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The effect of lipopolysaccharide on lipid bilayer permeability of β-lactam antibiotics

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The lipid-bilayer permeability of cephalosporins was extensively suppressed by addition of lipopolysaccharide to liposomal membrane in proportion to the hydrophobicity of the drugs. This suggests that the polysaccharide chain layer contributes to the barrier function. The importance of the polysaccharide chain in the barrier function was also supported by the fact that the permeability to Rd-type lipopolysaccharide-containing liposomes showed essentially the same dependency on the hydrophobicity of the cephalosporins as that of the lipopolysaccharide-free liposomes. In this case the permeability of the cephalosporins was proportional to their hydrophobicity. Similar lipopolysaccharide effect was also observed in the permeation of penicillins.

β-Lactam *Outer membrane* *Lipopolysaccharide* *Permeability* *Liposome* *Hydrophobicity*

1. INTRODUCTION

In [1] we suggested the significance of the matrix region of outer membrane in permeation of penicillins into gram-negative bacteria through the porin is known as the main pathway for hydrophilic cephalosporins [2–4]. Phospholipid bilayer permeability of moderately hydrophilic penicillins was much higher than that of the cephalosporins having similar hydrophobicity [5,6]. On the other hand, the permeability of the phospholipid bilayer to methicillin, oxacillin and its derivatives lay between that of moderately hydrophilic penicillins and cephalosporins [6]. However, these penicillins exhibited weak antibacterial activity against gram-negative bacteria due to their low outer membrane permeability [7]. This difference in the permeation of the phospholipid bilayer and outer membrane might be attributed to lipopolysaccharide (LPS) in the outer membrane. There are two distinct hypotheses for the barrier function of LPS. It is possible that the solidifying effect of LPS on the outer leaflet of the outer membrane may contribute to the barrier function [8–10], or that an additional layer composed of the polysaccharide chains of LPS in the outer membrane may play an important role in the exclusion of hydrophobic solutes [11].

Here we introduced LPS into the liposomal membrane and investigated the LPS effect on lipid bilayer permeability of β-lactams. If the former hypothesis is true, the reduction of the permeability of β-lactams through the LPS-containing liposomal membrane should be independent of the degree of the hydrophobicity of the molecule. On the other hand, if the latter is true, the permeability of hydrophobic β-lactams through the LPS-containing liposomal membrane should be reduced more than that of hydrophilic one.

We used mainly cephalosporins as the drugs for studying lipid-bilayer permeability because a series of cephalosporins having different hydrophobicity were easily obtainable. Cephalosporins permeate the intact outer membrane mainly via the porins. However, they also show the phospholipid bilayer permeability according to their hydrophobicity,

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although the dependency was smaller than that of penicillins [6].

2. MATERIALS AND METHODS

2.1. *β-Lactam antibiotics*

β-Lactam antibiotics were a generous gift from the following pharmaceutical companies: cephaloridine, Torii Pharmaceutical Co. (Tokyo); cephalexin, Toyama Chemical Co. (Tokyo); cefazolin, Fujisawa Pharmaceutical Co. (Osaka); cephalosporin-C, benzylpenicillin and ampicillin, Meiji Seika Co. (Tokyo); sulbactam, Pfizer Taito Co. (Tokyo).

2.2. *Preparation of phospholipids, LPS and β-lactamase*

Bacterial phospholipids were extracted from *E. coli* CS109 [1] as in [12] and purified by silica gel column chromatography. Cardiolipin was obtained from Sigma. Intact LPS and Rd-type LPS lacking a greater part of polysaccharide chain, were isolated from *E. coli* YA21 and its deep rough mutant YA21-6 [13], respectively, as in [14]. The concentration of LPS was determined from its phosphorus content on the assumption that the number of phosphorus atoms per molecule of intact LPS and deep rough LPS is 4 and 3, respectively [15]. Cephalosporinase and penicillinase was prepared from *Citrobacter freundii* GN346 and *E. coli* ML1410 RGN823, respectively, as in [16].

2.3. *Preparation of liposomes*

β-Lactamase-enclosing liposomes were prepared from *E. coli* phospholipids and cardiolipin (Sigma) in the presence of *C. freundii* cephalosporinase as in [5]. β-Lactamase-enclosing liposomes containing LPS were prepared as follows. A mixture of 4 mg of the phospholipids and 0.37 mg cardiolipin in a test tube was dried from chloroform solution to a thin film using nitrogen gas then placed under vacuum for 2 h. Indicated amounts of water suspension of LPS were added to the thin film, mixed by bath sonication and the suspension was freeze-dried. Then 0.4 ml phosphate-buffered saline (PBS, pH 7.0) containing 2.8 nmol cephalosporinase was added unless otherwise stated. The mixture was briefly sonicated with a Branson water bath sonicator and quickly frozen in solid CO_2/acetone and then slowly thawed at room temperature. The resulting suspension was again briefly sonicated. The enzyme outside the liposomes was irreversibly inactivated by an excess of sulbactam 500 molar unless otherwise stated and the inactivated enzyme was removed by gel filtration as in [5].

2.4. *Measurement of β-lactam uptake into liposomes*

Uptake of β-lactams into liposomes was assayed by measuring hydrolysis of the drugs by the enzyme entrapped inside the liposomes as in [5,6].

2.5. *Determination of internal volume of liposomes*

The volume inside the liposomes was determined by measuring the entrapped glucose as in [17]. Liposomes were prepared as described above except that β-lactamase was replaced by 100 mM glucose. Excess glucose outside the liposomes was removed by dialysis against 500 ml PBS (pH 7.0) for 2 h with 4 changes of the buffer. The amounts of glucose entrapped inside the liposomes were spectrophotometrically determined by the change in 340 nm absorbance indicating reduction of NADP in the presence of hexokinase, glucose-6-phosphate dehydrogenase, ATP and the necessary cofactors when the liposomes were disrupted by Triton X-100.

2.6. *Reversed-phase thin-layer chromatography*

The hydrophobic character of the β-lactam antibiotics was expressed by the R_f value which was measured by reversed-phase thin-layer chromatography (TLC) [5]. A smaller R_f value indicates a higher hydrophobicity of the molecule.

3. RESULTS AND DISCUSSION

3.1. *Estimation of the permeability parameter*

The permeability parameter was calculated on the basis of Fick's law of diffusion as described in [5,6]. In those papers, the parameter was expressed as $min^{-1} \cdot \mu M\ lipid^{-1}$, which is equivalent to cm^3/min per nmol lipid. This mode of expression is only valid when the compositions of the liposomes remain unchanged, since the internal volume increased probably due to the decrease in a degree of stacking of the lamellar on addition of

LPS [18], which brought about an increase in the surface area of the liposomes. In fig.1, the internal volume of the liposome was expressed as nl/nmol hydrocarbon chain residue, since phosphatidylethanolamine, cardiolipin and LPS contain 2, 4 and 6 hydrocarbon chains per molecule, respectively [15]. When 0.04 nmol LPS/nmol phospholipid was added to the liposome, the internal volume increased by a factor of about 1.7. When the LPS content was 0.04–0.13 nmol, the internal volume remained constant, while the internal volume again decreased when LPS content was above 0.13 due to the formation of micelle. Therefore, the internal volume of liposomes is a good measure for the surface area of the liposomes under an LPS content of 0.13. Here, the permeability parameter was expressed as cm^3/min per nl liposomal internal volume.

3.2. *The effect of Mg^{2+} on the permeability parameter*

Although phospholipid bilayer permeability of β-lactam antibiotics was independent of Mg^{2+} concentration in the assay mixture, the permeability of the drugs to LPS-containing liposomes was significantly influenced by the Mg^{2+} concentration outside the liposomes (fig.2). Under magnesium-free conditions, the permeability of cephalosporins to LPS-containing liposomes was higher than that of the liposomes without LPS. This phenomenon might be caused by a loosening of the molecular packing in the bilayer due to ionic repulsion between fixed negative charges of LPS molecules. In the case of intact cells, it is known that the removal of Mg^{2+} by EDTA results in an abnormal increase in the bacterial susceptibility to hydrophobic agents due to a structural defect of the outer membrane [19–21]. Therefore Mg^{2+} are presumed to be necessary for stabilizing the normal molecular packing of the LPS-containing lipid-bilayer. When magnesium was added to the assay mixture, the permeability of cephalosporins to LPS-containing liposomes decreased until the permeation rate became constant at a magnesium chloride concentration of 5 mM (fig.2). In the following experiments, the permeability to LPS-containing liposomes was measured in the presence of 10 mM magnesium chloride.

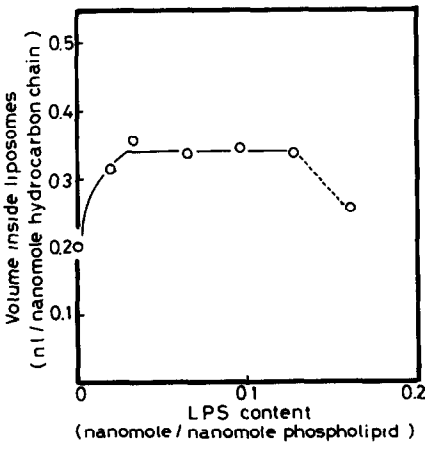

Fig.1. Dependence of the internal volume of liposomes on LPS content. The volume inside the liposomes was determined as described in section 2.

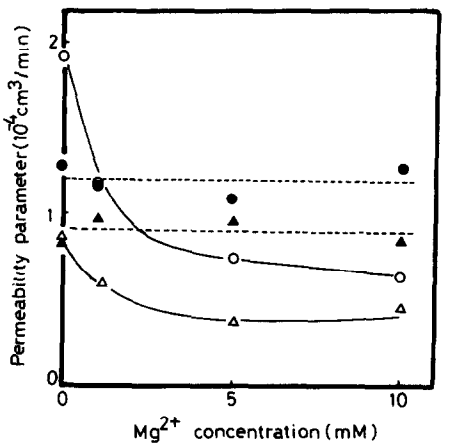

Fig.2. The effect of Mg^{2+} on the permeability of cephalosporins. Liposomes were prepared in magnesium free buffer as described in section 2. The indicated concentration of magnesium chloride was added to the assay mixture. LPS content of LPS-containing liposomes was 0.13 nmol per nmol phospholipid. (○, ●; (△, ▲) Permeability of cephaloridine and cefazolin, respectively. (○, △; ●, ▲) Permeability to the LPS-containing liposomes and the liposomes free from LPS, respectively. Permeability was expressed as cm^3/min per nl liposomal internal volume.

3.3. *The effect of hydrophobicity of β-lactams on their permeability parameter to LPS-containing liposomes*

The permeability of cephalosporins to the liposomes composed of phospholipids alone in-

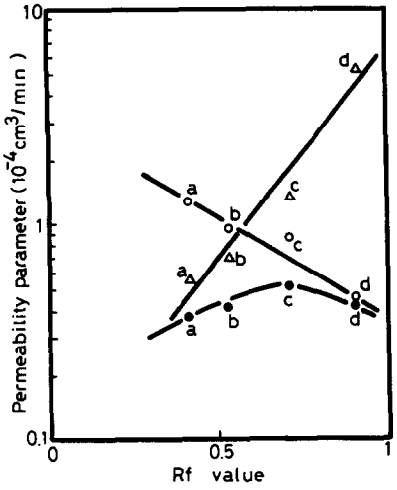

Fig.3. Dependence of the permeability of cephalosporins on hydrophobicity of the molecules. The permeability and R_f value of cephaloridine (a), cephalexin (b), cefazolin (c) and cephalosporin-C (d) was measured as described in section 2. (○, ●) Permeability to the liposomes free from LPS and the liposomes containing 0.13 nmol LPS/nmol phospholipid, respectively. (△) Calculated permeability parameter to hypothetical polysaccharide chain layer described in the text. Permeability parameter was expressed as in fig.2.

creased with increasing hydrophobicity (fig.3) whereas their permeability to LPS-containing liposomes showed a complex dependency on their hydrophobicity. In the presence of LPS, the permeability of cephalosporins decreased in proportion to their hydrophobicity. As a result, a moderately hydrophilic cephalosporin, cefazolin, showed the best permeability to the liposomes containing LPS among the cephalosporins tested. This phenomenon can be well interpreted if one assumes that the thick hydrophilic layer composed of the polysaccharide chain region of LPS is stacked on the hydrophobic layer composed of hydrocarbon chains. In such a stacked layer system, the overall permeability parameter (P_t) can be expressed as a reciprocal of the sum of reciprocals of the permeability parameters of individual layers as follows:

$$1/P_t = 1/P_s + 1/P_h$$

where P_s and P_h represent the permeability parameters of the polysaccharide chain region and hydrocarbon chain region, respectively. As a first approximation, P_h is equal to the phospholipid bilayer permeability. We could thus estimate the permeability parameter of the polysaccharide chain region (P_s). P_s values of 4 representative cephalosporins are plotted against the R_f value in fig.3. The P_s value was clearly proportional to the hydrophilicity of molecule. This result is reasonable when the hydrophilic nature of the polysaccharide chain region is taken into consideration.

The effect of LPS on the permeability of penicillins was also measured by employing two representative penicillins; i.e., benzylpenicillin and ampicillin. In the presence of LPS, benzylpenicillin which has a higher hydrophobic property than ampicillin more significantly reduced its permeation to the liposome than ampicillin (table 1). The LPS effect on the penicillins was essentially the same as that found in the case of cephalosporins.

3.4. *The permeability of β-lactams to liposomes containing Rd-type lipopolysaccharide lacking the greater part of the polysaccharide chain*

Liposomes were made from phospholipids and the Rd-type LPS which was isolated from a deep rough mutant of *E. coli*. In these liposomes, the dependency of the permeability of cephalosporins on their hydrophobicity was essentially similar to that of liposomes composed of phospholipid alone (fig.4). This observation also supported the assumption that the polysaccharide chain region of

Table 1

The effect of LPS on the permeability of penicillins[a]

Penicillins	R_f value	Permeability parameter (cm^3/min)[b]	
		PL alone	PL + LPS[c]
Benzylpenicillin	0.35	92.7×10^{-5}	37.0×10^{-5}
Ampicillin	0.57	57.5×10^{-5}	40.6×10^{-5}

[a] Penicillin permeability was measured by using penicillinase-enclosing liposomes. Penicillinase outside the liposome was inactivated by 500 molar excess of clavulanic acid. The other conditions for assay were the same as those for cephalosporins

[b] This parameter is expressed per nl liposomal internal volume

[c] LPS content is 0.13 nmol/nmol phospholipid

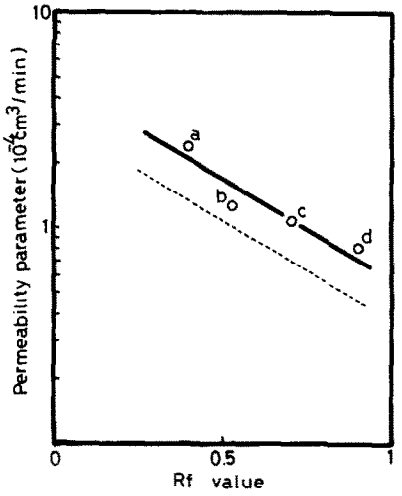

Fig.4. Dependence of the permeability of cephalosporins to the liposomes containing 0.06 nmol Rd-type LPS per nmol phospholipid on the hydrophobicity of the molecule. The assay mixture contained 10 mM magnesium chloride. Symbols are: a, cephaloridine; b, cephalexin; c, cefazolin; d, cephalosporin-C. Permeability parameter was expressed as in fig.2. (– – –) Dependency of the permeability of the cephalosporins to LPS-free liposomes.

intact LPS acts as a permeability barrier against hydrophobic molecules. Plots of the permeability to the liposomes containing Rd-type LPS against the R_f value of the drugs was somewhat higher than corresponding plots obtained by the liposomes free from LPS. In the liposomes containing Rd-type LPS, the molecular packing may be looser than that in the liposomes free from LPS because of the exposed negative charge of Rd-type LPS.

The results obtained here strongly support the idea that LPS, based on its polysaccharide chain region, acts as a selective barrier against the permeation of hydrophobic β-lactams.

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