Treatment of Experimental Leishmaniasis with the Immunomodulators Imiquimod and S-28463: Efficacy and Mode of Action

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Leishmaniasis is an infectious disease caused by different species of *Leishmania* protozoa and is ranked among the six most important tropical infectious diseases by the World Health Organization, with about 12 million people currently infected and 350 million people at risk of infection [1, 2]. Visceral leishmaniasis is currently a major cause of morbidity in a number of countries, most notably India and Sudan [2]. *Leishmania* protozoan parasites are transmitted to humans through the bite of the infected sandfly. Within the sandfly vector, *Leishmania* exist as flagellated promastigotes; however, in the mammalian host, the promastigotes differentiate into nonflagellated amastigotes, which specifically multiply within the phagolysosomal vacuoles of macrophages [3]. Cutaneous leishmaniasis is the most common form of the disease and results in skin lesions, which can last for several months or years, at the site of infection. Cutaneous leishmaniasis is caused by several species of *Leishmania* species, including *Leishmania major* [2, 3]. Visceral leishmaniasis (also known as Kala azar) is associated with infection by *Leishmania donovani*. Visceral leishmaniasis causes fever, weight loss, emaciation, and an enlarged liver and spleen, and is associated with a high rate of mortality if not treated [2, 4].

There is a need for new, effective, and less toxic treatments for leishmaniasis, an infectious disease caused by *Leishmania* protozoa and is the major cause of suffering and morbidity in much of the developing world. Imiquimod, an immune-response modifier, has recently been approved by the Food and Drug Administration for the treatment of genital warts caused by human papillomaviruses. Imiquimod initiates a local immune reaction, including the stimulation of macrophages, resulting in resolution of human papillomavirus infection and regression of the viral lesion. Since imiquimod activates a number of immune cells, including macrophages, which are the only host cells of *Leishmania* species, an investigation was done to determine whether it induces leishmanicidal properties in infected macrophages in vitro and in vivo in a mouse model. Imiquimod and a related compound, S-28463, effectively stimulated leishmanicidal activity in macrophages; moreover, imiquimod stimulated signal transduction associated with inducing nitric oxide synthesis in macrophages.
caused by a viral infection, we were interested in investigating whether it could stimulate leishmanicidal activity in infected macrophages and to determine its mechanism of action. To that end, we examined the ability of imiquimod and its related compound S-28463 to stimulate leishmanicidal activity in infected macrophages in vitro and to reduce the severity of infection in vivo in experimental cutaneous leishmaniasis. We also examined their roles in induction of nitric oxide (NO) and the AP-1- and NF-κB-associated signal transduction pathways in macrophages.

Materials and Methods

Infection of macrophages with L. donovani. The IS2D and Ethiopian LV9 strains of L. donovani were used in this study. The IS2D strain could be cultured continuously in culture medium, and the LV9 strain was maintained by continuous passage in female golden Syrian hamsters (Charles River Canada, St. Constant, Canada). Amastigotes were isolated from the heavily infected hamster spleen, as previously described [10]. Bone marrow–derived macrophages (BMM) were obtained from femurs of 6- to 8-week-old female BALB/c mice (Charles River Canada), as previously described [11, 12]. Quiescent BMM (10^6 cells/mL) in polystyrene tubes were infected with amastigotes at a ratio of 10:1 amastigotes per macrophage for 12 h. BMM were washed 3 times at low speed to remove noningested parasites and then resuspended in RPMI-1640 complete medium. Infection levels were determined by examination, by use of a microscope, of Giemsa-stained cytocentrifuge preparations [12].

Imidazoquinoline induction of leishmanicidal activity in vitro. The imidazoquinoline compounds, imiquimod and S-28463, were provided by R. Miller (3M Pharmaceuticals). An in vitro infection assay was performed as described previously [12]. BMM were infected with L. donovani IS2D or LV9 amastigotes overnight and then treated with increasing doses (0, 10, and 100 ng/mL and 1 μg/mL of imiquimod or with increasing doses (0, 5, 10, and 100 ng/mL of S-28463. The effect of S-28463 and imiquimod on macrophage leishmanicidal activity was determined by counting, using a microscope, intracellular parasites at days 1, 2, 3, 4, and 5 after treatment. L. donovani IS2D stationary-phase promastigotes or axenic amastigotes (10^6 cells/mL) in screw-capped polystyrene tubes were treated with 0, 5, 10, and 100 ng/mL S-28463. Parasite numbers were determined at days 1, 2, 3, 4, and 5 after treatment.

Imiquimod induction of leishmanicidal activity in vivo. Two groups of 5 female 6- to 8-week-old BALB/c mice (22–28 g) were infected by injection in the right hind footpad with 5 × 10^6 stationary-phase L. major (Friedlin V9) promastigotes. After infection, 1 group of mice was treated with 5% imiquimod cream (Al dara), and the other group was treated with placebo cream every 3 days. The 5% imiquimod cream and placebo cream were applied evenly over the infected area of the footpad. At week 3 of infection, infected mice were killed and lesion development was photographed and measured with a dial caliper (Peacock, Japan) and expressed as the increased thickness of the footpad on the infected right hind foot compared with that of the uninfected left hind foot, as previously described [13]. Alternatively, following infection, the lesions were allowed to develop for 2 weeks, at which time the swelling of the footpad was apparent, but the ulceration had not yet developed. At this time, treatment was initiated and given every 3 days for an additional 2 weeks. Parasite burdens were determined by identifying motile promastigotes, using a microscope, after serial dilution of footpad homogenates and calculating the geometric mean (log_10) and SE of the last positive reciprocal dilution for each group, as previously described [14].

Measurement of NO production and inhibition of NO synthesis with Nω-monomethyl-L-arginine (L-NMMA). The level of NO produced by L. donovani-infected and noninfected BMM treated with 0, 10, and 100 ng/mL and 1 μg/mL imiquimod or 0, 5, 10, and 100 ng/mL S-28463 or 100 U/mL recombinant murine IFN-γ (rmIFN-γ) plus 100 ng/mL lipopolysaccharide (LPS) was determined by measuring NO_2^- production using the Griess reaction according to the microassay as previously described [15]. In brief, 50 μL of the culture supernatants was incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-naphthyl-ethylenediamine dihydrochloride in 2.5% H_3PO_4) for 10 min at room temperature. The absorbance was measured at 550 nm, and sodium nitrite (NaNO_2) was used as the standard.

For the inhibition of NO synthesis, L. donovani LV9–infected BMM were treated with medium alone or medium containing 100 U/mL IFN-γ and 100 ng/mL LPS or 100 ng/mL S-28463, with or without 500 μM of L-NMMA (Calbiochem, La Jolla, CA) to inhibit NO synthesis. Cultures were incubated for 72 h at 37°C. The effect of L-NMMA on macrophage leishmanicidal activity was determined by visual counting of intracellular parasites after Giemsa-stained cytocentrifuge preparations as previously described [12].

Northern blot analysis of inducible NO synthase (iNOS) and c-fos mRNA. Normal BMM (10^6 cells/sample) were treated with 100 ng/mL S-28463 or 100 U/mL rmIFN-γ plus 100 ng/mL LPS for the iNOS mRNA–induced positive control or 100 ng/mL LPS for the c-fos mRNA–induced positive control. The negative control for c-fos and iNOS mRNAs were untreated BMM. Total cellular RNA was subjected to Northern blot analysis, as previously described [11, 16]. The mouse iNOS probe was the 4.1-kb NotI fragment from pmmac-NOS (provided by Charles J. Lowenstein, Johns Hopkins University School of Medicine, Baltimore). The c-fos probe consisted of the 1.3-kb PvuII-BglII fragment from pFBH-1. To ensure that equal amounts of RNA were analyzed, we stripped blots, rehybridized them with a radiolabeled cDNA probe for actin mRNA (1.25-kb PstI of pBA-1), washed them, and again subjected them to autoradiography. When quantifying by scanning densitometry, we used multiple exposures to ensure that all signals were within the linear response range of the film.

Analysis of activated signal transducer and activator of transcription-1 (STAT1). BMM were treated with 100 ng/mL S-28463 or 200 U/mL rmIFN-γ for various times; control BMM incubated in parallel were left untreated. In brief, after stimulation, ~5 × 10^6 cells/mL were washed once with ice-cold PBS plus 0.1 M NaCl, pH 8.0, 0.5% NP-40, 10% glycerol, 0.1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2 μg/mL pepstatin, 1 μg/mL chymostatin, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl) for 20 min at 4°C. Cell lysates were centrifuged at 13,000 g at 4°C for 15 min to remove
nuclei. Cytoplasmic fractions were precleared with 40 μL of normal rabbit serum and adjusted for the protein concentration, using a protein assay kit (Bio-Rad, Richmond, CA). STAT proteins were immunoprecipitated with 10 μL of anti-STAT1 p84/p91 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 2 h and followed by complexing with 20 μL of agarose plus protein A/G at 4°C for 2 h. Agarose-bound immune complexes were pelleted by microcentrifugation at 13,000 g for 10 min and washed 3 times with lysis buffer plus 0.1 M M Na2VO4. The immune complexes were boiled for 5 min in SDS-polyacrylamide gel electrophoresis sample buffer and run on 10% SDS-polyacrylamide gels and transferred to nitrocellulose (Bio-Rad). Membranes were blocked in PBS containing 10% fetal bovine serum, 0.05% Tween 20, and 0.1% M M Na2VO4, and then reacted with a monoclonal antibody specific for phosphotyrosine (PY20; Signal Transduction Laboratories, rabbit serum and adjusted for the protein concentration, using a protein assay kit (Bio-Rad, Richmond, CA). STAT proteins were determined over 5 days. As shown in figure 1E and 1F, there was no indication that S-28463 was directly toxic to either promastigotes or amastigotes, even when used at high concentrations. Similar data were obtained with imiquimod (data not shown). Taken together, the data from figure 1 show that imiquimod and S-28463 were not directly toxic to infected and noninfected BMM was followed by the release of NO. As shown in figure 2B, 24 and 48 h after treatment with S-28463, there was a substantial release of NO at levels comparable to what was induced by IFN-γ and LPS. In contrast, unstimulated cells produced only a basal level of iNOS mRNA. The data are also significant in demonstrating that these compounds stimulate leishmanicidal activity in macrophages in the absence of any other cell types. Having demonstrated that imiquimod and S-28463 could induce leishmanicidal activity in macrophages, we began to examine the possible mechanisms of action. Because imiquimod and S-28463 are related compounds, and S-28463 induced greater leishmanicidal activity in macrophages, we used S-28463 to examine the mechanism of action of the imidazoquinolines. It has been previously established that NO release has a potent antimicrobial activity and was active through stimulating a local immune response [7–9]. It was therefore important to determine whether imiquimod or S-28463 had any direct toxic effect against L. donovani promastigotes or amastigotes. Axenic promastigotes and amastigotes were cultured in increasing concentrations of S-28463, and the number of parasites was determined over 5 days. As shown in figure 2E and 2F, there was no indication that S-28463 was directly toxic to either promastigotes or amastigotes, even when used at high concentrations. Similar data were obtained with imiquimod (data not shown). Taken together, the data from figure 1 show that imiquimod and S-28463 were not directly toxic to Leishmania species but did display leishmanicidal activity in the context of infected macrophages, arguing that these compounds induced a leishmanicidal activity in the macrophage. These data are also significant in demonstrating that these compounds stimulate leishmanicidal activity in macrophages in the absence of any other cell types.

### Results

**In vitro studies.** We first determined whether imiquimod or its related compound, S-28463, could stimulate leishmanicidal activity in macrophages. BMM prepared from BALB/c mice were infected overnight with 2 L. donovani strains, LV9 or 1S2D, as described in Materials and Methods. Infected BMM were then treated with increasing amounts of imiquimod or S-28463, and the level of infection was determined for 5 consecutive days. At the concentrations used, imiquimod and S-28463 had no effect on the viability of the macrophages, as determined by trypsin blue exclusion. As shown in figure 1A–1D, both imiquimod and S-28463 demonstrated leishmanicidal activity in both strains of L. donovani; however, at lower concentrations, S-28463 was more potent than imiquimod.

Previous studies had shown that imiquimod had no direct antimicrobial activity and was active through stimulating a local immune response [7–9]. It was therefore important to determine whether imiquimod or S-28463 had any direct toxic effect against L. donovani promastigotes or amastigotes. Axenic promastigotes and amastigotes were cultured in increasing concentrations of S-28463, and the number of parasites was determined over 5 days. As shown in figure 1E and 1F, there was no indication that S-28463 was directly toxic to either promastigotes or amastigotes, even when used at high concentrations. Similar data were obtained with imiquimod (data not shown). Taken together, the data from figure 1 show that imiquimod and S-28463 were not directly toxic to Leishmania species but did display leishmanicidal activity in the context of infected macrophages, arguing that these compounds induced a leishmanicidal activity in the macrophage. These data are also significant in demonstrating that these compounds stimulate leishmanicidal activity in macrophages in the absence of any other cell types.

### Table 1. Inhibition of macrophage leishmanicidal activity by N6-monomethyl-l-arginine (L-NMMA).

<table>
<thead>
<tr>
<th>Macrophage cultured with</th>
<th>No. of Leishmania donovani/100 macrophages</th>
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<tr>
<td>Medium alone</td>
<td>192 ± 12</td>
</tr>
<tr>
<td>IFN-γ + LPS</td>
<td>30 ± 1a</td>
</tr>
<tr>
<td>IFN-γ + LPS + L-</td>
<td>193 ± 2</td>
</tr>
<tr>
<td>NMMA</td>
<td></td>
</tr>
<tr>
<td>S-28463</td>
<td>34 ± 7a</td>
</tr>
<tr>
<td>S-28463 + L-NMMA</td>
<td>179 ± 8</td>
</tr>
</tbody>
</table>

**NOTE.** L. donovani LV9-infected macrophages were treated with medium alone or medium containing 100 U/mL interferon (IFN-γ) and 10 ng/mL lipopolysaccharide (LPS) or 100 ng/mL S-28463 with or without 500 μM of L-NMMA. At 72 h, effect of treatments and L-NMMA on intracellular killing was determined by counting no. of intact parasites in 100 macrophages. Results are mean ± SE. Similar results were obtained from 3 separate experiments. Results were analyzed by use of Student’s t test.

a P < 0.05 compared with macrophages cultured with medium alone.
Figure 1. Imiquimod and S-28463 induce leishmanicidal activity in infected macrophages (MΦ). Bone marrow-derived macrophages were infected with either Leishmania donovani strain LV9 (A, C) or L. donovani strain 1S2D (B, D) and then treated with increasing concentrations of S-28463 (A, B) or imiquimod (C, D), as indicated. Data are mean ± SE of 3 independent experiments. S-28463 is not directly toxic against L. donovani 1S2D promastigotes or amastigotes cultured in absence of macrophages. Axenic promastigotes (E) or amastigotes (F) were cultured as described in Materials and Methods and treated with increasing concentrations of S-28463. Data are mean ± SE of 3 independent experiments.

LPS in the noninfected BMM. In the infected cells, there was a reduced but clearly detectable level of NO release after treatment with S-28463. These data show that infection with L. donovani could reduce but not completely inhibit S-28463–mediated release of NO. Similar results were obtained with imiquimod; however, there was a lower level of NO released in both the noninfected and infected cells after imiquimod treatment than was obtained with S-28463 treatment (data not shown). This is consistent with the data presented in figure 1, which also showed that S-28463 had more potent macrophage leishmanicidal activity than imiquimod.

To determine whether the S-28463–mediated leishmanicidal
activity was due to NO synthesis, we determined whether inhibition of NO synthesis with L-NMMA could inhibit the S-28463–mediated leishmanicidal activity. As shown in table 1, treatment of infected BMM with IFN-γ and LPS or with S-28463 induced leishmanicidal activity, and this was effectively reversed in the presence of the NO synthesis inhibitor L-NMMA. Taken together, these data argue that imiquimod and S-28463 could induce leishmanicidal activity in macrophages to a large extent through stimulating the expression of the iNOS gene and subsequent synthesis of NO.

S-28463–mediated signal transduction. We next examined the mechanism by which S-28463 stimulates the expression of the iNOS gene. It has been previously established that the iNOS promoter contains binding sites for the AP1 transcription factor and has multiple binding sites for STAT1 and NF-κB transcription factors, which are required for the maximum response to IFN-γ and LPS, respectively [20, 21]. We therefore examined whether S-28463 induces signal transduction responses leading to the activation of AP1, STAT1, and NF-κB. For the induction of AP1, we examined the induction of c-fos mRNA encoding the c-fos protein, which is a component of the AP1 transcription complex. In this experiment, LPS was used as a positive control for induction of c-fos mRNA induction. As shown in figure 3A, both S-28463 and LPS induced a rapid and transient expression of c-fos mRNA in BMM compared with that in unstimulated cells, which had very little detectable c-fos mRNA.

We next examined the NF-κB signal transduction pathway, which is involved in the expression of a number of cytokine genes and the iNOS gene in macrophages [20]. In quiescent cells, NF-κB is sequestered in the cytoplasm by tightly bound

![Figure 2.](image)

**Figure 2.** A. Northern blot analysis of inducible nitric oxide synthase (iNOS) mRNA expression induced by S-28463 in macrophages. Bone marrow–derived macrophages (BMM) were treated with S-28463 or combination of interferon-γ and lipopolysaccharide (IFN-γ + LPS), and levels of iNOS mRNA and actin mRNA were determined at 6 and 8 h after stimulation, as indicated. Levels of iNOS mRNA relative to level of actin mRNA are shown in densitometric analysis of Northern blot film. Similar results were obtained in 2 independent experiments. B. Induction of nitric oxide (NO) release by S-28463 in normal and *Leishmania donovani* 1S2D–infected macrophages. BMM were treated with increasing amounts of S-28463 or, as positive control, combination of IFN-γ + LPS, and production of NO was determined 24 and 48 h after stimulation, as described in Materials and Methods. Untreated BMM served as negative control for NO release. Note that there was no detectable NO release in untreated cells.
inhibitory proteins called IkBs [22]. Phosphorylation of IkB-α by the IkB kinase results in the release and subsequent translocation of NF-κB to the nucleus, where it binds to specific enhancer sequences, which results in the expression of a variety of genes, including the iNOS gene. To determine the effect of S-28463 on the NF-κB–associated pathway, we treated BMM with 100 ng/mL S-28463 and determined the phosphorylation of IkB-α. As a positive control for phosphorylation of IkB-α, we treated BMM with 10^4 U/mL TNF-α. As shown in figure 3B, both S-28463 and TNF-α induced phosphorylation of IkB-α, as evident from 30 min to 3 h after stimulation. These data argue that S-28463 could stimulate the NF-κB–associated signal transduction pathway.

It has been established that the iNOS promoter also contains several binding sites for the STAT1 transcription factor [20], which becomes activated through phosphorylation by the Jak family of kinases. Therefore, we next determined whether S-28463 could induce signal transduction associated with activation of the STAT1 transcription factor by determining whether S-28463 induced phosphorylation of STAT1. As a positive control for induction of phosphorylation, we treated BMM with IFN-γ. As shown in figure 3C, under conditions in which IFN-γ induced phosphorylation of the p91 STAT1 protein, S-28463 failed to do so. Phosphorylated STAT1 was detected at 15 and 30 min after stimulation with IFN-γ but not with S-28463. These data show that under conditions in which IFN-γ could activate the Jak-STAT1 signal transduction pathway in these macrophages, S-28463 could not.

Taken together, the data from the signal transduction analysis show that S-28463 induced the expression of the iNOS gene.

Figure 3. A. Northern blot analysis of c-fos gene expression induced by S-28463. Bone marrow–derived macrophages (BMM) were treated with S-28463 or lipopolysaccharide (LPS), and levels of c-fos mRNA and actin mRNA were determined by Northern blot analysis at 0.5, 1, and 3 h after stimulation (upper panel). Levels of c-fos mRNA relative to actin mRNA levels were determined by densitometric analysis (data not shown). Similar results were obtained in 2 independent experiments. B. Stimulation of phosphorylation of IkB-α by S-28463. BMM were treated with S-28463 or tumor necrosis factor (TNF)-α as positive control for stimulation of phosphorylation of IkB-α. Cell extracts were prepared at indicated times following stimulation, and levels of phosphorylated IkB-α (phospho-IkB-α) and total IkB-α were determined in each sample by Western blot analysis, as indicated. Note that 0 time point represents negative control, where cells were not treated, and there was little detectable phosphorylated IkB-α in these cells. Similar results were obtained in 4 independent experiments. C. S-28463 does not stimulate phosphorylation of signal transducer and activator of transcription-1 (STAT1) in macrophages. BMM were treated with S-28463 or interferon (IFN)-γ as positive control for stimulating phosphorylation of p91 STAT1. Control cells represent untreated cells. Cell extracts were prepared at 15 and 30 min, and level of phosphorylated STAT1 was determined as described in Materials and Methods. As shown, under conditions where IFN-γ induced phosphorylation of STAT1, S-28463 treatment was unable to do so. Similar results were obtained in 4 independent experiments.
gene, which could be due in part to the activation of the API and NF-κB transcription factors but not to the Jak-STAT1-associated signal transduction pathway.

**In vivo studies.** The above experiments, which were done on homogeneous macrophage cultures, revealed that imiquimod and S-28463 could induce macrophage-mediated leishmanicidal activity. This could result from the induction of signal transduction pathways associated with the synthesis of NO, which is toxic to the *Leishmania* intracellular parasite. However, it was important to determine whether these compounds were also capable of inducing leishmanicidal activity in vivo.

One of the most widely used experimental models for *Leishmania* infection is to infect the footpad of BALB/c mice with *L. major*, which results in swelling and skin ulceration that are characteristic of human cutaneous leishmaniasis. In general, however, cutaneous leishmaniasis caused by *L. major* progresses much faster in the BALB/c mouse model than in humans, as this mouse strain is very susceptible to *L. major*.

We therefore determined whether treatment with 5% imiquimod cream could reduce the severity of cutaneous leishmaniasis in BALB/c mice. Five-percent imiquimod cream was used in this experiment because it has been approved by the FDA for topical application in humans for cutaneous genital warts caused by human papillomaviruses. Initially, 2 groups of 5 BALB/c mice were infected in the right hind footpad with 5 × 10⁶ *L. major* promastigotes. After infection, 1 group was treated with 5% imiquimod cream, and the other group was treated with placebo cream (carrier cream minus imiquimod). Mice were treated at 3-day intervals for 3 weeks, as detailed in Materials and Methods.

Three weeks after infection, we terminated the experiment and recorded the size, level of infection, and appearance of the footpad lesions. As shown in figure 4, the imiquimod-treated footpads were significantly smaller than the control-treated footpads, indicating a reduced level of infection. Consistent with this observation, there were fewer parasites in the imiquimod-treated footpads (geometric mean [log₁₀] ± SE, 2.795 ± 0.598; *P* < .05, Student’s *t* test) than in the control-treated infected footpads (geometric mean [log₁₀] ± SE, 5.242 ± 0.238). However, the most striking effect was apparent in the overall appearance of the surface of the imiquimod-treated footpads compared with that of the placebo control–treated footpads. The imiquimod-treated footpads did not develop ulcerated red surface lesions, as is typical in this mouse model and as is seen in the placebo control–treated mice (figure 5). As shown in table 2, none of the imiquimod-treated mice developed the ulcerated skin lesions (shown in figure 5), whereas all of the placebo control–treated mice developed surface lesions of various sizes. This is perhaps not surprising since the 5% imiquimod cream was applied topically to the footpad surface, and this is where it appeared to have the greatest beneficial effect. However, it was also apparent that the treatment did not completely clear the infection, possibly because the imiquimod could not penetrate deeper beyond the footpad, where parasites are also actively proliferating.

In a second experiment, we delayed treatment for 2 weeks after infection until the swelling of the infected footpad was apparent. Treatment was then administered every 3 days for a 2-week period. Under these conditions, the results were very similar to those shown in figures 4 and 5, and, again, there was a significant inhibition of surface ulceration (table 3). The in vivo experiments in the experimental model were consistent with the in vitro experiments in showing that imiquimod was capable of inducing leishmanicidal activity.

**Discussion**

The purpose of this study was to determine whether the imidazoquinoline compounds, imiquimod and S-28463, could induce leishmanicidal activity and, if so, to explore potential mechanisms of action. The principal observation in this study was that these compounds have macrophage-mediating leishmanicidal activity both in vitro and in vivo. The imidazoquinolines did not demonstrate direct toxic effects against axenic promastigotes or amastigotes but could induce the expression of the iNOS gene and release of NO from both infected and noninfected macrophages. These observations argue that the
macrophage leishmanicidal activity induced by imidazoquinolines is due to a large extent to their ability to stimulate the release of NO. One of the most significant aspects of this research is that these analyses were performed with a small molecule, which has now been approved by the FDA for the treatment of a common viral cutaneous infection.

In this study, we also began to examine the mechanism by which the imidazoquinolines stimulate expression of the iNOS gene. Evidence was presented that S-28463 could stimulate the activation of the AP1- and the NF-κB–associated signal transduction pathways but not the Jak-STAT1–associated pathway in macrophages. This is particularly significant because it argues that activation of the Jak-STAT1 signal transduction–associated pathway is not required for induction of leishmanicidal activity in macrophages. This is noteworthy because resolution of infection is dependent on IFN-γ–mediated induction of leishmanicidal activity in macrophages, and the Jak-STAT1 pathway is believed to be a major response to IFN-γ.

It is also possible that the expression of the iNOS gene could be due in part to an autocrine mechanism, since S-28463 can induce TNF-α [9], which also can induce NO release [17]. These results are also consistent with previous observations that imiquimod can stimulate NF-κB DNA binding to the HIV-1 long terminal repeat NF-κB site in human monocytes [23].

Although this study focused on signal transduction associated with expression of the iNOS gene, it is likely that the imidazoquinolines can induce additional signal transduction pathways and biochemical changes in the macrophages, and it will also be of interest to define the imiquimod receptor on

Table 2. Surface lesions on footpads of BALB/c mice after treatment with 5% imiquimod cream.

<table>
<thead>
<tr>
<th>Group</th>
<th>Surface lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo cream</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1 small lesion</td>
</tr>
<tr>
<td>2</td>
<td>2 small lesions</td>
</tr>
<tr>
<td>3</td>
<td>1 big lesion</td>
</tr>
<tr>
<td>4</td>
<td>1 big lesion</td>
</tr>
<tr>
<td>5</td>
<td>1 big lesion</td>
</tr>
</tbody>
</table>

NOTE. Two groups of 5 BALB/c mice were infected in the right hind footpad with 5 × 10⁶ stationary-phase of Leishmania major (Friedlin V9) promastigotes. After infection, mice were treated with 5% imiquimod cream or placebo (see Materials and Methods). Lesions were scored according to the following system: none = no visible lesion, small lesion = open lesion ≤ 2.5 mm in diameter, big lesion = open lesion > 2.5 mm in diameter. Similar results were obtained from 3 separate experiments.

Figure 5. Imiquimod inhibition of surface ulceration in Leishmania major–infected mouse footpads. BALB/c mice were infected with L. major in the right hind footpad. Treatment with 5% imiquimod cream or placebo cream was initiated after infection and continued every 3 days for 3 weeks. Appearance of typical 5% imiquimod cream–treated and placebo control–treated footpads is shown.
macrophages. We are examining further the mechanism(s) by which imiquimod activates leishmanicidal activity in macrophages and whether it can mediate the release of IL-12.

In the in vivo experimental cutaneous leishmaniasis model, topical treatment with 5% imiquimod cream (compared with placebo cream) significantly reduced the severity of the lesions caused by *L. major*. In this experimental model, the infection progresses very rapidly throughout the infected foot. In comparison, a typical human cutaneous *L. major* infection progresses more slowly and is largely restricted to the surface. Topical treatment with 5% imiquimod cream had the greatest beneficial effect on the surface of the footpad. This may be because the imiquimod activated the macrophages closest to the surface where it was applied and could not penetrate as far as the inner regions of the foot. We would therefore expect the infection to spread further if treatment were stopped in this animal model. Nevertheless, the imiquimod-treated footpads demonstrated significantly less swelling due to infection, fewer parasites, and, most striking, the inhibition of surface ulceration.

On the basis of these observations, it would be interesting to determine whether 5% imiquimod cream could limit the spread or reduce the time of ulceration in human cutaneous leishmaniasis. Surface ulceration is a major complication in human cutaneous leishmaniasis because it results in disfiguring scarring, usually in exposed areas of the body, such as the face. It would also be interesting to carry out reverse transcription–polymerase chain reaction analysis on cutaneous lesions after treatment with 5% imiquimod cream to examine cytokine gene expression in regressing or progressing lesions. Such studies should be done in the future.

Since leishmaniasis is a disease largely restricted to developing countries, a major consideration is the cost associated with drug development. Five-percent imiquimod cream has been approved by the FDA for topical application in the treatment of cutaneous genital warts caused by human papillomaviruses, which is among the most common sexually transmitted diseases in the United States. If 5% imiquimod cream did show significant efficacy and safety, then this would be a definite advantage under the present circumstance, in which funds for developing new drugs to treat tropical diseases are limited. Moreover, the use of a safer topical application could also reduce the use of antimonials, which would significantly reduce the toxicity and side effects associated with the treatment of this disease.

Acknowledgments

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References


