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# Interaction of Polycationic Antibiotics with *Pseudomonas aeruginosa* Lipopolysaccharide and Lipid A Studied by Using Dansyl-Polymyxin

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**A fluorescent derivative of polymyxin B (dansyl-polymyxin) was used to study the interaction of polycations with lipopolysaccharide (LPS) and lipid A from *Pseudomonas aeruginosa*. Dansyl-polymyxin became bound to LPS and lipid A sites, including $Mg^{2+}$-binding sites, resulting in a 20-fold enhancement of fluorescence. A Hill plot of the binding data showed that the binding of dansyl-polymyxin to LPS was cooperative ($n$ = 1.98) and of high affinity ($S_{0.5}$ = 0.38 μM). The maximal binding capacity of LPS was approximately four molecules of dansyl-polymyxin per mol of LPS. The dansyl-polymyxin interaction with lipid A displayed similar kinetics ($n$ = 2.26; $S_{0.5}$ = 0.38 μM), and the maximal binding capacity was approximately 2 mol of dansyl-polymyxin per mol of lipid A. A variety of polycationic compounds, including gentamicin, streptomycin, and polymyxin B, as well as $Mg^{2+}$, were able to displace dansyl-polymyxin bound to LPS or to lipid A. Marked differences both in terms of the degree of displacement and in terms of the amount of competing polycation required to displace a given amount of dansyl-polymyxin were observed. Addition of excess polymyxin B resulted in displacement of all of the dansyl-polymyxin, demonstrating that only polymyxin-binding sites were being probed. Our data demonstrate that polymyxin B binds to multiple sites on LPS, including sites which bind aminoglycoside antibiotics and other polycationic compounds.**

*Pseudomonas aeruginosa* is presently recognized as one of the leading causes of death from gram-negative septicemia in North America (4). A major factor contributing to the success of this organism as an opportunistic pathogen is its intrinsic resistance to antibiotics. A proposed basis for antibiotic resistance in *P. aeruginosa* is the low rate of antibiotic permeation across the outer membrane due to the intrinsic barrier provided by the highly negatively charged lipopolysaccharide (LPS) and the poor functioning of the major porin protein F (16). Despite the major barrier presented by the outer membrane of *P. aeruginosa*, polycationic antibiotics like aminoglycosides are able to cross the membrane to some extent and have been used successfully in therapy (4).

While porin proteins provide one pathway for antibiotics to cross the outer membrane, workers in our laboratory have proposed the self-promoted pathway for antibiotic uptake as an alternative means for polycationic antibiotics (e.g., aminoglycosides and polymyxins) to pass through the outer membrane of *P. aeruginosa* (5, 6, 15). The results of previous studies of the interaction of polymyxin B with the outer membranes of other bacteria (20) were consistent with the proposal that polymyxin B uptake occurs via the self-promoted uptake pathway, as described for *P. aeruginosa* (5). The self-promoted uptake model requires that the polycationic antibiotic must first bind to LPS, resulting in the displacement of $Mg^{2+}$ and disruption of the stabilizing effect afforded by the $Mg^{2+}$ cross-bridging of adjacent LPS molecules in the outer membrane. The resultant permeabilized outer membrane then allows uptake of the interacting polycationic antibiotic; hence, the polycations promote their own uptake across the outer membrane. This model is supported by the facts that polymyxin B is known to interact with the LPS from *Salmonella typhimurium* (9) and that the $Mg^{2+}$ chelator EDTA is capable of permeabilizing the outer membrane of *P. aeruginosa* (15), presumably by removing $Mg^{2+}$ bound to LPS. To provide more direct evidence for this hypothesis, we examined the ability of a variety of polycationic compounds, including $Mg^{2+}$, to compete for binding to LPS with a fluorescent derivative of the polycationic antibiotic polymyxin B sulfate, dansyl-polymyxin, which was originally developed by Newton (14). Our results indicated that *P. aeruginosa* LPS has multiple binding sites for dansyl-polymyxin and that $Mg^{2+}$ and other polycations are able to displace dansyl-polymyxin from only some of these sites.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *P. aeruginosa* PAO1 strain H103 was used throughout this study; this strain has been described previously (15). The cells were grown in 1% Proteose Peptone no. 2 (Difco Laboratories, Detroit, Mich.) medium. Fresh medium was inoculated with an overnight culture at a final dilution of 1:20, and the resulting culture was grown with vigorous aeration at 37°C to an optical density at 600 nm of approximately 0.8.

**LPS and lipid A isolation.** LPS was isolated as described by Darveau and Hancock (3). The isolated LPS was extracted twice with an equal volume of chloroform-methanol (2:1) to remove the trace amounts of sodium dodecyl sulfate and phospholipids which resulted from the isolation procedure (3). The residual chloroform was removed by placing the extracted LPS in an evacuated desiccant chamber for about 1 h. The LPS level was determined by measuring the LPS-specific saccharide 2-keto-3-deoxyoctonate as described previously (18), except that we used 15 min of hydrolysis in $H_2SO_4$ and extraction of the chromophore in butanol-HCl (4:1). To calculate the molarity of LPS, we assumed that each *P. aeruginosa* LPS molecule contained two reactive 2-keto-3-deoxyoctonate residues. Lipid A (as a

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triethylamine salt) was obtained from isolated LPS by using mild acid hydrolysis as previously described (12) and was measured on a dry weight basis. To calculate the molarity of lipid A, we assumed a molecular weight of 1,926.

**Dansyl-polymyxin binding experiments.** Dansyl-polymyxin was prepared as described by Schindler and Teuber (20) and was quantitated by dinitrophenylation (1). The fluorescence of dansyl-polymyxin bound to LPS was measured by using a model 650-10S fluorescence spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) set at an excitation wavelength of 340 nm and an emission wavelength of 485 nm (13). Binding assays were performed by recording the fluorescence after the addition of portions of dansyl-polymyxin to cuvettes containing LPS (0.18 to 0.5 μM 2-keto-3-deoxyoctonate) in 1 ml of 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.0). The amount of dansyl-polymyxin bound to LPS was determined by using the following equation: amount of dansyl-polymyxin bound = $(f_0/F_{max}) \times$ concentration of dansyl-polymyxin in the cuvette, where $F_{max}$ was the level of fluorescence when all of the added dansyl-polymyxin was bound to LPS and was determined by measuring the fluorescence of a given concentration of dansyl-polymyxin in the presence of excess LPS (300 μg/ml) and $f_0$ was the observed fluorescence of the same concentration of dansyl-polymyxin when it was added to subsaturating LPS concentrations (1 to 3 μg/ml). The amount of dansyl-polymyxin free in solution was determined by subtracting the amount of dansyl-polymyxin from the total amount of dansyl-polymyxin added to a cuvette.

**Binding inhibition experiments.** Inhibitors of dansyl-polymyxin binding were titrated into cuvettes containing 3 μg of LPS or 0.63 μg of lipid A and 2 μM dansyl-polymyxin in 1 ml of 5 mM sodium HEPES buffer (pH 7.0), and the decrease in the observed fluorescence (percent inhibition) was recorded. The maximum inhibition by a given compound was calculated as the extrapolated *y* intercept of a plot of the reciprocal of percent inhibition as a function of the reciprocal of the inhibitor concentration. The *x* intercept gave the value for $-1/I_{50}$ ($I_{50}$ was the concentration which resulted in 50% of maximal inhibition at the LPS and dansyl-polymyxin concentrations used).

**Chemicals.** The chemicals used were of the highest quality commercially available, and most of the chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Terbium$^{3+}$ was obtained from ICN Pharmaceuticals Inc., K & K Lab Div., Plainview, N.Y., and HEPES buffer was obtained from Calbiochem-Behring Corp., La Jolla, Calif. Polymyxin B nonapeptide was prepared as described elsewhere (23).

## RESULTS

**Dansyl-polymyxin binding to LPS at an $Mg^{2+}$-binding site.** Previously (13), we reported that a fluorescent derivative of polymyxin B, dansyl-polymyxin, was able to bind to the outer surface of a polymyxin-resistant strain of *P. aeruginosa*, resulting in enhanced fluorescence of the dansyl group. Identical results were observed when whole cells of the parent strain were used (data not shown). In addition, dansyl-polymyxin bound to purified LPS (13), resulting in an enhancement of fluorescence of approximately 20-fold and a characteristic blue shift in the emission spectrum from 520 to 485 nm. These findings were confirmed in this study over the full range of dansyl-polymyxin concentrations which we used (Fig. 1). The emission peak of bound dansyl-polymyxin (485 nm) did not change over the entire range of the

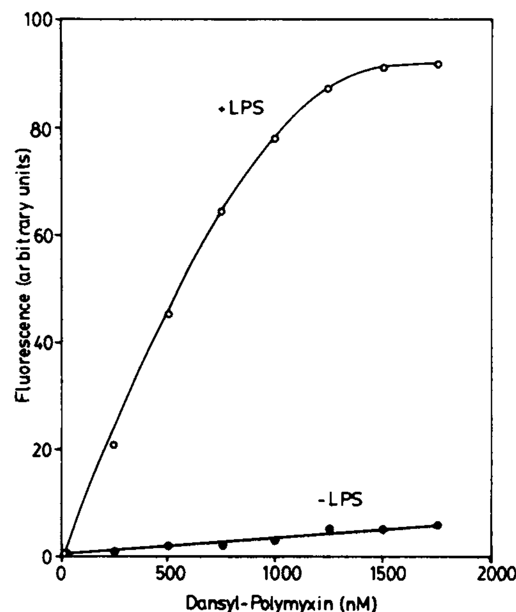


FIG. 1. LPS-dependent fluorescence of dansyl-polymyxin. Dansyl-polymyxin was titrated into a cuvette containing 1 ml of 5 mM HEPES buffer (pH 7.0) and 3 μg of *P. aeruginosa* LPS (○) or HEPES buffer alone (●).

dansyl-polymyxin–LPS titration curve (data not shown), which included conditions of both excess LPS and excess dansyl-polymyxin. In addition, there was no major change in the polarization of the fluorescence throughout the titration curve. These results indicate that the environment of the dansyl group of all bound dansyl-polymyxin molecules was similar. By measuring the increase in fluorescence at 485 nm, we were thus able to monitor the binding of dansyl-polymyxin to LPS and examine polycation-LPS interactions. Because we proposed that polycationic compounds interact at an $Mg^{2+}$-binding site on LPS (5, 6), we examined the ability of dansyl-polymyxin to compete with $Mg^{2+}$ for binding to LPS. When dansyl-polymyxin was titrated into a solution containing purified LPS, an increase in fluorescence was observed until all of the dansyl-polymyxin-binding sites on the LPS had been filled (Fig. 2A). A portion of the dansyl-polymyxin bound to LPS could be displaced by adding $Mg^{2+}$ ions to the same cuvette (Fig. 2B). This inhibition by $Mg^{2+}$ was reversed by subsequent addition of the $Mg^{2+}$ chelator EDTA (Fig. 2C), which preferentially removed $Mg^{2+}$, allowing dansyl-polymyxin to rebind to the LPS. The results of this experiment strongly suggested that dansyl-polymyxin was bound to an $Mg^{2+}$-binding site on the LPS molecule.

**Binding of dansyl-polymyxin to multiple sites on LPS.** Polymyxin B is an amphipathic molecule which possesses a charged cyclic heptapeptide head group with a tripeptide tail to which a hydrophobic fatty acid residue is attached. The presence of the highly positively charged peptide portion probably accounts, in part, for the ability of this molecule to bind to the LPS molecule as a result of charge-charge interactions with negatively charged residues on the LPS (2). A plot of the amount of bound dansyl-polymyxin as a function of the amount of dansyl-polymyxin added (Fig. 1) gave a curve which suggested that binding was saturated at higher concentrations. The data did not fit to a simple Scatchard plot (not shown), but fitted well to a Hill plot

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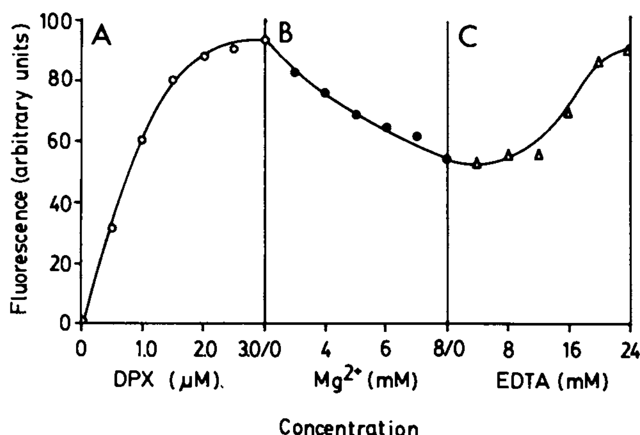


FIG. 2. Inhibition of dansyl-polymyxin binding to LPS by $Mg^{2+}$ and reversal of inhibition by EDTA. LPS isolated from *P. aeruginosa* strain H103 was present in a cuvette at a concentration of 1 μg/ml in 5 mM HEPES buffer (pH 7.0). Dansyl-polymyxin was added in portions to the final concentrations indicated on the *x* axis, and the fluorescence emission at 485 nm was measured after each addition. After a final concentration of 3.0 μM dansyl-polymyxin was reached, $MgCl_2$ was added to the same cuvette in portions, and the fluorescence emission was determined after each addition. When the final concentration of $Mg^{2+}$ was 8 mM, EDTA was titrated into the cuvette to the final concentrations indicated on the *x* axis. DPX, Dansyl-polymyxin.

(Fig. 3), suggesting that there was cooperative interaction of dansyl-polymyxin with LPS. The slope of this Hill plot ($n$), which was the Hill number and provided a minimal estimate of the number of interaction sites, was 1.98 (Table 1). The $x$ intercept ($S_{0.5}$), which provided a measure of the affinity of LPS for dansyl-polymyxin, was 0.38 μM (Table 1).

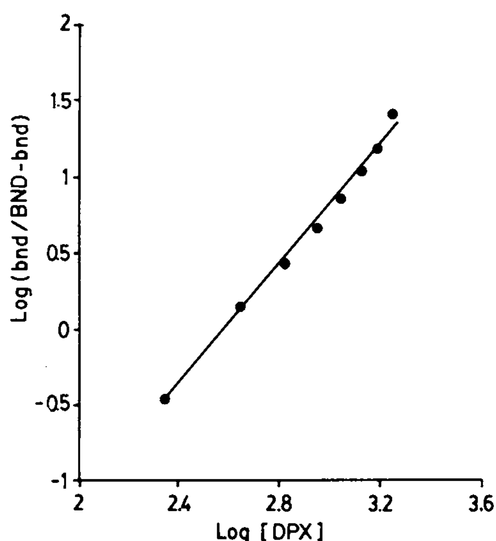


FIG. 3. Hill plot of dansyl-polymyxin binding to *P. aeruginosa* LPS. Dansyl-polymyxin was titrated into a solution containing LPS as described in the legend to Fig. 1. The amount of bound dansyl-polymyxin (bnd) was calculated as described in Materials and Methods. The maximum fluorescence (BND) was determined experimentally as the amount of fluorescence observed when the LPS was completely saturated with dansyl-polymyxin, as revealed by a lack of an increase in fluorescence upon further additions of dansyl-polymyxin. The data are the averages of four experiments. DPX, Dansyl-polymyxin.

TABLE 1. Kinetics of binding of dansyl-polymyxin to LPS and lipid A[a]

| Compound | $S_{0.5}$ (μM) | $n$ | Maximum amt of dansyl-polymyxin bound (mol/mol of LPS) |
|---|---|---|---|
| LPS | 0.38 | 1.98 | 4.36 |
| Lipid A | 0.38 | 2.26 | 1.98 |

[a] The results were derived from plots similar to those shown in Fig. 1 and 3. For each set of results, data obtained in three or four independent experiments were averaged prior to drawing Hill plots. Correlation coefficients of 0.99 were obtained by linear regression of the data points used for the Hill plots.

Demonstration of cooperative kinetics strongly suggested that more than a single site on each LPS molecule bound dansyl-polymyxin. Indeed, when saturating amounts of dansyl-polymyxin were added to a given amount of LPS (experiments were performed by using several different concentrations of LPS), a maximum of approximately four bound dansyl-polymyxin molecules per LPS molecule was observed (Table 1).

To determine which portion of the LPS molecule bound dansyl-polymyxin, lipid A was prepared from LPS by mild acid hydrolysis. Titration of lipid A with dansyl-polymyxin demonstrated similar kinetics ($n$ = 2.26; $S_{0.5}$ = 0.38 μM) (Table 1), but a maximum of only two bound dansyl-polymyxin molecules per LPS molecule was observed (Table 1).

**Inhibition of dansyl-polymyxin binding to LPS by polycations.** Hancock et al. (6, 7, 10) have proposed that polycationic compounds are able to disrupt the outer membrane permeability barrier by displacing the $Mg^{2+}$-binding site on LPS. We examined the interaction of these so-called permeabilizers (6) with LPS by measuring the ability of a variety of these compounds to compete with dansyl-polymyxin for binding of the LPS. Our data (Table 2) indicated that there was a marked difference in the abilities of the compounds to compete with dansyl-polymyxin for binding to LPS, both in terms of concentration and in terms of degree of competition. For example, polymyxin displaced 100% of the bound dansyl-polymyxin, while gentamicin displaced only 63% of the probe (Fig. 4). Of the compounds tested, only polymyxin B and the polyamino acids poly-L-lysine and poly-L-ornithine completely displaced bound

TABLE 2. Inhibition by polycations of dansyl-polymyxin binding to LPS and lipid A[a]

| Polycation | LPS | | Lipid A | |
|---|---|---|---|---|
| | $I_{50}$ (μM) | Maximal inhibition (%) | $I_{50}$ (μM) | Maximal inhibition (%) |
| Poly-L-lysine | 0.05 | 100 | 0.04 | 67 |
| Poly-L-ornithine | 0.64 | 100 | 0.48 | 77 |
| Polymyxin B | 2.2 | 100 | 3.9 | 100 |
| Terbium$^{3+}$ | 16.0 | 68 | 12.5 | 61 |
| Polymyxin B nonapeptide | 107 | 77 | 177 | 83 |
| Gentamicin | 590 | 63 | 1,340 | 75 |
| Streptomycin | 4,370 | 80 | 2,220 | 55 |
| $Mg^{2+}$ | 29,300 | 63 | 16,800 | 90 |

[a] The $I_{50}$ and maximal inhibition values were determined as described in Materials and Methods.

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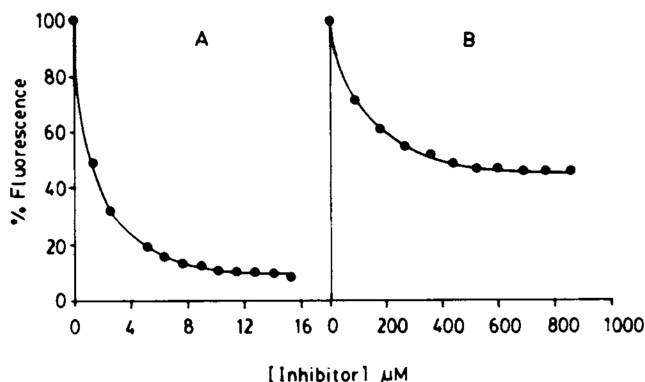


FIG. 4. Differential inhibition of dansyl-polymyxin binding to LPS by polymyxin B and gentamicin. Polymyxin B (A) or gentamicin (B) was titrated into a cuvette containing 3 µg of LPS and 2 µM dansyl-polymyxin, and the decrease in fluorescence was recorded. Maximum inhibition was calculated as described in Materials and Methods.

dansyl-polymyxin from LPS. Polymyxin B nonapeptide, a delipidated derivative of polymyxin B, displaced only 77% of the bound dansyl-polymyxin. Interestingly, the apparent affinity of polymyxin B nonapeptide for LPS and lipid A was markedly lower than the affinity of its parent compound, polymyxin B, suggesting that the fatty acyl tail of polymyxin B plays an important role in LPS binding.

Similar experiments were performed with lipid A (Table 2). The relative abilities of polycations to displace dansyl-polymyxin from lipid A reflected the abilities of the cations to displace dansyl-polymyxin from LPS, and, except in the case of gentamicin, the $I_{50}$ values obtained were never more than twofold different for a given compound.

## DISCUSSION

Our data suggest that dansyl-polymyxin is an excellent probe for the interaction of polymyxin B and other polycations with LPS. Dansyl-polymyxin bound to LPS could easily be distinguished from free dansyl-polymyxin on the basis of its high fluorescence (Fig. 1). Furthermore, dansyl-polymyxin bound only to LPS or lipid A sites that normally bind unmodified polymyxin B since it could be completely displaced from these sites by adding excess polymyxin B. The affinity of dansyl-polymyxin for LPS (and lipid A), as estimated from the $S_{0.5}$ value (0.38 µM) (Table 1), was almost identical to the affinity of *S. typhimurium* LPS for polymyxin B (0.3 µM), as measured by Schindler and Osborn in experiments in which the LPS itself was tagged fluorescently (19). However, we observed more than four molecules of dansyl-polymyxin bound per *P. aeruginosa* LPS molecule (Table 2), whereas only one or two binding sites per *S. typhimurium* molecule were observed in the study of Schindler and Osborn (19). Presumably, this difference may be due to the much higher levels of phosphate present in *P. aeruginosa* LPS (12 to 18 mol/mol of LPS) (9) than in *S. typhimurium* LPS (4 to 7 mol/mol of LPS) (19), since phosphate is the major putative cation-binding site in LPS (14, 19).

The kinetics of binding of dansyl-polymyxin to LPS and lipid A suggested that there was cooperative interaction (Table 1 and Fig. 3). There are two possible explanations for this. One is that there is classical positive cooperativity in that binding of dansyl-polymyxin to one site on LPS or lipid A enhances the binding of dansyl-polymyxin to another site. We do not favor this explanation since the displacement of dansyl-polymyxin by polymyxin (and other polycations) was noncooperative and could be fitted to classical, simple binding kinetics. The second possibility is that the addition of dansyl-polymyxin in low concentrations changes the structure of LPS aggregates to a form which is better able to interact with dansyl-polymyxin. The complex aggregation patterns of LPSs (2) and the ability of polymyxin at modest concentrations to disrupt these aggregates (11, 20) have been well documented for the LPSs of other bacteria. This possibility is attractive since the proposed cooperative interaction of dansyl-polymyxin with LPS would mirror the cooperative permeabilization of *P. aeruginosa* outer membranes by polymyxin (B. Loh and R. E. W. Hancock, unpublished data). Futhermore, it is almost certain that the interaction of polymyxin with the outer membrane of intact *P. aeruginosa* cells involves a disruption of the aggregate structure of LPS in the outer membrane since the outer monolayer of this membrane has been shown to bleb out as a result of polymyxin treatment (8, 11, 20).

The competitive displacement of dansyl-polymyxin by other polycations, including aminoglycosides (Table 2), strongly suggests that some or all of the sites which bind dansyl-polymyxin are also capable of binding these polycations. In general, the $I_{50}$, which provides a relative measure of the affinity of these sites for the different polycations, increased (i.e., the affinity decreased) as the valence of the ion decreased. The one exception to this general scheme was terbium, a bulky trivalent ion, which had a higher affinity for LPS and lipid A than polymyxin B nonapeptide, a pentavalent ion. Nevertheless, all of the polycations tested had substantially higher affinities for LPS than $Mg^{2+}$, which is the major cell envelope-associated cation in $Mg^{2+}$-grown cells (17). Furthermore, we observed a strong relationship between the ability of these polycations to permeabilize outer membranes (7) and their apparent affinity for LPS (Table 2). In addition, the demonstration that the aminoglycosides gentamicin and streptomycin can interact with the LPS is consistent with the self-promoted uptake hypothesis (5–7, 10).

The simple inhibition kinetics and 100% maximal inhibition observed by using polymyxin as an inhibitor (Fig. 4 and Table 2) suggest that the approximately four polymyxin-binding sites of *P. aeruginosa* LPS have equivalent affinities for polymyxin. The results of dansyl-polymyxin binding experiments in which lipid A was used were also consistent with this proposal (Table 1). In contrast, most other polycations were capable of displacing only 55 to 80% of the dansyl-polymyxin. The simplest explanation for this is that not all of the polymyxin-binding sites have equivalent affinities for these competing polycations. Such heterogeneity of LPS binding sites was predicted on the basis of certain anomalous data obtained during studies on the ability of different polycations to permeabilize outer membranes (7).

The data presented here localize at least two of the dansyl-polymyxin-binding sites to lipid A. The existence of such sites on lipid A is logical since, in addition to its positively charged groups which could interact with the negatively charged phosphates, dansyl-polymyxin also has a fatty acyl tail, which could interact with the lipid A fatty acyl region. Nevertheless, since polymyxin can displace 100% of the dansyl-polymyxin, it is clear that the dansyl group per se does not result in the "creation" of extra polymyxin-binding sites. Native LPS clearly binds more dansyl-polymyxin (Table 1), and since each LPS molecule contains a single lipid A moiety, there must be two or three other high-affinity

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polymyxin-binding sites in *P. aeruginosa* LPS in addition to those found on lipid A. The nature of these binding sites is unknown at present, but we speculate that they may be 2-keto-2-deoxyoctonate- or heptose-associated phosphate moieties. We are currently attempting to isolate and characterize mutants that are resistant to outer membrane-permeabilizing cations in an attempt to further characterize the polycation-binding sites.

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