The Impact of Penicillinase on Cefamandole Treatment and Prophylaxis of Experimental Endocarditis Due to Methicillin-Resistant *Staphylococcus aureus*

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β-lactams active against methicillin-resistant *Staphylococcus aureus* (MRSA) must resist penicillinase hydrolysis and bind penicillin-binding protein 2A (PBP 2A). Cefamandole might share these properties. When tested against 2 isogenic pairs of MRSA that produced or did not produce penicillinase, MICs of cefamandole (8–32 mg/L) were not affected by penicillinase, and cefamandole had a ⩾40 times greater PBP 2A affinity than did methicillin. In rats, constant serum levels of 100 mg/L cefamandole successfully treated experimental endocarditis due to penicillinase-negative isolates but failed against penicillinase-producing organisms. This suggested that penicillinase produced in infected vegetations might hydrolyze the drug. Indeed, cefamandole was slowly degraded by penicillinase in vitro. Moreover, its efficacy was restored by combination with sulbactam in vivo. Cefamandole also uniformly prevented MRSA endocarditis in prophylaxis experiments, a setting in which bacteria were not yet clustered in the vegetations. Thus, while cefamandole treatment was limited by penicillinase, the drug was still successful for prophylaxis of experimental MRSA endocarditis.

Most methicillin-resistant staphylococci produce both penicillinase and a new, low-affinity penicillin-binding protein called PBP 2A [1]. This additional membrane polypeptide confers intrinsic resistance to virtually all β-lactam antibiotics and is thought to ensure peptidoglycan assembly when normal staphylococcal PBPs are blocked by β-lactam drugs. Indirect evidence for this hypothesis was provided both by cell wall analysis of methicillin-susceptible and -resistant staphylococci [2] and by site-specific mutation of the PBP 2A active serine residue [3]. These experiments suggested that PBP 2A was a transpeptidase and thus resembled other bacterial PBPs. Moreover, when methicillin-resistant staphylococci were grown in the presence of high methicillin concentrations, they switched from the synthesis of a normal peptidoglycan (in the absence of the drug) to the production of a poorly cross-linked and structurally distinct cell wall (in the presence of methicillin) that was likely to result from the uninhibited activity of PBP 2A [4]. Therefore, it is not astonishing that certain β-lactams with relatively good PBP 2A affinity have a demonstrable activity against methicillin-resistant staphylococci [5–9].

The potential usefulness of such compounds was first observed with ''old-fashioned'' molecules such as penicillin G, ampicillin, and amoxicillin [5–9]. These molecules appeared to be effective in the treatment of experimental endocarditis due to penicillinase-negative isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and were equivalent to control therapy with vancomycin in both rat and rabbit experiments. Against penicillinase-producing isolates, however, successful treatment required the addition of critical amounts of penicillinase inhibitors, such as sulbactam or clavulanate, to protect the drugs from penicillinase-induced hydrolysis [5, 9]. Thus, anti-MRSA β-lactams should combine the abilities both to bind to PBP 2A and to resist penicillinase-mediated degradation if they were to be considered for clinical use.

An experimental carbapenem (L-695,256) sharing these properties has recently demonstrated good in vivo activity in the rabbit model of MRSA endocarditis [7]. However, such molecules are not yet available for treatment in humans. Among nonexperimental β-lactams, on the other hand, the cephalosporin cefamandole might possess these characteristics. Cefamandole has a good penicillinase stability and an excellent activity against methicillin-susceptible staphylococci [10]. In addition, cefamandole has relatively low MIC for MRSA and was reported to be effective in the treatment of MRSA soft tissue infections in humans [11, 12]. In the present experiments, we further investigated the efficacy of cefamandole against a series of isogenic pairs of MRSA that produced or did not produce penicillinase. Investigations included in vitro determinations of drug susceptibility and PBP 2A affinity as well as in vivo therapeutic and prophylactic studies in the rat model of experimental endocarditis.

Materials and Methods

Microorganisms and growth conditions. The bacterial isolates used in this study are described in table 1. COL and P8 were 2 clinical isolates of MRSA expressing, respectively, homogeneous and heterogeneous resistance to methicillin [5, 13]. COL was penicillinase-negative, while P8 was penicillinase-positive. A penicillinase-producing transformant of COL was obtained by DNA transformation with the penicillinase-encoding plasmid pI524. A

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Table 1. Phenotypes of methicillin resistance and penicillinase (Bla) production and MICs of various antibiotics for test organisms.

		Homogeneous resistant		Heterogeneous resistant	
Antibiotic	MSSA Bla$^-$*	COL$^-$	COL$^+$	P8$^-$	P8$^+$
Methicillin	1	>128	>128	64	64
Flucloxacillin	0.5	64	64	32	32
Penicillin G	0.004	8	>128	4	>128
Amoxicillin	0.125	16	>128	8	>128
Amoxi-Clav†	0.125	16	64	8	8
Cefuroxime	1	>128	>128	32	32
Cefamandole	0.5	16	32	2	4
Sulbactam	>128	>128	>128	>128	>128
Cefam-Sul‡	0.5	16	16	2	2
Vancomycin	2	2	2	2	2

* Methicillin-susceptible *S. aureus* (MSSA) strain RN 2677 used as control.
† Amoxicillin and clavulanate combined in 5/1 (wt/wt) ratio.
‡ For MIC determination, cefamandole was combined with constant concentration of 4 mg/L sulbactam. Table indicates MICs of cefamandole.

penicillinase-negative derivative of P8 was obtained by temperature-induced loss of the penicillinase-encoding plasmid during growth at 43°C [5]. For the sake of clarity, these pairs of bacteria are referred to as COL$^-$/COL$^+$ and P8$^-$/P8$^+$ (for penicillinase-negative and penicillinase-positive isolates, respectively). RN2677 is a methicillin-susceptible laboratory isolate of *S. aureus* used as a control in in vitro experiments [5, 13]. Unless otherwise stated, bacteria were grown at 35°C in tryptic soy broth (TSB; Difco, Detroit) with aeration or on tryptic soy agar (TSA; Difco) supplemented with 2% NaCl. Plates were routinely supplemented with penicillinase (Bacto-Penase concentrate; Difco; 2000 U/mL final concentration) to avoid antibiotic carryover. Stocks were kept at −70°C in TSB supplemented with 10% (vol/vol) glycerol.

Antibiotics and chemicals. Cefamandole and vancomycin were obtained from Eli Lilly (Indianapolis); cefuroxime was obtained from Glaxo Pharmaceuticals (London); penicillin G was obtained from Hoechst-Pharma (Zurich); [^{3}H]penicillin (9 mCi/mL; 117 mg/L) was provided by Merck Sharp & Dohme (Rahway, NJ); sulbactam was obtained from Pfizer (Orsay, France); and methicillin, flucloxacillin, amoxicillin, and amoxicillin-clavulanate were obtained from Beecham Research Laboratories (Brockham Park, UK).

Antibiotic susceptibility, population analysis profile, and in vitro time-kill curves. MICs of antibiotics were determined by a previously described broth macrodilution method [14] in Mueller-Hinton broth (Difco) supplemented with 2% NaCl; 10^5 cfu/mL was used as inoculum. The MIC was defined as the lowest antibiotic concentration inhibiting visible bacterial growth after 24 h of incubation at 35°C. The phenotypic expression of β-lactam resistance was determined by spreading large bacterial inocula (10^9 cfu) as well as smaller inocula (10^6, 10^5, and 10^3 cfu) onto TSA plates containing 2% NaCl and 2-fold serial dilutions of antibiotics [5]. The numbers of colonies growing on the plates were enumerated after 48 h of incubation at 35°C. Population analysis profile curves were generated by plotting the numbers of colonies growing on the plates against the concentrations of antibiotic in the plates. In vitro time-kill curves were made as described [6].

Titration of PBP 2A. The presence of PBP 2A was determined in membrane fractions of bacterial lysates of the penicillinase-negative COL$^-$, as previously described [13]. In brief, 75-μL portions of membrane suspensions containing 4 mg/mL protein were incubated for 10 min at 37°C with 25 μL of a 1:10 (wt/wt) solution of [^{3}H]penicillin (77 mCi/mg) and cold penicillin to a final concentration of 2.5 mg/L [^{3}H]penicillin/mL of membrane suspension. The reaction was stopped by the addition of a 100-fold excess of cold penicillin, and the membranes were dissolved in the detergent sarkosyl. The membrane proteins were separated by SDS-PAGE, and the [^{3}H]penicillin-labeled PBPs were visualized by fluorography [5]. The binding affinities of methicillin, cefamandole, and amoxicillin for PBP 2A were assessed by measuring their ability to compete for the binding of [^{3}H]penicillin to PBPs [5]. Aliquots of membrane suspensions were distributed into series of tubes containing 2-fold serial dilutions of the competitor and incubated for 10 min at 37°C before [^{3}H]penicillin was added. The tubes were incubated for another 10 min at 37°C and processed as described. Intensities of the PBP 2A bands were quantified by densitometry by use of the ImageQuant version 3.3 software (Molecular Dynamics, Sunnyvale, CA). Binding affinities of the competing drugs were derived from densitometry quantification and expressed as the drug concentration inhibiting binding of [^{3}H]penicillin by 50% and 90% (IC_{50} and IC_{90}).

Penicillinase stability of antibiotics in vitro. The ability of cefamandole, methicillin, and amoxicillin to resist penicillinase-induced degradation was measured in broth cultures by use of a described bioassay [15]. Both pairs of MRSA P8 and COL, producing or not producing penicillinase, were used in this test.

Production of endocarditis and infusion pump installation. Catheter-induced sterile aortic vegetations were produced in rats as previously described [16]. At the same time, an intravenous (iv) catheter was inserted via the jugular vein into the superior vena cava and connected to a programmable infusion pump (Pump 44; Harvard Apparatus, South Natick, MA) to deliver the antibiotics [6]. The pump was set to deliver a volume of 0.2 mL of saline/h to keep the catheter open until the onset of therapy. No iv catheters were placed in the control animals.

Bacterial endocarditis was induced 24 h after catheterization by iv challenge of the animals with 0.5 mL of saline containing 10^5 cfu of either of the test organisms. This inoculum was 10 times larger than the minimum inoculum producing endocarditis in 90% of the untreated rats.

Treatment of experimental endocarditis. Antibiotic therapy was started 12 h after bacterial challenge and lasted for 3 days. The drugs were administrated at changing flow rates with the pump to produce either of the following kinetics in the serum of rats: simulation of sequential iv administration of 3 g of cefamandole given 4 times a day to humans [17, 18]; continuous iv infusion of cefamandole producing constant serum concentrations of 100 mg/L (this concentration was chosen because it inhibited at least 90% of PBP 2A in vitro; see Results); and simulation of sequential iv administration of 1 g of vancomycin given twice daily to humans [6]. This required total amounts of antibiotics of, respectively, cefamandole at 250 mg/kg of body weight/24 h (for the two cefamandole regimens) and vancomycin at 23.2 mg/kg of body weight/12 h. In certain experiments, cefamandole was combined with

sulbactam, which was administered via a separate pump and which simulated in rats the human pharmacokinetics produced by administration of 1 g of the drug given 4 times a day [9]. This required the administration of sulbactam at 50 mg/kg of body weight/6 h.

Prophylaxis of experimental endocarditis. Antibiotic prophylaxis was started 1 h before bacterial challenge and lasted for 1 day [19]. Kinetics of antibiotics in the serum of rats were as follows: simulation of sequential iv administration of 1 g of flucloxacillin given 4 times a day to humans [20], simulation of sequential iv administration of 3 g of cefamandole given 4 times a day to humans (as above), continuous iv infusion of cefamandole producing constant serum levels of 100 mg/L (as above), or simulation of sequential iv administration of 1 g of vancomycin given twice daily to humans (as above). Total amount of drug given to the animals was 42.3 mg of flucloxacillin/6 h. Amounts of drug for the other regimens were as described above.

Antibiotic concentrations in serum. Concentrations of antibiotic in serum were determined in groups of 4–9 uninfected or infected rats. Serum levels in infected animals came from internal controls for adequate drug delivery in therapeutic experiments. Blood was drawn by puncturing the periorbital sinuses of the animals at several time points during and after antibiotic administration. Antibiotic concentrations were determined by an agar diffusion assay with antibiotic medium 1 (Difco) and *Bacillus subtilis* ATCC 6633 as the indicator organism for cefamandole, flucloxacillin, and vancomycin and *Acinetobacter calcoaceticus* as the indicator organism for sulbactam. The diluent was pooled rat serum. The limits of detection of the assays were 0.3 mg/L for cefamandole, 0.3 mg/L for flucloxacillin, 0.6 mg/L for vancomycin, and 3.12 mg/L for sulbactam. The linearity of the standard curves was assessed by a regression coefficient of $\geq$0.995, and intraplate and interplate variations were $\leq$10%.

Evaluation of infection. In therapeutic experiments, the control rats were sacrificed at the time of treatment onset (i.e., 12 h after inoculation) to measure both the frequency and the severity of valvular infection at the start of therapy. Treated rats were sacrificed 18–24 h after the last antibiotic dose, at least 12 h after the trough level of drug in serum was reached. At that time, no residual antibiotic could be detected in the blood. In prophylaxis experiments, control rats were killed 24 h after bacterial challenge, whereas rats receiving prophylaxis were killed after 3 days (i.e., 2 days after the end of antibiotic administration). The valvular vegetations were sterilely dissected, weighed, homogenized in 1 mL of saline, and serially diluted before being spread on penicillinase-containing plates for colony counts. Quantitative blood cultures and spleen cultures were done in parallel. The numbers of colonies growing on the plates were determined after 48 h of incubation at 35°C. Bacterial densities in the vegetations were expressed as $\log_{10}$ colony-forming units per gram of tissue. The dilution technique permitted the detection of $\geq$2 $\log_{10}$ cfu/g of vegetation. For statistical comparisons of differences between the median vegetation bacterial densities of various treatment groups, culture-negative vegetations were considered to contain 2 $\log_{10}$ cfu/g.

Statistical analysis. Fisher's exact test was used to compare the rates of valvular infection. Bonferroni's correction was used for multiple-group comparison. Median bacterial densities in the vegetations of various treatment groups were compared by the nonparametric Mann-Whitney Wilcoxon unpaired test or the Kruskal-Wallis one-way analysis of variance on ranks with subsequent pairwise testing by the Dunn's method. Overall, differences were considered significant at $P \leq .05$ by use of two-tailed significance levels.

Results

Antibiotic susceptibility, population analysis profile, and time-kill curves. Table 1 shows the MICs of various antibiotics for the 4 test organisms. All of the organisms were highly resistant to methicillin and flucloxacillin. Amoxicillin had a relatively good activity against the penicillinase-negative derivatives but required the addition of clavulanate to be active against the penicillinase-producing organisms. In contrast, the MIC of cefamandole remained essentially unaffected by penicillinase production or by combination with sulbactam.

Figure 1 shows the population analysis profile of the homogeneously resistant COL^- and its penicillinase-producing derivative COL^+. As in the MIC determinations, amoxicillin was relatively effective against the penicillinase-negative version of the organisms but lost its activity in the presence of penicillinase. In contrast, cefamandole was active against both penicillinase-negative and penicillinase-positive isolates. The same observation was made with the penicillinase-negative and penicillinase-positive versions of the heterogeneously resistant P8. Moreover, when an additional panel of 10 unrelated clinical isolates of penicillinase-producing MRSA were tested, none of them grew on agar plates containing >32 mg/L cefamandole, while they grew on plates containing up to 1000 mg/L methicillin, flucloxacillin, or amoxicillin (data not shown). This shows that the relatively good anti-MRSA activity of cefamandole could be extended beyond the limit of the test bacteria used in the present experiments.

Time-kill experiments were done with antibiotic concentrations readily achieved in human serum. Cefamandole was bactericidal against all 4 test organisms, as shown by viability losses of $\geq$3 $\log_{10}$ cfu/mL after 24 h of exposure to drug at 100 mg/L. Amoxicillin (40 mg/L) was bactericidal only against the penicillinase-negative derivatives and required the addition of clavulanate to kill penicillinase-producing isolates. In contrast, neither methicillin nor flucloxacillin killed or inhibited any of the isolates when used at peak concentrations achievable in human serum during iv therapy (i.e., 100 mg/L).

Determination of PBP 2A affinity. The drug concentrations inhibiting 50% and 90% of [^{3}H]penicillin labeling of PBP 2A were determined in membrane fractions of the penicillinase-negative strain COL^-. The IC_{50} and IC_{90} of methicillin were 350 and 4000 mg/L, respectively. In comparison, these values were $\geq$40-fold lower for cefamandole and amoxicillin: 8 and 100 mg/L for cefamandole and 4 and 90 mg/L for amoxicillin. These results were reproducible on repeating the experiments several times with independent batches of cell membranes. Thus, the relatively good in vitro activity of cefamandole and

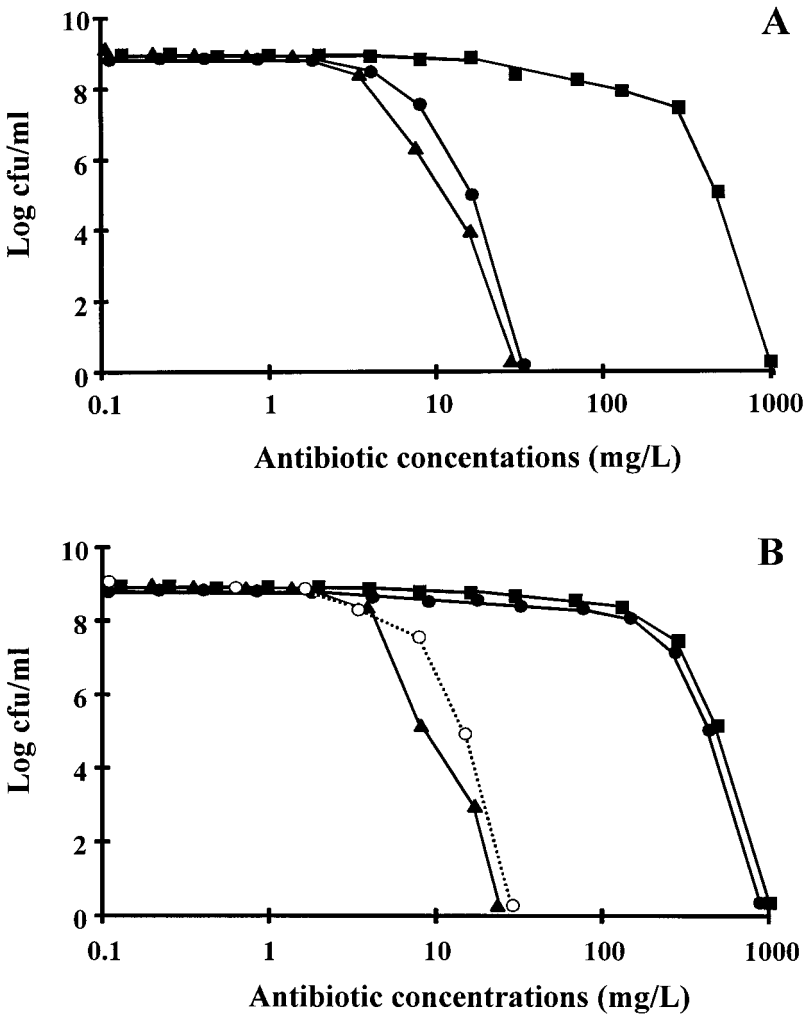

Figure 1. Population analysis profile of penicillinase-negative and homogeneously methicillin-resistant strain COL^- (**A**) and its penicillinase-producing transformant COL^+ (**B**). Various sizes of bacterial inocula were spread on agar plates containing increasing concentrations of either methicillin (■), cefamandole (▲), or amoxicillin (●). **B** also shows results with amoxicillin-clavulanate (○) against penicillinase-producing strain COL^+. Points indicate no. of colonies growing on plates after 48 h of incubation at 35°C.

amoxicillin against MRSA correlated with a greater PBP 2A affinity of these compounds compared with that of methicillin.

Treatment of experimental endocarditis due to penicillinase-negative MRSA. The homogeneously methicillin-resistant but penicillinase-negative strain COL^- was tested in these experiments. Two cefamandole regimens mimicking drug concentrations achievable in the sera of humans were tested, one simulating standard 4 times daily administration of 3 g of cefamandole, and one consisting of continuous infusion of the drug. Figure 2 depicts the serum kinetics of these cefamandole regimens (figure 2A) and their therapeutic results after 3 days of treatment (figure 2B). Human-like kinetics produced by sequential treatment failed to cure the animals, in spite of repeated high peak serum levels of the drug (~600 mg/L). Since cefamandole had a short serum half-life, it was possible that transient antibiotic peaks might not compensate for the prolonged periods of drug levels below the MIC and that continuous drug infusion might perform better than sequential administration. Indeed, figure 2 shows that continuous infusion producing steady serum concentrations of cefamandole of ~100 mg/L (dashed line) successfully cured valvular infections and was equivalent to or better than control treatment with vancomycin. Therefore, continuous infusion of cefamandole was used in the next series of experiments.

Treatment of experimental endocarditis due to penicillinase-producing MRSA. A second series of experiments investigated the impact of bacterial penicillinase production on the outcome of cefamandole therapy in vivo. Both the penicillinase-negative and -positive versions of strains COL and P8 were tested in parallel. Figure 3 shows that continuous infusion of cefamandole successfully treated animals infected with the β-lactamase–negative organisms. However, although cefamandole was not affected by penicillinase in susceptibility tests in vitro, the antibiotic was significantly less effective against the penicillinase-producing version of the strains in vivo. This observation raised questions about the stability of cefamandole to penicillinase in this condition.

Penicillinase stability of cefamandole. One reason for the discrepancy between in vitro and in vivo results might be related to an inoculum effect. As illustrated in figure 4, penicil-

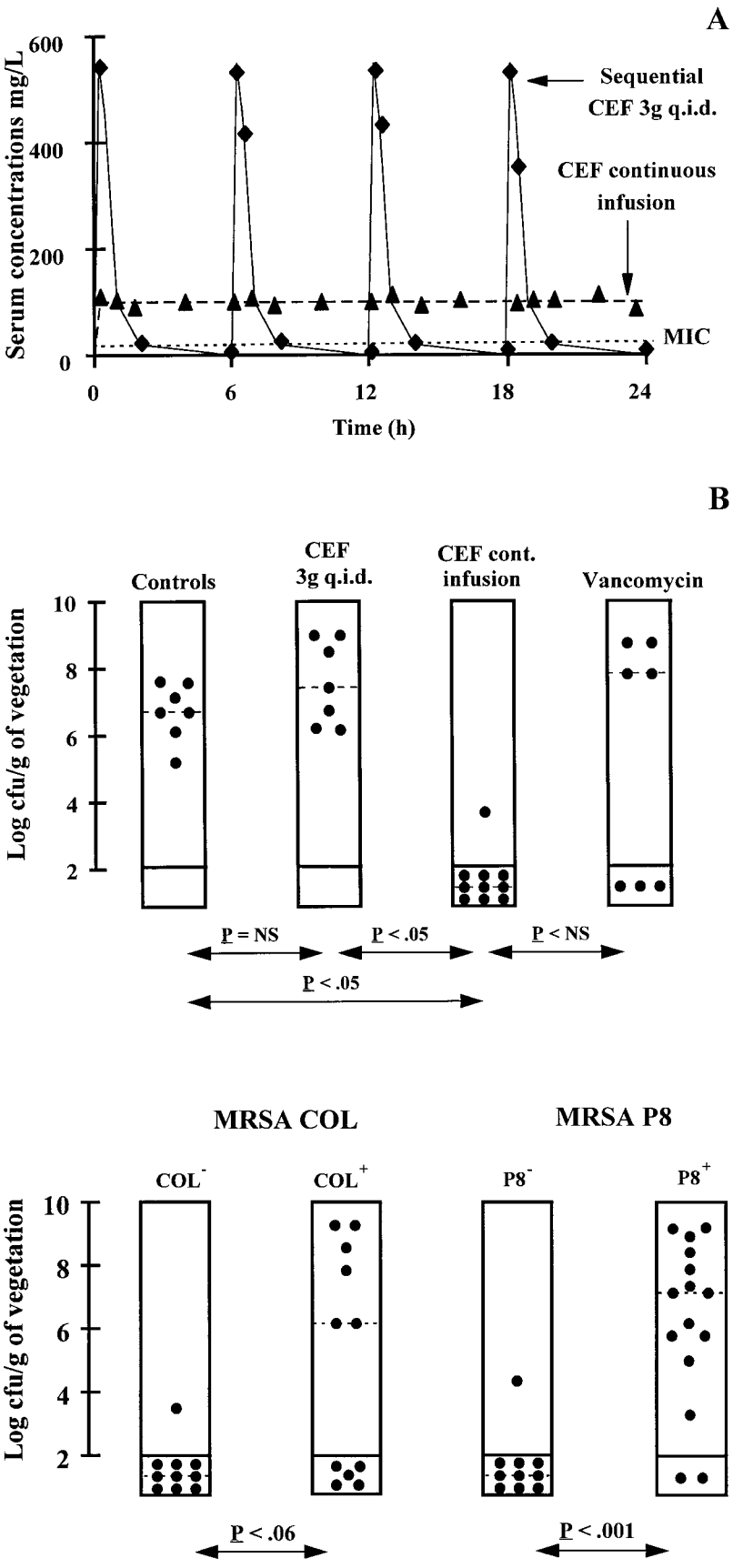

Figure 2. Kinetics of cefamandole in serum of rats (**A**) and results of treatment of experimental endocarditis due to penicillinase-negative strain COL^- (**B**). **A** depicts cefamandole kinetics that either simulated human pharmacokinetics produced by sequential administration of 3 g of drug 4 times daily (CEF 3g q.i.d.) or produced constant antibiotic levels of 100 mg/L (CEF continuous infusion). Dotted line in **A** indicates MIC of cefamandole for test organism. In **B**, treatment groups are indicated at tops of columns. Each dot indicates bacterial density in vegetations of single animal. Dashed lines indicate median value in each treatment group. Statistical differences between groups were determined by Kruskal-Wallis 1-way analysis of variance on ranks. Differences in median values among treatment groups were significant ($P <$ .001). Differences between specific groups, as determined by Dunn's method, are indicated at bottom. NS = not significant.

Figure 3. Treatment of experimental endocarditis with cefamandole given in continuous infusion (producing constant serum levels of 100 mg/L) in rats infected with penicillinase-negative and penicillinase-positive versions of strains COL and P8. Untreated controls were all heavily infected and are not shown. Each dot in columns indicates bacterial density in vegetation of single animal; dashed lines indicate median value in each treatment group. Statistical differences between pairs of groups were determined by Mann-Whitney-Wilcoxon unpaired test and Fisher's exact test. Both tests gave concordant results. Therefore, only highest *P* values given by either test are shown at bottom.

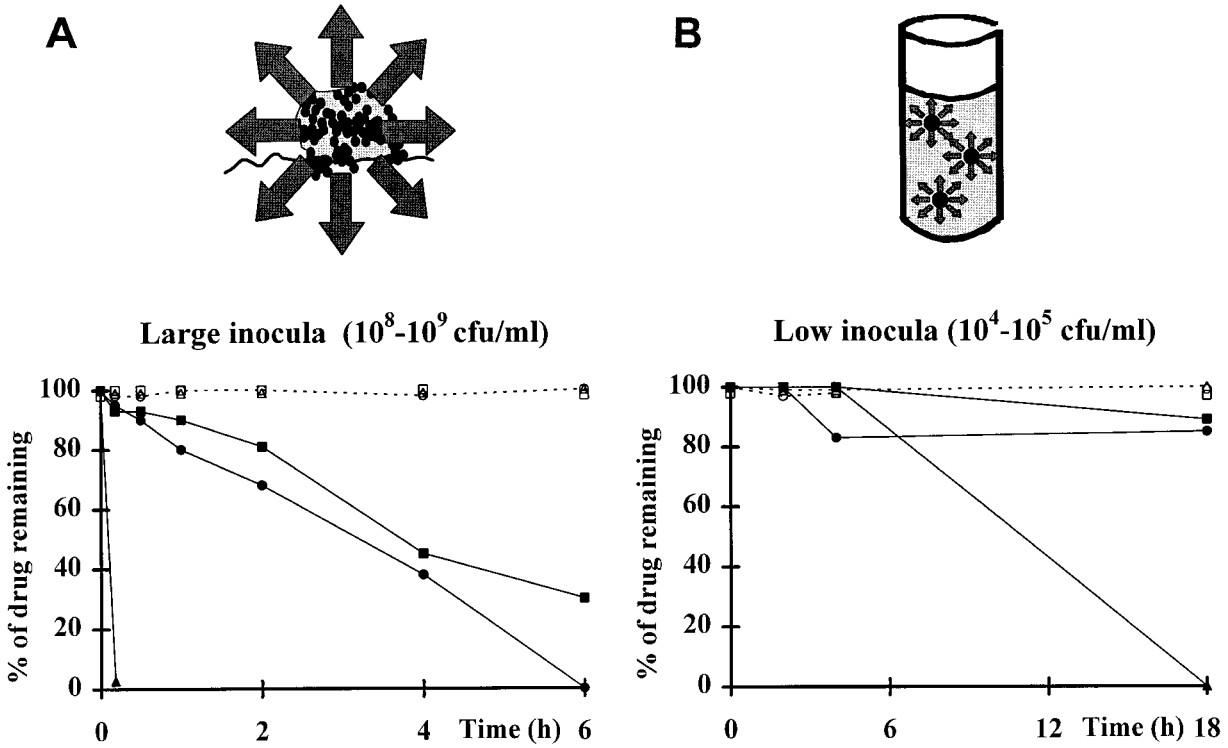

Figure 4. Model for inoculum-dependent degradation of β-lactams operating in infected vegetations (**A**) compared with situation in MIC test tubes (**B**). Upper part of **A** illustrates bacterial clusters packed in infected vegetations, surrounded by large concentrations of penicillinase (arrows). Graph in **A** shows in vitro degradation of amoxicillin (triangles), cefamandole (circles), and methicillin (squares) exposed to large bacterial inocula of either strain COL^+ (penicillinase-producer; closed symbols) or COL^- (penicillinase-negative; open symbols). **B** shows results of similar experiments with lower inoculum sizes, to mimic situation in MIC test tubes. Model is similar to that proposed by Goldman and Petersdorf [15].

linase concentrations might be higher around bacterial clusters packed in infected vegetations (figure 4A) than around single organisms suspended in broth cultures or spread on agar plates (figure 4B). Since cefamandole is not entirely immune to penicillin-induced hydrolysis [21], degradation of cefamandole might become an issue when switching from the in vitro susceptibility tests to deep-seated infections in vivo. A possible inoculum effect was investigated in vitro by testing the stability of cefamandole and other antibiotics exposed to broth cultures containing various bacterial counts of either strain COL^+ or strain COL^-. In the presence of large bacterial numbers (i.e., 10^8–10^9 cfu/mL; figure 4A, graph), cefamandole and methicillin lost almost 50% of their original activity within 3 h of exposure. Accordingly, amoxicillin barely resisted a few minutes in this experiment. In contrast, the three antibiotics were perfectly stable when exposed to broth cultures of the penicillinase-negative strain COL^-. In the presence of lower bacterial numbers (i.e., 10^5–10^6 cfu/mL; figure 4B, graph), on the other hand, both cefamandole and methicillin were quite stable for up to 18 h, whereas amoxicillin was progressively degraded over this time. Similar results were obtained when the pair of penicillinase-producing and -nonproducing MRSA $P8^+$ and $P8^-$ were used instead of COL (data not shown). Therefore, this supports the possibility that large bacterial densities and penicillinase concentrations in the vegetations might result in significant degradation of cefamandole at the infected site. This hypothesis was further tested by using the penicillinase inhibitor sulbactam in the next experiments.

Prevention of penicillinase-induced degradation of cefamandole by sulbactam. If the above assumption is correct, then combination of cefamandole with a penicillinase inhibitor, such as sulbactam, might restore its antibacterial efficacy. This was tested both in vitro and in vivo. First, large concentrations of sulbactam (400 mg/L) successfully prevented degradation of cefamandole in vitro, as tested by an assay similar to that presented in figure 4 that used the penicillinase-producing strain COL^+ as test bacterium. In a typical experiment, the residual activity of cefamandole exposed alone to the culture was 76% after 2 h of incubation and 0% after 4 h and 6 h of incubation. In the presence of sulbactam, in comparison, the residual activity of cefamandole was 100% at 2 h, 60% at 4 h, and 33% at 6 h.

Second, combination of cefamandole with sulbactam in vivo also restored the drug efficacy. Sulbactam administration produced a peak concentration in the serum of rats (mean ± SD of 3 animals) of 39.9 ± 11.5 mg/L at 30 min, followed by

concentrations of 30.8 ± 1 mg/L at 1 h, 9.8 ± 0.1 mg/L at 2 h, and undetectable values at 6 h. Figure 5 indicates that administration of sulbactam at this dose 4 times a day reestablished the therapeutic efficacy of continuous infusion of cefamandole, as if it were used against the penicillinase-negative version of the organism (see figure 3). Thus, taken together, these experiments strongly argue in favor of a direct role of in situ production of penicillinase as a cause of cefamandole treatment failure against experimental endocarditis due to penicillinase-producing MRSA.

Prophylaxis of experimental endocarditis. Although cefamandole was ineffective against experimental endocarditis due to penicillinase-producing MRSA, it might be successful for prophylaxis of such infection. Indeed, prophylaxis more closely resembles the test tube situation than does established infection, because prophylactic drugs are given while bacteria are circulating in the blood and only beginning to colonize the cardiac lesions [22, 23]. Figure 6 shows that cefamandole successfully prevented endocarditis due to the penicillinase-producing strain COL^+, whether it was administered sequentially as in humans or in continuous infusion, producing constant serum levels of drug of 100 mg/L. Vancomycin prophylaxis was also effective, whereas flucloxacillin completely failed to prevent infection. These results further support the hypothesis that the local concentration of penicillinase around the bacteria might be a critical factor determining antibiotic efficacy.

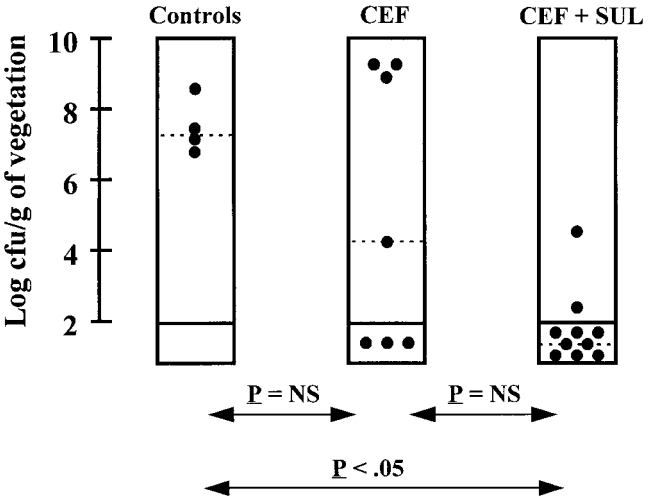

Figure 5. Treatment of experimental endocarditis with cefamandole given to rats in continuous infusion (producing constant serum levels of 100 mg/L) either alone (CEF) or in combination with sulbactam (CEF + SUL) at doses simulating human pharmacokinetics produced by administration of 1 g of drug 4 times a day. Rats were infected with penicillinase-producing, homogeneous methicillin-resistant strain COL^+. Each dot indicates bacterial density in vegetation of single animal. Dashed lines indicate median value in each treatment group. Statistical differences between groups were determined by Kruskal-Wallis 1-way analysis of variance on ranks ($P < .014$) with pairwise comparison between specific groups by Dunn's method and Fisher's exact test with Bonferroni's correction. Both tests gave concordant results. NS = not significant.

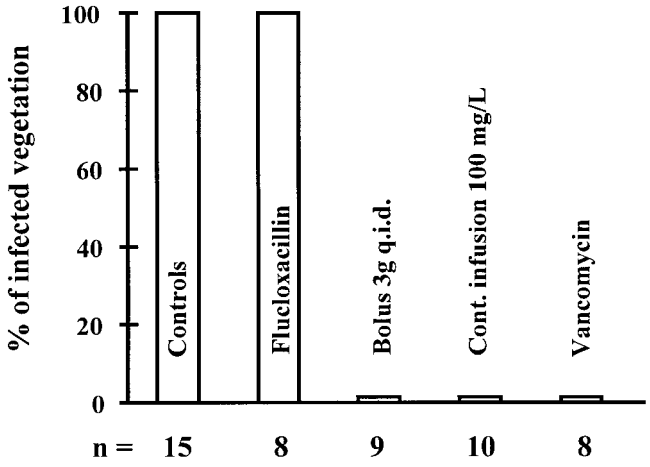

Figure 6. Cefamandole prophylaxis of experimental endocarditis due to penicillinase-producing strain COL^+. Prophylaxis regimens are indicated. No. of animals in each group are shown at bottom.

Discussion

The present studies highlight two pharmacodynamic limitations of cefamandole in the treatment of experimental endocarditis: one relatively trivial, which relates to the antibiotic dosing regimen, and a second, more fundamental, that points to the essential role of penicillinase in β-lactam resistance of MRSA. First, treatment of experimental endocarditis due to penicillinase-negative MRSA could not be achieved by sequential drug administration, because repeated transient peaks of cefamandole in the serum were unable to ensure prolonged supra-MIC drug concentrations in vivo. It is well established that β-lactam drugs must be maintained above growth-inhibiting concentrations to be effective [24, 25], and this limitation was easily overcome by using continuous infusion of the drug.

Second, treatment of endocarditis due to penicillinase-positive MRSA could not be achieved even by continuous drug infusion, presumably because cefamandole was inactivated in situ by bacterial penicillinase produced in the vegetations. This limitation was not expected, because large doses of the relatively penicillinase-stable cefamandole were assumed to overcome penicillinase-induced hydrolysis in vivo. However, complementary experiments clearly demonstrated an inoculum-dependent degradation of cefamandole by penicillinase, which could be prevented by coadministration of a penicillinase inhibitor such as sulbactam. This supports the possibility that large amounts of the enzyme surrounding bacterial clusters in the vegetations could protect the microorganisms from the drug and confirms a previous hypothesis by Goldman and Petersdorf [15], who postulated that the poor activity of cefazolin against experimental endocarditis due to a penicillinase-producing

strain of *S. aureus* could be due to degradation of the drug at the infected site. Moreover, it was further supported by the fact that any of the cefamandole regimens that failed in therapeutic experiments were highly effective in the prophylaxis studies, a setting more closely resembling the test tube than does the vegetation situation (see figure 4). Therefore, while the slow inactivation of cefamandole by penicillinase did not affect the results of in vitro susceptibility tests, it was clearly responsible for treatment failure in vivo.

The observation is important because it underlines the fact that in vitro susceptibility tests might not be predictive of in vivo results. Moreover, the lack of cefamandole efficacy against penicillinase-producing strains also highlights the everlasting role of penicillinase in β-lactam resistance of staphylococci. Besides MRSA, penicillinase may also adversely affect the efficacy of anti-staphylococcal β-lactams against methicillin-susceptible *S. aureus*. For example, combination of cefoperazone with sulbactam was more effective than cefoperazone alone against experimental endocarditis due to methicillin-susceptible *S. aureus* in rabbits, presumably because sulbactam protected cefoperazone from penicillinase-induced degradation [26]. Another example is borderline methicillin-resistant *S. aureus,* which have increased methicillin MICs because of penicillinase overproduction [27]. Although these organisms are not clinically relevant, they were able to decrease the efficacy of ampicillin-sulbactam treatment of experimental endocarditis due to borderline methicillin-resistant *S. aureus* in rats [28]. Therefore, production of penicillinase is not harmless, even when supposedly penicillinase-stable drugs are used to treat methicillin-susceptible *S. aureus*.

In the case of MRSA, penicillinase may represent a primary restriction to the possible use of existing β-lactams against infections due to such bacteria. Indeed, several studies have validated the fact that β-lactams with a relatively good PBP 2A affinity, such as penicillin, ampicillin, and amoxicillin, could successfully treat experimental MRSA infections, provided that they could escape penicillinase-induced degradation [5–9]. These observations were recently extended to methicillin-resistant *Staphylococcus epidermidis,* which also produce PBP 2A [6, 29]. However, although human-like kinetics of amoxicillin-clavulanate could successfully treat experimental MRSA endocarditis in rats [5], experiments in rabbits showed that combinations of ampicillin-sulbactam or penicillin-sulbactam tended to be less effective against infections due to penicillinase-producing than -nonproducing MRSA [9, 30]. Therefore, these results raised concern about the potential safety of penicillin–penicillinase inhibitor combinations for therapeutic use against penicillinase-producing MRSA in humans.

The present experiments with cefamandole further emphasize the crucial role of penicillinase as a troublemaker in the system. Figure 4 is an oversimplified illustration of the subtle confrontation between bacterial factors and antibiotics in the vegetation environment. On the side of the bacteria, rapid induction of penicillinase and production of large amounts of the enzyme are crucial for the survival of the organisms. On the side of the antibiotic, slow diffusion into the vegetation, strong penicillinase-inducing capacity, and low penicillinase stability are playing against the drug. Some of these characteristics can be defined, at least on the antibiotic side. However, the interplay of these factors in vivo may be difficult to predict. The reproducible therapeutic success of certain β-lactams against penicillinase-negative MRSA, on the other hand, supports the fact that PBP 2A can be blocked in vivo. Nevertheless, β-lactams with improved PBP 2A affinity would certainly gain in anti-MRSA efficacy and act in conjunction with their stability to penicillinase against these organisms. Therefore, both better PBP 2A affinity and penicillinase stability are important in the development of future β-lactams active against MRSA.

Taken together, the present observations show that while cefamandole demonstrates both relatively good PBP 2A affinity and anti-MRSA activity in vitro, it may fail against MRSA infections in vivo because of production of bacterial penicillinase. This is in contradiction to a report suggesting that cefamandole might be effective for treatment of soft tissue infections due to MRSA in humans [11] and raises caution concerning this indication. On the other hand, however, 1-day prophylaxis with cefamandole could effectively prevent experimental MRSA endocarditis, presumably because individual production of penicillinase by circulating bacteria is low. Cefamandole was known to be effective for prophylaxis in cardiovascular surgery before the methicillin resistance era [31, 32]. It might now be considered for prophylaxis against MRSA and methicillin-resistant *S. epidermidis* as well, especially when the use of vancomycin is prohibited to avoid the selection of vancomycin-resistant enterococci. Additional studies are warranted to further assess this prophylactic efficacy.

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