ENZYMATIC INACTIVATION OF GENTAMICIN C COMPONENTS BY CELL-FREE EXTRACT FROM *KLEBSIELLA PNEUMONIAE*

Sir:

Gentamicin C, a broad spectrum antibiotic complex, has been isolated from submerged culture of *Micromonospora purpurea*[1] and their chemical structure was described by COOPER *et al.*[2~4]. Purpurosamine, a component of gentamicin C, is a novel compound and contains no hydroxyl groups in the ring. It was reported that several aminoglycosidic antibiotics were inactivated by the strains of bacteria resistant to the drugs. The inactivating enzymes were known to be capable of specifically phosphorylating[5~10] antibiotics at the 3-hydroxy position of the amino sugar or acetylating kanamycin on the amino group of the amino sugar[5,11]. Similarly, a kanamycin-phosphorylating enzyme from a resistant strain of *Pseudomonas aeruginosa* inactivated paromomycin, mannosylparomomycin, aminodeoxykanamycin and neomycin, but did not lividomycin[12] and gentamicin C components, which were devoid of hydroxy group at position 3 of the amino sugar[13]. We also reported that the enzyme from gentamicin-resistant *P. aeruginosa* inactivated gentamicin C components by the mechanism involving the formation of an acetylated product[14].

Present studies have disclosed that gentamicin C components are inactivated by another mechanism involving the formation of adenylylated products of gentamicin C components. Gentamicin-resistant strains of *Klebsiella pneumoniae* 3020, 3694 and *P. aeruginosa* 99 were used and they were all clinical isolates. Drug resistance of *K. pneumoniae* 3694 was found to be transmissible by conjugation and was transferred to *Escherichia coli* ML 1410. This transmissible drug resistance factor R 99 was capable of conferring 6-drug resistance including sulfanilamide, chloramphenicol, tetracycline, streptomycin, kanamycin and gentamicin C. The organisms were cultured in heart infusion (HI) broth containing 0.1% glucose with shaking at 30°C. The cells at the logarithmic growth phase were harvested by centrifugation, washed three times with M/10 TMK solution (M/10 tris buffer containing 0.06 M KCl, 0.01 M magnesium acetate and 0.006 M 2-mercaptoethanol, pH 7.8) and resuspended in the same solution. The cell suspension was disrupted by a supersonic apparatus at 20 Kc for 10 minutes. After treatment with deoxyribonuclease (4 mcg/ml) at 30°C for 15 minutes, the sonicated suspension was centrifuged at $10,000 \times g$ for 20 minutes. The supernatant thus obtained was subjected to further centrifugation at $105,000 \times g$ for 60 minutes and the supernatant was dialyzed against TMK solution. The solution was designated the S-105 fraction.

As shown in Tables 1 and 2, the S-105 fractions obtained from *K. pneumoniae* 3020, 3694, and *E. coli* ML 1410 R_{100}^+ inactivated gentamicin C components in the presence of adenosinetriphosphate (ATP). Requirements for the inactivation of gentamicin C by the S-105 fraction from *E. coli* ML 1410 R_{100}^+ is shown in Table 1, gentamicin C components being inactivated in the presence of ATP but did not in the presence of acetylcoenzyme A in place of ATP.

Next, isotopic investigation on the incorporation of γ-^{32}P-labeled ATP, 8-^{14}C-labeled ATP and ^{14}C-labeled acetate into antibiotics was performed to disclose the inactivation mechanism (Table 2). The S-105 fractions from *K. pneumoniae* 3020, 3694 and *E. coli* ML 1410 R_{100}^+ inactivated 3 components of

Table 1. Requirements for the inactivation of gentamicin C by the cell-free extract of *E. coli* ML 1410 R_{100}^+

Reaction mixture	Percent of inactivation		
	C_1	C_2	C_{1a}
Complete system*	99.7	99.5	96.0
-(S-105)	0	0	0
-ATP	5	0	0
-ATP+acetyl CoA**	0	0	0

The inactivating reaction was carried out at 30°C for 1 hour and the remaining activity of the antibiotics was assayed by a paper disk method using *Bacillus subtilis* as test organism. The standard deviation of the bioassay was found to be less than 10%.

* Complete system consisted of 0.3 ml of the S-105 fraction (20 mg protein per ml), 0.1 ml of 40 mM ATP, 0.1 ml of 1 mM antibiotic and 0.5 ml of TMK solution.

** ATP was replaced by 0.1 ml of 12 mM acetyl CoA.

Table 2. Incorporation of isotope-labeled compounds into gentamicin C components in the process of their inactivation

S-105 fraction	MIC [a] (mcg/ml)	Gentamicin C components	P system [b]			A system [c]	
			32_P [d] cpm	14_C [e] cpm	Inactivation [f] %	14_C [g] cpm	Inactivation [f] %
K. pneumoniae 3020	>200	C_1	0	1,334	100	17	100
		C_2	6	573	100	17	100
		C_{1a}	0	607	91	3	100
K. pneumoniae 3694	100	C_1	0	2,056	98.8	9	99.1
		C_2	0	423	98.4	12	75
		C_{1a}	5	404	62	37	48
E. coli ML 1410 R_{100}^+	25	C_1	0	1,450	99.3	2	100
		C_2	0	686	98.3	2	99.1
		C_{1a}	0	519	94.8	7	89
E. coli ML 1410	0.2	C_1	0	8	0	3	0
		C_2	0	12	0	0	0
		C_{1a}	0	25	0	5	0
P. aeruginosa 99	>200	C_1	8	7	0	601	68
		C_2	0	12	0	397	60
		C_{1a}	0	0	0	631	92

The reaction was carried out at 30℃ for 60 minutes and then 10 μl of the reaction mixture was spotted on the phosphocellulose paper (about 1 cm², Whatman P 81), washed with water and dried. The paper was counted in a Tri-Carb scintillation counter (Packard) with a toluene-based fluid.

a): Minimum inhibitory concentration of gentamicin C complex.

b): The P system consisted of the following materials; 50 μl of TMK solution, 10 μl of 1 mM each antibiotic solution, 10 μl of 40 mM (50 μci/ml) ATP containing ^{32}P-labeled ATP or of 40 mM (35 μci/ml) ATP containing ^{14}C-labeled ATP and 30 μl of the S-105 fraction (20 mg protein per ml).

c): The A system consisted of the following materials; 30 μl of TMK solution, 10 μl of 1 mM each antibiotic solution, 10 μl of 40 mM ATP, 10 μl of 2 mM coenzyme A and 10 μl of ^{14}C-acetate (100 μci/ml).

d): Counts of the incorporated γ-^{32}P of ATP into antibiotics.

e): Counts of the incorporated ^{14}C-labeled component of ATP into antibiotics.

f): Number indicates the inactivation (%) of each component of gentamicin C in the same reaction mixture without labeled coenzymes.

g): Counts of the incorporated ^{14}C-labeled acetic acid into antibiotics.

gentamicin C complex in the presence of ATP, demonstrating the incorporation of ^{14}C-labeled component of ATP into gentamicin C but not of ^{32}P. In the A system shown in Table 2, the incorporation of ^{14}C-labeled acetic acid into gentamicin C was not seen in the inactivation of the drugs by these fractions. From the data, the inactivation of gentamicin C was considered to be caused by the formation of an adenylylated product.

Previous studies[14] indicated that the S-105 fraction from *P. aeruginosa* 99 inactivated gentamicin C components in the presence of acetylcoenzyme A by the mechanism involving the formation of acetylated product. This was confirmed further by the incorporation of ^{14}C-labeled acetic acid into gentamicin C (Table 2). The inactivated position of each of the gentamicin C components is still being elucidated and will be described elsewhere.

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