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Treatment of Experimental Staphylococcal Infections: Effect of Rifampin Alone and in Combination on Development of Rifampin Resistance

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Rifampin is a potentially useful anti-staphylococcal agent, but resistance develops frequently when the drug is used alone. The efficacy of rifampin, trimethoprim, and a penicillin alone or in combination was examined in mice with acute or subacute infections. Mice were infected intraperitoneally with penicillin-susceptible *Staphylococcus aureus*. Survival after penicillin therapy was only 9.1% in contrast to survival after rifampin therapy which was 68% ($P < 0.001$). No rifampin-resistant *S. aureus* were isolated from peritoneal fluid or heart blood samples from dead animals in these short-term experiments. Rifampin was ineffective (survival, 4.8%) for infections instituted with rifampin-resistant strains. Long-term experiments were conducted after intravenous injection of 4×10^8 *S. aureus*. Forty percent of the animals survived after methicillin therapy; 77% survived after rifampin therapy ($P < 0.001$). However, 40% of those animals that died after rifampin therapy died with rifampin-resistant organisms. No animal dying in groups treated with a combination of rifampin and trimethoprim (85% survival) or rifampin and methicillin (79% survival) died with rifampin-resistant organisms. Thus, rifampin combined with a penicillin or trimethoprim was effective in preventing the development of rifampin-resistant strains.

Rifampin is highly active in vitro against *Staphylococcus aureus*, being bactericidal at concentrations from 0.0031 to 0.0125 μg/ml of culture medium (7). Rifampin has also exhibited a high order of activity against experimental staphylococcal infections in mice and was superior to penicillin in three model systems (3, 6). This may be related to its ability to kill intracellular staphylococci (4, 6). Resistance of staphylococci to rifampin occurs, however, with an incidence of approximately 1 out of every 10^8 colony-forming units and may compromise therapeutic efficacy (2) even though these mutants may be less virulent than their susceptible antecedents (5). The present study was designed to examine the problem of rifampin resistance in the therapy of experimental staphylococcal infections and the role of combination therapy in preventing development of this resistance.

MATERIALS AND METHODS

Animals. White 27- to 30-g male mice (strain ICR/DUB, Flow Research, Dublin; Virginia Institute for Cancer Research) were used.

Organisms. The rifampin-susceptible Wood 46 strain of *S. aureus* (W46, ATCC 10832) and its rifampin-resistant variant (W46/Rif) were used in this study. W46 is coagulase positive, beta-hemolytic, penicillin susceptible, and mouse virulent. The rifampin-resistant variant of W46 was obtained by incubating 10^9 organisms in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 10 μg of rifampin for 18 h. A 0.1-ml amount of this now cloudy broth culture was added to fresh broth containing 100 μg of rifampin per ml. Subculture of 0.1 ml onto the surface of sheep blood agar plates containing 10 μg of rifampin per ml resulted in a stable rifampin-resistant variant (W46/Rif). Equal numbers of W46/Rif grew on pour plates prepared with 10 μg of rifampin per ml or plates prepared without rifampin. Stock cultures were maintained on refrigerated 5% sheep blood agar plates and were passed weekly. Inocula for intraperitoneal infection were prepared by resuspending the washed pellet from 9.9 ml of an 18-h Trypticase soy broth culture in 1 ml of 0.9% saline. A 0.3-ml portion containing 0.6×10^9 to 2.1×10^9 bacteria was injected into each mouse. For intravenous infections, the washed pellet from 9.9 ml of an 18-h Trypticase soy broth culture was resuspended in 1.5 ml of 0.9% saline. A 0.1-ml amount containing 3×10^8 to 5×10^8 bacteria was injected into the tail vein of each mouse. Bacterial counts were quantitated for each experiment by serial dilution and pour plate techniques.

W46 and W46/Rif are both coagulase positive, beta-hemolytic, and penicillin susceptible. The W46/Rif strain is less virulent for mice and produced less catalase (5). Combinations of W46 and W46/Rif were prepared by combining washed aliquots of each organism.

In vitro measurements of susceptibility to antimicrobial agents. Assays of minimal inhibitory concentration for rifampin, penicillin, methicillin, and

trimethoprim were performed by a serial twofold dilution technique in heart infusion broth with an inoculum of 10^5 organisms. The minimum bactericidal concentration was defined as the lowest concentration of antimicrobic in the minimum inhibitory concentration assay from which no more than five colonies grew from an 0.01-ml sample streaked on a blood agar plate (8).

The bacteriostatic and bactericidal activities of combinations of rifampin with penicillin, methicillin, or trimethoprim and of penicillin or methicillin with trimethoprim were measured by the checkerboard technique (1). Synergism or antagonism was defined as a two-tube change compared to the activity of the drugs alone. Combinations were tested against 10^5 W46 or W46/Rif *S. aureus* in heart infusion broth. Bacteriostatic and bactericidal endpoints were determined 24 and 48 h after incubation (1). Ranges of concentration of drugs used were as follows: rifampin, 0.00049 to 128 μg/ml; methicillin, 0.0156 to 32 μg/ml; penicillin, 0.0125 to 12.8 μg/ml; and trimethoprim, 0.03125 to 64 μg/ml.

Assessments of therapeutic activities. (i) Acute infections. Intraperitoneal infections were induced in 680 mice by injection of 0.6×10^9 to 2.1×10^9 staphylococci in 0.3 ml of 0.9% saline. A total of 264 mice received W46, 252 received W46/Rif, and 164 received a 1:1 mixture of the strains. At 105 min after inoculation of bacteria, mice were randomly divided into groups and intraperitoneal injections of antimicrobial agents were given. All antimicrobial agents were administered once in 0.2-ml portions. Each mouse received either 0.4 mg of rifampin (13.3 mg/kg), 0.6 mg of trimethoprim (20 mg/kg), or 7.8 mg of penicillin (260 mg/kg) which corresponds to per kilogram doses recommended for seriously ill children. In some experiments animals received either rifampin plus trimethoprim, rifampin plus penicillin, or penicillin plus trimethoprim. Control animals received no antimicrobial agents.

Of the 264 mice inoculated with W46, 56 were untreated, 44 received rifampin, 30 received trimethoprim, 44 received penicillin, 30 received rifampin and trimethoprim, 30 received penicillin and trimethoprim, and 30 received rifampin and penicillin.

Of the 252 mice inoculated with W46/Rif, 48 were untreated, 42 received rifampin, 30 received trimethoprim, 42 received penicillin, 30 received rifampin and trimethoprim, 30 received penicillin and trimethoprim, and 30 received rifampin and penicillin.

Of the 164 mice inoculated with a mixture of W46 and W46/Rif, 33 were untreated, 25 received rifampin, 20 received trimethoprim, 25 received penicillin, 20 received rifampin plus trimethoprim, 20 received penicillin plus trimethoprim, and 21 received rifampin plus penicillin.

Groups of mice were injected at 18 different times with representatives of treatment and control groups included. Mice were housed in groups of five in 29- by 17-cm plastic cages. Ill mice were not attacked by their cage mates under these conditions. Survival was determined at 24 h since animals surviving for 24 h rarely died after that point. Heart blood and peritoneal fluid samples were obtained as the animals died. Peritoneal fluid (0.1 ml) and heart blood (0.05 ml) were serially diluted, and pour plates were prepared with Trypticase soy agar with and without 1 μg of rifampin per ml to quantitate numbers of rifampin-susceptible and rifampin-resistant organisms.

(ii) Subacute infections. Subacute intravenous infections were induced by tail vein injection with 3×10^8 to 5×10^8 staphylococci in 0.1 ml of 0.9% saline. Three days after inoculation, mice were randomly divided into groups, and intraperitoneal injections of antimicrobial agents were given. Each mouse received either 0.4 mg of rifampin, 0.6 mg of trimethoprim, or 7.8 mg of methicillin. In some experiments animals received either rifampin plus trimethoprim, rifampin plus methicillin, or methicillin plus trimethoprim. Antimicrobial agents were given once daily for 10 days, and control groups remained untreated.

A total of 684 mice were injected at 10 different times. For 242 mice injected with W46, treatment was as follows: 30 were untreated; 44 received rifampin; 30 received trimethoprim; 30 received methicillin; 40 received rifampin plus trimethoprim; 30 received methicillin plus trimethoprim; and 38 received rifampin plus methicillin. For 230 mice injected with W46/Rif, treatment was as follows: 50 were untreated; 30 received rifampin; 30 received trimethoprim; 30 received methicillin; 30 received rifampin plus trimethoprim; 30 received methicillin plus trimethoprim; and 30 received rifampin plus methicillin. For 212 mice injected with a 1:1 mixture of the two strains, treatment was as follows: 32 were untreated; 30 received rifampin; 30 received trimethoprim; 30 received methicillin; 30 received rifampin plus trimethoprim; 30 received methicillin plus trimethoprim; and 30 received rifampin plus methicillin. Mice were observed for 17 days after completion of treatment. Heart blood was cultured from animals that were observed to die during the day, and numbers of rifampin-resistant and rifampin-susceptible organisms were quantitated.

Rifampin (CIBA Chemical Co., Summit, N.J.) for injection was prepared by dissolving the drug in methanol and diluting it with 0.9% saline for a final concentration of 1% methanol in 0.2 ml of saline. Trimethoprim (Hoffman-LaRoche, Inc., Nutley, N.J.) was dissolved in 0.1 N lactic acid and diluted with 0.9% saline to a final concentration of 1% 0.1 N lactic acid in 0.2 ml of saline. This amount of methanol or lactic acid was nontoxic to the mice. Penicillin (Sigma Chemical Co., St. Louis, Mo.) and methicillin (Sigma) were prepared by dissolving powder in sterile 0.9% saline.

RESULTS

In vitro measurements of susceptibility to antimicrobial agents. The susceptibilities of the organisms are shown in Table 1. Both strains were highly susceptible to penicillin and methicillin. Trimethoprim was bacteriostatic, W46 was susceptible to rifampin, and W46/Rif was highly resistant. No antagonism or synergism was seen with any of the combinations; all the combinations tested showed an indifferent reaction.

Assessments of therapeutic activities. (i) Acute infection. Results of therapeutic activity

TABLE 1. *Susceptibility of S. aureus W46 and W46/Rif to antimicrobial agents*[a]

S. aureus strain	Antimicrobial agent	Minimum inhibitory concn (μg/ml)	Minimum bactericidal concn (μg/ml)
Rifampin-susceptible W46	Rifampin	0.00391	0.0156
	Trimethoprim	0.5	>32
	Methicillin	4.0	4.0
	Penicillin	0.16	2.5
Rifampin-resistant W46/Rif	Rifampin	>128	>128
	Trimethoprim	0.5	>32
	Methicillin	4.0	4.0
	Penicillin	0.16	2.5

[a] Susceptibility to antimicrobial agents was determined in heart infusion broth with an inoculum of 10^5 organisms.

TABLE 2. *Assessment of therapeutic activities: acute infection*[a]

Intraperitoneal inoculum *S. aureus*	Drug dose (mg/mouse)			Survivors		Rifampin-resistant isolates/total	
	Rif	Pen	TMP	No./total	%	Heart blood	Peritoneal fluid
2.1×10^9 W46				4/56	7.1	0/5	0/5
	0.4			30/44	68	0/5	0/5
		7.8		4/44	9.09	0/5	0/5
			0.6	1/30	3.3	0/5	0/5
	0.4	7.8		30/30	100	0/5	0/5
	0.4		0.6	24/30	80	0/5	0/5
		7.8	0.6	2/20	6.7	0/5	0/5
1.2×10^9 W46/RIF *S. aureus*				11/48	23	5/5	5/5
	0.4			2/42	4.8	5/5	5/5
		7.8		13/42	31	5/5	5/5
			0.6	2/30	6.7	6/6	6/6
	0.4	7.8		10/30	33	6/6	6/6
	0.4		0.6	1/30	3.3	6/6	6/6
		7.8	0.6	7/30	23	6/6	6/6
3.0×10^8 W46 plus 3.0×10^8 6/ W46/Rif				7/33	21	4/4	4/4
	0.4			4/25	16	3/3	3/3
		7.8		16/25	64	2/2	2/2
			0.6	5/20	25	5/5	5/5
	0.4	7.8		12/21	57	4/4	4/4
	0.4		0.6	3/20	15	5/5	5/5
		7.8	0.6	11/20	55	5/5	5/5

[a] Mice were infected intraperitoneally with $\simeq 10^9$ rifampin-susceptible organisms. At 105 min after infection, mice were treated with either rifampin (Rif), trimethoprim (TMP), penicillin (Pen), or rifampin plus trimethoprim, rifampin plus penicillin, or penicillin plus trimethoprim. Survival was recorded 24 h after infection.

against acute infections are shown in Table 2. Survival was markedly enhanced by treatment with rifampin. With the W46 rifampin-susceptible strain, all the regimens containing rifampin were significantly better than the control ($P < 0.001$, chi square with Yates continuity correction). Neither trimethoprim nor penicillin was significantly better than no therapy. Rifampin plus penicillin was significantly better than rifampin alone ($P = 0.00175$).

Studies performed with W46/Rif, the rifampin-resistant variant, showed no significant increase in survival with any therapy. The rifampin-resistant strain was less virulent, as can be seen by 23% survival of untreated animals at 24 h versus 7.1% for the rifampin-susceptible *S. aureus* ($P = 0.045$). For infection induced with a mixture of rifampin-susceptible and rifampin-resistant *S. aureus*, penicillin alone, penicillin plus trimethoprim, and rifampin plus penicillin were all better than no therapy ($P = 0.0025$, 0.027, 0.016, respectively).

Peritoneal fluid and heart blood obtained from dead animals infected with rifampin-susceptible staphylococci grew *S. aureus*, none of which was resistant to rifampin. All 67 animals cultured that died after infection with rifampin-resistant staphylococci alone or in combination grew rifampin-resistant staphylococci from peritoneal fluid and heart blood.

(ii) Subacute infection. Results of therapeutic activity against subacute infections are shown

TABLE 3. *Assessment of therapeutic activities: subacute infection*[a]

Intravenous inoculum *S. aureus*	Drug dose (mg/mouse)			No. inoculated	No. of survivors (%)		Rifampin-resistant isolates/total in heart blood
	Rif	Meth	TMP		End of treatment	30 days after inoculation	
5.0×10^8 W46				30	2 (6.7)	2 (6.7)	0/5
	0.4			44	35 (79)	34 (77)	4/10
		7.8		30	15 (50)	12 (40)	0/5
			0.6	30	7 (23)	5 (17)	1/5
	0.4	7.8		38	32 (84)	30 (79)	0/7
	0.4		0.6	40	35 (87)	34 (85)	0/5
		7.8	0.6	30	14 (47)	12 (40)	0/5
3.3×10^8 W46/Rif				50	18 (36)	16 (32)	5/5
	0.4			30	15 (50)	14 (47)	5/5
		7.8		30	27 (90)	26 (87)	3/3
			0.6	30	17 (57)	14 (47)	3/3
	0.4	7.8		30	25 (83)	25 (83)	3/3
	0.4		0.6	30	15 (50)	11 (37)	4/4
		7.8	0.6	30	24 (80)	23 (77)	3/3
2.5×10^8 W46 plus 2.5×10^8 W46/Rif				32	27 (84)	21 (66)	5/5
	0.4			30	23 (77)	20 (67)	2/2
		7.8		30	25 (83)	23 (77)	2/2
			0.6	30	20 (67)	17 (57)	4/4
	0.4	7.8		30	28 (93)	28 (93)	2/2
	0.4		0.6	30	21 (70)	18 (60)	2/2
		7.8	0.6	30	26 (87)	23 (77)	2/2

[a] Mice were injected intravenously with washed staphylococci. Three days after infection, groups of mice were treated with either methicillin (Meth), trimethoprim (TMP), rifampin (Rif), or rifampin plus methicillin, rifampin plus trimethoprim, or methicillin plus trimethoprim daily for 10 days. Thirty mice were left untreated.

in Table 3. Regimens containing rifampin were effective for infections caused by W46 ($P < 0.001$). Therapy with methicillin was more effective than no therapy ($P = 0.006$), but rifampin therapy was more effective than methicillin therapy ($P = 0.0026$). Results were different when infections were induced with W46/Rif. Rifampin therapy was ineffective, and methicillin therapy and all regimens containing methicillin were most effective (methicillin versus rifampin, $P = 0.0027$). W46/Rif was less virulent, as can be seen by 32% survival of untreated animals at 30 days versus 6.7% for W46 ($P = 0.0187$). Regimens containing methicillin were also most effective for the therapy of intravenous infections induced with a mixture of W46 and W46/Rif.

Forty-two animals were cultured after intravenous infection with W46. No rifampin-resistant organisms were found in the untreated group or in the groups treated with either methicillin, rifampin plus trimethoprim, methicillin plus trimethoprim, or rifampin plus methicillin. However, 4 of 10 animals in the group treated with rifampin had rifampin-resistant staphylococci in their blood. Of those with positive cultures for rifampin-resistant organisms, more than 90% of bacteria present were resistant. In addition, one of five animals treated with trimethoprim demonstrated 80% of the isolates resistant to rifampin. Twenty-six animals were cultured after dying from intravenous infection with rifampin-resistant organisms, and all heart blood isolates were rifampin resistant.

Nineteen animals were cultured after dying from infections induced with a 1:1 mixture of rifampin-resistant and rifampin-susceptible staphylococci. Rifampin-resistant organisms were isolated from all of these animals.

DISCUSSION

Therapy with rifampin alone for rifampin-susceptible staphylococcal infections induced by the peritoneal route was highly effective. No rifampin-resistant variants were isolated from any of the mice treated in these acute experiments, despite the fact that the inoculum of over 10^9 organisms would be expected to contain these variants. In contrast, when mice were infected intravenously with rifampin-susceptible *S. aureus*, rifampin-resistant organisms were isolated from heart blood of 4 out of 10 animals succumbing to this subacute infection. There are several possible explanations for the emergence of rifampin-resistant strains in animals infected subacutely but not in those infected acutely. The

combination of both time and antibiotic pressure may be necessary for the resistant organisms to achieve significant population size, especially since the resistant organisms are less virulent in both mouse models.

Results of treatment with more than one antimicrobial agent were examined to see whether development of resistance was diminished. Combination therapy for infections caused by a single organism can be advantageous for three reasons.

(i) The combination may be truly synergistic (i.e., penicillin plus streptomycin for enterococcal endocarditis).

(ii) The combination may be additive and may enable more effective antimicrobics to be administered with diminished toxicity (i.e., triple sulfonamides).

(iii) The combination may diminish or abolish the development of resistance (i.e., isoniazid plus ethambutol for tuberculosis).

We failed to show any synergism in vitro, but use of combinations was effective in preventing development of resistant strains. In our studies we found that combination therapy was superior to therapy with rifampin alone for intraperitoneal infections and for intravenous infections induced with rifampin-resistant organisms or mixtures of rifampin-resistant and rifampin-susceptible organisms. It is possible that synergism occurred in vivo as an explanation for the good response to combinations.

The superiority of rifampin for therapy of staphylococcal infections in mice may be due to the ability of rifampin to kill intracellular organisms or to another property of the drug. Rifampin is highly active in vitro against nearly all wild strains of staphylococci. In previous studies we showed that despite the attainment of serum antimicrobial concentrations many times higher than the minimum bactericidal concentration for the penicillins, therapy with rifampin was clearly superior (3).

Since development of rifampin resistance could be avoided by administering another antistaphylococcal agent in combination with rifampin, rifampin plus another agent should be evaluated as therapy for staphylococcal infection in humans.

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