Lipopolysaccharide Outer Core Is a Ligand for Corneal Cell Binding and Ingestion of *Pseudomonas aeruginosa*

Tanweer S. Zaidi, Suzanne M. J. Fleiszig, Michael J. Preston, Joanna B. Goldberg, and Gerald B. Pier

**Purpose.** *Pseudomonas aeruginosa* has been observed to be adherent to and inside epithelial cells during experimental corneal infection. The authors identified bacterial ligands involved in adherence and entry of *P. aeruginosa* into corneal epithelial cells.

**Methods.** In vitro gentamicin survival assays were used to determine the intracellular survival of a panel of *P. aeruginosa* mutants. Strains (10^6 to 10^7 colony-forming units) were added to primary cultures of rabbit corneal epithelial cells (~10^5/well) for 3 hours, nonadherent bacteria were washed away, and extracellular bacteria were killed with gentamicin. The antibiotic was then washed away, and epithelial cells were lysed with 0.5% Triton X-100 to release internalized bacteria. Bacterial association (sum of bound and internalized bacteria) was measured by the omission of gentamicin. Similar assays were carried out with whole mouse eyes in situ.

**Results.** A lipopolysaccharide core with an exposed terminal glucose residue was found to be necessary for maximal association and entry of *P. aeruginosa* into corneal cells. Bacterial pili and flagella were not involved. Mutants of *P. aeruginosa* strains that do not produce an LPS core with a terminal glucose residue had a significantly lower level of association with (~50%) and ingestion by (>90%, P < 0.01) corneal cells than did strains with this characteristic. Complementation of the LPS production defect by plasmid-borne DNA returned association and ingestion to near parental levels. Lipopolysaccharides and delipidated oligosaccharides with a terminal glucose residue in the core inhibited bacterial association and entry into corneal cells. Experiments using *P. aeruginosa* LPS mutants and corneal cells on whole mouse eyes confirmed the role of the LPS core in cellular entry.


*Pseudomonas aeruginosa* is the pathogen most commonly involved in bacterial keratitis associated with the use of contact lenses.1,2 This form of corneal infection is rapidly progressive, is difficult to treat, and can cause visual impairment.1,3,4 *P. aeruginosa* is generally considered an extracellular pathogen, but we have previously demonstrated that it is able to enter and survive inside corneal cells in culture and in experimentally infected mice.5,6 The entry into rabbit corneal cells during experimental contact lens-associated infection7 and after corneal injury also has been reported.8 These observations may explain why antibiotic therapy for *P. aeruginosa* keratitis is often ineffective. The ability to invade host cells is a virulence factor of some bacterial species, including those in the genera of *Salmonella*, *Shigella*, *Yersinia*, and *Neisseria*.9,10

The ingestion by corneal epithelial cells of *P. aeruginosa* is related closely to the organism's ability to attach to these cells.5 In addition, some strains of *P. aeruginosa*, such as the commonly used 19660 strain, are highly cytotoxic and readily kill corneal cells after attachment and ingestion, rendering any ingested bacteria susceptible to gentamicin killing.5 Therefore, ingestion of cytotoxic *P. aeruginosa* strains is not readily measured by the gentamicin-exclusion assay. Some re-
TABLE 1. Strains of *Pseudomonas aeruginosa* and Plasmids Used in This Study

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Description</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>PAO1</td>
<td>Wild-type strain, LPS-smooth, serogroup O5</td>
<td>19, 24</td>
</tr>
<tr>
<td>PAOB1</td>
<td>Elastase (<em>lasB</em>)-negative mutant, <em>lasB::Ω</em></td>
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<tr>
<td>AK1012</td>
<td>Derivative of PAO1 LPS-defective, complete-core plus 1 O side-chain repeat unit</td>
<td>19, 24</td>
</tr>
<tr>
<td>AK1012 algC::tet</td>
<td><em>algC</em> mutant of PAO1 LPS-rough, incomplete-core O side-chain deficient</td>
<td>15</td>
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<tr>
<td>PAC1R</td>
<td>Wild-type strain, LPS-smooth, serogroup O3</td>
<td>18</td>
</tr>
<tr>
<td>PAC557</td>
<td>LPS-defective, complete-core derivative of PAC1R O side-chain deficient</td>
<td>18</td>
</tr>
<tr>
<td>PAC611</td>
<td>Core-defective, O side-chain-expressing derivative of PAC1R</td>
<td>18</td>
</tr>
<tr>
<td>PAC605</td>
<td>LPS-defective, incomplete-core derivative of PAC1R O side-chain deficient</td>
<td>18</td>
</tr>
<tr>
<td>PAC1R algC::tet</td>
<td><em>algC</em> mutant of PAC1R, LPS-rough, incomplete-core O side-chain deficient</td>
<td>15</td>
</tr>
<tr>
<td>6294</td>
<td>Corneal isolate, serogroup O6</td>
<td>17</td>
</tr>
<tr>
<td>6487</td>
<td>Corneal isolate, serogroup O6</td>
<td>Bascom–Palmer Eye Institute (Miami)</td>
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<tr>
<td>PAK</td>
<td>Wild-type strain, LPS-smooth, serogroup O6</td>
<td>S. Lory, Seattle</td>
</tr>
<tr>
<td>PAK-<strong>fitC</strong>—</td>
<td>Nonmotile, mutation in flagellin gene, <em>fitC::Tn5G</em></td>
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<tr>
<td>PAK-<strong>fitA</strong>—</td>
<td>Nonmotile, mutation in <em>rpoF</em> (<em>fitA</em>) gene, <em>fitA::Tn5G</em></td>
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<td>PAK-NP</td>
<td>Nonadherent mutant, <em>pilA::tet</em></td>
<td>27</td>
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<tr>
<td>pLAFR1</td>
<td>Tc' broad-host-range plasmid</td>
<td>52</td>
</tr>
<tr>
<td>pLPS1 algC</td>
<td><em>algC</em> gene cloned in pLAFR1</td>
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</tr>
<tr>
<td>pLPS188 algC</td>
<td><em>algC</em> gene cloned in pUCP18</td>
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LPS = lipopolysaccharide.

Searchers have proposed that lipopolysaccharide (LPS), pili, or both mediate the adherence of *P. aeruginosa* to corneal tissue, binding specifically to gangliotetraosyl-ceramide (asialo GM1). Others, however, dispute whether asialo GM1 is present in the corneas of rabbits and humans. Whether pili, LPS, or asialo GM1 is involved in ingestion of *P. aeruginosa* by corneal cells is unknown.

To identify the *P. aeruginosa* ligand(s) involved in entry into corneal cells, we tested a variety of mutant strains for their ability to invade primary cultures of rabbit corneal epithelial cells. We found that the core oligosaccharide fraction of the bacterial LPS was a dominant bacterial ligand involved in corneal cell entry.

**METHODS**

**Preparation of Bacteria**

The bacterial strains and plasmids used in these experiments, along with their phenotypes and sources, are listed in Table 1. Bacteria were grown overnight at 37°C on a trypticase soy agar plate. The inoculum was prepared by resuspension of bacteria into Ham’s F-12 medium to an appropriate optical density. The actual colony-forming units (cfu) of bacteria added to a given assay was determined by conventional methods of diluting and plating cells for enumeration.

**Cell Cultures**

Primary cultures of rabbit epithelial cells were prepared as previously described. In brief, rabbit eyes were obtained from a commercial supplier (Pel Freeze Biologicals, Rogers, AR) and washed in saline; the corneas were excised and then rinsed in Hanks’ balanced salt solution. The epithelium was removed after treatment with Dispase II (Boehringer Mannheim, Indianapolis, IN) in SHEM medium. All reagents, apart from nutrient mixture Ham’s F-12 (HyClone Laboratories, Logan, UT) and Eagle’s minimal essential medium (Whittaker Bioproducts, Walkersville, MD), were obtained from Sigma Chemical (St. Louis, MO). Epithelial cells were seeded into tissue culture wells (diameter, 15 mm; Costar, Cambridge, MA) in the presence of SHEM medium and cultured at 37°C in 5% CO₂. When epithelial cells became 95% to 100% confluent, they were used in uptake experiments.

**Association and Ingestion Assays**

Cultured corneal epithelial cells (~10⁵/well) were incubated with varying numbers of cfu of *P. aeruginosa*
for 3 hours at 37°C in 5% CO₂; subsequent washing removed nonadherent bacteria. For quantifying (in cfu) bacterial association with the cells, Triton X-100 (0.5%) was added to wells to lyse epithelial cells, and the total number of P. aeruginosa organisms was determined by serial dilution and plating for bacterial enumeration. For measurement (in cfu) of the number of P. aeruginosa organisms invading cells, gentamicin (200 μg/ml) was added to cultures after completion of the association step to kill remaining extracellular bacteria. After 2 hours, cells were washed thoroughly to remove the antibiotic, and Triton X-100 (0.5%) was added to lyse the cells and release intracellular P. aeruginosa that survived antibiotic treatment. A viable count of the surviving (ingested) bacteria released from the cells was performed. In all assays, control wells were included wherein Triton X-100 was added to cells and was followed by 200 μg gentamicin/ml to insure that this level of antibiotic could kill all the bacteria. In addition, we determined that corneal cells did not take up a sufficient amount of gentamicin to kill 50 to 100 cfu of P. aeruginosa, which represented <0.001% of the gentamicin added to the wells.

Ingestion by Epithelial Cells on Intact Mouse Corneas

Corneal surfaces of C57/B16 mouse eyes were injured by scratching with a 26-gauge needle. All animals were treated in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The eyes were then enucleated and placed in a petri dish containing wet cotton. Each eye was inoculated with 10⁶ cfu of P. aeruginosa and was left at 37°C for 3 hours. After this incubation period, the corneas were washed extensively to remove nonadherent bacteria, and a viable count of the remaining adherent bacteria was performed after homogenization of some of the corneas in tryptic soy broth containing 0.5% Triton X-100. This count gave us the number of bacteria associated with the cornea (total of adherent and ingested bacteria).

In an assessment of the level of ingestion of P. aeruginosa, additional corneas were washed and then treated with gentamicin for 1 hour to kill extracellular bacteria. The antibiotic was washed away, the corneas were homogenized in 0.5% Triton X-100 (as described above), and a viable count was performed to determine the number of internalized bacteria.

Preparation of Lipopolysaccharide

Lipopolysaccharide was obtained from P. aeruginosa strains PAC557, which contains a complete LPS core but no O-side chains, and PAC1R algC::tet, a genetically derived strain that expresses an incomplete core oligosaccharide and no O-side chains on the LPS. After growth on agar plates for 24 hours, bacteria were suspended in 10% sodium lauryl sarcosine to lyse the cells, debris was removed by centrifugation at 20,000g for 15 minutes, and LPS was recovered from the supernatant by the addition of four volumes of 95% ethyl alcohol. After 18 hours at 4°C, the precipitate was collected by centrifugation and resuspended in PBS, and this solution was ultracentrifuged for 3 hours at 100,000g. The LPS pellet obtained from the ultracentrifugation was resuspended in PBS; it was digested with DNase and RNase (0.1 mg/ml) plus 4 μM CaCl₂ and 4 μM MgCl₂ overnight at 37°C and then with 0.1 mg of Pronase/ml for 2 hours at 56°C. The suspension was ultracentrifuged again to pellet the LPS. This material was redissolved in water and lyophilized.

Core oligosaccharide fragments lacking lipid A (delipidated LPS) were obtained by hydrolysis of LPS in 1% acetic acid for 3 hours at 95°C. The precipitated lipid A was removed and the supernatant neutralized for use in uptake assays. Inhibition of uptake by LPS and oligosaccharides was measured by the addition of the inhibitor to the ingestion assay mixture.

Statistical Analyses

Simple regression was used to analyze the interactions of bacterial inoculum (log transformed) with association and ingestion by epithelial cells. Analysis of variance (ANOVA) and the F-test were used to determine the significance level. Lines with slopes whose 99% confidence intervals (CI) did not overlap were considered significantly different. The 99% CI was used to account for multigroup comparisons (i.e., application of the Bonferroni correction to the significance level). Parametric statistics were used to analyze normally distributed data (either raw or log-transformed results), including the unpaired and paired t-test for two-group comparisons and ANOVA for comparisons of three or more groups. The Fisher PLSD statistic was used to determine which pairs of observations among groups of three or more observations were significantly different from each other at the 0.05 significance level. For these analyses, the Statview SE+ Graphic software program (Abacus Concepts, Berkeley, CA) was used on a Macintosh computer (Apple, Cupertino, CA).

RESULTS

Effect of Bacterial Inoculum on Adherence and Ingestion by Epithelial Cells In Vitro

Before studying the ability of mutant strains of P. aeruginosa derived from different parental backgrounds to invade rabbit corneal epithelial cells, we determined the effect of varying the bacterial inoculum on epithelial cell association and ingestion; the information enabled us to adjust the number of cfu of bacteria added to cells to achieve comparable levels of ingestion with different strains and to compare laboratory strains.
Pseudomonas aeruginosa LPS and Corneal Cell Ingestion

FIGURE 1. Effect of bacterial inoculum on Pseudomonas aeruginosa association with (upper lines) and ingestion by (lower lines) rabbit corneal epithelial cells in primary cultures. Each point represents the mean of three or four determinations, and error bars show the standard deviations. All slopes for ingestion and association had nonoverlapping 99% confidence intervals, indicating that they differ at a level of $P < 0.01$. Note that the range of the $x$-axis is different for strains PAK and PAC1R than for the other three strains.

(i.e., PAO1, PAC1R, and PAK) with low-passage clinical isolates from corneal infection (i.e., 6294 and 6487). Results are presented in Figure 1. When the log$_{10}$ of the inoculum was plotted against the log$_{10}$ of bacteria associated with and ingested by corneal cells, a dose-dependent increase in both parameters was noted for all five strains, with correlation coefficients ($R^2$ values) $\geq 0.942$. For all five strains, the slopes of the lines for association were steeper than the slopes of the lines for ingestion, and the two slopes differed significantly from each other (nonoverlapping 99% CI). In general, the number of cfu invading the cells was approximately 5% to 10% of the number associated with corneal cells, regardless of the inoculum used. The exception involved strain PAC1R, for which the figure was generally <1%. This strain had the second highest slope for association (0.111) but a very low slope for ingestion (0.0005), indicating strong association with but poor ingestion by corneal cells.

In all cases, it was clear that there was a direct relationship between the level of bacterial association with the cells and subsequent ingestion by the cells. Strains PAK and PAC1R were ingested poorly by corneal cells, with the lowest slopes for this measurement. These strains required a >10-fold higher inoculum to achieve the same level of uptake as occurred with the other three strains (Fig. 1). Strains PAO1, 6294, and 6487 differed significantly from each other in regard to epithelial cell entry, with nonoverlapping 99% CI for the slopes of the dose-response relationship of inoculum to ingestion. It is interesting that strains PAO1 and 6294 are highly infective in the murine corneal-scratch model of ulcerative keratitis, whereas strains PAK and PAC1R are not pathogenic in this model.
which is needed for the production of a complete LPS with isogenic derivatives expressing only a rough, partial-P. aeruginosa

The structure of the LPS cores of both its parental strain and isogenic strain PAC611 indicated that a terminal glucose residue linked to N-

ability to be ingested by rabbit corneal cells in vitro

Plasmid-borae Gene

Identification of the Lipopolysaccharide
Structure Needed for Corneal Cell Ingestion

The structure of the LPS cores of P. aeruginosa strains PAC1R and PAO1 have been determined (Fig. 2), and isogenic strains differing in the production of various parts of the LPS core have been derived,18,19 which would allow us to define precisely the LPS structure promoting association and invasion with corneal cells. In addition, we had available genetic constructs of strains PAC1R and PAO1 wherein the gene, algC, encoding tetracycline (tet) resistance into the coding part of this gene (strains PAC1R algC"• tet and PAO1 algC"• tet15). Assessment of these strains for their ability to inhibit corneal cell association and ingestion using Lipopolysaccharide and the Delipidated Core

Inhibition of Association and Ingestion Using Lipopolysaccharide and the Delipidated Core Oligosaccharide

The above results indicated that the exposed core oligosaccharide on LPS was the bacterial ligand used for corneal cell association and ingestion. To evaluate this hypothesis further, we tested the ability of intact LPS and delipidated core oligosaccharide from P. aeruginosa PAC557 to inhibit the ingestion by corneal cells of P. aeruginosa PAO1 in vitro. As shown in Figure 4, the addition of \( \geq 10 \mu g \) of intact LPS/ml or \( \geq 1 \mu g \) of delipidated LPS per milliliter to bacterial suspensions during the ingestion assay significantly inhibited uptake of P. aeruginosa by corneal cells (\( P \leq 0.028 \), ANOVA, repeated measures). Doses of 50 to 100 \( \mu g \) of intact or delipidated LPS of P. aeruginosa strain PAC557 per milliliter were optimal for this purpose.

Intact and delipidated LPS were next evaluated for their ability to inhibit corneal cell association and ingestion of wild-type, LPS-smooth strains of P. aeruginosa and of strains expressing an intact LPS core but devoid of O-side-chain expression. For a control, we used LPS isolated from P. aeruginosa strain PAC1R algC"• tet, which makes an incomplete LPS core. As shown in Figure 5, the addition of 100 \( \mu g \) of intact or delipidated core LPS from P. aeruginosa PAC557 per milliliter to bacterial suspensions during the association and ingestion assays significantly inhibited corneal cell association and uptake of P. aeruginosa strains (\( P < 0.01 \), ANOVA). In contrast, LPS and delipidated LPS from P. aeruginosa PAC1R algC"• tet (incompletemcore LPS) failed to inhibit significantly either association or uptake in vitro. To confirm that it is the core oligosaccharide portion of the LPS that promotes uptake of P. aeruginosa, we tried to inhibit ingestion with
purified, high molecular weight O-polysaccharide side chains from *P. aeruginosa* strains PACIR and PAO1. Neither of these preparations inhibited bacterial ingestion (data not shown).

**Role of Lipopolysaccharide Core Oligosaccharide in Adherence and Ingestion by Corneal Cells on Intact Mouse Eyes**

To correlate our in vitro findings with the pathophysiology in an intact eye, we studied association and ingestion by epithelial cells on the scratch-injured corneas of enucleated whole mouse eyes of *P. aeruginosa* strains expressing smooth, complete-core, or incomplete-core LPS. We used enucleated eyes because strains expressing LPS-core oligosaccharides in the absence of O antigens are killed rapidly by complement factors shortly after inoculation into the eye of a live animal.17 Using the enucleated intact eye, we confirmed that *P. aeruginosa* strains expressing a complete-core oligosaccharide adhered well to corneal tissues and were ingested by epithelial cells at a level comparable to that documented for LPS-smooth parental strains (Fig. 6). As in cell cultures, the strains expressing an incomplete LPS core had significantly reduced ingestion by epithelial cells on scratch-injured corneas of whole mouse eyes ($P \leq 0.02$, ANOVA). Interestingly, in these experiments, the association with intact corneal tissues of strain PAC605 expressing an incomplete LPS core was reduced, but not significantly, from the parental level. In addition, strain AK1401, expressing a complete-core oligosaccharide and a single O-antigen repeat, had a comparable level of association with the intact cornea, as did parental strain PAO1, but it had a significantly reduced level of entry into corneal cells compared with this strain. These results likely reflect a variable effect of LPS-core on adherence and entry of *P. aeruginosa* into mouse corneal cells because of multiple receptor–ligand interactions, differences between rabbit and mouse cells, and differences between in situ and in vitro situations.

**DISCUSSION**

The initial interaction of a bacterial pathogen with components on the mucosal surfaces of humans is a key part of the pathogenic process. The outcome of this interaction will determine whether infection leads to disease or to clearance of the pathogen. Many pathogens use an array of surface factors to attach to host tissues, including pili, flagella, matrix-binding proteins, nonpilus adhesins, and LPS oligosaccharides.26–30 The host counters with mucous secretions, glycoprotein barriers, phagocytes, immunoglobulins, and inhibitors of bacterial binding.31–33 In corneal infections caused by *P. aeruginosa*, pili and LPS have been reported to mediate bacterial adherence to corneal cells,12,34 but there is controversy about the epithelial cell receptor involved in adherence.35,36 Studies
in our laboratory\textsuperscript{5,6} during the past few years also have suggested that in addition to epithelial cell binding, uptake could be another important element in the pathogenesis of \textit{P. aeruginosa} corneal infections. To assess the hypothesis that bacterial ingestion by corneal epithelial cells is an important feature of the pathogenic process, we have been conducting a series of investigations to define bacterial and host factors involved in ingestion. In this report of our studies, using in vitro primary cultures of rabbit corneal epithelial cells and enucleated whole mouse eyes, we identify the outer core of the bacterial LPS as a ligand promoting binding to and ingestion by corneal cells of \textit{P. aeruginosa}. This finding is consistent with the reports of Fletcher et al\textsuperscript{11} and Gupta et al\textsuperscript{12} indicating a role for \textit{P. aeruginosa} LPS in adherence to corneal tissues. Gupta et al\textsuperscript{12} also proposed that the corneal cell receptor for \textit{P. aeruginosa} LPS is asialo GM\textsubscript{1}, which is thought to be a receptor for pathogenic bacteria in many host tissues.\textsuperscript{13,57,58} In addition, Gupta et al\textsuperscript{12} reported that \textit{P. aeruginosa} pili bind to asialo GM\textsubscript{1}; however, we found no role for pili in the ingestion of this organism by cultured rabbit corneal epithelial cells. We did note that bacterial uptake was much more dependent on outer-core LPS than was association (i.e., adherent plus ingested bacteria); thus, adherence probably involves bacterial ligands in addition to LPS.

Because the interaction of a bacterium with an epithelial cell is a complex process involving multiple receptor–ligand interactions, we first characterized the ability of five strains of \textit{P. aeruginosa} to adhere to and be ingested by cultured rabbit corneal epithelial cells. We found substantial differences among the five strains in the slopes of the lines relating increasing bacterial inoculum to epithelial cell association and ingestion. In general, however, association and ingestion were parallel phenomena: Those strains with larger slopes for association also had larger slopes for ingestion; the exception was strain PACIR, which had a slope for association approximately 10-fold higher than that of strain PAK, but it had a 10-fold lower slope for ingestion. This information enabled us to adjust the inocula of the different bacterial strains so that their levels of association and ingestion were comparable in studies with cultured rabbit corneal epithelial cells.

In spite of strain-related variability, the experiments reported here consistently showed that the outer-core oligosaccharide of \textit{P. aeruginosa} LPS is one of the ligands promoting bacterial association and ingestion in this system. In addition, the LPS outer-core
TABLE 2. Complementation of the Incomplete LPS Core Genetic Defect in *Pseudomonas aeruginosa* Strain AK1012 Restores Epithelial Cell Association and Ingestion

<table>
<thead>
<tr>
<th>Strain*</th>
<th>LPS Phenotype</th>
<th>cfu (± SEM) Associating</th>
<th>cfu (± SEM) Ingesting</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>Complete</td>
<td>2.8 x 10^5 ± 3.1 x 10^4</td>
<td>9.3 x 10^5 ± 1.8 x 10^5</td>
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<tr>
<td>PAO1algC::tet</td>
<td>Incomplete</td>
<td>6 x 10^5 ± 1.6 x 10^4†††</td>
<td>5.0 x 10^5 ± 1.4 x 10^5†††</td>
</tr>
<tr>
<td>AK1012(pLAFR1)</td>
<td>Incomplete</td>
<td>1.1 x 10^5 ± 4.0 x 10^4†††</td>
<td>6.0 x 10^5 ± 1.1 x 10^5†††</td>
</tr>
<tr>
<td>AK1012(pLPS1)</td>
<td>Complete</td>
<td>2.4 x 10^5 ± 4.1 x 10^4§</td>
<td>5.4 x 10^5 ± 2.7 x 10^5§</td>
</tr>
<tr>
<td>AK1012(pLPS188)</td>
<td>Complete</td>
<td>3.0 x 10^5 ± 3.5 x 10^4§</td>
<td>9.1 x 10^5 ± 1.6 x 10^5§</td>
</tr>
</tbody>
</table>

* Approximately 10^8 cfu of bacteria were added to each well.
† Strains PAO1 algC::tet and AK1012(pLAFR1) are significantly less well associated with and ingested by corneal epithelial cells than are strains with a complete LPS (strains PAO1, AK1012[pLPS1] and AK1012[pLPS188], *P* < 0.001, ANOVA; *P* < 0.01, Fisher PLSD for all pairwise comparisons).
‡ Strain PAO1 algC::tet had a significantly lower epithelial cell association, but not ingestion, than did strain AK1012(pLAFR1) (*P* < 0.01, Fisher PLSD).
§ The two complemented strains of AK1012 are not significantly different in their association and ingestion properties from the parental strain PAO1.

LPS = lipopolysaccharide; cfu = colony-forming unit.

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*Pseudomonas aeruginosa* LPS and Corneal Cell Ingestion

oligosaccharide is needed for full ingestion of *P. aeruginosa* by cells on enucleated mouse corneas. Using two series of isogenic strains of *P. aeruginosa* with well-characterized LPS-defective variants, 18,19,24 as well as genetically defined LPS-defective variants created by the insertion of a tetracycline-resistance cartridge into the algC gene, 15,20 we clearly showed that those strains expressing a complete outer-core oligosaccharide associated with and were ingested by cultured epithelial cells most efficiently. Complementation of the LPS-core synthesis defect in *P. aeruginosa* strain AK1012 by the cloned algC gene restored association with and ingestion to parental levels. Purified complete-core LPS and complete-core oligosaccharide inhibited association with and ingestion by cultured corneal cells of various *P. aeruginosa* strains. These findings parallel those found in studies 39 with respiratory epithelial cell cultures and are consistent with a role for lipooligosaccharide in the ingestion by cultured epithelial cells of *Neisseria gonorrhoeae*. 40

Unlike most gram-negative bacteria, *P. aeruginosa* produces an LPS with O-side chains attached to only 10% to 30% of the outer core residues—an arrangement that leaves the majority of the outer-core oligosaccharide exposed. 41,42 This arrangement likely accounts for the ability of the LPS outer-core structure of *P. aeruginosa* to serve as a ligand to epithelial cells. The nature of the receptor on the epithelial cell is not defined clearly; as noted above, controversy surrounds the report that asialo GM1 is present on corneal epithelial cells and serves as a receptor for *P. aeruginosa* LPS. 35,36 In addition, the tetrasaccharide portion of asialo GM1 could be present on the surface of a cell either as part of this glycolipid or as an oligosaccharide substituent on a glycoprotein. Thus, the exact nature of the epithelial cell target for *P. aeruginosa* LPS-mediated adherence requires further study.

The identification of the bacterial ligand promoting epithelial cell ingestion would normally lead to experiments that evaluate the ability of isogenic bacterial mutants that vary in their phenotypic expression of the ligand to cause disease in an appropriate setting. However, this approach is not feasible in this situation because *P. aeruginosa* strains expressing only an incomplete-core LPS are highly sensitive to the bactericidal effects of complement and, therefore, are

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![FIGURE 4. Inhibition of ingestion of *Pseudomonas aeruginosa* PAO1 by corneal cells in the presence of either intact complete-core lipopolysaccharide (LPS) or delipidated (95°C, 3 hours, 1% acetic acid) complete-core oligosaccharide isolated from *P. aeruginosa* strain PAC557. Points represent means, and error bars represent the standard errors. Doses of ≥ 10 µg of either intact or delipidated core LPS per milliliter significantly inhibit ingestion (P = 0.028 and 0.025, respectively, analysis of variance, repeated measures; P < 0.05, Fisher PLSD for pairwise comparisons of doses of 10, 50, and 100 µg of either inhibitor per milliliter with no inhibitor).](image-url)
Structure of inhibitor added
• None
B Complete core
& Incomplete core
cfu Ingested by epithelial cells
Strain: PAC1R PAC657 PAC1R PAC657 PAC1R PAC557 PAC1R PAC557
Inhibitor: Intact IPS Dellpldated LPS Intact LPS Dellpldated LPS
cfu associating with epithelial cells
800000
Strain: PAO1 AK1401 PAO1 AK1401 PAO1 AK1401 PAO1 AK1401
Inhibitor: Intact LPS Dellpldated LPS Intact LPS Dellpldated LPS
FIGURE 5. Inhibition of association with (left) and ingestion by (right) rabbit corneal cells of strains of Pseudomonas aeruginosa expressing either smooth lipopolysaccharide (LPS; PAC1R and PAO1) or complete-core LPS (PAC557 and AK1401): effect of addition to the assays of no inhibitor, intact LPS, or delipidated LPS. Bars represent the means, and error bars represent the standard errors. The strain used in each experiment and the type of inhibitor (intact or delipidated LPS) are shown on the x-axis of each graph. Analysis of variance was used to compare the number of colony-forming units (cfu) of P. aeruginosa associating with or invading corneal cells under the three circumstances (P ≤ 0.03). Pairwise comparisons among the three groups treated with inhibitors (whose structures are indicated in the legend) were all < 0.05 (Fisher’s PLSD statistic). The number of cfu of P. aeruginosa added varied from $10^6$ to $2 \times 10^7$ cfu/well of epithelial cells, depending on the strain and on whether association or ingestion was being measured.

FIGURE 6. Association and ingestion by corneal epithelial cells on scratch-injured, enucleated mouse eyes of isogenic lipopolysaccharide (LPS)-derivatives of Pseudomonas aeruginosa strains of the PAC1R series (upper graph) and the PAO1 series (lower graph). For both series of LPS derivatives, ingestion was significantly (P ≤ 0.04, ANOVA of log-transformed results) less for the incomplete-core strains compared with the LPS smooth strains. Only for the PAO1 series was association significantly (P < 0.002, ANOVA of log-transformed results) less for the strain expressing an incomplete-core LPS. Bars represent geometric means, and error bars represent the SEM. *Results are significantly different from the wild-type strain; † results are significantly different from the strain expressing a complete-core LPS. Corneas were inoculated with $\sim 10^8$ colony-forming units of each strain. ANOVA = analysis of variance.

these processes. Most bacterial interactions with mammalian cells are multifactorial, and multiple surface proteins and other structures logically would be expected to participate in this interaction. Numerous investigations have suggested that bacterial pili are prominent ligands for binding epithelial cells, whereas other studies have indicated that nonpili adhesins produced by P. aeruginosa also mediate bacterial binding to both cells and mucins. In the studies described here, epithelial cell ingestion of bacterial strains that lack a complete outer-core LPS generally was reduced by >90% from levels documented for isogenic wild-type strains, although we cannot exclude the possibility that, by altering the structure of

virtually avirulent in models of corneal infection. The same point applies to strains that express a complete-core LPS but lack O-side chains. In addition, because LPS-core oligosaccharides are involved in adherence of P. aeruginosa to corneal cells and because strategies that interrupt their production or activity would affect epithelial cell adherence as well as ingestion, it is difficult to determine the specific contribution of ingestion to pathogenesis. Thus, alternative strategies will be needed to illuminate the role of epithelial cell ingestion in the pathogenesis of P. aeruginosa corneal infection.

Although these results indicate that the LPS outer core of P. aeruginosa is a ligand for this organism’s binding to and ingestion by corneal cells, it is likely that other receptor–ligand interactions contribute to
Pseudomonas aeruginosa LPS and Corneal Cell Ingestion

the LPS on these strains, we also altered the expression or presentation of other prominent surface factors. The addition of purified complete-core LPS or oligosaccharide generally inhibited ingestion by approximately 50%, but we would not expect these types of experiments to achieve the same level of reduction in binding and ingestion as was achieved by use of bacterial mutant strains. Of note is the recent report⁶⁰ that N. gonorrhoeae requires a complete oligosaccharide substituent on lipid A to invade epithelial cells fully, yet the production of proteins conferring the opacity phenotype onto these bacteria is required for epithelial cell ingestion.⁶⁵ Taken together, these findings indicate that specific receptor–ligand interactions involving bacterial surface LPS carbohydrates and receptors on epithelial cells contribute to the initial encounter of a bacterial cell and a host mucosal surface. Further studies are required to determine how best to evaluate the importance of LPS outer core-mediated internalization of P. aeruginosa into epithelial cells in the pathogenesis of corneal infections. Such studies must account for the co-dependsyony of epithelial cell adherence with ingestion and the role of LPS O side chains in conferring serum resistance onto P. aeruginosa.

Key Words
bacterial pathogenesis, corneal ulceration, epithelial cell ingestion, lipopolysaccharide, Pseudomonas keratitis

References


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