THE POLYMYXIN SENSITIVITY AND THE PHOSPHATIDYL ETHANOLAMINE CONTENT OF THE *VIBRIO CHOLERAE* MEMBRANES

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The phospholipid composition of three different strains of *Vibrio cholerae* was determined by quantitative two-dimensional thin-layer chromatography. The polymyxin sensitivity of the whole organisms or of the liposomes derived from the total phospholipids of these organisms depended solely on the phosphatidyl ethanolamine content of the system concerned and could be quantitatively related by the equations

$$Y_1 = 1.074\ X_1 - 9.828$$

and $$Y_2 = 1.22\ X_2 - 34.47$$

where Y represents the maximum lysis (%)/hr and X, the % phosphatidyl ethanolamine content of the system concerned, the suffix 1 and 2 corresponding to the liposomal and the whole organisms respectively. The analysis revealed the requirement of a threshold amount of phosphatidyl ethanolamine for the expression of polymyxin action on the system concerned.

The mechanism of action of the membrane-active antibiotic polymyxin B has been investigated recently by several authors[1~7]. HSUCHEN and FEINGOLD[5] reported that polymyxin sensitivity increased in proportion to the molar percentage of *Escherichia coli* phospholipid present in the liposomal membrane. Since phosphatidyl ethanolamine is the major phospholipid constituent in the *E. coli* phospholipid, the authors concluded that phosphatidyl ethanolamine represented a target molecule responsible for the polymyxin sensitivity of biological membrane. IMAI *et al.*[6], further reported that liposomes prepared from *E. coli* phospholipid, a mixture of purified phosphatidyl ethanolamine and cardiolipin, a mixture of phosphatidyl ethanolamine and phosphatidyl glycerol were extremely sensitive to polymyxin while those prepared from total lipids of *Streptococcus sanguis*, total lipids of sheep erythrocyte membranes, a mixture of egg lecithin and negatively charged amphiphatic molecules were less sensitive to the action of this antibiotic. However the presence or absence of any direct quantitative relation between the phosphatidyl ethanolamine content and the polymyxin sensitivity of any biological membrane which contains other phospholipids also, has not been analysed so far. The present work aims at investigating the above aspect of the polymyxin action on three different strains of *Vibrio cholerae* organism and also on the liposomes derived from the total phospholipids of these organisms.

Materials and Methods

Bacterial growth and lysis

Vibrio cholerae strains OGAWA 154 (classical), INABA 569B (classical) and V21/2 (boitype El Tor) were used in this study. Bacteria were harvested from mid log phase growth in nutrient broth containing 1% beef extract, 1% bacto-peptone (Difco) and 0.5% NaCl, (pH being adjusted to 8.0) by centrifugation, washed in 0.85% saline and resuspended in tris-HCl buffer containing 0.145 M NaCl (pH 7.4). The desired amount of polymyxin B was then added to this bacterial suspension kept at 37°C (initial optical density being adjusted to about 0.30) and lysis was followed by noting the change in the optical

density at 650 nm with time with the help of a Bausch and Lomb Spectronic 20 Spectrophotometer. The percentage lysis was calculated by considering the initial O.D. to represent 0% lysis.

Isolation and estimation of phospholipids

Lipid from *V. cholerae* organisms in the resting phase of growth was isolated following in general the methods of KANFER and KENNEDY[8] and BLIGH and DYER[9]. Phospholipids were isolated from the total lipid fraction mostly by repeated precipitation with ice-cold acetone[5,10] and also by column chromatography on silicic acid (Chromatographic grade; 100~200 mesh size) for comparison and/or further purification[11,12]. There was no significant difference in the results as between the two methods of isolation[13]. All extraction procedures were carried out, as far as practicable, under a nitrogen atmosphere[13]. Phospholipids were stored under nitrogen at −20°C.

Identification and estimation of different phospholipids were done by two-dimensional thin-layer chromatography using chloroform - methanol - 7 N NH_4OH (65: 30: 4, v/v) followed by chloroform - methanol - acetic acid - water (170: 25: 25: 6, v/v) as the developing solvents in the two runs respectively[12,14,15]. Identification of individual phospholipid components was done from (1) a comparison with the chromatographic pattern of standards obtained from M/s. Sigma Chemical Company, U.S.A., (2) the measured Rf values and also (3) by using different staining agents, *viz.*, iodine vapour and 1% starch solution[16], 50% sulphuric acid[16], rhodamin B (0.05%) in ethanol[17,18], ammonium molybdate-perchloric acid spray[17,18], 0.5% chromic sulphuric acid reagent[18], phosphate stain of VASKOVSKY and KOTETSKY[19], 0.25% ninhydrin in acetone - lutidine (9: 1) for amino groups[20] and periodate SCHIFF reagent[21,22] for vicinal hydroxyl groups.

For quantitation, measured amounts of extracts were spotted by using micro-pipettes. The individual spots obtained after development and after staining with iodine vapour were scraped off carefully and the phosphorus contents were estimated colorimetrically by the FISKE and SUBBA ROW method[23]. An identical amount of silica gel scraped from a blank thin-layer chromatoplate was subjected to the phosphorus estimation and used as reference or control.

Preparation of liposomes

Liposomes were prepared following in general the method described previously[24,25,26]. The marker entrapped was 0.145 M K_2CrO_4 in tris-HCl buffer, pH 7.4. Lipid phosphorus was estimated by the method of FISKE and SUBBA ROW[23]. Chromate leakage was followed spectrophotometrically using its absorption maximum at 375 nm by the Zeiss Spectrophotometer PMQ II.

Action of polymyxin on the liposomes

Polymixin was dissolved in tris-HCl (pH 7.4) containing 0.145 M NaCl to give a stock solution of desired antibiotic concentration. One-ml aliquots of the liposome suspension were mixed with the appropriate amounts of the polymyxin stock solution in small test tubes and incubated at 37°C for different periods of time. Usually the reaction was completed in about 30 minutes.[5,7]. One-ml aliquots were then dialysed against 10 ml of tris-HCl buffer (pH 7.4) containing 0.145 M NaCl. The dialysis was carried out with shaking at 37°C for 1 hour. Leakage of marker from the liposomes under the action of polymyxin or Triton-X-100 was determined after 1 hour by noting the optical density at 375 nm of the respective dialysis against a reference containing the dialysate obtained in an identical manner from untreated liposomes. Percentage leakage was obtained by considering the Triton-X (10%, v/v) induced leakage as 100. The detergent usually released the total amount of marker sequestered in the liposome[5,26].

Results and Discussion

By the action of polymyxin B *V. cholerae* cells underwent rapid lysis upto the first 20 minutes as indicated by the decrease in optical density at 650 nm. The reaction however ended within 60 minutes[7]. The three different strains of *V. cholerae* used in this work exhibited varying degrees of susceptibility to the action of polymyxin B. While the *V. cholerae* strain OGAWA 154 was most sensitive (70% lysis resulting after 1 hour by the action of 20 μg/ml of polymyxin), *V. cholerae* strain V21/2 (biotype El

Table 1. Phospholipid compositions of different *V. cholerae* strains.

The figures indicate the amounts of the different phospholipids as percentage of the respective total phospholipid. Standard deviations were calculated from measurements on four different batches of phospholipid preparation.

V. cholerae strain	Cardiolipin (CL)	Phosphatidyl glycerol (PG)	Phosphatidyl ethanolamine (PE)	Lysophosphatidyl ethanolamine (Lyso-PE)	Phosphatidyl serine (PS)
OGAWA 154 (Classical)	4.76±0.71	13.47±1.27	80.30±2.69	2.80±1.10	—
INABA 569B (Classical)	6.0 ±1.0	17.50±2.5	72.50±2.5	4.24±0.76	1.38±0.07
V21/2 (El Tor)	17.85±1.54	25.07±1.78	51.04±0.30	4.69±1.07	2.98±0.05

Fig. 1. Lysis of *V. cholerae* cells under the action of increasing concentrations of polymyxin.

Lysis was observed in each case after 60-minute treatment of the cells with the antibiotic at 37°C. ●, *V. cholerae* strain OGAWA 154; ○, *V. cholerae* strain INABA 569B; ▲, *V. cholerae* (El Tor) strain V21/2.

Each experimental point was obtained as the average of three independent measurements.

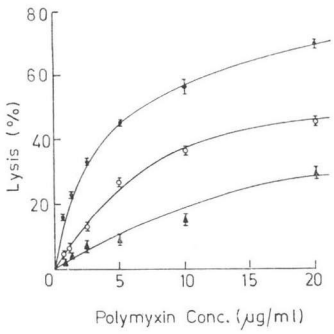
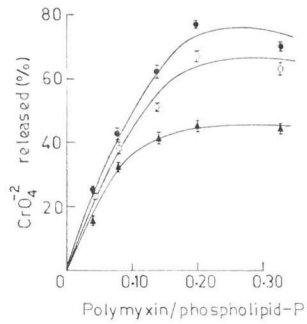


Fig. 2. CrO_4^{--} released from the liposomes derived from the total phospholipids of different *V. cholerae* strains under the action of increasing amounts of polymyxin.

The lysis was observed in each case after 1-hour treatment.

The symbols refer to the liposomes derived from the phospholipids of the strain OGAWA 154 (●), INABA 569B (○) and V21/2 (▲). Each experimental point was obtained as the average of three independent measurements.



Tor) was least sensitive (only 30% lysis resulting under identical condition) to the action of this antibiotic (Fig. 1). Quantitative thin-layer chromatographic data (Table 1) on the phospholipid composition of different strains revealed that the strain OGAWA 154 contained the maximum amount of phosphatidyl ethanolamine (80.3%) while the strain V21/2 contained the least amount of the same (51.04%). This indicated that even the extents of lysis of the different *V. cholerae* cells under the action of polymyxin could be correlated directly with their phosphatidyl ethanolamine contents.

The polymyxin induced leakage of chromate from the liposomes derived from the total phospholipids of different strains of *V. cholerae* increased very rapidly upto the first 10 minutes and then slowed down leading to a plateau within 30 minutes[7] the susceptibility of the liposomes to the action of polymyxin again depended directly on the phosphatidyl ethanolamine content of their membrane (Fig. 2). The amount of chromate released increased, in any case, with the increasing value of the ratio of polymyxin to phospholipid phosphorus and levelled of when the ratio reached 0.20 or higher values.

Fig. 3 shows that the susceptibility of the liposomes or the whole cells of *V. cholerae* was linearly

related with the phosphatidyl ethanolamine content of the system concerned. The equations relating the percentage lysis and the percentage phosphatidyl ethanolamine (PE) content in the two cases were derived from the experimental data by the least square method and are given as follows

$$Y_1 = 1.074X_1 - 9.828 \quad (1)$$

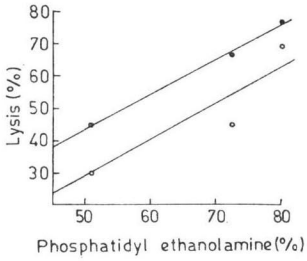
and

$$Y_2 = 1.22X_2 - 34.47 \quad (2)$$

where Y represents maximum lysis (%)/hr and X the PE(%) contents of the systems concerned. The suffix 1 and 2 refer to the liposomes and the whole cells of *V. cholerae* respectively. It is interesting to note that the slopes of the two least square lines are almost the same and the small difference could be ascribed to the experimental errors, indicating thereby that the liposomal system and the whole cells behaved almost similarly towards the action of this antibiotic. The extent of lysis however was lower for the whole cells, which is understandable in view of the much greater complexity of the structure of the whole cell envelopes (cell wall and plasma membrane) as compared to the liposomal ones. It is also interesting to note that none of the least square lines described by the equations 1 and 2 passed through the origin indicating the existence of a threshold in the amount of phosphatidyl ethanolamine required for the expression of polymyxin action. The threshold amounts (as percentage of total phospholipid content of the system) of phosphatidyl ethanolamine (PE) needed for the expression of polymyxin action were 9.15 (%) and 28.3 (%) for the liposomal system and the whole cells respectively, as calculated from the equations of the respective least square lines. It is of interest to note in this respect that the requirement of a threshold amount of phosphatidyl ethanolamine in the membrane was also indicated by the earlier workers[5,6] from other considerations.

Fig. 3. Least square lines describing relations between the degree of maximum lysis (%)/hour and the phosphatidyl ethanolamine content (%) of i) the liposomes derived from the phospholipids of different *V. cholerae* strains (●–●) and ii) different *V. cholerae* organisms (○–○). In each case the minimum polymyxin concentrations which yielded maximum lysis of the system after 1 hour (as indicated in Figs. 1 and 2) were used.

The solid lines represent the calculated least square lines and the open and solid circles the experimental points.



Quantitative analysis of the phospholipid composition of different *V. cholerae* strains as presented in Table 1 revealed that the strain possessing greater amount of phosphatidyl ethanolamine contained lesser amount of any other phospholipid in a comparative scale. A critical examination of the amounts of other phospholipids in the three strains thus indicated that the polymyxin sensitivity was not significantly affected by any of these other phospholipids but depended solely on the phosphatidyl ethanolamine content of the system concerned. This is consistent with the previous finding that polymyxin sensitivity increased in proportion to the molar % of *E. coli* phospholipid present[5]. Also significant in this respect is the finding of IMAI *et al.*[6] that the degree of susceptibility to the antibiotics was much smaller in liposomes with lecithin than in those with PE even though they had the same amount of cardiolipin.

Since the plot in Fig. 3 indicated that there was a positive correlation between the phosphatidyl ethanolamine content and the polymyxin sensitivity of the membrane, these data were subjected to a detailed statistical analysis as shown in Table 2. For the liposomal system, the correlation coefficient (calculated from the values of the regression coefficients) was positive and very nearly one. Signifi-

Table 2. Statistical analysis of the data presented in Fig. 3 indicating the significance of the correlation between the phosphatidyl ethanolamine content and polymyxin sensitivity of the membranes.

System	Regression coefficients[1]		Correlation coefficient	Significance	
	m_1	m_2	$\gamma = \sqrt{m_1 \times m_2}$	't' value[2]	Probability (P)
Liposomal system	1.074	0.929	+0.999	22.845	$\simeq 0.03$
Bacterial system	1.219	0.700	+0.924	2.415	>0.10

1. Regression coefficients refer to the regression lines $Y = m_1X + c_1$ and $X = m_2Y + c_2$;
2. $t = \gamma\sqrt{n-2} / \sqrt{1-\gamma^2}$, where n = number of samples (for small sample)

cance test indicated that the correlation was significant to about 3% level. The statistical analysis thus showed that there was a positive and significant correlation between the phosphatidyl ethanolamine content and polymyxin sensitivity of the liposomal membrane. The correlation was however not significant for the bacterial system under study even though the correlation coefficient was quite high (0.924). It is apparent that the levels of significance could be improved further with larger number of data.

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