cluster of differentiation 3; CD4: Cluster of differentiation 4; CD8: Cluster of differentiation 8; CD4/CD8: Ratio of CD4 and CD8 sub populations of T lymphocytes; T0: Before antimony treatment ; T1: After antimony treatment; HIV/AIDS: Human immune deficiency virus/Acquired immunodeficiency syndrome; WHO: World Health Organization; LABIMED: Laboratorios de Investigación Médica (Spanish); CUMETROP: Centro Universitario de Medicina Tropical (Spanish).

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### Tables

#### Table 1. Demographic data of cutaneous Leishmaniasis patients and its controls.

| Parameters          | Patients (n=44) | Controls (n=30) |
|---------------------|----------------|-----------------|
| Age (years)<sup>a</sup> | 29 (13 – 50)   | 43 (15 – 62)    |
| Female<sup>b</sup>   | 17 (39)        | 10 (33)         |
| Male<sup>b</sup>     | 30 (68)        | 19 (63)         |

<sup>a</sup> Median (range); <sup>b</sup> Frequency (percentage)

#### Table 2. Changes observed in biological markers before and after intervention in cutaneous Leishmaniasis patient groups.

| Parameters                  | T0        | T1        | p Values (Wilcoxon test) |
|-----------------------------|-----------|-----------|--------------------------|
| Albendazole group (n=22)    |           |           |                          |
| Eosinophil*                 | 4 (7)     | 8 (12)    | 0.276                    |
| CD4/CD8 T cells ratio       | 2.2 (0.6) | 1.8 (0.3) | 0.034                    |
| IgE (IU/mL)                 | 798 (559) | 799 (376) | 0.836                    |
| TNF-α (pg/mL)               | 16 (8)    | 19 (24)   | 0.207                    |
| IL-17 (pg/mL)               | 50 (12)   | 48 (22)   | 0.689                    |
| No Albendazole group (n=22) |           |           |                          |
| Eosinophil*                 | 6 (11)    | 7 (11)    | 0.433                    |
| CD4/CD8 T cells ratio       | 2.1 (0.8) | 1.8 (0.6) | 0.068                    |
| IgE (IU/mL)                 | 757 (248) | 824 (419) | 0.293                    |
| TNF-α (pg/mL)               | 16 (6)    | 16 (5)    | 0.212                    |
| IL-17 (pg/mL)               | 51 (11)   | 45 (10)   | 0.656                    |

Data are expressed as the median and IQR.

* Data correspond to count of cells expressed in percentage; T0= before intervention, T1= ten days after intervention; Level of significance < 0.05
Table 3. Comparison of biological markers presented by cutaneous Leishmaniasis patients and control subjects.

| Parameters                  | Patients (n=44) | Controls (n=30) | p Values T1-T0 vs. Controls* |
|-----------------------------|-----------------|-----------------|-----------------------------|
|                             | T0              | T1              |                             |
| Eosinophil<sup>a</sup>     | 4(8)            | 8(11)           | 3(3)                        | 0.01                        |
| CD4/CD8 T cells ratio       | 2.2(0.6)        | 1.8(0.3)        | 1.5(1.1)                    | 0.008                       |
| IgE (IU/mL)                 | 795(265)        | 805(372.25)     | 189(98)                     | 0.001                       |
| TNF-α (pg/mL)               | 16(6)           | 17(6)           | 21(14.5)                    | 0.001                       |
| IL-17 (pg/mL)               | 51(11)          | 48(12)          | 48(10.5)                    | 0.001                       |

Data are expressed as the median and IQR.

<sup>a</sup> Data correspond to count of cells expressed in percentage; *Mann-Whitney test Level of significance < 0.05

Figures
Clinical characteristics of cutaneous Leishmaniasis lesions in Albendazole and Non Albendazole groups (expressed as percent of occurrence). T0 = Before antimony treatment; T1 = Ten days after completion of antimony treatment. Comparison between groups (Chi square test, p≥0.05)
Figure 2

Changes of the lesion area of patients with cutaneous Leishmaniasis in Albendazole and Non Albendazole groups. Comparison T1 vs.T0 (Wilcoxon test, p=0.001 for both groups).

Comparison between groups (Mann-Whitney test, p=0.003)

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