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

María Fernanda García Bustos[1], Alejandra Beatriz Barrio[1], Cecilia Maria Parodi Ramoneda[1], Federico Ramos[1], María Celia Mora[1], Jacinto Convit[2] y Miguel Angel Basombrío[1].

[1]Instituto de Patología Experimental, Facultad de Ciencias de la Salud, Universidad Nacional de Salta. Salta, Argentina.
[2]Instituto de Biomedicina, Universidad Central de Venezuela, Ministerio del Poder Popular para la Salud. Caracas, Venezuela.

Keywords: leishmaniasis, immunology, treatment.

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.

Corresponding author: María Fernanda García Bustos. Instituto de Patología Experimental (UE CONICET), Facultad de Ciencias de la Salud, Universidad Nacional de Salta. Avenida Bolivia 5150 (4400), Salta Capital, República Argentina. Phone-Fax: (+54) 3874255333. E-mail: mfbustos@unsa.edu.ar.

Correlatos inmunológicos de curación en el primer paciente con Leishmaniasis Cutánea Americana tratado con inmunoterapia en Argentina. Reporte de un caso.

Invest Clin 2011; 52 (4): 365 - 375

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.

Recibido: 29-03-2011. Aceptado: 22-07-2011

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,

9), to effectively counteract the evasive nature of this intracellular parasite.

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/m^2 (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,

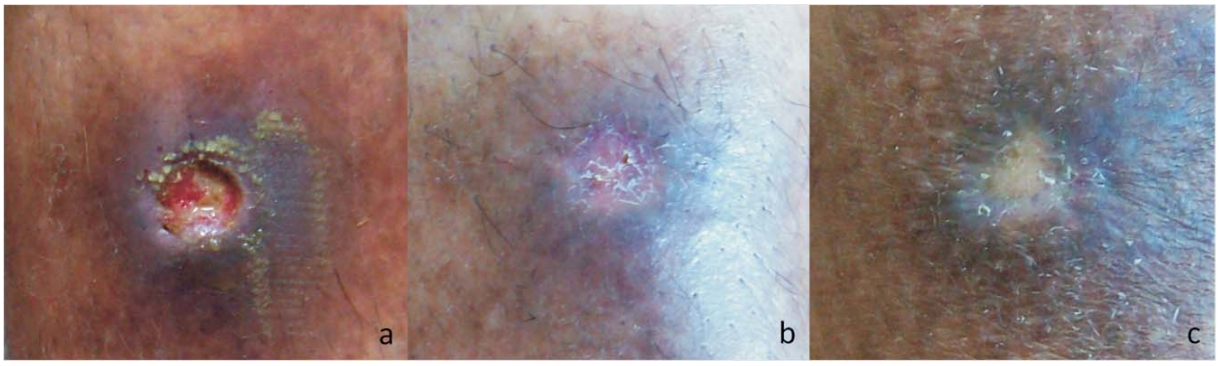

Fig. 1. Skin lesion before treatment (a), before receiving the second IT dose (b) and 24 months after finishing treatment (c).

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 6.25×10^6 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).

The DNA sample was also subjected to Polymorphism Specific-PCR (PS-PCR) for identification of *Leishmania* species. PS-PCR were performed in two steps. In the first step DNA samples were amplified with primers V1-V2 and L1-L2 for identification of the subgenus *Viannia (V.)* and *Leishmania (L.)*, respectively. In the second step, specific primers were used for species identification level (2, 13, 14). A 78 bp band identifying the *Leishmania* subgenus (Fig. 2) and a 62 bp band, characteristic of infection by *L. (L.) amazonensis* were detected (Fig. 3).

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 6×10^9 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

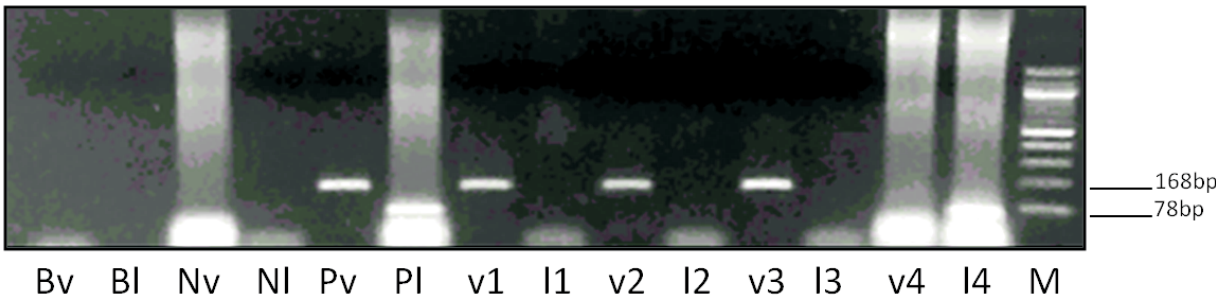

Fig. 2. Agarose gel showing amplification with V1-V2 (v) and L1-L2 (l) primers for the *Leishmania* subgenus identification. Bv and Bl: blank control without DNA. Nv: negative control for v, M2269 strain (*L. (L.) amazonensis*). Nl: negative control for l, M2903 strain (*L. (V.) braziliensis*). Pv: positive control for v, M2903 strain. Pl: positive control for l, M2269 strain. v1-l1 to v3-l3 samples positive for the *Viannia* subgenus showing the 168 bp band. v4-l4: case report sample showing the 78 bp band corresponding to the *Leishmania* subgenus. M: 100bp molecular marker.

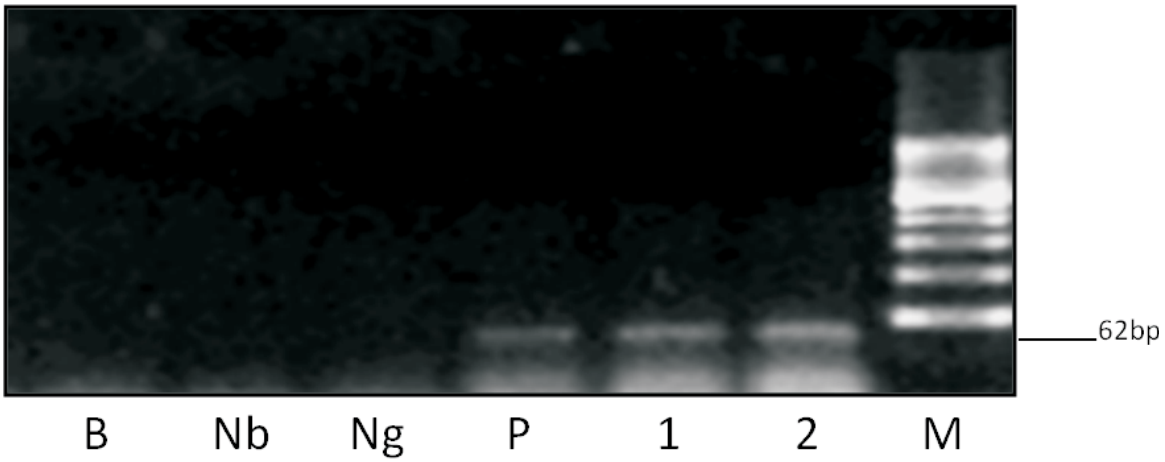

Fig. 3. Agarose gel showing amplification with A1-A2 primers for the *L. (L.) amazonensis* sp. identification. B: blank. Nb: negative control M2903 strain (*L. (V.) braziliensis*). Ng: negative control, M 4147 strain (*L. (V.) guyanensis*). P: positive control, M2269 strain (*L. (L.) amazonensis*). 1-2: case report sample showing the 62 bp band corresponding to *L. (L.) amazonensis*. M: 100bp molecular marker.

arm, and 1 dose in the other) of 6×10^8 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, exudating 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

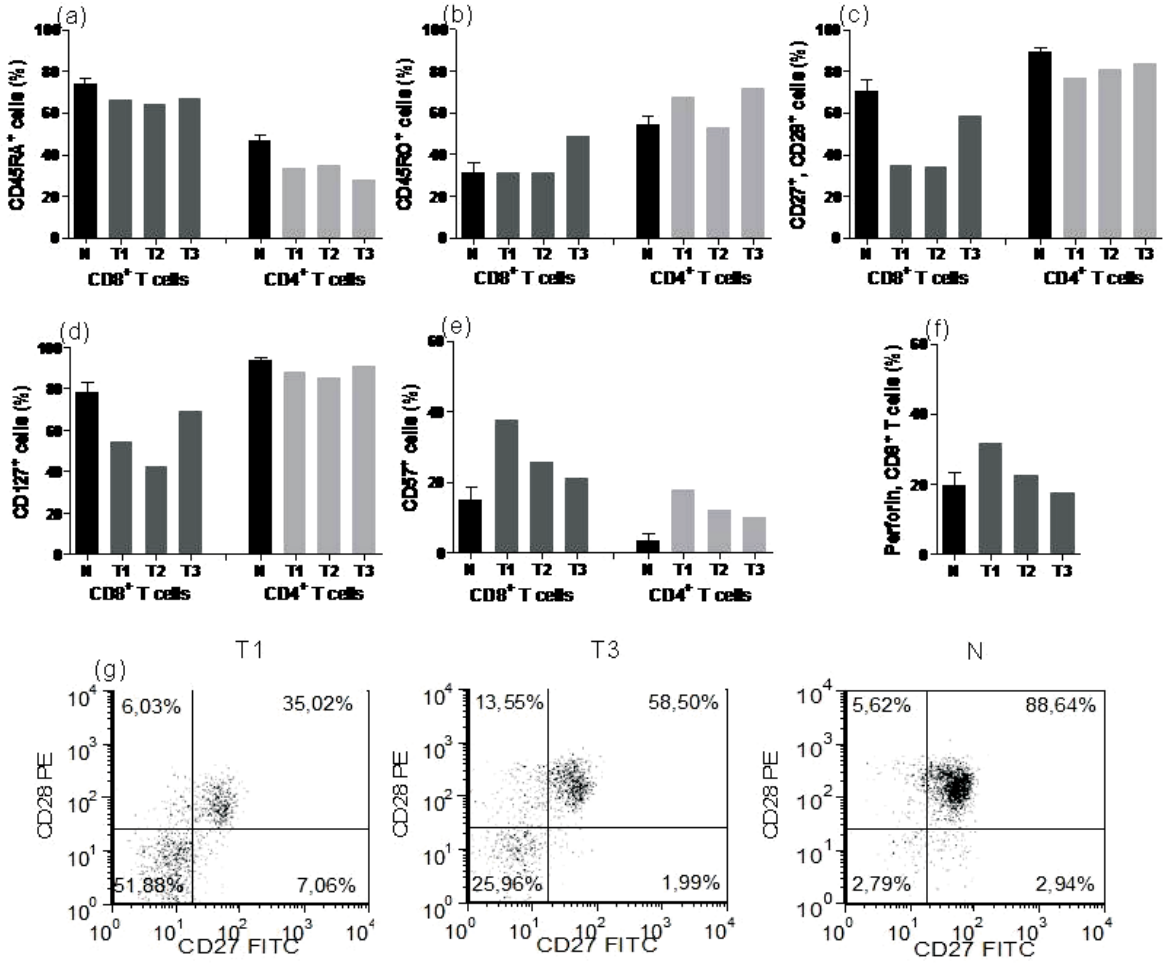

Fig. 4. Expression of CD27, CD28 and other memory, differentiation and senescence markers on peripheral CD4+ and CD8+ T lymphocytes at different stages along the patient's follow up (T1= before initiation of immunotherapy (IT); T2= 3 months after IT; T3= 1 year after IT) and for normal controls (N, n= 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).

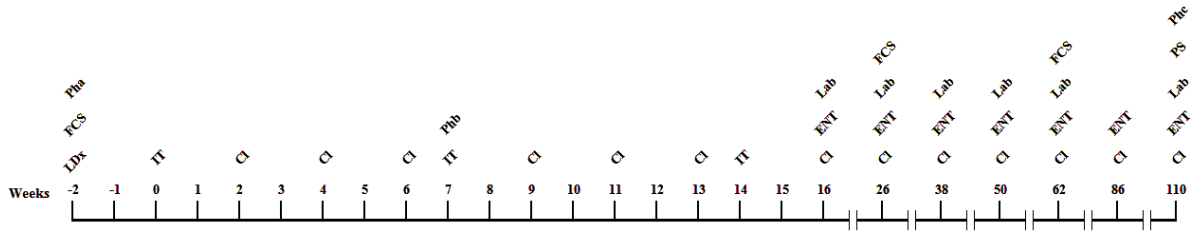

Fig. 5. Timeline showing the sequence of events that occurred before and after the first dose of immunotherapy. LDx: *Leishmania* diagnosis (smears, PCR, culture and Montenegro intradermal reaction). FCS: Flow citometry sample. Pha, Phb and Phc: Photos a, b and c of figure 1. IT: immunotherapy. Cl: Clinical 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 leishmaniotic 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.

ACKNOWLEDGEMENTS

The authors thank the Florencio Fiorini Foundation, Roemmers Foundation, Baron Foundation, CONICET (National Research Council) and National Commission "Salud Investiga" (National Health Ministry) for financial support. They also thank the Services of Dermatology and Otorhinolaryngology of the San Bernardo Hospital, Dr. Gloria Chalabe (in charge of the Dermatologic Diseases of Sanitary Interest Program, Salta Health Ministry), Dr. Alberto Gentile (Director of Epidemiology, Salta Health Ministry), Mrs. María Eugenia Gallinoto (Research Assistant, Institute of Biomedicine, Central University of Venezuela) and Dr. Luis Parada (Vice-Director of the Institute of Experimental Pathology, National University of Salta), for support and guidance.

REFERENCES

1. **Llanos-Cuentas A, Tulliano G, Araujo-Castillo R, Miranda-Verastegui C, Santamaria-Castrellon G, Ramírez L, Lazo M, De Doncker S, Boelaert M, Robays J, Dujardin J, Arévalo J, Chappuis F.** Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous Leishmaniasis in Perú. Clin Infect Dis 2008; 46: 223-231.
2. **Barrio AB, García Bustos MF, Mora MC, Parodi C, Ramos F, Moreno S, Basombrío MA.** Identification by PS-PCR of Leishmania Species and its Correlation with Clinical, Epidemiologic, and Therapeutic Characteristics in Salta, Argentina. Revista Argentina de Salud Pública 2009; 1: 30-33.
3. **Zerpa O, Ulrich M, Blanco B, Polegre M, Ávila A, Matos N, Mendoza I, Pratlong F, Ravel C, Convit J.** Diffuse cutaneous leishmaniasis responds to miltefosine but then relapses. Br J Dermatol 2007; 156: 1328-1335.
4. **Mayrink W, Carvalho Botelho AC, Araújo Magalhães P, Batista SM, Oliveira Lima A, Genaro O, da Costa CA, de Melo MN, Marques Michalick MS, Williams P, Dias M, Teixeira Caiaffa W, do Nascimento E, Machado-Coelho GLL.** Immunotherapy, immunochemotherapy and chemotherapy for American Cutaneous Leishmaniasis treatment. Rev Soc Bras Med Trop 2006; 39: 14-21.
5. **Convit J, Ulrich M, Zerpa O, Borges R, Aranzazu N, Valera M, Villarroel H, Zapata Z, Tomedes I.** Immunotherapy of American Cutaneous Leishmaniasis in Venezuela during the period 1990-99. Trans R Soc Trop Med Hyg 2003; 97: 469-472.
6. **Convit J, Rondón A, Ulrich M, Bloom B, Castellanos P, Pinardi ME, Castés M, Garcia L.** Immunotherapy versus chemotherapy in localized cutaneous leishmaniasis. Lancet 1987; 1: 401-405.
7. **Convit J, Castellanos PL, Ulrich M, Castés M, Pinardi ME, Rodriguez N, Bloom B, Formica S, Valecillos L, Bretaña A.** Immunotherapy of Localized, Intermediate, and Diffuse Forms of American Cutaneous Leishmaniasis. J Infect Dis 1989; 160: 104-115.
8. **Castés M, Moros Z, Martínez A, Trujillo D, Castellanos PL, Rondón AJ, Convit J.** Cell-mediated immunity in localized cutaneous leishmaniasis patients before and af-

ter treatment with immunotherapy or chemotherapy. Parasite Immunol 1989; 11: 211-222.
9. **Cabrera M, Blackwell JM, Castés M, Trujillo D, Convit J, Shaw MA.** Immunotherapy with live BCG plus heat killed *Leishmania* induces a T helper 1-like response in American cutaneous leishmaniasis patients. Parasite Immunol 2000; 22: 73-9.
10. **Barrio A, Mora MC, Ramos F, Moreno S, Samsón R, Basombrío MA.** Use of kDNA-based polymerase chain reaction as a sensitive and differentially diagnostic method of American Tegumentary Leishmaniasis in disease-endemic areas of Northern Argentina. Am J Trop Med Hyg 2007; 77: 636-639.
11. **Frank FM, Fernández MM, Taranto NJ, Cajal SP, Margni RA, Castro E, Thomaz-Soccol V, Malchiodi EL.** Characterization of human infection by *Leishmania spp*. in the Northwest of Argentina: immune response, double infection with *Trypanosoma cruzi* and species of *Leishmania* involved. Parasitology 2003; 126: 31-39.
12. **Boaventura VS, Cafe V, Costa J, Oliveira F, Bafica A, Rosato A, de Freitas LAR, Brodskyn C, Barral-Netto M, Barral A.** Short Report: Concomitant Early Mucosal and Cutaneous Leishmaniasis in Brazil. Am J Trop Med Hyg 2006; 75: 267-269.
13. **Marco JD, Bhutto AM, Soomro FR, Baloch JH, Barroso PA, Kato H, Uezato H, Katakura K,** Korenaga **M, Nonaka S, Hashiguchi Y.** Multilocus enzyme electrophoresis and cytochrome B gene sequencing-based identification of *Leishmania* isolates from different foci of Cutaneous Leishmaniasis in Pakistan. Am J Trop Med Hyg 2006; 75: 261-266.
14. **Mimori T, Matsumoto T, Calvopiña MH, Gómez EA, Saya H, Katakura K, Nonaka S, Shamsuzzaman SM, Hashiguchi Y.** Usefulness of sampling with cotton swab for PCR-diagnosis of cutaneous leishmaniasis in the New World. Acta Trop 2002; 81: 197-202.
15. **Convit J, Ulrich M, Polegre MA, Ávila A, Rodriguez N, Mazzedo M, Blanco B.** Therapy of Venezuelan Patients with Severe Mucocutaneous or Early Lesions of Diffuse Cutaneous Leishmaniasis with a Vaccine Containing Pasteurized *Leishmania* Promastigotes and Bacillus Calmette-Guerin. Preliminary Report. Mem Inst Osw Cruz 2004; 99: 57-62.
16. **Albareda MC, Laucella SA, Alvarez MG, Armenti AH, Bertochi G, Tarleton RL, Postan M.** *Trypanosoma cruzi* modulates the profile of memory CD8+ T cells in chronic Chagas' Disease patients. Int Immunol 2006; 18: 465-471.
17. **Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL.** Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med 2002; 8: 379-385.
18. **Appay V, Rowland-Jones SL.** Lessons from the study of T-cell differentiation in persistent human virus infection. Semin Immunol 2004; 16**:** 205-212.
19. **Armijos RX, Weigel MM, Calvopina M, Hidalgo A, Cevallos W, Correa J.** Safety, immunogenicity, and efficacy of an autoclaved *Leishmania amazonensis* vaccine plus BCG adjuvant against New World Cutaneous Leishmaniasis. Vaccine 2004; 12: 1320-1326.
20. **Bergan JJ, Schmid-Schönbein GW, Coleridge Smith PD, Nicolaides AN, Boisseau MR, Eklof B.** Chronic Venous Disease. N Engl J Med 2006; 355: 488-498.
21. **Costa JML, Netto EM, Vale KC, Osaki NK, Tada MS, Marsden PD.** Spontaneous healing of cutaneous *Leishmania braziliensis braziliensis* ulcers. Trans R Soc Trop Med Hyg 1987; 81: 606.
22. **Silveira FT, Blackwell JM, Ishikawa EA, Braga R, Shaw JJ, Quinnell RJ, Soong L, Kima P, McMahon-Pratt D, Black GF, Shaw MA.** T cell responses to crude and defined leishmanial antigens in patients from the lower Amazon region of Brazil infected with different species of *Leishmania* of the subgenera *Leishmania* and *Viannia*. Parasite Immunol 1998; 20: 19-26.

23. **Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R.** Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 2006; 439: 682-687.
24. **Zhang SY, Zhang Z, Fu JL, Kang FB, Xu XS, Nie WM, Zhou CB, Zhao M, Wang FS.** Progressive CD127 down-regulation correlates with increased apoptosis of CD8 T cells during chronic HIV-1 infection. Eur J Immunol 2008; 39(5):1425-1534.
25. **Hinrichs CS, Gattinoni L, Restifo NP.** Programming CD8+ T cells for effective immunotherapy. Curr Opin Immunol 2006; 18: 363-370.
26. **Laucella SA, Postan M, Martin D, Hubby Fralish B, Albareda MC, Alvarez MG, Lococo B, Barbieri G, Viotti RJ, Tarleton RL.** Frequency of interferon-gamma-producing T cells specific for *Trypanosoma cruzi* inversely correlates with disease severity in chronic human Chagas Disease. J Infect Dis 2004; 189: 909-918.
27. **Bustamante JM, Bixby LM, Tarleton RL.** Drug-induced cure drives conversion to a stable and protective CD8+ T central memory response in chronic Chagas' Disease. Nat Med 2008; 14: 542-550.