Treatment of Experimental Endocarditis Due to Methicillin-Susceptible or Methicillin-Resistant *Staphylococcus aureus* with Trimethoprim-Sulfamethoxazole and Antibiotics That Inhibit Cell Wall Synthesis

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Using two strains of *Staphylococcus aureus*, one susceptible and one heterogeneously resistant to methicillin, for which MICs and MBCs of trimethoprim-sulfamethoxazole (TMP-SMX) were 0.06 and 0.06 µg/ml and 0.06 and 0.25 µg/ml, respectively (concentrations are those of TMP), we studied the efficacies of TMP-SMX and cloxacillin, teicoplanin, and vancomycin for treatment of experimental staphylococcal endocarditis. Rabbits were treated with dosages of TMP-SMX selected to achieve concentrations in serum equivalent to that obtained in humans treated for *Pneumocystis carinii* pneumonia. The overall mortality rate of rabbits treated with TMP-SMX was 84% at day 3, not different from that of the control groups ($P > 0.1$). No sterile vegetations were observed to be present in control groups or in animals treated with TMP-SMX. However, 26, 60, and 75% of rabbits treated with teicoplanin, cloxacillin, and vancomycin, respectively, showed sterile vegetations. For methicillin-susceptible *S. aureus* (MSSA), the mean vegetation counts were not significantly different between the control group and the group treated with TMP-SMX ($P > 0.1$). For methicillin-resistant *S. aureus* (MRSA), treatment with TMP-SMX was more effective than no therapy, decreasing the number of organisms in vegetations ($P < 0.01$). For both strains, therapy with cloxacillin and therapy with teicoplanin or vancomycin were significantly more effective than therapy with TMP-SMX. Despite high concentrations of teicoplanin in serum which exceeded MBCs for staphylococci more than 50 times at the peak and 10 times at the trough, therapy with cloxacillin or vancomycin was superior to therapy with teicoplanin against both MSSA and MRSA. These data do not support the use of TMP-SMX in treatment of endocarditis and other severe staphylococcal infections with high bacterial counts.

Staphylococcus aureus remains an important cause of both uncomplicated and complicated bacteremic infections and is the leading cause of infectious endocarditis in many medical centers around the world (23, 30). Antimicrobial therapy of staphylococcal infections has become more troublesome because of the recent emergence and spread of methicillin-resistant *S. aureus* (MRSA) strains. Previous reports have documented an increased prevalence of MRSA in hospitals in the United States and Europe (3, 18), and there is at present a scarcity of effective antimicrobial regimens for these strains (12).

Beta-lactam antibiotics alone or in combination with aminoglycosides are regarded as first-choice therapy for treatment of methicillin-susceptible *S. aureus* (MSSA) endocarditis, and vancomycin therapy is recommended for patients with penicillin allergies and for those with serious infections caused by MRSA, including endocarditis (19). However, several recent reports have shown suboptimal clinical outcome in patients with staphylococcal endocarditis treated with vancomycin (13, 16). For these reasons, a continuous search for alternative antimicrobial drugs is of paramount importance.

In vitro, most strains of MRSA are sensitive to trimethoprim-sulfamethoxazole (TMP-SMX), with MICs ranging from 0.05-1.0 to 0.4-8.0 µg/ml (15), and as determined by time-kill

studies, the combination shows a rapid bactericidal effect at concentrations four times the MIC (32). In addition, synergistic bactericidal activity has been shown in a model of disseminated infection in mice (9). However, to the best of our knowledge, results of treatment of endocarditis caused by MRSA in an experimental model have not been published. Because this model can be considered a rigorous test of antimicrobial efficacy, it may provide valuable information on the potential role of TMP-SMX in the treatment of endocarditis and other severe infections caused by MRSA.

MATERIALS AND METHODS

In vitro studies. Two strains of *S. aureus* isolated from patients with endocarditis were used; one strain was MSSA, and the other was a heterogeneous MRSA strain. The microorganisms were stored frozen in defibrinated sheep blood at −70°C. Before being tested, each strain was thawed and subcultured in tryptic soy agar containing 5% sheep blood. A microdilution method was used for susceptibility testing (22). Inocula were prepared from broth cultures in the log phase of growth to yield an inoculum size of 5.5×10^5 CFU of staphylococci per ml and were inoculated into serial twofold dilutions of antimicrobial agent in Mueller-Hinton broth that was supplemented with 5% hemolyzed horse blood for TMP-SMX testing. The supplement of 5% lysed horse blood contains sufficient thymidine phosphorylase to inactivate thymidine in the media. For cloxacillin testing, cation-supplemented Mueller-Hinton broth with 2% NaCl was used. Subcultures were made for confirmation of purity and quantitation of the inoculum size. Wells containing the inoculum in serial dilutions of antimicrobial agents were incubated for 24 h at 35°C in room air. The MIC was defined as the lowest concentration of antimicrobial agent in broth which did not permit visible growth of staphylococci. The MBC was determined by subculture of 100 µl of broth from a control well, the first well containing growth, and from all wells without visible growth and was defined as the lowest concentration of antibiotic that killed ≥99.9% of the original inoculum (27).

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Time-kill studies were performed at 37°C in 10 ml of Mueller-Hinton broth with inocula of 10^7 to 10^8 CFU of staphylococci per ml (27). For TMP-SMX testing, Mueller-Hinton broth supplemented with 5% lysed horse blood was used. Samples (100 μl) were taken at 0, 4, and 24 h and quantitatively subcultured onto blood agar. Tests were performed in triplicate, and the results are expressed as mean values.

Animal studies. Experimental aortic valve endocarditis was established in New Zealand White rabbits (weight, 2 to 3 kg) by modifications of the method described by Perlman and Freedman (21). In brief, animals were anesthetized with a mixture of ketamine and xylazine injected intramuscularly (i.m.). An incision was made in the neck, and the right carotid artery was exposed. The artery was ligated distally, and a sterile polyethylene catheter was inserted into the artery through a small incision and advanced proximally across the aortic valve into the left ventricle. A pressure-sensitive monitoring device was attached to the distal end of the catheter to ensure that the catheter tip crossed the aortic valve and entered the left ventricle. The end of the catheter was sealed and tied to the carotid artery, and the wound was closed over the catheter with surgical clips. The catheter was left in place throughout the experiment.

Twenty-four hours after the insertion of the catheter, 1 ml of broth containing 10^7 to 10^8 CFU of *S. aureus* per ml was injected into a peripheral ear vein. The presence of endocarditis was confirmed by a blood culture yielding staphylococci obtained before initiation of antimicrobial therapy.

Antimicrobial therapy was started 24 h after intravenous injection of *S. aureus*. Animals were placed into treatment groups as follows. For MSSA, the control group included 10 animals that received no antimicrobial therapy, the cloxacillin group consisted of 12 animals that were treated i.m. with 200 mg of cloxacillin per kg of body weight three times a day, the vancomycin group consisted of 12 animals treated intravenously with 25 mg of vancomycin per kg of body weight twice a day (b.i.d.), the teicoplanin group consisted of 12 rabbits that received i.m. therapy with 30 mg of teicoplanin per kg of body weight b.i.d., and the TMP-SMX group consisted of 12 animals that received i.m. therapy with a fixed drug combination (1:5) which consisted of 50 mg of TMP plus 250 mg of SMX per kg of body weight, divided in three daily doses.

For MRSA, the control group included 10 animals that received no therapy, the vancomycin group consisted of 14 animals that received 25 mg of vancomycin per kg of body weight intravenously b.i.d., the teicoplanin group consisted of 15 animals that were treated with 30 mg of teicoplanin per kg of body weight i.m. b.i.d., and the TMP-SMX group consisted of 11 animals that received i.m. therapy with 50 mg of TMP plus 250 mg of SMX per kg of body weight, divided in three daily doses.

Antimicrobial therapy was given for 3 days. At the end of the treatment period and at least 12 h after administration of the last dose of antibiotics, animals were sacrificed by intravenous injection of sodium pentobarbital. The chest was opened, the heart was excised and opened, and the aortic valve vegetations were removed aseptically. In order to determine whether and to what degree endocardial infection was present, the entire valve and vegetations were weighed and homogenized in 0.9 ml of Mueller-Hinton broth by using a stomacher device. The number of CFU of *S. aureus* per gram of vegetation was quantitated by a pour plate method with tryptic soy agar. The results were expressed as $\log_{10}$ CFU of staphylococci per gram of valve vegetation. Because all the undiluted vegetation homogenate was cultured, as little as 5 to 10 CFU of vegetation per g could be detected. Sterile vegetations were assigned a value of 0 $\log_{10}$ unit, corresponding to 1 CFU/g.

Only animals with positive blood cultures and catheters positioned across the aortic valve at autopsy that received at least a full day of antimicrobial therapy (three doses of cloxacillin or TMP-SMX or two doses of teicoplanin or vancomycin) were included in the final analysis. Randomly selected colonies recovered from vegetations of rabbits treated with TMP-SMX were immediately taken for MIC determinations in antibiotic-free plates.

Measurement of concentrations of antimicrobial agents in serum. Pharmacokinetic studies were performed with uninfected control animals to estimate dosages of TMP required to obtain peak levels of about 3 to 5 μg/ml, equivalent to those achieved during oral or parenteral therapy in humans (11, 31). Blood samples were taken from rabbits with endocarditis on the second day of therapy 60 min after administration of antibiotics and just before the next dose to measure the peak and trough levels of antimicrobial agents. Concentrations of vancomycin in serum were measured by using TDX (Abbott Laboratories), and concentrations of cloxacillin and teicoplanin in serum were measured by a bioassay with *Bacillus subtilis* ATCC 6633 (1). Levels of TMP in serum were tested by a bioassay using *Bacillus pumilus* as the test strain (4).

Analysis of results. Differences in mean $\log_{10}$ CFU of staphylococci per gram of vegetation were analyzed statistically by using the Kruskall-Wallis nonparametric test. In addition, the Mann-Whitney test was used when two groups were compared. The statistical analysis was performed with SPSS software. For comparison of two means, a P value of <0.05 was considered significant.

RESULTS

In vitro microbiologic studies. The MICs and MBCs of the antibiotics for both strains used in the experimental design are shown in Table 1, and rates of decrease in the number of *S.*

TABLE 1. Antibiotic susceptibilities of two strains of *S. aureus* used in the experimental model of aortic endocarditis

Antibiotic	MIC/MBC (μg/ml) for:	
	MSSA	MRSA
Cloxacillin	0.25/0.5	>16/>16
TMP-SMX (1:20)[a]	0.06/0.06	0.06/0.25
Teicoplanin	0.25/1	0.25/1
Vancomycin	1/1	1/2

[a] Concentrations listed are the concentrations of TMP.

aureus organisms per milliliter of Mueller-Hinton broth in the time-kill studies are shown in Tables 2 and 3. With a high inoculum of MSSA, each antibiotic produced only a modest decrease in the number of microorganisms at 4 h (reductions from 0.9 to 1.2 CFU/ml in the inoculum). After 24 h of incubation, the mean counts of staphylococci did not differ between different antibiotics. For MRSA, after 4 h of incubation the reduction in the inoculum was also small (reductions from 0.4 to 1 CFU/ml in the inoculum). After 24 h of incubation, the reduction in the mean count was greater for TMP-SMX and vancomycin than for teicoplanin.

Therapeutic studies. In the control groups of rabbits, which were not treated with antimicrobial agents, the rate of mortality due to aortic staphylococcal endocarditis was 90% on the third day. In the experiments conducted with MSSA, this high mortality rate was reduced to 0, 8, and 0% by treatment with cloxacillin, teicoplanin, and vancomycin, respectively. In the experiments conducted with MRSA, spontaneous mortality was reduced to 13 and 7% by therapy with teicoplanin and vancomycin, respectively. Remarkably, treatment with TMP-SMX showed a much lower capacity to keep the rabbits alive during the complete period of treatment. Thirty-one animals were used in the therapeutic studies with TMP-SMX. Eight (29%) did not survive the first day of treatment and were not included in the final analysis. Of 23 rabbits that at least completed a full day of antimicrobial therapy, only 5 (21.7%) survived the entire period of scheduled treatment (nine doses). Of those, two were infected with MSSA and three were infected with MRSA. The rest of the animals received three doses (five rabbits), four doses (three rabbits), six doses (six rabbits), or eight doses (four rabbits). The overall mortality rate for animals treated with TMP-SMX was 84% at day 3, not significantly different from that observed for the control groups ($P >$ 0.1).

Table 4 and Table 5 show the results of treatment of experimental endocarditis caused by MSSA and MRSA and the mean peak and trough levels of antimicrobial agents in blood. No sterile vegetations occurred in the control groups or the groups of animals treated with TMP-SMX. However, 26, 60, and 75% of animals treated with teicoplanin, cloxacillin, and

TABLE 2. Time-kill studies of MSSA used in the experimental model of aortic endocarditis

Antibiotic (μg/ml)	$\log_{10}$ CFU/ml at time (h):		
	0	4	24
None [control]	8.00	9.10	9.93
Cloxacillin (20)	8.00	6.80	5.10
TMP-SMX (5-25)	8.00	7.10	5.65
Teicoplanin (15)	8.00	6.90	5.46
Vancomycin (15)	8.00	7.10	5.20

TABLE 3. Time-kill studies of MRSA used in the experimental model of aortic endocarditis

Antibiotic (µg/ml)	Log$_{10}$ CFU/ml at time (h):		
	0	4	24
None [control]	7.50	8.42	9.50
TMP-SMX (5-25)	7.50	7.15	4.78
Teicoplanin (15)	7.50	7.10	5.60
Vancomycin (15)	7.50	6.56	3.94

vancomycin, respectively, showed sterile vegetations. For MSSA, the mean vegetation counts were not significantly different between the control group and the group of rabbits treated with TMP-SMX ($P > 0.1$). However, for the MRSA strain, treatment with TMP-SMX was significantly more effective than no therapy in reducing the number of organisms in the vegetations ($P < 0.01$). Resistance to TMP-SMX among *S. aureus* strains isolated from vegetations after therapy was not found.

For MSSA, both cloxacillin and vancomycin were superior to teicoplanin in reducing the number of microorganisms in cardiac vegetations. No significant difference was observed between cloxacillin and vancomycin treatments ($P > 0.05$). For MRSA, after 3 days of therapy the mean vegetation count for rabbits treated with vancomycin was significantly lower than that observed for animals treated with teicoplanin ($P < 0.01$).

DISCUSSION

Most clinical isolates of *S. aureus*, including methicillin-resistant strains, are sensitive to TMP-SMX (15), and supposedly the combination acts synergistically to produce a bactericidal effect on staphylococci both in vitro and in vivo (9, 25). For these reasons, TMP-SMX is being considered as an effective alternative to vancomycin in the treatment of infections caused by MRSA, including cases of endocarditis (12, 28). However, only a few anecdotal reports have suggested that TMP-SMX is valuable in the treatment of staphylococcal infections (2, 29). A double-blind comparative trial found vancomycin superior to TMP-SMX in efficacy and safety for treatment of intravenous drug users with staphylococcal infections (17). The cure rate obtained with TMP-SMX was 86%, and all treatment failures observed occurred in patients with infections caused by MSSA. Therefore, the authors of that report recommended TMP-SMX as an alternative to vancomycin for selected cases of infections caused by MRSA (17).

Our experiments were deliberately done with a high infecting inoculum to assess the efficacies of TMP-SMX and other antimicrobial agents in reducing mortality and bacterial populations during acute adverse conditions. Under such conditions, therapy with TMP-SMX was unable to lower the rate of mortality due to the infection and only a minority of the treated animals survived the 3 days of programmed therapy. Although for animals infected with MSSA no difference in the bacterial counts in vegetations was observed between the untreated group and the group treated with TMP-SMX, the combination drug produced a significant decrease in the number of MRSA organisms in aortic vegetations in comparison with the number in the control group. Both cloxacillin treatment for MSSA infection and treatment with vancomycin and teicoplanin for MRSA infection were superior to therapy with TMP-SMX.

To the best of our knowledge, only one experimental study has addressed the comparative efficacy of TMP-SMX versus other antimicrobial agents against *S. aureus* in vivo (24). Using the experimental model of meningitis, Scheld et al. showed that TMP-SMX killed *S. aureus* at a slower rate and more incompletely than nafcillin did (24).

There are a number of possible reasons for the lack of activity of TMP-SMX in this experimental model of staphylococcal endocarditis and for the apparent discrepancy between results of MIC studies and in vivo results. The MIC for microorganisms isolated from vegetations of animals which were TMP-SMX treatment failures was identical to that determined for the original strain; therefore, the development of resistance during therapy is improbable. Insufficient levels of the antimicrobial agents in serum and a lack of penetration into the vegetations are also unlikely possibilities. Although there are no data on the diffusion of TMP into cardiac vegetations, the drug is widely distributed in most human tissues, including the prostate gland, and cerebrospinal fluid (20). Optimal dosages of TMP-SMX for the treatment of staphylococcal infections have not been formulated. In this study, dosages of TMP-SMX were chosen in order to obtain serum drug levels in rabbits equivalent to those obtained in adults treated with intravenous TMP-SMX for serious infections (31). These dosages achieved peak levels of TMP in serum exceeding 20 to 80 times the MBCs for the infecting strains and at least doubled the MBCs at trough levels. Therefore, although the antibiotic concentrations needed to exhibit an in vivo antibacterial effect in vegetations could be much higher than the concentrations active in vitro (14), the possibility that higher levels of TMP in serum could have caused a greater antimicrobial effect seems remote.

The possibility of an in vivo inoculum effect could be the most plausible explanation. Previous studies showed that the MBC of TMP-SMX for staphylococci did increase when the inoculum was raised from 10^5 to 10^7 CFU per ml (24). In our studies, the kinetics of killing of staphylococci assessed by the

TABLE 4. Results of treatment of experimental endocarditis due to MSSA

Antimicrobial agent	No. of animals/ no. of sterile vegetations	Peak/trough (µg/ml) (mean ± SD)	Log$_{10}$ CFU/g of vegetation (mean ± SD)
None (control)	10/0		9.74 ± 0.81
TMP-SMX	12/0	4.75 ± 1.4/0.5 ± 0.3	9.33 ± 0.41[a]
Teicoplanin	12/3	59 ± 10/12.6 ± 4.7	5.49 ± 1.78[b]
Cloxacillin	10/6	65 ± 15/2.6 ± 1.3	2.28 ± 1.71[c]
Vancomycin	10/8	44 ± 7/2.8 ± 1.2	1.56 ± 1.19[d,e]

[a] $P = 0.1$ for TMP-SMX versus the control.
[b] $P < 0.01$ for teicoplanin versus the control or TMP-SMX.
[c] $P < 0.01$ for cloxacillin versus the control or teicoplanin.
[d] $P < 0.01$ for vancomycin versus the control or teicoplanin.
[e] $P = 0.3$ for vancomycin versus cloxacillin.

TABLE 5. Results of treatment of experimental endocarditis due to MRSA

Antimicrobial agent	No. of animals/ no. of sterile vegetations	Peak/trough (µg/ml) (mean ± SD)	Log$_{10}$ CFU/g of vegetation (mean ± SD)
None (control)	10/0		10.1 ± 0.81
TMP-SMX	11/0	4.86 ± 1.3/0.8 ± 0.4	9.27 ± 0.38[a]
Teicoplanin	15/4	53 ± 12/12.8 ± 8.5	5.04 ± 1.83[b]
Vancomycin	14/10	48 ± 14/3.5 ± 1.6	3.14 ± 1.65[c]

[a] $P < 0.01$ for TMP-SMX versus the control.
[b] $P < 0.01$ for teicoplanin versus the control or TMP-SMX.
[c] $P < 0.01$ for vancomycin versus the control, TMP-SMX, or teicoplanin.

time-kill method with high inocula showed a slow and modest decrease in the number of microorganisms, which predicted a suboptimal in vivo effect. These observations may be most relevant because the conditions for the interaction between the antibiotics and staphylococci in cardiac vegetations surely were even worse, with a greater number of microorganisms in fibrin clots.

The clinical correlates of these experimental observations may be evident only in infections such as endocarditis, characterized by the presence of large numbers of microorganisms in tissues. A few patients with endocarditis due to *S. aureus* have been treated with TMP-SMX alone or in combinations with other antimicrobial agents (2, 17, 25, 26, 29). Markowitz et al. treated 11 patients with tricuspid endocarditis caused by MSSA or MRSA with TMP-SMX, and the infection was apparently eradicated in 7 (17), a cure rate that, although much lower than that achieved by therapy with antibiotics that inhibit cell wall synthesis, still was considerable. However, tricuspid endocarditis may be easier to cure than left-sided disease because fewer microorganisms are present in cardiac vegetations (bacterial densities are approximately 300 times lower) and the infection shows a spontaneous evolution towards sterilization and cure (5, 10). Therefore, it was not entirely unexpected that treatment with TMP-SMX did show some activity in this situation. We believe that although the results of these experiments cannot be directly extrapolated to therapy of human infections, they do not encourage the use of TMP-SMX in the treatment of endocarditis and other serious staphylococcal infections characterized by the presence of large numbers of microorganisms in the tissues.

Previous studies done with rabbits with experimental endocarditis have shown that the efficacy of teicoplanin compared favorably both with that of nafcillin for infection by an MSSA strain and with that of vancomycin for infection by an MRSA strain (7). In the present experiments, despite the high peak and trough levels of teicoplanin in serum attained during therapy, which were equivalent to those achieved in humans treated with high doses of teicoplanin, cloxacillin and vancomycin were more effective than teicoplanin in decreasing the number of MSSA or MRSA organisms in cardiac vegetations after 3 days of treatment. The reasons for this suboptimal activity of teicoplanin remain unknown. Cremieux et al. have shown that teicoplanin concentrates at the periphery of the vegetations and diffuses poorly into the center (8). The high-level protein binding of teicoplanin may be a factor in the reportedly poor penetration of the drug into vegetations. It has been suggested that high trough concentrations of teicoplanin in serum (10 times the MIC or higher) may be necessary to overcome the barrier to penetration in order to determine an optimal bactericidal activity (6). Our observations suggest that, besides the serum drug concentrations, other factors, such as the relatively low bactericidal rate of teicoplanin at high bacterial densities, may be relevant in determining the efficacy of this antibiotic in vivo.

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