COMBINED ACTION OF SULBENICILLIN AND GENTAMICIN

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Nine laboratory standard strains and 29 clinically isolated strains of *Pseudomonas aeruginosa* were employed for the studies of combined use of sulbenicillin and gentamicin *in vitro*. Increased antibacterial activity by the combined use of the two drugs over the activity of either drug alone was observed against all the strains tested. Bactericidal activity against *P. aeruginosa* N 18 was enhanced by combining the two drugs. Synergistic activity of the two drugs was also observed in protective studies against intraperitoneal infection in mice by several *Pseudomonas* strains.

Sulbenicillin is a broad spectrum semisynthetic penicillin that possesses *in vitro* and *in vivo* antibacterial activity against *Pseudomonas aeruginosa*.[1,2] Clinical trials indicated that sulbenicillin was a useful drug for certain *Pseudomonas* infections.[3] Gentamicin is an aminoglycoside antibiotic which is proved to be active against Gram-positive and Gram-negative bacteria including *P. aeruginosa*.[4,5] The combined use of gentamicin with several other antibiotics were studied.[6,7] BRUMFITT *et al.*[8] reported for the first time that the enhancement of antibacterial activity against *P. aeruginosa* was found in the combined use of carbenicillin and gentamicin. Several laboratory and clinical studies[9~23] also revealed that the combined use of these two drugs was more effective against *P. aeruginosa* and its infections. On the other hand, MCLAUGHLIN and REEVES[24] reported the inactivation of gentamicin by the combined use with carbenicillin *in vitro* and *in vivo*. Many discussions have been given to these problems until today.

The present paper reports the synergistic activity of sulbenicillin and gentamicin in *in vitro* antibacterial studies and protective studies of combined use of the two drugs against intraperitoneal *Pseudomonas* infection in mice.

Materials and Methods

1. Antibiotics: Disodium sulbenicillin used was a commercial sample of Takeda Chemical Industries, Ltd., Osaka, Japan. Gentamicin sulfate in commercial grade ("Gentacin") was perchased from Schering Corporation, U.S.A. The antibiotics were dissolved in distilled water.

2. Animals: Four week old male ddy-SLC mice weighing 19~23 g were used.

3. Strains: Laboratory standard strains; *P. aeruginosa* N 18, D 363, U 31, P 1, P 3, P 5, P 6, P 8, P 10 and NC-5 were supplied by Dr. Y. HONMA, the Institute of Medical Science, the University of Tokyo. *P. aeruginosa* sp. which was maintained in our laboratory for a long time was also used. Clinical isolates of *P. aeruginosa* were made available through the courtesy of Miss Y. SHIMIZU, the Central Clinical Laboratory, Osaka University Hospital.

4. Antibacterial studies *in vitro*: Antibacterial activity was determined by the agar-dilution method using Trypticase soy agar (TSA) (BBL) as test medium. The test organisms were cultivated overnight in King A* broth and the cultures were diluted 10-fold in distilled water. One

* King A broth: Peptone 20 g, magnesium chloride 1.4 g, ammonium sulfate 10 g, glycerin 1 g, distilled water 1,000 ml, adjust to pH 7.2.

Table 1. Susceptibility of *P. aeruginosa* to sulbenicillin (SB-PC) and gentamicin (GM)

Organism	MIC in mcg/ml	
	SB-PC	GM
P. aeruginosa N 18	1.56	0.78
P. aeruginosa D 363	25	3.125
P. aeruginosa U 31	50	6.25
P. aeruginosa sp.	50	6.25
P. aeruginosa NC-5	200	12.5

Inoculum size: One loopful of bacterial suspension (10^8/ml).

loopful of a suspension of test organism was streaked on each assay plate. The bacterial growth was observed after overnight incubation at 37°C. A checkerboard method which assorted two-fold serial dilution of antibiotic was used for the test of combined activity. The combined action index was calculated by the number of plates which showed no visible growth of the test organisms on TSA contained the sub-effective concentration of antibiotic at several ratios.

5. Bactericidal test: The viability of *P. aeruginosa* N 18 in the presence of the drug was determined by the colony count technique. An overnight culture of test organism in King A broth was suspended in 10^3 times volume of Trypticase soy broth (TSB) (BBL), and sulbenicillin, gentamicin and combination of the two drugs were added in dose of one-half of the minimum inhibitory concentration.

6. Protective test: *P. aeruginosa* N 18, D 363, U 31, sp. and NC-5 cultivated overnight in King A broth were diluted to the concentration of 10^{-3}, 10^{-1}, 10^{-2}, 10^{-2} and 10^{-6} with 5% mucin, respectively. The mice were injected intraperitoneally with 0.5 ml of the bacterial suspension. Antibiotic solution was given in a single subcutaneous administration at a different site immediately after challenge. All the strains employed were susceptible to sulbenicillin and gentamicin, respectively. Susceptibilities of these organisms to two drugs are shown in Table 1.

Results

1. Antibacterial Activity

In vitro susceptibility of *P. aeruginosa* N 18 to sulbenicillin, gentamicin and combination of the two drugs at various concentrations is shown in Table 2. The growth of *P. aeruginosa* N 18 was inhibited by sulbenicillin and gentamicin at the concentrations of 1.56 and 0.4 mcg/ml, respectively, and the organism has grown with various degree at lower concentration than that above described. However, on the medium containing 0.2 mcg/ml of gentamicin, the growth of the organism was inhibited at the concentration of 0.4 mcg/ml of sulbenicillin, and on the medium containing 0.1 mcg/ml of gentamicin, it was inhibited at the concentration of 0.78 mcg/ml of sulbenicillin. In this case, the combined action index was 3.

The combined activity of sulbenicillin and gentamicin was tested against 9 laboratory standard strains and 29 strains isolates from the clinical materials of *P. aeruginosa*. As shown in Tables 3 and 4, the combined action of sulbenicillin and gentamicin was observed on all the strains tested, and the combined action index of each test strain varied.

Table 2. Enhancement of activity against *P. aeruginosa* N 18 to sulbenicillin and gentamicin.

	mcg/ml	Sulbenicillin					
		0	0.1	0.2	0.4	0.78	1.56
Gentamicin	0	+++	+++	+++	++	±	−
	0.05	+++	+++	++	+	±	−
	0.1	+++	+++	++	+		−
	0.2	++	++	±			−
	0.4	−	−	−	−	−	−

2. Bactericidal Action

The minimum inhibitory concentration of sulbenicillin and gentamicin in TSB against *P. aeruginosa* N 18 was 3.125

Table 3. Combined action of sulbenicillin and gentamicin against *P. aeruginosa* standard strains

Orgnism	Combined action index
P. aeruginosa N 18	3
P. aeruginosa D 363	3
P. aeruginosa NC-5	8
P. aeruginosa P 1	5
P. aeruginosa P 3	9
P. aeruginosa P 5	10
P. aeruginosa P 6	6
P. aeruginosa P 8	3
P. aeruginosa P 10	9

Fig. 1. Bacteticidal action of sulbenicillin, gentamicin and the combinations against *P. aeruginosa* N 18.

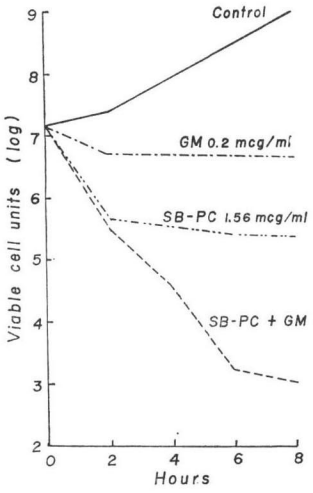


Table 4. Combined action of sulbenicillin and gentamicin against clinically isolated strains of *P. aeruginosa*

Strain No.	Combined action index	Strain No.	Combined action index
1	3	39	7
4	12	40	11
5	6	41	6
7	6	43	4
8	7	44	10
9	1	45	7
14	4	47	6
15	3	48	12
17	5	52	9
21	4	54	9
25	5	55	6
28	2	56	6
31	7	57	6
34	8	59	4
35	7		

and 1.56 mcg/ml, respectively. From these obervations, the bactericidal action of sulbenicillin, gentamicin and the combined use of the two drugs was tested at the concentrations of 1.56 and 0.78 mcg/ml of sulbenicillin and gentamicin, respectively. The killing curves obtained by sulbenicillin, gentamicin and the combined use of the two drugs on *P. aeruginosa* N 18 are shown in Fig. 1. In the case of the single use of sulbenicillin, the decrease of viable cell numbers was observed in the first 2 hours, and thereafter, remained unchanged. No significant changes of viable cell numbers

Table 5. Protective effect of sulbenicillin, gentamicin and the combinations on *P. aeruginosa* N 18 infection in mice

		Sulbenicillin (mg/kg)						
		0	5	10	20	40	80	160
Gentamicin (mg/kg)	0	0/20*	1/20	2/20	9/25	10/20	13/20	15/15
	0.3125	0/15	2/15	4/15	14/20	8/10	5/5	5/5
	0.625	0/20	12/20	13/20	20/25	9/10	10/10	5/5
	1.25	15/25	10/15	18/20	14/15	10/10	10/10	5/5
	2.5	24/25	12/15	9/10	15/15	10/10	10/10	5/5
	5	25/25	5/5	5/5	10/10	10/10	10/10	5/5

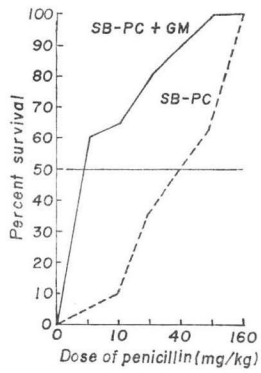
* Survived/Total

Table 6. Protective effect of gentamicin on mice infected with several *Pseudomonas* strains

Organism	Exp. No.	Dose of gentamicin (mg/kg)					
		0	3.125	6.25	12.5	25	50
P. aeruginosa D 363	I	0/5*	0/5	0/5	3/5	5/5	5/5
	II	0/5	0/5	0/5	2/5	4/5	5/5
	III	0/5	0/5	0/5	0/5	4/5	5/5
P. aeruginosa U 31	I	0/5	0/5	0/5	0/5	2/5	5/5
	II	0/5	0/5	0/5	0/5	1/5	5/5
	III	0/5	0/5	0/5	0/5	3/5	5/5
P. aeruginosa sp.	I	0/5	0/5	0/5	0/5	4/5	5/5
	II	0/5	0/5	0/5	0/5	4/5	5/5
P. aeruginosa NC-5	I	0/5	0/5	0/5	1/5	3/5	5/5
	II	0/5	0/5	0/5	2/5	2/5	4/5

* Survived/Total

Fig. 2. Survival rates of *P. aeruginosa* N 18 infected mice treated by sulbenicillin alone and the combinations of sulbenicillin and gentamicin (0.625 mg/kg).

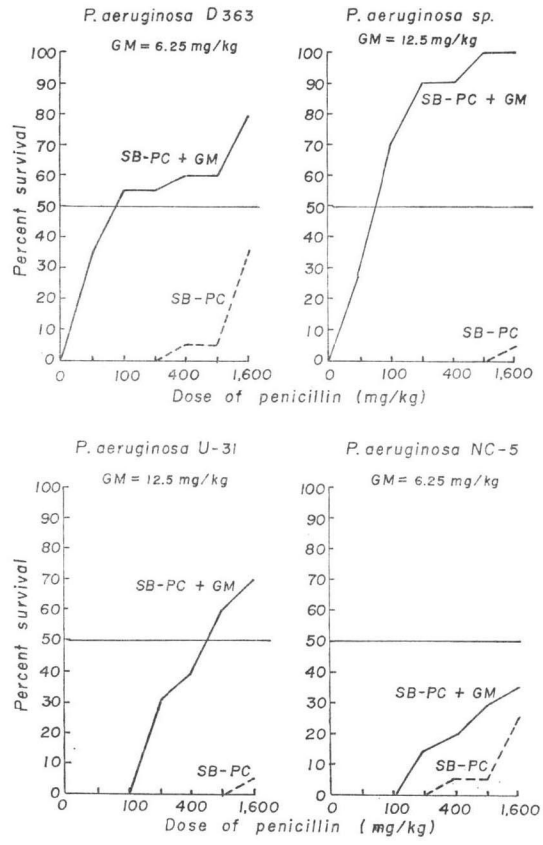


were observed at the indicated concentration of gentamicin. Marked killing activity was demonstrated in the combination of the two drugs. The viable cell numbers were decreased to about 10^{-4} of the initial viable cell numbers 6~8 hours after incubation.

3. Protective Effect

Mice infected intraperitoneally with *P. aeruginosa* N 18 were treated with sulbenicillin, gentamicin and the combined use of the two drugs. Survival rates of each group which had been treated with each of the single drug and the combination of the two drugs are

Fig. 3. Survival rates of *P. aeruginosa* infected mice treated by sulbenicillin alone and the combinations of sulbenicillin and gentamicin.



summarized in Tables 5 and 6. The doses which produced a survival percentage of more than 50 % by the single treatment of sulbenicillin and gentamicin were 40 mg/kg and 1.25 mg/kg, respectively, and respective ED_{50} values of sulbenicillin and gentamicin were 37.8 mg/kg and 1.2 mg/kg. Twelve of 20 mice (60 %) survived by the combined treatment of 5 mg/kg of sulbenicillin and 0.625 mg/kg of gentamicin which did not permit survival of the infected mice, and 14 of 20 mice (70 %) survived by the combined treatment of 20 mg/kg of sulbenicillin and 0.325 mg/kg of gentamicin. By combined treatment of 0.625 mg/kg and 0.3125 mg/kg of gentamicin, the ED_{50} value of sulbenicilin was decreased to 16.4 mg/kg and 6.5 mg/kg, respectively. The survival rate of mice treated by the combined use of sulbenicillin and 0.625 mg/kg of gentamicin and sulbenicillin alone is shown in Fig. 2.

Since the apparent combined action of sulbenicilin and gentamicin was observed in mice infected with *P. aeruginosa* N 18, the combined activity of the two drugs was tested on the intraperitoneal infection in mice by several other *Pseudomonas* strains. Protective effect of single dose gentamicin on infected mice is shown in Table 6. The highest dose of gentamicin which did not protect the mice infected with *P. aeruginosa* D 363, U 31, sp. and NC-5 was 6.25, 12.5, 12.5 and 6.25 mg/kg, respectively. These gentamicin doses were then combined with sulbenicillin. Protective activity of the combined use of sulbenicilin and gentamicin was compared with that of the single use of sulbenicillin. Seven days after the administration, marked increase in survival rate was observed after the combined treatment of sulbenicillin and gentamicin, as shown in Fig. 3, while few or no animals survived after the single administration of sulbenicillin.

Discussion

Pseudomonas infections have increased during the recent past. Gentamicin and polymyxin B are known as active antibiotics against *P. aeruginosa*. Sulbenicillin and carbenicillin are also active against *P. aeruginosa* and the toxicity of the penicillins are very low. SMITH *et al.*[16] and YUCE and ROOYEN[22] have shown synergistic effect of the combined use of carbenicillin and gentamicin against pseudomonas infections clinically. NUNNERY[17] and COOPER *et al.*[14] indicated that the combined use of carbenicillin and gentamicin lowered the toxicity and reduced the treatment period of gentamicin. On the other hand, MCLAUGHLIN and REEVES[24] found the inactivation of gentamicin by carbenicillin. WAITZ *et al.*[23] demonstrated that the combined use of gentamicin and carbenicillin *in vitro* resulted inactivation of gentamicin, the rate of which is time, temperature and medium dependent. Furthermore, they demonstrated the protective activity of combined use of the two drugs in mice.

The present studies indicated that the combined use of sulbenicillin and gentamicin enhanced the antibacterial activity *in vitro* and protective activity against lethal *Pseudomonas* infection in mice.

Unstable spheroplast of *P. aeruginosa* induced by carbenicillin, and L-phase variant of *Staphylococcus aureus* were more susceptible to gentamicin than the parent strains. These observations suggested that the increased activity by the combined use of sulbenicillin and gentamicin was attributable to the increase of penetration of gentamicin into the cells in consequence of the disorder of the synthesis system of cell wall. The inactivation of gentamicin by sulbenicillin took rather long time as in the case of gentamicin by carbenicillin. For example, about 20 % of gentamicin activity was lost following the incubation in phosphate buffer or horse serum which contained sulbenicillin at the concentrations of 400 mcg/ml and gentamicin 10 mg/ml at 37°C for 24 hours. The penetration of the drug into cells was relatively fast[28] and the

amount of drug in the cell increased in proportion to the time of exposure.[29] The synergistic activity of surbenicillin and gentamicin both *in vitro* and *in vivo* observed in the present studies will be explained by the finding that gentamicin was inactivated by sulbenicillin slower than the synergistic effect.

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