ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1995, p. 2631–2634 Vol. 39, No. 12
0066-4804/95/$04.00+0

Autolysis of Methicillin-Resistant *Staphylococcus aureus* Is Involved in Synergism between Imipenem and Cefotiam

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Received 28 October 1994/Returned for modification 9 December 1994/Accepted 26 July 1995

Imipenem-induced autolysis and the activity of imipenem plus cefotiam were studied in 16 strains of methicillin-resistant *Staphylococcus aureus* (MRSA). The degree of imipenem-induced autolysis and the rate of synergistic action of imipenem plus cefotiam varied among strains and did not correlate with susceptibility to either imipenem or cefotiam. However, the degree of autolysis correlated well with susceptibility to the synergistic action of imipenem plus cefotiam. In methicillin-susceptible *S. aureus* strains, both imipenem-induced autolysis and the synergistic activity of the combined drugs were less than those observed in MRSA strains. Differences in the degree of autolysis were not due to differences in autolytic enzyme production. The autolysis of imipenem-pretreated MRSA was enhanced further by cefotiam, while treatment of cells in the reverse order did not enhance autolysis. These findings indicate that cell wall impairment in MRSA is caused by exposure to imipenem but not to cefotiam and that this difference in drug actions results in synergism between imipenem and cefotiam. The possible participation of penicillin-binding proteins PBP 2′ and PBP4 in the observed effect is discussed.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an increasingly common cause of nosocomial infections. Therapeutic options for MRSA infections are very limited because MRSA strains often are resistant not only to β-lactam antibiotics but also to macrolides, aminoglycosides, and fluoroquinolones (3, 21). Methicillin resistance of *S. aureus* is mediated by an acquired, chromosomally located resistance determinant, *mecA*, which produces a low-affinity penicillin-binding protein (PBP) named PBP 2′ or PBP 2a (6, 20). Any compounds capable of inhibiting PBP 2′ may be highly effective in the eradication of MRSA; however, such compounds are not yet used in practice (2). In this context, investigations into the effects of drugs used in combination may be worthwhile. Several combinations including β-lactam–β-lactam combinations have been reported to have an effect against MRSA (9, 19).

We previously reported that combinations of imipenem with a β-lactam antibiotic such as cefotiam exhibited potent synergistic activity against MRSA (13–15). It was especially noteworthy that the clinical response rate with the combination of imipenem plus cefotiam was greater than 75% (14).

PBP 4 and imipenem-induced autolysis presumably play an important role in the mechanism of action of this combination, because the same type of effect was observed between a β-lactam antibiotic having a strong affinity to PBP 4 and another β-lactam antibiotic having no affinity to PBP 4 (13). Best et al. (1) reported that the autolysins of *S. aureus* participated in the bactericidal activity of β-lactam antibiotics. Qoronfleh and Wilkinson (17) reported that inhibition of PBP 4 led to a poor cross-linkage of peptidoglycan (PG) and that such a hypo-cross-linked PG was susceptible to autolytic enzyme. Extending their work, we investigated the relationship between the synergistic effect of imipenem plus cefotiam and autolysis induced by imipenem.

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MATERIALS AND METHODS

Bacterial strains. The 16 clinical strains of MRSA used in the study were obtained during 1989 and 1990 in different hospitals located in different regions of Japan (14). Two pairs of isolates, each pair isogenic for *mecA*, were used (see Table 2). Strain MS353 was a typical methicillin-susceptible *S. aureus* (MSSA) strain. Strain BB5918 was a clinical isolate of heterogeneous MRSA. Strain BB6216 was a mutant lacking *mecA* that was obtained by the replica method after 2 days of incubation of *S. aureus* BB5918 at 41°C. Strain BB6218 was a transductant of *S. aureus* MS353 into which the plasmid pMS555 carrying the *mecA* gene was transduced by using the S1 *tet* phage (10).

In vitro susceptibility tests. Methicillin and imipenem were obtained from Banyu Pharmaceutical Co., Ltd., Tokyo, Japan. Cefotiam was obtained from Takeda Chemical Industries, Ltd., Osaka, Japan. The MICs for the test organisms were determined by the agar dilution method with Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) supplemented with 2% NaCl at 35°C. The in vitro effect of combining two antibiotics was measured quantitatively by a checkerboard agar dilution method as described previously (13). The synergistic effect of two drugs was represented by the synergistic ratio (SR), which was the reciprocal of the minimum fractional inhibitory concentration (FIC) index (11).

Analysis of PBPs. Antibiotic affinity to the PBPs of *S. aureus* BB5918 was determined by competition assay with [^{14}C]benzylpenicillin. Membranes were first incubated with various concentrations of nonradioactive antibiotics (0.01 to 100 μg/ml) for 30 min at 30°C. Then, [^{14}C]benzylpenicillin was added to a final concentration of 50 μg/ml. The samples were incubated for an additional 30 min. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8.0% acrylamide, 0.06% bisacrylamide) and were then visualized by fluorography. The concentration of each antibiotic required for 50% saturation of the PBPs was estimated by measuring the band with a densitometer (Shimadzu, Kyoto, Japan).

Imipenem-induced autolysis. Test strains were grown in 200 ml of tryptic soy broth (Difco Laboratories) at 37°C with shaking at 100 rpm to an A_{585} of 0.4. Subinhibitory concentrations of imipenem were added to sterile conical tubes (Becton Dickinson Labware, Cockeysville, N.J.) containing 20 ml of the broth culture. Further incubations were continued for 3.5 h. The growing cells were collected by centrifugation (15,000 × *g* for 10 min) and were washed twice with 0.1 M KCl–0.01 M $MgCl_2$–0.01 M Tris-HCl buffer (pH 7.0) (lytic buffer) to remove the antibiotic. The cells were resuspended in the same buffer containing 0.1% agarose (Agarose 1600; Wako Pure Chemical Industries, Ltd., Osaka, Japan), which was prewarmed at 37°C and was placed in a cuvette. The A_{585} was adjusted from 0.8 to 1.2. After immediately chilling to solidify the mixture, A_{585}

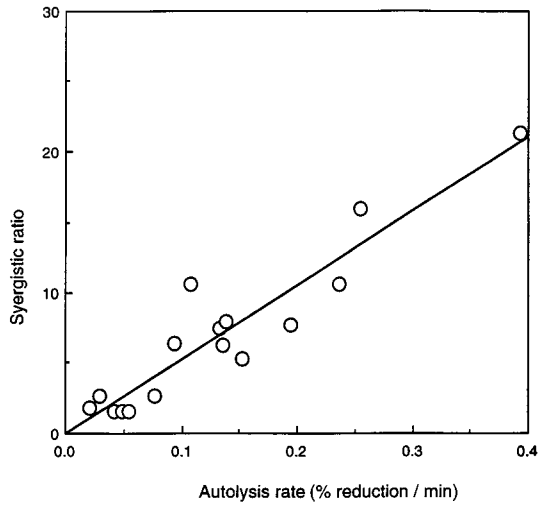

FIG. 1. Relationship between the autolysis rate induced by one-half the MIC of imipenem and the SR of imipenem plus cefotiam against 16 strains of MRSA.

readings were taken in a spectrophotometer (U3210; Hitachi, Ltd., Tokyo, Japan) equipped with 6 a cell positioner (Hitachi, Ltd.) at intervals during incubation at 30°C for 5 h. The degree of individual autolysis at time t was calculated from the following equation: autolysis at t (percent) = $100 - (As_t/As_0)/(Ac_t/Ac_0)$, where As_t is the A_{585} of the sample at time t, As_0 is the A_{585} of the sample at time zero, Ac_t is the A_{585} of the control at time t, and Ac_0 is the A_{585} of the control at time zero. The A_{585} reading of the cell suspension without antibiotic treatment was used as the control. Autolytic rates were represented as the initial autolytic rate at time zero, which was extrapolated from the simple least-squares regression of the autolytic rate (percent reduction of A_{585} per minute) at time t versus t.

Detection of the *mecA* gene. The *mecA* gene was detected by PCR. The synthetic oligonucleotides used as primers were 5′-AGATTGGGATCATA GCGTCA-3′ corresponding to nucleotides 374 to 394 and 5′-GAAGGTATC ATCTTGTACCC-3′ corresponding to the complementary nucleotides 632 to 652 in the *mecA* gene (16). PCR was performed with an automated thermal cycler (DNA thermal cycler 480; Perkin-Elmer Cetus, Norwalk, Conn.) (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min for 25 cycles).

Autolysin extraction. The freeze-thaw extraction procedure described by Huff et al. (8) was used to extract autolytic enzyme (autolysin). Two pairs of strains isogenic for *mecA* grown in 200 ml of tryptic soy broth (Difco) at 37°C for 20 h were harvested by centrifugation at 15,380 × *g* for 5 min at 4°C. The cells were then washed twice with the lytic buffer (pH 7.0) by centrifugation. The cells were resuspended in 2.0 ml of the same buffer and were frozen at −20°C for 24 h. The freeze-thaw extraction was repeated twice, and the cells were removed by centrifugation (56,000 × *g* for 10 min at 4°C). The protein content of the autolysin extracts was determined by the method of Lowry et al. (12).

Assay for autolytic enzyme activity. Freeze-dried cells of *S. aureus* BB5918 were used as the substrate for lytic enzyme. Cells grown in 1 liter of tryptic soy broth containing 0.78 μg of imipenem per ml at 37°C for 3.5 h were harvested by centrifugation (4,000 × *g* for 20 min at 4°C). The cells were resuspended in 50 ml of the lytic buffer (pH 7.0). To inactivate endogenous autolytic enzymes, the resuspended cells were boiled for 10 min and were then freeze-dried. The freeze-dried cells were suspended in 1 ml of enzyme solution to an A_{585} of 1.0 in cuvettes, and the A_{585} readings were taken with a model U3210 spectrophotometer (Hitachi, Ltd.) at intervals during incubation at 30°C.

Effects of a staggered regimen on autolysis. From the results of preliminary checkerboard titration, the lowest FIC index (0.133) was observed with the combination of 0.1 μg of imipenem per ml and 25 μg of cefotiam per ml. In the staggered regimen experiment, one-eighth of the concentrations of imipenem and cefotiam mentioned above were used for both pretreatment and the autolysis assay. The lysis of cells previously exposed to a single antibiotic was measured in the presence of an additional antibiotic. The harvested cells grown in the presence of 0.0125 μg of imipenem per ml were washed with the lytic buffer to remove imipenem. Autolysis of the cells was monitored in the presence of 3.13 μg of cefotiam per ml. In the reverse order, the lysis of cells grown in the presence of 3.13 μg of cefotiam per ml was monitored in the presence of 0.0125 μg of imipenem per ml. Control experiments were performed (i) with pretreatment with a subinhibitory concentration (one-half the MIC) of imipenem alone, (ii) with pretreatment without antibiotic in the presence of imipenem, and (iii) with pretreatment without antibiotic in the absence of antibiotic.

TABLE 1. Affinities of imipenem and cefotiam for the PBPs of MRSA strain BB5918

PBP	IC_{50} (μg/ml)[a]	
	Imipenem	Cefotiam
1	0.031	0.041
2′	>100	>100
2	0.36	0.13
3	0.14	0.09
4	0.012	100

[a] IC_{50}, 50% inhibitory concentration.

RESULTS

Relationship between the degree of autolysis and the synergistic activity of imipenem in combination with cefotiam. There were no significant relationships between the degree of resistance to imipenem and either the SR of imipenem plus cefotiam or the autolysis rates of the cells grown in the presence of subinhibitory concentrations (one-half the MIC) of imipenem (data not shown).

However, the SR of imipenem plus cefotiam and the autolysis induced by the subinhibitory concentration of imipenem were found to correlate linearly (Fig. 1). The correlation coefficient was 0.87. These facts suggest that the autolysis induced by imipenem was responsible for the synergism.

PBP affinity to imipenem and cefotiam. The results of PBP-binding assay are presented in Table 1. The 50% inhibitory concentration of imipenem bound to PBPs 1, 2, and 3 were similar to those of cefotiam. Neither compound showed an affinity for PBP 2′, which indicated a lack of antibacterial activity against MRSA.

Effect of *mecA* on autolysis. To examine the influence of *mecA* on synergism and autolysis, we constructed two pairs of strains isogenic for *mecA* (Table 2). The presence of *mecA* in the sets of isogenic strains was determined by PCR. *mecA* was not detected in the methicillin-susceptible strains MS353 and BB6216. In contrast, *mecA* was detected in BB5918 as being chromosomally located and in BB6218 as being located on a plasmid. The autolytic patterns of methicillin-resistant BB5918 and methicillin-susceptible MS353 are shown in Fig. 2, together with those of the isogenic strains, strains BB6218 and BB6216. The degree of autolysis depended on the concentration of imipenem. Significant autolysis was observed in the $mecA^+$ strains BB5918 and BB6218 rather than in the strains lacking *mecA*, strains MS353 and BB6216, at concentrations less than one-half the MIC of imipenem. Thus, the strains

TABLE 2. *S. aureus* strains used in the study and relevant properties

Strain	Relevant genotype	Relevant phenotype[a]	Origin or reference
MS353	*mecA* lacking	Mc^s	Clinical isolate
BB6218	$mecA^+$, pMS555[b]	Mc^r	This study; transductant of MS353 with pMS555 carrying $mecA^+$
BB5918	$mecA^+$	Mc^r	Clinical isolate (16)
BB6216	*mecA* lacking	Mc^s	This study; *mecA*-defective mutant from BB5918

[a] MC^s, methicillin susceptible; MC^r, methicillin resistant.
[b] pMS555, S1 *tet*-defective phage plasmid carrying the *mecA* gene.

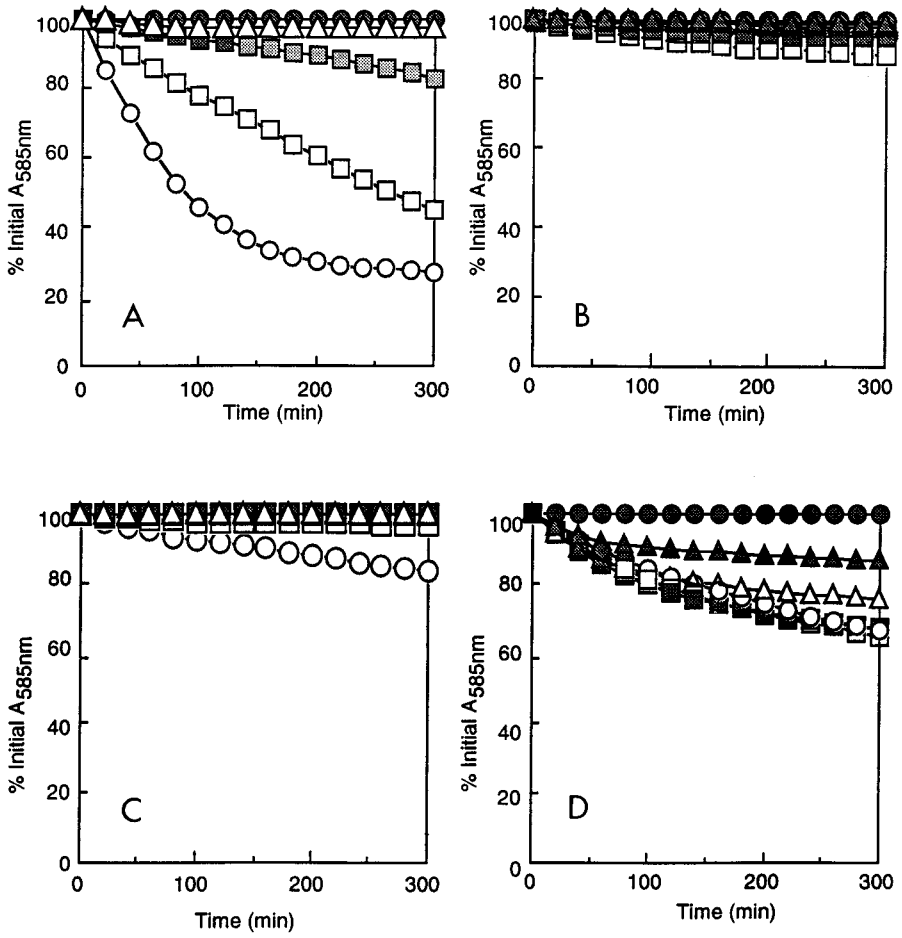

FIG. 2. Imipenem-induced autolytic profiles of two sets of isogenic strains. *S. aureus* BB5918 (*mecA*$^+$) (A) and *S. aureus* BB6218 (*mecA*$^+$) (D) were MRSA. *S. aureus* BB6216 (lacking *mecA*) (B) and *S. aureus* MS353 (lacking *mecA*) (C) were MSSA. Symbols: ●, control; ○, 1/2 the MIC; □, 1/4 the MIC; ▨, 1/8 the MIC; △, 1/16 the MIC; ◬, 1/32 the MIC. The MICs of imipenem for *S. aureus* BB5918, *S. aureus* 6218, *S. aureus* 6216, and *S. aureus* MS353 were 12.5, 0.39, 0.1, and 0.05 μg/ml, respectively. The control was pretreated without antibiotic.

carrying *mecA* were more susceptible to imipenem-induced autolysis than the *mecA*-deficient isogenic strains.

Production of autolytic enzyme. To distinguish whether the autolysis seen in the *mecA*$^+$ strains was due to alterations in the cell wall structure, which autolysin attacks, or to alterations in the autolytic enzyme content of these strains, autolysin activities in extracts of these pairs of strains were determined. As shown in Table 3, there was no difference in the activities of autolysin extracted from the autolysis-susceptible *mecA*$^+$ and autolysis-insusceptible *mecA*-lacking strains.

Effects of a staggered regimen on autolysis. As shown in Fig. 3, the cells pretreated with imipenem lysed rapidly in the presence of cefotiam. This autolysis was not caused by cefotiam alone, since the concentration of cefotiam (one-eighth the MIC) was far lower than the MIC. In contrast, the lysis of cells pretreated with cefotiam was not enhanced by the presence of imipenem. Pretreatment with imipenem alone also caused a slight acceleration of autolysis, suggesting that imipenem pretreatment made the cell wall sensitive to autolytic enzyme.

DISCUSSION

We previously proposed that PBP 4 may play an important role in the synergistic effect of the combination of imipenem and cefotiam against MRSA because synergy was found when a compound having a strong affinity to PBP 4, such as imipenem, was combined with a compound having no affinity to PBP 4, such as cefotiam (13).

This hypothesis was strongly supported by the present study because an in vitro combination effect occurred not only

TABLE 3. Effects of *mecA* on synergism, degree of autolysis, and autolytic enzyme activity

Strain	*mecA* gene status	Combination effect (μg/ml)		Minimum FIC index	Degree of autolysis (%/min)[a]	Autolytic enzyme activity (A_{585}; mg of protein/h)
		[IPM][b]/MIC of IPM alone	[CTM][c]/MIC of CTM alone			
MS353	−	0.006/0.05	0.1/0.39	0.376	0.106	0.0401
BB6218	+	0.006/0.39	6.25/50	0.14	0.323	0.0437
BB5918	+	0.1/12.5	25/200	0.133	0.786	0.0025
BB6216	−	0.025/0.1	0.39/1.56	0.5	0.039	0.0015

[a] The degree of autolysis was calculated by simple least-squares regression of A_{585} at time zero/A_{585} versus at time t and was expressed as a percentage of control autolysis.
[b] [IPM], concentration of imipenem when the FIC index was minimum.
[c] [CTM], concentration of cefotiam when the FIC index was minimum.

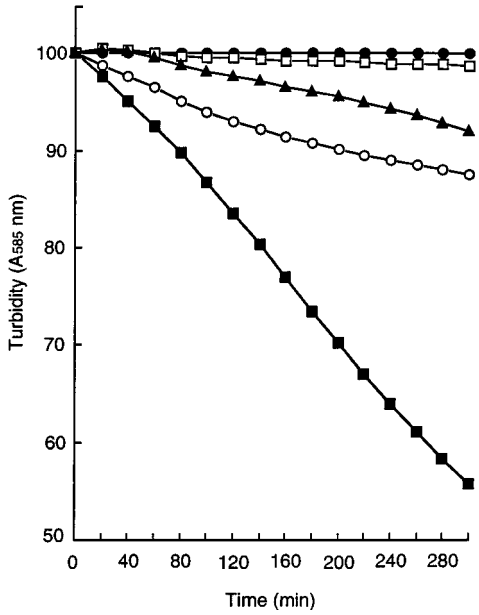

FIG. 3. Effects of autolysis of *S. aureus* BB5918 on a staggered regimen. Symbols: ●, autolysis in the absence of antibiotic in cells pretreated without antibiotic (control); □, autolysis in the presence of imipenem (0.0125 μg/ml) in cells pretreated without antibiotic; ▲, autolysis in the presence of imipenem (0.0125 μg/ml) in cells pretreated with cefotiam (3.13 μg/ml); ○, autolysis in the absence of antibiotic in cells pretreated with imipenem (0.0125 μg/ml); ■, autolysis in the presence of cefotiam (3.13 μg/ml) in cells pretreated with imipenem (0.0125 μg/ml).

against MRSA but also against MSSA (Table 3). PBP 4 is known to be responsible for the second cross-linkage of PG (18, 22). It is thought that imipenem inhibited the function of PBP 4 and caused incomplete formation of the second cross-links in the cell wall (7). Bacteria having an incomplete cell wall are susceptible to cefotiam, which attacks the cell wall by PBP 1, 2, or 3. This speculation was supported by the staggered experiment, which demonstrated that initial treatment with imipenem made the bacteria highly susceptible to the lytic action of cefotiam, while such an effect did not occur when the bacteria were exposed to the drugs in the reverse order.

The most important finding in the present study is a clear correlation between the synergistic effect of imipenem plus cefotiam against MRSA and the degree of autolysis induced by imipenem. This finding indicated that autolysis induced by imipenem is related to the mechanism of the synergistic effect of imipenem plus cefotiam. The present study revealed that autolysis induced by imipenem is caused by impairment of the cell wall but not by increasing amounts of autolysin (Table 3). This impairment of the cell wall presumably was caused by the inhibition of PBP 4. Consistent with the results reported by Gustafson et al. (5), imipenem-induced autolysis was stronger in *mecA*$^+$ strains than in isogenic strains lacking *mecA* (Table 3). The mechanism of the synergistic effect of imipenem plus cefotiam against MSSA is thought to be a decrease in the degree of cross-linkage of PG by inhibition of PBP 4. In contrast, in MRSA, the accumulation of abnormal PG (increasing amounts of PG monomers and dimers and decreasing amounts of oligomers) susceptible to autolysin by the action of PBP 2′ (4) occurred concomitantly with a decrease in the degree of cross-linkage of PG by inhibition of PBP 4. Therefore, the observed degree of autolysis and SR was greater in MRSA than in MSSA.

ACKNOWLEDGMENT

We thank Terutaka Hashizume for performing the PBP analysis.

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