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Mechanism of Resistance to Some Cephalosporins in *Staphylococcus aureus*

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The mechanism of resistance to some cephalosporins in *Staphylococcus aureus* strains was investigated with high-pressure liquid chromatography and nuclear magnetic resonance spectrometry. Drug inactivation by penicillinase was found to be the main mechanism of resistance to cefazolin, cephaloridine, and cephalothin in *S. aureus*.

Cephalosporins (CEPs) have been widely used for the treatment of staphylococcal infections. Recently, an increase in the number of CEP-resistant *Staphylococcus aureus* strains has been reported (1, 10). In a previous paper, we suggested that penicillinase (PCase) might play a part in the mechanism of resistance to cefazolin (CEZ) in resistant *S. aureus* owing to the fact that there is a positive correlation between the minimal inhibitory concentrations (MICs) of CEZ and the specific activities of β-lactamase to penicillin G in 40 CEZ-resistant strains. In this study, the mechanism of resistance to some CEPs in *S. aureus* was further examined with high-pressure liquid chromatography and nuclear magnetic resonance (NMR) spectrometry.

S. aureus strains SA1 and SA159 were used as CEP-resistant bacteria which were selected from 40 CEZ-resistant strains clinically isolated between 1979 and 1980 (11). Strains SA7, isolated during the same period, and MS353, reported to be plasmid free (8), were used as the susceptible strains. MICs were determined by an agar dilution method (6; Table 1).

Bacterial growth in broth containing CEZ, cephaloridine (CER), cephalothin (CET), or cefmetazole (CMZ) was determined turbidimetrically. During bacterial growth in drug-containing broth, 0.1-ml samples were withdrawn every hour to determine the drug concentration by high-pressure liquid chromatography (Fig. 1). An overnight broth culture of bacteria was diluted with fresh broth and shaken at 37°C. After 2 h, 0.3 ml of the culture was inoculated into 9.6 ml of broth containing one-eighth the MIC of CEZ and shaken for 30 min. A 0.1-ml amount of each drug solution was added to the CEZ-induced culture to make the concentration twice the MIC of CEZ, CER, CET, or CMZ. Bacterial growth was determined turbidimetrically. A sample of 1.0 ml of the culture was taken every hour and centrifuged at 10,000 rpm for 15 min, and the supernatant was filtered with a membrane filter (pore size, 0.45 μm). For high-pressure liquid chromatographic determination, 10 μl of the filtrate was used. A high-pressure pump (model 6000A), a radialpack C_{18} column (8 mm by 10 cm), and a UV detector (model 440; all from Japan Waters Co., Ltd., Tokyo, Japan) were used and a wavelength of 254 nm was adopted. Generally, 0.02 M KH_2PO_4–acetonitrile (85:15) was used as the mobile phase, but a solution of 0.02 M KH_2PO_4–methanol (60:40) was also used for CET analysis. In the broth containing CEZ with strain SA1 or SA159, the drug concentration rapidly decreased within 2 to 3 h, after which bacterial growth occurred. Decreases in drug concentration and bacterial growth of these strains in CER-containing broth were more rapid than those in CEZ-containing broth. In broth containing CET, there was an inverse relationship between bacterial growth of these strains and decrease in drug concentration. Strains SA7 and MS353 did not cause a decrease in drug concentration in broth containing CER or in broth containing either CEZ or CET. Bacterial growth did not occur in broth containing CMZ with any of the strains tested. After 5 h of incubation in broth containing CEZ with strain SA1, the culture was filtrated through a membrane filter to remove bacterial cells. The filtrate inactivated the drug. From the results, it was assumed that a decrease in drug concentration in the culture was due to inactivation by the enzyme produced by the drug-resistant bacteria.

Hydrolysis of CEPs by drug-resistant strain SA1 was then analyzed by a modified method of NMR spectrometry (7). Briefly, 20-ml samples of the CEZ-induced culture (see above) were collected by centrifugation, washed with 0.1 M

TABLE 1. MICs for strains used in this study

S. aureus strain	MIC (μg/ml) of[a]:							
	PCG	ABPC	MCIPC	MDIPC	CEZ	CER	CET	CMZ
SA1	50	50	0.4	0.4	6.25	3.13	6.25	6.25
SA159	100	50	1.56	0.4	50	6.25	6.25	12.5
SA7	1.56	1.56	0.2	0.1	0.1	0.1	0.1	0.78
MS353	<0.1	<0.1	0.2	0.2	0.1	0.1	0.2	0.4

[a] PCG, Penicillin G; ABPC, ampicillin; MCIPC, cloxacillin; MDIPC, dicloxacillin.

phosphate buffer (pH 5.8), and lyophilized. The lyophilized cells, 50 mg of CEZ or CER, and 0.4 ml of 0.1 M phosphate buffer solution in D_2O were placed in a test tube for NMR determination and allowed to stand at 37°C. NMR spectrum analysis was carried out with a 90-MHz NMR spectrometer (EM-390; Varian Associates, Inc.). Lyophilized cells of strain SA1 or MS353 were incubated with CEZ or CER in an NMR tube as described above. A decrease in integrated values for the 6-H and 7-H proton signals of the β-lactam ring was observed after incubation for 20 h with strain SA1. The kinetics of hydrolysis of CEZ and CER with strain SA1 and drug-susceptible strain MS353 are shown in Fig. 2. The hydrolysis rates of CEZ were 57.5 and 3.4% with strains SA1 and MS353, respectively. With strain SA1, CER hydrolysis occurred more rapidly than did CEZ hydrolysis, the hydrolysis rate of CER being 2.7 times that of CEZ. Under the same conditions, CET hydrolysis was not observed; however, a visible change in the β-lactam ring of CET was observed in the NMR spectrum when ingredients of the reaction mixture were changed. The hydrolysis rate of CET with strain SA1 was calculated as 7.6% when the background hydrolysis (28.3%) of the β-lactam ring of CET with MS353 was subtracted. The lyophilized cells from 20 ml of the CEZ-induced culture, 10 mg of CET, and 0.4 ml of Penassay broth (Difco Laboratories, Detroit, Mich.) were mixed in an NMR tube.

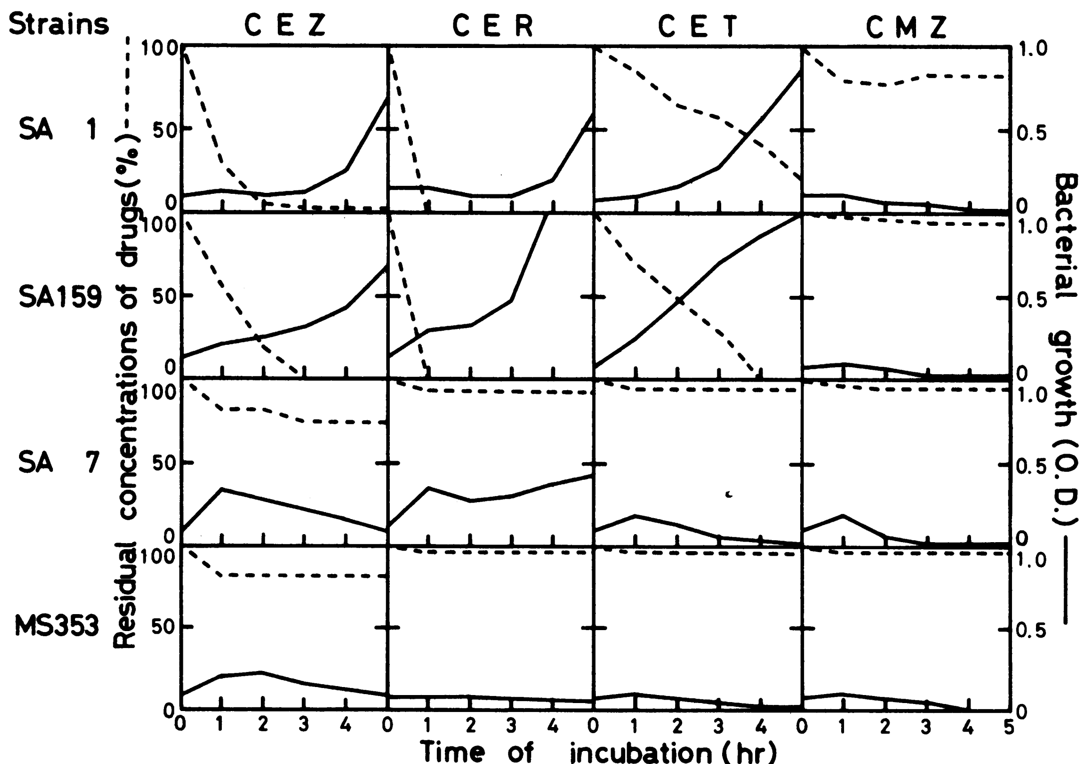

FIG. 1. Bacterial growth of CEP-resistant and -susceptible strains in broth containing CEZ, CER, CET, and CMZ and changes in drug concentrations in culture over time.

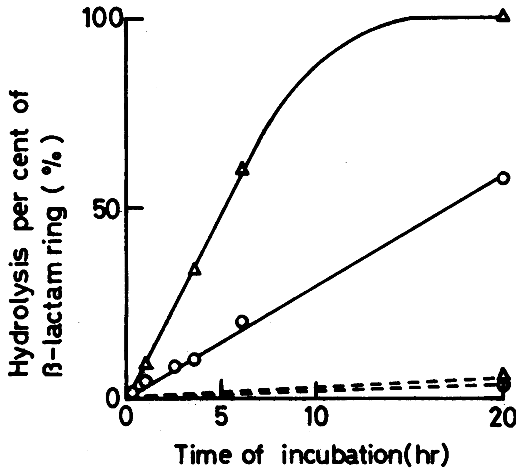

FIG. 2. Kinetics of hydrolysis of the β-lactam ring of CEZ or CER by NMR spectrometry. Lyophilized cells of strain SA1 (———) or MS353 (- - - - -) were incubated with CEZ (O) or CER (Δ) in an NMR tube as described in the text.

The NMR spectra of the suspension were measured after incubation for 20 h at 37°C. The background hydrolysis (28.3%) of the β-lactam ring of CET with strain MS353 was subtracted to obtain values of 7.6 and 0% for strains SA1 and MS353, respectively.

The β-lactamase produced by the drug-resistant strains has been found to be a PCase type from the substrate profiles, suggesting that resistance determinants to CEZ and penicillin G are governed by a plasmid, since both of the resistance determinants were irreversibly coeliminated (11). From these findings, it was concluded that CEZ, CER, or CET was more or less hydrolyzed even by the low hydrolyzing ability of PCase produced by CEP-resistant strains. In contrast, CMZ, which is known to be resistant to β-lactamase, remained in the broth without losing its antibacterial activity in these experiments, since it did not become hydrolyzed. It has been thought that the CEP resistance mechanism of *S. aureus* is not related to PCase (9) but is related to a change in penicillin-binding proteins (2–5). Our results indicate, however, that inactivation of CEPs by PCase is an important mechanism of resistance.

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