Vaccination against *Pseudomonas aeruginosa* Pneumonia in Immunocompromised Mice

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Immunocompromised patients are highly susceptible to infection with *Pseudomonas aeruginosa*. Our laboratory previously showed that intranasal administration of an attenuated *Salmonella* strain expressing the *P. aeruginosa* lipopolysaccharide O antigen was effective in clearing bacteria and preventing mortality in wild-type mice after intranasal challenge. We were interested in investigating the efficacy of this vaccine strategy in immunocompromised mice. Mice rendered leukopenic or neutropenic by intraperitoneal treatment with cyclophosphamide (Cy) or RB6-8CS5 antibody, respectively, were more susceptible to *P. aeruginosa* pneumonia than their nontreated counterparts, demonstrating 50% lethal doses several logs lower than that in wild-type mice. This hypersusceptibility was also associated with bacterial dissemination to the liver and spleen and increased lung permeability in Cy mice. Vaccination of the mice prior to treatment resulted in better survival and lower bacterial loads compared to vector-immunized mice. Although the treatments had no effect on antibody titers, this level of protection was still lower than that seen in untreated vaccinated mice. Administration of antibodies directly to the site of infection at the time of bacterial delivery prolonged survival and lowered bacterial loads in the immunocompromised mice. These results demonstrate the importance of white blood cells while still suggesting a critical role for antibodies in protection against *P. aeruginosa* infection.

*Pseudomonas aeruginosa* is an important opportunistic pathogen that is associated with infections in cystic fibrosis patients, burn patients, patients on ventilators, and contact lens wearers. Another important infection group consists of immunocompromised individuals, such as those undergoing chemotherapy or AIDS patients (11, 14, 17). Infections in these patient populations are difficult to treat, partly due to the innate antibiotic resistance of *P. aeruginosa* and partly due to the immunocompromised status of the patient. A particular problem associated with pneumonia in immunocompromised patients is bacteremia, which allows the bacteria to spread to other organs (11, 14, 17).

Recently, host factors contributing to dissemination of *P. aeruginosa* were investigated with a gastrointestinal model of colonization leading to infection (12). Mice previously infected with *P. aeruginosa* in the intestinal tract and then rendered neutropenic had increased dissemination and mortality. Two methods of inducing neutropenia were used in that study (12): treatment with cyclophosphamide (Cy) and treatment with the RB6-8CS5 antibody specific for the Ly6 antigen on the surface of neutrophils (9). Neutropenic mice were also used by Vance et al. (24) as a model to monitor dissemination of type III secretion system (TTSS) mutants of *P. aeruginosa*.

Previous work in our laboratory characterized a vaccine that confers serotype-specific protection against *P. aeruginosa* challenge (5). The vaccine consists of *Salmonella enterica* serovar Typhimurium strain SL3261, an attenuated *aroA* mutant (10), expressing the entire O antigen locus from a *P. aeruginosa* serogroup O11 strain (18). Intranasal vaccination with this *Salmonella* strain conferred complete protection in mice with challenge doses of five times the 50% lethal dose (LD$_{50}$) of both cytotoxic and noncytotoxic *P. aeruginosa* serogroup O11 strains. Administration of antibodies from vaccinated mice directly into the nasal passageway and lungs of infected mice was also able to confer protection when administered up to 6 h after infection (5). Here we investigate the efficacy of this live, attenuated vaccine in protecting leukopenic and neutropenic mice from *P. aeruginosa* infection.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** For challenge, the noncytotoxic *P. aeruginosa* strain 9882-80 (2) or cytotoxic strain PA103 (13) was used. Bacteria were grown on tryptic soy agar (TSA) plates for 10 to 12 h, then resuspended in sterile phosphate-buffered saline (PBS) to an optical density at 650 nm (OD$_{650}$) of 0.5, and diluted to the desired dose for infection. *Salmonella enterica* serovar Typhimurium strain SL3261, an attenuated *aroA* mutant (10) containing the plasmid pLPS2 expressing serogroup O11 (vaccine), and SL3261 containing the cloning vector pLAFR1 (vector) were used for immunization (4). For vaccination, an overnight culture was diluted 1:1,000 in fresh LB with tetracycline selection (10 μg/ml) and grown to an OD$_{650}$ of 0.5. Bacteria were then washed and resuspended in PBS.

**Immunocompromised mouse models.** Mice were made leukopenic or neutropenic by treatment with Cy (Sigma) or the RB6-8CS antibody, respectively, as described previously (12). Treatment of vaccinated mice began on day 28 after vaccination. Briefly, for leukopenia, mice were given Cy (150 mg/kg of body weight) by intraperitoneal (i.p.) injection every other day over a course of 5 days (days 1, 3, and 5). One day after the last injection, mice were challenged with *P. aeruginosa*. The RB6-8CS hybridoma cell line was kindly provided by Gerald Pier (Brigham and Women’s Hospital, Boston, MA). The cells were grown and the antibody purified at the Lymphocyte Culture Center at the University of Virginia. Mice were given 0.2 mg of the antibody i.p. 1 day prior to infection.

To collect blood, mice were placed under a heat lamp and blood was collected from the tail vein into a capillary tube coated with EDTA (IRIS International, Westwood, MA). Samples were analyzed on the Hemavet 850 within 6 to 7 h of collection to detect the levels of blood lymphocytes, neutrophils, and total white blood cells.

**Intranasal vaccination.** The University of Virginia Animal Care and Use Committee approved all animal protocols. Female BALB/c mice, 5 to 6 weeks

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old (Harlan Laboratory [Indianapolis, IN] or Jackson Laboratory [Bar Harbor, ME]), were immunized intranasally (i.n.), as described previously (5). Prior to vaccination, mice were anesthetized by i.p. injection of 0.2 ml of xylazine (1.3 mg/ml) and ketamine (6.7 mg/ml) in 0.9% saline. For active vaccination, mice were given 1 × 10^8 CFU of either SL3261/pLP2S (vaccine sera) or SL3261/pLAFR1 (vector) i.n. in a 20-μl volume (10 μl per nostril).

**Bacterial challenge and passive vaccination.** Bacterial challenge and passive vaccination were performed in Cy-treated mice on day 6 (24 h after the last injection) and in RB6-8C5 mice 24 h after injection. For bacterial challenge, anesthetized mice were given 20 μl of *P. aeruginosa* strain 9882-80 prepared as described above. For passive vaccination, mice were given PBS or sera collected from mice vaccinated with either SL3261/pLP2S (vaccine sera) or SL3261/pLAFR1 (vector sera) diluted 1:10 in PBS. Ten μl of sera or PBS was given i.n. (5 μl per nostril) followed immediately by a suspension of 10 μl of *P. aeruginosa*.

In all experiments, infected mice were closely monitored for morbidity and mortality.

**Collection of sera.** Sera were collected as described previously (4). Briefly, blood was obtained by nicking the tail vein of mice; blood sat at room temperature for about 4 h and was then placed at 4°C overnight. Serum was removed from the red blood cell pellet and was spun at 1,700 rpm for 10 min to remove contaminating red blood cells. Samples were stored at –80°C.

**Detection of bacterial load.** Bacterial loads were quantified in mice by collecting nasal wash (NW) or bronchoalveolar lavage fluid (BAL) or by harvesting organs after infection. Prior to sample collection, mice were anesthetized with CO2 asphyxiation. For the BAL, a catheter was placed in the trachea and 1 ml PBS plus 1% bovine serum albumin (PBS-B) was instilled into the lung and then extracted. For the NW, a catheter was placed at the oropharyngeal opening and 0.5 ml PBS-B was used to flush the nasal passage. Samples from infected mice were serially diluted and plated for determination of CFU/ml. Lungs, livers, and spleens were aseptically removed, weighed, and then homogenized in 1 ml PBS-B. Homogenized organs were serially diluted in PBS-B and then plated onto TSA plates for determining bacterial numbers; final results were expressed as CFU/g of tissue.

**Evans blue lung permeability assay.** After infection, mice were injected i.p. with 200 μl of a 1% Evans blue dye (EBD; Alfa Aesar, Ward Hill, MA) solution. Four hours after this injection, mice were sacrificed and blood, BAL, and spleens were collected. BAL and homogenized spleen samples were diluted and plated onto TSA plates to determine bacterial loads. BAL samples were spun at 4,000 rpm for 10 min to remove cells, and blood samples were spun at 1,700 rpm for 10 min to remove red blood cells. Results are expressed as the percentage of blue dye in the BAL compared to the sera, as determined by the OD_{500}.

**ELISA analysis.** Enzyme-linked immunosorbent assay (ELISA) analysis was performed on sera, NW, and BAL as previously described (4). Briefly, 96-well microtiter plates were coated with *P. aeruginosa* strain PA103, incubated overnight at 4°C, washed with PBS plus 0.05% Tween 20 (PBS-T), blocked with PBS-B, and then washed with PBS-T again. Samples to be analyzed were then incubated on plates overnight at 4°C and washed with PBS-T. Secondary antibody solution (1:5,000 in PBS-B) was then added and incubated at 37°C for 1 h. Plates were developed in the dark for 1 h with 1 mg/ml 4-nitrophenyl phosphate in substrate buffer (24.5 mg MgCl2, 48 ml diethanolamine per 500 ml; pH 9.8); development was stop by adding 50 μl 3 M NaOH. Plates were read using a plate reader at 405 nm. Data were collected using the SOFTmax PRO software (Molecular Devices Corp., Sunnyvale, CA) and then transferred to GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA) for analysis.

**Statistics.** Statistical analyses were performed using GraphPad Prism version 4.0. Titers for the ELISA data were determined by using linear regression of the reciprocal dilution for the average of two replicates. The endpoint titer is the x intercept of this line. Kaplan-Meier survival curves were used to graph the survival data, and differences were analyzed by the log-rank test. Differences in bacterial loads and titers were analyzed using the Mann-Whitney U test for two groups and the Kruskal-Wallis one-way analysis of variance for multiple groups. LD_{50} values were calculated based upon the method of Reed and Muench (20).

## RESULTS

**Effect of Cy treatment on susceptibility to *P. aeruginosa* infection.** BALB/c mice were treated with Cy to render them leukopenic. Blood was taken from these mice on days 1, 3, and 6, and the number of total white blood cells, lymphocytes, and neutrophils was determined (Table 1). Mice receiving Cy had significantly less lymphocytes, neutrophils, and overall white blood cells than mice receiving the saline control (*P < 0.0001 for all cell types; Mann-Whitney U test).

Mice treated with Cy were extremely susceptible to infection with *P. aeruginosa* strain 9882-80, having median survivals of about 2 days following infection with doses ranging from 10 to 100 CFU. To quantitatively determine the increased susceptibility of these Cy-treated mice to *P. aeruginosa* infection, the LD_{50} was calculated from data collected for the survival curve shown in Fig. 1. The LD_{50}, about 20 CFU, is 6 logs lower than the 2 × 10^7 CFU seen in untreated BALB/c mice infected with the same strain (4, 5).

Bacterial loads in the lungs and organs were quantified in Cy-treated mice infected with 200 CFU of strain 9882-80 (Fig. 1). At 24 h, these mice had high numbers of bacteria in their lungs (12,1720 CFU/g in lungs. These mice also had bacteria disseminated in liver (12,1720 CFU/g), spleen (24,981 CFU/g), and nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU in nasal passages (Fig. 1B and C): 2.022 × 10^7 CFU/g.

**TABLE 1. Effect of Cy and RB6-8C5 treatments on white blood cell counts in BALB/c mice**

<table>
<thead>
<tr>
<th>Expn group</th>
<th>WBC</th>
<th>NE</th>
<th>LY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy, b day 1</td>
<td>9.411 ± 3.615</td>
<td>1.009 ± 0.6191</td>
<td>7.741 ± 2.866</td>
</tr>
<tr>
<td>Saline, day 1</td>
<td>12.33 ± 0.7075</td>
<td>1.246 ± 0.2768</td>
<td>10.13 ± 0.7558</td>
</tr>
<tr>
<td>Cy, day 3</td>
<td>3.66 ± 1.228</td>
<td>0.686 ± 0.4121</td>
<td>2.631 ± 0.7553</td>
</tr>
<tr>
<td>Saline, day 3</td>
<td>7.132 ± 3.454</td>
<td>0.7100 ± 0.7124</td>
<td>5.976 ± 2.578</td>
</tr>
<tr>
<td>Cy, day 6</td>
<td>1.417 ± 0.5177</td>
<td>0.2256 ± 0.1839</td>
<td>1.059 ± 0.3115</td>
</tr>
<tr>
<td>Saline, day 6</td>
<td>11.85 ± 1.994</td>
<td>1.809 ± 0.4062</td>
<td>9.239 ± 1.448</td>
</tr>
<tr>
<td>RB6-8C5, c</td>
<td>ND *</td>
<td>0.1821 ± 0.3865</td>
<td>2.504 ± 0.9555</td>
</tr>
<tr>
<td>PBS, day 1</td>
<td>ND</td>
<td>0.9100 ± 0.1577</td>
<td>5.998 ± 1.483</td>
</tr>
</tbody>
</table>

\* Total white blood cell (WBC), neutrophil (NE), and lymphocyte (LY) counts as determined using a Hemavet 850. Values are means ± standard errors.

\* Cy (150 mg/kg) was given on days 1, 3, and 5, and blood was drawn on days 1, 3, and 6 for quantification of WBC.

\* RB6-8C5 antibody was given i.p. to mice (2.0 mg) 1 day before the blood was drawn for quantification.

\* ND, not determined.

**Lung permeability in Cy mice.** The dissemination of *P. aeruginosa* strain 9882-80 to the spleen and liver was surprising, since this strain lacks ExoU, classifying it as noncytotoxic (2). In fact, strain 9882-80 has been previously shown in our laboratory to not disseminate readily to the spleen or liver at much higher challenge doses than were used in the Cy-treated mice (data not shown). To determine whether this dissemination was due to changes in lung permeability in the presence of Cy, we used EBD, which binds to serum albumin, to evaluate vascular leakage into the lungs (16). Prior to investigations in Cy-treated mice, the effects of bacterial infection in nonimmunocompromised mice were assessed. BALB/c mice were infected with either 2.5 × 10^5 CFU or 8 × 10^5 CFU of the cytotoxic strain PA103 or with 8 × 10^5 CFU of the noncytotoxic strain 9882-80. The LD_{50} values for these bacterial strains had been previously calculated to be 1 × 10^5 CFU and 2 × 10^5 CFU, respectively (4, 5). Mice were infected with EBD solution 12 hours after infection. After an additional 4 h, the mice were sacrificed, BAL and serum were collected from each mouse, and the amount of EBD in the BAL was compared to...
The mice infected with the cytotoxic strain PA103 showed an increased percentage of dye in the lungs that was dependent on the infectious dose: mice given the higher dose had more EBD in their BALs. The mice infected with the noncytotoxic strain 9882-80 had less dye in their lungs than was seen with either PA103 dose, but levels were still above the background levels seen in mice given PBS instead of *P. aeruginosa*. These data correlate with other stud-

![FIG. 1. Effect of *P. aeruginosa* infection after Cy treatment in BALB/c mice. (A) Survival of Cy-treated mice infected with *P. aeruginosa* strain 9882-80. Symbols: square, control; diamond, 100 CFU; triangle, 50 to 75 CFU; circle, 10 to 20 CFU. The control represents saline-treated mice infected with 10^6 CFU of strain 9882-80. Median survival in hours: Cy treatment and 100 CFU, 48 h; Cy treatment and 50 to 75 CFU, 42 h; Cy treatment and 10 to 20 CFU, 49 h. (B and C) Bacterial loads in the NW and BAL (B) and lungs, liver, and spleen (C) of Cy-treated mice 24 h after infection with 200 CFU of strain 9882-80 are shown.

![FIG. 2. Lung permeability in BALB/c mice. (A) Lung permeability of BALB/c mice 16 h after infection with 8 \times 10^5 CFU (PA103H), 2.45 \times 10^5 CFU (PA103L) of strain PA103, or 8 \times 10^5 CFU of strain 9882-80. Symbols show statistical significance (P < 0.05; Mann-Whitney U test) on comparison to PA103H (•) and 9882-80 (#). (B) Lung permeability of Cy-treated mice 24 h after infection with 2 \times 10^5 CFU (Cy high) or 1,500 CFU (Cy low) of strain 9882-80. Symbols show statistical significance (P < 0.05; Mann-Whitney U test) on comparison to Cy high (•) and Cy low (#). (C) Percent dye in lungs correlated to CFU/ml in lungs of Cy-treated infected mice (P = 0.0002; Spearman correlation). Lung permeability is expressed as the percentage of dye in BAL to dye in sera of mice as determined by the OD_{600}.

The amount in serum (Fig. 2). The mice infected with the cytotoxic strain PA103 showed an increased percentage of dye in the lungs that was dependent on the infectious dose: mice given the higher dose had more EBD in their BALs. The mice infected with the noncytotoxic strain 9882-80 had less dye in their lungs than was seen with either PA103 dose, but levels were still above the background levels seen in mice given PBS instead of *P. aeruginosa*. These data correlate with other stud-

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ies that examined lung permeability and the TTSS and indicate that although the noncytotoxic strain, 9882-80, is capable of causing lung damage, cytotoxic strains, which contain ExoU, like PA103, cause more damage (2, 7, 15).

After characterizing the lung permeability in immunocompetent mice, vascular permeability was measured in the Cy-treated mice (Fig. 2). The lungs of Cy-treated mice instilled with PBS had no greater vascular leakage than saline-treated mice given PBS. Cy-treated mice infected with strain 9882-80 were shown to have a dose-dependent vascular leakage at 24 h. There was significantly more blue dye in the lungs of mice infected with the higher dose (2 × 10^5 CFU) compared to mice infected with the lower dose (1,500 CFU), PBS, or the saline-treated control group (P < 0.05; Mann-Whitney U test). Even the mice infected with the lower dose had a significantly higher percentage of dye in their lungs compared to the saline-treated control group (P < 0.05; Mann-Whitney U test). The bacterial load in the lungs correlated with the percentage of dye in BAL, as the more-blue BAL samples had more bacteria present (P < 0.0002; Spearman correlation). The mice also had bacteria detectable in their spleens at similar levels as that seen in the Cy-treated infected mice (Fig. 1C). These results show that Cy treatment in conjunction with P. aeruginosa infection by a noncytotoxic strain, but not Cy treatment alone, can increase lung permeability.

Efficacy of vaccination after Cy treatment. We previously showed that vaccination of immunocompetent mice with attenuated Salmonella expressing the P. aeruginosa O antigen fully protected them from P. aeruginosa in a pneumonia challenge model (5). We wanted to investigate the efficacy of this vaccine in an immunocompromised mouse model. Mice were vaccinated and Cy treatment began on day 28 postvaccination. After treatment, mice were challenged with strain 9882-80, and survival and dissemination of the bacteria in these mice was monitored (Fig. 3). Mice given the vaccine prior to Cy treatment and infected with 300 CFU survived longer than mice immunized with the vector, with median survivals of 70.5 h and 49 h, respectively (P < 0.05; log-rank test). The median survival for Cy-treated vaccine-immunized mice infected with 30 CFU was higher than the vector-immunized group, 120.25 h compared to 49 h, although the difference in the survival curves was not significant (P = 0.1108; log-rank test). After infection with 350 CFU, the Cy-treated vaccine-immunized mice also had lower bacterial loads 24 h after infection in the BAL, lungs, liver, and spleen (P < 0.05; Mann-Whitney U test). These results suggest that vaccination prior to Cy treatment prolongs the survival of these mice and slows the bacterial growth compared to vector-immunized mice.

Antibody response in vaccinated Cy-treated mice. Although protection was seen in the vaccinated mice that received Cy treatment, the mice were still more susceptible than was previously seen in immunocompetent vaccinated mice. To ensure that this change in susceptibility was due to the depletion of immune cells, and not from any differences in the immune response of these mice, the level of antibodies was analyzed after treatment (Fig. 4). One day after the final Cy treatment, BAL and sera were collected from mice and analyzed by ELISA. The Cy-treated mice had the same serum immunoglobulin G (IgG) and IgM titers as well as the same IgG subtype concentrations as did the saline-treated control mice. The BAL also had the same antibody reactivity between the

![FIG. 3. Survival and bacterial loads in immunized Cy-treated mice. (A) Survival of immunized Cy-treated mice infected with 300 CFU (triangle) or 30 CFU (square) of strain 9882-80 (n = 4 mice/group). Open symbols, vaccine immunized; closed symbols, vector immunized; X, control. The control represents vaccine-immunized Cy-treated mice instilled with PBS instead of bacteria. Median survival in hours: vaccine immunized and 300 CFU, 120.25 h; vaccine immunized and 30 CFU, 70.5 h; vector immunized and 300 CFU, 49 h; nonimmunized and 30 CFU, 49 h. Curves for the same doses are significantly different from each other (P < 0.05; log-rank test). (B and C) Bacterial load in NW and BAL (B) and lungs, liver and spleen (C) of vaccine-immunized Cy-treated mice 24 h after infection with 350 CFU of strain 9882-80. Symbols: circles, vector immunized; triangles, vaccine immunized. *, statistical significance compared to the vector-immunized group (P < 0.05; Mann-Whitney U Test).]
two treatment groups. Consequently, the antibody profile is unchanged as a result of Cy treatment.

**Passive vaccination in Cy-treated mice.** Since actively vaccinated mice seemed to be partially protected from infection, we further investigated whether antibodies applied directly at the site of infection would affect the disease outcome. Mice were treated with Cy and then given either vaccine sera or vector sera or PBS at the time of infection with strain 9882-80. As shown in Fig. 5, the mice that received vaccine sera had significantly fewer bacteria in their lungs than mice that received either vector sera or PBS; they also had less bacteria in their NW compared to mice given PBS ($P_{\text{H11021}} < 0.01$; Mann-Whitney U test). Although not significant in all cases, there does appear to be a trend toward less dissemination to the liver and spleen in the mice receiving the vaccine sera compared to mice receiving either vector sera or PBS. Specifically, there were significantly fewer bacteria in the livers of mice given vaccine sera compared to mice given vector sera ($P < 0.05$; Mann-Whitney U test). This suggests that although both neutrophils and lymphocytes are needed for protection, the addition of antibodies can slow the infection, delaying bacterial replication in the lungs and, consequently, dissemination to other organs.

**Vaccine efficacy in mice made neutropenic with RB6-8C5.** Since Cy treatment had effects on both the neutrophils and lymphocytes and, as a chemotherapy agent, could have other effects in the mouse, we continued investigations using the RB6-8C5 antibody, which is more specific for depleting neutrophils (9). Mice were treated i.p. with the RB6-8C5 antibody 1 day prior to infection to remove the neutrophils; white blood cell counts are reported in Table 1. The mice treated with the RB6-8C5 antibody had significantly fewer neutrophils than PBS-treated control mice ($P_{\text{H11021}} < 0.05$; Mann-Whitney U test). Interestingly, the lymphocyte levels in the RB6-8C5-treated mice were also significantly lower than in the PBS-treated control mice, although there was still a significantly higher amount than in the Cy-treated mice ($P < 0.0001$; Mann-Whitney U test). Mice that received the RB6-8C5 antibody prior to infection displayed a similar hypersusceptibility as the Cy mice to infection with strain 9882-80 and also had an LD$_{50}$ of about 20 CFU. The median survival times of these RB6-8C5-treated
mice infected with 50 CFU or 100 CFU of strain 9882-80 were 85 and 52 h, respectively (Fig. 6A).

In order to determine the effect of eliciting neutropenia subsequent to vaccination, we immunized mice and then treated them with RB6-8C5. After infection with 200 CFU of strain 9882-80, the vaccine-immunized mice survived longer than the vector-immunized mice, with median survivals of 47.5 h in vector-immunized mice while the vaccine-immunized median survival was undefined (Fig. 6B) \( (P < 0.05; \text{log-rank test}) \). Vaccinated mice treated with the RB6-8C5 antibody and then infected with 2,000 CFU also had significantly fewer bacteria in the BAL, lungs, liver, and spleen (D) of RB6-8C5-treated vaccinated mice 24 h after infection with 2,000 CFU of strain 9882-80. Symbols: circles, vector immunized; triangles, vaccine immunized. *, statistical significance compared to vector-immunized group \( (P < 0.05; \text{Mann-Whitney U Test}) \).

Passive vaccination was also performed in these neutropenic mice. Mice treated with RB6-8C5 and then given sera from vaccine- or vector-immunized mice at the time of infection were monitored for survival and bacterial loads. As shown in Fig. 8, RB6-8C5-treated mice receiving vaccine sera at the time of infection with 200 CFU of strain 9882-80 had significantly better survival than mice receiving PBS with the bacteria \( (P < 0.01; \text{log-rank test}) \) and had median survivals of 21 h, 24.25 h,
and undefined for the PBS-, vector sera-, and vaccine sera-treated mice, respectively. The only median survival that could be calculated for the passively vaccinated mice infected with 64 CFU of strain 9882-80 was for the group that received the vector sera, which had a median survival of 38 h; the survival curves were not significantly different at this infectious dose (P = 0.2873; log-rank test). Bacterial colonization of the respiratory tract and dissemination to the liver and spleen were also analyzed in these mice (Fig. 8). The mice receiving vaccine sera also had less bacteria in the NW, BAL, lungs, liver, and spleen compared to those that received the vector sera or PBS. The lungs and liver CFU counts were significantly different between the groups (P < 0.05; Kruskal-Wallis). The vaccine sera-treated mice had significantly less bacteria in lungs than vector sera-treated mice (P < 0.05; Mann-Whitney U test). Thus, the neutropenic mice behaved quite similarly to the Cy-treated mice after P. aeruginosa infection and showed increased survival following active and passive vaccination.

**DISCUSSION**

We found that rendering mice immunocompromised makes them hypersusceptible to P. aeruginosa pneumonia. This is characterized by a decrease in the LD$_{50}$ after i.n. challenge, with as few as 20 CFU of a noncytotoxic P. aeruginosa strain causing lethality; it was also noted that there were high numbers of bacteria found in the lungs, livers, and spleens of these mice. This susceptibility was decreased, based on better survival and lower bacterial loads, if mice were vaccinated prior to immunocompromise and infection or if, after immunocompromise, antibodies were given to mice at the time of infection.

Dissemination of P. aeruginosa from the gastrointestinal tract has been previously observed in immunocompromised mice. Koh et al. (12) showed that P. aeruginosa could colonize the gastrointestinal tract of immunocompetent mice but did not readily cause bacteremia or disseminate. However, administration of Cy to these infected mice resulted in bacteremia, dissemination, and death in the mice given either cytotoxic or noncytotoxic strains of P. aeruginosa PAO1. Administration of the RB6-8C5 antibody also resulted in increased mortality in mice that had been previously colonized with P. aeruginosa, again independent of the cytotoxic phenotype of the colonizing strain. Mortality was also shown to be dependent on the dose of Cy given, as higher Cy doses resulted in increased mortality by strains lacking ExoU. Complete mortality was seen with all strains at doses of Cy of ≥125 mg/kg (12), the dose of Cy that was used for our experiments. We also saw that after the Cy treatment, a low infectious dose of a noncytotoxic P. aeruginosa strain resulted in mortality and increased dissemination and bacteremia, compared to our previous findings with immunocompetent mice (unpublished data).

Dissemination of P. aeruginosa in a pneumonia challenge model in neutropenic mice was previously investigated by Vance et al. (24). They used this model to evaluate the virulence of TTSS mutants, performing competitive infections using both wild-type and mutant strains. Dissemination to the spleen and blood was seen in Cy-treated and doubly Cy- and RB6-8C5-treated mice infected with the noncytotoxic P. aeruginosa strain PAO1, but all data were expressed as competitive indices, so the actual numbers of bacteria that disseminated are not known (24). Consequently, we do not know how well the level of dissemination we observed correlates with what was previously seen by Vance and colleagues.

Cytotoxic strains of P. aeruginosa that express the phospholipase ExoU (15, 22) have been shown to disseminate more

**FIG. 8.** Passive vaccination following RB6-8C5 treatment. (A) Survival of RB6-8C5-treated mice given vector sera (circles), PBS (squares), or vaccine sera (triangles) i.n. concurrent with 200 CFU (open symbols) or 60 CFU (closed symbols) of strain 9882-80. Median survival in hours: vector sera treated and 60 CFU, 38 h; PBS treated and 200 CFU, 21 h; vector sera treated and 200 CFU, 24.25 h; all other median survivals were undefined. (B and C) Bacterial loads in NW and BAL (B) and in lungs, liver, and spleen (C) 24 h after RB6-8C5-treated mice received vector sera (circles), PBS (squares), or vaccine sera (triangles) at the time of infection with 1,000 CFU of strain 9882-80.
readily than noncytotoxic strains that express ExoS and are also associated with a higher patient mortality rate (21). However, Ader et al. showed that strains lacking ExoU that still have an intact TTSS are still more virulent, causing higher mortality and bacterial burdens as well as a more robust neutrophil influx and cytokine response in the lung, compared to P. aeruginosa strains that have a completely nonfunctional TTSS (1). Here, we were able to replicate the pattern of increased dissemination as well as increased lung permeability by the cytotoxic ExoU-containing strain PA103 compared to the noncytotoxic strain, 9882-80. When mice given Cy were infected with our challenge strain, 9882-80, a high degree of dissemination was seen, despite the fact that 9882-80 lacks ExoU (2).

Cy-treated mice infected with strain 9882-80 had bacteria present in the BAL and spleen at 24 h at similar levels both in the presence and absence of the Evans blue dye. Concurrent with the dissemination, the lungs of Cy-treated mice infected with strain 9882-80 were more permeable than uninfected controls. This effect appears to be dependent on the dose of bacteria given, as the number of CFU detected in the BAL correlated with the amount of blue dye found in the BAL. The higher levels of vascular leakage in the Cy-treated mice infected with strain 9882-80 compared to nontreated mice also infected with 9882-80 were not related to the Cy treatment itself, as Cy-treated mice given PBS instead of bacteria had no differences in lung permeability compared to control, saline-treated mice instilled with PBS instead of bacteria. This leads to the conclusion that it is the accumulation of bacteria in the lungs that results in the dissemination to the spleen and liver.

The efficacy of other P. aeruginosa vaccines has been investigated in immunocompromised mouse models. Active vaccination against OprF or OprI was shown to increase survival and raise the LD50 of Cy-treated mice to i.p. challenge of P. aeruginosa (25). Vaccination with a Salmonella enterica serovar Dublin strain expressing P. aeruginosa OprI was able to protect about 10 to 20% of Cy-treated vaccinated mice from oral challenge with P. aeruginosa (23). However, these mice received eight vaccinations or boosters to achieve this low level of protection, while our mice received only one vaccination. Administration of monoclonal antibodies to P. aeruginosa lipoproteins was shown to raise the LD50 of a subcutaneous challenge in mice rendered leukopenic by Cy treatment (8). Passive vaccination strategies using transfer of rabbit serum to SCID mice showed that mice could be completely protected from an i.p. P. aeruginosa challenge dose of up to 500 CFU for one epitope (25). Also, serotype-specific antiligopolysaccharide antibodies were found to raise the LD50 of a challenge directed into an incision on the backs of leukopenic mice (3). This protection at low bacterial challenge doses is consistent with what we saw in our experiments. These experiments were encouraging in that protection, although sometimes at low levels, could be achieved in immunocompromised mice, although none of these prior studies looked at a pneumonia challenge model. Here we have shown that neutrophils are required for the complete protection from P. aeruginosa challenge seen with the vaccine, although low levels of protection remain in vaccinated neutropenic mice.

Vaccination prior to rendering the mice immunocompromised made them less susceptible to the P. aeruginosa infection. However, this protection was still not to the same level as would be seen in wild-type mice. This is due to the loss of the immune cells, as the antibody response was unaffected by either treatment. The Cy treatment affects both neutrophils and lymphocytes, while the RB6-8C5 treatment is more specific for neutrophils but still causes a small decrease in lymphocyte numbers. Further investigations will look at the role of lymphocytes in protection, which could validate that the changes in protection in the RB6-8C5-treated mice are from the lack of neutrophils.

The presence of antibodies seems to be enough to slow the infection, although some degree of protection could be from the few remaining lymphocytes or neutrophils in these immunocompromised mice. When antibodies are added directly to the site of infection, the mice fare even better than when they have been vaccinated, suggesting that it is indeed antibodies in the lung that are slowing the progression of the disease and allowing the mice to survive longer. Work by others showed that intravenous administration of antibodies or vaccination of sheep with an O antigen-specific vaccine prior to infection with P. aeruginosa was able to prevent dissemination but not lung injury, while administration of antibodies to the respiratory tract was able to prevent both dissemination and lung injury (19). This could be because the circulating antibodies need lung damage to enter the lungs, while the administered antibodies are present directly at the site of infection before the lung damage occurs. These data correlate with what was seen in our immunocompromised mouse model. We have not investigated the lung permeability of the Cy-treated mice with our vaccination strategies to see if the results mirror what was seen in the sheep.

Either of our vaccination strategies could be a potential treatment in the immunocompromised patient population, particularly the passive administration of antibodies at the site of infection. Although neither active nor passive vaccination strategies were able to completely protect the mice, they did manage to slow the progression of disease. If these treatments were used in conjunction with antibiotic therapy in an immunocompromised patient, the outcome would likely be better. We have not tested this possibility in our mouse infection model. We also did not test cytotoxic strains in our immunocompromised mouse models. We have previously seen that cytotoxic strains are more virulent than noncytotoxic strains, having lower LD50 and also having a more rapid progression of disease and earlier dissemination. The infection process of cytotoxic strains in immunocompromised mice could be different, as such strains are likely to cause lung damage and disseminate to other organs at a faster rate than the noncytotoxic strain we tested. However, the presence of specific antibodies could also slow the progression of disease.

Our vaccine is to a single O antigen serogroup, and many serogroups have been implicated in disease. Although there are at least 20 serogroups of P. aeruginosa, not all of them are implicated in disease, making an O antigen-specific vaccine within reach. Analysis of acute and chronic lung isolates of P. aeruginosa yielded a higher prevalence of strains of serogroups O6, O1, O11, and O4 (6). Interestingly, strains belonging to serogroup O11 had a much higher prevalence of cytotoxicity than other serogroups and were also associated with a high mortality rate (6).
Here we have shown that treating mice with Cy renders them leukopenic and highly susceptible to \textit{P. aeruginosa} infection, characterized by increased lung permeability and decreased LD50. This susceptibility is also seen in mice rendered neutropenic by RB6-8C5 and can be reduced in both groups of immunocompromised mice by administration of our vaccine prior to treatment. Administration of antibodies directly to the lungs at the time of infection seems to afford better protection to the mice and could be combined with antibiotic therapies in clinical settings.

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\textbf{REFERENCES}