ANTI-MICROBIAL EFFECT OF COMBINATIONS OF COLISTIN METHANESULFONATE AND CHLORAMPHENICOL

II. *IN VIVO* EFFECT

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Mice infected with a virulent strain of *Escherichia coli* were treated by multiple injections with colistin methanesulfonate or chloramphenicol, or with a combination of the two drugs. It was observed that, with certain combination ratios, the potency of the combined drugs was 5~10 times higher than the potency, expected from an additive effect of both drugs. A slight elevation of toxicity by combination of the drugs was also observed. No synergistic effect of a combination of both drugs was observed in *Staphylococcus aureus* infections.

It has been found that there is a synergistic anti-microbial effect of colistin methanesulfonate (CLM) and chloramphenicol (CP) on the growth inhibition of gram-negative rods *in vitro*[1]. The present paper reports the results of comparative therapeutic experiments of acute bacterial infections by single and combined treatment with these drugs.

Materials and Methods

Bacterial strains: *Escherichia coli* GN 2411, an isolate from focus of a patient, was obtained from the Research Committee of Gram-Negative Bacteria (Chief, Dr. SHUNJI ISHIYAMA). The maximum growth-allowing dose (MAC) of this strain, determined by the agar plate technique was 0.8 mcg/ml for CLM and 3.2 mcg/ml for CP, indicating that this strain is sensitive to both drugs. After 5 passages of this strain through mice, it was lyophilized and stored under vacuum in ampules at 4°C. The 50% lethal dose (LD_{50}) of this stock strain was found to be 3.8 mcg (1.2×10^7 viable cells) when it was administered intraperitoneally into mice. The amount of bacteria was determined colorimetrically referring to a standard dry weight-optical density curve[2].

Staphylococcus aureus KS 185, maintained in our laboratory, is also a strain of human origin. The MAC of this strain was 50 and 3.2 mcg/ml for CLM and CP respectively, indicating that the strain was sensitive to both drugs. After 3 successive animal passages, the strain was also lyophilized and stored under vacuum. The LD_{50} was about 6 mcg (2×10^7 viable cells) when mice were infected intravenously.

Infection: Male and female *DDN* mice (weighing 16~20 g, average 18.2 g with a standard deviation of 0.32 g) were used[2]. They were fed a mouse diet (NF1 tablets, Toyo Yeast Co.) and given tap water *ad libitum*. Room temperature was maintained at 22~24°C. They were infected intraperitoneally with 25 mcg of *E. coli* GN 2411, and death

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was recorded over a period of 2 days. In the untreated control group, 63 out of 64 mice died within 2 days after the infection. In the case of *S. aureus* infection, the infection dose was 50 mcg and the route was via the tail vein. Death rate was recorded over a period of 5 days after infection. All 24 mice in the untreated control group died within 3 days.

Treatment and evaluation of drugs: Samples of CLM, containing 400 mg potency per gram (Kayaku Antibiotics Research, Co., Ltd., Tokyo) and of CP containing 990 mg potency per gram (Sumitomo Chemical Industry, Ltd., Osaka) were used. Infected mice were treated by multiple subcutaneous injections with these drugs dissolved in 0.1~0.2 ml of a 0.85 % NaCl solution 1, 6, 12 and 24 hours after the infection. Each group used for the treatment with a specific drug dose consisted of 23~96 mice. According to the method of LITCHFIELD and WILCOXON[3], the 50 % effective dose (ED_{50}) and its confidence limits were calculated from the survival rate. Using the formula presented in their paper, all values were calculated using a microcomputer (Programma 101, Olivetti, Ltd., Italy), but for reason of convenience, without using their calculation table.

Acute toxicity of drugs was determined by subcutaneous injection into mice. The LD_{50} was calculated from the death rate at the 7th day after administration using the same statistical method mentioned above. Synergism of therapeutic or toxic effect of a combination of the drugs was determined from the value of synergistic ratio (SR), which represented a grade of the synergistic activity of the two drugs[1]. The SR and its 95 % confidence limits were determined by the method described in a preceeding report[1].

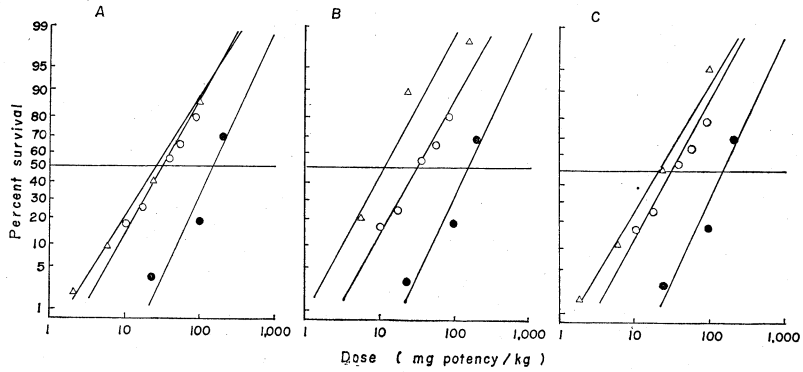
Results

Protection against *E. coil* Infection

After an intraperitoneal infection of mice with *E. coli* GN 2411, they were treated by subcutaneous injections with either single or combined drugs to compare their protective effects. In the single therapy with CP and in the combined therapy with both drugs, linear relationships were observed between the probits of survival rates and the exponents of doses (Fig. 1). However, in the case of single CLM therapy, the dose-responses fitted better a straight line relationship when doses were plotted on a decimal scale instead of logarithmic scale. This may be due to a difference in the mode of *in vivo* action of CLM compared with that of CP. However, near the ED_{50}, an almost

Fig. 1. Survival rates of mice infected with *E. coli* and treated with CLM or CP, or with their combination.

Mice were infected intraperitoneally with 25 mcg (about 7 LD_{50}) of *E. coli* GN 2411-5. Sixty minutes, 6, 12 and 24 hours after infection, they were treated by subcutaneous injections with various doses of CP (closed circles) or CLM (open circles), or their combination (triangles). The combination ratio was 34 : 1,000 in A ; 100 : 1,000 in B ; 200 : 1,000 in C.



straight line relationship existed between the dose and the survival rate, when the former was plotted logarithmically. For this reason the results obtained with doses of CLM lower than 5 mcg/kg were omitted, and each ED_{50} thus calculated is shown in Table 1. The regression lines in Fig. 1 obtained by the method of least squares agree well with the plotted points as examined by chi-square test at $P=0.05$. These regression lines were also found to run parallel to each other by the chi-square test ($P=0.05$) (Table 1).

Table 1. Effect of single and combined drug administration on an *E. coli* infection in mice

Group	Drug	Combination ratio	Determined ED_{50} (D_d) (mg/kg)	Confidence limits ($P=0.05$)	Linearity	
					χ^2	P
1	CLM		34.5	29.0～41.0	4.44	0.05
2	CP		176	130～239	6.54	0.05
3	CLM+CP	34 : 1,000	27.3	18.6～40.2	0.54	0.05
4	CLM+CP	100 : 1,000	11.5	6.3～19.8	3.23	0.05
5	CLM+CP	200 : 1,000	20.2	12.5～32.6	3.43	0.05

Table 2. Synergistic ratio of therapeutic effect of combined drugs

Combination ratio of CLM and CP	Hypothetical ED_{50} (D_a)* (mg/kg)	Confidence limits ($P=0.05$)	Synergistic ratio (D_a/D_d)	Confidence limits ($P=0.05$)
34 : 1,000	155	130～184	5.3	3.4～8.1
100 : 1,000	127	107～150	10.3	7.7～14
200 : 1,000	102	87～121	4.7	2.9～7.4

* Hypothetical ED_{50} in which additive effect of combined drugs is assumed.

To determine whether the combined drugs give an additive or synergistic effect, we calculated the synergistic ratio (SR). The SR of the therapeutic effect is a ratio of experimentally determined potency of the combined drug over a hypothetical potency in which additive effect of both drugs is assumed[1]. In each case, the SR was larger than the error function f_{SR}[1], showing that the difference in the experimentally determined potency was significantly higher than the hypothetical one at 5 % level[3]. Thus it is evident that both drugs act synergistically when used in combination for the treatment of experimental *E. coli* infection in mice.

Protection against *S. aureus* Infection

When mice were infected with *S. aureus* and treated with various amounts of CP, an ED_{50} of 168 mg/kg, with confidence limits of 27 to 310 mg/kg was obtained. To the 96 mg/kg of CP (the confidence limit 62～149 mg/kg) corresponding to 31 % of the effective dose, varying doses of CLM were added. Mice infected with *S. aureus* were treated with these combined preparations of drugs in order to investigate whether the potency of these combinations is higher than that of CP alone. As shown in Table 3, survival rates of mice in the combined treatment groups fell within a range of 22.1 to 40.3 % which corresponded to the expected survival rate of single CP treatment. This means that protective potency of CP is not affected by a combination with CLM.

Table 3. Effect of single and combined drug administration on the *S. aureus* infection in mice

Dose (mg/kg)		Survival rate (%)
CLM	CP	
0	28	20, 20, 40
0.37	28	30
1.5	28	45
6	28	25, 60
24	28	32
6	0	10
24	0	35
0	0	0

Each group consisted of 16～20 mice.

Toxicity of Combined Drugs

The LD_{50} values of single and combined drugs, determined by subcutaneous injection of various amounts of drugs to mice, are shown in Table 4. The experimentally determined toxicity (expressed as reciprocal of LD_{50}) of combined drugs was twice as high as the hypothetical toxicity, which was expected from an additive toxic effect of both drugs. Therefore, it seems that CLM and CP have a synergistic toxic effect on mice.

Table 4. Subcutaneous toxicity of CLM, CP and both (ratio 1 : 5)

Group	Drugs	LD_{50} (mg/kg)	Confidence limits ($P=0.05$)	Synergistic ratio	Confidence limits ($P=0.05$)
Determined	CLM	230	193～273		
Determined	CP	4,850	4,550～5,160		
Determined	CLM+CP	543	444～664	2.1	1.7～2.4
Hypothetical*	CLM+CP	1,110	956～1,290		

* Hypothetical LD_{50} assuming additive toxicity.

Discussion

Various simplified methods have been used for the calculation of the ED_{50} of drugs. In the present study, however, it was necessary to determine a possible synergistic action of combined drugs. For this reason a quantitative estimation of potency ratio such as SR was required. We preferred the method of LITCHFIELD and WILCOXON, because of its suitability for this purpose and its convenience in calculating the results by means of the microcomputer. When the ratio of CLM to CP was 34 : 1,000, the experimentally determined potency was found to be 7.3 times higher than the calculated potency expected from an additive effect of both drugs (Table 2). It has been shown by *in vitro* experiments that the highest synergistic effect occurred at the combination ratio of CLM to CP of 1 : 1 to 1 : 5. The potency ratio was never higher than 3 not even at the optimum ratio of the combination[1]. Preliminary experiments *in vivo*, however, gave only a weak synergism at these combination rates of the drugs. It is assumed that this difference of response *in vitro* and *in vivo* may be due to a difference in concentration level of both drugs in the blood of animals.

In the present study, a synergism was observed not only in the therapeutic effect but also in the toxic action of combined drug. However, the SR of toxicity was lower than that of the therapeutic effect of combined drugs. The differences between both SR values are considered to be significant when their confidence limits are taken in account. These results suggest the possibility of clinical application of a combined treatment with both drugs. However, as only the acute toxic effect of drug combinations was studied, therefore the safety of such a combinated treatment must be confirmed by further studies.

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