Therapy of Experimental Murine Brucellosis with Streptomycin Alone and in Combination with Ciprofloxacin, Doxycycline, and Rifampin

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Received 14 October 1992/Returned for modification 24 March 1993/Accepted 19 August 1993

The in vivo efficacy of streptomycin (STR), doxycycline (DOX), rifampin (RIF), ciprofloxacin (CIP), and their combinations was evaluated for a Brucella melitensis experimental infection in a mouse model. Animals were infected with 2 x 10^4 to 4 x 10^6 CFU of B. melitensis intraperitoneally on day 0 and were randomized to receive, starting on day 7, STR alone at 75, 150, or 300 mg/kg of body weight per day intraperitoneally or DOX at 6 mg/kg/day orally, RIF at 3 mg/kg/day orally, or CIP at 200 mg/kg/day orally, each of the last three drugs alone or in combination with STR at 75, 150, or 300 mg/kg/day, for 14 days. Therapy failure (defined as nonsterile spleens) was observed in all animals treated with STR at all doses and with CIP given as monotherapy. Mean log CFU isolated from the spleens remaining infected following monotherapy with STR or CIP were not different from those in control mice. RIF at a low dose did not have an effect on cure rates; however, a reduction in CFU relative to the CFU in untreated animals was obtained. DOX at low levels achieved a 35% cure rate and a reduction in CFU in animals not cured. All animals treated with DOX or RIF combined with any STR dose were cured, but none of the animals receiving the STR-CIP combinations was cured, and the splenic CFU remained similar to those in the controls. These results demonstrate that the combinations DOX-STR and RIF-STR are synergistic against B. melitensis, while the combination STR-CIP is indifferent and ineffective in the management of acute murine brucellosis. The results also appear to support the clinical superiority of combination drug therapy over monotherapy.

The "traditional" therapy of human brucellosis includes a combination of tetracyclines and streptomycin (STR) (7, 12). More recently, doxycycline (DOX) and rifampin (RIF) have been proposed as better agents by the joint FAO/WHO Expert Committee on Brucellosis (9) and have largely replaced the STR-tetracycline regimen. Despite the extensive cumulative clinical experience of therapy trials for human brucellosis, only in the past 7 years have controlled clinical trials been performed to compare the efficacy of the proposed combinations (1, 3, 5, 17, 21).

According to a recent comprehensive review, clinical outcome with combinations of antibiotics appeared to be superior to that with single agents (8). There are, however, no controlled data comparing the efficacy of each component of these regimes used alone to the efficacy of the suggested combinations.

The animal model of brucellosis in mice was previously used by other investigators and by our group to evaluate the efficacy of various antibiotics in the management of acute brucellosis (4, 13–16, 19, 20). In the present study, we investigated the efficacy of antibiotic combinations as well as their components in murine brucellosis.

MATERIALS AND METHODS

Bacteria. Brucella melitensis standard smooth strain 16 M, provided by M. Banai, National Brucella Reference Laboratory, Beit Dagan Veterinary Institute, Beit Dagan, Israel, was used for the inoculation of mice. The organism was cultured on brucella agar (Difco) at 37°C to the logarithmic phase and stored at 4°C until use. Identification of B. melitensis colonies isolated posttherapy infected spleens was based on typical colony patterns, Gram staining, and growth characteristics.

Antibiotics. STR (Teva, Ramat Gan, Israel), ciprofloxacin (CIP; Bayer AG, Leverkusen, Germany), DOX (Vibramycin; Pfizer, Karlsruhe, Germany), and RIF (Ciba-Geigy, Basel, Switzerland) were used. Antibiotic solutions were prepared according to manufacturers' instructions immediately before each administration. MICs against the study strain and against brucellae isolated from infected animals were determined by the broth microdilution method in microtiter trays. MBCs were determined according to standard procedures (22). The oral doses of DOX and RIF were selected on the basis of previous experimental data (20) and were chosen to yield subtherapeutic levels in blood, needed to determine a synergistic effect.

Animals. Adult white male ICR mice (Lewenberg, Yokneam, Israel) averaging 20 to 30 g were used. Mice were housed according to therapeutic groups and provided with food and water ad libitum.

Inoculation. B. melitensis was grown in brucella broth for 72 h to the logarithmic phase. The culture was adjusted (on the basis of viable counts) to yield 4 x 10^8 to 8 x 10^6 CFU/ml. Inoculation was performed by injecting 0.5 ml of culture containing 2 x 10^8 to 4 x 10^9 organisms in saline intraperitoneally (i.p.). Mice were then randomly assigned to treatment and control groups. The effect of high-dose STR (300 mg/kg of body weight per day) was intentionally studied only in two or three animals. The control group included 27 mice.

At the conclusion of the scheduled therapy period (day 21), treated and control animals were weighed and sacrificed under ether anesthesia, and their spleens were aseptically
removed, weighed, and homogenized in 1.0 ml of sterile saline. The homogenates (0.1 ml) were diluted in saline at decimal dilutions and seeded on brucella agar plates. Plates were incubated at 37°C for 72 to 96 h, and *B. melitensis* colonies were counted. Each procedure was performed in triplicate, and the results were averaged and expressed as a decimal logarithm. If no bacterial growth was apparent after 4 days of incubation, the plates were incubated for an additional 3 days before being considered sterile. No growth was considered a bacteriological cure.

**Therapeutic groups.** (i) **Monotherapy.** (a) *i.p.* antibiotics. Following an incubation period of 7 days, mice were randomly distributed to a single administration of 75 mg/kg/day was administered *i.p.* in one or two divided daily doses to 16 mice. STR at 150 mg/kg/day and STR at 300 mg/kg/day were introduced in a single administration to 10 and 2 mice, respectively.

(b) **Oral antibiotics.** CIP at 200 mg/kg/day, DOX at 6 mg/kg/day, and RIF at 3 mg/kg/day were administered with drinking water to groups of 6, 20, and 11 animals, respectively, starting on day 7 following inoculation and continuing for 14 days.

(ii) **Combination therapy.** Oral CIP (200 mg/kg/day) was given in combination with STR to a total of 23 animals. STR was administered *i.p.* at doses of 75, 150, and 300 mg/kg/day to 10, 10, and 3 mice, respectively. Low-dose DOX (6 mg/kg/day) was given orally in combination with STR to a total of 21 mice. STR was administered *i.p.* at 75 mg/kg/day to 8 mice, at 150 mg/kg/day to 10 mice, and at 300 mg/kg/day to 3 mice. RIF at 3 mg/kg/day was combined with the same three STR *i.p.* doses in nine, nine, and three mice.

**Drug levels.** From each group of infected animals treated with STR or CIP alone, blood was drawn by orbital puncture at 30 min and at 1, 2, 4, 6, 8, 12, and 24 h following drug administration. Blood was soaked onto a paper disc, which was placed on agar seeded with the appropriate microorganism for the bioassay of antibiotic concentrations. STR concentrations were determined on *B. subtilis* ATCC 6633-seeded plates. CIP concentrations were determined with *B. subtilis* ATCC 6633-agar dilution. DOX and RIF levels were not determined since, according to our previous data, at the doses at which they were used, levels in blood were low and below the sensitivity of the bioassay used (20).

**Data evaluation.** Four parameters were evaluated as indicators of therapeutic responses: (i) weight of animals at the end of treatment; (ii) weight of spleens at the end of treatment; (iii) cure, defined as the sterilization of an animal's spleen; and (iv) reduction of the CFU of *brucellae* cultured from the homogenized spleens.

**Statistical analysis.** The cure rate was evaluated statistically by use of the chi-square test. A comparative analysis between mice treated with the different regimens and non-treated mice for mean log CFU, mouse body weights, spleen weights, and the ratios of body weights to spleen weights was performed by use of the Fisher exact test. The limit of the level of significance was considered to be $\leq 0.01$.

**RESULTS**

**MICs.** In no case in which *brucellae* were isolated from the spleens of treated animals did a change in the MICs occur. The MICs and MBCs were the same as those in Table 1 of reference 20.

**Drug levels.** Serum CIP levels obtained at random samplings twice during the course of therapy from four animals each time ranged from 1.5 to 3 $\mu$g/ml, with a mean of 2.16 $\mu$g/ml (20). STR concentrations measured following *i.p.* injections of 37.5, 75, 150, and 300 mg/kg/day at various time intervals are presented in Fig. 1. DOX and RIF (6 and 3 mg/kg/day, respectively) yielded levels in blood below the detection limit of the specific bioassay used (20).

**Therapeutic outcome.** (i) **Body weights.** Of the whole group studied, 54 infected mice were cured and 103 remained infected. The difference in weights between healthy and infected animals was not statistically significant ($P > 0.01$). The difference between mouse weights depended largely on the therapy rather than the infection. For example, the lowest mean body weight (31.5 g) was observed in the group treated successfully with STR at 75 mg/kg/day and DOX at 6 mg/kg/day. The highest mean body weight (40.5 g) was observed in mice treated with CIP at 200 mg/kg/day.

(ii) **Spleen weights.** The mean weight of 101 sterile spleens was 267.7 mg, and that of 53 infected spleens was 405.9 mg ($P < 0.01$).

(iii) **Cure rates and log CFU.** Data for monotherapy are presented in Table 1, and those for combination therapy are presented in Table 2.

(a) **Control mice.** Of a total of 27 untreated control mice, 26 remained infected 28 days following inoculation (96.3% infected). The mean log number of CFU isolated from infected spleens of these 26 mice was 5.3.

| TABLE 1. Failure rate and log CFU in mice treated by monotherapy* |
|------------------|-------------------------------|-----------------|-------------------|-------------------|
| **Treatment group (route)** | **Doses (mg/kg/day)** | **No. infected/total no. tested** | **% Failure (P value relative to control)** | **Mean log CFU (P value relative to control)** |
| Control          | 0                             | 26/27           | 96.3 (NS)         | 5.3 (NS)           |
| STR (i.p.)       | All                           | 26/28           | 93 (NS)           | 5.48 (NS)          |
|                  | 75                            | 14/16           | 87.5 (NS)         | 5.46 (NS)          |
|                  | 150                           | 10/10           | 100 (NS)          | 5.55 (NS)          |
|                  | 300                           | 2/2             | 100 (NS)          | 5.3 (NS)           |
| CIP (p.o.)       | 200                           | 5/6             | 83 (NS)           | 5.53 (NS)          |
| DOX (p.o.)       | 6                             | 13/20           | 65 (S)            | 3.5 (S)            |
| RIF (p.o.)       | 3                             | 9/11            | 82 (NS)           | 3.8 (S)            |

* p.o., oral, S, significant ($P \leq 0.01$); NS, not significant.
(b) Monotherapy. A total of 28 mice were treated with three dose regimens of STR injected i.p. Sixteen animals received 75 mg/kg/day (9 in one dose and 7 in two divided doses), 10 received 150 mg/kg/day, and 2 received 300 mg/kg/day (the high-dose STR group was intentionally very small, since the toxic effects of STR at this dose were unpredictable and it was unclear whether the animals would survive therapy for 14 days). Fourteen of the 16 mice treated with low-dose STR (75 mg/kg/day) remained infected, yielding a failure rate of 87.5%, a value not statistically different from that of the controls. All mice infected with 150 or 300 mg/kg/day remained infected (100% failure rate). Since there was no significant difference (P > 0.01) in cure rates among all the STR regimens used, all three dose groups could be combined as a single therapeutic group, with an overall failure rate of 96% (22 of 23 infected).

The mean numbers of CFU grown per infected spleen were 5.46, 5.55, and 5.3 in the 75-, 150-, and 300-mg/kg/day STR treatment groups, respectively, values not statistically different from that of the controls (5.3) (P > 0.01).

Of the six mice treated with oral CIP at 200 mg/kg/day, five remained infected, for an 83.3% failure rate, a value not significantly different from that of control animals (P > 0.01).

From the spleens of the five infected mice treated with CIP at 200 mg/kg/day, a mean log number of CFU of 5.53 was recovered (P > 0.01 in comparison with the control value of 5.3).

Seven of 20 mice treated with DOX at 6 mg/kg/day were cured, for a 65% failure rate, a value significantly better than the control value (P < 0.01). For the 13 infected mice receiving DOX at 6 mg/kg/day, the mean log number of CFU of isolated brucellae was 3.5 (the control value was 5.3).

When analyzed by a one-way analysis of variance (Scheffe test), DOX alone was significantly more effective than either the control or STR alone (P < 0.01).

Nine of 11 mice treated with RIF remained infected, for an 82% failure rate, a value not significantly different from that of control animals (P > 0.01). For the nine infected mice in this group, the mean log number of spleenic CFU was 3.8 (the control value was 5.3), so RIF alone was significantly more effective than either the control or STR alone (P < 0.01).

c) Combination therapy. Since there was no significant difference (P > 0.01) in cure rates among all the STR regimens used, all three dose groups could be combined as a single therapeutic group.

Combinations of CIP at 200 mg/kg/day with STR at 75, 150, and 300 mg/kg/day were studied with 23 mice; 1 mouse in this group was cured at the end of therapy (96% failure rate for the whole group). One hundred percent failure (10 of 10 and 3 of 3 infected) was observed in mice treated with CIP plus STR at 75 and 300 mg/kg/day, respectively, and 90% failure (9 of 10 infected) was observed in mice treated with CIP plus STR at 150 mg/kg/day. The mean log numbers of CFU were 5.8, 5.7, and 5.9, respectively, for infected spleens from mice treated with STR at 75, 150, and 300 mg/kg/day in addition to CIP at 200 mg/kg/day. There was no significant difference between therapeutic groups and the control group (P > 0.01).

Of 21 mice treated with all three DOX (6 mg/kg/day)-STR combinations, only 1 remained infected at the end of therapy (5% failure rate). This sole failure was observed in one of eight mice treated with DOX plus STR at 75 mg/kg/day (12.5% failure rate). None of the other 13 mice treated with DOX-STR remained infected.

One of 21 mice treated with RIF at 3 mg/kg/day in combination with any of three STR doses remained infected (5% failure rate). This single failure was observed in a mouse given RIF and STR at 75 mg/kg/day. None of the nine mice treated with STR at 150 mg/kg/day or the three mice treated with STR at 300 mg/kg/day in combination with RIF at 3 mg/kg/day were infected.

Log CFU as a parameter of cure was not applicable in the latter two groups, since only one infected spleen was observed for each of those therapeutic combinations.

**DISCUSSION**

The results of the present investigation demonstrate the inefficacy of DOX and RIF alone at low concentrations (6 and 3 mg/kg/day, respectively) and of CIP alone at a high concentration (200 mg/kg/day) administered orally for 2 weeks to cure experimental murine brucellosis. The failure of low doses of RIF and DOX to achieve complete cure was previously demonstrated by us with the same model (20). The failure of CIP in this model is in accord with its deficient activity in time-kill curve experiments (18) and the high relapse rate observed with CIP therapy in cases of human brucellosis. Lang et al. described relapses for 4 of 6 patients (11), and Al Siabi reported relapses for 4 of 15 patients (27%) and an even higher relapse rate for bacteremic patients (4 of 6 relapsed) (2). The same trend was reported by Khuri-Bules and Shaker (10).

STR administered i.p. at all doses also failed to achieve cure or even reduce colony counts. Despite having highly infected spleens, with a log number of CFU of about 5 per spleen, the infected mice had body weights similar to those of the uninfected mice. This unexpected experimental finding may be attributable to the strain of mice used by us, since other, previously published studies demonstrated an appreciable weight loss in infected mice (4, 13–16, 19).

Since STR showed excellent in vitro activity in time-kill experiments, the lack of penetration of STR as well as other aminoglycosides into eukaryotic cells might explain the discrepancy between the in vitro results and the animal data. CIP failed to cure the animals, despite its excellent penetration into cells. The reason may be its inactivation either by the acid pH of the phagolysosome containing the brucellae.

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**TABLE 2. Failure rate and log CFU in mice treated by combination therapy**

<table>
<thead>
<tr>
<th>Treatment group (route)</th>
<th>Dose (mg/kg/day)</th>
<th>No. infected/total no. tested</th>
<th>% Failure (P value relative to the control)</th>
<th>Mean log CFU (P value relative to the control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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</tr>
<tr>
<td></td>
<td>0</td>
<td>26/27</td>
<td>96.3 (NS)</td>
<td>5.3 (NS)</td>
</tr>
<tr>
<td>CIP (p.o.)-STR (i.p.)</td>
<td>200/All</td>
<td>22/23</td>
<td>96 (NS)</td>
<td>5.8 (NS)</td>
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<tr>
<td></td>
<td>200/75</td>
<td>10/10</td>
<td>100 (NS)</td>
<td>5.8 (NS)</td>
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<tr>
<td></td>
<td>200/150</td>
<td>9/10</td>
<td>90 (NS)</td>
<td>5.7 (NS)</td>
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<tr>
<td></td>
<td>200/300</td>
<td>3/10</td>
<td>100 (NS)</td>
<td>5.9 (NS)</td>
</tr>
<tr>
<td>DOX (p.o.)-STR (i.p.)</td>
<td>6/All</td>
<td>1/21</td>
<td>5 (S)</td>
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<tr>
<td></td>
<td>6/75</td>
<td>0/10</td>
<td>12.5 (S)</td>
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<td>6/150</td>
<td>0/10</td>
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<tr>
<td></td>
<td>6/300</td>
<td>0/3</td>
<td>0 (S)</td>
<td></td>
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<tr>
<td>RIF (p.o.)-STR (i.p.)</td>
<td>3/All</td>
<td>1/21</td>
<td>5 (S)</td>
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<tr>
<td></td>
<td>3/75</td>
<td>1/9</td>
<td>11 (S)</td>
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<td></td>
<td>3/300</td>
<td>0/3</td>
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*p.o., oral.—, a single animal. S, significant (P ≤ 0.01); NS, not significant.*
or, at a later stage, fusion of the phagosome with the rough endoplasmic reticulum, forming a vacuole with a more neutral pH but possibly with a lower affinity for trapping quinolones (6).

The present results also demonstrate the superiority of drug combinations containing DOX and STR and containing RIF and STR (5% failure rates with both combinations at the end of therapy). Even though no controlled clinical trials were conducted to compare the efficacy of single-drug versus combination therapy, it was clinically apparent that higher primary cure rates and lower relapse rates were obtained with combination therapy than with single-drug therapy (8).

In test tubes, STR-minocycline and STR-RIF demonstrated a synergistic effect in time-kill curves (18). The combination of STR and CIP was also synergistic in time-kill curves (18), an effect that could not be obtained in the present study, probably because of the low pH, which inactivated CIP, in the phagolysosome that contained the organisms.

The mechanism of the superiority of DOX-STR and RIF-STR over the single components and over STR-CIP remains unclear. It is possible that RIF and DOX facilitate the entry of STR into the cell organelle that contains the ingested organisms or change the local conditions in a manner that allows STR to exert its activity and to produce a synergistic effect. Studies designed to resolve these issues are under way.

We conclude that it seems unwarranted to use CIP either alone or in combination with STR as therapy for acute brucellosis. DOX-STR and RIF-STR regimens appear to be synergistic and therefore offer potential advantages in the management of brucellosis.

REFERENCES
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