ANTIBACTERIAL ACTIVITY OF β-LACTAM ANTIBIOTICS AGAINST *STAPHYLOCOCCUS AUREUS*: EFFECT OF INOCULUM SIZE AND β-LACTAMASE STABILITY

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Clinical isolates of *Staphylococcus aureus* from 13 hospitals in Japan were divided into 66 strains of methicillin-sensitive *Staphylococcus aureus* (MSSA ; MIC≦3.13 μg/ml : inoculum size 10^6 and 10^8 CFU/ml) and 53 strains of methicillin-resistant *Staphylococcus aureus* (MRSA ; ≧12.5 μg/ml : 10^6 CFU/ml). The MICs of five β-lactam antibiotics against the MSSA and MRSA strains were determined with inoculum sizes of 10^6 and 10^8 CFU/ml.

In the MSSA strains, the MICs of ampicillin, cefaclor, and cefazolin, in that order, shifted towards the resistant side with an inoculum size of 10^8 CFU/ml as compared with 10^6 CFU/ml. But those of methicillin and cefmetazole were unchanged.

In the MRSA strains, the MICs of all the β-lactam antibiotics tested shifted towards the resistant side according to the increase in inoculum size.

Substrate profiles in β-lactamase from *S. aureus* were determined. Ampicillin, cefaclor, and cefazolin, in that order, were hydrolyzed. The results reflected the effect of the inoculum size on the MICs against the MSSA strains.

Key words : *S. aureus*, Inoculum size, β-lactamase, MSSA, MRSA

INTRODUCTION

Since 1980, MRSA strains have been frequently isolated from clinical specimens. UBUKATA[1], UTSUI[2] and HARTMAN[3] have independently reported that the resistance of MRSA against β-lactam antibiotics was due to the production of penicillin-binding protein 2′ (PBP 2′). Before that, however, β-lactamase had been considered the main mechanism of resistance against β-lactam antibiotics in *S. aureus*. Most of the recent clinical isolates of *S. aureus*, irrespective of MRSA and MSSA, have produced β-lactamase when tested with nitrocephin. We therefore think it important to distinguish between β-lactamase and PBP 2′ as the cause of the resistance. In this paper we elucidate the contribution of β-lactamase to the resistence of *S. aureus* against β-lactam antibiotics.

MATERIALS AND METHODS

Bacterial strains. 119 *S. aureus* strains were isolated from 13 hospitals in Japan from 1985 to 1986.

Antibiotics. All antibiotics were kindly provided : methicillin (Banyu), benzylpenicillin, ampicillin, cloxacillin (Meiji Seika), cefmetazole (Sankyo), cefazolin (Fujisawa), cafaclor, cefamandole (Shionogi), piperacillin (Toyama Chimical), cephaloridine (Nihon Glaxo).

Determination of MICs. Minimum inhibitory concentrations (MICs) were determined by the two-fold agar dilution method. For preculture and MIC determination, sensitivity test broth (Nissui) and sensitivity disk agar (Nissui) were used. Precultures were incubated for 20 h at 30°C. One loopful (about 5 μl) of 10-fold diluted preculture (10^8 CFU/ml) or 1000-fold diluted preculture (10^6 CFU/ml) was inoculated on agar plates containing various concentrations of antibiotics with a Microplanter (Sakuma Seisakusho). The plates were incubated for 18 h at 37°C. The MIC was defined as the lowest antibiotic concentration which inhibited visible bacterial growth.

Stability of β-lactamase. Cells of *S. aureus* strains were disintegrated by ultrasonication and centrifuged at 15,000×g. The supernatant fluid was used as the crude enzyme. Hydrolysis of β-lactam antibiotics was assayed by a modification of the microiodometric method[4].

Table 1. Influence of culture temperature on the preculture viable cell count

Strain \ Preculture	Viable cell count ($\times 10^9$ CFU/ml)	
	30°C, 20 h	37°C, 20 h
S. aureus 209 P	1.3	0.98
MS353	1.3	0.48
M-12	1.1	0.095
BI-1	1.4	2.3
BI-6	0.91	0.65
BI-9	1.2	0.58
BI-110	1.2	0.42
BI-186	1.1	0.33
BI-354	0.62	0.38
BI-355	1.1	0.13
BI-359	0.73	0.67
BI-364	0.82	0.44
BI-369	0.69	0.81
BI-373	2.5	0.15

Medium : Sensitivity Test Broth (Nissui)

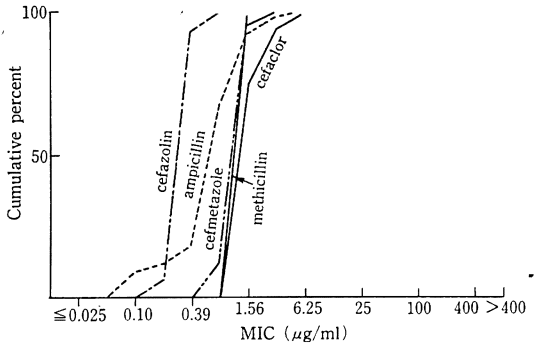

Fig. 1. Antibacterial activity against MSSA, 66 strains (10^6 CFU/ml)

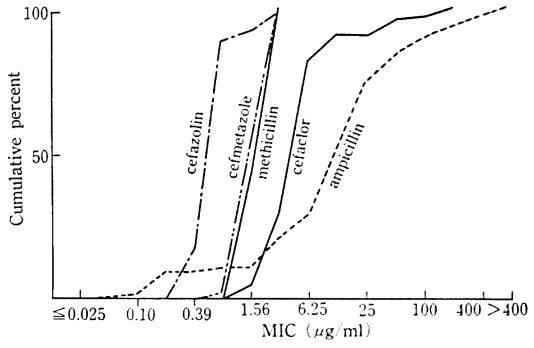

Fig. 2. Antibacterial activity against MSSA, 66 strains (10^8 CFU/ml)

RESULTS

Influence of culture temperature on the preculture viable cell count. In a previous report Laverdiere et al. showed the effect of inoculum size on the MICs of β-lactam antibiotics against *S. aureus*[5]. In determining the MIC, it is therefore important to prepare the inoculum size exactly.

Viable cell counts of the precultures which were incubated at 30°C or 37°C for 20 h for 14 *S. aureus* strains selected at random are shown in Table 1. In the preculture at 30°C the viable cell counts of all strains tested were about 1×10^9 CFU/ml. On the other hand, at 37°C those of several strains decreased to about 1×10^8 CFU/ml. The preculture condition for the MIC determination was therefore set up at 30°C for 20 h.

MICs against MSSA. The MICs of five β-lactam antibiotics, namely, methicillin, ampicillin, cefazolin, cefaclor, and cefmetazole, against 66 MSSA strains were determined. The MIC distributions at 10^6 and 10^8 CFU/ml are shown in Figs. 1 and 2.

At 10^8 CFU/ml, as compared with 10^6 CFU/ml, the curves of the MIC distributions of ampicillin, cefaclor, and cefazolin, in that order, were gently-sloping and shifted towards the resistant side. On the other hand, those of methicillin and cefmetazole were unchanged by the inoculum size.

MICs against MRSA. The MICs of the same five β-lactam antibiotics against 53 MRSA strains were determined. The MIC distributions at 10^6 and 10^8 CFU/ml are shown in Figs. 3 and 4. In MRSA the MIC distributions of all five β-lactam

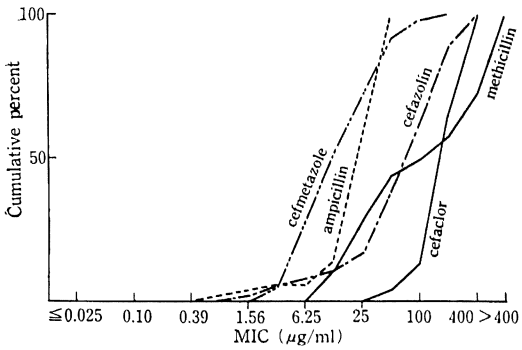

Fig. 3. Antibacterial activity against MRSA, 53 strains (10^6 CFU/ml)

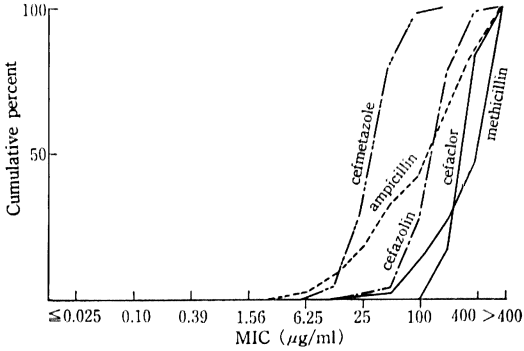

Fig. 4. Antibacterial activity against MRSA, 53 strains (10^8 CFU/ml)

Table 2. Substrate profile of β-lactamase from *S. aureus*

β-lactamase source / Substrate	Relative rate of hydrolysis*			
	MS15009/pI258	BI-40 (MSSA)	M-12 (MRSA)	BI-52 (MRSA)
Benzylpenicillin	100	100	100	100
Ampicillin	160	160	130	130
Piperacillin	230	200	210	190
Cloxacillin	0.23	0.21	0.21	0.23
Methicillin	>0.1	>0.1	>0.1	>0.1
Cefaclor	13	13	16	13
Cephaloridine	0.58	0.53	0.38	0.51
Cefazolin	0.31	0.24	0.27	0.29
Cefamandole	>0.1	>0.1	>0.1	>0.1
Cefmetazole	>0.1	>0.1	>0.1	>0.1

* Relative rate of hydrolysis is expressed as the percentage of hydrolysis of benzylpenicillin. Assay : microiodometric method.

antibiotics were influenced by the inoculum size and shifted towards the resistant side.

Stability to β-lactamases. The stability of β-lactam antibiotics to a typical β-lactamase produced by *S. aureus* (plasmid pI258-mediated β-lactamase) and β-lactamases from one clinical MSSA strain and two clinical MRSA strains were determined. The relative rate of hydrolysis is shown in Table 2.

These enzymes had the same substrate profile. Ampicillin was the most unstable, and subsequently cefaclor, cephaloridine, cefazolin, in that order, were hydrolysed, though methicillin and cefmetazole were not.

DISCUSSION

There are known to be two resistance mechanisms to β-lactam antibiotics in *S. aureus*. As to β-lactamase, our experiments on MSSA showed that the more unstable the β-lactam antibiotic to β-lactamase, the more the MIC was influenced by the inoculum size. We therefore assume that β-lactamase is the main resistance factor in MSSA.

In MRSA, two factors, both PBP 2′ and β-lactamase, may be involved in the resistance. Methicillin and cefmetazole were little hydrolyzed by β-lactamase, but their MICs against the MRSA strains were changed to some extent by the inoculum size. In MRSA, the MICs of all β-lactam antibiotics were influenced greatly by the inoculum size. UBUKUTA has suggested the reason for this is the production of inducible PBP 2′[1,6]. We think it is also important that highly-methicillin-resistant isolates (>400 μg/ml) can be selected easily on methicillin-containing agar plates from methicillin-intermediate-resistant strains (50 μg/ml)[7].

In this study we determined the stability of β-lactam antibiotics against β-lactamases originating from MSSA and MRSA. No difference was found in the substrate profiles of both enzymes, whose production was inducible. By comparing the affinity to and the inducibility of PBP 2′ of β-lactam antibiotics under the same conditions, their antibacterial activity against MRSA strains can be

explained more clearly.

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黄色ブドウ球菌に対する各種 β-ラクタム剤の抗菌力 —接種菌量の影響と β-ラクタマーゼ安定性について

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臨床材料より分離した黄色ブドウ球菌 119 株を，methicillin 感受性株 66 株（MSSA；MIC≦3.13 μg/ml：接種菌量 10^6 並びに 10^8 CFU/ml），methicillin 耐性株 53 株（MRSA；MIC≧12.5 μg/ml：10^6 CFU/ml）の二群に分け，それぞれについて各種 β-ラクタム剤の MIC を接種菌量 10^6，10^8 CFU/ml の二点を取って測定した。

MSSA については 10^6 に比して 10^8 接種で ampicillin＞cefaclor＞cefazolin の順で MIC の耐性側への移行がみられたが，cefmetazole, methicillin は接種菌量による MIC の変動，すなわち耐性化がみられなかった。

MRSA については 10^6 に比して 10^8 接種ですべての β-ラクタム剤で MIC の耐性側への移行がみられた。

各薬剤の黄色ブドウ球菌由来の β-ラクタマーゼに対する相対加水分解速度を測定したが，ampicillin＞cefaclor＞cefazolin の順に分解され，MSSA における接種菌量の影響を反映する結果が得られた。

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