CONCISE COMMUNICATION

Radical Cure of Experimental Cutaneous Leishmaniasis by the Bisphosphonate Pamidronate

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The effects in vivo of the bisphosphonate drug pamidronate, used in bone resorption therapy, were investigated in an experimental model of cutaneous leishmaniasis. Pamidronate at an intraperitoneal dose of 10 mg/kg/day for 5 days effects a radical cure of cutaneous leishmaniasis in Balb/c mice, as evidenced by long-term disappearance of lesions; disappearance of amastigotes in lesion sites, as determined by histopathological analysis and cultivation of material obtained from lesions; and polymerase chain reaction analysis of necropsy material, using probes specific for kinetoplast DNA. Pamidronate is, therefore, a new lead compound for the synthesis of drugs effective against cutaneous leishmaniasis.

There are ~ 2 million new cases annually of cutaneous, mucosal, or visceral leishmaniasis, a disease caused by at least 20 *Leishmania* species. Therapy is unsatisfactory [1], and the need for new, inexpensive drugs effective against leishmaniasis is clear.

A number of bisphosphonates have recently been shown to have significant activity against the proliferation of *Leishmania donovani* and other parasites in vitro [2]. Several bisphosphonates are potent inhibitors of bone resorption and are in clinical use for the treatment and prevention of osteoporosis, Paget's disease, hypercalcemia caused by malignancy, tumor metastases in bone, and other ailments [3]. Selective action on bone is based on the binding of the bisphosphonate moiety to the bone mineral and the inhibition of the osteoclast's farnesyl pyrophosphate synthase (FPPS) [3]. In the trypanosomatid *Trypanosoma cruzi*, FPPS has been postulated to be the major target of the bisphosphonate drugs [4], and an expressed FPPS from this parasite has been shown to be potently inhibited by bisphosphonates [4]. In this work, we investigated the effectiveness of the bisphosphonate pamidronate (Aredia; Novartis) in curing experimental cutaneous leishmaniasis in vivo.

Materials and Methods

Culture methods and treatment of mice. Leishmania mexicana amazonensis parasites (clone 12-D3) were obtained, as described elsewhere [5]. Promastigotes were grown to the stationary phase in SDM-79 medium (pH 7.2) containing 10% fetal calf serum. One million parasites were injected subcutaneously into the footpads of 8-10-week-old Balb/c mice. After 2-3 months, lesions of measurable size developed. Mice were randomly sorted into groups of 5-10 prior to dosing via intraperitoneal injections administered daily. Lesion size was then recorded once a week by measuring footpad swelling, using a caliper. Average lesion size was calculated as the sum of the differences obtained between infected and uninfected footpads, divided by the number of mice. Data on lesion progression were analyzed for statistical significance by use of analyses of variance and Student's t tests for all animals studied. A result was considered to be significant at P < .05. Necropsy material was cultivated in a diphasic medium at 28°C [6]. Two days after inoculation, parasites were counted in an hemocytometer. If no parasites were detected, the cultures were kept for 15 more days; if they remained negative, they were discarded. This method has been shown to detect Leishmania parasites in biopsy material of 42% of patients clinically diagnosed to have leishmaniasis [6].

Histopathology. Samples were fixed in 10% formaldehyde for 24 h and then were processed for histopathology. Sections 4 mm in diameter were stained with hematoxylin-eosin.

Polymerase chain reaction (PCR) method. The primers 5'-GAGGCCCCGAGCTTTGACC-3' and 5'-GGTGTAAAATAG-GGGCGGATGCTCTG-3' were designed in accord with the sequence of conserved regions of kinetoplast DNA (kDNA) of *L. mexicana* [7]. This method has been shown to be highly specific and to provide high sensitivity, since it can detect up to 10 femtograms of kDNA [7], and has been shown to detect *Leishmania*

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Figure 1. Effects of different doses of intraperitoneal pamidronate on *Leishmania mexicana amazonensis* lesions in mice. Mice showed decreases in lesion size after 1 dose (10 mg/kg) or 3 doses (10 mg/kg/ day), reactivation of lesions after 5 doses (5 mg/kg/day), and disappearance of lesions after 5 larger doses (10 mg/kg/day) of pamidronate. Statistical analysis (using analysis of variance and Student's *t* tests) indicated that there was a significant difference between the treated (10 mg/kg/day) and the control groups after week 2 (P < .05).

kDNA in biopsy material of 98% of patients with a clinical diagnosis of leishmaniasis [6]. The primers used were in a reaction mixture containing 100 ng of total DNA extracted from the homogenized whole footpad from each animal, 2 m*M* each dNTP, $10 \times$ reaction buffer, and 2 m*M* MgCl₂ [7]. The PCR was carried out as described elsewhere [7]. The length of the expected amplification product was 700 bp [7].

Results

We carried out 4 trials of the activity of pamidronate, using different doses; figure 1 shows a summarizing experiment. The only effect with a 10 mg/kg 1- or 3-day treatment was a small reduction in the lesion size. Control animals had to be euthanized by week 7, because the lesions became ulcerated. At a dose of 5 mg/kg for 5 days, the lesions were significantly smaller and did not ulcerate. However, in all 10 mice, lesions reactivated 7 weeks after initiation of the experiment. Similar recurrence of lesions after completion of treatment of cutaneous leishmaniasis with amphotericin B has been reported elsewhere [8]. At 10 mg/ kg for 5 days, mice showed a significant reduction in lesion size, starting 2 weeks after initiation of treatment. These relatively large lesions appeared to be fully resolved by week 10. DNA detection by PCR and culture of necropsy material were both positive until week 9, although the number of parasites after a 2-day culture decreased from 7×10^4 /mL at week 5 to 6×10^{3} /mL at week 7, 3×10^{3} /mL at week 8, and 2×10^{2} /mL at week 9. PCR and culture detection of parasite DNA were both negative, however, by week 13, at which point the 5 remaining animals were killed. This improvement without further therapy may reflect the immunostimulatory effect that has been attributed to pamidronate [9].

Figure 2 shows that samples from the footpads of representative pamidronate-treated animals (treatment with 10 mg/kg for 5 days; lanes 1, 3, 5, 6, 7, and 8) did not have the expected amplification band, although there was a strong amplification of the expected size in samples from control animals (lanes 2 and 4). Examples of the histopathology of the footpads of treated and untreated animals after completion of the experiment (treatment with 10 mg/kg for 5 days) are shown in figure 3. Control mice (figure 3B) showed numerous amastigotes inside the macrophages (figure 3D, arrows), in contrast to treated mice (figure 3A), which showed no parasites (figure 3C). To be certain that pamidronate was able to exert a radical cure in this experimental model of leishmaniasis, we maintained the mice alive for a long period of time in 2 additional experiments, in which we obtained a similar reduction in lesion size, using 10 mg/kg of pamidronate in either a 5-day or a 7-day treatment. Relapse was not observed in any of the treated animals (11 mice from the 2 experiments) after 5 or 7 months of observation, respectively.

Discussion

The total regression of lesions observed in all animals after completion of treatment with pamidronate suggests that the compound has leishmanicidal activity in this experimental model. Pamidronate did not produce apparent toxic effects in the mice treated with a 10 mg/kg/day dose for 5 days, as indicated by daily observation of their weight, activity, and appearance. However, pamidronate is known to have nephrotox-



Figure 2. Polymerase chain reaction (PCR) image of necropsy material from footpads of mice, showing band of expected size in lanes 2, 4 (untreated mice), and 10 (positive control; *Leishmania mexicana amazonensis* genomic DNA) and its absence in necropsy material from footpads of treated mice (*lanes 1, 3, 5, 6, 7, and 8*). *Lane 9*, Negative control for PCR mixture alone; MW, molecular weight marker.



Figure 3. Effects of intraperitoneal pamidronate on *Leishmania mexicana amazonensis* lesions in mice and on their histopathology. Shown are lesions of one of the treated (A) and one of the control (B) mice in an experiment using a dosage of 10 mg/kg/day for 5 days, 7 weeks after completion of treatment. *Bars*, 1 cm. Histopathological analysis (*C* and *D*) showed a significant no. of parasites in untreated mice (arrows and inset in panel *D* show amastigotes inside macrophages), in contrast to the absence of parasites in treated mice (*C*). *Bars*, 10 μ m (hematoxylineosin staining).

icity in mice at doses >20 mg/kg administered once, as evidenced by polyuria and kidney swelling [10]. A study in rats [11] has also demonstrated a nephrotoxic effect of pamidronate. Since the effective dose of pamidronate against cutaneous leishmaniasis in mice (10 mg/kg/day for 5 days) is higher than the dose that has been administered to humans without toxic side effects (60 mg/dose, administered every 2 weeks for 16 weeks) [12], it is possible that currently approved clinical regimens of pamidronate may not be sufficiently high to cure human cutaneous leishmaniasis. However, our results do suggest that the bisphosphonate pamidronate could be a useful lead compound for the synthesis of new drugs against this disease.

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