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Susceptibility of Methicillin-resistant *Staphylococcus aureus* Clinical Isolates to Various Antimicrobial Agents. IV. Aminoglycoside-modifying Enzyme AAC(6')/APH(2") is Responsible for Arbekacin-resistance Enhanced by Bleomycin

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ABSTRACT

Resistance patterns against various antimicrobial agents including β-lactams, aminoglycosides, tetracyclines, fluoroquinolones, macrolides were examined for 58 strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated at Hiroshima University Hospital from April to November 1992. All the MRSA strains produced type II-coagulase but not β-lactamase.

Regarding aminoglycoside-modifying enzymes, 7 strains (12%) appeared to be producing aminoglycoside 4',4"-adenyltransferase AAD(4',4") encoded by *aad*D without coproduction of bifunctional aminoglycoside 6'-acetyltransferase/2"-phosphotransferase AAC(6')/APH(2") encoded by *aac*A-*aph*D (referred to as tobramycin-resistant type, TOB^r). The remaining 51 strains (88%) were phenotypically producers of both enzymes (i.e., mix-resistant type, Mix^r). AAD(4',4"), encoded by *aad*D which was reported to be closely linked with bleomycin (BLM)-resistance determinant, could be seen in 100% MRSA strains and ca. 90% strains expressed AAC(6')/APH(2"). BLM endowed Mix^r-type but not TOB^r-type MRSA strain with enhanced resistance to arbekacin (ABK) dose-dependently, presumably by modifying the production of AAC(6')/APH(2"). The manifestation of ABK-resistant phenotype by Mix^r-type MRSA required the coexistence of BLM. Therefore, ABK must be administered carefully to cure MRSA infection in patients who have been treated with BLM.

Key words: *Methicillin-resistant Staphylococcus aureus (MRSA), Antibiotic resistance, AAC(6')/APH(2"), Arbekacin, Bleomycin*

The low-affinity penicillin binding protein (PBP), designated PBP 2'[22], PBP 2a[6] or MRSA PBP[19], encoded by methicillin (DMPPC)-resistance determinant *mec*A, is responsible for the intrinsic resistance of DMPPC-resistant *Staphylococcus aureus* (MRSA) to β-lactams. Furthermore, in most cases MRSA strains are cross-resistant to a variety of antimicrobial agents such as aminoglycosides, tetracyclines, macrolides and fluoroquinolones[13]. For the acquisition of multi-drug resistance, the *mec*-related region which is absent from DMPPC-susceptible *S. aureus* (MSSA) has played a pivotal role[5].

The phenotype of MRSA resistance to aminoglycoside antibiotics was determined by three inactivating enzymes: bifunctional aminoglycoside 6'-acetyltransferase/2"-phosphotransferase AAC(6')/APH(2") encoded by *aac*A-*aph*D, 4',4"-adenyltransferase AAD(4',4") encoded by *aad*D and 3'-phosphotransferase APH(3') encoded by *aph*A[10,14,21]. The *mec*A and *aad*D determinants appear to be linked, as seen by coordinate elimination in MRSA predominant in Japan[22]. Furthermore, it is highly possible that an integrated form of a known plasmid pUB110 carrying both *aad*D[17] and bleomycin (BLM)-resistance determinant (*ble*)[18] is resident in the *mec* region[5,23].

Arbekacin (ABK) lacks 3'- and 4'-hydroxy groups and is a poor substrate for AAC(6')/APH(2") due to the existence of (*S*)-4-amino-2-hydroxybutyl group. Given these structural features, few cases of ABK resistance have been reported since ABK was introduced

into clinical usage as a potent chemotherapeutic against MRSA infections in late 1990[8,9,11,20]. The ABK resistance was attributed to the enhanced activity of AAC(6')/APH(2") in those strains[11,15].

Recently, it was reported that BLM acts as a transcriptional inducer of the *neo* [APH(3')II determinant]-*ble* (BLM-resistance determinant)-*str* (SM-phsophotransferase determinant) operon of the transposon Tn5 in Tn5-containing *Escherichia coli*, increasing the resistance level to amikacin (AMK)[2]. AMK is a poor substrate for APH(3')II just as ABK is a poor substrate for AAC(6')/APH(2"). Therefore, we sought to examine whether BLM can increase the resistance to ABK in Mix^r-type MRSA expressing AAD(4',4") (i.e., the strain becomes resistant to BLM) and AAC(6')/APH(2") in an analogy with AMK resistance in Tn5-containing *E. coli*.

MATERIALS AND METHODS

Bacteria: Fifty-eight MRSA strains were isolated at Hiroshima University Hospital between April and November 1992. DMPPC-susceptible *S. aureus* FDA 209P was used as a reference.

Antibiotics: The antibiotics used and their manufacturers or distributors were as follows: DMPPC, erythromycin (EM) and lincomycin (LCM) (SIGMA Chemical Co.); flomoxef (FMOX), tobramycin (TOB) and vancomycin (VCM) (Shionogi & Co., Ltd.); isepamicin (ISP) (Asahi Chemical Ind., Ltd.); clarithromycin (CAM) (Taisho Pharmaceutical Co., Ltd.); tetracycline (TC) and minocycline (MINO) (Lederle Japan, Ltd.); fosfomycin (FOM) and ABK (Meiji Seika Kaisha, Ltd.); nosiheptide (NH) (Mitsubishi Kasei Corporation); josamycin (JM) (Yamanouchi Pharmaceutical Co., Ltd.); ofloxacin (OFLX) and levofloxacin (LVFX) (Daiichi Pharmaceutical CO., LTD.); temafloxacin (TMFX) (Tanabe Seiyaku Co., Ltd.); kanamycin (KM), dibekacin (DKB), AMK and gentamicin (GM) (Inst. Microb. Chem.); chloramphenicol (CP), benzylpenicillin (PCG) and neomycin (NM) (Wako Pure Chemical Ind., Ltd.); rifampicin (RFP) (Kanto Chemical Co., Inc.); streptomycin (SM) (Irvin Scientific); BLM (Kayaku Co., Ltd.).

Antimicrobial activity: The minimum inhibitory concentration (MIC) was measured unless otherwise specified by two-fold agar dilution method with Mueller-Hinton agar (MHA, DIFCO Laboratories). Test strains grown overnight at 37°C in 5 ml of Mueller-Hinton broth (MHB, DIFCO Laboratories) were 10^2-fold diluted with fresh MHB, and about 5×10^3 CFU was applied with multipoint plating apparatus on a surface of agar plate. The plates were incubated at 37°C for 20 hr.

The production of β-lactamase by individual MRSA strains was monitored by overlaying iodostarch agar supplemented with PCG on bacterial growth.

Effect of BLM on ABK resistance: The discs charged with serial 2-fold dilutions of BLM (200–31.25 μg) were placed on MHA plates containing 10^4 CFU/ml of MRSA. The plate, inoculated with strain 9710 (*aad*D/*aac*A-*aph*D), was supplemented with 3.13 μg/ml ABK, and that for strain 3712 (*aad*D) was supplemented with 0.63 μg/ml ABK. The effect of BLM on the MIC of ABK was studied by 2-fold broth microdilution method with MHB[9].

RESULTS

Fifty-eight MRSA strains were classified as: resistant, moderately resistant or susceptible to each antimicrobial agent depending on their MICs according to the definitions of Maple et al[13] and the British Society for Antimicrobial Chemotherapy[3]. The resistance patterns of all the MRSA strains against 25 antimicrobial agents as well as the MIC distribution of individual compounds are shown in Table 1. The antimicrobial agents, to which more than 95% strains were resistant, included: DMPPC, FMOX, KM, TOB, DKB, AMK, ISP, NM, EM, CAM, TC, MINO, FOM, OFLX, LVFX and TMFX. GM (resistance, 88%) was slightly less effective than these antibiotics and 22–24% strains were resistant to JM, LCM and CP. No resistance was observed against ABK, SM, VCM, NH and RFP. The coagulases produced by MRSA strains were unexceptionally classified into type II. The hydrolysis of PCG was not observed with all the strains.

The resistance phenotypes of MRSA to aminoglycoside antibiotics determined by three inactivating enzymes, in particular AAC(6')/APH(2") and AAD(4')[21], were shown in Table 2. All the strains expressed AAD(4') either exclusively (TOB^r type, 7 strains) or in combination with AAC(6')/APH(2") (Mix^r type, 51 strains). However, there was no strain expressing AAC(6')/APH(2") but not AAD(4',4") (referred to as GM-resistant type, GM^r type).

As for the resistance to tetracyclines, TC and MINO, it was shown that fifty-five strains are resistant to both antibiotics with a TC^{r}MINO^r phenotype (Tet M-type[16], Table 1), whereas the remaining 3 strains are susceptible to both with a TC^{s}MINO^s phenotype. However, there was no strain with a TC^{r}MINO^s phenotype (Tet K-type[16]).

The resistance to the macrolide-lincosamide-streptogramin B (MLS) group of antibiotics, which was first described in *S. aureus*[4] and is now common in this and other bacteria[1,12], was

Table 1. Incidence of antibiotic resistance in the MRSA strains isolated at Hiroshima University Hospital from April to November 1992 (58 strains)

Antimicrobial agent	Resistant strains		Moderately resistant strains		Susceptible strains		*S. aureus* FDA 209P (MIC, μg/ml)
	No. of strains (MIC, μg/ml)	%	No. of strains (MIC, μg/ml)	%	No. of strains (MIC, μg/ml)	%	
Methicillin (DMPPC)	58 (≧25)	100	0	0	0	0	(0.10)
Flomoxef (FMOX)	56 (≧12.5)	97	2 (1.56–3.13)	3	0	0	(0.20)
Kanamycin (KM)	58 (≧50)	100	0	0	0	0	(0.39)
Tobramycin (TOB)	58 (≧50)	100	0	0	0	0	(0.10)
Dibekacin (DKB)	50 (12.5–100)	86	8 (1.56–6.25)	14	0	0	(0.20)
Gentamicin (GM)	51 (50–100)	88	0	0	7 (0.20–0.39)	12	(0.20)
Amikacin (AMK)	58 (6.25–25)	100	0	0	0	0	(1.56)
Isepamicin (ISP)	38 (25–50)	66	20 (6.25–12.5)	34	0	0	(0.78)
Arbekacin (ABK)	0	0	0	0	58 (0.39–1.56)	100	(0.39)
Neomycin (NM)	58 (25–100)	100	0	0	0	0	(0.39)
Streptomycin (SM)	0	0	0	0	58 (1.56–6.25)	100	(3.13)
Erythromycin (EM)	39 (≧25)	67	19 (6.25–12.5)	33	0	0	(0.20)
Clarithromycin (CAM)	33 (≧25)	57	25 (3.13–12.5)	43	0	0	(0.10)
Josamycin (JM)	14 (>100)	24	0	0	44 (0.20–0.39)	76	(0.10)
Lincomycin (LCM)	14 (≧100)	24	0	0	44 (0.39–0.78)	76	(0.39)
Tetracycline (TC)	55 (50–100)	95	0	0	3 (0.20–0.39)	5	(0.20)
Minocycline (MINO)	55 (12.5)	95	0	0	3 (0.05–0.10)	5	(0.10)
Fosfomycin (FOM)	55 (>100)	95	2 (12.5–5.25)	3	1 (1.56)	2	(1.56)
Vancomycin (VCM)	0	0	0	0	58 (0.78–1.56)	100	(0.78)
Ofloxacin (OFLX)	58 (≧6.25)	100	0	0	0	0	(0.20)
Levofloxacin (LVFX)	58 (6.25–50)	100	0	0	0	0	(0.10)
Temafloxacin (TMFX)	58 (6.25–25)	100	0	0	0	0	(0.10)
Nosiheptide (NH)	0	0	0	0	58 (0.003–0.025)	100	(0.006)
Rifampicin (RFP)	0	0	0	0	58 (0.012–0.10)	100	(0.05)
Chloramphenicol (CP)	13 (50)	22	0	0	45 (3.13–6.25)	78	(1.56)

Table 2. Distribution of aminoglycoside modifying enzymes in the MRSA strains

Phenotype	Aminoglycoside-modifying enzyme	Number of strains
$KM^sTOB^sGM^sAMK^sABK^sNM^sSM^s$	—	0
$KM^rTOB^sGM^sAMK^sABK^sNM^rSM^s$	APH(3')	0
$KM^rTOB^rGM^sAMK^rABK^sNM^rSM^s$	ADD(4', 4") ± APH(3')	7
$KM^rTOB^rGM^rAMK^sABK^sNM^sSM^s$	AAC(6')/APH(2")	0
$KM^rTOB^rGM^rAMK^sABK^sNM^rSM^s$	AAC(6')/APH(2") + APH(3')	0
$KM^rTOB^rGM^rAMK^rABK^sNM^rSM^s$	AAC(6')/APH(2")+AAD(4', 4") ± APH(3')	51
	Total ----------	58

Table 3. Type distribution of MLS resistance in the MRSA strains

Phenotype	Type of resistance[a)]	Number of strains
$EM^rCAM^rJM^rLCM^r$	Constitutive	14
$EM^rCAM^rJM^sLCM^s$	Inducible	44
	Total ----------	58

a) When MLS resistance is inducible, the MRSA strains are resistant to 14-membered macrolides (e.g., EM, CAM), but not to 16-membered macrolides (e.g., JM) and lincosamides.

constitutive ($EM^rCAM^rJM^rLCM^r$ phenotype[7]) in 14 strains and inducible ($EM^rCAM^rJM^sLCM^s$ phenotype[7]) in 44 strains (Table 3).

The growth of Mix^r-type MRSA strain 9710 (*aad*D/*ble*/*aac*A-*aph*D) was observed surrounding discs charged with 31.3–200 μg of BLM on an agar plate containing 3.13 μg/ml ABK (Fig. 1A). The higher the concentration of BLM, the bigger the bacterial growing zone. In contrast, no growth of TOB^r-type MRSA strain 3712 (*aad*D/*ble*) was observed at 0.63 μg/ml ABK irrespective of the concentrations of BLM (Fig. 1B). The MICs of BLM for both strains were higher than 200 μg/ml. As shown in Table 4, the MICs of ABK for strain 9710 were in a range of 8–16 μg/ml (or moderately resistant concentrations) in the presence of BLM at 10–80 μg/ml, whereas only a marginal effect of BLM on the MICs of ABK was observed in the case of strain 3712.

DISCUSSION

Although MRSA strains moderately resistant to ABK (MICs, 6.25–25 μg/ml) have been reported on rare occasions in clinical facilities in Japan[11],

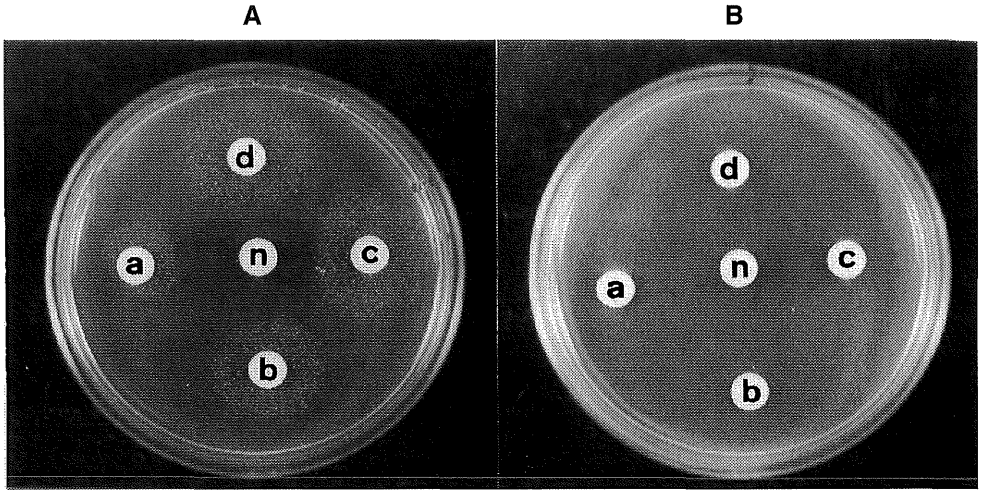

Fig. 1. Induction by BLM of growth of TOB^r- and Mix^r-type MRSA strains on ABK agar plates.
MHA plate, supplemented with 3.13 μg/ml ABK, was inoculated with MRSA strain 9710 (*aad*D/*aac*A-*aph*D, A). The plate for strain 3712 (*aad*D, B) was supplemented with 0.63 μg/ml ABK. Both plates were incubated at 37°C for 20 hr. Amounts of BLM are: a, 31.3 μg; b, 62.5 μg; c, 125 μg; d, 250 μg; n, control with no BLM.

Table 4. MICs of ABK for Mix^r- and TOB^r-type MRSA strains in the presence of doubling concentrations of BLM

BLM conc. (μg/ml)	MIC (μg/ml) of ABK	
	Strain 9710	Strain 3712
0	4	0.5
10	8	1
20	16	1
40	16	1
80	16	1

The MICs of ABK were measured by the broth microdilution method in MHB in the absence or presence of 10–80 μg/ml BLM after 20 hr at 37°C.

highly resistant strains (MICs, $\geqq$50 μg/ml) have not been isolated up to date. Okamoto et al established ABK-resistant variant from GM^r-type MRSA strain *in vitro* and proved that the ABK-resistance could be accounted for by the enhanced expression of *aac*A-*aph*D gene[15]. We also attempted to isolate a highly ABK-resistant MRSA variant from ABK-susceptible GM^r-type MRSA strain by repeated treatment with increasing concentrations of ABK. Although the MIC of 50 μg/ml was finally attained, the *in vitro* resistant variant could not grow as fast as a parental strain even under ABK-free conditions (unpublished data). The intracellular accumulation of AAC(6')/APH(2"), therefore, might be disadvantageous for the growth of MRSA, precluding the appearance of highly ABK-resistant strains *in vivo*.

As shown in Fig. 1, BLM increased ABK resistance by presumably enhancing the intracellular content of AAC(6')/APH(2") dose-dependently. This phenomenon requires the existence of BLM, because the isolate from the BLM-dependent ABK-resistant colony was no longer resistant to ABK in the absence of BLM (data not shown). These results raise the possibility of a decreased antimicrobial efficacy of ABK against Mix^r-type MRSA in patients treated with BLM or the related agents.

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