BIOCHEMICAL STUDIES ON GENTAMICIN RESISTANCE

Sir:

Two major mechanisms of resistance to aminoglycosidic antibiotics have been reported. One is a change in one of the 30S ribosomal proteins, which is the site of action of the antibiotics. Genetically it is related to chromosomal changes[1,2]. The other is the production of antibiotic-inactivating enzymes, which are found in *Escherichia coli* carrying an R factor and in other organisms. The inactivated products are adenylstreptomycin, streptomycin-3′-phosphate, kanamycin-3′-phosphate, 6′-N-acetylkanamycin, and adenylspectinomycin[3~9].

The primary site of action of gentamicin is in the ribosomes, as in the case of other aminoglycosidic antibiotics[11~13]. The biochemical basis of gentamicin resistance has been studied with gentamicin-resistant clinical isolates of *Pseudomonas aeruginosa*. The results are presented in this publication.

Gentamicin C_1, C_{1a}, C_2 and C complex were generously given by Dr. M. J. WEINSTEIN, Schering Research Division, Bloomfield, and gentamicin A by Dr. C. P. SCHAFFNER, Rutgers University, New Brunswick, N. J.

Pseudomonas aeruginosa H-9 was obtained from Dr. Y. HOMMA, Institute of Medical Science, University of Tokyo. The minimal inhibitory concentration (MIC) of gentamicin was 6 mcg/ml. The six gentamicin-resistant strains of *P. aeruginosa* (#54, 146, 207, 209, 290 and 360) were kindly supplied by Dr. J. A. WAITZ, Schering Research Division, Bloomfield, N. J. They were isolated at Grady Memorial Hospital in Atlanta, Georgia from burn wound infections. All the strains are resistant to gentamicin with MIC values higher than 100 mcg/ml.

The preparation of ribosomes and 105,000 $\times g$ supernatant fraction, and the method of amino acid incorporation into polypeptide were essentially the same as described in a previous report[2].

Significant incorporation of ^{14}C-leucine with endogenous mRNA and of ^{14}C-phenylalanine with poly U were demonstrated with the ribosomes and 105,000$\times g$ supernatant obtained from all the 7 strains of *P. aeruginosa*. Gentamicin C complex inhibited protein synthesis in the system from gentamicin-sensitive strain H-9 but did not significantly affect protein synthesis in the systems from gentamicin-resistant strains #54, 146, 207, 209, 290 and 360. The results are summarized in Table 1.

For the purpose of determining whether the ribosomes or supernatant is responsible for the gentamicin resistance or sensitivity, the ribosomes from gentamicin-resistant organisms were combined with the supernatant from the gentamicin-sensitive organism and *vice versa*, and the effects of gentamicin C complex on polyphenylalanine synthesis with poly U were examined. The antibiotic was observed to exert no significant activity on polypeptide synthesis, when the ribosomes from gentamicin-resistant organisms (#54, 146, 207, 209 and 360) and the supernatant fraction from the gentamicin-sensitive organism H-9 were involved in the system. However, gentamicin C inhibited polypeptide synthesis, when the ribosomes from gentamicin-sensitive strain H-9 and the supernatant fractions from

Table 1. Effects of gentamicin C complex on protein synthesis in cell-free systems obtained from the resistant and sensitive organisms

Pseudomonas aeruginosa	Incorporation of leucine with native mRNA		Incorporation of phenylalanine with poly U	
	−GM	+GM	−GM	+GM
#54	2.55	2.50	3.54	3.89
146	3.62	3.87	3.03	3.05
207	8.01	8.35	9.45	9.15
209	5.65	5.94	5.97	5.56
290	6.20	5.64	7.13	6.34
360	3.39	3.08	4.02	4.55
H-9	7.32	2.98	9.08	2.17

The number represents $\mu\mu$moles of ^{14}C-amino acid incorporated into the hot TCA-insoluble fraction. In the case of leucine incorporation, the reaction mixture contained, in 0.5 ml: ribosomes 1 mg, 105,000$\times g$ supernatant 0.5 mg, ATP 1 μmole, GTP 0.05 μmole, phosphoenolpyruvate 10 μmoles, pyruvate kinase 20 μg, ^{14}C-leucine 0.2 μc, 19 different amino acids 0.3 μmole each, and gentamicin C complex 5 μg. For phenylalanine incorporation, it contained: the ribosomes 0.5 mg, the preincubated 105,000$\times g$ supernatant 0.5 mg, ATP 1 μmole, GTP 0.05 μmole, phosphoenolpyruvate 10 μmoles, pyruvate kinase 20 μg, ^{14}C-phenylalanine 0.2 μc, poly U 40 μg and gentamicin C complex 5 μg. The buffer consisted of $MgCl_2$ 10 mM, NH_4Cl 50 mM, 2-mercaptoethanol 6 mM, and Tris-HCl 10 mM, pH 7.6. The reaction mixtures were incubated at 37°C for 20 minutes.

Table 2. Effects of gentamicin C complex on polypeptide synthesis in the systems, combining the ribosomes or soluble fractions from the resistant organisms and those from the sensitive organism

Systems		Incorporation of phenylalanine with poly U		
Ribosomes	105,000 $\times g$ sup.	−GM	+GM	% inhibition
# 54	H-9	2.78*	2.45	12
146	〃	3.71	3.59	3
207	〃	7.54	6.10	9
209	〃	5.89	5.90	0
290	〃	5.03	4.53	10
360	〃	4.11	3.58	13
H-9	# 54	6.98	1.87	73
〃	146	7.06	1.69	76
〃	207	7.41	2.30	69
〃	209	7.12	2.85	60
〃	290	6.31	1.77	72
〃	360	7.65	2.83	63

* The number represents $\mu\mu$moles of phenylalanine incorporated into the hot TCA-insoluble fraction. The assay method was the same as described in Table 1.

Table 3. Effects of extracts of the resistant organisms on gentamicins in the presence of ATP

Organisms	% Activity remained		
	Gentamicin C_1	Gentamicin C_{1a}	Gentamicin C_2
# 54	90	108	100
146	105	110	95
207	85	90	93
209	103	110	110
290	110	105	100
360	110	103	105

The reaction mixture consisted of the 105,000 $\times g$ supernatant 2 mg protein/ml, ATP 20 mM, antibiotics 0.2 mM, KCl 156 mM, Mg acetate 10 mM, 2-mercaptoethanol 15.6 mM and Tris-HCl 260 mM, pH 7.5. It was incubated at 37℃ for 5 hours. The reaction was terminated by heating at 80℃ for 5 minutes. It was diluted in phosphate buffer, pH 7.8, and assayed by a disc-plate method, using *Staphylococcus aureus* as the test organism[10].

gentamicin-resistant strains were used. The results (Table 2) show that the resistance to the gentamicin C complex in these organisms resides in the ribosomes.

The enzymatic activity of the 105,000 $\times g$ fraction from gentamicin-resistant organisms, which might phosphorylate, adenylate or acetylate gentamicin, were investigated, following the method employed with kanamycin[5~9]. As presented in Tables 3 and 4, no significant inactivation of gentamicin C_1, C_{1a} or C_2 by the cell extracts was observed in the presence of ATP and/or acetyl CoA.

The kanamycin-phosphorylating enzyme was obtained from *P. aeruginosa* H-9 by the

Table 4. Effects of extracts of the resistant organisms on gentamicins in the presence of acetyl-CoA

Organisms	% Activity remained		
	Gentamicin C_1	Gentamicin C_{1a}	Gentamicin C_2
# 54	103	110	102
146	110	103	110
207	95	105	110
209	93	95	105
290	105	108	100
360	100	105	95

The reaction mixture consisted of the 105,000 $\times g$ supernatant 3.6 mg protein/ml, acetyl-CoA 0.1 mM, ATP 4 mM, antibiotics 0.2 mM, KCl 156 mM, Mg acetate 10 mM, 2-mercaptoethanol 15.6 mM and Tris-HCl 260 mM, pH 7.5. It was incubated at 37℃ for 5 hours. The assay method was the same as described in Table 3.

Table 5. Effects of extracts of *Pseudomonas aeruginosa* H-9 on gentamicins

Antibiotics	% Activity remained
Gentamicin A	0
Gentamicin C_1	100
Gentamicin C_{1a}	100
Gentamicin C_2	100
Kanamycin	0

The reaction mixture consisted of a fraction of *Pseudomonas* extract 3 mg protein/ml ATP 20 mM, antibiotic 0.2 mM, KCl 156 mM, Mg acetate 10 mM, 2-mercaptoethanol 15.6 mM, and Tris-HCl 260 mM, pH 7.5. It was incubated at 37℃ for 5 hours. The assay method was the same as described in Table 3.

method which includes ammonium sulfate precipitation and chromatography with Sephadex G 100 and DEAE Sephadex A-50[7,8]. It was observed to inactivate gentamicin A and kanamycin in the presence of ATP, but did not significantly affect the activity of gentamicin C_1, C_{1a} or C_2 (Table 5).

Gentamicin A contains the paromamine moiety in its molecule. It has been reported that the 2-amino-2-deoxyglucose moiety of paromamine is phosphorylated by the kanamycin-phosphorylating enzyme in the presence of ATP, resulting in formation of paromamine-3′-phosphate. However, instead of 2-amino-2-deoxyglucose, gentamicin C_1, C_{1a} and C_2 contain the purpurosamine moiety, which lacks a hydroxyl group in the C-3 position. The differing sensitivity of gentamicin A and of the C group to the kanamycin-phosphorylating enzyme may be due to these structural differences.

In summary, the biochemical basis of

gentamicin resistance in the six clinically isolated strains of *P. aeruginosa* is a change in sensitivity of the ribosomes. No significant inactivation of the gentamicin C complex was demonstrated with the cell extracts in the presence of ATP and acetyl-CoA. The present results are in accordance with the fact that R-factor-mediated gentamicin resistance of high levels has not been observed and the levels of resistance mediated are very low. The present study also confirms that the primary action of gentamicin C is localized in the ribosomes. The kanamycin-phosphorylating enzyme affects gentamicin A but not gentamicin C group antibiotics.

NOBUO TANAKA*

Institute of Microbiology
Rutgers University,
The State University of New Jersey
New Brunswick, New Jersey, U.S.A.

The author deeply appreciates the cooperation of Dr. CARL P. SCHAFFNER during the present study.

(Received July 28, 1970)

References

1) OZAKI, M.; S. MIZUSHIMA & M. NOMURA : Identification and functional characterization of the protein controlled by streptomycin-resistant locus in *E. coli*. Nature 222 : 333～339, 1969
2) MASUKAWA, H. : Localization of sensitivity to kanamycin and streptomycin in 30S ribosomal proteins of *Escherichia coli*. J. Antibiotics 22 : 612～623, 1969
3) TAKASAWA, S.; R. UTAHARA, M. OKANISHI, K. MAEDA & H. UMEZAWA : Studies on adenylstreptomycin, a product of streptomycin inactivation by *E. coli* carrying the R factor. J. Antibiotics 21 : 477～484, 1968
4) OZANNE, B.; R. BENVENISTE, D. TIPPER & J. DAVIES : Aminoglycoside antibiotics : Inactivation by phosphorylation in *Escherichia coli* carrying R factor. J. Bact. 100 : 1144～1146, 1969
5) UMEZAWA, H.; M. OKANISHI, R. UTAHARA, K. MAEDA & S. KONDO : Isolation and structure of kanamycin inactivated by a cell free system of kanamycin-resistant *E. coli*. J. Antibiotics, Ser. A 20 : 136～141, 1967
6) KONDO, S.; M. OKANISHI, R. UTAHARA, K. MAEDA & H, UMEZAWA : Isolation of kanamycin and paromamine inactivated by *E. coli* carrying R factor. J. Antibiotics 21 : 22～29, 1968
7) DOI, O.; M. OGURA, N. TANAKA & H. UMEZAWA : Inactivation of kanamycin, neomycin and streptomycin by enzymes obtained in cells of *Pseudomonas aeruginosa*. Appl. Microbiol. 16 : 1276～1281, 1968
8) DOI, O.; S. KONDO, N. TANAKA & H. UMEZAWA : Purification and properties of kanamycin-phosphorylating enzyme from *Pseudomonas aeruginosa*. J. Antibiotics 22 : 273～282, 1969
9) DOI, O.; M. MIYAMOTO, N. TANAKA & H. UMEZAWA : Inactivation and phosphorylation of kanamycin by drug-resistant *Staphylococcus aureus*. Appl. Microbiol. 16 : 1282～1284, 1968
10) ODEN, E. M.; H. STANDER & M. J. WEINSTEIN ; Microbiological assay of gentamicin. Antimicr. Agents & Chemoth.-1963 : 8～13, 1964
11) DAVIES, J.; L. GORINI & B. D. DAVIS : Misreading of RNA codewords induced by aminoglycoside antibiotics. Molec. Pharmacol. 1 : 93～96, 1965
12) MILANESI, G. & O. CIFERRI : Studies on the mechanism of action of gentamicin. Effect on protein synthesis in cell-free extracts of *Escherichia coli*. Biochemistry 5 : 3926～3935, 1966
13) HAHN, F. E. & S. G. SARRE : Mechanism of action of gentamicin. J. Inf. Dis. 119 : 364～369, 1969
14) SMITH, D. H. : R factors for aminoglycoside antibiotics. J. Inf. Dis. 119 : 378～387, 1969

* Present address : Institute of Applied Microbiology, University of Tokyo, Tokyo. This work was carried out during the tenure of a National Science Foundation Senior Foreign Scientist Fellowship.