Durable Cure for Tuberculosis: Rifalazil in Combination with Isoniazid in a Murine Model of Mycobacterium tuberculosis Infection

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Rifalazil (formerly known as KRM-1648) in combination with isoniazid has been found to be more active than rifampin/isoniazid. Administration of rifalazil/isoniazid for 12 weeks resulted in continued apparent sterilization of organs 6 months after cessation of therapy. In this study we evaluated the durability of rifalazil/isoniazid treatment. Female CD-1 mice were infected with Mycobacterium tuberculosis ATCC 35801 (strain Erdman). Rifalazil and isoniazid were given in combination for 6 and 12 weeks; no mycobacteria could be cultured from spleens and lungs at both the 6-week and 12-week time points. After completing treatment, groups of mice treated with rifalazil/isoniazid for 6 or 12 weeks were observed without any additional treatment. These observation groups were compared to groups of rifalazil/isoniazid-treated mice (6 and 12 weeks) given dexamethasone for 7 and 8 weeks, respectively. Modest regrowth was noted in the spleens and lungs of the group treated with rifalazil/isoniazid for 6 weeks. Regrowth in the 6-weeks group was enhanced slightly by treatment with dexamethasone. In contrast, no regrowth was noted in the 12-weeks rifalazil/isoniazid group, and treatment with dexamethasone did not result in any regrowth.

New compounds, such as rifalazil (previously known as KRM-1648) and rifapentine, have been evaluated in murine tuberculosis models for their ability to improve antimycobacterial therapy [1, 2]. We have shown that rifalazil in combination with isoniazid rendered the bacteria nonculturable after 6 weeks of therapy, but regrowth of Mycobacterium tuberculosis was detected 3 months after the cessation of therapy [3]. Rifalazil/isoniazid treatment for 12 weeks rendered the bacteria nonculturable after the completion of therapy and throughout a 6-month observation period [4]. In contrast, 24 weeks of therapy with rifampin-isoniazid was required to achieve the same result (authors’ unpublished data).

Despite this nonculturable state, the M. tuberculosis DNA sequence IS6110 was detected by PCR in lung and spleen samples after 12 weeks of rifalazil/isoniazid therapy and during a 6-month observation period [5]. It could not be determined whether the PCR products detected were from bacteria that were intact and latent or from remaining M. tuberculosis DNA fragments. McCune et al. [6] described a model in which mice were infected with M. tuberculosis and treated with isoniazid-pyrazinamide for 12 weeks. This treatment rendered the bacteria nonculturable but viable. Such organisms are defined as latent. There is no method available for determining whether drug treatment sterilizes the bacteria or shifts them into a latent state. If the bacteria were latent, then reactivation might occur in time or with immunosuppression [6].

The current study was undertaken to determine whether the addition of dexamethasone, which has been shown to abrogate the ability of murine macrophages to kill M. tuberculosis [7], could stimulate reactivation of M. tuberculosis after 6 or 12 weeks of rifalazil/isoniazid therapy.

Materials and Methods

Five- to 6-week-old female outbred CD-1 mice (Charles River, Wilmington, MA) were infected intravenously with M. tuberculosis ATCC 35801 (strain Erdman) through a caudal vein. The organism was grown in modified 7H10 broth (pH, 6.6; 7H10 agar formulation, with agar and malachite green omitted) supplemented with 10% OADC enrichment (Difco, Detroit) and 0.05% Tween 80, adjusted to 200 Klett units per mL (Klett-Summerson colorimeter; Klett Manufacturing, Brooklyn, NY), and stored at −70°C. The stock culture was thawed on the day of infection and diluted in modified 7H10 broth to 1 Klett unit per mL (≈ 5 × 10^7 cfu/mL). The inoculum size was determined by titration in triplicate on 7H10 agar plates (BBL Microbiology Systems, Cockeysville, MD) supplemented with 10% OADC enrichment. The plates were incubated at 37°C in ambient air for 4 weeks before counting. Each mouse received ~ 3 × 10^7 viable organisms suspended in 0.2 mL of modified 7H10 broth.

Rifalazil was obtained from Kaneka (Osaka, Japan), and isoniazid was purchased from Sigma Chemical (St. Louis). Rifalazil/isoniazid treatment was started 1 week after infection and administered 5 days per week for 6 and 12 weeks at 20 mg/kg and 25
mg/kg, respectively. There were 8 mice per group. Mice treated in parallel for 6 and 12 weeks were maintained for ~2 months of observation (without treatment).

Mice in the control groups (untreated, infected mice) were killed 1, 3, 7, and 13 weeks after infection to compare with the mice in the treatment groups. Dexamethasone (purchased from Elkins-Sinn, Cherry Hill, NJ) was given 5 days per week at 1.5 mg/kg. One group was given dexamethasone 24 h after infection for a period of 3 weeks. Another group was treated with dexamethasone after 3 weeks of infection for 4 weeks. Other groups were given dexamethasone for 7 or 8 weeks after 6 or 12 weeks of rifalazil/isoniazid therapy, respectively.

Rifalazil was dissolved in dimethylsulfoxide (DMSO), then diluted in distilled water. The final concentration of DMSO was 0.5% at the time of administration. Isoniazid and dexamethasone were dissolved in distilled water. All drugs were given orally by gavage in a 0.2-mL volume.

Three to 5 days after the treatment phase was completed, the mice were killed by CO2 inhalation. Control mice were also killed at this time. Spleens and right lungs were aseptically removed and ground in a tissue homogenizer. The number of viable organisms was determined by serial dilution and titration on 7H10 agar plates supplemented with 10% OADC enrichment. For the mice in the groups that received rifalazil/isoniazid for 6 and 12 weeks (including observation groups), the entire volume of organ homogenate was plated (0.1-mL aliquots) to determine the number of culturable mycobacteria per organ. The plates were incubated at 37°C in ambient air for 4 weeks.

The viable cell counts were converted to logarithms. To determine statistical significance for groups treated with dexamethasone and for untreated controls, cell counts were evaluated by a Student’s t-test for equality of means.

Results

Dexamethasone was administered for 3 weeks after infection in order to determine whether dexamethasone could enhance *M. tuberculosis* infection in a murine model. Mice that received dexamethasone for 3 weeks after infection had 7.14 ± 0.54 cfu (mean log cfu/organ ± SD) in the spleens, and 7 of 8 mice had >9.0 cfu in the lungs (1 of 8 mice had 8.45 cfu in the lung). Control mice infected for 3 weeks had 5.66 ± 0.17 cfu in the spleens and 7.33 ± 0.58 cfu in the lungs. The growth of *M. tuberculosis* was significantly greater in mice that received dexamethasone than in untreated controls (*P* < .02 for the spleens; *P* values could not be calculated for the lungs).

To determine the effect dexamethasone treatment had on an established *M. tuberculosis* infection in mice, treatment was started after 3 weeks of infection. Treatment was continued for 4 weeks. Mice that received dexamethasone after 3 weeks of established infection (no treatment) had 7.34 ± 1.08 cfu in the spleens and 8.73 ± 0.50 cfu in the lungs. Control mice infected for a total of 7 weeks had 5.14 ± 0.61 cfu in the spleens and 6.57 ± 0.57 cfu in the lungs. Treatment with dexamethasone even after the immune response had been established significantly increased *M. tuberculosis* growth in the lungs, in comparison with growth in the lungs of control mice (*P* < .05). The growth of *M. tuberculosis* in spleens was not statistically different (*P* > .05). This may be due to the high variability in cfu within the dexamethasone treatment group.

To determine whether regrowth of *M. tuberculosis* would occur after antituberculosis therapy, mice were treated for either 6 or 12 weeks with rifalazil/isoniazid and observed for 7 or 8 weeks, respectively. These observation groups were either untreated or treated with dexamethasone. Six weeks of rifalazil/isoniazid therapy led to clearance of bacteria from both the lungs and spleens (table 1). During the 7-week observation period, regrowth was observed in the spleens of 3 of 8 mice and in the lungs of 1 of 8 mice. When mice were treated with dexamethasone for 7 weeks after the 6-week treatment period, regrowth was slightly enhanced in the spleens (3.65 ± 0.46 cfu with dexamethasone, vs. 2.46 ± 0.42 cfu without dexamethasone). Twelve weeks of rifalazil/isoniazid rendered the bacteria nonculturable in both the spleens and lungs of infected mice. There was no regrowth observed after 8 weeks of observation. The administration of dexamethasone for 8 weeks after 12 weeks of rifalazil/isoniazid treatment did not result in regrowth of *M. tuberculosis*.

Discussion

New antimycobacterial drugs are being developed to improve the therapy for tuberculosis. Rifalazil, a rifamycin derivative, has shown superior in vitro activity (MIC, 0.00047 mg/mL) against *M. tuberculosis* ATCC 35801 [8]. This in vitro activity was paralleled by its enhanced in vivo activity against *M. tuberculosis* in the murine model [1, 4, 8].

Previous experiments demonstrated that rifalazil in combination with isoniazid could lead to “sterilization” of *M. tu-

<table>
<thead>
<tr>
<th>Group by treatment regimen</th>
<th>Spleen</th>
<th>Lung</th>
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<tbody>
<tr>
<td>1 w control</td>
<td>5.9 ± 0.65</td>
<td>4.71 ± 0.87</td>
</tr>
<tr>
<td>6 w control</td>
<td>5.14 ± 0.61</td>
<td>6.57 ± 0.57</td>
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<tr>
<td>6 w Rlz/INH</td>
<td>0</td>
<td>0</td>
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<tr>
<td>6 w Rlz/INH, then 7 w obs</td>
<td>2.46 ± 0.42</td>
<td>3.04</td>
</tr>
<tr>
<td>6 w Rlz/INH, then 7 w Dex</td>
<td>3.65 ± 0.46</td>
<td>3.09 ± 0.12</td>
</tr>
<tr>
<td>12 w control</td>
<td>4.64 ± 0.46</td>
<td>5.97 ± 0.99</td>
</tr>
<tr>
<td>12 w Rlz/INH</td>
<td>0</td>
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<tr>
<td>12 w Rlz/INH, then 8 w obs</td>
<td>0</td>
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NOTE. Dex, dexamethasone; INH, isoniazid; obs, observation; Rlz, rifalazil

* Duration of the control treatment, drug treatment, and/or observation phase.
* Three of 8 mice exhibited regrowth in the spleen; 1 of 8 exhibited regrowth in the lung.
* Three of 8 mice exhibited regrowth in the spleen; 2 of 8 exhibited regrowth in the lung.
berculosis after 12 weeks of treatment [4]. PCR evaluation at that time revealed the existence of amplifiable *M. tuberculosis* DNA, even though the organism could not be cultured [5]. This finding led to the question of whether reactivation could be achieved by immunosuppressive treatment with dexamethasone. Dexamethasone can increase the susceptibility of mice to mycobacteria; it has been shown to exacerbate *Mycobacterium avium* infections in a murine model [9] and to abrogate the ability of murine macrophages to kill *M. tuberculosis* [7]. Incubation of murine macrophages (chronically infected with *Mycoplasma bovis* BCG) in the presence of dexamethasone resulted in the recovery of increased numbers of viable BCG [10].

In the present study we were able to show that dexamethasone treatment could promote the growth of *M. tuberculosis* at the beginning of an infection and after an infection has been established. This result indicated that an immunosuppressed state could be achieved with dexamethasone treatment. In order to determine whether we could stimulate regrowth of *M. tuberculosis* after rifalazil/isoniazid therapy, we treated mice with dexamethasone. Previous experiments that used an inoculum size of $5.2 \times 10^7$ cfu showed regrowth after 6 weeks of rifalazil/isoniazid therapy (after a 3-month observation period) [3]. In this study mice were infected with $3.0 \times 10^5$ cfu to determine whether a sustained nonculturable state (no regrowth after the observation period) could be reached after 6 weeks of rifalazil/isoniazid therapy. Regrowth after a 7-week observation period was observed in mice treated with rifalazil/isoniazid for 6 weeks. Mice treated with dexamethasone during the observation period had a slight increase in regrowth in the spleen. Subsequent studies performed in our laboratory have shown that the duration of dexamethasone treatment may not be long enough. When mice were infected with a low dose of *M. tuberculosis*, a chronic infection was established. The organism does not increase in number but remains stable. When dexamethasone was administered for 8 weeks, there was a slight increase in bacterial growth (authors’ unpublished data). It is our belief that the number of viable, nonculturable (latent) organisms remaining after 6 weeks of treatment with rifalazil/isoniazid was small, and the duration of dexamethasone treatment would need to be much longer to yield a significant increase in bacterial growth.

In contrast to 6 weeks of rifalazil/isoniazid therapy, 12 weeks of treatment did not lead to regrowth after an 8-week observation period. Dexamethasone treatment during the observation period did not stimulate regrowth of *M. tuberculosis*. It may be of interest in subsequent experiments to lengthen the dexamethasone treatment after 12 weeks of rifalazil/isoniazid therapy, in order to determine if increasing the duration would lead to regrowth.

Successful treatment of tuberculosis in humans is defined by an inability to culture mycobacteria from the patient. Recent evidence has shown that DNA-based amplification assays may be misleading because amplifiable nucleic acids may persist for long periods beyond the point at which cultures become negative [11]. Because bacterial mRNA has a short half-life, the detection of mRNA, such as Ag85B, may be a better indication of whether organisms are alive or dead [12]. In subsequent studies it would be of interest to determine whether mRNA could be detected after rifalazil/isoniazid treatment.

Dexamethasone has many general immunosuppressive effects. It can effect both T cell responses and macrophage responses. Although in this model dexamethasone did not stimulate reactivation after 12 weeks of rifalazil/isoniazid treatment, it is unclear whether a more specific form of immunosuppression would lead to reactivation.

References