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EFFECT OF POLYMYXIN B ON LIPOSOMAL MEMBRANES DERIVED FROM *ESCHERICHIA COLI* LIPIDS

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SUMMARY

The specificity of the action of polymyxin B was studied using liposomes as a model membrane system. Liposomes prepared from total lipids of Gram-negative bacteria *Escherichia coli*, a mixture of purified *E. coli* phosphatidylethanolamine and cardiolipin and a mixture of phosphatidylethanolamine and phosphatidylglycerol, were extremely sensitive to polymyxin while those prepared from lipids of Gram-positive bacteria *Streptococcus sanguis*, lipids of sheep erythrocyte membranes, mixtures of egg lecithin and negatively charged amphiphatic molecules, were less sensitive to the action of the antibiotic. Chlolesterol was shown to suppress the polymyxin-induced response in liposomes.

INTRODUCTION

The peptide antibiotic, polymyxin B, has potent bactericidal activity against most Gram-negative bacilli. There have been several reports on the mode of action of the polymyxins [1–4]. It is generally believed that this antibiotic interacts with membrane phospholipids to cause irreversible breakdown of the permeability barrier of the membranes. However, the identification of the composition and structure of membranes required for polymyxin susceptibility is still obscure.

Quite recently, HsuChen and Feingold [5] reported that polymyxin had a significant effect on liposomes derived from *E. coli* lipids. They mentioned further that incorporation of lecithin into liposomes with *E. coli* phospholipids reduced their susceptibility to polymyxin. It was concluded from these observations that the polymyxin susceptibility of biological membranes required both the presence of phosphatidylethanolamine and a threshold density of these molecules on the membrane Their results are, however, rather indirect, because liposomes used by them contained always 50 mole % of cholesterol and in some cases dicetyl phosphate was also incorporated.

In the present experiment where liposomes were prepared from *E. coli* total lipids without any supplement, the results obtained should give more direct information about the mechanism of polymyxin action. Our conclusion on the structural

requirement for polymyxin susceptibility is somewhat different from that of HsuChen and Feingold [5].

MATERIALS AND METHODS

Lipids. Lipids were extracted from *E. coli* K12 (W3110) (stationary phase), *Strep. sanguis* A30 (stationary phase) and sheep erythrocytes membranes [6] by the methods of Folch et al. [7]. Neutral lipids were removed from total lipid fractions of *E. coli* and *Strep. sanguis* by silicic acid chromatography. Thin-layer chromatograms demonstrated that the main phospholipid of *E. coli* was phosphatidylethanolamine, taking in about 80 % of the total phospholipids, whereas phosphatidylglycerol (about 5–8 %) and cardiolipin (about 10–15 %) were also present. Phosphatidylethanolamine, phosphatidylglycerol and cardiolipin were purified from the phospholipid fraction of *E. coli* by DEAE–cellulose and silicic acid chromatography [8, 9] Lecithin and phosphatidylethanolamine of egg yolk was prepared by chromatography on alumina and silicic acid. Fatty acid composition of egg lecithin and *E. coli* phosphatidylethanolamine were analyzed by gas-chromatography. The fatty acids of egg lecithin were as follows: 38.7 % of C16:0, 2.9 % of C16:1, 11.8 % of C18:0, 34.2 % of C18:1 and 12.4 % of C18:2. Those of *E. coli* phosphatidylethanolamine were 3.4 % of C14:0, 47.2 % of C16:0, 5.9 % of C16:1, 31.8 % of C17Δ, 6.2 % of C18:1 and 5.5 % of C19Δ. Cholesterol, dipalmitoyllecithin, dicetyl phosphate and stearylamine were purchased from commercial sources as described previously [10–12].

Preparation of liposomes and assaying for polymyxin B sensitivity. Liposomes were prepared by the method developed by Kinsky et al. [13] with slight modification [10]. The method of assay for glucose release was also similar to that of Kinsky et al. [13] except that $CaCl_2$ was omitted and final concentration of $MgCl_2$ in the assay solution was 1 mM. The chemicals and enzymes for the assay were obtained from commercial sources. Polymyxin B was purchased from Sigma Chemical Company, St. Louis, Mo., USA.

RESULTS

Capacities of liposomes derived from E. coli lipids to trap glucose

Total lipid fraction of *E. coli* K12 could be swollen in 0.3 M glucose and could trap glucose. Phospholipid fraction could be swollen more easily than the total lipid fraction, although the amount of glucose trapped in liposomes with the phospholipids was almost the same as those obtained in liposomes derived from the total lipid fraction (Table 1). The incorporation of up to 10 mole % dicetyl phosphate into the phospholipids did not influence the amount of glucose trapped in liposomes. Phosphatidylethanolamine purified from *E. coli* as well as from egg yolk did not swell in the isotonic glucose solution. Addition of more than 10 mole % of cardiolipin or more than 17 mole % of phosphatidylglycerol to the phosphatidylethanolamine made lipids swell easily. These liposomes trapped enough glucose and released little glucose in the assay solution (less than 10 % within 30 min). On the contrary, addition of dicetyl phosphate or synthetic cardiolipin derivative whose fatty acid was palmitic acid did not make phosphatidylethanolamine swell. It was further noticed that phosphatidyl-

TABLE I

CAPACITIES OF LIPOSOMES DERIVED FROM *E. COLI* LIPIDS TO TRAP GLUCOSE

Liposomes derived from *E. coli* lipids were prepared at room temperature. After dialyzing for 2 h at room temperature, the amount of trapped glucose was assayed enzymatically. The amount of trapped glucose is expressed as moles glucose/mole lipid phosphorus.

Source of liposomes	Incorporated by:	Appearance	Amount of trapped glucose (mole/moleP)
E. coli K12 (W3110) total lipids	—	well suspended	1.85
E. coli K12 (W3110) phospholipids	—	well suspended	1.68
E. coli K12 (W3110) phospholipids	Dicetyl phosphate (10 mole%)	well suspended	1.41
Egg yolk phosphatidylethanolamine	—	not well suspended	0.007
E. coli phosphatidylethanolamine	—	not well suspended	0.005
E. coli phosphatidylethanolamine	Dicetyl phosphate (10-50 mole%)	not well suspended	(0.69–0.98)**
E. coli phosphatidylethanolamine	Synthetic cardiolipin derivative* (10 mole%)	not well suspended	(2.0)**
E. coli phosphatidylethanolamine	*E. coli* cardiolipin (2 mole%)	well suspended in 0.3 M glucose not well suspended in saline	0.24
E. coli phosphatidylethanolamine	(5 mole%)		0.52
E. coli phosphatidylethanolamine	(10 mole%)		2.16
E. coli phosphatidylethanolamine	(20 mole%)		2.40
E. coli phosphatidylethanolamine	(30 mole%)		2.51
E. coli phosphatidylethanolamine	(50 mole%)		(1.92)**
E. coli phosphatidylethanolamine	*E. coli* phosphatidylglycerol (10 mole%)	well suspended	0.07
	(17 mole%)		1.1
E. coli phosphatidylethanolamine			
E. coli phosphatidylethanolamine			
E. coli phosphatidylethanolamine	(20 mole%)		1.47
E. coli phosphatidylglycerol	—		extremely leaky
E. coli cardiolipin	—		extremely leaky

* Bis (dipalmitoyl-D,L-glycerylphosphoryl)-1,3-propanediol disodium salt [18]

** Too leaky to determine reliable amount of trapped glucose

glycerol, cardiolipin and an equimolar mixture of phosphatidylethanolamine and cardiolipin formed leaky liposomes in the present experimental condition. Thus, it was impossible to use these liposomes for further experiments on the polymyxin sensitivity.

Effect of polymyxin B on liposomes

The effects of polymyxin B on glucose release from liposomes prepared with total phospholipids of *E. coli*, lipid fraction free from neutral lipids of *Strep. sanguis* and total lipids of sheep erythrocytes membranes are given in Fig. 1A. Liposomes prepared with *E. coli* phospholipids were the most sensitive to the antibiotic while those from *Strep. sanguis* phospholipid fraction were less sensitive. Liposomes with the erythrocyte membrane lipids were resistant to the action of the polymyxin. Thus, the selective action of polymyxin B, which showed lytic activity to intact *E. coli* cells but not to *Strep. sanguis* nor sheep erythrocytes, could be partly duplicated in very simple liposome systems. Kinetics of glucose release by a small concentration of polymyxin B (2.28 μg/ml) were compared at room temparature between liposomes of *E. coli* lipids and those of *Strep. sanguis* lipids (Fig. 1B). At this concentration of the antibiotic, about 70 % of glucose was released from liposomes with *E. coli* phospholipids after 30 minutes incubation, while an almost negligible amount of marker was lost from those with *Strep. sanguis* lipids. Liposomes with total lipid fraction of *E. coli* showed almost the same sensitivity to polymyxin B as those with the phospholipid fraction. In the case of liposomes with *Strep. sanguis* lipids, liposome could not be prepared from total lipid fraction. Therefore, polymyxin sensitivity was determined only in liposomes of the lipid fraction free from neutral lipids.

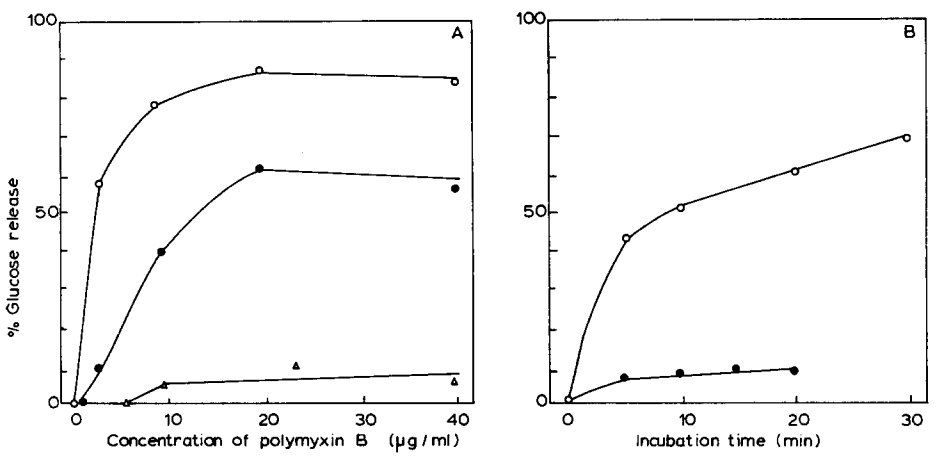

Fig. 1. Release of glucose from liposomes by the action of polymyxin B. A, Effect of polymyxin B concentration on the marker glucose release. An aliquot of liposomes (2.5 μl) prepared from *E. coli* phospholipids (○—○), *Strep. sanguis* lipids (●—●) and sheep erythrocytes membranes lipids (△—△) were incubated with various amounts of polymyxin B at room temperature for 30 min. B, Kinetics of the glucose release. Liposomes were incubated with polymyxin B (2.28 μg/ml) for various times. The symbols (○ and ●) represent release of glucose induced by polymyxin B from liposomes prepared from *E. coli* and *Strep. sanguis* lipids respectively.

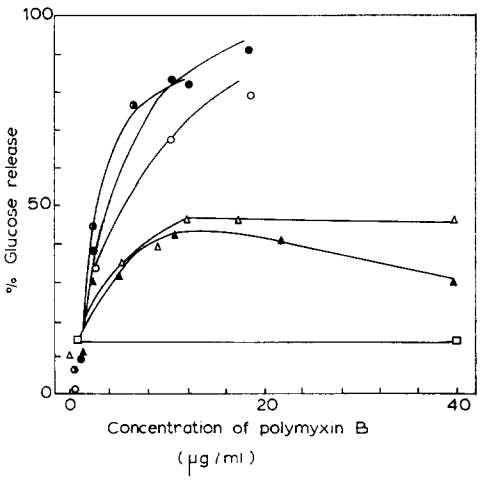

Fig. 2. Effect of polymyxin B on liposomes prepared with various lipids. Liposome composition: ○—○, *E. coli* phosphatidylethanolamine and cardiolipin (molar ratio, 10:1); ●—●, phosphatidylethanolamine and cardiolipin (5:1); ◑—◑, phosphatidylethanolamine and phosphatidylglycerol (5:1 △—△, egg lecithin and *E. coli* cardiolipin (10:1); ▲—▲ egg lecithin and dicetyl phosphate (10:1) □—□, egg lecithin and stearylamine (10:1). Experimental conditions were identical to those described for figure 1A.

Liposomes containing 10 parts of phosphatidylethanolamine and 1 part of cardiolipin or those containing 5 parts of phosphatidylethanolamine and 1 part of phosphatidylglycerol showed almost equal sensitivity to polymyxin B as liposomes with *E. coli* phospholipids (Fig. 2). When the amount of cardiolipin or phosphatidyglycerol incorporated into liposomes was increased, susceptibility to polymyxin did not significantly change. There was little difierence of sensitivity to polymyxin between liposomes containing phosphatidylglycerol and those containing cardiolipin. Fig. 2 also indicates that liposomes with the mixture of egg lecithin and cardiolipin or the mixture of egg lecithin and dicetyl phosphate were sensitive to polymyxin. The degree of susceptibility to the antibiotic was, however, much smaller in liposomes with lecithin than in those with phosphatidylethanolamine even when they had the same amount of cardiolipin. The difference seems unlikely to be due to the fatty acid composition, because the fatty acid composition of *E. coli* phosphatidylethanolamine was shown to be rather similar in the ratio of saturated fatty acid/unsaturated fatty acid to that of egg lecithin (see Materials and Methods.)

The presence of cholesterol in liposomes derived from *E. coli* phospholipids, egg lecithin or dipalmitoyl lecithin supressed their susceptibility toward polymyxin B (Fig. 3). The effect of cholesterol incorporation may result from reduction of phosphate groups in the liposomal bilayers. Experiments were also performed to determine how the amount of negative charge influences polymyxin-susceptibility. Table II shows that incorporation of increasing amounts of dicetyl phosphate did not have a significant enhancing effect on polymyxin sensitivity of liposomes with 50 mole % of cholesterol. Cholesterol may hinder the polymyxin molecule from penetrating into membranes by condensing the lipid bilayer, since cholesterol is well known to have the condensing effect on phospholipid bilayer [10, 14].

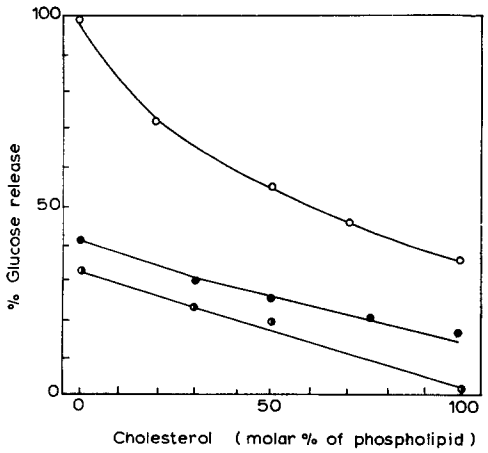

Fig. 3. Effect of cholesterol incorporation into liposomes of various phospholipids on the extent of glucose released by polymyxin B at 9 μg/ml. Liposomes with the antibiotics were incubated at room temperature for 30 min. The results with *E. coli* phospholipids were represented by ○—○; of dipalmitoyl lecithin (containing 10 mole% of dicetyl phosphate) by ◑—◑; of egg lecithin (containing 10 mole% of dicetyl phosphate) by ●—●. Experimental conditions were the same as those described for Fig. 1.

TABLE II

EFFECT OF POLYMYXIN B ON LIPOSOMES WITH DIFFERENT AMOUNTS OF DICETYL PHOSPHATE

Polymyxin B action on liposomes was measured from the amount of marker glucose released as described in the text. The concentration of Polymyxin added was 9 μg/ml.

Liposome composition (molar ratio)			Percentage of glucose released by polymyxin B
Egg lecithin	Dicetyl phosphate	Cholesterol	
2	0.1	0.3	37.0
2	0.1	1.0	20.9
2	0.2	1.0	13.6
2	0.3	1.0	13.3

Dipalmitoyl lecithin liposomes showed the lowest sensitivity to polymyxin B among liposomes tested in the present experiment. The fact suggests that membrane fluidity may have some influence on polymyxin sensitivity.

DISCUSSION

According to Haest et al [15], addition of 50 mM KCl to the films of the *E. coli* phospholipids did not results in a spontaneous swelling and dispersion of the lipids in the water phase. They postulated that the failure of the spontaneous swelling

might be due to phosphatidylethanolamine which was the main phosholipid in the bacterial lipids. In fact, Papahadjopoulos et al. [16] noticed that phosphatidylethanolamine did not give completely closed structures in KCl solutions. In our experiments, it was demonstrated that the total lipid and the phospholipid fractions extracted from a wild strain of *E. coli* (K12) could swell and be dispersed in 0.3 M glucose. The liposomes thus prepared were able to trap glucose. In agreement with the observation of Papahadjopoulos et al. [16], phosphatidylethanolamine from *E. coli* or egg yolk did not swell in 0.3 M glucose. Addition of 10– 20 mole % of cardiolipin or 20 mole % of phosphatidylglycerol to the phosphatidylethanolamine improved the situation; these mixtures formed stable liposomes. It is worthwhile to mention that the compositions of phosphatidylethanolamine and acidic phospholipids are almost equal to those observed in the total lipids of *E. coli*.

Few [2] has previously demonstrated with monolayers that polymyxin interacted with phosphatidylethanolamine but not with lecithin. As described above, HsuChen and Feingold [5] also obtained the results indicating that phosphatidylethanolamine is essential for membrane to interact with polymyxin B. In the present experiment, lecithin liposomes were sensitive to the antibiotic in the presence of negatively charged amphiphatic molecules such as dicetyl phosphate or cardiolipin, although the sensitivity of lecithin liposomes to the antibiotic was weaker than that of liposomes prepared from phosphatidylethanolamine. The result indicates that phosphatidylethanolamine may not be essential for the interaction of membranes with the antibiotic. A negative charge was shown to be required for the sensitivity to polymyxin, since liposomes with stearylamine as a charged molecule did not show any reactivity with the antibiotic. (Fig. 2). There was no appreciable difference between the sensitivity of liposomes containing cardiolipin and that of liposomes containing phosphatidylglycerol or dicetyl phosphate, indicating that the structure of negatively charged molecule was not strictly important. Therefore, it is possible to conclude that membranes which contain phosphatidylethanolamine as a main component and a certain amount of acidic phospholipid such as cardiolipin or phosphatidylglycerol may be most susceptible to polymyxin. Poor susceptibility of *Strep. sanguis* to polymyxin may be explained from our preliminary observation that it contains only a small amount of phosphatidylethanolamine. Alternatively, glycolipids detected as main components of *Strep. sanguis* lipids may exert a protective effect against polymyxin B action.

Incorporation of cholesterol into phospholipid bilayers inhibited permeability increase induced by several lytic reagents such as cytochrome *c*, hemoglobin [17] and lysolecithin [11]. Polymyxin action to liposomes was also inhibited by incorporaration of cholesterol. Resistance of liposomes derived from erythrocytes lipids and of animal cells to polymyxin possibly may be due to a protective effect of cholesterol.

The relation of effect of several reagents, such as lysolecithin [11] and Prymnesin [12], on liposomes to membrane fluidity was previously reported. The action of polymyxin B to liposomal membranes was also shown to be influenced by the fatty acid composition, suggesting that membrane fluidity might control the susceptibility to the antibiotic.

ADDENDUM

Yamaguchi, A. and Anraku, Y. have also independently obtained liposomes derived from *E. coli* lipids without any supplement.

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