Combined Interleukin-12 and Topical Chemotherapy for Established Leishmaniasis Drastically Reduces Tissue Parasitism and Relapses in Susceptible Mice

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Leishmaniasis, a major disease listed in the World Health Organization’s Tropical Disease Research Program [1], is an important public health problem because of its high prevalence and the lack of efficient prophylactic programs and adequate treatment. Cutaneous leishmaniasis is characterized by the development of localized cutaneous lesions, which may heal themselves. However, depending on the Leishmania species and the patient’s immune status, diffuse cutaneous or mucocutaneous leishmaniasis may occur [2].

Systemic administration of pentavalent antimony antimycotic compounds, amphotericin B, or pentamidine remains the conventional therapy for all forms of leishmaniasis [3], despite the spectrum of clinical manifestations and the toxicity associated with these compounds. Topical formulations containing paromomycin sulfate (PA) are successful in promoting local healing in BALB/c mice and in patients with Leishmania infection [4–10]. However, an important drawback of these and other therapeutic approaches is the recurrence of lesions and the possible dissemination of parasites [3, 11–13]. In 2 studies, 10%–13% of subjects did not respond to PA topical treatment [14, 15]. In BALB/c mice infected with Leishmania major and treated with topical PA, relapses usually are seen ≥70 days after treatment [4, 16, 17]. The reasons for nonresponse to chemotherapy or recurrence of lesions are not completely understood. Nevertheless, the lack of an appropriate immune response to control the proliferation of the remaining parasites may in part explain recrudescence after specific chemotherapy.

Interleukin (IL)–12 and interferon (IFN)–γ are important cytokines in promoting protective immunity against Leishmania infection [18–23]. In addition, if given during the early stages of infection, combined recombinant (r) IL-12 treatment and antimonal therapy inhibit appearance and progression of cutaneous lesions in BALB/c mice by promoting the development of a Th1 protective immune response [23]. In this study, we used a combined local rIL-12 and topical PA therapy to treat BALB/c mice infected with L. major that displayed fully developed lesions, an experimental set that closely corresponds to the clinical setting.

Materials and Methods

Parasites, mice, and infection. L. major strain MHOM/IL/80/ Friedlin was maintained in Schneider’s modified medium [24]. Female 6-week-old BALB/c mice were infected with 105 late–log phase promastigotes of L. major at the shaved rump.

rIL-12 and PA formulations. The rIL-12 and PA used in this study were provided by Genetics Institutes and by Pharmacia and Upjohn, respectively. The oily and aqueous phases of the oil and water (O/W) cream were prepared separately by mixing paraffin oil, cetyl alcohol, and glycerol stearate (oily phase) and PEG-40

Received 12 September 2000; revised 20 February 2001; electronically published 9 May 2001.

Financial support: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), CNPq/Programa de Auxílio ao Desenvolvimento Científico e Tecnológico–Subprograma de Biotecnologia (62.0106/95-6), CNPq/Pronex, and Fundação de Amparo à Pesquisa do Estado de Minas Gerais. A.P.F., C.A.P.T., L.A.M.F., and R.T.G. are CNPq research fellows. F.A.A.C. is a graduate student with a Coordenação de Aperfeiçoamento do Pessoal de Ensino Superior scholarship.

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The Journal of Infectious Diseases 2001; 183:1646–52

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hydrogenated castor oil and distilled water (water phase). PA dissolved in water was incorporated into the O/W cream to a final concentration of 5%. The PA concentration was determined by microbiologic assay.

**Therapy schedule.** Mice were distributed into groups of 6–8 animals. Crusts and necrotic material were removed from lesions before topical application. We applied the O/W cream (50 μL) over the lesions twice a day for 12 days. Control groups received either O/W cream with no PA or perilesional injections of rIL-12. Four doses of rIL-12 (500 ng each) were administered every third day as 2 perilesional injections of 250 ng each. The 4 rIL-12 doses were given to 1 group during PA treatment (DT) and to another after PA treatment (AT) when the animals showed no signs of ulceration. An additional group received 8 doses of rIL-12: 4 doses DT and 4 doses AT (DT/AT). Lesion diameters were measured weekly by caliper for 112 days. Mice also were evaluated for the presence of nodules and metastases. At 120 days, 4 animals from each group were killed for parasite quantification and immunologic and histopathologic analysis.

**Parasite quantification.** The number of viable parasites at the site of infection or in the spleen was determined by a limiting-dilution assay, as described elsewhere [25]. In brief, skin or spleen fragments were excised and homogenized in Schneider’s modified medium. After serial dilutions in 96-well plates, samples were incubated at 23°C. Each well was examined, and the number of parasites was determined from the highest dilution at which parasites could grow for 7 days.

**ELISA for parasite-specific total IgG, IgG1, and IgG2a isotypes.** Parasite-specific IgG, IgG1, and IgG2a were measured by ELISA as described elsewhere [26]. Plates were sensitized with 1 μg of soluble *Leishmania* antigens (SLAs) and were blocked and treated successively with 1:100 dilutions of mouse serum samples and 1:5000 dilutions of peroxidase-labeled antibodies specific to mouse total IgG, IgG1, or IgG2a isotypes (Sigma Chemical). Reactions were developed with H2O2 and o-phenylene diamine. Optical densities were read at 490 nm.

**Measurement of IFN-γ and IL-4 levels.** Single-cell preparations from spleen tissue were plated in triplicate in 24-well plates at 2 x 10⁶ cells/mL and were cultured in the presence of 20 μg/mL SLAs [18] or mitogen (concanavalin A) for 48 h. IFN-γ and IL-4 in supernatants were assessed by specific ELISA by using InterTest mouse IFN-γ or IL-4 ELISA kits (Genzyme).

**Analysis of reverse-transcriptase (RT) polymerase chain reaction (PCR) products.** RNA was extracted with RNAzol solution (Cinna/Biotecx), as described elsewhere [27], from skin fragments excised at the site of infection. Total RNA was reverse transcribed using Moloney murine leukemia virus RT (Gibco BRL) and was used as a template in PCR. The primer sequences, PCR product size, number of cycles, and annealing temperature used in cDNA amplifications were as follows: for hipoxantine phosphoribosyl transferase (HPRT), GTTGGTACAGGCCAGACCTTGTG and GATTCACTTGGGTGCCCTATCCAGGC; and, for IFN-γ, GGTTGACATGAAATCTCTGCAGAGC and CGCTGGACCTTGGTGGTGGACC, 237 bp, 29 cycles, 64°C. The increases in IFN-γ and IL-4 gene expression were determined by normalization of the densitometric values obtained for PCR products of infected and treated animals over the values obtained for HPRT expression in the skin of noninfected mice.

**Histopathology and immunocytochemical study.** Spleen and skin fragments were embedded in paraffin and stained by hematoxylin-eosin. For immunocytochemical analysis, paraffin samples were stained by the biotin-streptavidin peroxidase immunostaining technique as described by Sternberger [28] with modifications. We used immune anti-amastigote rabbit serum diluted 1:100 as primary antibody. A rabbit anti-mouse biotin-labeled antibody (Dako streptavidin kit) was used as a secondary antibody. Reactions were revealed with diaminobenzidine H2O2, and sections were counterstained with Mayer’s hematoxylin. Negative controls were normal rabbit serum or PBS. For quantification of *L. major* amastigotes in infected cells, defined spots rather than diffuse staining were counted in 10 random light microscopy fields examined on spleen fragments of 4 animals from each experimental group.

**Statistical analysis.** We used the unpaired Student’s t test to compare parasite loads and levels of IFN-γ, IL-4, or IgG isotypes determined by RT-PCR and/or ELISA. Differences were considered statistically significant at *P* < .05. We compared lesion size among experimental groups by using the Kruskal-Wallis nonparametric test.

**Results**

**Association of rIL-12 with PA topical therapy reduces relapses in *L. major*-infected BALB/c mice.** Augmentation of the efficacy of topical treatment by its association with rIL-12 was determined in a standard model of antileishmanial drug testing (**L. major*-infected BALB/c mice) [4]. For topical delivery, we used an O/W cream containing 5% PA. This formulation shows enhanced in vitro percutaneous absorption and similar in vivo activity against *L. major* infection in BALB/c mice when compared with ointment containing 15% PA (authors’ unpublished data).

As shown in figure 1A, lesion size developed progressively in the control group (O/W without PA). No significant differences were observed between this group and the group that received rIL-12 alone (data not shown). In animals treated with PA, lesion size decreased progressively independent of any association with rIL-12, and, at day 30 after the start of treatment, lesions were completely resolved. However, 86 days after cessation of therapy, lesions recurred in all animals given PA only. By the end of the study, lesions had recurred in 100% of the mice given PA alone, in 50% of those given rIL-12 AT or DT, and in only 25% of those given rIL-12 DT/AT (figure 1B). In addition, a slower increase in lesion size was observed in animals given the PA/rIL-12 therapy. By the end of the study, lesions were significantly smaller (*P* < .05) in PA/rIL-12- and PA-treated animals (table 1). Although no statistically significant differences were observed in lesion size among the groups that received PA/rIL-12 therapy, animals given the DT/AT regimen historically had smaller lesions than animals given rIL-12 AT or DT.

**Association of rIL-12 with PA topical therapy significantly**
alters immunity in L. major–infected BALB/c mice. To evaluate whether the combined therapy affected immunity in mice that had healed, we compared levels of IL-4 and IFN-γ produced by spleen cells of control mice, PA-treated mice, and mice healed after treatment with the combined therapy under the different regimens (DT, AT, and DT/AT). Spleen cells from PA/rIL-12–treated animals produced significantly more IFN-γ \( (P < .05) \) than did spleen cells from control mice or mice treated with PA or rIL-12 alone (data not shown). In contrast, decreased levels of IL-4 \( (P < .05) \) were observed in the PA/rIL-12–treated mice (figure 2B). No significant differences were found in cytokine levels among the healed animals treated with the combined therapy under the different regimens (data not shown). In addition, decreased levels of IgG1 antibodies, which are indicative of Th2 responses [29], were detected in mice treated with the PA/rIL-12 DT/AT regimen (figure 2C). No significant differences were found in IgG2a antibody levels.

**Table 1.** Average lesion size and number of viable parasites in the spleen and at the site of infection 120 days after start of treatment in mice treated with paromomycin sulfate (PA) and recombinant interleukin-12 (rIL-12).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lesion size, mean mm ± SD(^a)</th>
<th>Log of no. of viable parasites (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>Control</td>
<td>17.02 ± 0.56</td>
<td>9 ± 0.44</td>
</tr>
<tr>
<td>O/W PA</td>
<td>9.65 ± 2.10</td>
<td>3 ± 2.35(^c)</td>
</tr>
<tr>
<td>O/W PA + rIL-12 DT</td>
<td>3.8 ± 1.53 (5.71 ± 0.83)</td>
<td>2 ± 1.03(^d)</td>
</tr>
<tr>
<td>O/W PA + rIL-12 DT/AT</td>
<td>1.62 ± 0.89 (3.87 ± 0.5)</td>
<td>(\theta)(^d)</td>
</tr>
<tr>
<td>O/W PA + rIL-12 AT</td>
<td>3.96 ± 1.49 (5.28 ± 0.67)</td>
<td>1 ± 0.34(^d)</td>
</tr>
</tbody>
</table>

NOTE. Similar results were obtained in 2 different experiments. AT, after PA treatment; DT, during PA treatment; O/W, oil and water cream.

\(^a\) Average lesion size was determined from 8 animals per group. Nos. in parentheses indicate mean lesion size among animals with a recurrence of lesions while receiving PA/rIL-12 treatment.

\(^b\) Parasite quantification in 4 animals per group.

\(^c\) Significant vs. control (O/W without PA) group.

\(^d\) Significant vs. control (O/W without PA) group or vs. PA-treated group.

**Figure 1.** Course of *Leishmania major* infection in BALB/c mice given paromomycin sulfate (PA) and recombinant interleukin-12 (rIL-12) therapy. A, Groups of 8 infected mice treated with topical applications of 5% PA in oil and water (O/W) cream, subcutaneous injections of rIL-12, or a combination. Control mice received O/W cream without PA. No significant differences were observed between the control group and the group given rIL-12 only. Each point represents the mean \( \pm SD \) lesion diameter in each group and includes data for healed animals and for those with recurrence of lesions. The arrow indicates the end of topical treatment. B, Percentage of mice with recurrence of lesions in each group. Results are from 1 of 2 representative experiments. AT, after PA treatment; DT, during PA treatment.
Figure 2. Parasite-specific immune response in BALB/c mice infected with Leishmania major and treated with paromomycin sulfate (PA)/recombinant (r) interleukin (IL)–12 therapy. Interferon (IFN)–γ (A) and IL-4 (B) were assayed in 48-h culture supernatants of splenocytes stimulated with soluble Leishmania antigens of control, PA-treated, and PA/rIL-12–treated mice healed 120 days after the start of therapy. Each bar indicates the mean (±SD) cytokine level determined for 4 mice per group. No significant differences were found among the healed mice given different PA/rIL-12 treatment regimens. C, Parasite-specific IgG isotypes in serum samples from treated and control mice. Bars indicate mean (±SD) levels in 8 mice per group. *Statistically significant differences (P < .05).

Table 2. Occurrence of metastases, spleen hypertrophy, hyperplasia of splenic pulp, and inflammatory responses among experimental groups 120 days after treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metastases</th>
<th>Inflammatory reaction at skin</th>
<th>Spleen hypertrophy</th>
<th>Hyperplasia of splenic pulp</th>
<th>Amastigotes in spleen cells, mean no. ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Present</td>
<td>+++</td>
<td>Present</td>
<td>+++</td>
<td>36.5 ± 4.10</td>
</tr>
<tr>
<td>PA</td>
<td>Present</td>
<td>+</td>
<td>Present</td>
<td>+</td>
<td>18.2 ± 4.53</td>
</tr>
<tr>
<td>PA/rIL-12 DT/AT</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>+</td>
<td>3.5 ± 1.85</td>
</tr>
</tbody>
</table>

NOTE. AT, after PA treatment; DT, during PA treatment; PA, paromomycin sulfate; rIL-12, recombinant interleukin-12.

a Metastases at tail, joints, and feet.
b Determined at site of infection by microscopic analysis of hematoxylin-eosin–stained tissue sections.
c Discrete, moderate, or intense inflammatory reaction or hyperplasia of splenic pulp is indicated by +, ++, or ++++, respectively.
d Spleen was 5–6 times larger than in noninfected mice.
e Determined as described in Materials and Methods.
f No significant differences were found among the healed animals that had received PA/rIL-12 under different regimens.

the skin and spleen of the healed animals treated with the combined rIL-12 therapy were significantly lower than those in control mice or in mice given PA only. Histopathology (table 2) and Leishmania–specific immunocytochemical analysis at the infection site (data not shown) revealed heavily infected macrophages in mice treated with PA. In contrast, mice treated with the PA/rIL-12 DT/AT regimen had normal skin histopathology with no infected macrophages. Leishmania–specific immunostaining of the spleen also confirmed the presence of significantly more infected macrophages in control or PA-treated animals (table 2). In contrast, very few infected macrophages were detected in PA/rIL-12–treated mice. Thus, there was a direct correlation between tissue parasitism in the spleen and skin and the size and frequency of the lesions that recurred in mice given PA or combined therapy. Of note, no parasites were recovered from the skin or spleen of the animals treated with the DT/AT regimen, even 120 days after the start of therapy (table 1). Thus, animals treated with the DT/AT regimen appeared to be clinically and parasitologically distinct from those treated with the DT or AT regimen.

Parasite loads also correlated with significant differences in the development of metastases and spleen hypertrophy (table 2). Control and PA-treated mice displayed spleen hypertrophy and metastases at various sites. In contrast, the healed PA/rIL-12–treated mice had no signs of nodules, metastases, or spleen hypertrophy. The spleen histopathology revealed an intense hyperplasia of splenic pulp with granuloma formation in control and PA-treated animals. In contrast, PA/rIL-12–treated mice had a moderate reaction of splenic pulp (table 2).

rIL-12 with PA topical therapy significantly decreased inflammatory reaction at the infection site. An intense inflammatory reaction, characterized by the presence of mononuclear cells
Figure 3. Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of interferon (IFN)-γ, interleukin (IL)-4, and hypoxantine phosphoribosyl transferase (HPRT) mRNA levels at infection and 120 days after the start of treatment. Total RNA was extracted and reverse transcribed. RT-PCR reactions were done with primers specific to IFN-γ, IL-4, and HPRT gene sequences. HPRT expression was used to control RNA content and integrity in each sample. Left, Representative results for mice treated with paromomycin sulfate (PA) and recombinant (r) IL-12 and for control and noninfected mice. Right, Fold increase in cytokine mRNA gene expression was determined as described in Materials and Methods. AT, after PA treatment; +Ct, control positive for IL-4 and IFN-γ; DT, during PA treatment.

and infected macrophages, was observed in tissue fragments from the lesions that recurred in PA-treated mice (table 2). In contrast, in healed animals treated with the combined DT/AT regimen, skin histopathology was normal, with no inflammation or parasites at the infection site. RT-PCR showed consistently decreased levels of IL-4 and IFN-γ mRNAs (P < .05) at the infection site in healed PA/rIL-12-treated animals, when compared with control or PA-treated animals (figure 3). The IL-4 and IFN-γ mRNA levels were not significantly different in groups that had received PA/rIL-12 and in noninfected controls. Thus, our results suggest that PA/rIL-12 therapy completely abolished cytokine expression at the infection site.

**Discussion**

The basis of host refractoriness to specific therapy against *Leishmania* species is poorly understood, and acquired or natural resistance of parasites should be considered. However, association between the patient’s immune status and treatment failure or relapse after specific chemotherapy have also been demonstrated [2, 3, 30, 31]. A positive correlation between relapses and negative skin tests [32, 33] or low in vitro proliferation of CD4 and CD8 T cells [33] was observed in patients treated for cutaneous leishmaniasis. Systemic or local administration of cytokines (e.g., IFN-γ or granulocyte-macrophage colony-stimulating factor) combined with antimonial therapy results in more-rapid resolution of cutaneous and mucocutaneous leishmaniasis [34, 35], which are associated with higher levels of IFN-γ. Moreover, relapses after clinical cure are frequently observed in persons with visceral or diffuse cutaneous leishmaniasis, which are associated with specific anergy to leishmanial antigens and low IFN-γ levels [2, 3, 31]. In such persons, temporary healing after conventional therapy is associated with increased IFN-γ levels [36].

In susceptible BALB/c mice, although healing and a drastic reduction of parasite loads occur after treatment, replication of the remaining viable parasites leads to dissemination of parasites and relapse [4, 17]. This may be due to the development of a predominant Th2 immune response that is unable to control *L. major* replication after chemotherapy [17, 22, 23, 37]. In contrast, relapses are not seen after PA topical treatment of naturally resistant animal models [38] that are self-healing and that develop protective immunity after cure.

Because IL-12 induces the IFN-γ production by NK and T cells, its adjunct effect on chemotherapy has been investigated in fungal [39, 40] and parasite infections [41]. In experimental visceral leishmaniasis, administration of rIL-12 converted subtherapeutic doses of antimonial agents from weakly to strongly leishmanicidal [42]. For BALB/c mice infected with *L. major*, augmentation of the efficacy of conventional therapy by local or systemic administration of rIL-12 has been demonstrated [23]; however, the experimental set involved relatively low parasite burdens because therapy was administrated before lesions had developed. Since treatment of patients is generally initiated after complete development and ulceration of lesions, we hypothesized that topical treatment of fully developed lesions with PA would promote a reduction in parasite loads significant enough to allow rIL-12 treatment to interfere in the outcome...
of disease. Our results indicate that the concomitant use of rIL-12 treatment and topical PA therapy can significantly alter the course of infection by decreasing the incidence of relapses and the dissemination of parasites.

To investigate the mechanism of action of rIL-12 in controlling relapses in PA-treated mice, we analyzed the systemic and local immune responses in mice from different groups. Spleen cells from rIL-12/PA–treated mice produced higher levels of IFN-γ and lower levels of IL-4, compared with animals treated with PA or rIL-12 alone. Consistent with the decreased IL-4 production in PA/rIL-12–treated animals, we also observed a diminished level of circulating parasite-specific IgG1a antibodies. The expression of IL-4 mRNA was also diminished in the skin of healed PA/rIL-12–treated animals given the DT/AT regimen.

Another interesting finding of our study was that systemic leishmaniasis (as indicated by visceralization and metastases) was controlled in mice treated with PA/rIL-12. This finding could be explained by the leishmanicidal effect of the combined therapy, which limits tissue parasitism at the infection site, the major source for parasite spread to other sites. El-On et al. [43] suggested that relapses in BALB/c mice after topical therapy are due to migration of parasites from internal organs. In this context, rIL-12, when administered with PA, would help clear parasites that migrate from the site of infection, by redirecting the host T lymphocytes to a Th1 immune response, and thus would prevent relapse.

In this study, we found evidence that the association of rIL-12 to topical PA therapy can significantly reduce the recurrence of lesions and the dissemination of Leishmania parasites to other tissues. These data confirm and extend previous observations on the synergistic effect of rIL-12 as an adjunct immunotherapeutic agent in experimental leishmaniasis. However, because of the spectrum of clinical manifestations and the diversity of immunologic patterns associated with leishmaniasis, the clinical significance of these findings requires additional investigation. It is conceivable that this therapy may offer a greater benefit in cases of impaired cell-mediated immune responses and low levels of IFN-γ [2], which constitute immunologic markers of disease progression and in which failure of conventional chemotherapy is a common threat.

References

23. Nabors GS, Afonzo LCC, Farrel JP, Scott P. Switch from a type 2 to a type 1 T helper cell response and cure of established Leishmania major infection.


