The Penetration of Antibiotics into the Antibiotic Resistant Bacterium Pseudomonas aeruginosa: Interaction of Polycationic Antibiotics at a Common Site on the Lipopolysaccharide

R.A. MOORE - R.E.W. HANCOCK

Pseudomonas aeruginosa is presently recognized as one of the leading causes of death from Gram-negative septicemia in North America. A major factor contributing to the success of this organism as an opportunistic pathogen is its intrinsic resistance towards antibiotics. We have proposed that the basis for this intrinsic antibiotic resistance is a low rate of antibiotic permeation across the outer membrane. This is apparently due to a low proportion of the major porin protein F (less than 1% of the 200,000 molecules of protein F per cell) forming open channels *in vivo*[6]. The self promoted pathway for antibiotic uptake has been proposed by our laboratory as an alternative means for polycationic antibiotics (e.g. aminoglycosides and polymyxins) to pass through the outer membrane of *P. aeruginosa* and (in the case of polymyxin B) other Gram-negative bacteria. Because this pathway offers an alternative route for antibiotic uptake, we believe that a close examination of its properties may yield valuable information which may be applied towards future antibiotic design.

Prior work in our laboratory has shown that the outer membrane of *P. aeruginosa* can be permeabilized by a variety of polycationic compounds[1,2,3]. Permeabilization by these compounds leads to enhanced passage across the outer membrane of a chromogenic β-lactam (nitrocefin)[1,3], a protein, lysozyme[1,3], and the hydrophobic fluorophor, N-phenyl-napthylamine (NPN)[3]. Since these polycationic agents render the outer membrane permeable to other compounds it seems reasonable that the uptake of polycationic antibiotics is accoplished by an initial permeabilization of the outer membrane by the antibiotic and subsequent diffusion through the resulting destablized outer membrane. Our model of permeabilization requires that the polycationic antibiotic must first bind to the Mg^{2+}-binding site of the LPS thus displacing Mg^{2+} and destroying the stabilizing effect of Mg^{2+} cross-bridging of adjacent LPS molecules (*Figure 1*). This model is supported by the observation that the Mg^{2+} chelator EDTA is capable of permeabilizing the outer membrane of *P. aeruginosa*[5] presumably by removing Mg^{2+} bound to LPS. In addition, a single point mutation which renders *P. aeruginosa* resistant to polymyxin B also renders the cell resistant to a variety of aminoglycosides and to the permeabilizing effects of EDTA[5].

To determine whether there is a common site for polycation binding to LPS, we measured the ability of various polycationic antibiotics, as well as Mg^{2+}, to compete for binding to LPS with a fluorescent derivative of the polycationic antibiotic polymyxin B sulfate. A fluorescente derivative of polymyxin B was synthesized by conjugating dansyl-Cl to polymyxin B sulfate. Binding of dansyl polymyxin was monitored by following the increase influorescence emission at 420 nm as dansyl polymyxin became bound to LPS[4]. The criteria for a binding site common to dansyl polymyxin and other polycations was a decrease in observed fluorescence upon addition of the competitor compound. A common binding site for Mg^{2+} and polymyxin B on LPS was suggested by the observation that addition of Mg^{2+} to a solution containing LPS and dansyl polymyxin resulted in the reduction of the fluorescence caused by dansyl polymyxin binding to LPS (*Figure 2*). Inhibition by Mg^{2+} of dansyl polymyxin binding to LPS was reversed upon addition of the Mg^{2+} chelator EDTA (*Figure 2*). As shown in *Table 1*, a variety of polycationic compounds were able to successfully compete with dansyl polymyxin for binding to LPS. The most effective of these competitors was poly-L-lysine. EDTA was not able to displace dansyl polymyxin bound to LPS although this compound is an effective permeabilizer of the outer membrane of *P. aeruginosa*[2]. Streptocmycin was a less effective competitor for binding to LPS than gentamicin, a result which reflected the relative abilities of these aminoglycosides to permeabilize outer membranes[1,3]. Nevertheless, all of the compounds able to effectively permeabilize the outer membrane of *P. aeruginosa* (as previously reported[4]) had an apparently higher affinity for LPS than did Mg^{2+} since lower concentrations were required for 50% inhibition of fluorescence (*Table 1*).

The results presented here provide evidence that aminoglycosides and other polycationic compounds function to permeabilize the outer membrane of *P. aeruginosa* via a mechanism which first involves binding of the compound to the Mg^{2+} bind-

Department of Microbiology, University of British Columbia, Vancouver V6T 1W5, British Columbia, Canada.

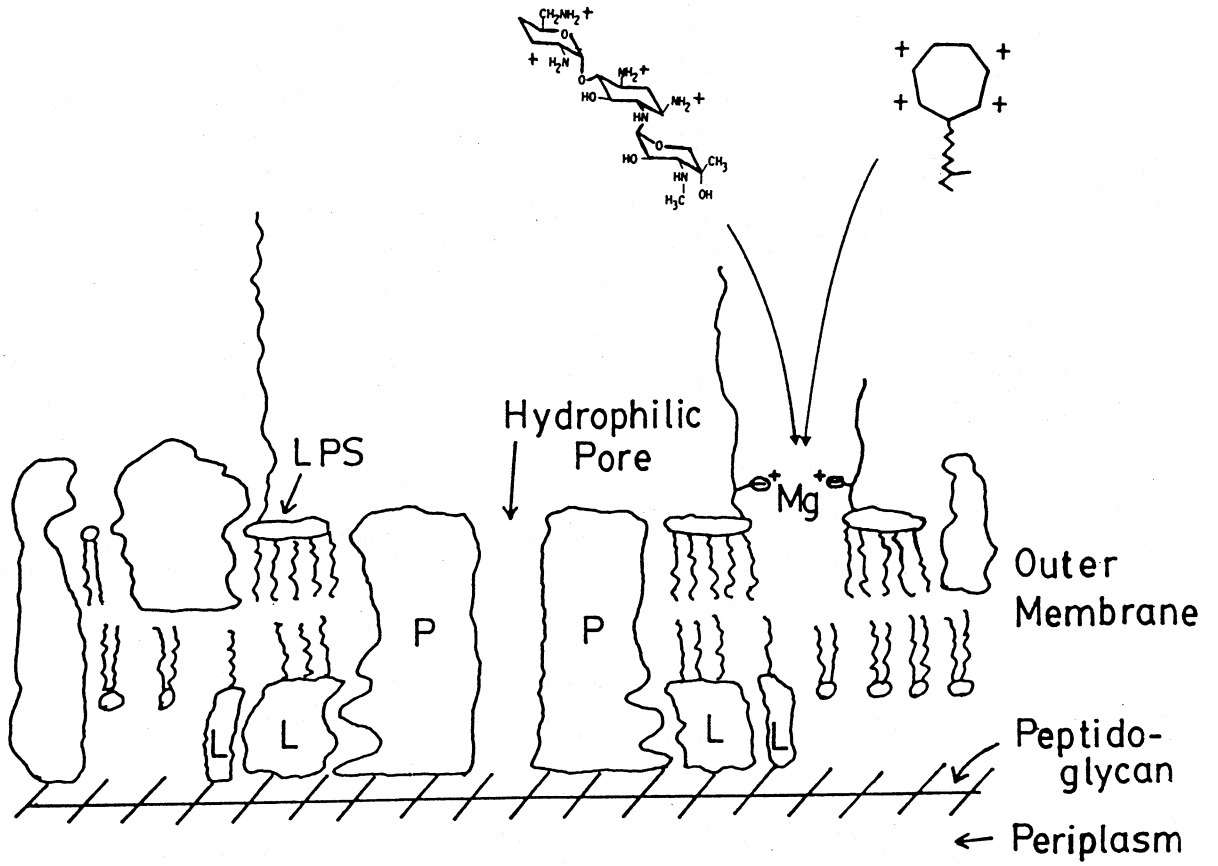

Figure 1 - A model for the self promoted uptake across the *Pseudomonas aeruginosa* outer membrane of polycationic aminoglycosides and polymyxins. Displacement of Mg^{2+} by the polycation leads to the disruption of Mg^{2+} crossbridging of LPS. Thus destabilized, the membrane is now permeable to a variety of compounds including lysozyme, β-lactam antibiotics, hydrophobic fluorescent compounds and presumably the polycations themselves. Other hydrophilic antibiotics probably pass through the outer membrane via the hydrophilic channels formed by porin proteins.

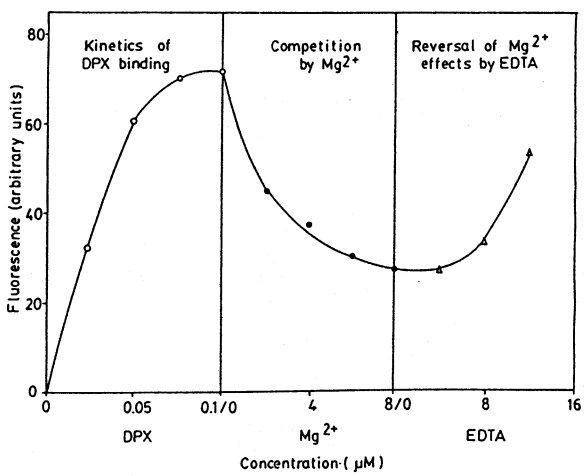

Figure 2 - Inhibition of dansyl polymyxin (DPX) binding to LPS by Mg^{2+} and reversal of inhibition by EDTA. LPS isolated from *P. aeruginosa* strain H103 was at a concentration of 1 μg/ml in Hepes buffer, 5 mM, pH 7.0. Dansyl polymyxin was added in aliquots to the final concentrations indicated on the X-axis and the fluorescence emission at 420 nm measured after each addition. After the final concentration reached 0.1 μM dansyl polymyxin, Mg^{2+} was added to the same cuvette in aliquots and the fluorescence emission determined after each addition. When the final concentration became 8 μM Mg^{2+}, EDTA was titrated in to the final concentrations indicated on the X axis.

TABLE 1 - *Inhibition of dansyl-polymyxin binding to LPS by various permeabilizers**

Permeabilizer	Concentration giving 50% inhibition of relative fluorescence (micromolar)
Mg^{2+}	870
Gramicidin S	525
Streptomycin	170
Gentamicin	12
Polymyxin B	3
Poly-L-Ornithine	0.03
Poly-L-Lysine	0.01

* LPS concentration was 1 microgram/ml (=0.083 micromolar) assuming a MW of 12.000. The dansyl-polymyxin concentration was 1.38 micromolar. Fluorescence was measured using a Perkin-Elmer 650-10S fluorescence spectrophotometer.

ing site of LPS. This data is therefore consistent with the self promoted uptake hypothesis.

ACKNOWLEDGMENTS - This work was financially supported by the Canadian Cystic Fibrosis Foundations.

REFERENCES

[1] HANCOCK R.E.W., RAFFLE V.J., NICAS T.I.: *Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in Pseudomonas aeruginosa.* Antimicrob. Agents Chemother., *19*, 777-785, 1981.

[2] HANCOCK R.E.W., WONG P.G.W.: *Compounds which increase the permeability of the Pseudomonas aeruginosa outer membrane.* Antimicrob. Agents Chemother., *26*, 48-52, 1984.

[3] LOH B., GRANT C., HANCOCK R.E.W.: *Use of the fluorescent probe 1-N-phenyl-napthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of Pseudomonas aeruginosa.* Antimicrob. Agents Chemother., *26*, 546-551, Oct. 1984.

[4] MOORE R.A., CHAN L., HANCOCK R.E.W.: *Evidence for two distinct mechanisms of resistance to polymyxin B in Pseudomonas aeruginosa.* Antimicrob. Agents Chemother., *26*, 539-545, Oct. 1984.

[5] NICAS T.I., HANCOCK R.E.W.: *Outer membrane protein Hl of Pseudomonas aeruginosa. Involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B and gentamicin.* J. Bacteriol., *143*, 872-878, 1980.

[6] NICAS T.I., HANCOCK R.E.W.: *Pseudomonas aeruginosa outer membrane permeability: Isolation of a porin protein F deficient mutant.* J. Bacteriol., *153*, 281-285, 1983.

Antibiotic Sensitivity in Lipopolysaccharide-Defective Mutants of Pseudomonas aeruginosa

P.M. MEADOW - P.L. WELLS

In the *Enterobacteriaceae*, the outer membrane forms a barrier that prevents many compounds from reaching the cytoplasmic membrane. Small hydrophilic compounds can diffuse freely through this barrier, but larger hydrophobic ones are excluded. Mutants of *Salmonella typhimurium* and of *Escherichia coli* that are sensitive to hydrophobic antibiotics have been isolated. The most common are deep rough (heptoseless) mutants[2], although others with apparently wild-type lipopolysaccharide (LPS) have been described recently[4]. In *Pseudomonas aeruginosa* where loss of the O-antigen is not associated with rough-smooth variation, LPS-defective mutants are harder to isolate and it has been difficult to identify its importance in response to antibiotics. The work described here was undertaken to determine whether the relative resistance of *P. aeruginosa* to antibiotics was related to LPS or to other outer membrane components. The strain selected for study was *P. aeruginosa* PACl which belongs to the 0:3 serotype. We aimed to isolate a series of LPS-defective mutants and to try to correlate changes in LPS and other outer membrane components in these mutants with antibiotic sensitivity.

Like the LPS of the *Enterobacteriaceae*, that of *P. aeruginosa* comprises three regions. The lipid A is similar except that β-hydroxymyristic acid is replaced by 10 and 12 carbon hydroxy acids. The polysaccharide portion can be divided into core, which is common or at least similar in all strains of *P. aeruginosa* and the side chains which are responsible for the heat-stable O-serotype. The repeating unit of the 0:3 strains consists of rhamnose, glucosamine, aminogalacturonic acid and bacillosamine, while the core contains rhamnose, glucose, galactosamine, alanine, heptose and KDO[3]. Following mutagenesis, a mutant of PACl defective in glucose metabolism was isolated and shown to lack the rhamnose of the core as well as the O-antigenic side chains. This mutant, PC556, was approximately ten times more sensitive than its parent strain to car-

Biochemistry Department, University College London, Gower Street, London WCIE 6BT, England.