Therapeutic Evaluation of Minocycline and Tetracycline for Mixed Anaerobic Infection in Mice

GALE B. HILL
Departments of Obstetrics and Gynecology, and Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710

Received for publication 12 November 1976

Minocycline has demonstrated greater in vitro activity against anaerobic bacteria than its parent compound, tetracycline. In vivo therapeutic efficacy of the two drugs was tested against a mixed anaerobic infection in a mouse model. *Fusobacterium necrophorum* plus *F. nucleatum* injected intraperitoneally produced progressive intrahepatic and occasional extrahepatic abscesses, which were measured at autopsy. Three treatment regimens were tested, single daily doses of antibiotic being administered by oral gavage: four doses begun at 2 or 24 h after challenge and 14 doses begun 3 weeks after challenge when abscesses were well developed. Autopsy was not performed until several weeks after completion of treatment to assess long-term effects. Based on the number of mice without lesions, the median effective dose (ED₅₀) in milligrams per kilogram per dose for minocycline was significantly lower than that for tetracycline with each regimen tested. With the 2-h (immediate therapy) regimen and the 24-h-delayed therapy regimen, minocycline was 30 and 6 times, respectively, more effective against hepatic abscesses than tetracycline on a weight basis. With each antibiotic, abscesses outside the liver were more resistant to therapy, although again minocycline was more effective. In the treatment of developed abscesses (3-week-delayed regimen), minocycline was effective (ED₅₀ <16 mg/kg), whereas tetracycline was ineffective (ED₅₀ >256 mg/kg). Minocycline has demonstrated greater therapeutic efficacy in vivo than tetracycline in this experimental infection, which is similar, in certain aspects, to human anaerobic infection. These data support further evaluation of the clinical usefulness of minocycline.

Increased recognition of the importance of anaerobic bacteria in human infection has intensified the search for antibiotics active against these organisms (2–5). In the past, tetracycline has been effective in treatment of an aerobic infection, but a variety of medically significant anaerobic bacteria have become increasingly resistant to the drug. Presently, approximately two-thirds of clinical isolates of *Bacteroides fragilis* are resistant (1, 13). In vitro data indicate that minocycline, a tetracycline derivative, has significantly greater activity against anaerobic and aerobic bacteria than the parent compound or other analogues (1, 12). The drug also has improved pharmacokinetic characteristics. Since it is more lipophilic than other tetracyclines, it is readily absorbed by tissues and gives rise to higher tissue levels of antibiotic (7, 9). Improved efficacy of minocycline versus tetracycline has been demonstrated in vivo in treatment of experimental infections with tetracycline-resistant staphylococci and other aerobic pathogens in mice (10).

Since a broader choice of antibiotics active against anaerobic bacterial infection is desirable, in this study the therapeutic efficacy in vivo of minocycline was compared with that of tetracycline in a standardized anaerobic infection, consisting primarily of liver abscesses, in mice. The model infection, which has been described previously (6), mimics common characteristics of human anaerobic infection. This mixed infection with two anaerobic species produces deep-seated abscesses that are chronic and progressive within the viscera. The present evaluation of minocycline and tetracycline was directed towards determining therapeutic, in addition to prophylactic, efficacy.

MATERIALS AND METHODS

Bacterial culture. Human clinical isolates of *Fusobacterium necrophorum* NCDC 9432 and *Fusobacterium nucleatum* NCDC 10206 were maintained as frozen samples prepared from cultures of lyophilized stocks. For each experiment, a frozen sample was inoculated into fluid thioglycolate medium (no.
135C, Baltimore Biological Laboratories, Cockeysville, Md.) supplemented with 0.2% yeast extract, menadione (0.5 μg/ml), hemin (5 μg/ml), and 10% rabbit serum. After one further transfer, growth was harvested at 20 h. The concentration of viable bacteria used for infection was determined by spreading duplicate 0.1-ml samples of 10-fold dilutions of broth cultures on the surface of blood agar plates. These were incubated, and plates containing 30 to 300 colonies were counted. Plates were prepared with brucella agar (Baltimore Biological Laboratories) supplemented with menadione (0.5 μg/ ml), hemin (5 μg/ml), and 5% defibrinated sheep blood. All media were immediately placed under anaerobic conditions for storage after preparation. Inoculation, incubation (37°C), and harvesting of cultures were performed within an anaerobic glove chamber (Coy Manufacturing Co., Ann Arbor, Mich.) containing 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Antibiotics. Minocycline-hydrochloride and tetracycline-hydrochloride (Lederle Laboratories Division, American Cyanamid Co.) solutions were prepared fresh daily in sterile distilled water. Doubling dilutions of each antibiotic were administered to mice by oral gavage. The correct dose (range, 4 to 256 mg/kg) was determined by weighing each mouse before treatment and by administering 0.1 ml of antibiotic dilution per 10 g of body weight.

In vitro susceptibility tests. The minimum inhibitory concentration (MIC) of tetracycline and minocycline for F. necrophorum and F. nucleatum was determined by a broth dilution test (14). Each MIC test was performed in duplicate on two occasions.

Experimental animals. Male, inbred, A/J mice (The Jackson Laboratory, Bar Harbor, Me.) were used at 10 to 12 weeks of age. Animals were given laboratory chow and water ad libitum. They were held 2 weeks after shipment to stabilize their health before use in experiments.

Infection model. The infection model (6) is briefly outlined here as it pertains to this study. Broth cultures of F. necrophorum and F. nucleatum were mixed in a 1:1 ratio, and 0.5 ml was injected intraperitoneally into mice. Mucin was not utilized for these inocula. The number of organisms injected ranged from $1 \times 10^6$ to $5 \times 10^6$ throughout these experiments.

On the day after challenge, untreated control mice appeared ill, with a ruffled coat, eyes partially or totally closed, and an increased respiratory rate. At autopsy, mice revealed a heavy growth of both anaerobic species around the liver and particularly below the diaphragm. Many control animals had positive blood cultures with the injected anaerobic species. Depending on the challenge dose, some mice (infrequently to a maximum of approximately one-third in these experiments) died of septicemia and peritonitis within several days. Fewer deaths occurred after 2 to 3 weeks. If animals were sacrificed and autopsied at 1 week, intrahepatic and perihepatic lesions were primarily visible, but abscesses were also located in the abdomen, mesentery, or scrotum and occasionally in the thoracic cavity. Although some of the lesions outside the liver sponta-

neously resolved during the first 1 to 2 weeks after challenge, after approximately 3 weeks, the number of abscesses remained quite constant, and these exhibited progressive enlargement. After several weeks, the majority of lesions were intrahepatic, with fewer abscesses in other sites. At approximately 5 weeks, one to three intrahepatic abscesses, which measured between 4 and 8 mm in diameter, were usually present. These increased to approximately 1 cm in diameter by 8 to 10 weeks. The liver and particularly the spleen were ruffled. The injected anaerobic species were present within these abscesses in high concentration and were verified by Gram stain smears and aerobic and anaerobic culture. Sparse growth of other organisms was detected only rarely.

Experimental design and analysis. The therapeutic effectiveness of minocycline and tetracycline were evaluated as follows. Mice were challenged with the mixed Fusobacterium inoculum and were randomized into the following groups: antibiotic test groups consisting of 10 to 15 animals per antibiotic dilution and an untreated infection control group consisting of two to three times the number of animals in each antibiotic test group. Two groups of drug control mice (5 to 10 per group) were injected with sterile broth and were administered the highest concentration of each of the antibiotics, 256 mg/kg, on the same schedule as the drug-treated infected mice. The infected control animals were given water (oral gavage) on the same dosage schedule.

Three different antibiotic regimens were tested, each in duplicate on separate occasions. After completion of a drug regimen, animals were held an additional 2.5 to 4 weeks before the results of therapy were evaluated at autopsy. This delay permitted observation of more long-term effects of treatment versus nontreatment. The test and control animals were autopsied at the same time, and the number and size of abscesses present in each animal were recorded (pathology score). A score of 0 was assigned when no lesions were found. Although assignment of pathology scores, which consider both the number and size of abscesses present in each mouse rather than simply a positive or negative score for any lesions, is probably a more sensitive measurement, analysis of these data is more complicated. Since differences could be discerned by comparing the number of mice negative for lesions among the matched antibiotic groups, this information was used for probit analysis to obtain the median effective dose (ED₅₀) in milligrams per kilogram per injection for each antibiotic.

RESULTS

In vitro susceptibility. MICs expressed as micrograms of minocycline and of tetracycline per milliliter were: 0.0188 and 0.075, respectively, against F. necrophorum and 0.0375 and 0.15, respectively, against F. nucleatum.

Treatment immediately after challenge. The effect of administering antibiotic shortly after infection of mice with F. necrophorum...
and \( F. \) \( nucelatum \) was tested. The initial dose was administered 2 h after challenge, and three additional doses were given at 24-h intervals on consecutive days. Mice were autopsied 4.5 weeks after challenge. Animals were evaluated on the basis of: (i) liver abscesses and (ii) abscesses at any site (i.e., intra-abdominal, scrotal, thoracic, with or without concurrent liver abscess), since tetracycline is known to reach high concentrations in the liver. With 256 mg/kg, minocycline completely protected mice, and with 4 mg/kg, protection was 62% (Table 1). Corresponding doses of tetracycline protected 67 and 20%, respectively. At each dose, the antibiotics, in general, protected fewer mice when extrahepatic lesions were considered in addition to hepatic ones than when hepatic lesions alone were graded. The \( ED_{50} \) (Table 2) for minocycline was significantly less than that for tetracycline for liver abscesses alone or for abscesses at all sites.

Treatment delayed for 1 day after challenge. On the second antibiotic regimen, the effect of delaying initiation of therapy for 24 h after infection was tested. Again, four doses at 24-h intervals were administered. Mice were autopsied 3.5 weeks after challenge. With this 24-h-delayed regimen, higher doses of each drug were required to treat liver abscesses and, in particular, abscesses at all sites, but minocycline was significantly more effective by weight than tetracycline (Table 2). Minocycline was as effective for lesions at any site as was tetracycline for liver abscesses alone. Tetracycline was ineffective in preventing extrahepatic abscesses.

Treatment delayed for 3 weeks after challenge. The effect of allowing abscesses to develop for 3 weeks before initiating antibiotic therapy was tested. Animals were administered single daily doses for a 2-week period (14 treatments). Mice were autopsied 7.5 weeks after challenge. On this schedule, minocycline was effective (Table 2), whereas tetracycline was ineffective at the highest dose tested (256 mg/kg). This degree of effectiveness (minocycline) was not anticipated against well-established abscesses so that the lowest concentrations tested were 16 and 8 mg/kg in the two experiments. The \( ED_{50} \) of minocycline, therefore, is indicated in Table 2 as being less than 16 mg/kg, but the extrapolated \( ED_{50} \) for minocycline on this regimen was 1 mg/kg for treating liver abscesses and abscesses at all sites.

Splenetic enlargement. Infected mice, in general, had enlarged spleens. The splenic weights

### Table 1. Effect of minocycline and tetracycline on mixed \( F. \) \( necrophorum \) and \( F. \) \( nucleatum \) infection in mice

<table>
<thead>
<tr>
<th>Antibiotic*</th>
<th>Abcess site</th>
<th>% Mice negative for lesion* at dose (mg/kg per injection) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minocycline</td>
<td>Liver only</td>
<td>100 94 84 85 89 74 62</td>
</tr>
<tr>
<td></td>
<td>All lesions</td>
<td>100 88 84 85 89 53 52</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Liver only</td>
<td>67 53 47 67 40 33 20</td>
</tr>
<tr>
<td></td>
<td>All lesions</td>
<td>61 47 37 67 20 29 13</td>
</tr>
</tbody>
</table>

* Antibiotics administered by oral gavage to 2, 24, 48, and 72 h after challenge.

* Combined data from two tests, 10 to 15 mice per group in each test. Infected nontreated control mice: 29/33 (88%) positive for liver abscesses, 30/33 (91%) positive for abscesses at any site.

### Table 2. Activities of minocycline and tetracycline on three different treatment schedules against mixed \( F. \) \( necrophorum \) and \( F. \) \( nucleatum \) infection in mice

<table>
<thead>
<tr>
<th>Antibiotic*</th>
<th>Abcess site</th>
<th>Median effective dose* (mg/kg per injection) on schedule of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 Treatments</td>
<td>4 Treatments</td>
</tr>
<tr>
<td></td>
<td>(from 2 to 72 h)</td>
<td>(from 24 to 96 h)</td>
</tr>
<tr>
<td>Minocycline</td>
<td>Liver only</td>
<td>1.4 (0.03–4.4)</td>
</tr>
<tr>
<td></td>
<td>All lesions</td>
<td>3.6 (0.6–7.4)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Liver only</td>
<td>42.7 (15.5–186)</td>
</tr>
<tr>
<td></td>
<td>All lesions</td>
<td>89.1 (38.9–646)</td>
</tr>
</tbody>
</table>

* Antibiotics administered by oral gavage.

* Median effective doses were determined from combined data from two separate tests on each schedule. Numbers in parentheses are 95% confidence limits. A total of 20 to 25 mice were committed to each dilution of antibiotic tested.
of minocycline- and tetracycline-treated mice at autopsy were compared to test whether these data would serve as a further index of infection and of response to each antibiotic. To obtain the most critical analysis of any differences, the splenic weights compared were from mice administered concentrations of antibiotic that were in between the ED₅₀ values (for liver lesions) derived for minocycline and tetracycline with each treatment regimen (Tables 2 and 3). The splenic weights (Table 3) generally reflected the greater therapeutic activity of minocycline, as indicated by its lower ED₅₀ for each regimen. Minocycline-treated mice, in general, had significantly smaller spleens than those of tetracycline-treated mice administered equivalent doses. Although the combined data (two experiments) from mice administered 8 mg/kg on the immediate regimen (Table 3) were not significant, one of these duplicate experiments, when analyzed individually, demonstrated significantly smaller ($P = 0.05$, rank sum test) splenic weights for minocycline-treated mice compared with those given tetracycline.

**DISCUSSION**

The therapeutic regimens tested in the present studies were designed to simulate clinical usage of antibiotics, whereas drug efficacy studies are often performed with single doses of antibiotics being administered immediately after bacterial challenge. The immediate treatment regimen can be considered analogous to the rapid initiation of antibiotic therapy incident to sudden contamination of the peritoneal cavity after trauma or bowel surgery. The 24-h-delayed regimen was initiated at a time when mice had bacteremia and appeared quite ill. This regimen might represent the treatment of symptoms in postoperative infectious complications or the early stages of a ruptured viscus. The third therapy regimen (3-week delay) simulated the more extensive medical treatment required for a well-established disease process as, for example, a patient presenting with an intrahepatic or intra-abdominal abscess. Autopsies were delayed for several weeks after termination of therapy to allow for resolution of treated abscesses and redevelopment or an increase in size of inadequately treated lesions. The absence of lesions would, therefore, be related more to a permanent effect than to continued inhibitory activity.

On each of the three regimens, minocycline was demonstrated to be significantly more effective by weight than tetracycline. Although positive/negative data for lesions were used for analysis, it was apparent that the pathology scores closely paralleled these values. Minocycline-treated mice generally had fewer and smaller lesions than mice given the same dose of tetracycline. The lower ED₅₀ values of each antibiotic in treating only hepatic abscesses in contrast to abscesses at any site probably relate to the affinity of tetracyclines for the liver, resulting in higher antibiotic concentrations.

Wilkins and Smith (15) tested four antibiotics useful against anaerobic infections in a lethal subcutaneous infection model initiated in mice with a sheep foot rot strain of *F. necrophorum*. Different combinations of the route of

<table>
<thead>
<tr>
<th>Antibiotic regimen</th>
<th>Drug dose per injection (mg/kg)</th>
<th>Mean spleen wt at autopsy (g ± standard deviation)</th>
<th>$P^*$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minocycline</td>
<td>Tetracycline</td>
<td></td>
</tr>
<tr>
<td>Immediate therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.09 ± 0.05*</td>
<td>0.22 ± 0.07</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>8</td>
<td>0.13 ± 0.03</td>
<td>0.16 ± 0.05</td>
<td>$0.1 &lt; P &lt; 0.2$</td>
</tr>
<tr>
<td>24-h-delayed therapy</td>
<td>0.08 ± 0.05</td>
<td>0.13 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>0.16 ± 0.03</td>
<td>0.19 ± 0.10</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>3-week-delayed therapy</td>
<td>0.18 ± 0.04</td>
<td>0.24 ± 0.06</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>64</td>
<td>0.08 ± 0.04</td>
<td>0.15 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.12 ± 0.05</td>
<td>0.19 ± 0.03</td>
<td>$P &lt; 0.01$</td>
</tr>
</tbody>
</table>

* $P^*$ values by Wilcoxon and Mann-Whitney rank sum test on combined data from duplicate experiments.

* Mean splenic weights from mice, corresponding to each of the two (duplicate) experiments, are presented separately.
infection, antibiotics, and dosing schedules were tested. On the most effective regimen, tetracycline was more active (ED₅₀ of 5 mg/kg) compared with clindamycin (11.1 mg/kg), penicillin G (11.8 mg/kg), and lincomycin (52.9 mg/kg). Minocycline was not tested. Kuck (8) compared minocycline with other antibiotics in infections initiated with human clinical isolates of F. necrophorum alone. A subcutaneous model similar to that of Wilkins and Smith (15) and an intraperitoneal model similar to that of Hill et al. (6) were employed. Drug efficacy was based on the number of animals without macroscopic lesions 10 to 14 days after challenge. These lesions generally healed spontaneously, but occasionally, they were progressive. With the antibiotic regimens tested, minocycline was the most active drug on a weight basis, whereas clindamycin was more active on the basis of serum concentration. Doxycycline and ampicillin were much less active, and tetracycline, penicillin G, and cephalaxin were ineffective at the doses tested. Comparison of serum concentrations and the ED₅₀ values of minocycline and clindamycin in mice indicated that antibiotic levels readily achievable in humans would be effective against the organism. Although less effective than minocycline, tetracycline was found in the present studies and in tests by Wilkins and Smith (15) to be considerably more active than in Kuck's studies, in which the ED₅₀ was never less than 256 mg/kg, even with tetracycline administered twice daily. The basis for this difference is not clear, since the two strains of F. necrophorum tested by Kuck (8) were susceptible in vitro to tetracycline (0.25 and 0.06 µg/ml).

In the present studies, the greater activity of minocycline over that of tetracycline might be predicted on the basis of in vitro susceptibility data, which showed that, against both organisms, minocycline was approximately four times more active than tetracycline. The in vivo activity of an antibiotic, however, is often not predictable by in vitro susceptibility data, a point demonstrated by the present data and other studies (6, 15). In addition, Redin (10) has demonstrated that, with an equivalent oral dose, minocycline achieves approximately four times higher plasma concentrations than tetracycline. The activity of minocycline in the present study, however, was even greater with the immediate regimen and the 3-week-delayed regimen than would have been predicted on the basis of in vitro susceptibility and plasma level data.

The in vivo model utilized in this evaluation of drug efficacy was similar in certain aspects to infections in humans with anaerobic bacteria. Approximately one-half or more of liver abscesses involve anaerobes, and fusobacteria, particularly F. necrophorum (formerly designated as Sphaerophorus necrophorus), are common isolates (11). Extrahepatic abscesses within the abdomen have an even higher incidence, over 90%, of anaerobic bacteria (3). Although in vitro susceptibility tests have demonstrated less inhibition of anaerobic bacteria with minocycline compared with that observed with clindamycin or chloramphenicol, alternate drugs are often desirable. Minocycline may be clinically useful, therefore, in a variety of settings, but in vitro susceptibility should be ascertained before its use in serious disease. The present data further encourage the potential clinical usefulness of minocycline for anaerobic infections.

ACKNOWLEDGMENTS

The valuable technical assistance of Ouida Ayers and Clinton Moorman is gratefully acknowledged.

I thank Dennis Tolley and Lawrence Myers of the Department of Community Health Sciences (Biostatistics) for their help in the statistical analysis.

This investigation was supported by a grant from Lederie Laboratories, Pearl River, N.Y.

LITERATURE CITED


