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Potentiating Effect of Lysophosphatidylcholine on Antibacterial Activity of Polymyxin Antibiotics

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Asolectin, a commercial crude phosphatidylcholine, which is known to inhibit the antibacterial activity of polymyxins, was studied to identify the main inhibiting substance. It was separated into several fractions by using high performance liquid chromatography (HPLC) on LiChrosorb SI 60, and each fraction was tested for effect on the antibacterial activity of colistin sulfate, one of the polymyxin group antibiotics. Most of these fractions showed various degrees of inhibiting effect but, conversely, one fraction (corresponding to lysophosphatidylcholine) potentiated the antibacterial activity of colistin.

With authentic samples we confirmed that lysophosphatidylcholine showed a potentiating effect on the antibacterial activity of colistin, polymyxin B and polymyxin M. These results suggest that some detergents may be useful as potentiators of the antibacterial activity of polymyxins.

Keywords——lysophosphatidylcholine; colistin; polymyxin B; polymyxin M; phospholipid

Bliss, Chandler and Schoenbach[1] first reported that asolectin (a commercial crude phosphatidylcholine of soybean), interfered with the antibacterial activity of polymyxins. Subsequently, there have been many reports concerning the interaction between polymyxin and phospholipid; however, the results have shown some inconsistency, probably because the experimental conditions varied.

Using high performance liquid chromatography (HPLC), we separated asolectin into several components in an attempt to identify the main inhibiting substance. Each component was tested for effect on the antibacterial activity of colistin. Most of these fractions showed various degrees of inhibitory effect but, conversely, one fraction (corresponding to lysophosphatidylcholine) potentiated the antibacterial activity of colistin. Therefore, we investigated the potentiating effect on the antibacterial activity of colistin, polymyxin B and polymyxin M with authentic samples of lysophosphatidylcholine and the other phospholipids.

Experimental

Materials——Colistin sulfate was kindly supplied by Banyu Pharmaceutical Co. (Tokyo, Japan). Polymyxin B sulfate was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Polymyxin M hydrochloride was the generous gift from Professor A. B. Silaev, Moscow University, U.S.S.R. Asolectin was purchased from Associated Concentrates Inc., U.S.A., and the following compounds were from Sigma; phosphatidylcholine type III-E from egg-yolk (hexane), phosphatidylcholine type III-s from soybean (chloroform), phosphatidylcholine dipalmitoyl grade 1 cryst. synth. (99%), phosphatidylethanolamine type V from *E. coli* (98%) (chloroform–methanol (9 : 1)), phosphatidylinositol ammonium salt grade III from soybean (98%) (chloroform), phosphatidylglycerol ammonium salt grade 1 from egg-yolk (98%) (chloroform–methanol (98 : 2)), phosphatidyl-L-serine amorphous powder from bovine brain (98%), L-α-phosphatidic acid sodium salt from egg-yolk (95%), cardiolipin sodium salt from bovine heart (ethanol), lysophosphatidylcholine type IV from soybean (98%) and lysophosphatidylcholine type I from egg-yolk.

Test Organism and Preparations of Test Agar Plates and Cell Suspension——*E. coli* NIHJ, a standard strain maintained in our laboratory, was used for antibacterial activity testing by the cylinder plate method and the disk method, and for determination of 50% bactericidal concentration (BC_{50}) value.

For the cylinder plate method and the disk method, test agar plates were made by adding 1/100 volume of *E. coli* broth (incubated for 20 h in heart infusion broth (HIB) "Nissan") to liquidized sensitivity test agar "Eiken" containing 3% sodium chloride (pH 6.5) at 48 °C. Ten ml of this mixture was poured into each 9 cm Petri dish and allowed to gel.

The bacterial cell suspension for the measurement of BC_{50} value was prepared as follows: after overnight incubation, the bacteria in HIB broth were collected by centrifugation for 10 min at 8000 ***g***. The cells were suspended in phosphate-buffered saline (PBS) (pH 7.2) and centrifuged again. After repeating this saline procedure two times more, the bacteria were suspended again in PBS (pH 6.3) to give approximately 10^6 cell/ml (measured in terms of optical density).

Disk Method for Measurement of Antibacterial Activity——Twelve different phospholipids were dissolved in chloroform or ethanol–water (1 : 1); if necessary, a small amount of acid or alkali was added. For each phospholipid, two concentrations of sample solution (1000 and 250 μg/ml) were made. Then, paper disks (8 mm) were impregnated with 20 μl of each solution and dried for 2 h in the air stream of a clean bench. These dried paper disks were further impregnated with standard colistin solution (100 μg/ml) and put on the seeded (*E. coli*) agar plate. After 0.5 h of diffusion at 4 °C, they were incubated for 20 h and the diameters of the inhibitory zones were measured. To obtain the standard curve, paper disks impregnated with only 20 μl of colistin solution (200, 100, 50 μg/ml) were used. The results were recorded on semilogarithmic graph paper with the diameter of the inhibitory zone on the vertical axis and the concentration of colistin on the horizontal axis. This standard curve was used for the determination of the antibacterial activity in the phospholipid-treated paper disk.

Measurement of BC_{50}——Each phospholipid was dissolved in water, water–ethanol (1 : 1) or ethanol–ether (1 : 1) according to its solubility to give a concentration of 5 mg/ml. Colistin was dissolved in 0.01 M phosphate buffer (pH 6.3) in serial two-fold dilutions ranging from 50 to 0 μg/ml. Then 0.1 ml of each phospholipid solution was added to 1 ml of each dilution of colistin. After 15 min at room temperature, 0.9 ml of washed cell suspension of *E. coli* (approximately 10^6 colony-forming unit (CFU)) was added to each test tube. Immediately after mixing, the tubes were incubated for precisely 20 min at 25 °C. Then, they were each diluted 100 fold with HIB, and a 0.1 ml aliquot was plated on heart infusion agar. After overnight incubation at 37 °C, the colonies were counted. As a control, a blank test tube without colistin was treated in exactly the same manner. The bactericidal rate was calculated by comparing the number of colonies with and without colistin, and the BC_{50} value was determined by transfer to probit and graphing, using the Lichfield and Wilcoxon method arbitrarily.

Fractionation of Asolectin by HPLC——An HPLC pump, TRI ROTAR model V (Japan Spectroscopic, Ltd., Japan) equipped with a UVIDEC 100 IV variable-wavelength detector and a gradient programmer (model GP-A40), was used. A normal-phase column, LiChrosorb SI 60 (5 μm), was adopted. The column size for analysis was 200 × 4 mm i.d. and for preparation 250 × 20 mm i.d. The chromatographic system was programed for linear gradient elution using the two mobile phases described in the legend to Fig. 1. The percentage of solvent B in solvent A was increased from 4 to 100% as shown in Fig. 1.

Each peak was collected in a 10 ml test tube. In most cases, 0.1 ml of pyridine was added during collection to neutralize the sulfuric acid in the solvents. After removal of the solvents by evaporation *in vacuo*, residues were redissolved in 2 ml of chloroform–methanol (1 : 1) and washed with 2 ml of water. The upper phase was discarded

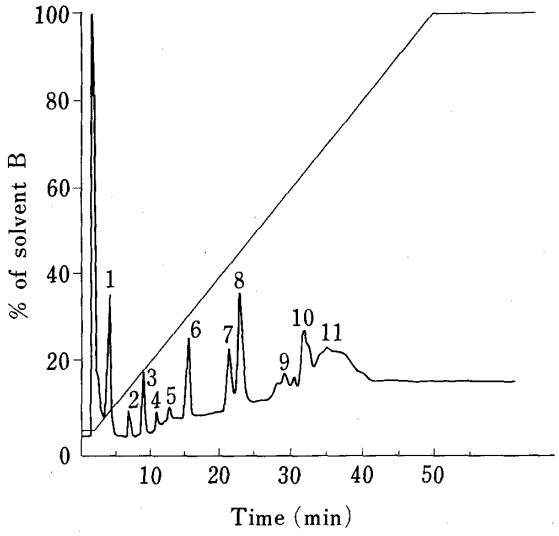

Fig. 1. HPLC Chromatogram of a Commercial Asolectin

Column, LiChrosorb SI 60 (250 × 4.6 mm i.d.); sample, 800 μg; mobile phase, hexane–isopropanol–water–sulfuric acid (solvent A, 97 : 3 : 0 : 0.025; solvent B, 50 : 48 : 1.9 : 0.1) linear gradient; flow rate, 1.8 ml/min; detector, UV 205 nm.

Peaks: 1, phosphatidic acid; 2, phosphatidylcholine; 3, phosphatidylethanolamine; 5, phosphatidylglycerol; 7, phosphatidylinositol; 11, lysophosphatidylcholine; 4, 6, 8, 9, 10, unknown.

after centrifugation and the lower phase was allowed to evaporate to 0.1 ml. This concentrated solution was analyzed by thin-layer chromatography (TLC) on a silica gel plate (solvent system, chloroform–methanol–28% aqueous ammonia–water (120 : 80 : 10 : 5)) and checked for effect on the antibacterial activity of colistin.

Results and Discussion

Separation of Asolectin Components by HPLC and Determination of Their Effects on the Antibacterial Activity of Colistin

In our preliminary experiment, we confirmed that the antibacterial activity of colistin was antagonized by asolectin proportionally to the concentration of asolectin in the agar medium. To identify the most active components of asolectin, we analyzed it by using a combination of a normal-phase silica column, LiChrosorb SI 60 (5 μm), and the mobile phase system described in the legend to Fig. 1, modifying a system reported by Yandrasitz *et al.*[2] Figure 1 shows the separation profile. By scaling up the chromatography system (using a large-bore column), the component of each peak could be isolated. To test these components for antagonistic effect, a cylinder plate method was used, with a separate agar plate containing each component. We expected that the diameter of the inhibitory zone of colistin would be decreased because of the antagonistic effect of components on the antibacterial activity of colistin. However, the peak 11 component increased the diameter of the inhibitory zone compared to that of the control agar plate without phospholipids. By comparing the *Rf* value (0.56) on TLC and the retention time (35 min) on HPLC with those of an authentic sample, this component was identified as lysophosphatidylcholine.

Effect of Commercial Phospholipids on the Antibacterial Activity of Colistin Using the Disk Method

In view of the unexpected result that lysophosphatidylcholine was not antagonistic but

TABLE I. Antagonistic and Potentiating Effects of Phospholipids on the Antibacterial Activity of Colistin Sulfate Estimated by the Disk Method

Pre-impregnated phospholipids (conc. of phospholipid 1000 μg/ml)	Diameter of inhibitory zone of colistin disk (20 μl of 100 μg/ml) (mean ± S.D., mm, $n=5$)	Relative potency of colistin obtained by standard curve method (μg/ml)
Lysophosphatidylcholine from egg-yolk	21.76 ± 0.65[a]	146
Lysophosphatidylcholine from soybean	20.68 ± 0.57[a]	118
Asolectin	16.14 ± 0.89	45.5
Phosphatidylcholine from egg-yolk	19.83 ± 0.53	98.0
Phosphatidylcholine from soybean	20.15 ± 0.68	105
Phosphatidylcholine dipalmitoyl synthesis	19.80 ± 0.84	97
Phosphatidylethanolamine from *E. coli*	20.15 ± 0.58	105
Phosphatidylinositol ammonium salt	11.95 ± 0.72	19.0
Phosphatidylglycerol ammonium salt	10.50 ± 0.48	14.0
Phosphatidylserine from bovine brain	13.68 ± 0.72	27.4
Phosphatidic acid sodium salt from egg-yolk	9.50 ± 0.41	11.5
Cardiolipin sodium salt	< 8.0	< 8.2
Without phospholipid		
Colistin 200 μg/ml	23.28	
100 μg/ml	20.00	
50 μg/ml	16.60	

a) $p<0.005$ (Student's t-test).

Table II. Effects of Phospholipids on the BC_{50} of Colistin Sulfate against *Escherichia coli*

Phospholipids (250 μg/ml)	BC_{50} of colistin (μg/ml) (confidence limits)
Without phospholipid	1.70 (1.59— 1.87)
Lysophosphatidylcholine from egg-yolk[a]	1.03 (0.98— 1.08)
Lysophosphatidylcholine from soybean[a]	1.26 (1.19— 1.34)
Asolectin[b]	10.3 (9.81—10.80)
Phosphatidylserine[c]	20.3 (19.15—21.52)
Cardiolipin (225 μg/ml)[d]	22.0 (20.95—23.10)
Phosphatidic acid from egg-yolk[e]	23.0 (21.50—24.61)

a) Both lysophosphatidylcholines were dissolved in water–ethanol (1 : 1). *b*) Asolectin was used as an emulsion in water. *c*) Phosphatidylserine was dissolved in water–ethanol (1 : 1) with addition of a small amount of sodium hydroxide. *d*) A commercial sample of cardiolipin in ethanol (4.5 mg/ml) was used. *e*) The sodium salt of phosphatidic acid was dissolved in water.

potentiated the antibacterial activity of colistin, eleven commercial phospholipid samples were tested for effect on colistin activity. Most of these samples were tested as solutions in ethanol, chloroform or hexane. When the dilution method for antibacterial activity was used, the antibacterial activity of the solvents themselves caused considerable problems in assessing the intrinsic activity of the phospholipids. Therefore, a paper disk method was adopted to circumvent this difficulty. A phospholipid preimpregnated disk was further impregnated with colistin solution. The potency of colistin in both the control disks and the phospholipid disks was calculated from the diameter of the inhibitory zone. Table I shows the relative activity of colistin plus various phospholipids determined by this method.

As can be seen, three kinds of phosphatidylcholine isolated from different sources had almost no effect. Phosphatidylethanolamine from *E. coli* was also almost ineffective. Phosphatidic acid and cardiolipin were most antagonistic, and other acidic compounds had various degrees of antagonistic effect: phosphatidylinositol, phosphatidylglycerol and phosphatidylserine had lower activity. In contrast, lysophosphatidylcholine showed a potentiating effect. In addition, lysophosphatidylcholine from egg-yolk was more active than that from soybean. Thus, we analyzed their constituent fatty acids by gas chromatography. A sample from egg-yolk contained palmitic acid (55%) and stearic acid (25%), while one from soybean contained palmitic acid (47%), palmitoleic acid (25%) and stearic acid (15%) as major fatty acids. Accordingly, the difference between the potentiating activities of the two samples might be due to the difference of the constituent fatty acids.

Effects of Phospholipids on the Bactericidal Activity of Colistin——To determine the BC_{50} of colistin and colistin plus phospholipid, serial two-fold dilutions of colistin were used. Although Few[3] expressed his results only as minus or plus for bacteria killed or not killed in each tube, we counted the number of surviving bacterial cells in each tube by plating on an agar plate. The BC_{50} value was based on bactericidal rate (transfer to probit) and colistin concentration.

In this experiment it was found that chloroform and hexane were bactericidal at a concentration of 5% or more. Therefore, several phospholipid samples dissolved in these solvents could not be tested. Table II summarizes the results.

Asolectin showed a moderate inhibitory effect on the bactericidal activity of colistin, while phosphatidylserine, cardiolipin and phosphatidic acid had strong inhibitory effects. On the other hand, two samples of lysophosphatidylcholine potentiated the bactericidal activity of colistin. These results parallel those obtained by the paper disk method. Thus, the bactericidal activity of these phospholipid plus colistin samples parallels their bacteriostatic

action.

Effects of Lysophosphatidylcholine on the Bactericidal Activities of Polymyxin B and Polymyxin M

The BC_{50} values of polymyxin B and polymyxin M were determined in the same manner as for colistin. The BC_{50} of polymyxin B plus egg-yolk lysophosphatidylcholine (250 μg/ml) was 0.9 (0.85—0.95) μg/ml, while that of polymyxin B alone was 1.8 (1.70—1.91) μg/ml. On the other hand, the BC_{50} value of polymyxin M plus lysophosphatidylcholine was 2.2 (2.08—2.33) μg/ml, and that of polymyxin M alone was 5.3 (5.00—5.62) μg/ml. In both cases, the bactericidal activity of the polymyxins was similarly potentiated by lysophosphatidylcholine.

In addition to Bliss's report,[1] there have been many other papers concerning the relationship between polymyxins and phospholipids.[3-6] However, no information is available about the interaction between polymyxin and lysophosphatidylcholine. Various workers have investigated the biological activity of lysophosphatidylcholine, its fusogenic action,[7] its stimulation of enzyme activity[8] and its detergent activity.[9] We consider that the potentiating effect of lysophosphatidylcholine on colistin activity might be associated with its detergent activity. Therefore, many kinds of detergent were examined for potentiating effects on colistin activity. These results will be reported in another paper.

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