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Fusidic Acid Alone or in Combination with Vancomycin for Therapy of Experimental Endocarditis Due to Methicillin-Resistant *Staphylococcus aureus*

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The usefulness of fusidic acid, alone or combined with vancomycin, was investigated for the therapy of experimental endocarditis caused in rabbits by a methicillin-resistant strain of *Staphylococcus aureus*. In vitro killing curves showed an indifferent interaction between the two antibiotics. In vivo, vancomycin alone was as effective as a vancomycin-fusidic acid combination ($P < 0.05$ versus control animals). No resistance to fusidic acid emerged during combination therapy. Fusidic acid alone was not effective. Resistance emerged in 5 of 12 animals treated with fusidic acid alone and was responsible for antibacterial failure. Fusidic acid alone was effective ($P < 0.001$) and did not select resistant strains if therapy was started when animals retained a smaller inoculum. We concluded that the vancomycin-fusidic acid combination exhibited no advantage over vancomycin alone in this model.

Methicillin-resistant *Staphylococcus aureus* is a major cause of nosocomial infection, and vancomycin remains the standard antimicrobial agent for the therapy of systemic infections due to methicillin-resistant *S. aureus*. However, given the slow rate of killing of vancomycin in vitro against staphylococci (1) and the moderate extravascular diffusion of this antibiotic, attempts have been made to combine vancomycin with other antibiotics with better pharmacodynamic and/or pharmacokinetic properties (3, 6). Unfortunately, most methicillin-resistant strains of *S. aureus* are now resistant to aminoglycosides, fluoroquinolones, and rifampin in many countries (12, 13). Fusidic acid is an antimicrobial agent which remains active in vitro against methicillin-resistant strains of *S. aureus*, despite several decades of clinical use in some countries (5, 16), and has been used successfully to treat severe staphylococcal infections (14). Thus, fusidic acid appears to be an attractive agent for combination with vancomycin for the therapy of methicillin-resistant staphylococcal infections (17). However, the in vitro and in vivo rationale for extensive use of this combination for therapy of infections due to methicillin-resistant strains of *S. aureus* is lacking. We therefore investigated the potential usefulness of fusidic acid, alone or combined with vancomycin, for the treatment of aortic endocarditis caused in rabbits by a methicillin-resistant strain of *S. aureus*.

S. aureus HM1054 was isolated from the blood of a patient with septicemia. This strain was resistant to methicillin. MICs and MBCs of vancomycin and fusidic acid were determined by the macrodilution method, with an inoculum of 5×10^5 CFU/ml (15). A disk susceptibility test was used to ensure that the surviving organisms remained susceptible to fusidic acid. The influence of serum on fusidic acid susceptibility was studied by the MIC determination performed in Mueller-Hinton broth with 50% normal rabbit serum or human serum.

Time-kill curves were used to test the bactericidal activities of fusidic acid and vancomycin, alone and in combination. Overnight cultures were diluted in glass tubes containing 10 ml of fresh Mueller-Hinton broth to yield an inoculum of 5×10^5 CFU/ml. The following concentrations were used: 8 μg/ml for vancomycin and 0.5, 8, and 32 μg/ml for fusidic acid. After 0, 3, 6, and 24 h of incubation at 37°C, serial dilutions of 0.1-ml samples were subcultured onto agar plates with a spiral plater and incubated for 24 h before CFU were counted. In preliminary experiments, antibiotic carryover was ruled out by plating samples of bacterial suspensions containing 10^1 to 10^3 CFU/ml in the presence or absence of antibiotics alone or in combination (15). Bactericidal activity was defined as an at least 10^3-fold decrease in the original inoculum (15). Synergism was defined as a ≥100-fold increase in killing at 24 h with the combination in comparison with the most active single drug (4). Antagonism was defined as a ≥100-fold decrease in killing at 24 h with the combination compared with the most active single drug alone (4). The results were the mean of two sets of experiments.

The frequency of spontaneous resistance to fusidic acid was determined by plating portions (0.1 ml) from an inoculum of approximately 10^{10} CFU/ml on Mueller-Hinton agar plates containing fusidic acid at concentrations of 1, 10, and 100 μg/ml. The plates were incubated at 37°C for 24 h. The MICs of fusidic acid against 3 to 10 resistant clones growing on antibiotic-containing agars were then determined. The frequency of spontaneous resistance was calculated by dividing the colony count on the fusidic acid-containing plates by the original inoculum and was the mean of two independent experiments.

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Aortic endocarditis was induced in rabbits as previously described (7). Twenty-four hours after catheter insertion, each rabbit was inoculated by ear vein with 10^6 CFU of *S. aureus* in 1 ml of 0.9% NaCl. Within 2 days after bacterial inoculation, approximately 30% of the animals died from sepsis. Untreated rabbits were sacrificed 48 h after bacterial inoculation and served as controls. The weights of the vegetations at that time ranged between 6 and 46 mg. To produce an infection with a smaller inoculum, additional experiments were performed with a bacterial inoculation of 2×10^4 CFU instead of 10^6 CFU. Forty-eight hours after inoculation, animals received one of the following regimens every 12 h for 4 days: vancomycin at 30 mg/kg intramuscularly, fusidic acid at 200 mg/kg subcutaneously, or a combination of both. Dose ranging with fusidic acid given subcutaneously (30 to 400 mg/kg) showed that 200 mg/kg was the largest dose tolerated and produced levels in serum comparable to those achieved in humans after administration of a 500-mg oral dose (20). Blood was sampled 1 and 12 h after the last antibiotic injection for determination of peak and trough bacteriostatic and bactericidal titers in serum and antibiotic levels in serum. Animals were killed, and colony counts in vegetations were determined as previously described (7). Portions (0.1 ml) from the undiluted suspension and the 1:10-diluted suspension of each vegetation were plated onto agar plates containing final concentrations of 0.12, 0.25, and 1 μg of fusidic acid per ml and incubated for 48 h at 37°C. Colony counts were made, and MICs were determined for colonies growing on antibiotic containing agar.

Bacteriostatic and bactericidal titers in serum were determined in a final volume of 1 ml in 50% Mueller-Hinton broth with an inoculum of 5×10^5 CFU/ml. Serial twofold dilutions were made (range, 1/2 to 1/256). After 24 h of incubation at 37°C, the bacteriostatic titer was defined as the highest dilution that prevented turbidity; 0.1-ml portions were then removed from all tubes and subcultured onto Mueller-Hinton agar plates. After 24 h of incubation at 37°C, the number of viable CFU was determined. A disk suceptibility test was used to ensure that the surviving organisms remained susceptible to fusidic acid. The bactericidal titer was defined as the highest dilution that killed at least 99.9% of the original inoculum.

Serum pharmacokinetic parameters were determined for fusidic acid in three infected rabbits after a single subcutaneous injection of 200 mg/kg. One milliliter of blood was sampled via a femoral catheter at 0.5, 1, 2, 4, 8, and 12 h after the injection. Serum elimination half-life was calculated by nonlinear regression from the terminal portion of the concentration-versus-time curve. Three additional infected rabbits were sacrificed 1 h after a single subcutaneous injection of 200 mg of fusidic acid per kg, and vegetations and blood were sampled for determination of fusidic acid concentrations.

Fusidic acid concentrations were measured in serum and in vegetations of animals by the agar diffusion method. The indicator organism was *S. aureus* 209P, and the medium was antibiotic medium no. 1 (Difco) plus 3% KH_2PO_4. Assays of vegetations and serum samples were performed with phosphate buffer (pH 6) and horse serum, respectively. Concentrations of vancomycin were measured by Enzymatic Immuno Assay (Syva, Biomérieux). The sensitivities of the assay were 0.1 and 0.5 μg/ml for fusidic acid and vancomycin, respectively.

Means of bacterial concentrations in vegetations from various groups of animals were compared by analysis of variance followed by a multiple comparison of means by Fisher's least-significant-difference procedure. The comparison of bacterial concentrations in vegetations from the two groups of animals infected with the smallest inoculum was done by an unpaired *t* test. The Fisher exact test was used to determine differences between proportions. A *P* value of <0.05 was considered significant. All of the results are expressed as means ± standard deviations.

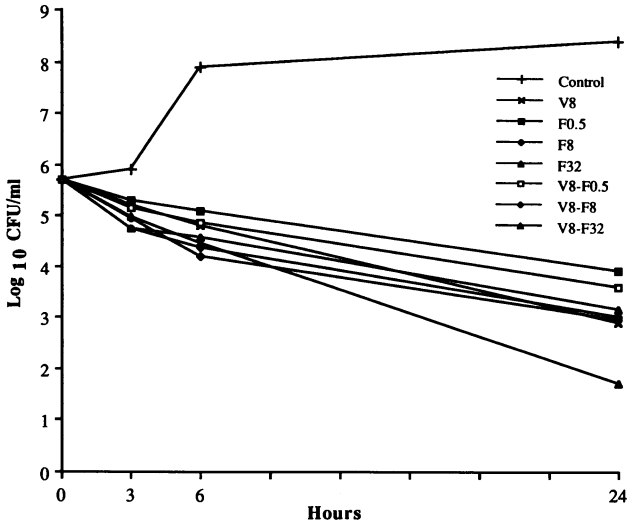

FIG. 1. In vitro killing rates in Mueller-Hinton broth of *S. aureus* HM1054 incubated with vancomycin (V at 8 μg/ml) or fusidic acid (F) at 0.5, 8, or 32 μg/ml, alone or in combination. The control was antibiotic-free medium.

The MICs and MBCs were 0.5 and ≥16 and 0.12 and 32 μg/ml for vancomycin and fusidic acid, respectively. When determined in the presence of rabbit serum, the MIC and MBC of fusidic acid increased to 2 and 64 μg/ml, respectively. The MIC of fusidic acid was 4 μg/ml when determined in the presence of human serum.

As shown in Figure 1, when fusidic acid was tested alone, a concentration effect was observed, with a bactericidal effect achieved at 24 h with the highest concentration tested only (4.0 $\log_{10}$ CFU/ml killing with fusidic acid at 32 μg/ml versus 2.8 and 1.8 $\log_{10}$ CFU/ml killing for the 8- and 0.5-μg/ml concentrations, respectively). When used in combination, fusidic acid (0.5 and 8 μg/ml) and vancomycin exhibited an indifferent interaction (killing difference of <0.5 $\log_{10}$ CFU/ml between single-drug and combination therapies). However, at the highest concentration tested (32 μg/ml), fusidic acid tended to show antagonism in combination with vancomycin (1.7 and 3.1 $\log_{10}$ CFU/ml at 24 h for fusidic acid alone and combined with vancomycin, respectively).

TABLE 1. In vitro selection of clones of *S. aureus* HM1054 resistant to fusidic acid

Concn of fusidic acid (μg/ml)	Frequency of resistant clones	No. of resistant clones at MIC (μg/ml) of:					
		8	16	32	64	128	>128
1	4×10^{-7}	6		1	3		
10	2×10^{-8}				6	4	
100	1×10^{-9}						3

TABLE 2. Results of different 4-day treatment regimens for rabbits infected with *S. aureus* HM1054 at different inoculum sizes

Treatment	Log_{10} CFU/g of vegetation (mean ± SD) (no. of vegetations with resistant clones/total no. of vegetations)	
	Large inoculum (10^6 CFU)	Small inoculum (10^4 CFU)
None (control)	8.2 ± 1.1 (0/6)	4.8 ± 0.3 (0/4)
Vancomycin	6.3 ± 1.2[a] (0/7)	ND[b]
Fusidic acid	7.1 ± 1.7 (5/12)	2.9 ± 0.3[c] (0/5)
Combination	6.2 ± 1.2[a] (0/6)	ND

[a] $P < 0.05$ in comparison with controls.
[b] ND, not done.
[c] $P = 0.0001$ in comparison with controls.

As shown in Table 1, the frequency of emergence of resistance decreased and the MICs for the resistant clones increased as the concentration of fusidic acid tested increased from 1 to 100 μg/ml. In the presence of 1 μg of fusidic acid per ml, resistant clones for which the MIC levels were low and high were selected.

After single injections of fusidic acid into three infected animals, the peak and trough levels in serum were 17 ± 11 and 2.6 ± 1.9 μg/ml, respectively; the elimination half-life of fusidic acid in serum was 4.5 ± 0.1 h ($n = 3$); the mean vegetation/serum ratio of the concentration of fusidic acid obtained 1 h after single injections into three rabbits was 0.71. The peak and trough levels in serum at the end of therapy were 57 ± 18 and 9 ± 9 μg/ml for fusidic acid and 27 ± 12 and 5 ± 3 μg/ml for vancomycin.

Both antibiotics, used either alone or in combination, produced an antibacterial activity in serum that was mainly bacteriostatic. Bacteriostatic and bactericidal activities in serum were not improved by addition of fusidic acid to vancomycin (data not shown).

As shown in Table 2, with the larger inoculum tested, control animals retained 8.2 ± 1.1 log_{10} CFU/g of vegetation when therapy was started; vancomycin alone was as effective as the vancomycin-fusidic acid combination. Fusidic acid alone was ineffective. When the smaller inoculum was tested, control animals retained 4.8 ± 0.3 log_{10} CFU/g of vegetation; fusidic acid significantly reduced bacterial concentrations in vegetations in comparison with this inoculum ($P = 0.0001$). No animal retained sterile vegetation with either inoculum.

In control animals, no clone resistant to fusidic acid could be detected with either inoculum. This result was in agreement with our in vitro findings, since less than 10^7 CFU was actually plated onto agars because of the weight of the vegetations. When the larger inoculum was tested, resistance emerged in 5 of 12 animals treated with fusidic acid alone. Fusidic acid MICs against the resistant clones ranged from 8 to 128 μg/ml. In four animals, resistant clones had fusidic acid MICs of 64 to 128 μg/ml and represented almost the entire bacterial population recovered from the vegetations. Among these four animals, three died before the end of the experiment. For the fifth rabbit, the resistant strain had a MIC of 8 μg/ml and represented less than 1% of the bacterial population recovered from the vegetation; this animal survived until the day of sacrifice. Animals with cardiac vegetations retaining resistant clones had significantly higher bacterial titers than did animals without resistant clones (8.5 ± 1.2 [$n = 5$] versus 6.1 ± 1.1 [$n = 7$] log_{10} CFU/g of vegetation for animals with and without emergence of resistant clones, respectively [$P < 0.01$]) and tended to die sooner (three of five animals died spontaneously versus none of seven animals with and without emergence of resistant clones, respectively [$P = 0.09$]). Animals that retained cardiac vegetations without resistant clones had significantly lower bacterial titers than did control animals ($P < 0.01$).

No resistance to fusidic acid emerged during combination therapy with the larger inoculum or with fusidic acid alone against the smaller inoculum.

Our study indicated that fusidic acid alone was not active and that the combination of fusidic acid and vancomycin was not more effective than vancomycin alone for therapy of a severe experimental staphylococcal infection induced with a large inoculum in vivo.

Emergence of resistant clones during therapy was the main reason for the lack of efficacy of fusidic acid alone for treatment of staphylococcal endocarditis. Emergence of resistance during therapy with fusidic acid alone for staphylococcal infection is a well-known phenomenon that has not always been associated with clinical failure (19). In our experimental study, however, emergence of resistance to fusidic acid was associated with decreased in vivo antimicrobial activity and decreased survival of animals. In contrast, with the smaller inoculum tested, the absence of emergence of resistance was associated with significant in vivo activity of fusidic acid alone. Therefore, the size of the bacterial inoculum was a major determinant of the probability of emergence of resistance to fusidic acid in vivo.

Fusidic acid alone was effective in rabbits that retained vegetations without resistant clones, whatever the inoculum size tested, despite high-level protein binding and weak bactericidal activity. However, these factors may explain the limited in vivo antimicrobial effect of fusidic acid alone, even in the absence of emergence of resistant clones. The influence of protein binding on the bacteriostatic activity of fusidic acid was confirmed by the 4- and 5-dilution increases in the MIC for the study strain when tested in the presence of rabbit or human serum, respectively. This result confirmed previous studies (2, 9, 10) and may be explained by the very high level of protein binding of this antibiotic (≥97%) reported in the literature (2, 18). This point, together with the weak in vitro bactericidal activity against the study strain, explained the lack of significant bactericidal titers in the sera from rabbits treated with fusidic acid alone.

The most important point was the lack of enhanced in vivo activity with the combination of fusidic acid and vancomycin compared with that of single antimicrobial regimens. Combination of vancomycin with fusidic acid actually suppressed the emergence of resistance to fusidic acid observed in animals treated with fusidic acid alone. However, this was not sufficient to produce "in vivo synergism" (6). The interaction of vancomycin and fusidic acid was indifferent in rabbit serum and in vitro. Indifference between these two antibiotics has already been reported with the checkerboard method (8), and antagonism between vancomycin and fusidic acid against *S. aureus* has previously been reported in vitro with the killing-curve method (11). Thus, the favorable pharmacokinetics of fusidic acid and the suppression of emergence of resistance when vancomycin was combined with fusidic acid were not sufficient to overcome the indifferent interaction observed between these two drugs in vitro, in contrast, for instance, to what has been reported with rifampin in combination with vancomycin (3, 6).

We concluded that the vancomycin-fusidic acid combination was indifferent in vitro and in vivo and therefore did not

exhibit any advantage over vancomycin alone in this model and that emergence of resistance to fusidic acid, responsible for bacteriological failure, occurred during monotherapy when a large inoculum was present at the start of treatment.

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