ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1998, p. 1278–1280 Vol. 42, No. 5
0066-4804/98/$04.00+0

A Triazine Dye, Cibacron Blue F3GA, Decreases Oxacillin Resistance Levels in Methicillin-Resistant *Staphylococcus aureus*

CHIHARU SHIRAI,[1,2] MOTOYUKI SUGAI,[2]* HITOSHI KOMATSUZAWA,[2] KOUJI OHTA,[2] MICHIO YAMAKIDO,[1] AND HIDEKAZU SUGINAKA[2]

Department of Internal Medicine II, Hiroshima University School of Medicine,[1] and Department of Microbiology, Hiroshima University School of Dentistry,[2] Hiroshima 734, Japan

Received 20 October 1997/Returned for modification 8 January 1998/Accepted 10 February 1998

Cibacron blue F3GA (CB) was found to reduce the MIC of oxacillin for methicillin-resistant *Staphylococcus aureus* (MRSA). This effect was not observed with methicillin-susceptible *S. aureus*. CB alters the resistance level of MRSA through a factor(s) other than *mecA*-related products, major autolysins, or *femAB* products. The exact target(s) of CB in causing the effect is unknown.

Cibacron blue F3GA (CB) is a triazinyl dye widely used as the affinity ligand for dye-ligand chromatography. CB is structurally similar to naturally occurring heterocycles, such as nucleoside phosphate, NAD^+, coenzyme A, and folic acid (1–3, 8). It has been demonstrated that CB specifically binds to nucleotide binding sites of kinases and dehydrogenases and that some of the enzyme activities are inhibited by CB (1, 4, 6, 7). The aim of this study was to investigate the effect of CB on the in vitro susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) to oxacillin. CB used in this study was from Sigma Chemical Co., St. Louis, Mo. (C 9534) and was formerly called reactive blue 2 (R 4502); it has an A-ring orthosulfonic acid (9). CB purified by reversed-phase high-pressure liquid chromatography according to the procedure described by Hanggi and Carr (9) behaved similarly to unpurified CB, and consequently CB was used without purification in this study. MICs were determined by a microdilution method (13), and population analysis was carried out as described elsewhere (12). CB alone was not inhibitory to staphylococcal strains when used at up to 2,500 μg/ml in the experiments. The effect of CB on in vitro susceptibility to oxacillin was evaluated with 28 MRSA and 10 methicillin-susceptible *S. aureus* (MSSA) strains. For all MRSA strains, the MIC of oxacillin was significantly reduced in the presence of CB concentrations of 39 μg/ml or higher (Fig. 1). Highly resistant MRSA strains appeared to be less susceptible to the sensitizing effect of CB, but 78 to 156 μg of CB per ml markedly reduced the MICs of oxacillin for those strains. We therefore employed 100 μg of CB per ml in further

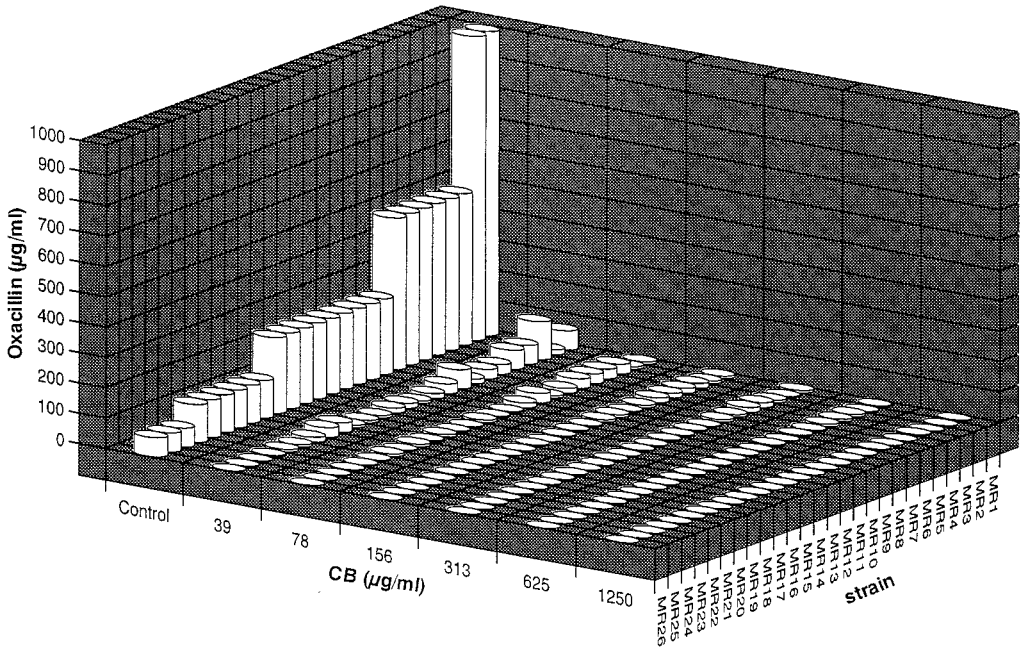

FIG. 1. Effect of CB on the susceptibilities of MRSA isolates to oxacillin. The column height indicates the MIC of oxacillin.

* Corresponding author. Mailing address: Department of Microbiology, Hiroshima University School of Dentistry, Kasumi 1-2-3, Minami-ku, Hiroshima City, Hiroshima 734-8553, Japan. Phone: 81-82-257-5637. Fax: 81-82-257-5639. E-mail: sugai@ipc.hiroshima-u.ac.jp.

TABLE 1. MICs of various antibiotics for MRSA in the presence and absence of CB

MRSA strain	MIC[a] (μg/ml) of:																			
	FOM		CS		VCM		BC		CP		PCG		DMPPC		MPIPC		CEZ		CCL	
	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
MR1	512	512	128	128	2	2	64	128	8	8	64	4	>512	4	>512	16	512	2	512	32
MR6	256	>512	128	64	4	4	64	128	8	4	32	8	>512	8	512	64	512	32	>512	>512
MR8	32	32	64	64	2	2	64	128	32	32	16	4	256	4	512	8	256	1	512	16
MR14	512	>512	128	64	4	1	64	128	8	8	16	16	64	8	256	2	128	<0.5	512	4
MR15	512	512	64	128	1	1	64	128	8	8	64	4	512	4	256	4	128	2	256	4
MR20	64	64	32	32	1	1	32	64	8	8	32	4	>512	4	128	8	256	2	512	32
MR24	16	16	64	32	1	1	32	32	16	16	32	<0.5	32	2	64	1	64	<0.5	256	16
MR26	16	16	64	32	4	1	16	32	16	16	64	8	32	2	64	2	128	<0.5	512	2

[a] MICs were tested in the absence (−) and presence (+) of CB. FOM, fosfomycin; CS, cycloserine; VCM, vancomycin; BC, bacitracin; CP, chloramphenicol; PCG, benzylpenicillin; DMPPC, methicillin; MPIPC, oxacillin; CEZ, cefazolin; CCL, cefaclor.

studies, unless otherwise noted. On the other hand, MICs of oxacillin for MSSA did not change at all in the presence of CB (data not shown). A population analysis of 28 MRSA strains and 2 MSSA strains was carried out in the presence and absence of CB. In most cases, the population curve of homogeneously or heterogeneously oxacillin-resistant MRSA shifted to the left in the presence of CB. On the other hand, population curves of MSSA did not change at all in the presence of CB and confirmed the results of MIC analysis. CB alone also had no effect on the population curve. This sensitizing effect of CB was observed only when β-lactam was used with CB (Table 1). Various triazinyl dyes related to CB were tested for an effect on the sensitivity of MRSA to oxacillin and were found to have a very weak effect on a limited number of strains (data not shown).

We further assessed the effect of CB on the bactericidal activity of oxacillin with *S. aureus* MR15. When CB was added to exponentially growing MR15, the cells grew in clusters as described previously (20). Measurement of CFU of the culture after brief sonication to disperse clusters revealed that CB did not affect the growth of MR15. Oxacillin at a concentration of 16 μg/ml also did not significantly affect the growth of strain MR15. On the other hand, coincubation of CB with oxacillin (16 μg/ml) completely inhibited growth but did not reduce the number of CFU. These results suggest that the sensitization effect of CB on MRSA cells is bacteriostatic.

We studied the effect of CB on the synthesis of penicillin-binding proteins (PBPs) in strain MR6-2 (β-lactamase free) (13). *S. aureus* cell membranes were prepared from cells grown in the presence or absence of CB. The binding of β-lactam antibiotics to PBPs was investigated with ^{14}C-labeled benzylpenicillin (10 to 30 Ci/mmol) (Amersham International, Bucks, United Kingdom) (5). The amounts of PBP2′ and PBP2 were not affected by the presence of CB in the culture. Next, the effect of CB on the binding of ^{14}C-labeled benzylpenicillin to PBPs was investigated. The binding study revealed that the kinetics also were not affected by CB.

CB has been shown to inhibit bacteriolytic enzyme activities of *S. aureus* (16, 19). To determine whether bacteriolytic enzymes are involved in the sensitizing effect of CB, we studied the effect of CB on the resistance levels of Lyt$^-$ mutants, which virtually lack major autolysins of *S. aureus* (the *atl* gene products), and of their parent, MRSA MR6 (13). Population analysis of two Lyt$^-$ mutants, Lyt-2 and Lyt-5, derived from MR6 indicated that the Lyt$^-$ phenotype had no effect on the levels of resistance of these strains to oxacillin. 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 (11, 14, 22). We studied the effect of CB on the resistance level of a prototype MRSA strain, N315, and its isogenic derivatives N315P (penicillinase negative) and N315-IR74 (*mecI mecR*::*tet*) (14). The population curve was shifted to the left irrespective of the status of the *mecI-mecR* element and the penicillinase plasmid. We studied whether CB could affect the structure of peptidoglycan as previously observed in studies demonstrating the lower sensitivity of *femAB* mutants to lysostaphin compared to that of the wild type (10, 15). We determined the susceptibilities of several MRSA strains grown in the presence or absence of CB to various bacteriolytic enzymes with different bond specificities, including 62-kDa *N*-acetylmuramyl-L-alanine amidase (18), 51-kDa endo-β-*N*-acetylglucosaminidase (17), and lysostaphin, by zymography (12). Regardless of whether the cells were grown in the presence or absence of CB, the minimum bacteriolytic doses of *N*-acetylmuramyl-L-alanine amidase, *N*-acetylglucosaminidase, and lysostaphin for the strains were identical.

In conclusion, our results suggested that CB alters the resistance level of MRSA through a factor(s) other than *mecA*-related products, major 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 methicillin resistance in *S. aureus.*

We thank Keiichi Hiramatsu, Juntendo University, for bacterial strains. We also thank Tadashi Oshida, Tanabe Seiyaku Co., Ltd., for helpful discussions and Masao Kuwabara, Hiroshima Prefectural Hospital, for encouragement.

This investigation was supported in part by a grant from the Scientific Research Funds (07557115 and 084557481) of the Ministry of Education, Science and Culture, Japan (1996 and 1997).

REFERENCES

1. **Ashton, A. R., and G. M. Polya.** 1978. The specific interaction of cibacron and related dyes with cyclic nucleotide phosphodiesterase and lactate dehydrogenase. Biochem. J. **175:**501–506.
2. **Baird, J. K., R. F. Sherwood, R. J. G. Carr, and A. Atkinson.** 1976. Enzyme purification by substrate elution chromatography from procion dye-polysaccharide matrices. FEBS Lett. **70:**61–66.
3. **Beissner, R. S., and F. B. Rudolph.** 1978. Interaction of cibacron blue 3G-A and related dyes with nucleotide-requiring enzymes. Arch. Biochem. Biophys. **189:**76–80.
4. **Biellmann, J.-F., J.-P. Samama, C. I. Bränden, and H. Eklund.** 1979. X-ray studies of the binding of cibacron blue F3GA to liver alcohol dehydrogenase. Eur. J. Biochem. **102:**107–110.
5. **Brown, D. F., and P. E. Reynolds.** 1980. Intrinsic resistance to β-lactam antibiotics in *Staphylococcus aureus*. FEBS Lett. **122:**275–278.

6. **Clonis, Y. D., M. J. Goldfinch, and C. R. Lowe.** 1981. The interaction of yeast hexokinase with procion green H-4G. Biochem. J. **197:**203–211.
7. **Clonis, Y. D., and C. R. Lowe.** 1980. Triazine dyes, a new class of affinity labels for nucleotide-dependent enzymes. Biochem. J. **191:**247–251.
8. **Edwards, R. A., and R. W. Woody.** 1979. Spectroscopic studies of cibacron blue and congo red bound to dehydrogenases and kinases, evaluation of dyes as probes of the dinucleotide fold. Biochemistry **18:**5197–5204.
9. **Hanggi, D., and P. Carr.** 1985. Analytical evaluation of the purity of commercial preparations of cibacron blue F3GA and related dyes. Anal. Biochem. **149:**91–104.
10. **Henze, U., T. Sidow, J. Wecke, H. Labischinski, and B. Berger-Bächi.** 1993. Influence of *femB* on methicillin resistance and peptidoglycan metabolism in *Staphylococcus aureus*. J. Bacteriol. **175:**1612–1620.
11. **Hiramatsu, K., E. Suzuki, H. Takayama, Y. Katayama, and T. Yokota.** 1990. Role of penicillinase plasmids in the stability of the *mecA* gene in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **34:**600–604.
12. **Komatsuzawa, H., M. Sugai, C. Shirai, J. Suzuki, K. Hiramatsu, and H. Suginaka.** 1995. Triton X-100 alters the resistance level of methicillin-resistant *Staphylococcus aureus* to oxacillin. FEMS Microbiol. Lett. **134:**209–212.
13. **Komatsuzawa, H., J. Suzuki, M. Sugai, Y. Miyake, and H. Suginaka.** 1994. The effect of Triton X-100 on the in-vitro susceptibility of methicillin-resistant *Staphylococcus aureus* to oxacillin. J. Antimicrob. Chemother. **34:**885–897.
14. **Kuwahara-Arai, K., N. Kondo, S. Hori, E. Tateda-Suzuki, and K. Hiramatsu.** 1996. Suppression of methicillin resistance in a *mecA*-containing pre-methicillin-resistant *Staphylococcus aureus* strain is caused by the *mecI*-mediated repression of PBP 2′ production. Antimicrob. Agents Chemother. **40:**2680–2685.
15. **Maidhof, H., B. Reinicke, P. Blümel, B. Berger-Bächi, and H. Labischinski.** 1991. *femA*, which encodes a factor essential for expression of methicillin resistance, affects glycine content of peptidoglycan in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. J. Bacteriol. **173:**3507–3513.
16. **Sugai, M., T. Akiyama, H. Komatsuzawa, Y. Miyake, and H. Suginaka.** 1990. Characterization of sodium dodecyl sulfate-stable *Staphylococcus aureus* bacteriolytic enzymes by polyacrylamide gel electrophoresis. J. Bacteriol. **172:**6494–6498.
17. **Sugai, M., H. Koike, Y.-M. Hong, Y. Miyake, R. Nogami, and H. Suginaka.** 1989. Purification of a 51 kDa endo-β-N-acetylglucosaminidase from *Staphylococcus aureus*. FEMS Microbiol. Lett. **61:**267–272.
18. **Sugai, M., H. Komatsuzawa, T. Akiyama, Y.-M. Hong, T. Oshida, Y. Miyake, T. Yamaguchi, and H. Suginaka.** 1995. Identification of endo-β-*N*-acetylglucosaminidase and *N*-acetylmuramyl-L-alanine amidase as cluster-dispersing enzymes in *Staphylococcus aureus*. J. Bacteriol. **177:**1491–1496.
19. **Sugai, M., K. Ooku, T. Akiyama, S. Inoue, S. Iseda, Y. Miyake, and H. Suginaka.** 1991. Suppression of penicillin-induced lysis of *Staphylococcus aureus* by cibacron blue 3G-A. FEMS Microbiol. Lett. **80:**151–154.
20. **Sugai, M., K. Ooku, T. Takata, Y. Miyake, and H. Suginaka.** 1990. A triazine dye, cibacron blue 3G-A induces *Staphylococcus aureus* to form giant clusters. FEMS Microbiol. Lett. **67:**175–178.
21. **Suzuki, E., K. Kuwahara-Arai, J. F. Richardson, and K. Hiramatsu.** 1993. Distribution of *mec* regulator genes in methicillin-resistant *Staphylococcus* clinical strains. Antimicrob. Agents Chemother. **37:**1219–1226.
22. **Tesch, W., C. Ryffel, A. Strässle, F. H. Kayser, and B. Berger-Bächi.** 1990. Evidence of a novel staphylococcal *mec*-encoded element (*mecR*) controlling expression of penicillin-binding protein 2′. Antimicrob. Agents Chemother. **34:**1703–1706.