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Treatment of Experimental Staphylococcal Osteomyelitis with Rifampin and Trimethoprim, Alone and in Combination

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Rifampin and trimethoprim were used alone and in combination in the treatment of chronic osteomyelitis due to *Staphylococcus aureus* in rabbits. Rifampin levels in infected bone were well above the minimum inhibitory concentration for the infecting strain of *S. aureus* for at least 4 h after injection. In contrast, trimethoprim levels in diseased bone were below the minimum inhibitory concentration as early as 1 h after injection. Trimethoprim or rifampin, administered alone for 14 days, were ineffective in sterilizing infected rabbit bones. The combination of rifampin plus trimethoprim was significantly more effective ($P < 0.005$) than either agents given alone for a comparable duration of time. Staphylococci isolated from the bones of rabbits treated with rifampin alone or rifampin plus trimethoprim were uniformly resistant to rifampin, but retained their susceptibility to trimethoprim.

Previous studies in an experimental model of staphylococcal osteomyelitis have shown that the use of single agents such as lincomycin, cephalothin, gentamicin, oxacillin, and rifampin was only moderately effective in sterilizing bone (7). More recent studies, in the same model, showed that combinations of oxacillin plus sisomicin or rifampin combined with an aminoglycoside or cephalothin were significantly more effective (7, 8).

One of the problems in the treatment of chronic osteomyelitis has been the need to administer antibiotics parenterally over a prolonged period. A major attraction of the use of rifampin is that the drug can be administered orally once or twice a day and that levels are attained in serum which should be high enough to maintain adequate levels in bone (11). However, because of the development of rifampin-resistant colonies, this agent cannot be recommended for use by itself. Trimethoprim, which can also be administered orally and which has activity against *Staphylococcus aureus*, was therefore tested as an antimicrobial agent to be used in combination with rifampin.

MATERIALS AND METHODS

Production of osteomyelitis. The technique for production of osteomyelitis has been described previously (6). New Zealand white rabbits (weight, 4 lb; ca. 1.8 kg) received an intramedullary injection into the tibia of sodium morrhuate and 3×10^6 colony-forming units of *S. aureus* (6). This strain of *S. aureus* is resistant to penicillin, erythromycin, and tetracycline and susceptible to oxacillin, cephalothin, gentamicin, rifampin, and trimethoprim.

Culture of bone. Cultures were obtained by techniques described previously (9).

Measurement of antibiotics in serum and bone. The cylinder plate method was used for the assay of antibiotics (4).

Serum assay. Rifampin (kindly supplied by John Eble, Dow Chemical Corp., Indianapolis, Ind.) was assayed as described previously (7); the minimum quantity of rifampin that could be measured was 0.1 μg/ml. For trimethoprim assay, standard curves were constructed from the sizes of zones of inhibition of reference standards tested with *Bacillus pumilus* (WRL-CN 607, kindly supplied by S. Bushby, Wellcome Research Laboratories). The assay was performed in Wellcome nutrient agar (Burroughs-Wellcome Laboratories, Research Triangle Park, N.C.) with the addition of thymidine phosphorylase (0.025 IU of enzyme per ml). The trimethoprim powder was dissolved in 0.5 N lactic acid. All sera and standards were further diluted in 50% normal rabbit serum and 50% 0.1 M phosphate buffer at pH 7.3. The minimum quantity of trimethoprim that could be measured was 0.01 μg/ml.

Bone assay. The bones were prepared as described previously (7). The minimum quantities of trimethoprim and rifampin that could be measured were 0.01 and 0.1 μg/ml of bone suspension, respectively; the amount of antibiotic was expressed as micrograms per gram of bone.

To determine whether trimethoprim or rifampin binds to bone powder, standards of each antibiotic were agitated for 4 h before assay either with suspensions of bone powder from uninfected rabbits or with buffer. No differences in zone sizes were seen, indicating that the antibiotics did not bind to bone powder in this assay.

Conduct of therapeutic trials. Rabbits were divided into groups and received the following regimens: (i) no antibiotic therapy; (ii) single daily injection of

rifampin at 40 mg/kg; (iii) four-times daily injection of trimethoprim, 40 mg/kg; (iv) single daily injection of rifampin, 40 mg/kg plus four-times-daily injections of trimethoprim, 40 mg/kg. All injections of antibiotics were subcutaneous. Therapy was instituted 14 days after infection and given for 7, 14, or 28 days. All rabbits were killed 70 days after infection. Recovery of any number of colony-forming units of *S. aureus* was considered to represent a positive bone culture. The assay is designed to detect as few as 1 or 2 colonies per ml of bone flush. Minimum inhibitory concentrations (MICs) were determined for all staphylococci recovered from bones of treated animals.

Radiological evaluation of severity of disease. Severity of disease was evaluated by a radiological technique described previously (9).

Determination of MIC. An inoculum (0.5 ml) containing either 10^5 or 10^7 *S. aureus* in Mueller-Hinton broth was added to 0.5 ml of serial dilutions of antibiotic standards. For trimethoprim, thymidine phosphorylase (0.25 IU of enzyme per ml) was added to the broth. The mixture was incubated at 37°C for 18 h and then observed for turbidity.

Synergism. The criterion used to designate a combination as synergistic was a fourfold or greater reduction in MIC for each agent.

Determination of rates of killing by antibiotics. An overnight culture of the infecting strain of *S. aureus* was diluted in Mueller-Hinton broth (containing thymidine phosphorylase at 0.25 IU of enzyme per ml) to yield a final concentration of 2×10^7 or 2×10^5 colony-forming units per ml. To this preparation was added either a single antibiotic or a combination of antibiotics; a sample was removed immediately for determination of the number of colony-forming units by a standard pour-plate technique. The bacteria-antibiotic mixture was incubated at 37°C, and at 6 and 24 h samples were removed for determination of the number of colony-forming units by pour-plate technique.

RESULTS

MIC determinations. With an inoculum of 10^5 organisms of the strain of *S. aureus* used to produce osteomyelitis, the MICs of rifampin and trimethoprim were 0.039 and 1.25 μg/ml, respectively. An increase of the inoculum to 10^7 organisms raised the MIC of trimethoprim to 2.5 μg/ml. With rifampin, an increase of the inoculum to 10^7 organisms raised the MIC to 1.25 μg/ml with frequent "skip tubes."

Synergism. The combination of rifampin plus trimethoprim showed antagonism when tested by checkerboard technique.

Rates of killing by antibiotics. There was no evidence of enhanced killing of *S. aureus* when rifampin and trimethoprim were combined in multiple different combinations of concentrations.

Measurement of antibiotic concentrations in serum and bone. Levels of rifampin and trimethoprim in serum and osteomyelitic bone are shown in Table 1. As in previous studies, rifampin levels in diseased bone remained well above the MIC for 4 h for the infecting strain of *S. aureus*. Not shown in the table are two rabbits sacrificed at 18 h and two sacrificed at 24 h after subcutaneous injection of rifampin (40 mg/kg); in these animals concentrations of rifampin in serum ranged from 0.6 to 0.9 μg/ml, and concentrations in diseased bone ranged from 0.2 to 0.3 μg/g. In contrast, the level of trimethoprim in bone was below the MIC for the infecting strain of *S. aureus* at the earliest time of measurement (1 h after injection). By 4 h, the level of trimethoprim in bone was approximately one-third the MIC, and by 6 h, it was approximately one-tenth the MIC.

Therapeutic trials. The results of therapy are shown in Table 2. Trimethoprim alone for 7 or 14 days and rifampin alone for 14 days were ineffective in sterilizing infected rabbit bones. The combination of rifampin plus trimethoprim for 7 days was significantly more effective ($P < 0.005$) than trimethoprim alone for 7 days. The combination of rifampin plus trimethoprim for 14 days was significantly more effective ($P < 0.005$) than either agent given alone for a comparable duration. Extension of the duration of therapy of the combination of rifampin and trimethoprim to 28 days was not significantly more effective in sterilizing infected rabbit bones than

TABLE 1. *Antibiotic concentration in serum and bone*[a]

Time after injection (h)	Drug concn after dose:			
	Rifampin, 40 mg/kg		Trimethoprim, 40 mg/kg	
	Serum (μg/ml)	Diseased bone (μg/g)	Serum (μg/ml)	Diseased bone (μg/g)
1	6.5 ± 1.7	1.6 ± 1.0	6.2 ± 1.6	0.85 ± 0.23
2	ND	ND	6.6 ± 2.4	0.85 ± 0.33
4	6.2 ± 2.1	1.8 ± 0.6	2.1 ± 0.6	0.38 ± 0.07
6	ND	ND	0.9 ± 0.3	0.14 ± 0.02

[a] Results are expressed as mean ± standard deviation. For each time interval after injection, six animals were studied. ND, Not done.

TABLE 2. *Results of treatment with rifampin and trimethoprim alone and in combination for experimental osteomyelitis due to S. aureus in rabbits*

Antibiotic[a]	Duration of therapy (days)	No. of animals[b]	Severity of disease[c]	Rabbits (%) with sequestra on day 70	Rabbits (%) with positive bone culture on day 70
None	—	20	2.8 ± 0.4	80	100
Trimethoprim	7	20	2.4 ± 0.7	45	95
Trimethoprim	14	20	2.5 ± 0.7	40	95
Rifampin	14	20	2.3 ± 0.7	40	80
Rifampin + trimethoprim	7	20	2.1 ± 1.0	25	50
Rifampin + trimethoprim	14	20	2.1 ± 0.8	20	35
Rifampin + trimethoprim	28	20	2.0 ± 1.0	20	25

[a] Rifampin was not tested in a 7-day regimen. See the text for dose of each antibiotic.

[b] Number of animals surviving to day 70; each group initially contained 25 animals.

[c] Radiological evaluation on day 70 based on a scale of 0 to 3 (mean ± standard deviation): 0 = no disease; 1 = minimal periosteal reaction, bone destruction limited to area proximate to site of injection; 2 = severe periosteal reaction, bone destruction limited to proximal half of tibia, new bone formation; 3 = severe periosteal reaction, bone destruction involving entire length of tibia, extensive new bone formation.

the same combination given for either 7 or 14 days.

MIC determinations on staphylococci recovered from bone. MIC determinations for rifampin and trimethoprim were performed on staphylococci recovered from the bones of rabbits in all groups. No evidence of resistance to trimethoprim was observed; in contrast, all organisms recovered from animals receiving either rifampin alone or rifampin in combination with trimethoprim were uniformly resistant to greater than 5 μg of rifampin per ml.

DISCUSSION

In the present study, treatment with either trimethoprim or rifampin was ineffective in sterilizing the bones of animals with staphylococcal osteomyelitis. The combination of rifampin and trimethoprim was significantly more effective than either agent alone. Rifampin-resistant organisms were consistently recovered from the bones of animals in which combination therapy failed.

The use of trimethoprim for the treatment of staphylococcal osteomyelitis is potentially attractive. The MIC of trimethoprim, when tested against several strains of staphylococci, has been reported to range from 0.2 to 1 μg/ml (2). The level of trimethoprim attained in bone in humans is not known; in rabbits, the bone level was approximately 13% of simultaneous serum levels, a ratio which approximates that for rifampin and sisomicin. Despite the relatively good penetration into bone, because of the high MIC of our organism (1.25 μg/ml), the levels of trimethoprim in bone in rabbits did not equal or exceed the MIC at any time after injection. It is conceivable that trimethoprim might have been more effective if tested against a strain of *S. aureus* that required a lower MIC or if the drug were given in higher dosage or more frequently.

The in vitro results of combining trimethoprim and rifampin indicated antagonism by checkerboard technique and no enhancement of killing in time-kill curves. Others have also reported antagonism between these two agents in in vitro testing (5); the interpretation of these results has been disputed by other workers (1, 10). In the therapeutic trials, the combination of rifampin and trimethoprim was significantly more effective than either agent alone. Since the presumed reason for this therapeutic success was the fact that trimethoprim is acting to inhibit or kill rifampin-resistant mutants, it is likely that any antibiotic that is effective against the infecting bacteria could serve this purpose. The combination of rifampin and trimethoprim was approximately as effective as the previously tested combinations of rifampin plus sisomicin or rifampin plus cephalothin (7). The only regimen previously tested that was significantly more effective than rifampin plus trimethoprim was the combination of three agents (rifampin, cephalothin, and sisomicin) (7).

In summary, the combination of rifampin plus trimethoprim (despite the theoretical disadvantages of using trimethoprim against an organism with relatively high resistance) was effective in the treatment of staphylococcal osteomyelitis in rabbits. The potential attractiveness of such a combination of antibiotics is that both can be administered orally; oral administration, if therapeutically effective, would allow for shorter periods of hospitalization and reduced costs.

Based on the animals studied, there is nothing to recommend the combination of rifampin and trimethoprim over the combination of rifampin plus cephalothin or sisomicin. Since the peak serum level of trimethoprim in humans after an oral dose of 160 mg is approximately 2 μg/ml (3), it is likely that the level in bone, with orally administered trimethoprim, will be below the MIC for many strains of staphylococci; this fact makes the combination of rifampin and trimethoprim theoretically less attractive for use as treatment for chronic staphylococcal osteomyelitis. Whether these experimental results and theoretical considerations can be extrapolated to the clinical situation in humans awaits controlled trials.

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LITERATURE CITED

1. **Arioli, V., and M. Berti.** 1979. Interaction between rifampicin and trimethoprim. J. Antimicrob. Chemother. **5**:113–114.
2. **Bushby, S,** 1969. Combined antibacterial action in vitro of trimethoprim and sulphonamides. Postgrad. Med. J. **45**(Suppl.):10–17.
3. **Bushby, S., and G. Hitchings.** 1968. Trimethoprim, a sulphonamide potentiator. Br. J. Pharmacol. Chemother. 33:72–90.
4. **Grove, D., and W. Randall.** 1955. Assay methods of antibiotics, a laboratory manual, vol. 26. Antibiotic monographs no. 2. Medical Encyclopedia, New York.
5. **Harvey, R.** 1978. Antagonistic interaction of rifampicin and trimethoprim. J. Antimicrob. Chemother. **4**:315–327.
6. **Norden, C.** 1970. Experimental osteomyelitis. I. A description of the model. J. Infect. Dis. **122**:410–418.
7. **Norden, C.** 1975. Experimental osteomyelitis. IV. Therapeutic trials with rifampin alone and in combination with gentamicin, sisomicin and cephalothin. J. Infect. Dis. **132**:493–499.
8. **Norden, C.** 1978. Experimental osteomyelitis. V. Therapeutic trials with oxacillin and sisomicin alone and in combination. J. Infect. Dis. **137**:155–160.
9. **Norden, C., and E. Keleti.** 1980. Experimental osteomyelins caused by *Pseudomonas aeruginosa*. J. Infect. Dis. **141**:71–75.
10. **Steward, D., and J. Eble.** 1979. Interaction between rifampicin and trimethoprim. J. Antimicrob. Chemother. **5**:114.
11. **Verbist, L., and A. Gyseler.** 1968. Antituberculous activity of rifampin in vitro and in vivo and the concentrations attained in human blood. Am. Rev. Respir. Dis. **98**:923–932.