RELEASE OF LIPOTEICHOIC ACID FROM *STAPHYLOCOCCUS AUREUS* BY TREATMENT WITH CEFMETAZOLE AND OTHER β-LACTAM ANTIBIOTICS

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The effect of cefmetazole on the growth together with the release of cellular lipoteichoic acid from cefazolin-resistant strains of *Staphylococcus aureus* was compared with that of cefazolin, cefotiam, cefoxitin and cefuroxime. Bacteriolytic actions were measured by turbidity and bactericidal actions were followed by viable cell count. Release of cellular lipoteichoic acid was measured by the radioactivity in the supernatant of the cultures. Cefmetazole exerted more potent effects on the bacterial growth and induced more marked release of cellular lipoteichoic acid from resistant strains as compared with other β-lactams.

In recent years resistant strains of *Staphylococcus aureus* susceptibility to cefazolin is 12.5 μg/ml or higher at an inoculum of 10^8 colony-forming units (cfu) per ml have been isolated from clinical materials at a proportion of about 10% at Tokyo Clinical Research Center[1,2]. These strains tend to be resistant to other cephalosporins, *e.g.*, cefotiam or cefuroxime. Cefmetazole, a cephamycin derivative, is more effective against these strains as compared with cefazolin, cefotiam, cefoxitin and cefuroxime.

It has been assumed that the bacteriolysis by β-lactam antibiotics is induced by the binding of β-lactams to the penicillin-binding proteins, indicating that the bacteriolysis is a consequence of the inhibition of cell wall biosynthesis[3,4]. Studies on bacterial autolysin [5,6,7] and its inhibitor, lipoteichoic acid revealed that lipoteichoic acids from some Gram-positive bacteria such as *Streptococcus faecalis*, *S. pneumoniae* or *S. aureus* inhibit the autolysin activity[8~12]. Lipoteichoic acids were also implicated in the triggering of bacterial autolysin activity so that the bacteriolytic and bactericidal effects of penicillin on some bacteria were brought about[7,9,10]. Spontaneous release of lipoteichoic acids from streptococci and lactobacilli in both exponential and stationary phases was reported[13,14]. In addition, stimulated release of cellular lipoteichoic acid from *S. pyogenes* or *S. sanguis* during the treatment with penicillin or other cell wall inhibitors was reported[15,16,17]. Similarly the release of lipoteichoic acid from *S. aureus* by treatment with oxacillin was reported[13].

In the present study bacteriolytic and bactericidal actions of cefmetazole against several resistant strains of *S. aureus* were examined, and the release of cellular lipoteichoic acid (secreted radioactivity from glycerol-labeled cultures) from these strains and from a sensitive strain after treatment with

cefmetazole was also investigated in comparison with other β-lactam antibiotics.

Materials and Methods

Chemicals

Cefmetazole (Sankyo Co., Ltd., Tokyo), cefazolin (Fujisawa Pharmaceutical Co., Osaka), cefotiam (Takeda Chemical Ind., Osaka), cefoxitin (Daiichi Seiyaku Chemical Co., Tokyo) and cefuroxime (Shin Nihon Jitsugyo Co., Tokyo) were commercially available. [^{14}C]-Labeled glycerol (11.8 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, Mass.).

Bacteria

Four cefazolin-resistant strains of *S. aureus* clinically isolated at Tokyo Clinical Research Center and one sensitive strain of *S. aureus* FDA209P were used.

Antibacterial Activity

Minimum inhibitory concentrations (MIC) of β-lactam antibiotics were determined by using two-fold dilutions in heart infusion agar (Eiken Chemical Co., Tokyo) with an inoculum of 10^8 cfu/ml.

Measurement of Bacteriolytic and Bactericidal Actions

One ml of cultures grown in Trypto-soy broth (TSB, Eiken Chemical Co.) for 20 hours at 37°C was added to 100 ml of fresh TSB and the culture fluids were incubated with shaking at 37°C for 2 hours. To these an antibiotic was added to a final concentration of 1, 10 or 100 μg/ml. Each culture was further incubated and the bacterial growth was measured at 0, 1, 2, 3, 4, 5 and 6 hours by following the change in turbidity (absorbance at 550 nm) with a spectrophotometer (Model 139, Hitachi Ltd., Tokyo). Simultaneously the viable cell number was counted at 0, 1, 2, 4 and 6 hours by the pour plate method.

Release of Cellular Lipoteichoic Acid

One-tenth ml of overnight cultures was inoculated into 10 ml of fresh TSB and the culture fluid was incubated with shaking at 37°C for 1 hour. Then 50 μl of [^{14}C]-labeled glycerol (5 μCi) was added to the cultures. Which were further incubated and harvested at late exponential phase (absorbance at 550 nm, 1.0). After three washes with isotope-free TSB, the cell pellets were resuspended in the same broth and a cell suspension was prepared to an absorbance of 0.2 (550 nm). An antibiotic was added into the cell suspension to a final concentration of 1, 10 and 100 μg/ml, and each culture was incubated with shaking at 37°C. Five hundred μl of the culture was removed at 0, 30, 60, 90 and 120 minutes and centrifuged at 12,000 rpm for 5 minutes. The resulting supernatant solution (250 μl) was added to a glass scintillation vial with 1 ml of Bio-solv (BBS-3, Beckman Instruments Inc., Fullerton, Calif.) as an aqueous solubilizer and 9 ml of scintillation fluid consisting of 8 g of 2,5-diphenyloxazole and 0.2 g of 2,2′-diphenylenbis(4-methyl-5-phenyloxazole) in 1 liter of toluene. The radioactivity in the supernatant was measured with a liquid scintillation spectrometer (Model LS-9000, Beckman Instruments Inc.).

Results

Bacteriolytic and Bactericidal Actions

The effect of cefmetazole or cefazolin on the growth of a resistant strain of *S. aureus* No. 109 is shown in Fig. 1. At 1 μg/ml both cefmetazole and cefazolin had little effect on turbidity or viability during 6 hours of incubation. These antibiotics at 10 μg/ml or 100 μg/ml did not reduce turbidity during the first 1 hour of incubation, but caused a reduction of viable cell number. After 2 hours of incubation bacteriolysis occurred with concentrations of 10 μg/ml and 100 μg/ml of cefmetazole and 100 μg/ml of cefazolin, and viability decreased 1,000-fold or more as compared with that at zero time. At a concentration of 10 μg/ml cefazolin exerted a growth inhibitory effect during the first

Fig. 1. Effect of cefmetazole and cefazolin on the growth of a resistant strain of *S. aureus* No. 109.

An antibiotic was added to exponentially growing cultures at zero time (shown by the arrow). Bacteriolytic action (A) was measured by turbidity, and bactericidal action (B) was followed by viable cell count at the times indicated. Symbols: (▽), control; (▲, △), 1 μg/ml; (■, □), 10 μg/ml; (●, ○), 100 μg/ml. Solid symbols, cefmetazole; open symbols, cefazolin.

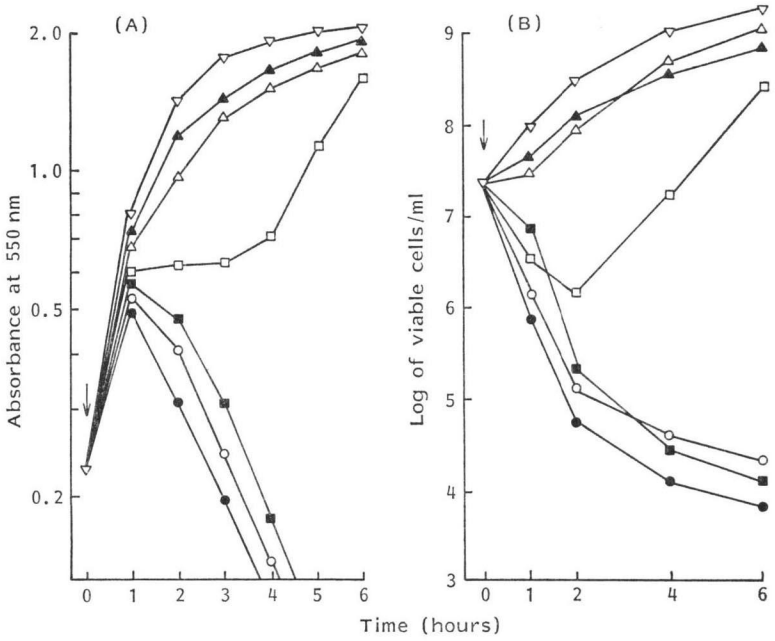


Fig. 2. Comparison of bacteriolytic (A) and bactericidal (B) activities against a resistant strain of *S. aureus* No. 109 at a concentration of 10 μg/ml of various antibiotics.

Symbols: (▽), control; (●), cefmetazole; (○), cefazolin; (■), cefotiam; (□), cefoxitin; (▲), cefuroxime.

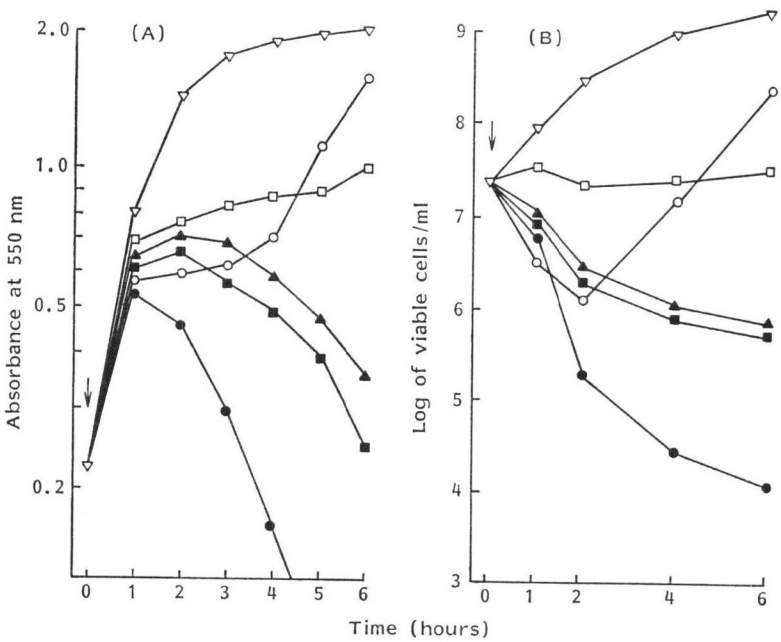


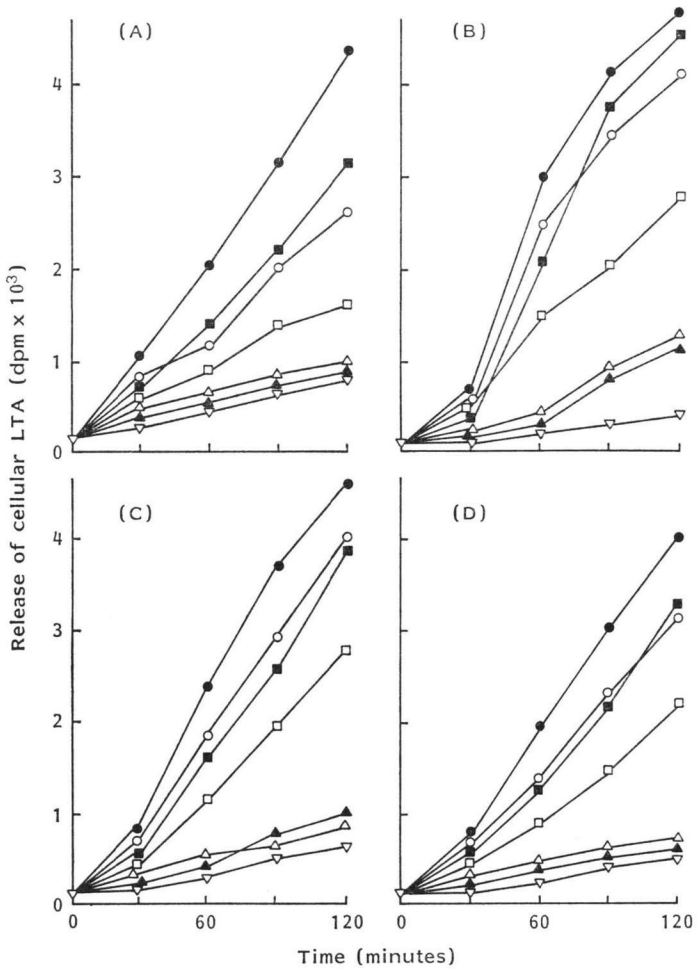
Table 1. MIC of five β-lactam antibiotics against several strains of *S. aureus*.

Strain No.	MIC (μg/ml)				
	Cefmetazole	Cefazolin	Cefotiam	Cefoxitin	Cefuroxime
32	6.25	50	25	25	100
109	6.25	25	6.25	12.5	12.5
120	6.25	6.25	6.25	12.5	12.5
169	6.25	12.5	6.25	25	12.5
FDA209P	0.78	0.20	0.20	1.56	0.78

Fig. 3. Release of cellular lipoteichoic acid from strains No. 32 (A), No. 109 (B), No. 120 (C) and No. 169 (D) of *S. aureus* by treatment with cefmetazole or cefazolin.

At the times indicated a portion of the culture was removed and centrifuged, and the radioactivity in the supernatant was measured.

Abbreviation: LTA, lipoteichoic acid. Symbols are the same as in Fig. 1.



2 hours, but thereafter regrowth of the cells was observed. In the cases of other three resistant strains (No. 32, No. 120, No. 169), although the susceptibility to cefazolin was different (Table 1), similar phenomena were observed, *i.e.*, cefmetazole at 10 μg/ml exhibited nearly the same activities as 100 μg/ml of cefazolin against the growth of these strains.

Fig. 4. Comparison of the releasing activity against a resistant strain of *S. aureus* No. 109 at a concentration of 10 μg/ml of various antibiotics.

Abbreviation: LTA, Lipoteichoic acid. Symbols are the same as in Fig. 2.

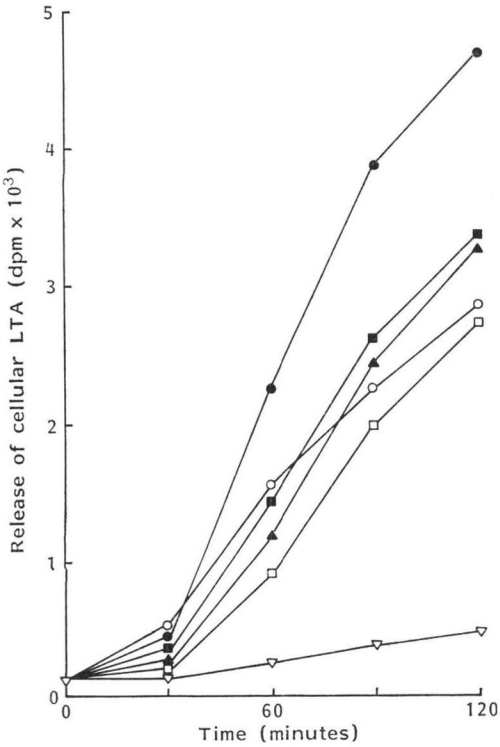
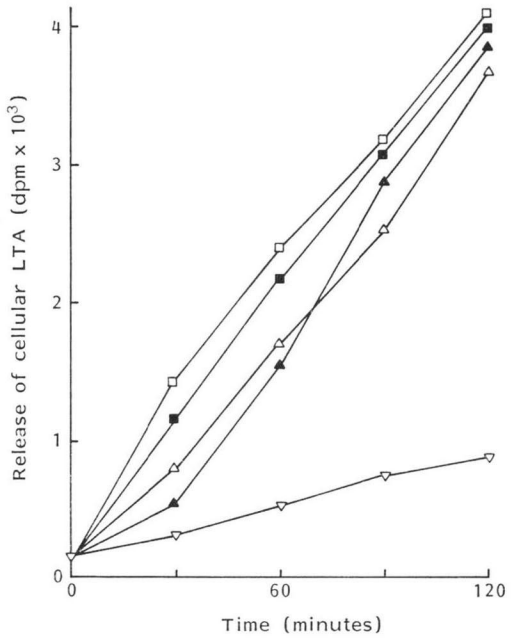


Fig. 5. Release of cellular lipoteichoic acid from a sensitive strain of *S. aureus* FDA209P by treatment with cefmetazole or cefazolin.

Abbreviation: LTA, lipoteichoic acid. Symbols: (▽), control; (▲, △), 1 μg/ml; (■, □), 10 μg/ml. Solid symbols, cefmetazole; open symbols, cefazolin.



The effect of 10 μg/ml of various antibiotics on the resistant strain No. 109 are shown in Fig. 2. Cefoxitin exhibited only static action during 6 hours of incubation. Both cefotiam and cefuroxime caused reduction of turbidity and viability to a lesser extent than did cefmetazole. From these results it is indicated that cefmetazole has more potent bacteriolytic and bactericidal activities against cefazolin-resistant strains of *S. aureus* than do cefazolin, cefotiam, cefoxitin or cefuroxime.

Release of Cellular Lipoteichoic Acid

The release of cellular lipoteichoic acid from four resistant strains of *S. aureus* by treatment with cefmetazole or cefazolin at concentrations of 1, 10 and 100 μg/ml, is shown in Fig. 3. In all strains release of cellular lipoteichoic acid increased with time in response to the indicated concentrations of cefmetazole or cefazolin. With these strains cefmetazole at 100 μg/ml induced the release of lipoteichoic acid to the highest degree, followed by cefazolin at 100 μg/ml, cefmetazole at 10 μg/ml and cefazolin at 10 μg/ml in descending order. The releasing activity of cefmetazole at 10 μg/ml was nearly the same as that of cefazolin at 100 μg/ml. At 1 μg/ml both cefmetazole and cefazolin induced no release.

Comparison of the releasing activity against the resistant strain No. 109 at 10 μg/ml of various antibiotics is shown in Fig. 4. Cefmetazole induced a more marked release from this strain than the other β-lactams.

The release from a sensitive strain of *S. aureus* FDA209P after treatment with cefmetazole or cefazolin is shown in Fig. 5. At 1 μg/ml both antibiotics induced a more marked release of cellular lipoteichoic acid from this strain than from the resistant strains. The release induced by cefmetazole was not significantly different from that by cefazolin at concentrations of 1 μg/ml and 10 μg/ml.

Discussion

It has been well known that bacterial autolysin is implicated in elongation, division and separation of a cell during bacterial growth[19,20,21]. ROGERS *et al.*[5] and TOMASZ *et al.*[6,7] reported that the lytic effect of penicillin was due to the action of autolytic enzymes in the cell, and CLEAVELAND *et al.*[8,9,22] reported that autolysin activity was inhibited by the lipoteichoic acid in streptococci. But little had been known about the physiological function of the lipoteichoic acid in *S. aureus*, and its role in the inhibition of cell lysis. Recently, SUGINAKA *et al.*[11] reported that the prevention of penicillin-induced lysis of *S. aureus* by lipoteichoic acid was due to the inhibition of autolytic activity of bacteria. Furthermore, the cells protected from penicillin-induced lysis were viable, but these were in static state. The growth of cells was not changed following the addition of lipoteichoic acid alone, indicating that the lipoteichoic acid had no influence on the peptidoglycan synthesis. Release of the cellular lipoteichoic acid, an autolysin inhibitor, from streptococci[15,16,17] and *S. aureus*[11,18] by treatment with penicillin has been reported. These facts suggest that the direct action of penicillin is merely stasis, and that the bacteriolysis is caused by the activation of autolysin, *i.e.*, triggering, through the release of cellular lipoteichoic acid.

In the present study cefmetazole exhibited more potent lytic and killing activities against several resistant strains of *S. aureus* than cefazolin or other β-lactam antibiotics. The release of cellular lipoteichoic acid was also observed by treatment with cefmetazole or other β-lactams. Cefmetazole induced more marked release of cellular lipoteichoic acid from resistant strains than did cefazolin, cefotiam, cefoxitin or cefuroxime, whereas, the releasing activity of cefmetazole against a sensitive strain was nearly the same as that of cefazolin. From these results we have concluded that cefmetazole induces a more potent activation of autolysin through the release of cellular lipoteichoic acid from resistant strains of *S. aureus*, and therefore cefmetazole has more potent bacteriolytic (killing) effects on these strains in comparison with cefazolin, cefotiam, cefoxitin and cefuroxime.

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