The Antituberculosis Drug Pyrazinamide Affects the Course of Cutaneous Leishmaniasis In Vivo and Increases Activation of Macrophages and Dendritic Cells

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Received 11 August 2009/Returned for modification 8 September 2009/Accepted 11 September 2009

Antileishmanial therapy is suboptimal due to toxicity, high cost, and development of resistance to available drugs. Pyrazinamide (PZA) is a constituent of short-course tuberculosis chemotherapy. We investigated the effect of PZA on Leishmania major promastigote and amastigote survival. Promastigotes were more sensitive to the drug than amastigotes, with concentrations at which 50% of parasites were inhibited (MIC50) of 16.1 and 8.2 μM, respectively (48 h posttreatment). Moreover, 90% of amastigotes were eliminated at 120 h posttreatment, indicating that longer treatments will result in parasite elimination. Most strikingly, PZA treatment of infected C57BL/6 mice resulted in protection against disease and in a 100-fold reduction in the parasite burden. PZA treatment of J774 cells and bone marrow-derived dendritic cells and macrophages increased interleukin 12, tumor necrosis factor alpha, and activation marker expression, as well as nitric oxide production, suggesting that PZA enhances effective immune responses against the parasite. PZA treatment also activates dendritic cells deficient in Toll-like receptor 2 and 4 expression to initiate a proinflammatory response, confirming that the immunostimulatory effect of PZA is directly caused by the drug and is independent of Toll-like receptor stimulation. These results not only are strongly indicative of the promise of PZA as an alternative antileishmanial chemotherapy but also suggest that PZA causes collateral immunostimulation, a phenomenon that has never been reported for this drug.

The leishmaniasis are a group of insect-transmitted parasitic diseases prevalent worldwide, endemic in 88 countries; 350 million people are at risk, and 12 million people are affected. Two million new cases are estimated to occur annually, although only 600,000 are officially reported (10). During the last two decades, it has become increasingly apparent that the leishmaniasis are much more prevalent than had been previously supposed. With human migration and vector expansion dramatically affecting the spread of disease, dramatic outbreaks have occurred in locations with previously low levels of infection (e.g., Kabul, Afghanistan, with more than 200,000 infected [22]). Immunologically naive individuals from the developed world traveling to areas of endemcity are particularly prone to infection. Leishmaniasis has been found among American soldiers deployed to the Middle East during both Gulf wars, current conflicts in Afghanistan, and Central America (3, 7, 12, 14, 18, 20, 24, 29). Civilians traveling into these areas are also at risk (2).

Currently, there are nearly 25 licensed compounds with antileishmanial effects, but only a few are used in humans. Drawbacks associated with conventional treatment with antimoniais and amphotericin B include high toxicity, differences in strain sensitivity, and resistance. Moreover, the expense of these drugs often precludes their use. As recently as 2004, liposomal amphotericin B, miltefosine, and paromomycin were identified by WHO/TDR as the three most promising drugs in the market. These drugs are not new: amphotericin B has been used extensively for decades as a second-line drug for treatment of leishmaniasis (in addition to its antifungal activity), miltefosine was developed long ago as an anticancer agent, and paromomycin is more than 50 years old. To date, these three agents, together with antimoniais and nonliposomal amphotericin B, are the reference chemotherapeutic agents for the leishmanias. Oral miltefosine has been shown to be as efficacious against leishmaniasis as the standard amphotericin B treatment in India; however, important side effects, such as teratogenicity, are associated with this drug (28). With this limitation, the need for safer, inexpensive, and widely available treatments continues to be one of the top research priorities for disease control (5).

In contrast to other possible strategies (“orphan drugs,” combinatorial chemistry, or rational design), we seek new indications for existing drugs, which can be a very fruitful route for drug discovery and development (6). Pyrazinamide (PZA), an essential constituent of short-course tuberculosis chemotherapy (11), was developed as an analog of nicotinamide and used in the late 20th century in the treatment of Mycobacterium tuberculosis. PZA and related analogs have also demonstrated activity against other Mycobacterium spp. (9, 21, 27). In myco-

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† Published ahead of print on 21 September 2009.
bacteria, we found that PZA inhibits the enzyme fatty acid synthase I (FASI) (33) by competitive inhibition of a NADPH binding site (25). By analogy, it could be proposed that PZA interferes with fatty acid synthesis in trypanosomatids. Although no genes homologous to the FASI gene have been identified in the _Leishmania_ genome, this parasite employs microsomal elongases in an iterative manner to synthesize fatty acids (17). This suggests that inhibition of fatty acid synthesis might be an attractive chemotherapeutic target in _Leishmania_ (16, 23).

In this article, we demonstrate that PZA has antileishmanial effect in vitro on both promastigotes and amastigotes. More importantly, PZA dramatically decreases lesion development and the parasite burden in _C57BL/6_ mice infected with _Leishmania major_. Finally, we show that PZA increases activation of infected macrophages and dendritic cells by increasing expression of costimulatory molecules and secretion of proinflammatory cytokines and nitric oxide. These results not only show that PZA constitutes a very promising alternative therapy for leishmaniasis but also suggest that the drug causes collateral immunostimulation.

**MATERIALS AND METHODS**

Mice. _C57BL/6_ mice (5 to 6 weeks of age) were purchased from Taconic (Germantown, NY). Toll-like receptor 2 (TLR-2)- and TLR-4-deficient mice were kindly provided by David Russell at Cornell University. All mice were maintained in the Baker Institute Animal Care Facility under pathogen-free conditions.

**Parasite and cell culture.** _L. major_ clone V1 (MHOM/UL80/Friedlin) promastigotes were grown at 26°C in medium 199 supplemented with 20% heat-inactivated fetal calf serum (FCS) (Gemini, Sacramento, CA), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50% FCS) (H9262). J774 murine cells were obtained from American Type Culture Collection (ATCC). J774 cells were cultured in RPMI 1640 supplemented as described above. DNA was isolated using standard phenol-chloroform extraction. DNA concentrations were estimated from the highest dilution at which promastigotes could be grown out after 7 days of incubation at 26°C.

**Amastigote and promastigote drug treatment.** Mid-log-phase (day 3 of culture) _L. major_ promastigotes were employed. Parasite concentration was adjusted to 10⁶ promastigotes/ml and parasites were seeded into 96-well plates in a volume of 100 µl (final concentration, 10⁵ promastigotes/well). PZA was tested in triplicate in a concentration gradient from 1,000 to 0.5 µg/ml and added to the wells containing the parasite in a volume of 100 µl. A negative control was included with wells containing only parasites and medium. The positive control consisted of amphotericin B (0.1 and 1 µg/ml; Sigma), as previously tested by us (19). After 48 h of incubation at 26°C, 10 µl of each well was diluted in 90 µl of the vital colorant (trypan blue in phosphate-buffered saline), and the parasites were quantified in a Neubauer chamber. Data were normalized as percentages of survival compared to data for untreated controls. The 50% lethal dose was extrapolated from the graph as the concentration of the products that inhibited the parasitic growth at 50% of the values of the negative control.

**Amastigotes were generated by infecting either _J774_ murine macrophages or bone marrow-derived macrophages or dendritic cells from _C57BL/6_ mice. Infections were carried out after cells were seeded in eight-well Labtek chambers (Thermo FIS (clone C17.8), IL-10 (clone JES5-16E3), and tumor necrosis factor alpha (TNF-α) (clone D19) and inducible nitric oxide synthase (iNOS) (clone NOS-IN). Incubations were carried out for 30 min on ice. All antibodies were purchased from BD Biosciences or eBioscience. For each sample, at least 50,000 cells were analyzed. The data were collected and analyzed using the CELLQuest software program and a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). In other experiments, supernatants from untreated cells were collected and assayed by enzyme-linked immunosorbent assay (ELISA) for the secretion of IL-12, IL-10, or TNF-α using commercial kits (Invitrogen). The levels of nitric oxide in treated and untreated cultures was also measured in supernatants using the Griess reagent (Invitrogen). Statistical analysis. Statistical analysis of the in vivo data used a one-way analysis of variance followed by Bonferroni’s post hoc test using the GraphPad Prism software program (San Diego, CA) (n = 7). Results were considered.
PZA has antileishmanial effect in vitro. The effect of PZA was first determined with promastigotes of *L. major* after 48 h. *L. major* promastigotes treated with PZA experienced a decrease in cell proliferation. The MIC$_{50}$ was established as 16.2 

\[ \text{g/ml} \] (16.1 \text{M}) (Table 1). Intracellular amastigotes appeared to be slightly more sensitive to the effect of PZA than the extracellular forms (MIC$_{50}$ = 10.2 \text{g/ml} or 8.2 \text{M}). Incubation of *L. major* with 1 \text{g/ml} amphotericin B (as a positive control) caused 100% mortality of promastigotes and 90% of amastigotes.

To determine the potential cytotoxicity of PZA toward the mammalian cell line, coincubation of PZA with uninfected J774 cells was also carried out for 48 h. Results showed that the drug was cytotoxic at 200 

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To determine the potential cytotoxicity of PZA toward the mammalian cell line, coincubation of PZA with uninfected J774 cells was also carried out for 48 h. Results showed that the drug was cytotoxic at 200 \text{g/ml}, resulting in mortality of 25% of the cell monolayer. The MIC$_{50}$ for mammalian cells was established as 425.6 \text{g/ml}. The control drug amphotericin B caused only 5% mortality of the cell monolayer at the concentration tested.

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To distinguish between leishmanioptastic and leishmanicidal effects, a time course survival curve was generated for J774 cultures infected with *L. major*. Figure 1 shows parasite loads in macrophages at 24, 48, and 120 h posttreatment with 100 

\[ \text{g/ml} \] PZA. Interestingly, about 90% of *L. major* parasites were efficiently eliminated from the cells after 120 h, indicating that PZA is a leishmanicidal drug.

**RESULTS**

**PZA significantly reduces clinical disease and parasite burdens in infected mice.** To assess the efficacy of PZA in vivo, C57BL/6 mice were intradermally infected with $5 \times 10^5$ *L. major* parasites in each ear ($n = 6$ mice; 12 ears) and treated orally with PZA at several concentrations (900 to 150 mg/kg of body weight). As shown in Fig. 2A, the oral administration of PZA produced a significant ($P < 0.0001$) reduction of the average lesion size in all treated groups compared with results for untreated mice at all time points. However, there were no statistical differences among the three experimental groups treated with different doses of PZA. Parasite burdens in the ears were determined at 5 weeks postinfection in the experimental groups. Figure 2B shows that treatment dramatically decreased the parasite burden in infected ears at week 6 (100-fold; $P = 0.008$) compared to results for the control. As before, no statistical differences were detected among the PZA-treated groups. Parasite burdens were also comparable among all experimental groups when determined after healing, at 12 weeks postinfection. Finally, PZA treatment was not toxic and did not affect the growth of the experimental animals since no significant differences in body weight were found at week seven postinfection (Fig. 2C).

**PZA increases J774 cell activation as well as release of proinflammatory cytokines and nitric oxide.** Because of the striking in vivo effect of PZA, we studied the effect of treatment on macrophages. First, we looked at activation markers and cytokine secretion by J774 cells (a murine macrophage cell line) following drug treatment (10 and 100 \text{M}) in the presence or absence of *L. major* infection. As a positive control, cells were exposed to a mixture of IFN-$\gamma$ and LPS. Figure 3 shows that drug treatment increased expression of the costimulatory molecules CD80, CD86, and MHC class II, suggesting that treatment alone increases the ability of the macrophage to present antigen. *L. major*-infected J774 cells downregulated the expression of costimulatory molecules and class II MHC molecules compared to that for infected, untreated controls, a phenomenon typically associated with *L. major* infection. Interestingly, treatment of *L. major*-infected cells with PZA rescued the ability of the cell line to upregulate all surface markers studied at the same level as that for cells treated with the drug alone.

We also determined the ability of J774 cells to produce cytokines in response to infection and/or treatment. Cells were infected, treated, or activated as described above. The amounts of the proinflammatory cytokines IL-12 and TNF-$\alpha$ (implicated in Th1 response and parasite killing) and the repressive

**TABLE 1. Effect of PZA on *L. major* and J774 cell survival**

<table>
<thead>
<tr>
<th>Group assessed</th>
<th>% Survival at PZA concn (\text{M}) of:</th>
<th>MIC$_{50}$ of PZA (\text{mg/ml} and \text{M})</th>
<th>% Survival with amphotericin B treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>12.5</td>
<td>50</td>
</tr>
<tr>
<td>Promastigotes</td>
<td>88 ± 15</td>
<td>50 ± 18</td>
<td>38 ± 14</td>
</tr>
<tr>
<td>Amastigotes</td>
<td>89 ± 12</td>
<td>47 ± 12</td>
<td>23 ± 10</td>
</tr>
<tr>
<td>Uninfected J774 cells</td>
<td>100 ± 10</td>
<td>100 ± 10</td>
<td>100 ± 12</td>
</tr>
</tbody>
</table>

* Amphotericin B (1 \text{M}) was included in the experiment as a control. After 48 h, parasite and cell survival was determined. Data are expressed as percentages of survival compared to results for the untreated control (100% survival). Values are means ± standard deviations of at least three independent determinations.

$^b$ = not determined.

significant when \( P \) values were $\leq 0.05$. MIC$_{50}$s were interpolated from curves generated by nonlinear regression analysis using GraphPad Prism.
cytokine IL-10 in the culture supernatants were determined by ELISA. The level of nitric oxide in the same supernatants was also determined. PZA treatment alone increased cytokine production, especially that of IL-12 and TNF-α, and NO release. Cytokine production was also increased in the wells treated with PZA and infected with *L. major* compared with that for infection with *L. major* alone, suggesting again that the immune response is enhanced by PZA in infected cells (Table 2). Treatment also slightly increased IL-10 production, although the response of the cell line to the drug was dominated by the release of proinflammatory factors. Finally, to test the specificity of the immune activation by PZA, we determined the effect that amphotericin B treatment had on activation and cytokine expression by J774 cells. Treatment of J774 cells with the drug did not result in activation at the doses tested (Fig. 4).

Bone marrow-derived macrophages and dendritic cells from C57BL/6 mice also release proinflammatory factors in response to PZA. The background of J774 cells is the susceptible BALB/c mouse strain. Because BALB/c susceptibility to *L. major* is mediated by its inability to initiate Th1 responses, we
tested the effect of the drugs on primary cells isolated from the resistant mouse strain C57BL/6.

We obtained bone marrow cells and grew them in the presence of the cytokines macrophage CSF or granulocyte-macrophage CSF to induce differentiation of macrophages or dendritic cells, respectively. In this experiment, we included the study of the immune response of dendritic cells because they are essential for the initiation the immune response against L. major. Table 3 shows that, as before, parasite infection inhibits the initiation of inflammatory responses by macrophages, as evidenced by their inability to produce cytokines or release nitric oxide. Again, this effect was rescued by the treatment of macrophages with PZA.

Dendritic cells infected with L. major were able to release IL-12, TNF-α, and nitric oxide following infection. However, this effect was greatly enhanced (10-fold) if PZA was added to the infected cells. Both dendritic cells and macrophages also increased the expression of costimulatory molecules (not shown). Together, these results suggest that PZA has immunostimulatory properties that may contribute to parasite killing beyond the leishmanicidal effect of the compounds.

The stimulatory effect of PZA is independent of TLR engagement. To confirm that the immunostimulatory effect of PZA is directly caused by the drug and is not due to contaminants (e.g., endotoxin) or other ligands that could cause cell activation via TLRs, we studied the effect of drug treatment on bone marrow-derived dendritic cells isolated from TLR-2/TLR-4 double knock-out mice. In this experiment, we treated the dendritic cells as described above in the presence or absence of L. major infection. Again, as a positive control, cells were primed with IFN-γ and LPS. Figure 5A shows that drug treatment increased expression of activation markers and MHC molecules in a dose-dependent manner in dendritic cells lacking TLR-2 and -4, suggesting that PZA treatment and not a contaminant was responsible for cell engagement.

FIG. 4. PZA increases proinflammatory cytokine production in J774 cells. IL-10, IL-12, TNF-α, and nitric oxide production were determined by ELISA (cytokines) or Griess test (nitric oxide) in J774 cells infected with L. major and treated with 10 or 100 μM PZA. *A group of uninfected cells was treated with 100 ng/ml LPS and 10 IU IFN-γ as a positive control of activation. Cytokine levels (± standard deviations) in L. major-infected macrophages and in untreated cells were also determined. Data were obtained from three independent experiments. *, statistically significant when compared with result for untreated, L. major-infected control group (P < 0.05).
The emergence of the leishmaniases and the lack of affordable therapy have necessitated the development of novel anti-leishmanial therapies. In this report, it has been shown that the clinical antituberculous drug PZA has activity against L. major both in vivo and in vitro. PZA is a drug that has been employed extensively, first having been used in the treatment of pulmonary tuberculosis in humans in 1949 (30). The use of a licensed, well-known drug for indications other than the treatment of tuberculosis could eliminate hurdles associated with the development of new anti-leishmanial antigens and provide therapeutically optimal alternatives for a disease for which chemotherapy is suboptimal. Moreover, PZA is an orally administered drug, therefore obviating the need for parenteral injections.

Our data show that PZA is efficient at controlling the growth of L. major in vitro. The MIC<sub>50</sub> is estimated to be 10 μM for promastigotes and 100.1 μM for amastigotes. These MIC<sub>50</sub>s are comparable to what was obtained by Klemens et al. (15) with a murine model of tuberculosis. Although intracellular amastigotes appeared to be less sensitive than promastigotes to the effect of the PZA at 48 h postinfection (10-fold increase in MIC<sub>50</sub>), an extended kinetic analysis revealed that PZA, employed at 100 μM, eliminated 90% of the parasites in cultured cells after 120 h of culture. This concentration is equivalent to MIC<sub>50</sub> determined by ELISA (cytokines) or Griess test (nitric oxide).

The mechanism of action of PZA is just beginning to be unraveled. We have previously reported that PZA inhibits the enzyme FASI (33) by competitive inhibition of a NADPH binding site (25). L. major lacks FASI but possesses microsomal elongases (17) that effect many of the same chemical transformations as FAS. Our in vitro, and especially our in vivo, results are consistent with our hypothesis that this pathway is indeed an optimal target for the development of antileishmanial drugs.

It has been proposed, however, that Leishmania parasites can survive with greater altered lipid profiles than trypanosomes and even acquire lipids from the hosting macrophage (23). Our results demonstrate that long-term treatment of leishmanial cultures with PZA resulted in almost complete elimination of parasites from macrophages. If fatty acid synthesis is nonessential in Leishmania or these organisms are highly resistant to lipid depletion, it is possible that parasite killing is not mediated exclusively by the direct effect of the drug on parasite survival and replication. Thus, the antileishmanial effect of PZA may be caused (or enhanced) by chemotherapeutic interaction with the macrophage. Our data show that J774 cells, as well as primary cells from C57BL/6 mice, upregulate activation markers and release cytokines following treatment with PZA, demonstrating that the drug enhances the immune response to L. major infection. This immunoenhancing effect was not replicated in cells treated with amphotericin B, despite publications reporting the immunostimulatory effect of the drug (8), or in cells deficient in TRL-2 and -4 receptors, confirming that immunostimulation is a PZA-specific event. This phenomenon would be especially desirable in situations where patients are immunocompromised. Because Leishmania/HIV coinfections have been extensively documented (1), the development of drugs that boost the immune system of the host may be extremely useful.

The data presented here together provide the grounds for further testing of PZA (and PZA analogs) as an antileishmanial drug, for the determination of the immune status of the L. major-infected mice following PZA treatment, and for extension of these screens to other models of Leishmania infection. This pioneering alternative may work as a novel chemotherapeutic approach to treating leishmaniasis, in particular visceral leishmaniasis, the most severe leishmanial disease.
ACKNOWLEDGMENT

This work was funded by the Baker Institute for Animal Health.

REFERENCES


