Case Report: Increase of NK Cells and Proinflammatory Monocytes Are Associated with the Clinical Improvement of Diffuse Cutaneous Leishmaniasis after Immunochemotherapy with BCG/Leishmania Antigens


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Abstract. Diffuse cutaneous leishmaniasis (DCL) is characterized by disseminated lesions and the absence of a specific cellular immune response. Here, the immunochemotherapy outcome of a patient with DCL from Amazonian Brazil infected with Leishmania (Leishmania) amazonensis is presented. After several unsuccessful chemotherapy treatment regimens and many relapses, a monthly immunotherapy scheme of L. amazonensis PH8 plus L. (Viannia) braziliensis M2903 monovalent vaccines associated with Bacillus Calmette-Guerin (BCG) was established, one round of which also included an M2903 vaccine associated with intermittent antimonial treatment. Temporary healing of all lesions was achieved, although Leishmania skin tests were negative and interferon γ was not detected in mononuclear cell cultures stimulated with Leishmania antigens. The frequencies of CD16+CD56+ NK cells (−2×) and CD14+CD16+ proinflammatory monocytes (−8×) increased in peripheral blood, and CD56+ lymphocytes were found infiltrating the lesions. An association between the increase of the frequency of innate immune system cells and the healing of lesions is shown, suggesting that this protocol of immunotherapy reduced the parasite load and activated NK cells and monocytes.

INTRODUCTION

Diffuse cutaneous leishmaniasis (DCL) is a rare disease characterized by the presence of non-ulcerative disseminated nodules that may cover several parts of the patient’s body. In the New World, it is caused by Leishmania complex species such as Leishmania (Leishmania) amazonensis, which is the causal agent in Brazil. Histopathologic analysis of the dermis of infected individuals shows a severe infiltration of vacuolated macrophages containing abundant parasites. A humoral immune response is detected in patients, but specific cell immunity is absent.1–3 There is no effective treatment for DCL, and relapses frequently occur even when immunotherapy is used in an attempt to induce a specific cellular immune response.4–6 A vaccine containing killed Leishmania promastigotes and Bacillus Calmette-Guerin (BCG) was described to improve healing and the immune response against Leishmania when used alone or in combination with chemotherapy to treat DCL patients.7,8 The BCG leads to the activation of innate immune system cells such as macrophages and natural killer (NK) cells.9 BCG increases NO, reactive oxygen intermediates, and tumor necrosis factor (TNF) production, all of which are important molecules for the leishmanicidal activity of macrophages.10–12 Besides TNF, other cytokines are released from BCG-activated monocytes/macrophages, and they are potent NK cell activators.13 BCG also directly activates NK cells, which are able to kill tumor cells, Leishmania-infected macrophages, and even free parasites,13,14 as well as being able to produce cytokines to activate monocytes/macrophages.14 Interferon γ (IFNγ), produced by NK cells, and TNF work synergistically to induce macrophage leishmanicidal activity and to control Leishmania infection.15,16

In this study, we report a transitory remission of all lesions in a patient with DCL treated with L. (L.) amazonensis plus L. (V.) braziliensis monovalent vaccines associated with BCG and with chemotherapy. A possible role of CD14+CD16+ monocytes and CD56+ NK cells in the healing is discussed.

MATERIALS AND METHODS

Patient description. MAB is a 22-year-old man, from Amazonian Brazil, who presented with cutaneous lesions at age 4; during the following 14 years, he received different schedules of treatment with pentavalent antimonial and pentamidine and showed periods of improvement and relapse. In 2003, he was assisted at the Tropical Medicine Hospital (Anuar Auad Hospital) in Goiânia, Goiás, Brazil. A clinical exam showed disseminated cutaneous lesions on the body, including papules, nodules (both infiltrating and vegetative), and ulcerative lesions.

Isolation of parasites, histopathologic examinations, and species identification. Informed consent was signed by the patient, and the protocols for human and animal procedures were approved by a local ethics committee (Ethics Committee in Animal and Human Research of the Clinical Hospital of Federal University of Goiás). Biopsy fragments of lesions were macerated and injected into the footpad of IFNγ−deficient C57Bl/6 mice for in vivo parasite isolation or cultured in Grace’s insect medium (Gibco BRL, Grand Island, NY) for in vitro isolation. Paraffin-embedded 3-μm tissue sections were stained using hematoxylin-eosin (H&E) or submitted to immunohistochemistry (IHC) with rabbit polyclonal antibodies anti-Leishmania spp. (kindly supplied by Dr. Luis Antônio Freitas, Bahia, Brazil). After incubation with these antibodies, sections were treated with streptavidin-biotin-peroxidase (kit-LSAB-plus; Dako, Glostrup, Denmark) and counterstained with Mayer hematoxylin. DNA was extracted from promastigotes,16 and small subunit ribosomal DNA (SSU rDNA) sequence amplification was performed according
to Uliana and others, using primers S1/S4. The nucleotide sequence of the resulting fragment of 520 bp was obtained directly by automatic sequencing using an ABI Big-Dye kit.

**Treatment regimens.** Treatment started in April 2003 with amphotericin B (Anforicin B, Cristália, Lindóia, SP, Brazil); liposomal amphotericin B (Ambisome; Gilead–United Medical, San Dimas, CA); pentavalent antimonial (N-methyl glucamine antimonate, Glucantime; Aventis, São Paulo, SP, Brazil); and rifampicin (Rifampicina; Lafpe, Recife, PE, Brazil) plus isoniazid (Isoniazida; Lafpe). In September 2004, the treatment regimen was supplemented with monthly doses of the monovalent *L. (L.) amazonensis* (IFLA/BR/1967/PH8, referred as PH8 vaccine) and *L. (V.) braziliensis* (MHOM/BR/1975/M2903, referred as M2903 vaccine) vaccines together with BCG (moreau strain; Fap, Rio de Janeiro, RJ, Brazil) until March 2007. The vaccine preparations used in this study were produced using well-defined World Health Organization reference strains and a methodology described in detail by Mendonça and others. The vaccines (600 μg) plus BCG (400 μg; the dose of BCG was decreased 100 μg each month until 100 μg, which was used until relapses, when the dose was increased again to 400 μg) were inoculated intradermally (ID) in three different places (300 μL each injection). The BCG was administered until obtaining of a positive protein purified derivative (PPD) reaction (30 months of use), and after that, he received the vaccines alone. During the immune stimulation (vaccine + BCG), he also underwent chemotherapy regimens of Glucantime, liposomal amphotericin B, and azithromycin (Azidromic; Royton, São Paulo, SP, Brazil). The patient also received an intermittent schedule of Glucantime plus M2903 vaccine. These procedures were based on Mayrink and others and Toledo and others.

**Skin tests and indirect immunofluorescence reaction.** The skin test was performed using antigen preparations from two different laboratories, both of which were a preparation of *L. (L.) amazonensis* (MHOM/BR/73/M2269) containing 40 μg/mL total nitrogen (ISEP, Curitiba, PR, Brazil and Federal University of Minas Gerais, Belo Horizonte, MG, Brazil). PPD (2 T.U./0.1 mL; Statens Serum Institut, Copenhagen, Denmark) was used to test the *in vivo* immune response to BCG. The antigens (0.1 mL) were injected ID into the forearm, and after 72 hours, the reaction was considered positive when induration at the site of injection was ≥ 5 mm. Peripheral blood was collected, and serum was submitted to an indirect immunofluorescence (IIF) reaction using promastigote forms of *L. (L.) amazonensis* (IFLA/BR/67/PH8) to detect specific IgG antibodies.

**Cytokine evaluation, flow cytometric analysis for NK cells and monocytes, and IHC for CD56 antigens.** Peripheral blood mononuclear cells were isolated using a Ficoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) and cultured (3 × 10^5/mL) in RPMI 1640 medium (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal calf serum (Gibco) for 72 hours (5% CO2, 37°C) without or with stimuli [phytohemagglutinin A (PHA); Sigma; 10 μg/mL whole antigens from *L. (L.) amazonensis* (IFLA/BR/67/PH8), *L. (V.) braziliensis* (MHOM/BR/1975/M2903) or patient isolate (MAB3) promastigotes, 2.1 parasite/cell ratio]. Supernatants were submitted to standard enzymatic immunoassays for detecting IFNγ (B133.1- and B133.5-biotinylated monoclonal antibodies) or TNF (B157.9- and B157.7-biotinylated monoclonal antibodies). Antibodies were kindly supplied by Dr. Luis Carlos Afonso (Federal University of Ouro Preto, Ouro Preto, MG, Brazil). All procedures were performed according to Toledo and others.

For immunophenotyping assay of peripheral blood leukocytes, 100-μL aliquots of whole peripheral blood collected in EDTA were added to 5 μL of monoclonal antibodies (mAbs) specific for human NK and monocyte cell surface markers, including anti-CD3-FITC and TC (Clone HIT3a), CD14-PE (TUK4), CD16-FITC (Clone 3G8), and CD56-PE (Clone MEM-188). Isotypic controls included mouse IgG1 (Clone G18-145) and IgG2 (Clone G18-145) labeled with FITC, PE, or TC. The mAbs were purchased from Dako (Carpinteria, CA), Becton Dickinson (Mountain View, CA), or Caltag (Burlingame, CA). After erythocyte lysis, cytofluorimetric data acquisition was performed in a Becton Dickinson FACScalibur instrument. CELLQuest software provided by the manufacturer was used for data acquisition and analysis. The analysis of CD3−CD16−CD56− and CD3+CD16−CD56− cells and additional analysis to quantify CD3+CD56− (CD56dim or CD56bright) cells were performed as described by Gaddy and Broxmeyer and Cooper and others. Finally, analysis of CD14+CD16+ monocytes was performed according to Ziegler-Heitbrok and others. Paraffin-embedded 3-μm tissue sections were treated with mAb against the CD56 antigen (123C3,5, mouse IgG1; 1/100; BioSB, Santa Barbara, CA). Afterward, the sections were incubated with streptavidin-biotin-peroxidase (Kit-LSAB-plus; Dako Cytomation, Glostrup, Denmark) and DAB (3,3-diaminobenzidine tetrahydrochloride; Sigma). For counterstaining, Mayer hematoxylin was used.

**RESULTS**

**Laboratory diagnosis, parasite isolation, and identification of species.** At the time of first treatment (April 2003), Patient MAB had disseminated lesions and a positive IIF reaction for IgG antibodies to *L. (L.) amazonensis* (titer = 640). Skin tests were negative for PPD and *L. (L.) amazonensis* antigens. On histopathologic examination of biopsy fragments, the dermis showed mononuclear cell infiltration composed mainly of macrophages, along with a few lymphocytes and plasma cells. Amastigote forms of the parasite were visualized inside macrophages in a high number. As shown in Figure 1, the SS rDNA sequences from two parasite isolates obtained from two different biopsy fragments (isolate MAB3, from 2003, and isolate MAB6, from 2006) were compatible with *L. (L.) amazonensis* sequences.

**Clinical and immunologic outcome during treatment.** Starting in April 2003, the patient received only chemotherapy, as shown in Table 1. Although there was some clinical improvement, most notably the size of the lesions, not all lesions healed completely, and relapses occurred soon after each treatment (~1 month). In these 14 months of chemotherapy, the patient received medication during almost 9 of the months. In September 2004, he started to receive the monovalent PH8 and M2903 vaccines plus BCG monthly. After 10 months of immunochemoerapy, the best clinical results throughout all therapeutic attempts were observed. Although some relapses occurred during the treatment, the interval between these relapses was increased (from 2 to 9 months), and lesions were less severe each time, with reduced size and infiltration. After 30 months of immunochemoerapy, the PPD became positive. From that point, the patient received monovalent PH8 and M2903 vaccines without BCG. By May 2007, 32 months
Immunochemotherapy: the patient received medication during almost 9 months of this 30-month period, increasing the intervals between March until May 2007. Liposomal amphotericin 2 mg/kg/day (total dose: 2,200 mg) Relapse (less severity) May 2006 Liposomal amphotericin 2 mg/kg/day (total dose: 1,400 mg) Relapse (less severity) March 2006 Pentavalent antimonial (20 mg/kg/day/30 days) Relapse (less severity) November 2005 Pentavalent antimonial (20 mg/kg/day/30 days) Relapse (less severity) September 2004 Azithromycin (500 mg/day) after March, only vaccine without BCG Clinical improvement† and relapses

Figure 1. MAB3 and MAB6 isolates are L. (L.) amazonensis. Ribosomal DNA was extracted, and primers S1 and S4 were used to amplify target molecules; 5 μL of amplification products was analyzed on a 2% agarose gel. Top: 1. L. (L.) amazonensis MHOM/BR/1973/M2269; 2. L. (L.) chagasi MHOM/BR/1972/LD; 3. L. (V.) braziliensis MHOM/BR/1975/M2903; 4. MAB3; 5. MAB6; 6. no DNA as template; 7. DNA size marker, HinIII-digested lambda DNA. Bottom: SSU rDNA sequences showing that MAB3 and MAB6 isolates present sequences compatible with L. (L.) amazonensis (LLA).

DISCUSSION

In this study, we described a patient with DCL treated with immunochemotherapy using Leishmania vaccines plus BCG for an extended period. The patient received several treatments during his 18 years of disease, and the treatment described here was the first that completely resolved, albeit temporarily, all his lesions. In addition, when the lesions reappeared during immunochemotherapy, each time they became less severe, leading to a significant improvement in the quality of life after many years of living with the disease. The side effects of immunotherapy were less severe than those of the chemotherapy, being restricted to inflammatory reactions at the injection site of vaccine plus BCG. Because the immunotherapy led to a reduction in the frequency of remissions and an increase in the intervals between them, less chemotherapy was necessary to control the lesions, which decreased the overall

| Table 1: Clinical evolution during treatment* |
| Period | Treatment | Clinical response |
| April until July 2003 | Amphotericin B 1 mg/kg/day (total dose: 1,700 mg) and liposomal amphotericin B 2 mg/kg/day (total dose: 2,760 mg) | Clinical improvement† and relapses |
| November 2003 | Pentavalent antimonial (20 mg/kg/day/30 days) | Clinical improvement and relapse |
| December 2003 until June 2004 | Rifampicin (600 mg/day) + isoniazid (400 mg/day) | Clinical improvement and relapses |
| Chemotherapy: the patient received medication for almost 9 months of this 14-month period, and he presented relapses every ~1 or 2 months | Pentavalent antimonial (20 mg/kg/day/30 days) BCG + vaccine† monthly until March 2007 | Clinical improvement and relapse (less severity§) |
| September 2004 | Pentavalent antimonial (20 mg/kg/day/30 days) + vaccine‡ | Clinical improvement and relapse (less severity) |
| November 2004 | Pentavalent antimonial (20 mg/kg/day/30 days) | Clinical improvement and relapse (less severity) |
| February 2005 | Pentavalent antimonial (20 mg/kg/day/30 days) | Clinical improvement and relapse (less severity) |
| May 2005 | Pentavalent antimonial (20 mg/kg/day/30 days) | Clinical improvement and relapse (less severity) |
| July 2005 | Liposomal amphotericin 2 mg/kg/day (total dose: 2,400 mg) | Clinical improvement and relapse (less severity) |
| November 2005 | Pentavalent antimonial (20 mg/kg/day/30 days) | Clinical improvement and relapse (less severity) |
| March 2006 | Liposomal amphotericin 2 mg/kg/day (total dose: 2,200 mg) | Relapse (less severity) |
| May 2006 | Liposomal amphotericin 2 mg/kg/day (total dose: 1,400 mg) | Relapse (less severity) |
| February 2007 | Liposomal amphotericin 2 mg/kg/day (total dose: 2,200 mg) | Relapse (less severity) |
| March until May 2007 | Azithromycin (500 mg/day) after March, only vaccine without BCG | Healing of all lesions; positive PPD (21 mm) |

*Patient was submitted to different schemes of chemotherapy plus immunotherapy.
†Clinical improvement: reduction in size of the lesions without complete healing.
‡Leishmania (L.) amazonensis PH8 and L. (V.) braziliensis M2903 monovalent vaccines with BCG (BCG was used until March 2007; for 30 months).
§Less severity: reduction in number and size of the lesions, and less infiltration of the lesions. For details, see Materials and Methods.
¶Leishmania (V.) braziliensis M2903 vaccine (three series with intermittent use of pentavalent antimonial).
side effects of the therapy and reduced patient suffering. Furthermore, the cost of the immunotherapy is low, and its use reduced the cost of chemotherapy.

**Leishmania (L.) amazonensis** PH8 and **L. (V.) braziliensis** M2903 monovalent vaccines were selected to induce both a species-specific immune response and also a cross-protective immune response. BCG has been used in an attempt to create a milieu favoring the development of a cellular immune response, especially by activating monocytes, macrophages, dendritic cells, and NK cells to cooperate with antigen-specific T lymphocytes. After several months of immunotherapy, despite the apparent clinical cure of all lesions, a *Leishmania*-specific cellular immune response was not detected. Our data are similar to previous results showing that patients with DCL not only present relapses, but frequently, they do not develop a specific cell immune response. In contrast, Convit and others showed that treatment of patients of DCL with a vaccine containing promastigotes of *L. (V.) braziliensis* and BCG leads to both a clinical cure and a positive *Leishmania* skin test, suggesting that it is possible to achieve an effective immunomodulation to improve specific cellular immune response in some patients with DCL.

We studied possible alterations in the blood frequency of monocytes and NK cells that can be directly activated by BCG and also by *Leishmania* promastigotes. When all lesions healed, we found an increase in CD16^+^CD56^+^ NK cell frequency (~2×). The subpopulation of CD56^dim^ NK cells was preferentially increased. This subpopulation represents the majority of NK cells in peripheral blood (~90% of NK cells), contains high cytotoxic activity, and migrates to inflammatory sites where these cells can interact with different cells of the immune system. In fact, we found CD56^+^ lymphocytes in healing lesions, suggesting an infiltration of CD56^+^ NK cells at these sites. Although there is not an unequivocal means to identify NK cells in tissue sections, the presence of CD56^+^ lymphocytes in patient lesions is suggestive of NK cells. Thus, we speculate that in infected sites, activated NK cells can lyse infected macrophages and parasites, and in addition, they can release IFNγ and TNF to activate macrophages for parasite elimination. Corroborating our data, it has been shown that IFNγ-producing NK cells increased in the DCL lesions and parasites decreased concomitantly after immunocombination therapy with pentamidine, allopurinol, and IFNγ.

We found a significant increase in the frequency of blood CD14^+^CD16^+^ monocytes, which are considered proinflammatory cells because they produce TNF, but not the anti-inflammatory cytokine interleukin-10. Whereas TNF is important
to activate macrophage leishmanicidal activity, a decrease in IL-10 production is associated with transient cure in DCL.\textsuperscript{31} It has been suggested that a high frequency of CD14\textsuperscript{+}CD16\textsuperscript{+} monocytes at diagnosis of localized cutaneous form of leishmaniasis could be associated with disease severity.\textsuperscript{32} Nevertheless, as seen in this case, CD14\textsuperscript{+}CD16\textsuperscript{+} monocytes can release TNF to activate NK cells;\textsuperscript{33} thus, these cells can help establish a distinct outcome in patients with DCL.

To our knowledge, this is the first description of NK cell and monocyte subset alterations in a patient with DCL treated with \textit{Leishmania} vaccines plus BCG and chemotherapy. Our results highlight the relevance of the activation of the innate immune system for \textit{Leishmania} control, especially when the acquired immune system is not effectively activated.

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