Antimony plus Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor Applied Topically in Low Doses Enhances Healing of Cutaneous Leishmaniasis Ulcers: A Randomized, Double-Blind, Placebo-Controlled Study

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Cutaneous leishmaniasis (CL) requires 2–6 months to heal. In an effort to reduce this healing time, we studied topically applied granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjunct to antimonial therapy. Ten patients received antimony plus topical GM-CSF, and 10 patients received antimony plus placebo (saline). GM-CSF was diluted for topical use and was applied 3 times weekly for 3 weeks (1–2 μg/cm^2/lesion). The mean ± SD healing time was 43 ± 14 days in the GM-CSF group and was 104 ± 79 days in the placebo group ($P = .043$). Ten (100%) of 10 patients in the GM-CSF group healed within 60 days, compared with 5 (50%) of 10 patients in the placebo group. Two of the patients in the placebo group required retreatment with antimony. In conclusion, topically applied GM-CSF is effective in the management of CL.

Cutaneous leishmaniasis (CL) caused by *Leishmania* species is a sandfly-transmitted protozoal disease endemic in many tropical countries of the Americas, Africa, and Asia. It has been estimated that 400,000 new cases occur each year worldwide [1]. In infections with *L. braziliensis,* an important etiologic agent in Brazil, the typical clinical manifestation is a single skin ulcer, localized predominantly in lower limbs [2]. Weeks to years after the onset of cutaneous disease, mucosal lesion(s) involving the nasal mucosa, palate, pharynx, and larynx may develop in ~5% of patients [3]. Pentavalent antimonials have been the treatment of choice for leishmaniasis for >50 years, despite the need for daily intravenous injections for 20–30 days, prolonged healing time (3–4 months), and serious side effects, which include pancreatitis, liver-enzyme abnormalities, and cardiac arrhythmia [4]. Alternative drugs, such as amphotericin B and pentamidine, are of even greater toxicity [5].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multipotential growth factor for bone marrow stem cells [6]. Both Th1 and Th2 lymphocyte subsets respond to GM-CSF [7]. In vitro, GM-CSF has been shown to activate macrophages that kill *Leishmania* pathogens [8]. In addition to its microbicidal activity, GM-CSF is known to stimulate scar formation and tissue-wound healing [9]. It has also been shown that GM-CSF administered in low doses has an effect on platelet function in bone marrow [10]. Moreover, it has been shown that (1) GM-CSF applied locally in low doses improves healing of chronic venous ulcers [11] and (2) another cytokine, keratinocyte growth factor–2, applied locally also accelerates healing of chronic venous ulcers [12]. Our previous results have shown that intralesional injections of GM-CSF (400 μg) reduce the healing time of CL ulcers by 50% [13]. The present study was designed to evaluate the effect that topically applied lower doses of recombinant human GM-CSF (90 μg) plus standard parenteral antimonial therapy have on the healing of CL ulcers, because, if low doses are effective, it becomes possible that this high-cost medication could be used in the treatment of this disease.

Patients and methods. Patients were referred to the health post of Corte de Pedra, Bahia, Brazil, an area where *L. braziliensis* infection is endemic. The health post is the reference center for the diagnosis and treatment of leishmaniasis for an area of ~7000 km^2 and a population of >500,000 persons.

The present study was a randomized, double-blind, placebo-controlled study in which the groups were selected from the patients presenting at the health post. The inclusion criteria were an age of 15–50 years and a diagnosis of CL within 60 days of the beginning of the cutaneous lesion, confirmed either by parasitologic (culture or histopathologic) examination or by positive results of at least 2 of the following: compatible histopathologic examination, serologic examination, or delayed-type hypersen-

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Table 1. Demographic and clinical data for patients with cutaneous leishmaniasis treated with antimony plus either placebo (saline) or recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) applied topically in low doses.

Treatment group, patient (sex)	Age, years	Size of lesion, mm	Duration of lesion, days	Montenegro skin test, mm	No. of antimony courses	Healing time, days
Antimony plus GM-CSF						
1 (M)	18	30	30	05	1	23
2 (F)	21	15	15	20	1	27
3 (M)	15	33	30	15	1	28
4 (F)	39	25	30	15	1	34
5 (M)	16	28	20	10	1	35
6 (M)	23	13	60	20	1	43
7 (F)	30	32	30	17	1	45
8 (M)	58	20	21	20	1	58
9 (M)	19	35	10	20	1	60
10 (M)	40	23	30	23	1	60
Mean ± SD	28 ± 14[a]	25 ± 8[a]	28 ± 14[a]	17 ± 40[a]	…	43 ± 14[b]
Antimony plus placebo						
1 (M)	40	20	30	12	1	21
2 (M)	17	20	20	11	1	35
3 (M)	37	30	15	12	1	48
4 (M)	19	30	60	15	1	55
5 (M)	30	30	40	12	1	62
6 (M)	22	15	30	22	1	100
7 (M)	33	20	30	19	1	102
8 (M)	57	26	24	12	1	103
9 (M)	15	22	30	13	2	215
10 (F)	15	28	60	9	3	256
Mean ± SD	29 ± 14[a]	24 ± 5[a]	34 ± 15[a]	14 ± 40[a]	…	104 ± 79[b]

NOTE. F, female; M, male.

[a] Difference between treatment groups was not significant ($P > .05$, Mann-Whitney U test).

[b] Difference between treatment groups was significant ($P = .043$, Mann-Whitney U test).

sitivity test (also called "the Montenegro skin test") to *Leishmania* antigen. The exclusion criteria were pregnancy, an age of <15 or >50 years, other associated acute or chronic illness, and a history of allergy to GM-CSF and/or antimony.

Written, informed consent was obtained from all patients >18 years old and from parents of younger patients, and the study was approved by the Ethics Committee of the Hospital Universitário Professor Edgard Santos, Bahia, Brazil. The patients who met the inclusion criteria were randomized by use of a randomization table; the randomization was performed by a statistician. The patients were then assigned to either the GM-CSF group (antimony [20 mg/kg of body weight daily for 20 days] plus GM-CSF) or the placebo group (antimony plus saline). GM-CSF was applied not by the physicians who performed the clinical follow-up of the patients but by a different person. Both the patients and the physicians who performed the clinical follow-up were blinded.

Commercially available GM-CSF (Leucomax; Novartis) was diluted in saline, to a final concentration of 10 μg/mL. The use of this commercially available GM-CSF, which was designed to be applied subcutaneously or intravenously, was based on information from a previous study showing that GM-CSF is active after dilution in saline and has an effect when applied topically for the treatment of chronic venous ulcers; it can be frozen at −18°C for several months [11]. This solution was aliquoted in 1-mL tubes.

Treatment was administered on an outpatient basis by the employees of the health post. The patients in the GM-CSF group were treated as follows: ulcers were cleansed with 0.9% sodium-chloride solution and were sprinkled with 1 mL of GM-CSF working solution (10 μg of GM-CSF/mL in 0.9% sodium-chloride solution) per 10 cm^2 of ulcer area, providing a final dose between 1 and 2 $\mu g/cm^2$ of ulcer area. A nonadhesive hydrophobic wound compress (Adaptic; Johnson & Johnson) was secured over the area with a cotton bandage and a short-stretch compression bandage (Colban; 3M). The GM-CSF working solution was reapplied and dressings changed 3 times/week, on Mondays, Wednesdays, and Fridays, for 3 weeks (for a total of 9 GM-CSF applications). The patients in the placebo group received saline applied locally instead of GM-CSF. All of the patients received intravenous pentavalent antimonial treatment (meglumin antimoniate; Roche) daily for 20 days, at 20 mg/kg of body weight.

For 6 months after treatment was initiated, the patients were evaluated every 15 days; afterward, the patients were evaluated every 2 months until 1 year of follow-up was completed. Two

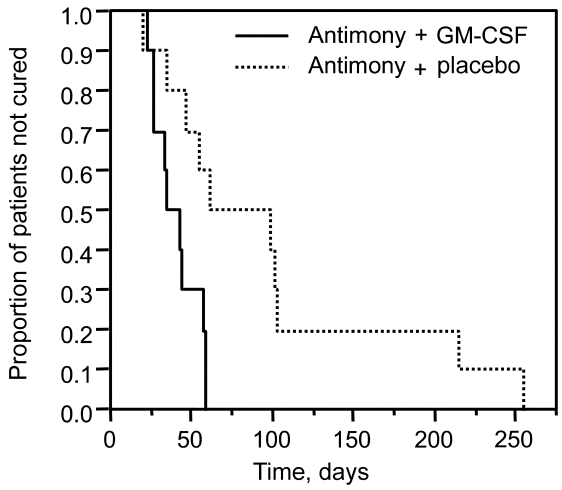

Figure 1. Kaplan-Meier curve showing the proportion of patients who were not cured. Patients were treated with antimony plus either placebo (saline) or recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) applied topically in low doses.

physicians independently examined the patients on all visits. To maintain the double-blind nature of the study, questions on possible side effects of the treatment were deferred to a third medical doctor who was conversant with the known side effects of GM-CSF (such as general malaise and myalgias). Cure was defined as complete reepithelialization of the ulcer. Treatment failure was defined as a persistent, nonhealing ulcer 3 months after the initiation of treatment; patients with the latter received an additional course of intravenous pentavalent antimonial therapy (20 mg/kg of body weight daily for 20 days).

Results. Twenty-two patients were initially assigned to the 2 groups. Two patients (1 in each of the groups) were excluded, because the topical medication was administered by use of a different wound compress. In total, 20 patients were followed, 10 each in the GM-CSF and placebo groups. All of these patients completed the study-treatment regimen and follow-up. As shown in table 1, the patients in the GM-CSF and placebo groups were similar (by the Mann-Whitney *U* test) with respect to the following parameters: sex, age, duration and size of ulcer, and Montenegro skin-test results. The histopathologic results of the lesion biopsies were compatible with leishmaniasis in all patients, and the amastigote form of *Leishmania* parasites was seen in the dermal infiltrate of 3 patients in the GM-CSF group and of 2 patients in the placebo group. Ulcers healed faster in the GM-CSF group than in the placebo group (mean ± SD, 43 ± 14 vs. 104 ± 79 days; $P = .043$). By day 45 after the initiation of treatment, the ulcers of 6 patients (60%) in the GM-CSF group had healed, compared with 2 (20%) in the placebo group. By day 60, the ulcers of all 10 patients in the GM-CSF group had healed; in contrast, the ulcers of only 5 (50%) of the patients in the placebo group had healed by day 60, and it was only after 256 days that the ulcers of all of the patients in the placebo group had healed (figure 1) ($P = .02$, Fisher's exact test). The calculated relative risk for complete healing at day 60 was 2 (95% confidence interval, 1.07–3.10) for the patients in the GM-CSF group versus the patients in the placebo group. Because of nonhealed ulcers 90 days after the initiation of treatment, 2 patients in the placebo group required a second course of antimony; in contrast, none of the patients in the GM-CSF group required a second course. No side effects were detected in the patients in the GM-CSF group. All of the patients were followed for at least 1 year, and no remission occurred.

Discussion. The present randomized, double-blind, placebo-controlled clinical trial has indicated that the use of GM-CSF, applied locally in low doses as an adjuvant to pentavalent antimonial therapy, significantly decreases the healing time of CL ulcers. Differences in the demographic characteristics between the randomized groups were not significant.

Potentially, GM-CSF may decrease the healing time of CL ulcers via 3 mechanisms: (1) an increase in the killing of parasites by direct activation of macrophages [8]; (2) enhancement of scar formation [11]; and (3) modulation of the immunologic response to the lesion site [14]. With respect to the first potential mechanism, a previous study has shown that GM-CSF activates macrophages that kill *Leishmania* parasites in vitro [8]. With respect to the second potential mechanism, GM-CSF has been described as improving healing and scarring of cutaneous lesions caused by chronic venous ulcers [11]. With respect to the third potential mechanism, it has been shown that the macrophage cell line U937 produces interleukin (IL)–10 in the presence of GM-CSF [14]. Patients with CL produce high levels of interferon-γ and tumor necrosis factor (TNF)–α that are important in the killing of the parasite. However, these cytokines may be up-regulated and may be responsible for inflammation and tissue damage. GM-CSF can induce these cytokines but can also induce IL-10, which could modulate the inflammatory response and tissue damage caused by the proinflammatory cytokines. Recently, pentoxifylline, an inhibitor of TNF-α production, has been successfully used to treat refractory mucosal leishmaniasis, which suggests that this disease is related to high inflammatory response [15]. Our data suggest that GM-CSF may influence the human immune response to *Leishmania* parasites by 1 or all of the above mechanisms.

A reduction in healing time from a mean of 104 to 43 days, without any remissions, reduces the need for medical supervision, improves treatment compliance, and reduces lost work days. Moreover, 1 vial of GM-CSF (300 μg) is enough to treat 3 patients by the means described in the present study—a total cost of <$20/patient. This is 5 times lower than the cost of treatment with intralesional GM-CSF injections ($100/patient), with equivalent clinical benefits. Some side effects, such as bone pain and myalgia, have been noted when GM-CSF has been

administered subcutaneously [13]; however, no side effects were observed in either the present study or in a previous trial that administered topically applied GM-CSF for the treatment of venous ulcers [11]. The only potential problem might be the inability of patients to apply the medication themselves, because the success of the present study was due to the administration of the medication at a health post, where the protocol was followed very carefully. At present, a topical preparation of GM-CSF is not commercially available, and we consider investment in the creation of a topical formulation—with the potential to provide more absorption and an easier means of application—to be very important. The use of a topical GM-CSF formulation to treat patients with CL would make treatment both easier and cheaper, with few or no side effects. Thus, the present study opens the possibility of both improving the drug arsenal for the treatment of CL ulcers and, in accordance with our previous results, reducing the dose of antimony and/or the duration of antimonial therapy. Furthermore, the combination of antimony plus GM-CSF may be more effective in severe cases of CL ulcers and in patients refractory to antimonial therapy.

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