A Live-Attenuated *Pseudomonas aeruginosa* Vaccine Elicits Outer Membrane Protein-Specific Active and Passive Protection against Corneal Infection

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Received 28 June 2005/Returned for modification 15 August 2005/Accepted 8 November 2005

*Pseudomonas aeruginosa* can cause sight-threatening corneal infections in humans, particularly those who wear contact lenses. We have previously shown that a live-attenuated *P. aeruginosa* vaccine given intranasally protected mice against acute lethal pneumonia in a lipopolysaccharide (LPS) serogroup-specific manner. In the current study, we evaluated the protective and therapeutic efficacies, as well as the target antigens, of this vaccine in a murine corneal infection model. C3H/HeN mice were nasally immunized with the vaccine (an *aroA* deletion mutant of strain PAO1, designated PAO1*ΔaroA*) or with *Escherichia coli* as a control and were challenged 3 weeks later by inoculating the scratch-injured cornea with *P. aeruginosa*. For passive prophylaxis and therapy, we utilized a serum raised in rabbits nasally immunized with PAO1*ΔaroA* or *E. coli*. Outcome measures included corneal pathology scores and, in some experiments, reductions in total and internalized bacterial CFU. We found that both active and passive immunization reduced corneal pathology scores after challenge with a variety of *P. aeruginosa* strains, including several serogroup-heterologous strains. Even when given therapeutically starting as late as 24 h after infection, the rabbit antiserum to PAO1*ΔaroA* was effective at reducing corneal pathology scores. Immunotherapy of established infections also reduced the numbers of total and internalized corneal *P. aeruginosa* bacteria. Experiments using absorbed sera showed that the protective antibodies are specific to outer membrane proteins. Thus, live-attenuated *P. aeruginosa* vaccines delivered nasally protect against corneal infections in mice and potentially can be used to prepare passive therapy reagents for the treatment of established *P. aeruginosa* corneal infections caused by diverse LPS serogroups.

*Pseudomonas aeruginosa* is the pathogen most frequently isolated in cases of contact lens-associated bacterial keratitis (1, 10, 38, 41), a rapidly progressive infection that can lead to permanent scarring of the cornea and loss of vision. Even when antibiotic treatment eradicates the offending pathogen, corneal damage from inflammation can still occur (24). Fluoroquinolones, a class of antibiotics frequently used for treatment of corneal infections in humans, are likely to become ineffective in the coming years judging from data on changing susceptibility patterns of *P. aeruginosa* corneal isolates (1, 44). These issues underscore the need for new vaccines or immunotherapies that might prevent or hasten resolution of this sight-threatening infection.

The development of vaccines for *P. aeruginosa* infections has been hindered by the complexity of the organism’s pathogenesis. Elaboration of a wide array of virulence factors and the propensity to infect many different tissues have made it difficult to determine which host immune effectors and which microbial factors need to be targeted for effective immunity (34). Antibodies directed to the serogroup-determining O antigen on the lipopolysaccharide (LPS) are highly protective in a number of different infection models, including keratitis (29), but production of an O antigen-based vaccine has been hindered by the extensive antigenic and chemical variation in these antigens combined with poor immunogenicity when multivalent vaccines have been formulated (5, 6, 26, 27).

We previously reported that nasal immunization of mice and rabbits with an unmarked *aroA* deletion mutant of the *P. aeruginosa* serogroup O2/O5 strain PAO1, designated PAO1*ΔaroA*, was safe and highly immunogenic and could protect mice against acute fatal pneumonia, although the protection did not extend beyond the O2/O5 serogroup strains (31, 33). In rats, nasal immunization has been reported to be the most efficacious route for protection against corneal infection (42). In the current study, we evaluated the protective efficacies against murine corneal infection of active and passive immunization with PAO1*ΔaroA* and of therapy with a rabbit antisera raised against PAO1*ΔaroA*. In striking contrast to the serogroup-specific protection seen in acute pneumonia, the PAO1*ΔaroA* vaccine was effective against corneal infections caused by diverse serogroups of *P. aeruginosa*. Additionally, the antiserum raised against PAO1*ΔaroA* was effective therapeutically even when started as late as 24 h after infection. Experiments using antisera absorbed with whole bacteria and outer membrane protein (OMP) preparations indicated that OMP antigens are the targets for the protective antibody in this setting.

**MATERIALS AND METHODS**

**Bacterial strains.** The strains used in this study are listed in Table 1.

**Active immunization.** Six- to eight-week-old C3H/HeN female mice were obtained from Charles River Laboratories (Wilmington, MA) and immunized by...
three intranasal (i.n.) applications of *P. aeruginosa* PAO1ΔaroA or *Escherichia coli* HB101 at 1-week intervals, as previously described (31). The *aroA* gene encodes the shikimate-pathway enzyme 5-enolpyruvylyshikimate 3-phosphate synthase, which is essential for biosynthesis of aromatic amino acids. Mice were anesthetized with ketamine and xylazine prior to i.n. immunization. The immunizing doses, all in 20-μl volumes, increased each week and were 10⁸ CFU per mouse the first week, 5 × 10⁹ CFU the second week, and 10⁹ CFU the third week. All animal experiments conformed to local and federal guidelines for the use of animals in research and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Preparation of antisera for passive immunotherapy.** New Zealand White rabbits (Millbrook Breeding Labs, Amherst, MA) were nasally immunized with PAO1ΔaroA or *E. coli* HB101 by initially using the same doses and schedule as for the mice. The immunizing doses for rabbits were given in volumes of 100 μl (50 μl per nostril). Starting 4 to 7 weeks after the third immunization, rabbits were given repeated i.n. doses of 10⁹ CFU every 2 to 4 weeks. Rabbits were anesthetized with a mixture of atropine, ketamine, and xylazine injected subcutaneously prior to each immunization. Sera for protection studies were obtained after a minimum of four boosting doses.

Antibodies in immune sera were absorbed by incubation with lyophilized *P. aeruginosa* strains (2 mg/ml) for 3 h at 4°C with gentle mixing by tumbling. Strains used for absorption included PAO1 (serogroup O2/O5), the parental strain of PAO1ΔaroA; 6294 (serogroup O6); 6354 (serogroup O8); AK44, an LPS-defective strain with no O antigen and a truncated outer core (see Table 1 for references). For the PAO1ΔaroA mutant, an LPS-defective strain with no O antigen and a truncated outer core; and the PAO1 strain Fisher IT-7 by overnight incubation at 4°C in 100 μl of phosphate-buffered saline. Following infection, eyes were anesthetized with a mixture of atropine, ketamine, and xylazine, and then eyes (one per mouse) were scratched and inoculated with the bacterial inoculum suspended in 5 μl of phosphate-buffered saline. Following infection, eyes were assigned a pathology score every 24 h according to the following scheme (3): 0, eye macroscopically identical to the uninfected contralateral control eye; 1, faint opacity partially covering the pupil; 2, dense opacity covering the pupil; 3, dense opacity covering the entire anterior segment; 4, perforation of the cornea, phthisis bulbi (shrinkage of the globe after inflammatory disease), or both. Generally, maximal pathology scores were observed 72 h after infection, so data for this time point are shown in all figures depicting corneal pathology scores except those involving therapy with serum given at 24, 48, and 72 h after infection, where pathology scores at 96 h after infection are listed.

For CFU determinations, mice were euthanized by carbon dioxide overdose 48 h after infection; then eyes were washed and either homogenized in trypsin soy broth containing Triton X-100 (0.5%) to enumerate total bacteria (adherent and internalized) or incubated with gentamicin (0.3 mg/ml) to kill extracellular bacteria, washed to remove the gentamicin, and homogenized in Triton X-100 to release intracellular bacteria for enumeration by serial dilution and plating, all as previously described (8, 46, 47). Control aliquots of cells were lysed in the presence of gentamicin and the lysates plated on trypsin soy agar to verify that intracellular bacteria were still susceptible to gentamicin at the concentration used.

**Preparation of OMPs for serum absorption.** A crude preparation of OMPs was isolated using published methods (39). Briefly, *P. aeruginosa* PAO1 or its *galU* mutant was grown to stationary phase at 37°C in chemically defined medium and harvested by centrifugation. The pellet was resuspended in water and broken by sonication. Unbroken cells were removed by centrifugation at 5,000 × g for 10 min. N-Laurylsarcosine, sodium salt (Sigma, St. Louis, MO), was added to the supernatant to a concentration of 2% and then incubated at 20°C for 30 min. The resultant suspension was centrifuged at 38,000 × g for 1 h. The membrane pellet was washed twice with water and then lyophilized. The protein content was estimated using the Bradford reagent. For absorption studies, 10 μg of dried OMP was added per ml of serum and allowed to absorb overnight at 4°C.

### Table 1. Strains used in this study

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<th>Strain</th>
<th>Description</th>
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<td>LPS-defective derivative of PAO1 (absent O antigen, incomplete core)</td>
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<td>AK44</td>
<td>LPS-defective derivative of PAO1 (absent O antigen, complete outer core)</td>
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<td>ExoU⁺ PAO1</td>
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RESULTS

Protection against corneal pathology by active and passive immunization. Active nasal immunization with PAO1 ΔaroA elicited a high level of protective efficacy against corneal pathology in P. aeruginosa-infected mice (Fig. 1A). The mice were fully protected against corneal pathology after challenge with the parental strain PAO1, its cytotoxic variant ExoU⁺ PAO1, and the serogroup-heterologous strains 6294 (serogroup O6) and 6206 (serogroup O11). Control mice were immunized with E. coli HB101. (B and C) Protective efficacy of passive immunization with a rabbit antiserum to PAO1 ΔaroA (or E. coli as a control) against corneal infection due to serogroup-homologous (B) and serogroup-heterologous (C) P. aeruginosa strains. Each data point represents one eye of one mouse. Corneal pathology scores are described in Materials and Methods. P values were obtained by the Kruskal-Wallis test with Dunn’s correction for pairwise comparisons in panel A and by the Mann-Whitney U test for panels B and C.

FIG. 1. (A) Protective efficacy of active nasal immunization with PAO1 ΔaroA against corneal infection due to the parental strain PAO1, its cytotoxic variant ExoU⁺ PAO1, and the serogroup-heterologous strains 6294 (serogroup O6) and 6206 (serogroup O11). Control mice were immunized with E. coli HB101. (B and C) Protective efficacy of passive immunization with a rabbit antiserum to PAO1 ΔaroA (or E. coli as a control) against corneal infection due to serogroup-homologous (B) and serogroup-heterologous (C) P. aeruginosa strains. Each data point represents one eye of one mouse. Corneal pathology scores are described in Materials and Methods. P values were obtained by the Kruskal-Wallis test with Dunn’s correction for pairwise comparisons in panel A and by the Mann-Whitney U test for panels B and C.

ExoU⁺ PAO1, which carries the ExoU cytotoxin and its chaperone on a plasmid and is far more virulent than the parental strain in the acute pneumonia model. For PAO1, the minimum 50% infectious dose (ID₅₀), defined by the proportion of mice achieving a pathology score of ≥2 in this model, was previously found to be 2.5 × 10⁵ CFU per eye, and the upper limit of the 95% confidence interval (CI) was found to be 2.5
× 10^5 CFU (28). Thus, we observed homologous protection at a dose 4,000-fold above the ID_{50}. No eye pathology was observed in the PAO1ΔaroA-immunized mice challenged with the invasive but noncytotoxic serogroup O6 strain 6294 (ID_{50}, 2 × 10^5 CFU; upper limit of the 95% CI, 4 × 10^5 CFU [29]). No significant protection was seen after challenge with the highly virulent cytotoxic serogroup O11 strain 6206 (ID_{50}, 3 × 10^5 CFU; upper limit of the 95% CI, 1 × 10^5 CFU [28]), although the lack of protection may have been due to the use of a very high challenge dose (16,000-fold above the ID_{50}). An uncorrected Kruskal-Wallis test suggested a trend toward protection (P = 0.12) even with this high challenge dose. It should be noted that the expression of challenge doses as multiples of the ID_{50} is problematic in the corneal infection model, since we have previously shown that similar numbers of bacteria are associated with the cornea when an inoculum of either 10^5 or 10^6 CFU is used, suggesting that there is a saturation point for infection (8). To test the duration of protection after active immunization, we challenged mice with strain 6294 (2 × 10^6 CFU) 7 weeks after the final immunization (4 weeks later than the timing of the challenge in the experiments described above) and again found significant (P < 0.05) protection (data not shown).

To ascertain whether passive immunization could be efficacious in reducing corneal pathology from P. aeruginosa infection, we administered to mice, via i.p. injection, antisera derived from rabbits nasally immunized with PAO1ΔaroA or E. coli HB101 as a control. Passive prophylaxis with a PAO1ΔaroA-specific antiserum was highly effective in preventing corneal infection due to serogroup-homologous strains (Fig. 1B) as well as serogroup-heterologous strains (Fig. 1C). There was high-level protection against the prototypic members of the O2/O5 serogroup (Fig. 1B), including strains with variable subtype epitopes (Fisher IT-3, Fisher IT-7, 170006, and 170007) as well as the cytotoxic variant of the parental strain (ExoU^+ PAO1). Remarkably, there was also significant protection against a number of serogroup-heterologous strains from diverse serogroups (Fig. 1C), including strains such as 6206 and 6077, which are cytotoxic due to expression of ExoU and, as noted above, are highly virulent in this model.

**Immunotherapy of established infection.** Because prophylactic administration of immune serum would not likely have clinical utility, we investigated the use of the rabbit antisera as therapy for established corneal infections. We first tested whether a single i.p. dose of antiserum at 4, 8, or 24 h after infection could ameliorate the corneal pathology by 72 h after infection with the parental strain PAO1 given as a challenge dose of 1.4 × 10^7 CFU per eye. The antiserum to PAO1ΔaroA given systemically as a single dose either 4 or 8 h after infection produced significant improvement in corneal pathology scores compared to the control antiserum, while protection was lost if the antiserum was given 24 h after infection (Fig. 2A). However, if additional doses were also given at 48 and 72 h after infection (i.e., doses at 24, 48, and 72 h after infection), therapeutic benefit was again seen, with significant reduction in corneal pathology scores not only after infection with the parental strain PAO1 (Fig. 2A) but also after infection with two LPS-heterologous strains, 6294 and 6077, and nearly significant (P = 0.06) benefit with a fourth heterologous strain, 6354 (Fig. 2B). In other experiments, a three-dose, early-therapy regimen

![FIG. 2. (A) Therapy of established corneal infections due to P. aeruginosa PAO1 using rabbit antiserum to PAO1ΔaroA (or E. coli as a control) administered at different times after infection. The inoculum was 1.4 × 10^7 CFU per eye. (B) Protective efficacy following therapeutic administration of rabbit antiserum to PAO1ΔaroA (or E. coli as a control) against corneal infection with heterologous strains. Antisera were given i.p. 24, 48, and 72 h after infection. (C) Protective efficacy of therapeutic administration of rabbit antiserum to PAO1ΔaroA (or E. coli as a control) against corneal infection with homologous and heterologous strains. Antisera were given i.p. 4, 8, and 24 h after infection. For all experiments, each data point represents one eye of one mouse. Corneal pathology scores are described in Materials and Methods. P values were obtained by the Mann-Whitney U test.](http://iai.asm.org/)
of PAO1ΔaroA-specific antiserum given at 4, 8, and 24 h after infection was effective as treatment for infection due to the parental strain PAO1 and its cytotoxic variant ExoU+ PAO1 as well as two heterologous strains, 6294 and 6354 (Fig. 2C). This therapeutic regimen (dosing at 4, 8, and 24 h) was studied with a limited number of strains due to its lack of clinical utility in light of doses starting so early after infection.

**Antibacterial effect of antiserum to P. aeruginosa**

**PAO1ΔaroA.** To assess whether the changes in corneal pathology scores after therapy were correlated with an antibacterial activity and whether the anti-PAO1ΔaroA serum changed the cellular uptake of bacteria during infection, we measured the total and intracellular CFU in infected eyes using gentamicin exclusion assays done 24 h after administration of therapeutic antiserum, which was 48 h after infection. In these studies, *E. coli* or PAO1ΔaroA antiserum was administered i.p. 24 h after infection with PAO1 (2 × 10^7 CFU per eye) or the heterologous strain 6294 (1 × 10^7 CFU per eye). As shown in Fig. 3, treatment of established corneal infection due to PAO1 with the PAO1ΔaroA antiserum led to roughly 1-log_10-lower total CFU and 2-log_10-lower internalized CFU than those for mice treated with the *E. coli* antiserum, and these differences were statistically significant (P < 0.01 by Mann-Whitney U tests). At this 48-h time point, corneal pathology scores were significantly lower in the anti-PAO1ΔaroA serum group (data not shown), even though pathology scores 72 h after infection were not significantly different in the experiments for which results are shown in Fig. 2A. Treatment of corneal infection due to the heterologous strain 6294 also produced significant reductions in both total and intracellular CFU compared to the control antiserum, and again the reductions in internalized CFU were of greater magnitude than those in total CFU (Fig. 3). The high levels of bacterial invasion noted in these experiments are consistent with our previous findings of 10 to 30% invasion 24 h after infection with strain 6294 in C57BL/6 mice (8, 47). These data suggest that the PAO1ΔaroA antiserum not only has an antibacterial effect but also inhibits internalization of the bacteria by the corneal epithelium, a process we have previously shown to be crucial for the pathogenesis of experimental *P. aeruginosa* corneal infections (46).

**Determination of protective antigens.** Having established that the PAO1ΔaroA antiserum had high levels of both prophylactic and therapeutic efficacy, we next sought to determine the protective antigen(s). Initial experiments evaluated the serogroup specificity of the protection to assess whether antibody to the LPS O antigen was an important immune effector, as we have found in the acute pneumonia model using this vaccine (33) and in the corneal infection model using monoclonal antibodies directed at the O antigen (29). Absorption of the PAO1ΔaroA antiserum with either the parental strain PAO1, the O antigen-deficient strain AK44, or the vaccine serogroup-heterologous strain 6294 (serogroup O6) or 6354 (serogroup O8) significantly diminished the prophylactic efficacy against corneal infection due to PAO1 or 6294 (Fig. 4A). Absorption of the PAO1ΔaroA antiserum with *E. coli* HB101 followed by infection with 6294 had no effect on prophylactic efficacy: corneal pathology scores were identical to those achieved by passive administration of the unabsorbed PAO1ΔaroA antiserum (not shown). To confirm further that the protective activity is not removed by absorption with O antigen, we absorbed the PAO1ΔaroA antiserum with purified O antigen from the serogroup O2/O5 strain Fisher IT-7. The O antigen from this strain is known to be highly cross-reactive with that of PAO1. Following absorption, titers of IgG to the purified PAO1 O antigen, as measured by enzyme-linked immunosorbent assay, were the same as those in the *E. coli*-specific antiserum (data not shown). As shown in Fig. 4C, removal of the O antigen-specific IgG in the PAO1ΔaroA antiserum did not alter the passive protective efficacy against strain 6294. Based on these results, the O antigen was not the protective antigen contained in the PAO1ΔaroA vaccine.

To evaluate whether the protective antigen(s) was a protein or a nonprotein entity, we absorbed the PAO1ΔaroA antiserum with protease- or heat-treated PAO1 and tested the prophylactic efficacies of these manipulated sera against corneal infection with strain 6294 (serogroup O6) or 6487 (serogroup O8). As shown in Fig. 4B, sera absorbed with protease-treated PAO1 had protective efficacy similar to that of unabsorbed sera, suggesting that the protective antigen(s) is protease sensitive. Heat treatment, however, did not destroy the antigen(s), as protection was lost when the PAO1ΔaroA antiserum was absorbed with heat-treated PAO1 (Fig. 4B). Taken together, these results suggested that the target antigens could be epitopes on heat-stable proteins or protein-containing molecules such as lipoproteins.

To clarify further the targets of the protective antibody elicited by the PAO1ΔaroA vaccine, we assessed whether antibody to the LPS outer core mediated protection. We hypothesized that such outer-core-specific antibodies might prevent binding of *P. aeruginosa* to the cystic fibrosis transmembrane conductance regulator on corneal epithelial cells, a critical step in the pathogenesis of *P. aeruginosa* corneal infection (46). We therefore absorbed the PAO1ΔaroA antiserum with a galU mutant.
of PAO1 that expresses an incomplete LPS core and no O antigen (7). This absorbed serum, which should retain antibody to the outer core, was not protective against corneal infection with strain 6294 (Fig. 5A). Thus, antibody to the outer LPS core did not play a role in protection.

We next tested whether the target antigen(s) resided in the OMP fraction. We used OMPs prepared from the galU mutant of PAO1 to avoid any contamination from LPS O antigen, which we initially thought might play a protective role based on our prior studies using monoclonal antibodies to LPS (29). As shown in Fig. 5B, absorption of the PAO1ΔaroA antiserum with OMPs from the PAO1 galU mutant abrogated the protective efficacy against infection with strain 6294, indicating that the target antigen or antigens reside in the outer membrane fraction. Importantly, as noted above absorption of the PAO1ΔaroA antiserum with E. coli HB101 did not change the protection against strain 6294, suggesting that the target epitopes are specific to P. aeruginosa and are not shared with E. coli.

DISCUSSION

The current studies show that active immunization with a live-attenuated aroA deletion mutant of P. aeruginosa strain PAO1 or administration of a rabbit antiserum raised against this strain can effectively prevent murine corneal infections caused by a number of P. aeruginosa strains of heterologous LPS serogroups and, in the case of the rabbit antiserum, can even treat these infections. In keeping with the lack of serogroup specificity, the results indicate that heat-stable, protease-sensitive antigens within the bacterial outer membrane are the targets of the protective antibody.

What is perhaps most remarkable about these findings is the striking contrast with our results using this vaccine in the setting of acute fatal pneumonia, in which serogroup-heterologous protection was not observed after either active or passive immunization (33). In fact, in those pneumonia experiments, passive immunization with rabbit IgG derived from the same
PAO1ΔaroA antisera used in the current studies protected against challenge only by ExoU+ PAO1 and not by PAO1 itself, likely due to the much higher challenge doses needed for lethality with the noncytotoxic strain PAO1. Lack of protection against PAO1-induced fatal pneumonia was seen despite the presence in the recipient mice of high levels of opsonic serum antibody at titers similar to the protective levels observed after active nasal immunization (33). We had shown in prior studies that the opsonic antibody in this rabbit antisera raised against PAO1ΔaroA is directed primarily against the LPS O antigen (31). Thus, while antibody to the O antigen appears to be the most important immune effector engendered by this live-attenuated vaccine in the setting of pneumonia, surprisingly it is not critical for protection against corneal infection.

One basis for this difference may be the lower doses of *P. aeruginosa* required in the corneal infection model than in the pneumonia model. Most strains of *P. aeruginosa* cause significant pathology in mouse eyes at doses of $<10^7$ CFU/eye, whereas significant lung infections generally require higher doses for most strains. Furthermore, we have shown that within 15 min of application of *P. aeruginosa* to the cornea of a scratch-injured mouse eye, 90% of the inoculum is undetectable, likely due to local defenses such as entrapment within the tear film and antimicrobial peptide activity (28). Thus, effective inocula of *P. aeruginosa* leading to highly significant corneal pathology likely range from $10^4$ to $10^5$ CFU/eye, when infecting doses of $10^3$ to $10^4$ CFU/eye are applied (28). Also, in the current studies we found that in the eyes of mice examined 48 h after infection and treated with the control antisera there were about $2 \times 10^7$ CFU/eye, a lower level of tissue infection than occurs in the lungs of nonimmune mice (31). Under these circumstances an antibody with less overall effective activity, such as those directed to OMP antigens, could show prophylactic and therapeutic benefits comparable to that of an antibody specific to LPS O antigens, which is effective at protecting against higher challenge doses or higher levels of bacteria that can be found in infected lung tissues.

The lack of essentiality of antibody to the O antigen in the setting of corneal infection is, in one sense, a beneficial situation, since protection against multiple serogroups of *P. aeruginosa* can potentially be attained with a single vaccine. While other studies of corneal (19), lung (25), and burn wound (22, 37) infections have shown significantly diminished potency of non-LPS-based vaccines compared with LPS-based vaccines, we observed protection against challenge doses well above the ID$_{50}$ of some strains in the current studies. These findings do not contradict prior results from our group (29) and others (19) showing strong protective efficacy of LPS O antigen-specific antibodies against *P. aeruginosa* corneal infection. In our prior studies (29), only LPS-specific immunity was evaluated, primarily using monoclonal antibodies. In serogroup-homologous challenge experiments, these antibodies were quite effective. Broad protection engendered by the PAO1ΔaroA vaccine in the current experiments suggests that when immunization elicits antibodies to a variety of surface antigens, they likely can synergize to diminish pathological consequences from *P. aeruginosa* corneal infection in a manner analogous to that of antibodies highly specific to LPS O antigens.

Vaccination for experimental corneal infections using whole-cell vaccines has had a somewhat checkered past. Early studies of *P. aeruginosa* keratitis in rats following immunization with phenol-killed *P. aeruginosa* suggested that prior immunization might lead to worse disease upon subsequent challenge (43). In those experiments, the unimmunized rats had bacteria and neutrophils in their corneas but little stromal degradation, while the immunized rats had no bacteria but massive corneal damage. Thus, active immunization strategies might stimulate antibody-mediated or T-cell-orchestrated corneal damage. Indeed, the host’s immune response during bacterial keratitis has been shown to play a critical role in the pathogenesis of the disease, perhaps more so than in any other infected tissue. Neutrophils have been found to migrate into the corneal stroma early after the onset of infection (16, 18). While the neutrophils are required for bacterial killing and removal from the tissue, their presence contributed to corneal damage in some studies (43). T cells, particularly T helper type 1 (Th1) cells, have also been shown to play a role in the pathogenesis of corneal infections (14). In the absence of T-cell infiltration into the cornea, neutrophils did not persist and stromal destruction was diminished (15).

More recently, however, and despite these potential issues, protective efficacy using active immunization was achieved in the studies of Thakur and coworkers (42), who evaluated various routes of immunization of rats with paraformaldehyde-killed *P. aeruginosa*. They found that, compared with oral and intra-Peyer’s patch routes, nasal immunization resulted in the fastest bacterial clearance. In comparison with unimmunized rats, polymorphonuclear leukocyte (PMN) infiltration in the eyes of immunized rats was higher early during the infection, while at later points the number of PMNs diminished in immunized rats but continued to increase in the unimmunized group (42).

In addition to whole-cell vaccines, a number of studies have evaluated other *P. aeruginosa* secreted and surface proteins as vaccine candidates for corneal infections. Rabbits immunized with purified protease preparations from *P. aeruginosa* using active or passive approaches were protected from corneal damage (19). Active, passive, or topical immunization of mice with purified *P. aeruginosa* flagella was also found to be beneficial in experimental keratitis (36). The same group used purified pili as a candidate vaccine and reported that immunization with pili heterologous to the infecting bacterial strain failed to protect against corneal infection despite the fact that heterologous pili were effective at decreasing bacterial binding to the cornea in vitro (35).

The ability of antibody to antigens in *P. aeruginosa* OMP preparations to protect against experimental corneal infections has been described by other laboratories. Moon and coworkers found that prophylactic intravenous administration of monoclonal antibodies directed against OMP H2 and/or F (also known as OprF) was protective against corneal damage (23). Only a single challenge strain was used in those studies, so the broadness of protection was not evaluated. Immunization with recombinant OprF or OprF peptides has been shown to elicit protective immune responses in a number of infection models other than keratitis (11, 30, 40) and has been studied in humans as well (21). Crystal and coworkers recently reported the use of an adenovirus vector expressing an OprF peptide in the viral capsid as a subcutaneously delivered vaccine against *P. aeruginosa* pneumonia by using the agar-bead model of murine
pneumonia to challenge the immunized mice (45). The OprF peptide used in those studies stimulated both specific antibody and T-cell responses. We are currently defining the specific antigens recognized by the PAO1ΔaroA antiseraum and will determine whether antibodies to OprF, or to this and many other OMPs (which we would expect to see elicited by immunization with a live-attenuated vaccine), are the critical target antigens.

The current study suggests that live-attenuated P. aeruginosa vaccines could be used to prepare passive therapy reagents that might be employed in the treatment of established P. aeruginosa corneal infections. The efficacy of postinfection treatment described here is important, because the clinical utility of prophylactic therapy or active immunization is questionable. As existing antimicrobials become less effective due to microbial resistance, and with few new gram-negative-specific antibiotics, phylactic therapy or active immunization is questionable. As existing antimicrobials become less effective due to microbial resistance, and with few new gram-negative-specific antibiotics, phylactic therapy or active immunization is questionable. As existing antimicrobials become less effective due to microbial resistance, and with few new gram-negative-specific antibiotics, phylactic therapy or active immunization is questionable.

A hideous mutation in a PA103 (serogroup O11) strain was shown to be required for a complete lipopolysaccharide core and repairs a secondary species. The current study suggests that live-attenuated vaccines could be used to prepare passive therapy reagents that might be employed in the treatment of established P. aeruginosa corneal infections. The efficacy of postinfection treatment described here is important, because the clinical utility of prophylactic therapy or active immunization is questionable. As existing antimicrobials become less effective due to microbial resistance, and with few new gram-negative-specific antibiotics, phylactic therapy or active immunization is questionable. As existing antimicrobials become less effective due to microbial resistance, and with few new gram-negative-specific antibiotics, phylactic therapy or active immunization is questionable. As existing antimicrobials become less effective due to microbial resistance, and with few new gram-negative-specific antibiotics, phylactic therapy or active immunization is questionable. As existing antimicrobials become less effective due to microbial resistance, and with few new gram-negative-specific antibiotics, phylactic therapy or active immunization is questionable. As existing antimicrobials become less effective due to microbial resistance, and with few new gram-negative-specific antibiotics, phylactic therapy or active immunization is questionable.


