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The Mechanism of Sensitizing Effect of a Triazine Dye, Cibacron Blue F3GA, on Methicillin-resistant *Staphylococcus aureus* to Oxacillin

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ABSTRACT

We recently found that a triazinyl dye, cibacron blue F3GA (CB), has a sensitizing effect on the in-vitro susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) to oxacillin (C. Shirai, M. Sugai, H. Komatsuzawa, K. Ohta, M. Yamakido, H. Suginaka, Antimicrob. Agents Chemother. 42: 1278–1280, 1998). Among nine triazinyl dyes tested, CB showed the strongest sensitizing effect. Population analysis demonstrated that CB reduced the resistance level of MRSA. In the presence of oxacillin at subinhibitory concentrations, CB inhibited the growth of MRSA, but its effect on the cells appeared to be bacteriostatic. Under experimental conditions, CB did not affect the amount of PBP2', binding of [^{14}C]benzylpenicillin to PBP2', or peptidoglycan susceptibilities to bacteriolytic enzymes. Autolytic enzyme-deficient MRSA mutants were equally as sensitive as the parent strain to the effect of CB on the susceptibility to oxacillin. CB affected the resistance level of MRSA irrespective of the status of the *mecI*/*mecR1* element and/or penicillinase plasmids. The sensitivities of several bacteriolytic enzymes to heat-inactivated MRSA cells were not affected when the cells were grown in the presence of CB. CB did not stimulate the release of lipoteichoic acid from the cells. These results suggest that the sensitization effect of CB cannot be simply explained by its effect on *mecA* related products, autolysins, *femAB* products or the release of lipoteichoic acid.

Key words: *MRSA, Cibacron Blue, Susceptibility, ß-lactam*

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become one of the most important nosocomial pathogens in the world and requires strict infection control. The resistance to virtually all ß-lactam antibiotics of MRSA is mediated by an extra penicillin binding protein (PBP) 2' (or 2A), which is encoded by *mecA*, a structural gene within the *mec* locus[17,36,40,57]. PBP2' has low affinity for ß-lactam antibiotics and is thought to be the only PBP still functioning in cell wall peptidoglycan synthesis at ß-lactam concentrations high enough to saturate the normal PBPs of *S. aureus*. The expression of methicillin resistance is classified into two types: homogeneous and heterogeneous[56]. The level of resistance varies among strains. Differences in the transcription or translation of *mecA* may affect the methicillin resistance level. *mecI-mecR1*, which are located upstream of *mecA*, and *blaI* were shown to regulate the production of PBP2'[19,22–24,38,55]. However, the amount of PBP2' does not always coincide with the resistance level, suggesting that genes other than *mecA*-related regulatory genes are involved in methicillin resistance. Indeed, several chromosomal sites which on inactivation by transposon insertion decrease methicillin resistance, were identified outside the *mecA* gene[4,6,14,37], including a series of *fem*s [5,18,21,30,34,39,59], *llm*[35], sigma factor[60] and *fmt*[26]. It is now apparent that genes other than these are involved in the expression of methicillin resistance.

Cibacron blue F3GA (CB) is a triazinyl dye widely used as an affinity ligand for dye-ligand chromatography (for review,[15,33,47]). CB is structurally similar to naturally occurring heterocycles such as nucleoside phosphate, NAD^+, coenzyme A and folic acid[1–3,16]. It has been demonstrated that CB specifically binds to the nucleotide binding site of kinases and dehydrogenases, and some of the enzyme activities were inhibited by CB[1,7,11,12].

We have recently found that CB has a sensitizing effect on the in-vitro susceptibility of MRSA to

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oxacillin[43]. The aim of this study was to investigate the effect of CB on the possible targets involved in the resistance mechanisms of MRSA to oxacillin.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Twenty seven clinical isolates of MRSA and two methicillin-sensitive *S. aureus* (MSSA), MSRN450 and MS108–4–1, were studied; methicillin resistance was defined as a MIC of > 4 μg/ml of oxacillin. N315 and its isogenic strains, N315P, a penicillinase plasmid-deleted strain of N315, and N315-IR74, which contains *tet* inserted into *mecI*, MR6, MR108–4, MS108–4–1 were gifts from Dr. Hiramatsu[31]. Autolysin-defective mutants, Lyt-2 and Lyt-5, were derivatives of MR6 after mutagenesis with ethylmethan sulfate as described[28]. Other MRSA strains and MSSA strains were from our laboratory stock.

Antibiotics and reagents

The antibiotics used in this study (and their respective suppliers) were as follows: 6-phenyl [1-^{14}C]benzylpenicillin (10–30 Ci/mmol) and [2-^{3}H]glycerol (0.5–1 Ci/mmol) (Amersham International, Buckinghamshire, England); oxacillin (MPIPC, Sigma Chemical Co., St Louis, MO). For the paper disk assay, an oxacillin disk (30 μg, Showa-disk, Showa Yakuhin Co. Ltd., Tokyo) was used. The triazinyl dyes used in this study were cibacron blue F3GA (CB, Sigma C9534); Reactive blue 4 (Sigma R9003); Reactive blue 5 (Sigma R9503); Basillen blue E3G (Sigma R5520); Reactive Brown 10 (Sigma R0385); Reactive Red 2 (Sigma R7138); Reactive Yellow 2 (Sigma R8003); Reactive Green 19 (Sigma R9253); Reactive Orange 14 (Sigma R8254). The lysostaphin was from Sigma. 51 kDa *N*-acetylglucosaminidase (GL) and 62 kDa *N*-acetylmuramyl-L-alanine amidase (AM) were purified as described[49,50]. Other reagents were obtained from commercial sources. CB used in this study is formerly assigned as Reactive blue 2 R4502, which has a ring "A" of ortho-sulfonic acid[10,20]. CB purified with reversed-phase HPLC according to the procedure described by Hanggi and Carr[20] behaved similarly to unpurified CB, and consequently CB was used without purification in this study.

Susceptibility testing

MICs were determined by a microdilution method at 37°C or 30°C[28]. The medium used was Trypticase Soy Broth (TSB, BBL Microbiology Systems, Cockeysville, MD) and the initial suspensions, prepared by diluting overnight broth cultures of the study strains, contained 10^8 cfu/L. The MIC was defined as the lowest concentration which prevented visible growth after incubation without shaking for 24 hr. MBCs were determined by subculturing aliquots from each clear well on an antibiotic-free trypticase soy agar (TSA, Difco, Detroit, MI) plate, as described[27]. Susceptibility was also tested using a disk assay. A filter paper disk containing 30 μg of oxacillin (Showa-disk) was placed on a heavily seeded lawn of MRSA culture on a TSA plate. The zone of growth inhibition was observed after overnight incubation of the plate.

Monitoring bacterial growth and viability

An overnight culture of *S. aureus* in 10 ml of TSB was diluted 1000-fold with fresh TSB, and 9.9 ml volumes of the diluted suspensions were shaken for 1 hr at 37°C. Small portions were transferred into TSB alone or TSB containing oxacillin at a concentration of 1024 or 16 μg/ml and/or 50 μg/ml of CB, which were then reincubated at 37°C. At 0.5 hr intervals, 0.1 ml aliquots were withdrawn and subjected to mild sonication (Taitek) to disperse cell-clusters. The samples were plated on TSA plates for determination of the number of viable bacteria by the pour-plate method[27].

Population analysis

Population analysis was carried out as described elsewhere[42]. Small portions of overnight cultures were plated on TSA containing various concentrations of oxacilllin with or without CB (100 μg/ml). In the case of Lyt$^-$ mutant, cultures were briefly sonicated using an ultrasonic disrupter (UR200, output level 4, Tomy Seiko, Tokyo, Japan) to disperse cell-clusters before plating. Complete dispersion of the cell-clusters was confirmed by using a phase contrast microscope. The number of colony forming units (cfus) was determined after 48 hr incubation at 37°C.

Susceptibility of heat-killed cells to bacteriolytic enzymes

The susceptibilities of heat-inactivated whole cells to lysostaphin, GL, and AM, were studied using the strain MR108–4 by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)[32,48]. Overnight cultures of *S. aureus* in TSB with or without CB were washed with 10 mM phosphate buffered saline (PBS, pH 7.0) twice, and boiled at 100°C for 30 min in PBS containing 4% SDS. Cells were washed with distilled water 6 times and lyophilized. Cells (0.5 mg dry weight/ml) were incorporated into the polyacrylamide gel solution before polymerization. Two-fold dilutions of bacteriolytic enzyme preparations were applied to the well. After electrophoresis, the activity of the bacteriolytic enzyme was detected, as described previously[48]. The minimal concentration to exhibit a detectable bacteriolytic band in the gel was defined as MBD (minimal bacteriolytic dose).

PBP analysis

PBPs were detected according to the method of Brown and Reynolds[8]. Staphylococci were grown in TSB to mid-logarithmic phase at 37°C with shaking. The suspensions of *S. aureus*, further incubated for 60 min with or without CB (100 μg/ml), were washed with 50 mM Tris HCl buffer (pH 7.0) and resuspended in the same buffer containing DNase (10 μg/ml) and lysostaphin (100 μg/ml). The suspensions were then incubated at 37°C until lysis was complete and cooled to 4°C. Membranes were recovered by centrifugation at 40,000 *g* for 30 min at 4°C, resuspended in 10 mM phosphate buffer (pH 7.0) and incubated with 6-phenyl[1-^{14}C]benzylpenicillin (10–30 Ci/mmol) for 10 min at 30°C. In some experiments, membranes were recovered from staphylococcal cells grown in TSB to mid-logarithmic phase as described above and incubated with various concentrations of 6-phenyl[1-^{14}C]benzylpenicillin in the presence or absence of CB (100 μg/ml) for 10 min at 30°C. Alternatively, the membranes were incubated with a fixed concentration of 6-phenyl[1-^{14}C]benzylpenicillin in the presence or absence of CB (100 μg/ml) for various incubation periods. The reaction was stopped by adding 4% SDS. The PBPs were separated by SDS-PAGE and detected by fluorography.

Assay of LTA, lipids and teichoic acid

S. aureus strain MR1, 7, 8, and 24 were grown to the exponential phase and labeled for approximately 6 generations with [2-^{3}H]glycerol (0.5–1 Ci/mmol) in TSB at 37°C. To measure the lipoteichoic acid (LTA) released into culture supernatant, the cells were harvested by centrifugation at 10,000 *g* for 10 min at 4°C, washed and resuspended in warm TSB containing 10 mM glycerol to an absorbance at 660 nm (A660) of 0.15. The suspensions were then incubated at 37°C for a further 30 min, after which oxacillin and/or CB were added: a suspension without additives (control) was also included. All of the suspensions were reincubated at 37°C and 1 ml samples were withdrawn at 0.5 hr intervals. These were centrifuged at 10,000 *g* for 10 min at 4°C and lipids were extracted from the supernatants with a mixture of chloroform and methanol in a 2:1 (v/v) ratio[13]. The radioactivity of LTA in the upper water phase was measured. To measure cellular LTA, lipids and teichoic acid, cells were radiolabeled as described above in the presence or absence of CB and/or oxacillin in TSB at 37°C. All of the suspensions were centrifuged at 10,000 *g* for 10 min at 4°C and lipids were extracted from the pellets with a mixture of chloroform and methanol in a 2:1 (v/v) ratio. The radioactivities in the upper water phase and in the lower phase were attributed to LTA and lipids respectively. The radioactivity in the intermediate insoluble phase was attributed to cell wall teichoic acid.

RESULTS

Effect of CB on the in vitro susceptibility of MRSA to oxacillin

CB alone was not inhibitory to staphylococcal

Table 1. MICs and MBCs of oxacillin for MRSA in the presence or absence of CB at 30°C and 37°C

Strain	MIC (μg/ml)				MBC (μg/ml)			
temp.	30°C		37°C		30°C		37°C	
CB	−	+	−	+	−	+	−	+
1	1024	16	1024	16	N.D.	N.D.	N.D.	N.D.
2	512	16	1024	8	512	512	1024	1024
3	1024	32	512	32	N.D.	1024	1024	1024
4	1024	64	512	64	N.D.	N.D.	N.D.	N.D.
5	1024	64	512	64	N.D.	1024	1024	1024
6	1024	64	512	32	N.D.	1024	1024	1024
7	512	32	512	32	N.D.	512	512	512
8	1024	16	512	8	N.D.	N.D.	N.D.	N.D.
9	256	64	256	32	N.D.	256	256	256
12	512	8	256	8	512	512	512	256
13	512	16	256	8	512	512	512	256
14	256	4	256	2	N.D.	N.D.	N.D.	N.D.
15	512	4	256	4	N.D.	N.D.	N.D.	N.D.
17	512	4	256	2	N.D.	512	512	256
18	256	4	128	4	256	256	128	128
20	128	8	128	8	N.D.	N.D.	N.D.	N.D.
21	128	4	128	4	128	128	128	128
22	256	8	128	8	256	128	128	128
24	128	2	64	1	N.D.	N.D.	N.D.	N.D.
25	64	2	64	2	64	64	64	16
26	64	4	64	2	N.D.	N.D.	N.D.	N.D.

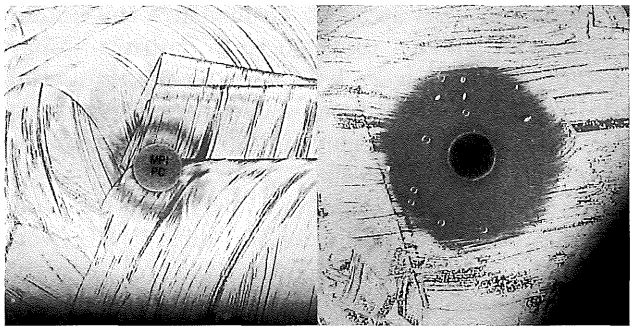

Fig. 1. Disk assay for oxacillin sensitivity of MRSA in the absence or presence of CB. A paper disk containing oxacillin was placed on a heavily seeded lawn of a strain MR12 culture on a Trypticase soy agar plate containing (a) none or (b) CB (100 μg/ml), and incubated overnight.

strains used in the experiments up to 2500 μg/ml. We have previously shown that MIC to oxacillin was significantly reduced in the presence of CB concentrations of 100 μg/ml or higher[43]. We therefore employed 100 μg/ml of CB for further studies unless otherwise noted. The influence of CB on the resistance to oxacillin was also studied at 30°C with 26 MRSA strains. At this lower temperature, the MICs were higher than at 37°C, as is typical of methicillin-resistant staphylococci. In the presence of CB, the strains became sensitive to oxacillin also under these conditions (Table 1). The MICs were reduced by factors from 8 to 128. CB was equally effective on the MIC of oxacillin at 37°C and 30°C. On the other hand, MBCs of oxacillin for MRSA did not change very much in the presence of CB at 37°C or 30°C. The sensitizing effect of CB on MRSA was also demonstrated by using a disk assay system. A paper disk containing oxacillin was placed on a heavily seeded lawn of a strain MR12 culture on the TSA containing 100 μg/ml of CB. The zone of inhibition was observed after overnight incubation, as shown in Fig. 1. Similar results were obtained with tested MRSA strains. The zone of inhibition further suggests that MRSA became sensitive to oxacillin in the presence of CB.

A population analysis of 28 MRSA strains and 2 MSSA strains was carried out in the presence or absence of CB (100 μg/ml). Typical effects upon the highly resistant MRSA and upon heterogeneously resistant MRSA are shown in Fig. 2. The resistance profile of highly resistant strains such

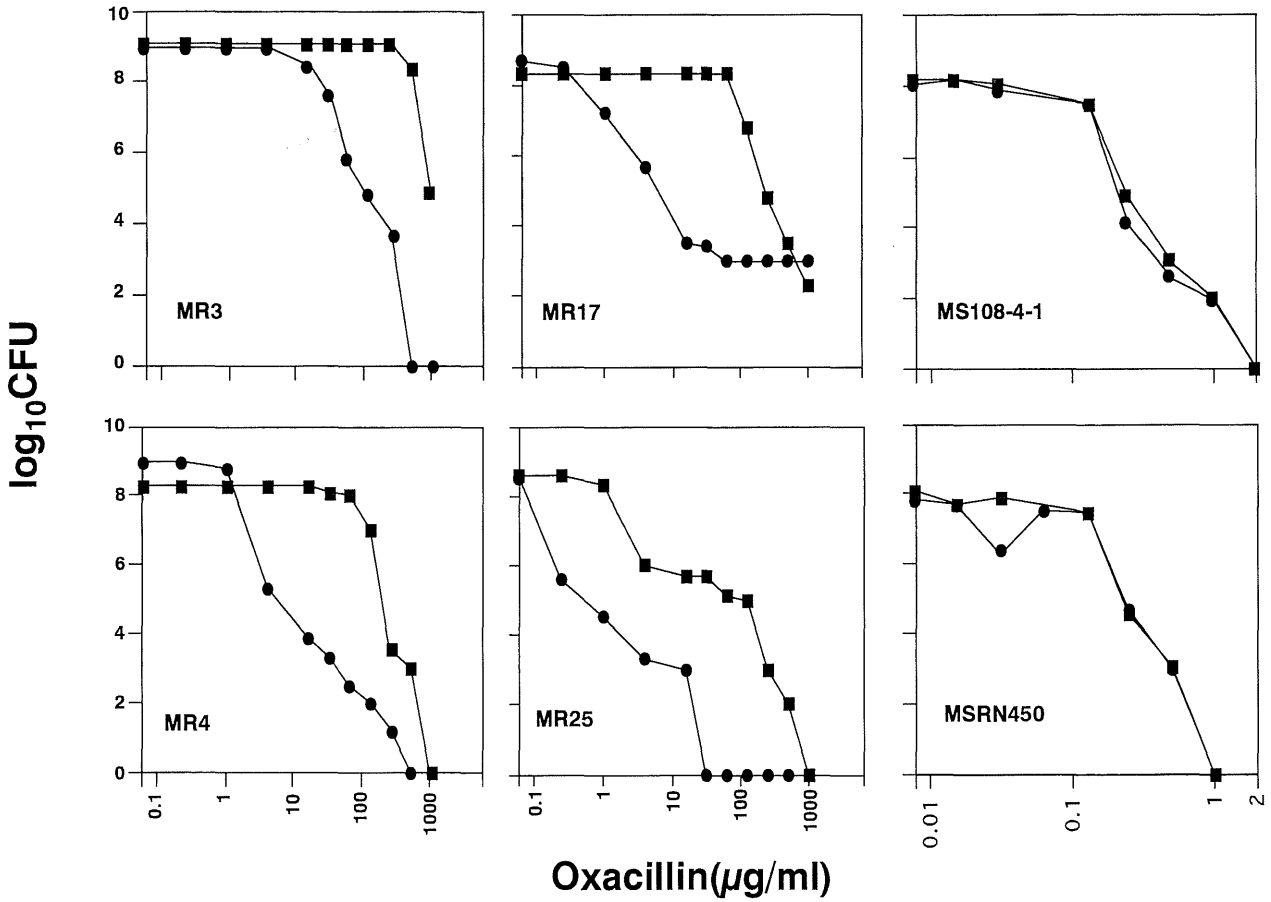

Fig. 2. The effect of CB on the population of MRSA and MSSA isolates. Bacterial cultures were plated on TSA containing various concentrations of oxacillin with or without CB (100 μg/ml). The cfu was determined after a 48 hr incubation at 37°C. ■, strain grown on TSA; ●, strain grown on TSA containing CB (100 μg/ml).

Table 2. MICs (μg/ml) of oxacillin for MRSA in the presence of dye.

strain	MR1	MR2	MR3	MR4	MR5	MR6	MR7	MR8	MR9	MR10	MR11	MR12	MR13
cont.	1024	1024	512	512	512	512	512	512	256	256	256	256	256
CB	16	8	32	64	64	32	32	8	32	16	16	8	8
BBE3G	512	512	512	512	256	512	256	256	256	256	256	128	256
RB4	512	512	512	512	512	512	512	512	256	256	256	256	256
RB5	512	512	512	512	512	512	512	512	256	256	256	256	256
RR2	1024	256	512	512	512	512	512	512	256	256	256	256	256
RY2	1024	1024	512	512	512	512	512	512	256	256	256	256	256
RB10	512	1024	512	512	512	512	512	512	256	256	256	256	256
RG19	1024	1024	512	512	512	512	512	512	256	256	256	256	256
RO14	512	512	256	256	128	256	256	256	128	128	256	128	128

strain	MR14	MR15	MR16	MR17	MR18	MR19	MR20	MR21	MR22	MR23	MR24	MR25	MR26
cont.	256	256	256	256	128	128	128	128	128	128	64	64	64
CB	2	4	2	2	4	4	8	4	8	4	1	2	2
BBE3G	16	64	64	64	128	32	64	32	128	32	4	64	2
RB4	64	64	128	256	64	128	128	128	12	128	16	32	16
RB5	64	64	64	64	128	64	128	128	128	128	8	64	8
RR2	32	64	128	256	64	64	128	128	128	128	16	32	8
RY2	32	32	64	256	128	128	128	128	128	128	16	32	16
RB10	32	32	128	256	128	64	128	128	128	128	16	32	16
RG19	32	32	128	256	128	128	128	128	128	128	16	64	32
RO14	8	16	128	256	16	64	64	64	64	64	4	32	64

CB, Cibacron Blue F3GA; BBE3G, Basilen Blue E3G; RB4, Reactive Blue4; RB5, Reactive Blue5; RR2, Reactive Red2; RY2, Reactive Yellow2; RB10, Reactive Brown10; RG19, Reactive Green 19; RO14, Reactive Orange 14.

as MR3, MR17 and MR4, was changed to that of heterogeneously resistant MRSA in the presence of CB. The population curve of heterogeneous resistant MRSA such as MR25 shifted to the left in the presence of CB. On the other hand, the population curves of MSSA did not change at all in the presence of CB and confirmed the results of MIC analysis.

CB belongs to a group of triazinyl dyes. We therefore studied the effect of various related triazinyl dyes on the susceptibility of MRSA to oxacillin. As shown in Table 2, dyes other than CB had a very weak sensitizing effect on a limited number of strains. On the other hand, CB showed the strongest sensitizing effect among dyes tested to all strains. It was noted that MR14, 24, and 26 were sensitive to all dyes tested.

Effect of CB on the growth and viability of *S. aureus* in the presence of oxacillin

We further assessed the effect of CB on the bactericidal activity of oxacillin using MR15. When CB was added to exponentially growing cells of MR15, cells grew in clusters as described[52]. Measurement of cfu of the culture after sonication revealed that CB did not affect the cell growth of MR15 (Fig. 3). Oxacillin at a concentration of 16 μg/ml did not significantly affect the cell growth of strain MR15 either. On the other hand, coincubation of CB with oxacillin (16 μg/ml) resulted in complete inhibition of cell growth (Fig. 3). Taken

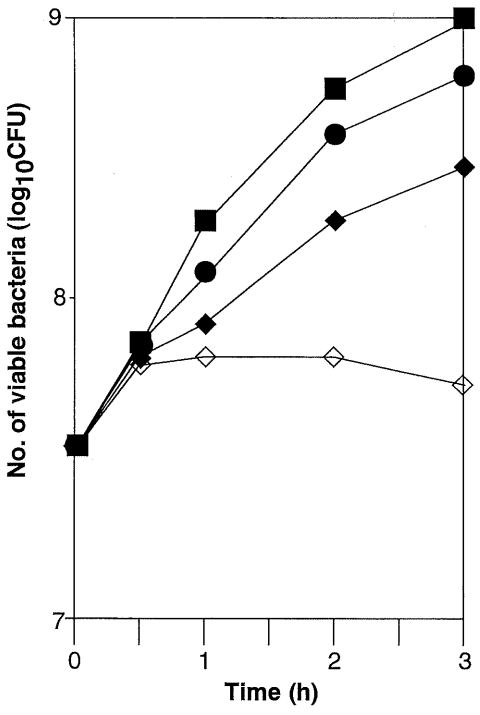

Fig. 3. The effect on the viability of *S. aureus* MR6 during incubation in the presence of no additives (control, ■), 16 μg of oxacillin/ml (◆), 16 μg of oxacillin and 100 μg of CB/ml (◇) or 100 μg of CB/ml (●). The effects were determined by monitoring changes in the number of viable bacteria.

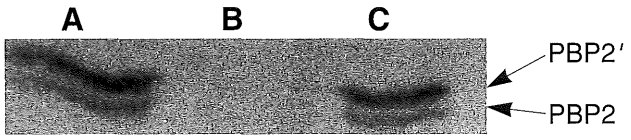

Fig. 4. Effect of CB on PBP profiles of *S. aureus* MR6 following growth in the presence of no additives (control) (A), oxacillin at a concentration of 32 μg/ml (B), 100 μg of CB/ml (C).

together, these results suggest that the sensitization effect of CB on MRSA cells is bacteriostatic.

Effect of CB on penicillin G binding to PBPs

We first studied whether coincubation of CB affects the amount of PBPs in staphylococcal cells. *S. aureus* cell membranes were prepared from cells grown in the presence or absence of CB (100 μg/ml). The binding of ß-lactam antibiotics to PBPs was investigated using [^{14}C]-labeled benzylpenicillin. The amounts of PBP2' and 2 were not affected by the presence of CB in the culture (Fig. 4). Next, the effect of CB on the binding of oxacillin to PBPs was investigated using [^{14}C]-labeled benzylpenicillin. First, the binding of [^{14}C]-labeled benzylpenicillin to the membrane was examined after incubation of the cell membranes suspended in buffer containing various concentrations of penicillin G in the presence of CB (100 μg/ml). The binding curve showed saturation type kinetics, which were similar to those obtained in the absence of CB (Fig. 5a). Second, the time-course binding of [^{14}C]-labeled benzylpenicillin to the membrane was assessed after incubation of cell membranes for various times in buffer containing [^{14}C]-labeled benzylpenicillin in the presence of CB (100 μg/ml). As shown in Fig. 5b, the binding kinetics were not affected by CB.

Effect of CB on putative factors other than PBPs involved in ß-lactam resistance.

CB has been shown to inhibit the bacteriolytic enzyme activities of *S. aureus*[48,51]. To know whether bacteriolytic enzymes are involved in the sensitizing effect of CB, we studied the effect of CB on the resistance level of lyt$^-$ mutants and their parent MRSA MR6. Population analysis of two lyt$^-$ mutants, Lyt-2 and Lyt-5, derived from MR6 indicate that the Lyt$^-$ phenotype had no effect on the resistance level of these strains to oxacillin (Fig. 6). Moreover, the mutants were as sensitive as the parent strains to the effect of CB on susceptibility to oxacillin.

The *mecI/mecR* element and penicillinase plasmids were shown to affect the resistance level of MRSA[24,31,53-55]. We studied the effect of CB on the resistance level of a prototype MRSA, N315 and its isogenic derivatives N315P (penicillinase -) and N315-IR74 (*mecI/mecR1::tet*)[31]. As shown in Fig. 6, the population curve was shifted to the left irrespective of the status of the *mecI/mecR1* element or penicillinase plasmid.

We studied whether CB could affect the structure of peptidoglycan as previously observed in the lower sensitivity of lysostaphin to *femAB* mutants[5,21,34]. *femAB* was found to be a locus essential for methicillin resistance in MRSA[5], and later shown to encode factors involved in the transfer of glycine into interpeptide bridges of *S. aureus*[21,29,34,45]. Mutations in *femAB* alter the susceptibility of *S. aureus* to lysostaphin[29,34,45]. We

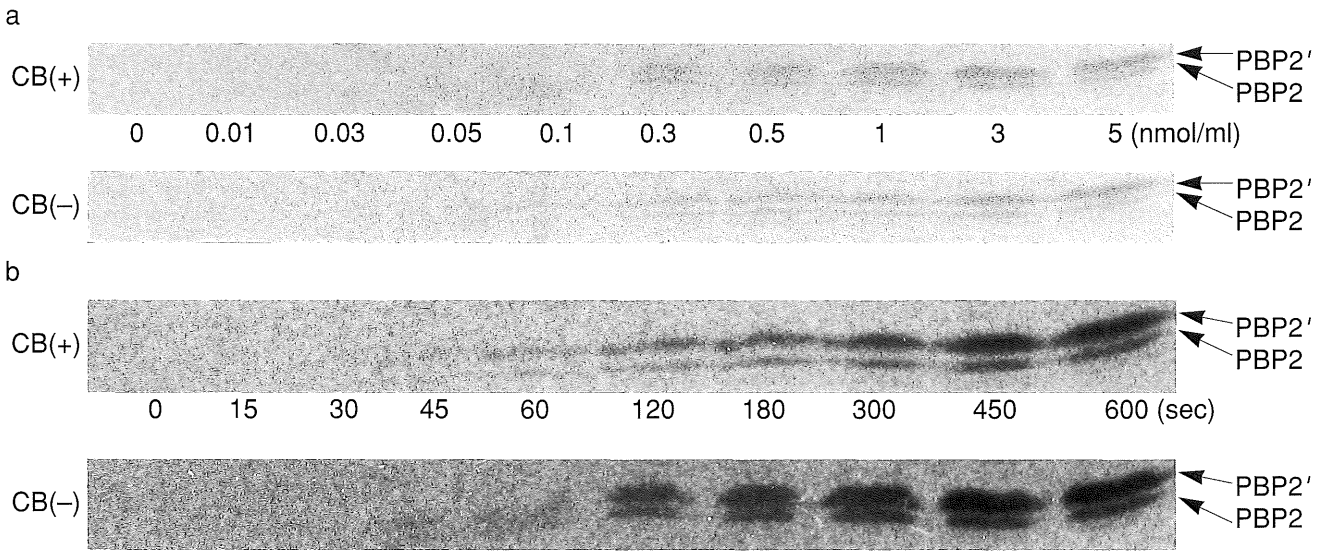

Fig. 5. Kinetics of binding of [^{14}C]labeled benzylpenicillin to the PBP2' in the absence (upper panel) or presence (lower panel) of 100 μg of CB/ml. a, dose dependence. Cytoplasmic membranes (1.2 mg of protein) of *S. aureus* MR6 were incubated with various doses of [^{14}C]-labeled benzylpenicillin for 10 min. b, time course. Cytoplasmic membranes (1.2 mg of protein) of *S. aureus* MR6 were incubated with 1 nmol/ml of [^{14}C]-labeled benzylpenicillin for various periods of time.

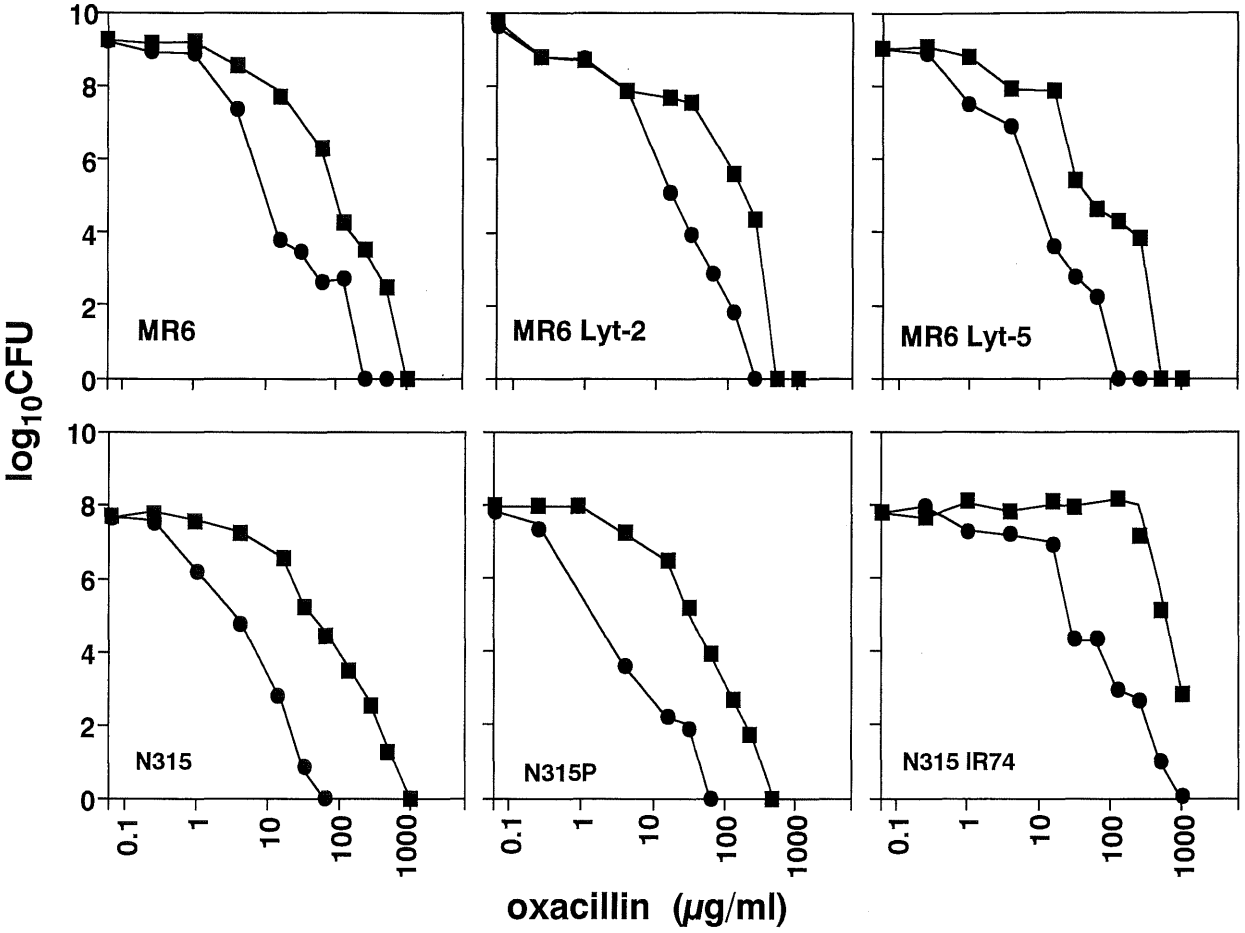

Fig. 6. The effect of CB on the population of MRSA. Bacterial cultures were plated on TSA containing various concentrations of oxacillin with or without CB (100 μg/ml). The cfu was determined after a 48 hr incubation at 37°C. ■, strain grown on TSA; ●, strain grown on TSA containing CB (100 μg/ml).

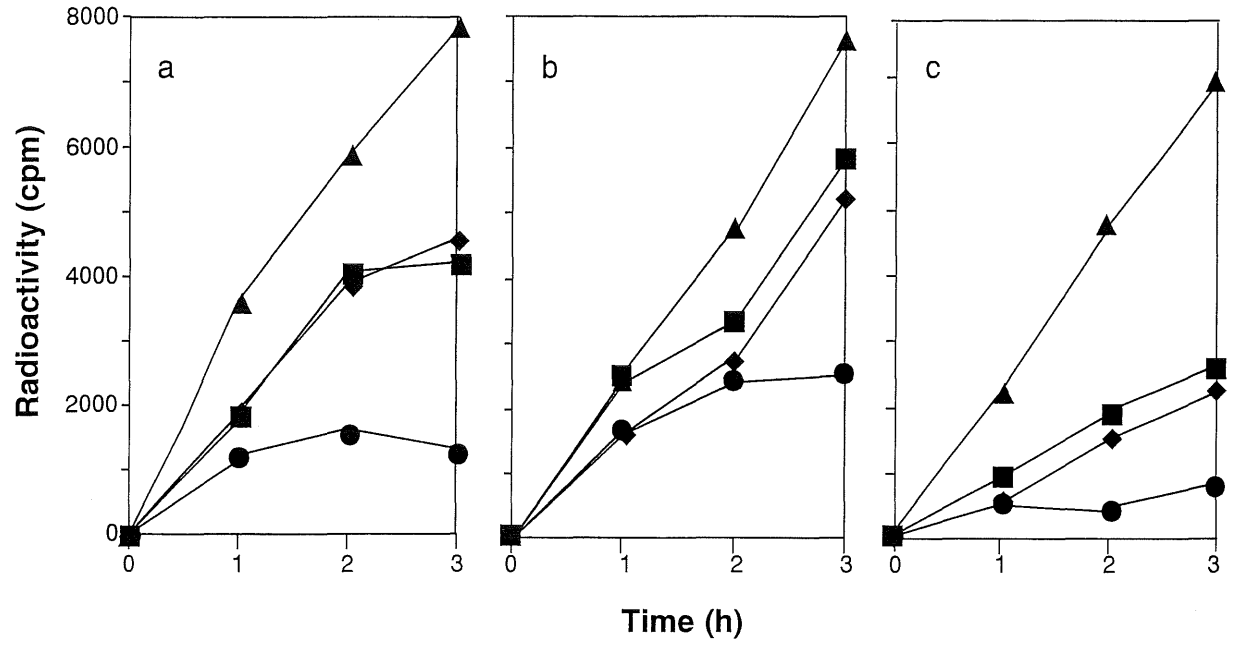

Fig. 7. The effect of CB on the release of lipoteichoic acid. Release of [2-^{3}H]glycerol-labelled LTA from *S. aureus* strain (a) MR5, (b) MR9 and (c) MR25 during growth in the presence of the following: no additives (control ●); oxacillin at MIC (MR5, 512 μg/ml; MR9, 256 μg/ml; MR25, 64 μg/ml)(▲); CB at 100 μg/ml (■); or both CB at 100 μg/ml and oxacillin at MIC in the presence of CB (MR5, 64 μg/ml; MR9, 32 μg/ml; MR25, 2 μg/ml)(◆).

determined the susceptibility of strain MR108–4 grown in the presence or absence of CB to various bacteriolytic enzymes with different bond specificities, including AM, GL and lysostaphin. Regardless of whether the cells were grown in the presence or absence of CB, the MBD of AM, GL, and lysostaphin for strain MR108–4 were 10, 80, and 1.25 ng respectively.

The nonionic detergents, polydocanol and Triton X-100, were shown to enhance the in-vitro activity of ß-lactam antibiotics to MRSA[9,27,28,54]. It has been demonstrated that such detergents stimulate the release of LTA from the bacteria although its relevance to the sensitization effect of nonionic detergent is unknown[9,28]. We studied whether CB could affect the release of LTA using strain MR5, 9, and 25. An MIC of oxacillin enhanced the release of LTA from the cells, as reported previously (Fig. 7)[28]. On the other hand, CB alone decreased the release of LTA and counteracted the increase of the release by oxacillin. We also investigated whether CB affects the amount of LTA, lipids and teichoic acid in the cells according to the procedure described in the Methods. CB at a concentration of 100 μg/ml did not significantly affect the amount of LTA, lipids or teichoic acid either (not shown).

DISCUSSION

The results presented in this study demonstrate that CB decreased the MIC of oxacillin to MRSA, whereas it did not affect the MBC. The combination effect of CB with oxacillin appeared to be bacteriostatic. Studies using commercially available related dyes suggested that the sensitizing effect is CB specific. Even Basilen Blue E3G, whose structure is very similar to CB (except that a sulfonic acid group in ring "A" was assigned to the meta- and/or para-position instead of the ortho-position as was the case with CB[20]), showed a very weak sensitizing effect. Stereochemistry of CB may be important for the sensitizing effect of CB.

Autolysins produced by bacteria have been proposed to play important physiological roles in various aspects of cell wall metabolism including insertion of new monomers, remodeling and turnover of peptidoglycan, cell division and separation (for review,[25,41,44,46,58]). Previous studies suggested that CB interferes with autolysin-mediated functions of *S. aureus*. CB inhibits cell separation of *S. aureus* and induces giant cell clusters[52]. CB also inhibits penicillin-induced autolysis of *S. aureus*[51]. CB has affinity to major autolysins, *atl* gene products of *S. aureus* and inhibits their bacteriolytic activities[48–50]. CB inhibits dispersion of cell-clusters by *atl* gene products in vitro[50]. We therefore studied whether autolysin is involved in the sensitizing effect of CB on MRSA to oxacillin. Two lyt$^-$ mutants which secrete virtually no detectable bacteriolytic enzymes were equally as sensitive to CB as the parent strain in their sensitizing effect. CB did not affect the amount of PBP2' in situ and capacity or kinetics of [^{14}C]-labeled benzylpenicillin binding to PBP2'. CB had a sensitizing effect on MRSA regardless of the status of the *mecI*/*mecR1* element or penicillinase plasmid in the cells. CB did not affect the susceptibility of *S. aureus* cells to various bacteriolytic enzymes including lysostaphin. These results suggest that autolysin(s), *mecA* related products and *femAB* are not involved in CB-mediated sensitization of MRSA to oxacillin.

In previous studies, the non-ionic detergents, polydocanol and Triton X-100 have been shown to decrease the resistance level of MRSA to ß-lactam antibiotics[9,27,28,54]. The sensitization mechanism of non-ionic detergent remains unclear, but it appears that the mechanism of CB is different from that of non-ionic detergent. The combination of oxacillin and non-ionic detergent significantly decreased the viable count suggesting that the effect is bactericidal[9,28]. On the other hand, the effect of the combination of oxacillin and CB was bacteriostatic. Triton X-100 reduces the resistance level of not only MRSA but MSSA to oxacillin though the extent of the reduction in MIC is slight[28]. On the other hand, CB affected the MIC level of MRSA but not MSSA to oxacillin. Polidocanol and Triton X-100 but not CB induced the release of LTA from the cells[9,28]. A highly methicillin resistant strain MR10, which is resistant to the sensitization effect of Triton X-100, decreased the resistance level to oxacillin in the presence of CB. The combination of CB and Triton X-100 synergistically reduced the resistance level of MR10 to oxacillin (not shown).

In conclusion, our results suggested that CB alters the resistance level of MRSA through factor(s) other than *mecA* related products, autolysins, or *femAB* products. Although the exact target(s) of CB in causing this sensitizing effect is not clear, it is likely that the target is involved in a critical metabolic pathway given that PBP2' is the only functional PBP. Further studies of the sensitization effect of CB may help to elucidate the molecular mechanism of the methicillin resistance of *S. aureus*.

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