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Diffusion of β-Lactam Antibiotics Through Liposome Membranes Containing Purified Porins

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A method to determine the diffusion of cephalosporins through porin pores in vitro was developed, using liposomes reconstituted from phospholipids, lipopolysaccharides, and purified porin trimers. With this method, the roles of several species of porin pores from *Escherichia coli* and *Salmonella typhimurium* in the diffusion of cephalexin, cephaloridine, and cephalothin were examined. Results clearly showed that porins from *E. coli* B and 39,000-molecular-weight porins from *S. typhimurium* formed the most efficient pores. Thus, these were considered to represent a single functional group. OmpF and OmpE porins of *E. coli* K-12 and 38,000-molecular-weight porins of *S. typhimurium* formed moderately efficient pores. OmpC porins of *E. coli* K-12 and 40,000-molecular-weight porins of *S. typhimurium* were the least efficient pore formers. The present method can be used to distinguish the role of individual porin pores in the diffusion of cephalosporins.

The outer membrane of gram-negative bacteria is located exterior to the cytoplasmic membrane and functions as a barrier to hazardous agents, such as bile, long-chain fatty acids, dyes, and surfactants (14). This function of the outer membrane protects bacteria from killing by certain antibiotics, such as macrolides, actinomycin D, rifampin, etc., although these antibiotics are active against EDTA-treated gram-negative bacteria (6). The diffusion of small hydrophilic molecules across the outer membrane is mediated via a molecular sieve which allows the diffusion of saccharides having molecular weights of up to 500 (4, 12). The protein complexes that confer the sieve property to reconstituted vesicle membranes were isolated and purified from the outer membrane of *Escherichia coli* and *Salmonella typhimurium* (8, 9). These pores are constructed from three identical subunits of the outer membrane proteins, known as porins (5, 11). Thus, the pores made of porins play a major role in the translocation of solutes across the outer membrane, with the exception of certain specific solutes (3, 14). Small hydrophilic antibiotics such as β-lactams are believed to diffuse through porin pores (13).

Zimmermann and Rosselet (24) and Sawai et al. (18) independently developed an assay method for the diffusion of β-lactam antibiotics through intact outer membranes, using strains which produce periplasmic β-lactamase. Nikaido et al. (15), using the above technique, demonstrated that mutant strains of *S. typhimurium* producing decreased amounts of porins were less permeable to cephaloridine (CER). However, the precise role of individual porin species in the diffusion of antibiotics has not yet been firmly established. Here, we have developed an in vitro method for determining the diffusion of β-lactam antibiotics through porin pores, using liposome membranes containing defined amounts of purified porin trimers and encapsulating purified β-lactamase in their intravesicular spaces. Using this method, the diffusion of CER, cephalexin (CEX), and cephalothin (CET) through the pores formed by various porin species was determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used were *E. coli* B, which produces B porin, and K-12 strains KY2201, KY2209 (17), and CE1212 (22), which produce OmpC, OmpF, and OmpE porins, respectively. Medium and growth conditions for all *E. coli* strains have been described previously (9). *S. typhimurium* strains SH5551, SH6377, and SH6017, producing 40,000-molecular-weight (40K), 39K, and 38K porins, respectively, were grown as reported earlier (20). *Citrobacter freundii* GN346 (19), which produces a cephalosporinase, was grown in veal infusion broth (BBL Microbiology Systems).

Purification of porins. Porins were purified by a procedure reported earlier (20), except gel filtration was performed with a Sephacryl S-300 column. The sample was concentrated by using an Amicon PM30 ultrafiltration membrane filter.

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Purification of cephalosporinase. *C. freundii* GN346 was grown for 2.5 h at 37°C with vigorous aeration, using a 10% inoculum. Cells were harvested by centrifugation and disrupted with a French pressure cell at 1,500 kg/cm^2. Crude extracts were obtained by centrifugation at 190,000 × *g* for 60 min at 4°C, applied onto a column of CM-Sephadex A50 (2.5 by 17 cm), and equilibrated with 0.01 M sodium phosphate buffer (pH 6.8), and the column was eluted with a linear gradient of NaCl (0 to 0.5 M) in 0.01 M sodium phosphate buffer (pH 6.8). A single peak of β-lactamase activity was eluted at around 0.13 M NaCl. This and neighboring fractions were pooled and kept frozen at −80°C after concentration and dialysis against a large excess of 0.05 M sodium phosphate buffer (pH 7.0).

Reconstitution of vesicle membranes. Reconstitution of vesicle membranes by sonic oscillation of a dried film of phospholipids and lipopolysaccharide (LPS) and with or without porin was done by a method described earlier (8), except the dried film was suspended in 100 μl of a solution containing β-lactamase (1,265 U μl^{-1} at 200 μM CET at 30°C) and 0.05 M sodium phosphate buffer (pH 7.0). The vesicle suspension was passed through a Sepharose CL-6B column (0.9 by 50 cm) equilibrated with the above-described buffer. Vesicles made from 5 μmol of phospholipids and 0.5 μmol of LPS entrapped 0.3% of added enzyme. Reconstitution of vesicle membranes by 1-*O*-*n*-octyl-β-D-glucopyranoside (octyl-glucoside) dialysis was done essentially by the method of Mimms et al. (7). Phospholipids (2 μmol) in chloroform were dried in a conical centrifuge tube under an N_2 gas stream and then kept in a desiccator under vacuum for 3 h. The dried lipid film was dissolved in a mixture containing 87.7 μl of 10% octyl-glucoside (30 μmol)–0.2 μmol of LPS–100 μl of β-lactamase (with the same activity as that described above) in 0.05 M phosphate buffer (pH 7.0) and with or without porins. The mixture was dialyzed (Spectropore membrane; cut-off molecular weight, 12,500) against 1,000 ml of 0.05 M phosphate buffer (pH 7.0) at 4°C for 18 h. The contents of the dialysis bag were passed through a Sepharose CL-6B column (0.9 by 50 cm) equilibrated with 0.05 M phosphate buffer (pH 7.0), and the column was eluted with the same buffer. The vesicle membranes formed by this technique entrapped about 1.5% of added β-lactamase.

Estimation of β-lactamase activity. β-Lactamase activity was assayed spectrophotometrically by following enzymatic rupture of the β-lactam ring at 255 nm at 30°C. The reaction mixture (1.0 ml) contained 100 μl of vesicles, 200 μM substrate, and 0.05 M sodium phosphate buffer (pH 7.0). The mixture, minus substrate, was prewarmed at 30°C for 8 min, and the reaction was started by adding 100 μl of substrate. Decrease of absorption at 255 nm was monitored with a Hitachi 200-20 spectrophotometer equipped with a temperature-controlled water circulator. Complete hydrolysis of 1.0 nmol of CER, CET, and CEX yielded decreases in $A_{255\ nm}^{1\ cm}$ of 0.012, 0.0079, and 0.0068, respectively.

Calculation of PA. Since the present assay method is based on Fick's law and the solute diffusion is measured indirectly by determination of enzymatic degradation of the substrates, the diffusion velocity (V) can be expressed as $V = PA(Co - Ci) = [(V_{max} \cdot Ci)/(K_m + Ci)]$, where P is the permeability coefficient, A is the surface area, and Co and Ci are substrate concentrations outside and inside the vesicles, respectively. V_{max} and Ci were calculated from enzymatic activity with the detergent-lysed vesicles and by the above equation, respectively. The permeability parameter (PA) was expressed as cubic centimeters per minute per 0.1 nmol of LPS per nanomole of phospholipids. Detailed discussions are given in earlier publications (21, 24).

Other methods. Phospholipids and LPS were extracted and quantified as described previously (8).

Antibiotics. β-Lactam antibiotics used throughout this study were reagent grade.

RESULTS

We have developed an in vitro method for the estimation of simple diffusion of certain molecules across liposome membranes (21), based on an earlier method for determination of β-lactam diffusion (24). We established an in vitro assay method for the diffusion of β-lactam antibiotics by a combination of the above two newly developed techniques, using vesicle membranes reconstituted from phospholipids, LPS, and porins and entrapping purified β-lactamase in their intravesicular spaces. Since the theoretical bases for this assay method are similar to those of previously published works (21, 24), we shall note only the points which are directly relevant to the present investigation. The K_ms of the cephalosporinase from *C. freundii* for CET, CER, and CEX were determined to be 10, 100, and 50 μM, respectively, at pH 7.0. The enzymatic activity of the cephalosporinase was not influenced measurably when 0.1% Tween 80 (added 8 min before the initiation of the enzyme reaction) was present in the reaction mixture. β-Lactamase activity determined with vesicles lacking porin (presumably owing to enzymes attached to the external surfaces of the vesicle membranes) appeared to be 2% of the total enzyme activity of detergent-lysed vesicles. The diffusion of cephalosporins through the lipid bilayer, presumably crossing the membranes by dissolving in the hydrophobic domain, was calculated to be 10% compared with that of the detergent-lysed vesicles. Therefore, 12% of the total enzyme activity was subtracted from the enzyme activity of intact vesicles, and 2% of the total enzyme activity was subtracted from that of the detergent-lysed vesicles. The enzymatic activity due to the diffusion of substrate through a nonporin route(s) was distinguished from that due to enzymes attached to the vesicle exterior as follows. The vesicles (minus porin and enzyme) were incubated with 0.1 ml of β-lactamase (1,265 U μl^{-1}) at 25°C for 30 min, and the enzymatic activity was determined with these vesicles after passing them through a column. The enzyme activity of the enzyme-loaded vesicles (lacking porin) was measured as described above. The enzyme activity of the vesicles

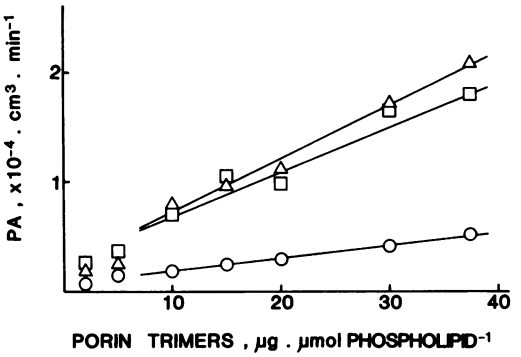

FIG. 1. Effect of porin concentration on diffusion of cephalosporins. Vesicle membranes were reconstituted with 5 μmole of phospholipids, 0.5 μmol of LPS, and purified B porin trimers by sonication and passed through a Sepharose CL-6B column (0.9 by 50 cm). β-Lactamase activity in intact and detergent-lysed vesicles was determined spectrophotometrically in a 1-ml cuvette at 30°C. Enzyme activity was expressed as nanomoles of substrate hydrolyzed per minute per nanomole of phospholipids per 0.1 nanomole of LPS. The PAs of the substrates at various porin concentrations per micromole of phospholipid are shown. Cephalosporins used as substrates were CET (○), CER (△), and CEX (□).

mixed with the enzyme was subtracted from that of the enzyme-loaded vesicles after adjusting the V_{max} and the vesicle concentrations. Leakage of β-lactamase from the vesicles was undetectably small for at least 2 h after passing the loaded vesicles through the column, while most of the permeability assay was completed.

Effect of porin concentration on the diffusion of cephalosporins. If it is assumed that cephalosporins cross membranes through porin pores, their diffusion should be proportional to the amount of porin added to the vesicle membranes. We have determined the diffusion of CER, CEX, and CET through the vesicle membranes containing porin trimers. The diffusion of CER, CEX, and CET is linearly related to the amount of porin added (Fig. 1), though linearity is somewhat poor for CER and CEX at low porin concentrations. This is probably owing to a population of vesicles lacking porin. It is evident from these results that these cephalosporins cross the membrane through porin pores.

Effect of substrate concentration on the diffusion of cephalosporins. Since the present assay method is based on Fick's law, the PAs of the membranes should not be influenced by external substrate concentration. We have examined this with the vesicles containing porin trimers with various concentrations of extravesicular CET (60 to 200 μM CET; 6- to 20-fold higher than the K_m). The PAs of the membranes were nearly constant when substrate concentrations of 80 to 200 μM were used (Fig. 2), suggesting that the assay method obeyed Fick's law. PAs with substrate concentrations of 60 and 70 μM were slightly deviated, suggesting that use of substrate concentrations that are close to the K_m of the enzyme should be avoided if possible. Since the present assay method seems to be usable for determining the diffusion of cephalosporins in vitro, we studied solute selectivity of porin pores formed by various porin species.

Diffusion of cephalosporins through vesicle membranes reconstituted from a single species of porin from *E. coli*. Diffusion of CER, CEX, and CET was determined by using vesicle membranes containing various amounts of porin, and PAs were calculated. B porin formed very efficient pores for the diffusion of all three cephalosporins (Fig. 3). The PA for CEX obtained with 50 μg of B porins, for instance, was 2.3, 16, and 3 times higher than those of OmpF, OmpC, and OmpE porin pores, respectively. OmpF and OmpE porin pores showed similar diffusion properties for these cephalosporins. OmpC porin pore is practically inactive for the diffusion of CEX and CET. Although OmpC porin pore allowed the diffusion of CER somewhat, it was the least efficient pore of the porins prepared from *E. coli* (Fig. 3C). Of the cephalosporins, CEX was the most permeable solute, followed by CER and CET for all porin pores; CER was the most permeable through OmpC porin pore. Therefore, the solute selectivity of various porins from *E. coli* is more or less similar, but the efficacy of solute diffusion is different among porins.

Diffusion of cephalosporins through vesicle membranes containing a single species of porin from *S. typhimurium*. The possibility of solute selectivity of porin pores from a single species of porin from *S. typhimurium* was investigated (Fig. 4). The vesicle membranes reconstituted

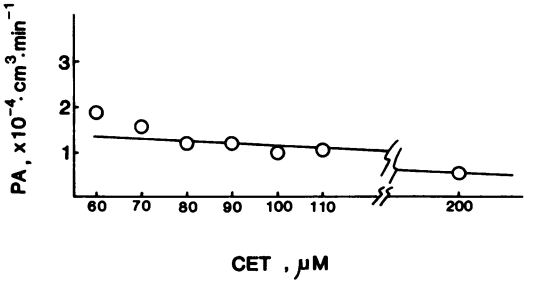

FIG. 2. Effect of substrate concentration on PA. Vesicles were formed from 5 μmol of phospholipids, 0.5 μmol of LPS, and 100 μg of B porin trimers, and the hydrolysis of CET was assayed as described in the text. PAs were calculated on the basis of an experiment in which 20 μg of porins per μmol of phospholipids per 0.1 μmol of LPS was used.

from 39K porins formed very efficient pores for the diffusion of CER, CEX, and CET, and the PAs for these drugs at 50 μg of porins appeared to be 5×10^{-4}, 3.4×10^{-4}, $2.3 \times 10^{-4} \cdot cm^3$ per min, respectively (Fig. 4B). These PAs are comparable to those of the most efficient pores of *E. coli*, B porins. The pore made of 40K porin was the least efficient of the *S. typhimurium* porins tested (Fig. 4C). The diffusion efficiency of 38K porin pore for these cephalosporins is somewhat intermediate between those of 39K and 40K porin pores (Fig. 4A). The diffusion of these cephalosporins through 38K porin pore was not influenced significantly by the amount of porin added, for reasons unclear at present. The PA of the membrane containing 39K porin pore for CER is 6 and about 60 times higher than those of 38K and 40K porin pore, respectively. An interesting observation is that 39K porin pores allowed very efficient diffusion of CER as compared with the other two drugs (Fig. 4B).

DISCUSSION

An in vitro method to determine the diffusion of cephalosporins, using liposome membranes reconstituted from phospholipids, LPS, and a homogeneous preparation of porins, was developed. Since the diffusion of cephalosporins was proportional to the amount of porin incorporated into vesicle membranes, and the diffusion of CET (and possibly of other substrates also) was not influenced significantly by changes in extravesicular substrate concentration, the assay method described here seems to be reliable and

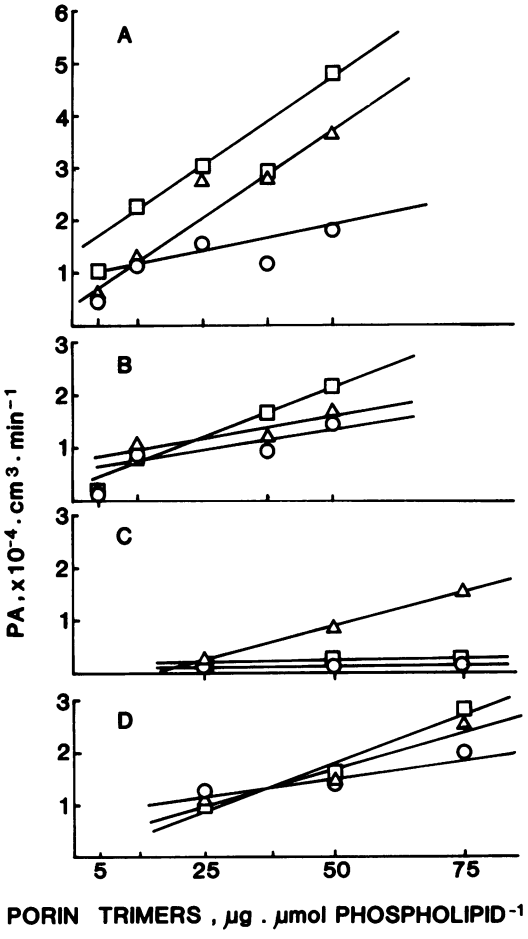

FIG. 3. Diffusion of cephalosporins through vesicle membranes reconstituted from a single species of porin trimer from *E. coli*. Vesicle membranes were reconstituted from 2 μmol of phospholipids, 0.2 μmol of LPS, and purified porin trimers by the detergent-dialysis method as described in the text. Diffusion of β-lactam antibiotics was determined as described above. PAs were calculated as described in the text and plotted versus the amount of porin trimer added per micromole of phospholipid. (A) B porin; (B) OmpF porin; (C) OmpC porin; (D) OmpE porin. Cephalosporins used as substrates were CET (○), CER (△), and CEX (□).

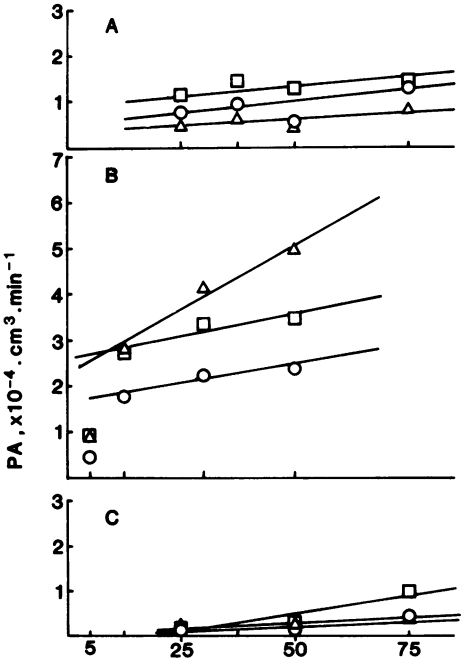

FIG. 4. Diffusion of cephalosporins through vesicle membranes reconstituted from a single species of porin trimers from *S. typhimurium*. Reconstitution of vesicle membranes and determination of substrate diffusion were carried out in a manner similar to that described in the legend to Fig. 3, except porin trimers from *S. typhimurium* were used. (A) 38K porin; (B) 39K porin; (C) 40K porin. Cephalosporins used as substrates were CET (○), CER (△), and CEX (□).

reproducible. Octyl-glucoside dialysis and sonication were employed for reconstitution of vesicle membranes and encapsulation of β-lactamase. Although these two methods gave essentially the same results, the former seems superior to the latter in two respects; the vesicles formed by the former method entrapped approximately fivefold-higher amounts of β-lactamase than the latter, and it provided more reproducible results.

To test the reliability of the present assay method, we determined the solute selectivity of pores made of a single species of porin, using three cephalosporins. The following results were obtained. (i) B porin from *E. coli* B and 39K porin from *S. typhimurium* showed similar permeability properties for the diffusion of cephalosporins; therefore, they were considered to belong to the same functional group. The pores made of B and 39K porin were the most efficient, followed by OmpF and OmpE porins. The role of OmpF porin in the diffusion of CER is consistent with results obtained with an *ompF*$^+$ *ompC* mutation in *E. coli* (23). (ii) The OmpC porin of *E. coli* K-12 and the 40K porin of *S. typhimurium* were negligibly diffusable to all cephalosporins except CER through OmpC porin pore, suggesting that these porins belong to the same functional group. (iii) Although OmpF and OmpE porins showed similar properties in this study, they belong to different functional groups. The reasons for this are that OmpE porin is coded for by the *pho* operon of *E. coli* (22) and has a greater than threefold-higher efficiency for the diffusion of *p*-nitrophenylphosphate and 4-methylumbelliferylphosphate than that of OmpF porin (N. J. Ishii and T. Nakae, unpublished data). Other workers have reported that OmpE porin serves as a poor diffusion pathway for CER; however, our present experiment did not support this conclusion (23). (iv) In general, CEX was the most permeable through many porin pores, followed by CER and CET. Therefore, solute specificity of these pores was relatively poor, and hence we assumed that the efficacy of solute diffusion through different species of porin pores is mainly determined by differences in the pore sizes and hydrophilicity (and possibly the electrochemical natures) of the solutes.

A study in which mutant strains of *S. typhimurium* producing only 38K or 40K porin were used led to the conclusion that 38K and 40K porins form the most efficient pores for the diffusion of CER (15). Our work, however, clearly shows that 40K porin is practically inactive for the diffusion of CER. This discrepancy may be explained by the fact that the diffusion of CER observed with the mutant strain was mediated via the residual amounts of 39K porin (the most efficient porin), since the mutant strain produced a significant amount of 39K porin under the growth conditions employed (10, 16). The in vitro assay method described here clearly distinguishes small differences that might be undetectable in an experiment in which whole cells are used.

As shown in Fig. 3 and 4, 39K and B porins formed the most efficient pores for the diffusion of all cephalosporins tested so far, for reasons not clear at present. However, our earlier experiments suggested that the pore diameters of *S. typhimurium* porins are larger than those of *E. coli* K-12 porins (1). Of the cephalosporins tested, CEX diffused most readily through three species of porin pores from *E. coli*, possibly owing to an additional positive charge in the CEX molecule. OmpC porin pore was an exception. These results confirmed earlier observations with a black-lipid membrane (2). However, the situation is not always the same in the case of *S. typhimurium* porins, since 39K porin best allowed the diffusion of CER. The reason for the high efficiency of diffusion by porin pores of CER, which has the largest molecules of the three drugs tested, is not clear. However, the hydrophilic nature of the molecule (24) may contribute to high efficiency of diffusion.

Although this assay technique provided an easy and reliable way of determining the diffusion of cephalosporins across vesicle membranes in vitro, application of the method to the determination of diffusion of penicillin derivatives was limited, even if penicillinase is encapsulated in the intravesicular spaces, because penicillin derivatives seem to penetrate vesicle membranes lacking porin, probably diffusing through the lipid domain of the lipid bilayer. (The hydrolysis rate of penicillin G with vesicle membranes made of phospholipids (porin minus) was 40 to 50% of that of detergent-lysed vesicle membranes [T. Nakae, unpublished data].) Attempts have been made to reduce the hydrolysis rate of penicillin G with porin-lacking membranes, using a synthetic phospholipid with a higher melting point, distearoyl phosphatidyl choline (58°C mp); the result showed that the hydrolysis of penicillin G with vesicle membranes lacking porin was reduced to 25% of that of detergent-lysed liposomes (Y. Kobayashi and T. Nakae, unpublished data). Therefore, it should be possible to determine the diffusion of penicillin derivatives with liposomes made of such a lipid.

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