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Short Communication

Combined action of polymyxin B and gramicidin Dubos on liposomes derived from phospholipids of *E. coli*

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Abstract

Liposomes derived from the total phospholipids of *E. coli* NCL 8879 strain are susceptible to polymyxin B. More pronounced disruption occurs in the presence of gramicidin Dubos. Gramicidin, at concentration levels which are ineffective against liposomes, potentiates the disruptive action of polymyxin B on liposomes which contain glycine and chromate as markers. The enhancement by gramicidin of the antimembrane activity of polymyxin B is compared with the potentiation effect seen *in vitro* with in tact cells.

Key words: Phospholipids, cell membrane, antibacterial activity.

1. Introduction

The primary site of action of the polycationic peptide antibiotic polymyxin B is considered to be the outer membrane of Gram-negative bacteria. Polymyxin is known to exert its antibiotic activity through interaction with phospholipids of the cell membrane[1–4]. The *in vitro* interaction of the antibiotic with phospholipids suggest that blebs formed on the surface of polymyxin treated Gram-negative bacteria or vesicles which are revealed are complexes of polymyxin B and phospholipids present in the lipopolysaccharide moiety of the outer membrane[5,6]. Further, the vesicles formed stain for polysaccharides. They possibly consist of lipopolysaccharides. Liposomes derived from the phospholipids of Gram-negative bacteria are disrupted by polymyxin B[7–10]. Our earlier findings of *in vitro* potentiation of the antibacterial activity of polymyxin B by gramicidin Dubos[11] has prompted us to examine the effect of gramicidin on liposome disruption by polymyxin B. The ability of gramicidin Dubos to potentiate the lytic action of polymyxin on liposomes has become evident.

. Experimental and results

.1. *Preparation of E.* coli *total phospholipids*

'ospholipids were isolated by the method of Kanfer and Kennedy[12], from *E. coli* NCL 8879) grown as below. The growth medium (pH 7·4) contained the following omponents (per litre): Peptone 10 gm, glucose 5 gm, sodium chloride 5 gm, dipotassium phosphate 4 gm, monopotassium phosphate 1·5 gm, and sodium deoxycholate ·7 gm. Cultures were incubated at 37° for 24 hr. Growth of the bacterial cells was neasured turbidimetrically.

Cultures were centrifuged and the cells were washed with saline. The cell pellets were uspended in saline so as to have a cell density equivalent to 0·5 optical density unit. Cell suspensions, usually 100 ml, were treated with trichloroacetic acid to a final concenration of 5 per cent (w/v). The precipitate, collected by centrifugation, was suspended n methanol (24 ml) and warmed at 55° for 15 minutes in a tightly stoppered vessel. Chloroform (48 ml) was added to the cooled suspension and after vigorous shaking was llowed to stand at room temperature overnight. The suspension was filtered through glasswool and the filtrate was equilibrated against an equal volume of aqueous 2 M KCl oy vigorous shaking. The chloroform phase was washed twice in similar fashion with 2 M KCl and finally with water. Phospholipids were precipitated from this extract by adding ice cold acetone. Phospholipid isolated was preserved at −20°, after estimating content in terms of phosphorus by the method of Fiske and Subbarow[13].

2.2. *Preparation of liposomes*

Liposomes were prepared by the method described by Sessa *et al*[14], and Sessa and Weissmann[15]. Phospholipids (100 μ moles phosphorus equivalent of phospholipid) were added in a chloroform solution to a round bottomed flask. The solution was evaporated to get a uniformly thin layer. Swelling solution of 50 ml (0·04 M potassium chromate or glycine in 0·05 M Tris–HCl buffer, pH 7·4) was added and left overnight. After the lipid film was removed by gentle shaking, the suspension was disrupted by sonication using 150 W MSE sonicator, for two minutes at 20 KC/sec. The liposome suspension was then dialysed against Tris–HCl buffer (pH 7·4) containing 0·145 M NaCl to remove the untrapped marker. The dialysis was continued till no significant amount of marker (chromate or glycine) could be detected in the dialysate so as to ensure the complete removal of untrapped marker. The final preparation of liposomes contained 2·0–2·1 μ moles phosphorus phospholipid/ml. Chromate was estimated spectrophotometrically using its absorption maxima at 375 nm and glycine by the ninhydin method[16].

2.3. *Lytic action of polymyxin B and polymyxin B-gramicidin combination on liposomes*

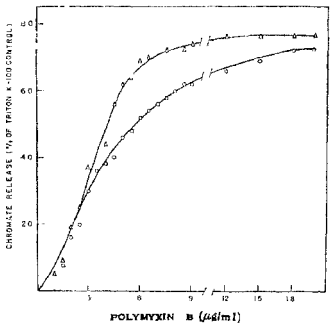
Stock solution of polymyxin B was prepared in Tris–HCl buffer (pH 7·4) containing 0·145 M NaCl. Different concentrations, ranging from 1·0 μg/ml to 40 μg/ml were added to one ml of the liposome suspension. The final volume of the assay was made up to 2 ml with Tris–HCl buffer. Another assay system was set up for polymyxin B-

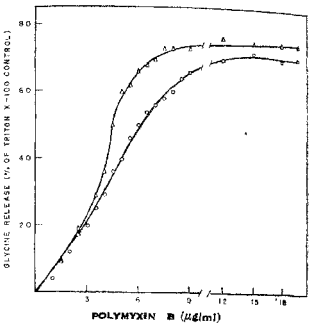
gramicidin combination, adding 10 μg/ml of gramicidin in all the assay tubes containing polymyxin B. Lytic experiments were also carried out with gramicidin alone (concentrations ranging from 10 to 30 μg/ml). Complete release of the marker was observed with Triton-X-100 (10 per cent, Vol/Vol). The suspensions were incubated at 37° for 1 hour and dialysed in small dialysis bags by putting them in 10 ml of Tris–HCl buffer (pH 7·4) containing 0·145 M NaCl. The integrity of control liposomes was checked by dialysis of suspension alone after incubation.

The effects of polymyxin B and polymyxin B-gramicidin combination on the disruption of liposomes containing chromate and glycine as markers, are shown in Figs. 1 and 2. Polymyxin B accelerated the leakage of markers as the concentration was increased from 1 μg/ml to 20 μg/ml. Lysis of liposomes almost attained a plateau at higher concentrations of polymyxin B. Gramicidin alone was ineffective in disrupting the liposome suspension at levels of 10 μg/ml and 15 μg/ml. The leakage of chromate and glycine was only 7–8 per cent even at a higher gramicidin concentration of 30 μg/ml. In glycine sequestered liposomes, the lytic action of polymyxin B was increased from 36, 50, 56, 60, 66 and 70 per cent to 50, 66, 70, 73, 73 and 76 per cent at polymyxin B concentrations of 5, 6, 7, 8, 9 and 12 μg/ml, respectively when 10 μg/ml of gramicidin was present (Fig. 1). Under the same conditions, the leakage of chromate was increased from 46, 52, 60, 62 and 66 per cent to 62, 69, 70, 72, 74 and 76 per cent (Fig. 2). It is also observed that potentiation by gramicidin of release of markers from liposomes by polymyxin B is prominent only at concentrations of polymyxin B above 2·5 μg/ml. Control liposome suspensions showed integrity for 8 hours.

3. Discussion

Phosphatidylethanolamine is an effective target site for polymyxin B in sensitize membranes[8,10,17]. The majority of Gram-negative bacteria are susceptible to polymyxin B because of their high content of phosphatidylethanolamine, 80 per cent of total phospholipid in membranes. Methylated phosphatidylethanolamine, phosphatidylglycerol and other phospholipids make up the remainder[12]. Phosph tidylethanolamine, isolated from *E. coli* or egg yolk alone cannot swell in marker solutions to form stable liposomes. For this, addition of 10 to 20 per cent cardiolipin or phosphatidylglycerol is reported as necessary[9,18]. Membranes which contain phosphatidylethanolamine as the main component and a certain amount of acidic phospholipids such as cardiolipin and phosphatidylglycerol are known to be more susceptible to polymyxin B[8]. In our experience, phospholipids extracted from *E. coli* NCL 8879 strain exhibited appreciable swelling in 0·04 M marker solutions and formed stable liposomes after sonication and dialysis. Such liposomes also showed a high degree of lysis in the presence of polymyxin B. It appears that phospholipids of the *E. coli* NCL 8879 strain used by us are satisfactory for use in model systems. Maximum disruption (76 per cent) occurred in the presence of 20 μg/ml of polymyxin B. While such liposome suspensions are not affected by gramicidin, the latter does potentiate the antimembrane activity of polymyxin B (Figs. 1 and 2). The extent of potentiation observed (16 per cent) is small, compared





FIGS. 1 and 2. Effect of polymyxin B and polymyxin B - gramicidin mixture on liposomes prepared from *E. coli* phospholipids. Released markers : Chromate (Fig. 1) and Glycine (Fig. 2). O—O Polymyxin B ; Δ—Δ Polymyxin B + gramicidin (10 μg/ml.

to enhancements (15 fold) previously observed[11] with in tact cells of *E. coli*. The greater susceptibility of in tact cells of *E. coli* to the polymyxin B - gramicidin combination, relative to the liposomes, may be due to either the unique spatial organization of phospholipids on the in tact cell surface or the possession of suitable binding sites for the potentiating antibiotic, namely, gramicidin. It is known that bacteria sensitive to polymyxin B do possess the ability to bind gramicidin[11].

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