Immunological correlates of cure in the first American Cutaneous Leishmaniasis patient treated by immunotherapy in Argentina. A case report.

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Abstract. A patient with localized cutaneous leishmaniasis due to Leishmania (Leishmania) amazonensis infection was treated with an antigen containing heat-killed L. (L.) amazonensis promastigotes plus BCG. Expression of T-cell differentiation, memory and senescence receptors markers were analyzed on T cell subpopulations, in order to establish the correlation between the percentages of expression of these receptors and his clinical status, at different stages of his follow up. The following case reports on the achievement of a successful clinical outcome with complete resolution after receiving immunotherapy. A thorough clinical and immunological follow up supporting the healing process of this patient’s lesion is presented in detail.
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Palabras clave: leishmaniasis, inmunología, tratamiento.

Resumen. Un paciente con leishmaniasis cutánea localizada producida por Leishmania (Leishmania) amazonensis fue tratado con un antígeno compuesto por promastigotes de L. (L.) amazonensis muertos por calor combinado con BCG. Se analizó la expresión de distintos receptores de diferenciación, de memoria y de senescencia en las subpoblaciones de células T, con el fin de establecer una relación entre los porcentajes de expresión de dichos receptores y la clínica del paciente en diferentes momentos del seguimiento. Se reporta en este caso un resultado exitoso, con resolución completa de la lesión después de recibir la inmunoterapia, y se presenta en detalle un seguimiento clínico e inmunológico completo durante el proceso de curación.


INTRODUCTION

Conventional therapy of American Cutaneous Leishmaniasis (ACL) is based on treatment regimens with pentavalent antimonials such as meglumine antimoniate. However, antimonial derivatives should be used with caution due to their potential systemic and local side effects, especially in patients with underlying heart disease and particularly, those with conduction and rhythm disorders. On the other hand, prolonged periods of treatment as well as the occurrence of cases with primary and secondary unresponsiveness occurring in neighbouring countries such as Perú (1, 2), severely restrain the use of these drugs and has prompted the quest for newer and safer therapeutic alternatives.

An alternative treatment option, partially available in Argentina, is amphotericin B, a nephrotoxic drug presenting severe adverse effects. The liposomal form of amphotericin B displays a very good record of efficacy and tolerability, but it is extremely expensive. Another alternative is miltefosine, the first oral drug approved for its use in visceral and cutaneous leishmaniasis. This drug is particularly useful, combining oral administration with high efficacy and low to moderate side effects. However, this drug is also very expensive for argentine patients and post-therapy relapses have been described in Leishmania (Leishmania) amazonensis cases (3), the species involved in this presentation.

Immunotherapy (IT) as an alternative therapeutic approach for ACL has been in use and widely recommended by some groups for a long time (4, 5). Studies carried out in Venezuela by Convit et al., using a vaccine containing Leishmania promastigotes along with BCG for treating patients affected by ACL revealed 90 to 95% clinical remission rates (5-7), with absent or minimal side effects, restricted to the injection site. The rationale behind this admixture of antigen plus BCG is based on the induction and consequent reinforcement of a persistent Th1-type response (8,
Several surface antigens, referred to as human leukocyte differentiation antigens, allow distinction among lymphocyte populations. Within the T lymphocyte subpopulation, CD4+ cells, which trigger the immune response cascade and CD8+ cells, which undertake the effector functions of cell-mediated immunity, are the two main cell types. CD8+ cells secrete several effector molecules, such as perforin. CD4+ lymphocytes differentiate into two main types, producing either Th1 or Th2-type cytokines. Th1 cells secrete mainly gamma-interferon (γ-IFN), associated with protection against intracellular pathogens. Th2 cells secrete mainly IL4 and IL5 and participate in allergic reactions and in protection from metazoan parasites. Different surface markers allow the characterization of functionally different CD4+ and CD8+ lymphocytes. The CD45 antigen, expressed on the T cell surface, presents two different isoforms, RA and RO. CD45RA is characteristic of naïve T cells, and C45RO is associated with memory T cells. The switch, changing the expression of the first into the second isoform, occurs as a consequence of antigenic stimulation and leads to populations able to respond to recall antigens. T lymphocytes evolve from the naïve to the responder state in a step-wise fashion, CD27, CD28 and CD127 surface markers are expressed in early differentiation stages. Whereas CD57 (and perforin, in T CD8+ cells) are expressed in terminal differentiation stages. Furthermore, the differentiation process progressively reduces the response to the antigen, a process known as cell senescence.

Studies in chronic infections indicate that the cell phenotype is driven by antigen load and time of exposure. In leishmaniasis, it is therefore relevant to follow patients suffering different clinical stages and to test T cell responses after specific treatment. Herein, we report the first case of ACL treated with IT in Argentina who reached a complete cure. The data from the clinical and immunological evaluation of the patient under treatment are also presented.

**CLINICAL REPORT**

A 40 year-old male patient living in the city of Salta, Argentina, presented with an ulcerous lesion on the right leg, which progressed over a period of 8 months prior to consultation. Initially nodular, the lesion gradually increased in size, evolving into a pustulous lesion, which later ulcerated. The patient was an avid fisherman who traveled frequently to the leishmaniasis-endemic area of Las Lajitas (latitude 24° 43’ 31.3” S, longitude 60° 11’ 45.4” W), located in the Anta department of the Province of Salta. Physical examination revealed a 10 × 9 mm round ulcer with sharply raised, infiltrated bluish borders present on the anterior aspect of the right leg (Fig. 1a). The lesion was tender and pruriginous, presenting a cobblestone ulcer bed with no signs of associated secondary infection. Additionally the patient had bilateral, prominent varicose tracts with edema, eczema, hyperpigmentation and lipodermatosclerosis of both ankles, reflective of a severe peripheral vascular disease. No other alterations were found in the physical examination, except for a general increase of subcutaneous fat and a body mass index > 40 kg/m² (morbid obesity).

**Laboratory findings**

Samples from the lesion were taken for bacteriological and mycological analysis in order to rule out entities on the differential diagnoses. Direct microscopic examination of samples smears, as well as cultures were all negative for cutaneous tuberculosis,
syphilis, and subcutaneous and systemic mycoses. Histopathological evaluation showed absence of neoplastic disease. In order to determine the possibility of leishmaniasis, samples from the ulcerous lesion were collected by scrapping with wood sticks and evaluated by Giemsa-stained smears and Leishmania-specific polymerase-chain reaction (PCR; 10). Additionally, aspirates from the lesion were cultured in appropriated growth-media for parasites and the Montenegro intradermal reaction (IDR) was performed according to standard protocols used in the laboratory (11). The IDR antigen consisted in a suspension of $6.25 \times 10^6$ promastigotes/mL autoclaved Leishmania mexicana pifanoi. Microscopic examination of the smears revealed the presence of intracellular amastigotes, whereas the IDR was positive and the PCR with generic Leishmania primers confirmed the infection with Leishmania sp. However, no growth was detected after 30 days of culture. Blood tests, including complete blood count and a comprehensive metabolic panel, were within the normal range. ELISA and indirect hemmaglutination for Chagas’ Disease were negative. Chest X rays revealed a moderate cardiomegaly but the electrocardiogram was unremarkable. An ear, nose and throat (ENT) videofibroscopic examination ruled out the presence of mucosal lesions (12).

Treatment scheme
Since this patient presented cardiovascular risk factors (cardiomegaly, obesity), we explored an alternative treatment to conventional chemotherapy. In fact, the patient was treated with IT, based on the administration of an antigen prepared by the Institute of Biomedicine (Central University of Venezuela, Ministry of Health and Social Development, Caracas, Venezuela). The antigen consists of a suspension containing $6 \times 10^9$ heat-killed L. (L.) amazonensis promastigotes/mL (MHOM/VE/84/MEL), pasteurized at 56 °C for 30 minutes. The patient received three IT doses, at 7 weeks intervals between doses. Each dose consisted of an intradermal injection, in the deltoid region (alternating 2 doses in one...
arm, and 1 dose in the other) of $6 \times 10^8$ pasteurized promastigotes and 0.075 mg of BCG vaccine (Statens Serum Institute, Copenhagen, Denmark) in a total volume of 0.12 mL (15). The use of this antigen was approved by the Ministry of Public Health, the Medical Association of Salta and by the Bioethics Committees of the Health Sciences Faculty, University of Salta and Faculty of Medicine of the University of Rosario de Santa Fe (Argentina). An informed and written consent was obtained from the patient.

**Clinical follow-up**

The patient was questioned for symptoms and subjected to physical examination every 15 days after the first IT dose, and up to 15 days after the last dose. Laboratory tests (complete blood count and compre-
hensive metabolic panel) and ENT plus general clinical examinations, were performed at 15 days and 3, 6, 9 and 12 months post treatment. ENT and full physical examination is now performed every 6 months until completing a 5-year follow up.

Laboratory results and ENT examinations remained unaltered within normal limits during the period of treatment. Physical examination, at the time of receiving his second dose, revealed a completely healed lesion (Fig. 1b), which remained so up to the last follow up appointment, 24 months post treatment (Fig. 1c). In this last clinical exam a biopsy from the lesion scar and a blood sample were taken for performing a PCR with Leishmania-specific generic primers, and the reaction did not detect DNA from Leishmania sp. in neither of these samples.

A subjective hyperthermia 48 hours after the first IT dose, as well as minor local signs and symptoms at the inoculation site were the only noted side effects, both attributable to the characteristic reactivity of the BCG vaccine. These local signs and symptoms consisted of initial erythema and induration, which appeared between 24 and 48 hours after inoculation and gradually rendered in the formation of a small pustule that fistulized, exuding purulent material 8 days after injection. The lesion was covered with a scab and surrounded with scalded skin. Thirty days after inoculation, a round, small (less than 1 cm in diameter) hyperpigmented scar persisted, evolving later into a hypopigmented, depressed lesion. The initial phlogosis was more intense and occurred sooner after the second and the third injections.

Immunological evaluation
Flow-cytometry analyses were performed on the CD4 and CD8 T cell populations from peripheral blood at different time points, to investigate the profile of specific surface and intracellular markers. Since different studies have shown a relationship between the clinical outcome of the disease and the differentiation stage of T cells (16-18), we determined the percentages of differentiation, memory and senescence receptors in these T cells. As control, 12 persons without history of leishmaniasis and free of any acute illness (N) were also studied. Samples from the patient were taken before receiving IT (T1), 3 months (T2) and 12 months post-treatment (T3). These analyses revealed that both CD4 and CD8 T cell subpopulations contained stable percentages of CD45RA+ cells along the study period (Fig. 4a). However, a tendency toward increasing CD45RO+ memory T cells, a year after finishing treatment was observed (Fig. 4b). Interestingly, the percentages of CD27+, CD28+ co-expression (Fig. 4c and 4g) and CD127+ T cells (Fig. 4d) were lower in the patient’s sample obtained before treatment when compared with the control group. Furthermore, increased expression of the senescence marker CD57 (Fig. 4e) and the cytolytic molecule perforin (Fig. 4f) was detected at that time point. These differences were more profound in CD8 T cells. These results suggest the presence of a late differentiated subset of cells in the patient’s sample that contrast with the early differentiated phenotype of the cells from the control group. As shown in Fig. 4, the percentages of the different markers began to progressively revert in later samples and one year after IT, the cytometric profile became similar to that of the control group. Fig. 5 shows the complete sequence of events relative to Day 0 (which corresponds to the first IT dose).

DISCUSSION
The efficacy of IT in ACL has been extensively evaluated, particularly by the groups of Convit et al. (5-7), who reported
Fig. 4. Expression of CD27, CD28 and other memory, differentiation and senescence markers on peripheral CD4+ and CD8+ T lymphocytes at different stages along the patient’s follow up (T1 = before initiation of immunotherapy (IT); T2 = 3 months after IT; T3 = 1 year after IT) and for normal controls (N, n= 12. Mean ± standard error). (a, b) Expression of CD45RA and CD45RO respectively. (c) Co-expression of CD27 and CD28. (d) Expression of CD127+ cells. (e) Expression of the senescence marker CD57. (f) Expression of perforin determined on permeabilized CD8+ T cells. (g) Dot plots of the CD8 region for the patient before receiving IT (T1) and 1 year after IT (T3), and one example for a normal control (N).

Fig. 5. Timeline showing the sequence of events that occurred before and after the first dose of immunotherapy. LDx: *Leishmania* diagnosis (smears, PCR, culture and Montenegro intradermal reaction). FCS: Flow citometry sample. Pha, Phb and Phc: Photos a, b and c of figure 1. IT: immunotherapy. Cl: Clinical examination. ENT: ear, nose and throat examination. Lab: Laboratory determinations. PS: PCR samples (peripheral blood and biopsy from the lesion scar).
90-95% cure rates of the cases. Also Castés et al., have studied the immunological response together with the clinical outcome in patients treated with IT. They have shown that the heat-treated *Leishmania* antigen combined with BCG activates T cells. This was reflected by an increase in the mitogen-induced lymphoproliferative response, as well as the up regulation of IL-2 (CD25+) receptor expression in peripheral lymphocytes, detected by an antibody to IL-2 surface receptor (8). More recent studies by Cabrera et al. (9) on cutaneous and mucosal leishmaniasis demonstrated that T cell activation in response to IT is antigen-specific, mediated by γ-IFN directed to both BCG and *Leishmania* antigens, and it is associated with clinical remission/cure. Both studies demonstrated that immunotherapy induces and reinforces a persistent Th1 response.

However, in spite of the increasing clinical and immunological evidence supporting the efficacy of IT for the treatment of leishmaniasis, doubts remain because of the high rates of spontaneous remission of leishmaniatic lesions. In our patient, the treatment of the disease with IT has been successful. Moreover, laboratory tests and the periodic clinical examination suggest that spontaneous remission was unlikely. Among the reasons supporting our conclusion, the antigen specificity deserves special consideration. The antigen inoculated in this patient consisted of promastigotes of the same species as those detected in the lesion, maximizing the antigenic identity between the immunizing and the target parasites, in contrast with other IT and vaccination studies (4, 5, 19). In this respect, the PCR analysis for the diagnosis is very convenient, not only because it increases the sensitivity of detection, but also because it provides useful information regarding the *Leishmania* species that should be used in IT treatment. Secondly, the success of IT is emphasized by the fact that healing occurred in spite of the adverse clinical background, namely, the vascular impairment presented by this patient (20). Thirdly and most important, the time taken for the clinical cure of the disease was much shorter than the average interval described by Convit et al. for IT period. In fact, the lesion was completely re-epithelized seven weeks after the first IT dose, and this is similar to the period required for obtaining a cure after conventional chemotherapy, which rarely exceeds 3 months for *L. (V) braziliensis*. Cures observed after this period are more connected with spontaneous remission than with retarded effect of therapy (21). Longer times to reach spontaneous healing were observed for localized cutaneous leishmaniasis produced by *L. (L) amazonensis*: even with treatment, some antigens would produce an inhibition of cellular immune responses (22).

On the other hand, we observed high consistency between clinical and immunological findings. Several studies on chronic pathologies caused by human immunodeficiency virus, hepatitis C, or *Trypanosoma cruzi* parasites, among others, indicate that the presence of memory T cells in early differentiation stages (CD27+, CD28+; CD127++; CD57−; Perforin−) is associated with stronger protective immunity and milder disease symptoms (16-18). Conversely, the presence of T cells in advanced differentiation stages (CD27−, CD28−; CD57++; Perforin+) is related to signs of senescence and more severe pathology (23-26). The reversion from a late or highly differentiated to an early differentiation phenotype could be associated to less parasitic load and good response to treatment (27). This patient presented an advanced T-cell differentiation phenotype at the time of initial diagnosis, but after the IT procedure, we detected by cytometric analysis a tendency towards a phenotype reversion, e.
g., a markers profile of an earlier T-cell differentiation phenotype. Furthermore, the percentages of the different cell populations were similar to those of the control group. This results clearly indicate that the patient had a good response to the proposed therapy.

In summary, the results obtained with this patient indicate that IT may be a safe, inexpensive and effective alternative for the treatment of ATL in our region. The efficacy of IT is remarkable, taking into account the long time required for self-healing of leishmaniotic ulcers. Obviously, distinction between slow self healing and rapid therapeutic response must be confirmed in additional patients by a phase II clinical trial.

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