ASTROMICIN (FORTIMICIN A)-RESISTANT BACTERIA IN JAPAN

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The susceptibility of 539 clinical isolates to astromicin (ASTM) and other aminoglycoside antibiotics was determined. Isolation frequency of ASTM-resistant strains among 443 *Enterobacteriaceae* species (130 *Serratia marcescens*, 94 *Proteus* sp., and others) was 2.9% (13 strains). Thirty-two strains (33.3%) among 96 glucose-nonfermentative gram-negative bacilli (53 *Pseudomonas aeruginosa*, and others) were resistant to ASTM. Twenty strains which showed various levels of ASTM resistance among 45 ASTM-resistant strains were selected for further studies. None of these 20 strains was found to produce ASTM-inactivation enzyme [AAC(3)-I or a so far unknown enzyme]. Fifteen among them were shown to possess one, two, or three kinds of aminoglycoside-inactivation

enzymes. ASTM resistance in 4 strains of the 20 was decreased in the concomitant presence of subinhibitory concentrations of ethylenediaminetetraacetic acid (EDTA). ASTM resistance of the remaining 16 strains was unchanged even by the presence of EDTA.

INTRODUCTION

Astromicin (fortimicin, KW-1070) is the most active member of the fortimicin complex of aminoglycoside antibiotics produced by fermentation of *Micromonospora olivoasterospora*[1]. It is a pseudodisaccharide-incorporating fortamine, a novel aminocyclitol[2]. This new antibiotic possesses a broad spectrum of activity against gram-positive and gram-negative bacteria, but it has relatively weak activity against *Pseudomonas aeruginosa* strains[3]. ASTM is effective against many aminoglycoside-resistant bacteria capable of producing aminoglycoside-inactivation enzymes except for AAC(3)-I[4].

The present studies have been carried out in an attempt to investigate, at the present stage, the isolation frequency of ASTM-resistant gram-negative isolates and the presence of aminoglycoside-inactivation enzymes in certain of these isolates. Furthermore, we carried out studies on the effects of subinhibitory concentrations of ethylenediaminetetraacetic acid (EDTA) on minimal inhibitory concentrations (MICs) of ASTM.

MATERIALS AND METHODS

Bacterial strains. A total of 539 gram-negative clinical isolates were obtained from 63 hospitals (medical organs of the authors and the related hospitals) in Japan between 1979 and 1980. These were random isolates from urine obtained by catheter or mid stream of different patients. Among 45 ASTM-resistant strains (Table 1, 2), 20 strains (Table 3) were chosen as the representative ASTM-resistant strains, showing various resistance-levels to ASTM. *Staphylococcus aureus* FDA 209 P was also used as a reference for the EDTA test[5].

Media. Lac-BTB (lactose-bromothymol blue) agar was used as a solid medium. Lac-BTB agar consisted of nutrient broth (10 g of beef extract, 10 g of peptone and 3 g of NaCl in 1 liter of distilled water) supplemented with 10 g of lactose, 80 mg of bromothymol blue and 15% of agar. For the antibiotic susceptibility tests, Mueller-Hinton medium (Nissui, Tokyo) was used. For the preparation of cell-free extracts, Medium B was used and consisted of 2 g of yeast extracts, 10 g of peptone, 8 g of $Na_2HPO_4 \cdot 12H_2O$, 2 g of KH_2PO_4, 1.2 g of $(NH_4)_2SO_4$, 2 g of glucose, 0.4 g of $MgSO_4 \cdot 7H_2O$ and 1 liter of distilled water.

Antibiotics and chemicals. The following drugs were used: gentamicin (GM), GM-C_1, GM-C_{1a}, GM-C_2, micronomicin (MCR), sisomicin (SISO), netilmicin (NTL), astromicin (ASTM), kanamycin (KM)-A, -B, -C, 3′,4′-dideoxykanamycin-B (DKB), tobramycin, neomycin C, paromomycin, lividomycin, amikacin (AMK), ribostamycin, butirosin B, spectinomycin (SPCM), streptomycin (SM), dihydrostreptomycin (DSM), and apramycin. Trilithium *S*-acetyl CoA, disodium adenosine 5′-triphosphate (ATP), and EDTA were all commercial products. The isotope-labeled compounds, *i.e.*, [γ-^{32}P]ATP(6.3 Ci/mmol), [8-^{14}C]ATP(54.2 mCi/mmol), and [1-^{14}C] *S*-acetyl coenzyme A (54.0 mCi/mmol) were purchased from the New England Nuclear Corp., Boston, Mass., USA.

Antimicrobial susceptibility tests. The MICs were determined by the agar dilution method. Overnight cultures of test strains were diluted to a final concentration of approximately 5×10^5 cells/ml, and 5 μl of each culture was inoculated by an inoculator (Microplanter, Sakuma, Tokyo) on Mueller-Hinton agar plates containing serial two-fold dilutions of the drug. MICs were recorded after 18 hours of incubation at 37℃. The criteria for resistance to aminoglycoside antibiotics were 12.5 μg/ml. The effects of EDTA were estimated in the same way (agar dilution method) whether in the presence or absence of a subinhibitory concentration (1/2 or 1/4 MIC) of EDTA.

Preparation of cell-free extracts. An organism derived from a single colony was grown at 37℃ in 1 liter of Medium B or Medium B supplemented with 0.4% KNO_3. The crude cell-free extracts were prepared from late logarithmic growing cells of tested strains. The cells were harvested by centrifugation, washed twice with TMK solution (0.01 M Tris-HCl buffer containing 0.01 M $MgSO_4 \cdot 6H_2O$, 0.06 M KCl, and 6 mM 2-mercaptoethanol, pH 7.5), and resuspended in the same buffer. The cell suspension was disrupted sonically

Table 1 Isolation frequency of ASTM-resistant strains

Organism	No. of isolates	No. of resistant strains	Isolation frequency of resistant strains (%)
E. coli	76	5	6.6
K. pneumoniae	46	0	0.0
K. oxytoca	17	1	5.9
Enterobacter sp.	36	0	0.0
Citrobacter sp.	22	0	0.0
P. mirabilis	33	2	6.0
indole (+) *Proteus* sp.	61	1	9.1
S. marcescens	130	4	3.1
Others (G^-)*	22	0	0.0
	(443)	(13)	(2.9)
P. aeruginosa	53	24	45.3
Pseudomonas sp.	28	5	17.9
A. anitratus	9	2	22.2
Others (GNF·G^-)†	6	1	16.6
	(96)	(32)	(33.3)
Total	539	45	8.3

* Other *Enterobacteriaceae* species. G^-, gram-negative bacteria.
† Other glucose-nonfermentative (GNF) gram-negative bacteria.

Table 2 Resistance pattern of ASTM-resistance strains

Organism	Resistance pattern*	No. of strains
E. coli	ASTM KM-A DKB AMK SPCM DSM	1
	MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	1
	GM MCR SISO ASTM KM-A DKB SPCM DSM	1
	GM MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	2
K. oxytoca	GM MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	1
P. mirabilis	ASTM KM-A DKB AMK SPCM DSM	1
	GM MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	1
P. rettgeri	GM MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	1
S. marcescens	NTL ASTM KM-A DKB AMK SPCM DSM	1
	GM MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	3
P. aeruginosa	ASTM KM-A SPCM DSM	8
	ASTM KM-A DKB SPCM DSM	3
	ASTM KM-A DKB AMK SPCM DSM	1
	GM MCR SISO NTL ASTM KM-A SPCM DSM	1
	GM MCR SISO NTL ASTM KM-A DKB SPCM DSM	1
	GM MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	3
	GM MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	7
Pseudomonas sp.	ASTM KM-A SPCM DSM	2
	GM MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	3
A. anitratus	NTL ASTM SPCM DSM	1
	GM MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	1
F. odoratum	GM MCR SISO NTL ASTM KM-A DKB AMK SPCM DSM	1

* Drugs tested were GM, MCR, SISO, NTL, ASTM, KM-A, DKB, AMK, SPCM, and DSM.

at 20 kHz for 5~10 minutes and then centrifuged at 30,000×g for 30 minutes at 4℃. The supernatant thus obtained (S-30 fraction) was used as the crude enzyme solution; its protein content was adjusted to 10 mg of protein per ml. Protein in the S-30 fraction was determined by the method of LOWRY et al.[6], using bovine serum albumin as a standard.

Enzyme assay. We examined the existence of enzymatic inactivation of aminoglycoside antibiotics by the method of OZANNE et al.[7], with three different buffers [acetate buffer (pH 6.0), Tris-maleate buffer (pH 7.0), or Tris-HCl buffer (pH 8.0)]. Inactivation of each aminoglycoside was attempted in each buffer for each extract. The reaction mixture consisted of 0.03 ml of S-30 fraction, 0.01 ml of 0.02 M $MgCl_2 \cdot 6H_2O$, 0.01 ml of 0.8 mM drug, 0.01 ml of [γ-^{32}P]ATP (50 μCi/ml) (APH system), [8-^{14}C]ATP (20 μCi/ml) (AAD system), or [1-^{14}C] *S*-acetyl coenzyme A (10 μCi/ml) (AAC system), 0.01 ml of 0.8 mM unlabeled ATP or 0.8 mM unlabeled *S*-acetyl coenzyme A, and 0.03 ml of 0.2 M buffer in a total volume of 0.1 ml. The experimental procedure followed was as described previously[8].

Classification of aminoglycoside-inactivation enzymes. Classification of aminoglycoside-inactivation enzymes was estimated by the substrate specificities[9,10] and the representative inactivation products shown below. Inactivated products of the representative aminoglycoside antibiotics (SISO, KM-A, DKB, and SM) were purified by column

Table 3 MICs of ASTM and GM for the representative ASTM-resistant strains (20 strains)

	MIC (μg/ml)	
	GM	ASTM
E. coli		
GN 12781	1.56	12.5
GN 12782	6.25	25.0
GN 12783	12.5	25.0
P. mirabilis		
GN 12784	6.25	25.0
GN 12786	1,600	25.0
P. rettgeri		
GN 12785	25.0	50.0
S. marcescens		
GN 12787	3.12	12.5
GN 12788	12.5	12.5
GN 12789	12.5	25.0
GN 12793	1,600	12.5
A. anitratus		
GN 12790	1.56	100
GN 12791	>1,600	800
F. odoratum		
GN 12792	>1,600	1,600
P. aeruginosa		
GN 12794	0.78	25.0
GN 12795	1.56	25.0
GN 12796	3.12	100
GN 12797	25.0	100
GN 12798	800	25.0
GN 12800	800	100
GN 12799	1,600	200

Table 4 Aminoglycoside-inactivation enzymes of representative ASTM-resistant strains

	Organism	Inactivation enzyme*
E. coli	GN 12781	AAD(3″)
P. mirabilis	GN 12786	AAC(3)-III, AAD(2″)
S. marcescens	GN 12787	AAC(6′)-III
	GN 12788	AAC(3)-III
		AAD(3″)
		APH(3′)-I
	GN 12789	AAC(3)-III
		AAD(2″)
		APH(3′)-III
	GN 12793	AAC(3)-III-IV
		AAD(2″)
		APH(3′)-III
A. anitratus	GN 12791	AAD(2″)
		APH(3′)-III, and APH(6)
F. odoratum	GN 12792	AAD(3″)
P. aeruginosa	GN 12794	APH(3′)-II
	GN 12795	APH(3′)-II
	GN 12796	APH(3′)-II
	GN 12797	APH(3′)-II, and APH(3″)
	GN 12798	AAC(3)-III
		AAD(3″)
		APH(3′)-II
	GN 12800	AAC(6′)-III
		APH(3′)-II, and APH(3″)
	GN 12799	AAC(6′)-IV
		AAD(3″)
		APH(3′)-II

* For details, see Materials and Methods.

Table 5 Effect of EDTA on the MICs of ASTM against ASTM-resistant strains

		Addition of EDTA (μmol/ml)	MIC(μg/ml) ASTM	MIC(μmol/ml) EDTA
E. coli	GN 12781	None	12.5 (1)*	3.80
		0.95	12.5	
	GN 12782	None	25.0 (1)	3.80
		0.95	25.0	
	GN 12783	None	25.0 (1)	3.80
		0.95	25.0	
P. mirabilis	GN 12784	None	25.0 (1)	3.80
		0.95	25.0	
	GN 12786	None	25.0 (2)	1.98
		0.95	12.5	
P. rettgeri	GN 12785	None	50.0 (1)	1.90
		0.48	50.0	
S. marcescens	GN 12787	None	12.5 (4)	121
		30.4	3.12	
	GN 12788	None	12.5 (1)	30.4
		15.2	12.5	
	GN 12789	None	25.0 (1)	15.2
		3.80	25.0	
	GN 12793	None	12.5 (2)	60.8
		15.2	6.25	
A. anitratus	GN 12790	None	100 (1)	1.90
		0.95	100	
	GN 12791	None	800 (2)	1.90
		0.95	400	
F. odoratum	GN 12792	None	1,600 (1)	3.80
		0.95	1,600	
P. aeruginosa	GN 12794	None	25.0 (1)	3.80
		0.95	25.0	
	GN 12795	None	50.0 (4)	1.90
		0.95	12.5	
	GN 12796	None	100 (1)	1.90
		0.95	100	
	GN 12797	None	100 (4)	1.90
		0.95	25.0	
	GN 12798	None	25.0(16)	7.60
		1.90	1.56	
	GN 12800	None	100 (1)	1.90
		0.95	100	
	GN 12799	None	200 (2)	3.80
		0.95	100	
S. aureus	FDA 209 P	None	0.39(1)	1.90
		0.48	0.39	

* The ratio of MICs of ASTM in the absence of EDTA to those in the presence of EDTA.

chromatography. The *N*-acetylated[11,12], *O*-adenylated[8,13], or *O*-phosphorylated[14,15] position of aminoglycoside antibiotics was estimated by paper electrophoresis and thin-layer chromatography in comparison with the following authentic samples: 3-*N*-acetylated SISO[11], 6′-*N*-acetylated KM-A[12], 2″-adenylated DKB[13], 3′-phosphorylated KM-A[15], 3″-adenylated SM and 6-adenylated SM[8], 3″-phosphorylated SM and 6-phosphorylated SM[14].

RESULTS

Isolation frequency of ASTM-resistant strains. The MICs of 539 gram-negative clinical isolates to aminoglycoside antibiotics were determined. The isolation frequency of ASTM-resistant strains (MIC, ≧12.5 μg of ASTM per ml) and their resistance patterns to aminoglycoside antibiotics are shown in Tables 1 and 2. As shown in Table 1, 45 strains (8.3%) among 539 isolates were found to be ASTM-resistant. Except for glucose-nonfermentative strains, only 13 strains (2.9%) among 443 isolates were resistant to ASTM. Antibacterial activity of ASTM against *P. aeruginosa* and other glucose-nonfermentative gram-negative bacilli was weak. Thirtytwo strains (33.3%) among them were resistant to ASTM.

As shown in Table 2, there were no strains resistant to ASTM alone among the ASTM-resistant strains. All were resistant to KM-A, SPCM, and DSM except for one strain of *A. anitratus*. More than one-half of the ASTM-resistant strains were highly and multiply resistant to the 10 aminoglycoside antibiotics tested.

Aminoglycoside-inactivation enzymes of the representative ASTM-resistant strains. Since 6′-epi-purpurosamine B residue of ASTM is similar to purpurosamine residue of GM-C_2, we examined the resistance correlation between ASTM and GM. Among 45 ASTM-resistant strains, we selected the 20 strains which showed different levels of resistance to ASTM and GM (from low- to high-level). The MICs of the 20 strains are shown in Table 3.

The aminoglycoside-inactivation enzymes of the representative ASTM-resistant strains are shown in Table 4. All strains tested were found to produce various aminoglycoside inactivation enzymes, but we could not detect any enzyme capable of inactivating ASTM.

In *P. aeruginosa* strains (GN 12794, GN 12795, GN 12796, GN 12797, GN 12798, GN 12800, and GN 12799), all strains were found to possess aminoglycoside-inactivation enzymes. APH (3′)-II was detected in every instance.

Effect of EDTA on the MICs of ASTM. Table 5 shows the MICs of ASTM against 20 ASTM-resistant strains and *S. aureus* FDA 209P in the presence of subinhibitory concentrations (1/2, or 1/4 MIC) of EDTA. ASTM resistance in 4 strains of the 20 was decreased by 4- or 16-fold in the presence of subinhibitory concentration of EDTA. On the other hand, ASTM resistance of the remaining 16 strains was scarcely decreased.

DISCUSSION

ASTM inhibited the growth of more than 90% of the clinical isolates of *Escherichia, Klebsiella, Enterobacter, Citrobacter, Proteus, Serratia,* and other *Enterobacteriaceae* species tested, at a concentration of 8 μg/ml[16]. In our study, the similar ratio was obtained in the clinical isolates of the above-mentioned *Enterobacteriaceae* species by ASTM at a concentration of 12.5 μg/ml. It was reported that AAC(3)-I was produced probably by R plasmids in multiple-resistant clinical isolates[17]. According to the results of our study, however, AAC(3)-I does not seem to be widespread in Japan. By any of our ASTM-resistant strains, ASTM was not inactivated, although various enzymes inactivating other aminoglycosides were detected.

In *S. marcescens* and *P. aeruginosa* strains, resistance levels to aminoglycoside antibiotics except ASTM were generally associated with the presence of inactivation enzymes.

It was reported that the addition of EDTA reduced the MICs of antibiotics in drug-resistant gram-negative bacteria due to damage of the permeability barrier of the outer membrane by chelation of the divalent cations[5].

We could not detect an ASTM-inactivation enzyme(s) among the 20 ASTM-resistant strains tested. ASTM resistance in 4 strains was decreased in the presence of subinhibitory concentrations of EDTA. ASTM resistance in the remaining 16 strains was little changed in the presence of EDTA.

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本邦における Astromicin (Fortimicin, KW-1070) 耐性菌について

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アミノグリコシド系抗生物質（AGs）である Astromicin(ASTM) は，その化学構造中に新規な fortamine 残基を有し，細菌学的特徴として AAC(3)-I 以外の AGs 不活化酵素に対し安定である。

今回，われわれは ASTM の現在時における細菌学的特徴を臨床分離株（1979 年から 1980 年に分離した 539 株）を用いて調べた。

（1） 443 株の腸内細菌（*S. marcescens*, 130 株；*Proteus* sp., 94 株；その他，219 株）のうち 13 株（2.9%）が ASTM 耐性菌（MIC, ≧12.5 μg/ml）であり，ASTM 耐性菌の出現頻度は低かった。

（2） 96 株のブドウ糖非発酵グラム陰性桿菌（*P. aeruginosa*, 53 株；その他，43 株）のうち 32 株（33.3%）が ASTM 耐性菌であった。この点，従来の結果とほぼ一致した。

（3） 上記 45 株（8.3%）の ASTM 耐性菌の中から代表 20 株を選び AGs 不活化酵素の有無を調べた。その結果，15 株に ASTM 以外の AGs 不活化酵素を認めたが，ASTM を不活化する酵素の存在は認められなかった。

（4） 上記代表 20 株の ASTM に対する感受性に及ぼす EDTA の同時併用効果を調べたところ，4 株において感受性が増大したが，残り 16 株ではほとんど変化しなかった。