Impacts of Dosing Frequency of the Combination Rifampin-Streptomycin on Its Bactericidal and Sterilizing Activities against *Mycobacterium ulcerans* in Mice

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Because of operational limitations, a significant proportion of the health centers at the peripheral level are able to provide treatment to Buruli ulcer patients with the combination rifampin (rifampicin)-streptomycin (RIF-STR) only five times weekly (5/7) instead of seven times weekly (7/7), as recommended. The objective of this experiment is to assess the impacts of various dosing frequencies of the combination on its bactericidal and sterilizing activities against *Mycobacterium ulcerans* in mice. The results demonstrate that the bactericidal activities did not differ significantly among five dosing frequencies of the combination, ranging from seven times to twice weekly, whereas the sterilizing activities differed widely. RIF-STR 7/7 was the only regimen that was able to sterilize the infection after 4 to 8 weeks of treatment; the sterilizing activities associated with reduced dosing frequencies were significantly diminished, and 8 weeks of 5/7 administration yielded a relapse rate greater than the generally accepted level of 5%. We recommend that the duration of treatment with 5/7 administration be prolonged beyond 8 weeks and that additional experiments with mice be carried out, with sufficient statistical power to compare the relapse rates of *M. ulcerans* infection between 8 weeks of 7/7 administration and 10 and 12 weeks of 5/7 administration of RIF-STR.

Daily administration of the combination rifampin (rifampicin)-streptomycin (RIF-STR) for 8 weeks is currently the only drug regimen recommended by the World Health Organization for the treatment of Buruli ulcer (14). In hospital settings, where daily treatment can be ensured, the regimen is highly effective (2, 3), and the 12-month relapse rate is negligible—only 1.44% (2). To expand the coverage of Buruli ulcer treatment in the field, delivery of RIF-STR is being decentralized to peripheral health centers. However, a significant proportion of these health centers operate only on weekdays and are therefore able to provide treatment to ambulatory patients only five times weekly (5/7) rather than seven times weekly (7/7), as recommended. It is not clear whether the administration of RIF-STR 5/7 is as effective as administration 7/7. Furthermore, for operational reasons, it is unlikely that every ambulatory patient will receive regular treatment with RIF-STR even during the weekdays. Therefore, it is important to determine the maximal irregularity of RIF-STR administration capable of yielding an acceptable relapse rate of *Mycobacterium ulcerans* infection within a fixed duration of treatment (e.g., 12 weeks, because of the possible adverse effects caused by longer durations of treatment with STR [1]). We have assessed the impacts of various dosing frequencies of RIF-STR on its bactericidal and sterilizing activities against *M. ulcerans* in mice.

**MATERIALS AND METHODS**

**Antimicrobial agents.** RIF was purchased from Gruppo Lepetit (Anagni, Italy) and STR from Panpharma (Fougères, France).

**Infection of mice with *M. ulcerans*.** As in our earlier experiments (5–8), the left hind footpad of each of 440 4-week-old female BALB/c mice was inoculated subcutaneously with 0.03 ml of a bacterial suspension containing 1.5 × 10⁹ CFU of *M. ulcerans* CU001.

**Treatment of mice.** Seven weeks after the mice were infected, by which time all of the mice demonstrated a lesion index (8) of 2 (i.e., definite inflammatory swelling limited to the inoculated footpad) or 3 (i.e., inflammatory swelling involving the entire inoculated foot), 10 mice were sacrificed for the enumeration of CFU in the inoculated footpad in order to establish the pretreatment (day zero) value. The remaining 430 mice were randomly allocated among six groups: one untreated control group of 40 mice and five treated groups of 50 to 90 mice each (Table 1). Treatment was begun immediately after randomization. Each treated group was treated with one of five regimens differing in the dosing frequency of the RIF-STR combination: either daily (7/7), five times weekly (5/7) (Monday and Thursday), four times weekly (4/7) (Monday, Tuesday, Thursday, and Friday), three times weekly (3/7) (Monday, Tuesday, and Thursday), or twice-weekly (2/7) (Monday and Thursday). RIF was suspended in 0.05% agar-distilled water and administered by gavage; STR was diluted with normal saline and administered by subcutaneous injection. The dosages on each day of treatment were 10 mg of RIF per kg of body weight and 150 mg of STR per kg of body weight, as in our earlier experiments (5–8).

At least 10 mice per group were sacrificed at regular intervals during treatment, as shown in Table 1. During treatment, 18 mice died from causes other than *M. ulcerans* infection. Only 202 mice were available for monitoring of the relapse of *M. ulcerans* infection after the completion of treatment (6–8): 17 to 20 mice from each of the groups that had been treated for 4, 6, or 8 weeks with RIF-STR administered either 7/7 or 5/7, for 8 or 10 weeks with RIF-STR 4/7, for 10 or 12 weeks with RIF-STR 3/7, or for 12 weeks with RIF-STR 2/7 (Table 2). These mice were held without treatment for an additional 28 weeks.

**Assessment of the severity of infection and the effectiveness of treatment.** The severity of infection and the effectiveness of treatment were assessed according to the mean log₁₀ CFU count per inoculated footpad. The sterilizing activity of the treatment was assessed in terms of the relapse rate of *M. ulcerans* infection during the 28-week posttreatment follow-up.

To enumerate CFU, the tissues of the footpad were removed aseptically at...
TABLE 1. Culture results during treatment of mice with various dosing frequencies of the combination RIF-STR

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>2 wks</th>
<th>4 wks</th>
<th>6 wks</th>
<th>8 wks</th>
<th>10 wks</th>
<th>12 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40</td>
<td>10/10</td>
<td>6.09 ± 0.19</td>
<td>0.8</td>
<td>0.8</td>
<td>0.35 ± 0.19</td>
<td>0.8</td>
</tr>
<tr>
<td>RIF-STR 77</td>
<td>90</td>
<td>10/10</td>
<td>6.64 ± 0.11</td>
<td>0.01</td>
<td>0.01</td>
<td>0.33 ± 0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>RIF-STR 57</td>
<td>90</td>
<td>10/10</td>
<td>7.06 ± 0.07</td>
<td>0.01</td>
<td>0.01</td>
<td>0.38 ± 0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>RIF-STR 27</td>
<td>90</td>
<td>10/10</td>
<td>7.20 ± 0.10</td>
<td>0.01</td>
<td>0.01</td>
<td>0.39 ± 0.04</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**Note**:
- The cultures were administered in batches of 10 mice per group.
- CFU count is given as the number of colony-forming units per inoculated footpad.
- The culture positivity rate was 10/10 and the CFU count was 5.72 ± 0.03 CFU per inoculated footpad.

**Results**:
- All mice were negative for the isolate CU001 (5) weeks after treatment with RIF-STR, indicating a significant reduction in the CFU count per inoculated footpad.
- A single colony was detected in one of the eight footpads; however, log10 CFU per inoculated footpad in the treated group was significantly lower than the pretreatment value.
- A regimen was considered bactericidal if the mean log10 CFU count per inoculated footpad in the treated group was significantly lower than the pretreatment value.
- The culture-positive relapse rate during the 28-week posttreatment follow-up was no greater than 5%.

**RESULTS**

Evolution of the lesion index and log10 CFU count of the inoculated footpads during treatment. Among the untreated control mice, the average lesion index continued to increase (Fig. 1); at the time point corresponding to 4 weeks after treatment, 3 of the 30 untreated mice had already succumbed to *M. ulcerans* infection. Inoculated footpads were invariably culture positive for *M. ulcerans*, and the mean log10 CFU count
per inoculated footpad had increased significantly and progressively over the pretreatment value (P < 0.01) (Table 1).

The average lesion index of every treated group was significantly lower than that of the untreated control group (Fig. 1), and no treated mouse died from *M. ulcerans* infection during treatment. The great majority of mice administered RIF-STR either 7/7, 5/7, or 4/7 became culture negative after 4 weeks of treatment; by contrast, a majority of the mice administered RIF-STR 2/7 remained culture positive after 8 or 10 weeks of treatment (Table 1). The log_{10} CFU counts of the 25 culture-positive treated mice (all from groups that had been administered RIF-STR either 4/7, 3/7, or 2/7) were rather low: 17 mice had 1 to 10 colonies, 6 had 11 to 20 colonies, 1 had 21 colonies, and 1 had 22 colonies. As a result, the mean log_{10} CFU counts of all treated groups were significantly lower than the pretreatment value (P < 0.01). There was no significant difference in the lesion index and the log_{10} CFU count among the five treated groups at most time points, with two exceptions: (i) during the first 5 weeks of treatment, the average lesion index of the RIF-STR 7/7 group was significantly lower than those of most of the other treated groups (P < 0.05) (Fig. 1); (ii) the rates of culture positivity of the groups treated with RIF-STR 2/7 for 8 and 10 weeks were significantly greater than those of the other treated groups at the same time points (P < 0.05) (Table 1). However, when treatment with RIF-STR 2/7 was continued to 12 weeks, only 2 of 10 treated mice remained culture positive, and the rate of culture positivity no longer differed significantly from those of the other treated groups (P > 0.05), assuming that the culture results of these groups would have remained the same as at the time of their final sacrifice (Table 1).

Relapse rates of *M. ulcerans* infection during the 28-week posttreatment follow-up. Among the 202 mice monitored for relapse of *M. ulcerans* infection after the completion of treatment, clear-cut culture-results were available for 195 mice. The cultures from seven mice were contaminated; these mice were excluded from the denominators for calculation of the rate of culture-positive relapse. As shown in Table 2, there were 28 culture-positive relapses: 27 relapses were associated with rebounds of the lesion index of the inoculated footpad to ≥3, while 1 relapse was not associated with a rebound of the lesion index but was revealed by a positive culture (6.9 × 10^{2} colonies) when the mouse was sacrificed at the end of the 28-week follow-up.

![FIG. 1. Evolution of the average lesion index during treatment of mice with various dosing frequencies of the RIF-STR combination.](http://aac.asm.org/)

### Table 2. Culture-positive relapse rates of *M. ulcerans* infection during 28-week posttreatment follow-up

<table>
<thead>
<tr>
<th>RIF-STR dosing frequency</th>
<th>No. of mice with culture-positive relapse/no. of mice examined (%) after treatment for:</th>
<th>Interval between end of treatment and rebound of lesion index for each individual relapse (wks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 wks</td>
<td>6 wks</td>
</tr>
<tr>
<td>7/7</td>
<td>0/17 (0)</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>5/7</td>
<td>4/17 (23.5)</td>
<td>3/15 (20.0)</td>
</tr>
<tr>
<td>4/7</td>
<td>12/20 (60)</td>
<td>11/20 (55)</td>
</tr>
<tr>
<td>3/7</td>
<td>4/16 (25.0)*</td>
<td>2/16 (12.5)</td>
</tr>
<tr>
<td>2/7</td>
<td></td>
<td>9/20 (45.0)</td>
</tr>
</tbody>
</table>

* One relapse after 10 weeks of treatment with RIF-STR 3/7 was not associated with rebound of the lesion index but was revealed by a positive culture (6.9 × 10^{2} colonies) when the mouse was sacrificed at the end of the 28-week follow-up.
As shown in Table 2, not a single culture-positive relapse was observed after treatment with RIF-STR 7/7 for 4 weeks or longer. However, the relapse rates were 23.5% (4/17), 20.0% (3/15), and 11.7% (2/17), respectively, after 4, 6, and 8 weeks of treatment with RIF-STR 5/7. The combined relapse rate after 4, 6, and 8 weeks of treatment with RIF-STR 5/7 was significantly greater by Fisher’s exact probability calculation than that after treatment with RIF-STR 7/7 (P = 0.001), indicating that the sterilizing activity of RIF-STR against M. ulcerans was significantly compromised by reducing the dosing frequency from 7/7 to 5/7 and that 8 weeks of RIF-STR 5/7 failed to sterilize M. ulcerans infection in mice. When the dosing frequency of RIF-STR was reduced further to 4/7 or 3/7 but the duration of treatment was prolonged to 10 weeks, the relapse rates were 16.7% (3/18) and 25.0% (4/16), respectively, both unacceptably high. Further prolongation of treatment to 12 weeks yielded a relapse rate of 5.6% (1/18) for the group administered RIF-STR 3/7 and a relapse rate of 45% (9/20) for the RIF-STR 2/7 group. Table 2 also demonstrates that, in the two groups that had been treated with RIF-STR 2/7 or 3/7, 10 of the 13 relapses occurred within 16 weeks after the end of treatment, whereas only 3 of the 14 relapses—a significantly smaller proportion—among the mice treated with RIF-STR 4/7 or 5/7 were observed during this time (P < 0.05). The intervals between the end of treatment and the rebound of the lesion index were significantly shorter for the first two groups than for the latter two groups, probably because, at the time the treatment was stopped, the numbers of persisting viable M. ulcerans organisms (i.e., “persisters”) in the first two groups were greater than those in the latter two groups, although this interpretation cannot be confirmed by cultivation.

Results of drug susceptibility tests of the 28 relapsing isolates of M. ulcerans. When drug susceptibility was tested by the proportion method, the CU001 isolate, used as a control, multiplied well on drug-free 10% oleic acid-albumin-dextrose-catalase-enriched 7H11 agar medium but not on drug-containing (4 μg/ml of either RIF or STR) 7H11 agar medium. Likewise, among the 28 relapsing isolates, 22 (79%) multiplied on drug-free medium, but none multiplied on drug-containg medium; 6 relapsing isolates failed to multiply on drug-free medium. When drug susceptibility was tested by genetic methods, no mutation was detected in the rpoB, rrs, or rpsl genes of any of the 28 relapsing isolates. Thus, all 28 relapsing isolates of M. ulcerans remained susceptible to RIF and STR.

DISCUSSION

Table 1 demonstrates that, in terms of the reduction of culture positivity and the mean log_{10} CFU count per inoculated footpad during treatment, the bactericidal activities of RIF-STR 5/7 and RIF-STR 7/7 against M. ulcerans were virtually identical. The bactericidal activities for the three remaining treated groups, i.e., those treated with RIF-STR either 4/7, 3/7, or 2/7, did not differ significantly from those for the former two groups, although conversion to culture negativity for the RIF-STR 2/7 group was delayed by a few weeks. Assuming that, at the beginning of treatment, the great majority of organisms in the M. ulcerans population were actively metabolizing and rapidly dividing, it seems reasonable to suspect that all five RIF-STR regimens were equally effective in containing and killing these organisms, thereby reducing or stopping the production of mycolactone. Consequently, during treatment with RIF-STR, as shown in Fig. 1, similar declines in the average lesion index in the inoculated footpads were observed for all treated groups. Therefore, it appears likely that clinical improvement of Buruli ulcer may occur not only during treatment with either 7/7 or 5/7 administration but also during treatment with a dosing frequency less than 5/7.

Nevertheless, the long-term efficacy of RIF-STR treatment with a dosing frequency less than 7/7 may be problematic, because the relapse rate is more closely linked to the sterilizing activity of treatment, i.e., the killing of the small number of “persisters,” which are believed to have brief periods of metabolic activity interspersed with periods of dormancy. Because only a tiny fraction of the persisters are killed after each dose of drug administered, the rate at which the persisters are eradicated is largely determined by the dosing frequency of the treatment: the lower the dosing frequency, the greater the number of surviving persisters and the higher the relapse rate. As shown in Table 2, RIF-STR 7/7 was the only regimen that sterilized the M. ulcerans infection after 4 to 8 weeks of treatment. This result is in accord with the observation that Buruli ulcer patients treated daily with RIF-STR for 8 weeks had only a negligible relapse rate (2). However, relapses occurred when the dosing frequency of RIF-STR was reduced to 5/7; after 8 weeks of RIF-STR 5/7, the relapse rate was 11.7%, higher than the generally accepted relapse rate of 5%, indicating that the sterilizing activity against M. ulcerans was significantly compromised by reducing the RIF-STR dosing frequency from 7/7 to 5/7. Further reduction of the RIF-STR dosing frequency to 4/7 or 3/7 yielded unacceptably high relapse rates even if the duration of treatment was prolonged to 10 weeks, indicating that a small increase in the duration of treatment was insufficient to compensate for the decline in sterilizing activity resulting from the reduction of the dosing frequency. Further prolongation of the duration of treatment to 12 weeks yielded a nearly acceptable relapse rate (5.6%) for the RIF-STR 3/7 group, but the relapse rate remained alarmingly high (45%) for the RIF-STR 2/7 group.

Decentralizing the delivery of the RIF-STR combination to health centers at the peripheral level is an important strategy for expanding the coverage of Buruli ulcer treatment. However, for operational reasons, a significant proportion of these health centers are able to provide treatment to ambulatory patients only five times weekly instead of seven times weekly. The current experiment has clearly demonstrated that the sterilizing activity against M. ulcerans infection of mice was significantly compromised by reducing the RIF-STR dosing frequency to 5/7 and that 8 weeks of administration of RIF-STR 5/7 failed to prevent an unacceptable rate of relapse of M. ulcerans infection. Because there is no easy solution to the operational difficulties of providing daily RIF-STR treatment at the peripheral health centers, a simpler strategy is to modify the treatment regimen. One possible approach is to change to an orally administered combination regimen, such as the combination of RIF with clarithromycin (6, 7), which does not require patients to be treated daily at the health center. However, despite an ongoing clinical trial that has demonstrated promising therapeutic effects of the RIF-clarithromycin combination for 20 patients with Buruli ulcer (A. Chauty and B. Ji,
unpublished data), because of the small sample size, none of the oral regimens can currently be employed as routine treatment in the field. Therefore, one must accept the reality that a significant number of health centers at the peripheral level can provide only RIF-STR 5/7 for treatment of Buruli ulcer. To compensate for the compromised sterilizing activity of this regimen against M. ulcerans, the duration of treatment with RIF-STR 5/7 must be prolonged. The design of the current mouse experiment did not permit direct determination of the appropriate duration of treatment with RIF-STR 5/7 that may yield an acceptable relapse rate. However, based on the observations that the relapse rate gradually declined as the duration of treatment was prolonged and that the relapse rate was reduced to 11.7% after treatment of mice with RIF-STR 5/7 for 8 weeks (Table 2), it appears reasonable to expect the relapse rate after 10 or 12 weeks of 5/7 administration to be below 5%. Given the practical difficulty of conducting a randomized, controlled clinical trial to compare the relapse rates after 7/7 and 5/7 administration of RIF-STR, an additional experiment with sufficient statistical power to compare the relapse rates of M. ulcerans infection in mice after 8 weeks of RIF-STR 7/7 to those after 10 and 12 weeks of RIF-STR 5/7 is warranted.

The current experiment did not provide direct evidence that the relapse rate in mice after RIF-STR treatment administered either 4/7 or 3/7 was significantly greater than that after treatment with RIF-STR 5/7. On the other hand, the facts that the relapse rates remained unacceptably high after 10 weeks of either 4/7 or 3/7 administration and were very high after 12 weeks of 2/7 administration suggest that nonadherence of patients to the administration of RIF-STR may further compromise the sterilizing activity of the treatment. Therefore, patients should be encouraged to adhere closely to 5/7 administration, and a second course of treatment should be considered for those patients who have missed a substantial number of doses of RIF-STR.

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REFERENCES