Virulence of *Pseudomonas aeruginosa* in a Murine Model of Gastrointestinal Colonization and Dissemination in Neutropenia

Andrew Y. Koh,^{1,2,3,4*} Gregory P. Priebe,^{1,3,5} and Gerald B. Pier¹

Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School,¹
Divisions of Hematology/Oncology² and Infectious Diseases,³ Department of Medicine, and Department of
Anesthesia, Critical Care Medicine,⁵ Children's Hospital, and Department of Pediatric
Oncology, Dana Farber Cancer Institute,⁴ Boston, Massachusetts

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Pseudomonas aeruginosa bacteremia in cancer patients develops from initial gastrointestinal (GI) colonization with translocation into the bloodstream in the setting of chemotherapy-induced neutropenia and GI mucosal damage. We established a reproducible mouse model of *P. aeruginosa* GI colonization and systemic spread during neutropenia. Mice received 2 mg of streptomycin/ml of drinking water and 1,500 U of penicillin G/ml for 4 days and then ingested 10⁷ CFU of *P. aeruginosa* per ml of drinking water for 5 days. After GI colonization levels were determined, cyclophosphamide (Cy) was then injected intraperitoneally (i.p.) three times every other day or an antineutrophil monoclonal antibody, RB6-8C5, was injected i.p. once. Dissemination was defined by the presence of *P. aeruginosa* in spleens of moribund or dead mice. In this mouse model, *P. aeruginosa* colonizes the GI tract and then disseminates systemically once Cy or RB6-8C5 is administered. The duration and intensity of neutropenia, related to Cy dose, was found to be a means to compare the virulence of different *P. aeruginosa* strains, as exhibited by comparisons of strains lacking or producing the virulence-enhancing ExoU cytotoxin. The lipopolysaccharide outer core polysaccharide and O side chains were critical in establishing GI colonization, and *P. aeruginosa* mutants lacking the *aroA* gene (necessary for synthesizing aromatic amino acids) were able to establish GI colonization but unable to disseminate. Both the colonization and dissemination phases of *P. aeruginosa* pathogenesis can be studied in this model, which should prove useful for evaluating pathogenesis, therapies, and associated means to control *P. aeruginosa* nosocomial infections.

Pseudomonas aeruginosa causes significant morbidity and mortality in immunocompromised hosts (1, 15, 44), particularly neutropenic cancer patients (16). Although currently gram-positive organisms account for 60 to 70% of all documented infections in febrile neutropenic cancer patients (17, 26), these infections, in general, are typically more indolent, and delays of 24 to 48 h in initiating antibiotic therapy are usually not detrimental (17, 46). Importantly, the incidence of *P. aeruginosa* bacteremia has decreased in solid tumor patients but not in patients with acute leukemia (8). In fact, despite its lower incidence, *P. aeruginosa* continues to cause a disproportionate degree of morbidity and mortality in this patient population (6, 15, 16).

The presumed mechanism for establishing *P. aeruginosa* bacteremia in cancer patients involves initial gastrointestinal (GI) colonization with subsequent translocation into the bloodstream in the setting of chemotherapy-induced neutropenia and GI mucosal damage (39). Leukemia patients who develop *P. aeruginosa* bacteremia have been found to have fecal cultures that are positive for the same strain of *P. aeruginosa* (18, 50). When fecal cultures of these patients showed the presence

of other potentially pathogenic gram-negative organisms (e.g., *Escherichia coli*, *Klebsiella* sp., etc.), *P. aeruginosa* was much more likely to translocate to the blood, even if the coinfecting gram-negative organism was more abundant (50). Since *P. aeruginosa* is usually not a part of the normal commensal human GI flora, a large proportion of *P. aeruginosa* infections in this patient population are hospital acquired (6, 18).

The bacterial pathogens that have been most frequently studied in bacterial translocation include members of the *Enterobacteriaceae* family, such as *E. coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* (4, 20), as well as enterococci (28). Surprisingly little is known about the pathogenesis of *P. aeruginosa* GI colonization and translocation, and although many of the findings described previously for other microbes may be generalized to *P. aeruginosa*, scant experimental data have been generated to either confirm or refute these generalizations. The fact that *P. aeruginosa* is a serious pathogen in surface or mucosal sites other than the GI tract (the eye [40, 47], the urinary tract [3], and the bronchial mucosa [27]) suggests that there may be common mechanisms that this organism uses to colonize these different surfaces. This leads to the obvious assumption that infection could be prevented if colonization of mucosal surfaces could be interrupted. Thus, any insight into the colonization mechanisms of *P. aeruginosa* could help us devise such strategies.

Previous work has shown that the treatment of mice with streptomycin in the drinking water allows for colonization of

* Corresponding author. Mailing address: Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 181 Longwood Ave., Boston, MA 02115. Phone: (617) 525-2665. Fax: (617) 525-2510. E-mail: Andrew_koh@dfci.harvard.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
PAO1	Wild-type strain, LPS smooth, noncytotoxic, chloramphenicol sensitive	M. Vasil
PAO1 ExoU ⁺	PAO1 (pUCP19 <i>exoU</i> spcU), cytotoxic variant of PAO1 carrying the ExoU gene and its chaperone gene, spcU, on plasmid pUCP19; Cb ^r	3
PAK	Wild-type strain, LPS smooth, noncytotoxic	S. Lory
PAK ExoU ⁺	PAK carrying ExoU gene on pUCP19 <i>exoU</i> spcU	S. Lory
PA103	Wild-type strain, LPS smooth, cytotoxic	Lab stock
PA14	Wild-type strain, LPS smooth, cytotoxic	F. Ausubel
15921	Wild-type strain, LPS-smooth, noncytotoxic	2
15921 ExoU ⁺	15921 (pUCP19 <i>exoU</i> spcU), cytotoxic variant of 15921 carrying the plasmid pUCP19 <i>exoU</i> spcU	2
PAO1 Δ galU	galU mutant of PAO1 in which the galU gene is disrupted by aaC1 cassette; Gm ^r	41
PAO1 Δ galU(pCD204)	PAO1 Δ galU containing the intact galU gene on plasmid pCD204; Cb ^r , Tc ^r	41
PAO1 Δ aroA(pUCP19)	aroA deletion mutant of PAO1, carrying empty vector pUCP19; Cb ^r	42
PAO1 Δ aroA(pMB1)	PAO1 Δ aroA containing the intact aroA gene on plasmid pMB1; Cb ^r	42
pMB1	pUCP18 plasmid containing the intact aroA gene; Ap ^r	42
pUCP19	Broad-host-range shuttle vector; Ap ^r , Cb ^r	41, 42
pCD204	pUCP18Tc carrying a 1,036-bp PCR fragment encompassing galU from PAO1 in the same orientation as the lac promoter; Tc ^r	42

^a Abbreviations: Ap, ampicillin; Gm, gentamicin; Cb, carbenicillin; and Tc, tetracycline.

the GI tract with *P. aeruginosa* (38) and that immunization against lipopolysaccharide (LPS) O side chain antigens can reduce mucosal colonization levels (37). However, subsequent work established that the previously published method did not result in consistent GI colonization with a variety of strains of *P. aeruginosa*. In this study, we established a more reproducible mouse model of *P. aeruginosa* gastrointestinal colonization and additionally evaluated the ability of different strains to undergo systemic spread during neutropenia; we found that this model allows us to define pathogen virulence factors (colonization and translocation factors) prior to and after the induction of neutropenia. We confirmed that in this mouse model, *P. aeruginosa* initially colonizes the gastrointestinal tract and then disseminates systemically once either cyclophosphamide (Cy) or an antineutrophil monoclonal antibody, RB6-8C5, is administered. Because the Cy-induced neutropenia is dose dependent and because the levels of GI colonization with various wild-type strains of *P. aeruginosa* were generally comparable, the Cy dose that was needed to elicit dissemination and ultimately death is another means to potentially classify strains based on differences in virulence. We also found that inducing neutropenia without GI mucosal damage (by use of an antineutrophil monoclonal antibody) was sufficient for inducing dissemination in our murine model. Finally, we were able to identify mutants that were unable to establish GI tract colonization and mutants that were able to colonize but unable to disseminate, indicating the utility of this animal model to study different aspects of the pathogenic process of *P. aeruginosa* in the setting of GI colonization and dissemination.

MATERIALS AND METHODS

Bacterial strains, recombinant plasmids, and vectors. The strains of *P. aeruginosa* and the recombinant plasmids and vectors used are listed in Table 1. *P. aeruginosa* strains were made streptomycin resistant (SR) by serial incubation in tryptic soy broth with increasing concentrations of streptomycin. Confirmation of streptomycin resistance was made by positive growth on ceftrimide agar with 2 mg of streptomycin/ml.

Murine model of antibiotic-induced GI tract colonization by *P. aeruginosa* and Cy-induced bacteremia. C3H/HeN mice (6- to 8-week-old females) were fed sterile water with 2 mg of streptomycin/ml and 1,500 U of penicillin G/ml for 4

days (21), after which stool was collected and homogenized in 1 ml of 1% protease peptone and 100 μ l of homogenate was plated on MacConkey agar to verify clearance of endogenous aerobic gram-negative GI flora. Mice were then fed sterile water with 1,500 U of penicillin G/ml and SR *P. aeruginosa* strains (approximately 10^7 CFU/ml) for 5 days (38). Water containing SR *P. aeruginosa* was changed after 2 to 3 days in order to maintain bacterial levels. After 5 days of exposure to bacteria, stool was again collected, homogenized in 1 ml of 1% protease peptone, serially diluted in this medium, and plated on cetrimide agar with streptomycin to measure levels of GI colonization with *P. aeruginosa*. Sterile water with 2 mg of streptomycin/ml and 1,500 U of penicillin G/ml was restarted and continued for the remainder of the experiment. *P. aeruginosa* GI colonization levels were checked and were not affected by reinitiation of antibiotic water. Each mouse was weighed, and a Cy dose ranging from 50 to 150 mg/kg of body weight was calculated. Accurate weighing and calculation of the Cy dose was found to be critical for obtaining reproducible results. Cy was then injected intraperitoneally (i.p.) three times every other day (11). Mice were monitored twice a day for morbidity and mortality over an additional 5 days. Moribund mice were euthanized, and dead mice were frozen at -20°C . Mice were later thawed, and spleens were resected, homogenized, and plated on MacConkey and cetrimide agars with 2 mg of streptomycin/ml. Oxidase-positive colonies on the cetrimide-plus-streptomycin agar were used for confirmation of dissemination of the initially colonizing *P. aeruginosa* strain. Death due to *P. aeruginosa* infection was recorded only if spleens yielded $>10^3$ CFU of *P. aeruginosa*/g.

Cy dose and effect on white blood cell counts. In order to determine both the depth and the duration of neutropenia and lymphopenia caused by Cy administration, mice were organized into groups based on Cy doses of 50, 75, 100, 125, and 150 mg/kg. Four mice from each Cy group were bled on nine consecutive days while undergoing i.p. administration of Cy as per the protocol listed above every other day (on days 0, 2, and 4). Blood sampled on day 0 was before the first Cy dose. Blood was collected into heparinized tubes, diluted 1:3 with phosphate-buffered saline (PBS) in order to achieve sufficient sample size, and taken to the hematology core laboratory at Children's Hospital, Boston, Mass. Complete and differential blood counts were run on an Advia 120 hematology analyzer set to analyze murine blood. The average white blood cell count, absolute lymphocyte count (ALC), and absolute neutrophil count (ANC) were calculated and graphed.

RB6-8C5 monoclonal antibody. In order to understand better the contribution of neutrophils to innate immune defense against *P. aeruginosa* after initial GI colonization, we tried to eliminate the Cy-induced GI mucosal damage and lymphopenia by using a rat anti-mouse neutrophil monoclonal antibody (MAb), RB6-8C5 (13, 14), to induce neutropenia. We obtained the RB6-8C5 hybridoma cell line from C. Czuprynski, University of Wisconsin. Hybridoma cells were tested for biological contaminants and were found to be pathogen free. Hybridoma cells were grown in cell culture (Dulbecco's modified Eagle's medium with 10% fetal calf serum). The RB6-8C5 MAb, a rat immunoglobulin G [IgG2b(κ)], was purified from cell culture supernatant by affinity chromatography (recombinant protein G agarose; Invitrogen). Purity was verified on a lithium dodecyl

sulfate-4 to 12% Tris-Bis gel (Invitrogen) stained with Coomassie blue. The MAb concentration of the sample was determined by enzyme-linked immunosorbent assay using microtiter plates (Immulon 2HB) sensitized with goat anti-rat IgG (diluted 1:250). The secondary antibody used was an alkaline-phosphatase goat anti-rat IgG (diluted 1:1,000). A standard curve using purified rat IgG was also generated.

To determine the depth and duration of neutropenia induced by RB6-8C5, mice were arranged into groups of four. Each mouse was given 0.2 mg of RB6-8C5 via i.p. injection. Each group of mice was bled on seven consecutive days, and blood samples were sent for complete blood count and differential analysis to the hematology laboratory at Children's Hospital. One group of mice was bled prior to the administration of MAb to establish a baseline.

The murine model described above was then employed, using the administration of RB6-8C5, rather than the Cy injections, to induce neutropenia. Each mouse was given 0.2 mg of RB6-8C5 (12, 13) via i.p. injection once and then monitored for morbidity and mortality for an additional 5 days.

Translocation studies. To definitively confirm that the *P. aeruginosa* colonizing the GI tract was translocating into the blood, mice were organized into the following five groups: group 1, no *P. aeruginosa* and 125 mg of Cy/kg/dose; group 2, SR PAO1 and no Cy; group 3, SR PAO1 ExoU⁺ and no Cy; group 4, SR PAO1 and 125 mg of Cy/kg/dose; and group 5, SR PAO1 ExoU⁺ and 75 mg of Cy/kg/dose. All mice from each of the groups were started on the GI colonization and dissemination protocol described above. Four mice from each group were sacrificed on days 0, 1, 3, 5, 6, 7, and 8 after the first Cy dose. Blood (100 μ l) was drawn via the tail vein (after ethanol cleaning) and spread plated on MacConkey plus streptomycin and ceftrimide plus streptomycin agar plates. Mice were then sacrificed. Mesenteric lymph nodes (MLN), spleens, and lungs were resected; organs were homogenized in 1 ml of 1% protease peptone; and 100 μ l of homogenate was spread plated on MacConkey plus streptomycin and ceftrimide plus streptomycin agar plates. Oxidase-positive colonies on the ceftrimide-plus-streptomycin agar were used as confirmation of the presence of *P. aeruginosa*. Serial dilutions and plating for bacterial enumeration were also done to obtain quantitative data (CFU per gram or CFU per milliliter).

Histological analysis of mouse ceca. C3H/HeN mice (6- to 8-week-old females) were divided into three groups and sacrificed after the following specific conditions were met: group A was given sterile water with 2 mg of streptomycin/ml and 1,500 U of penicillin G/ml for 5 days; group B was given streptomycin and penicillin water for 5 days, given 125 mg of Cy/kg i.p. three times every other day, and observed for 48 h after the last Cy dose; and group C was given streptomycin and penicillin water for 5 days, given 0.2 mg of RB6-8C5 i.p. once, and observed for 48 h after the RB6-8C5 dose. Cecae were resected and immediately fixed in Bouin's solution. Sections were stained with hematoxylin and eosin and reviewed by a veterinary pathologist.

Preparation of extracellular proteins and detection of ExoU. *P. aeruginosa* strains (PAO1, PAO1 ExoU⁺, PA103, PAK ExoU⁺, PA14, 15921, and 15921 ExoU⁺) were grown at 37°C overnight in tryptic soy broth with 10 mM nitrilotriacetic acid, a chelator known to induce the type III secretion apparatus (23). Supernatants were prepared from the cultures by centrifugation at 10,000 $\times$ g at 4°C for 30 min. Proteins present in the supernatants were precipitated by the addition of ammonium sulfate (50%) and left overnight at 4°C. Precipitated material was isolated by centrifugation at 10,000 $\times$ g at 4°C for 30 min. Protein pellets were resuspended in distilled H₂O and run through PD-10 desalting columns (Amersham Biosciences). Protein concentrations were determined by Bradford protein assay. For detection of the ExoU protein, polyvinylidene fluoride membranes were activated by soaking in methanol and equilibrated in PBS-1% bovine serum albumin-0.05% Tween. A slot blotter was used to load samples of 1, 5, 10, and 20 μ g of total extracellular protein material, and with strains PA14 and 15921 ExoU⁺, extracellular protein samples of 100, 500, 1,000, and 2,000 μ g were loaded as well. The membrane was blocked with PBS containing 5% skim milk at room temperature for 1 h. The primary antibody used was a polyclonal rabbit IgG to ExoU (2) diluted 1:100, and the blot was rotated at room temperature for 90 min. The secondary antibody was an anti-rabbit whole-molecule IgG conjugated to alkaline phosphatase diluted 1:50,000, which was left on the blot for 90 min at 37°C. After washing, a phosphatase substrate developer (KPL) was then added.

Statistical analyses. Survival data were analyzed by Fisher's exact test with Statview software (SAS Institute, Cary, N.C.). A comparison of GI colonization levels was analyzed by Mann-Whitney tests with Statview, and when multiple comparisons or more than two groups were analyzed, Bonferroni's correction to the significance level α was invoked.

FIG. 1. Gastrointestinal colonization levels of mice fed *P. aeruginosa* strains PAO1, PAO1 ExoU⁺, PAK, PAK ExoU⁺, 15921, 15921 ExoU⁺, PA103, and PA14 suspended in sterile drinking water with 1,500 U of penicillin G/ml for 5 days. Points represent results from individual animals, and horizontal lines represent the medians. $n = 16$ for each strain.

RESULTS

GI colonization by *P. aeruginosa*. Various *P. aeruginosa* strains were evaluated in the murine model, and most strains consistently colonized the normal-flora-depleted mouse GI tract (Fig. 1). As expression of the ExoU cytotoxin has been associated with more severe *P. aeruginosa* respiratory tract disease (22, 45), our analysis included two strains that expressed ExoU, PA14 and PA103, and additional strains expressing ExoU protein from a recombinant plasmid. Overall, the expression of ExoU had no effect on GI colonization levels.

Translocation by *P. aeruginosa*. We next determined the effect of delivering Cy on the translocation of GI-colonizing *P. aeruginosa* to the blood, MLN, spleen, or lungs. Control mice that were not colonized with *P. aeruginosa* but were treated with the 125 mg of Cy/kg/dose (group 1) did not grow *P. aeruginosa* from the blood, MLN, spleen, or lungs at any of the time points measured (Fig. 2). All mice in this group survived until the time of sacrifice, and none appeared ill or moribund. Mice that were colonized with *P. aeruginosa* PAO1 but not treated with Cy (group 2) did not show any evidence of translocation to the MLN. Interestingly, mice that were colonized with the PAO1 ExoU⁺ strain and not given Cy (group 3) did have *P. aeruginosa* in the MLN on days 0 and 3. Also of interest, mice from groups 2 and 3 showed sporadic evidence of bacteremia (group 2 on day 3; group 3 on days 0 and 3), but none of these mice looked moribund or died prior to their designated time of sacrifice. For both group 2 and group 3, there was no evidence of *P. aeruginosa* in the spleens or lungs at any of the time points. Mice that were colonized with strain PAO1 (group 4) and mice that were colonized with strain PAO1 ExoU⁺ (group 5) and given 125 mg of Cy/kg three times had blood, MLN, spleen, and lung cultures that were markedly positive for *P. aeruginosa* on days 6, 7, and 8. Many mice were moribund during this time period, and a number of mice died before their intended sacrifice time. Quantitative data (CFU per gram or CFU per milliliter) were also collected (data not shown): some mice were found dead on sacrifice days, and since the time of death was unknown, there may have been additional bacterial growth secondary to necrosis, so accurate

FIG. 2. Translocation of *P. aeruginosa* strains PAO1 and PAO1 ExoU⁺ to extraintestinal tissues. Groups of four mice were sacrificed on days 0, 1, 3, 5, 6, 7, and 8 after the first of three total Cy doses. Blood, MLN, spleens, and lungs from four animals were sampled, homogenized, and plated on MacConkey and cetrimide agar. Bars represent the percentage of four mice with a specific organ that tested positive for *P. aeruginosa* (oxidase-positive colonies on cetrimide agar).

determinations of CFU in tissues was not feasible in this setting.

At autopsy, we also analyzed the lungs for the presence of *P. aeruginosa* to determine whether aspiration of the bacteria in the drinking water had occurred or if the ingestion of feces with *P. aeruginosa* could have led to respiratory tract colonization. Only the lung homogenates from groups 4 and 5, which were infected with bacteria and treated with Cy, grew *P. aeruginosa* upon culture, likely due to the presence of the bacterium in the blood at this time. Thus, there was no evidence of *P. aeruginosa* colonizing the respiratory tract in the absence of sufficient neutropenia to induce hematogenous spread to this tissue.

Cy-induced neutropenia and lymphopenia. The depth and duration of neutropenia were found to be directly proportional to the dose of Cy (Fig. 3). Neutropenia (defined as an ANC of <500) was achieved with all dosing groups by day 4. Severe neutropenia (defined as an ANC of <100) was also seen with all doses of Cy. Notably, only with the lowest doses (50 and 75 mg of Cy/kg) was there any evidence of neutrophil recovery by day 8 (Fig. 3). ALCs followed the same general trends as the ANC (data not shown).

RB6-8C5-induced neutropenia. Mice were found to be neutropenic 24 h after administration of RB6-8C5 MAb and remained neutropenic for 5 days (Fig. 4). Interestingly, the ALC was also found to decrease during the same time period, reaching a nadir of 694 on day 2 but otherwise remaining above 1,000 (data not shown).

P. aeruginosa strains PAO1, PAO1 ExoU⁺, PAK, PAK ExoU⁺, and PA103 were used in our murine model, as well as a control group of mice receiving no *P. aeruginosa*, with the

sole modification of substituting one dose of 0.2 mg of RB6-8C5 MAb for the Cy doses. Mice were found to be colonized with levels of the respective *P. aeruginosa* strains comparable to what was found previously (Fig. 1). On day 5 after the RB6-8C5 MAb was given, two of eight mice in the PAO1 group remained alive, zero of eight mice in the PAO1 ExoU⁺ group remained alive, one of eight mice in the PAK group remained alive, three of eight mice in the PAK ExoU⁺ group remained alive, zero of eight mice in the PA103 group remained alive,

FIG. 3. Effect of Cy on neutrophil blood counts. C3H/HeN mice were administered Cy at 150, 125, 100, 75, and 50 mg/kg. The average absolute neutrophil count for four mice was used for each time point. Boxes around day numbers indicate days when Cy was administered.

FIG. 4. Effect of RB6-8C5 monoclonal antibody on neutrophil blood counts. C3H/HeN mice were administered 0.200 mg of RB6-8C5 per mouse on day 0. The average absolute neutrophil count of four mice was used for each time point.

and all eight mice in the no *P. aeruginosa* group remained alive (Table 2). The surviving mice received another 0.2-mg dose of RB6-8C5 MAb to maintain neutropenia. Five days later, only one mouse in the PAO1 group and one mouse in the PAK ExoU⁺ group remained alive, while all eight mice in the no *P. aeruginosa* group were still alive ($P < 0.001$; Fisher's exact test with Bonferroni's correction for comparing each of the five experimental groups to the one control group). *P. aeruginosa* dissemination was confirmed by the presence of oxidase-positive colonies growing from homogenates plated on cetrimide agar with streptomycin.

Histological analysis of mouse ceca. The histology of ceca obtained from mice given either antibiotics only, antibiotics plus Cy (three doses of 125 mg/kg every other day), or antibiotics plus RB6-8C5 MAb was reviewed by a veterinary pathologist. There was no evidence of any GI mucosal injury or damage in the group given antibiotic water only, the group given antibiotic water and Cy, or the group given antibiotic water and RB6-8C5 (<http://pierlab.bwh.harvard.edu/home/supplementarydata/>).

Determination of the relationship of Cy dose and virulence of *P. aeruginosa*. Because the expression of the ExoU cytotoxin is associated with increased virulence of *P. aeruginosa* in both animal models (2) and humans (22, 45), we chose to compare the effects of Cy dose on the survival of mice colonized with paired strains isogenic for the expression of ExoU and those carrying a control plasmid as well as other strains of *P. aeruginosa* known to produce ExoU. Groups of four mice were colonized with a *P. aeruginosa* strain (PA14, PA103, PAO1, PAO1 ExoU⁺, PAK, PAK ExoU⁺, 15921, or 15921 ExoU⁺), and then different groups received doses of 50, 75, 100, 125, or 150

mg of Cy/kg three times every other day. Prior to the first dose of Cy, GI colonization levels did not differ among any of the pairs of strains or among all of the strains and were comparable to those exhibited in Fig. 1 (data not shown). No mice died when three doses of 50 mg of Cy/kg were given (Table 3). In order for strain PAO1 to achieve 100% mortality, a dose of 125 mg of Cy/kg or higher was needed (Table 3), whereas strain PAO1 ExoU⁺ was able to cause 100% mortality with a dose of 75 mg of Cy/kg or higher. Strains PAK and PAK ExoU⁺ both caused 100% mortality with doses of 75 mg of Cy/kg or higher. Strains 15921 pUCP19 and 15921 ExoU⁺ were able to achieve 100% mortality with doses of 100 mg of Cy/kg or higher; however, strain 15921 ExoU⁺ did cause somewhat greater mortality at 75 mg of Cy/kg/dose ($P = 0.09$, Fisher's exact test). Both naturally occurring ExoU⁺ strains, PA14 and PA103, were able to achieve 100% mortality with doses of 75 mg of Cy/kg or higher (Table 3). Overall, strains expressing the ExoU cytotoxin were generally more virulent than strains not expressing this cytotoxin, except for strain PAK, wherein the parental strain was as virulent on its own as was the naturally occurring ExoU⁺ strain.

ExoU expression analyzed by immunoblot. To determine whether some of the differences in virulence among the ExoU⁺ strains were due to production of different levels of this protein, we probed culture supernates of the *P. aeruginosa* strains with an antiserum raised to ExoU. Applying 20 μ g or less of cell supernate proteins, the antiserum detected bands of the appropriate molecular size in supernates of cultures of strains PA103, PAO1 ExoU⁺, and PAK ExoU⁺ but not in supernates of strains PAO1, PA14, PAK, 15921, or 15921 ExoU⁺ (Fig. 5A). To determine why the extracellular proteins

TABLE 2. Survival of mice after administration of 0.2 mg of MAb RB6-8C5

Measurement	Survival after infection with:					
	No <i>P. aeruginosa</i>	PAO1	PAO1 ExoU ⁺	PAK	PAK ExoU ⁺	PA103
No. of survivors/no. challenged	8/8	1/8 ^a	0/8	0/8 ^b	1/8 ^c	0/8

^a Two mice were given an additional 0.2 mg of RB6-8C5 MAb on day 5.

^b One mouse was given an additional 0.2 mg of RB6-8C5 MAb on day 5.

^c Three mice were given an additional 0.2 mg of RB6-8C5 MAb on day 5.

TABLE 3. Survival of mice after administration of Cy

<i>P. aeruginosa</i> strain	No. of surviving mice/total no. of infected mice at a Cy dose (in mg/kg) of:				
	50	75	100	125	150
PAO1	4/4	4/4 ^a	4/4 ^a	0/4	0/4
PAO1 ExoU ⁺	4/4	0/4 ^a	0/4 ^a	0/4	0/4
PAK	4/4	0/4	0/4	0/4	0/4
PAK ExoU ⁺	4/4	0/4	0/4	0/4	0/4
15921 pUCP19	4/4	19/20 ^b	0/4	0/4	0/4
15921 ExoU ⁺	4/4	14/20 ^b	0/4	0/4	0/4
PA103	4/4	0/4	0/4	0/4	0/4
PA14	4/4	0/4	0/4	0/4	0/4

^a *P* = 0.03 by Fisher's exact *t* test comparing mice given the same dose of Cy and colonized by isogenic strains with and without ExoU expression.
^b *P* = 0.09 by Fisher's exact *t* test comparing mice given 75 mg of Cy/kg and colonized by isogenic strains with and without ExoU expression.

from *P. aeruginosa* strains 15921 ExoU⁺ and PA14 did not react with our ExoU antiserum, we increased the amount of supernate protein from the samples to 100 times that used in the initial experiment. In this setting, our antiserum was able to detect the appropriate-sized band in supernates of strain 15921 ExoU⁺ but still not in PA14 (Fig. 5A). Strain PA14 has previously been shown to be an ExoU-producing strain (34); we obtained the antiserum that Miyata et al. (34) had used previously (polyclonal rabbit IgG to ExoU) and performed the immunoblot again using the methods de-

scribed above. While this serum also did not detect ExoU bands in strain PA14 after 20 µg or less of supernate proteins was applied, when we increased the amount of supernate protein in the samples to 100 times that used in the initial experiment, proteins of the appropriate molecular size reacting with the antiserum to ExoU were detected (Fig. 5B). Apparently, strains 15921 ExoU⁺ and PA14 produce low levels of ExoU in vitro, but their virulence in our murine model is close to or at the level found to date for the more pathogenic ExoU⁺ strains, suggesting that there may be increased levels of ExoU made by these strains in vivo.

Effect of mutation in the *galU* gene on *P. aeruginosa* colonization. Previous work (37) in an earlier model of GI colonization indicated that immunity to *P. aeruginosa* LPS O antigens could reduce GI colonization levels of *P. aeruginosa*. To correlate this earlier finding with the contribution of LPS constituents to the virulence of *P. aeruginosa*, we created a strain with a mutation in the *galU* gene (41) that is needed for synthesis of the complete outer core of the LPS and attachment of O side chains but does not interfere with low-level elaboration of alginate. Since PAO1 Δ*galU* and its complemented counterpart are both gentamicin resistant (and the complemented strain is also tetracycline resistant), neither strain was made streptomycin resistant. In place of streptomycin, we used 0.25 mg of gentamicin/ml and penicillin G in the antibiotic water for initial GI decontamination. After water containing only the *P. aeruginosa* strains was used for 5 days, gentamicin and penicil-

FIG. 5. ExoU production by *P. aeruginosa* strains. (A) Immunoblot of concentrated culture supernatants grown in the presence of calcium chelator. Secreted proteins were precipitated with ammonium sulfate. The primary antibody was polyclonal rabbit IgG to ExoU (3) at a 1:100 dilution. Lane 9 contains supernate proteins from strain 15921 ExoU⁺ applied at 100 times the concentrations in lanes 1 to 8. (B) Immunoblot of concentrated culture supernatants produced as described in panel A, with a polyclonal rabbit IgG to ExoU prepared as described previously (34) as primary antibody. Lane 3 is extracellular ExoU produced by PA14, but extracellular proteins were added at 100 times the concentration used in the other lanes.

FIG. 6. Average CFU per gram of stool for PAO1 $\Delta galU$, the complemented strain PAO1 $\Delta galU$ (pCD204), the complemented mutant strain PAO1 $\Delta aroA$ (pMB1), and *aroA* mutant strain PAO1 $\Delta aroA$ on day -1 (before the first Cy dose) and for PAO1 $\Delta aroA$ on day +10 (day 6 after last Cy dose). Individual points show results for single mice, and horizontal lines represent the medians. *P* values were determined by a Mann-Whitney test.

lin were again placed in the drinking water. Strain PAO1 $\Delta galU$ failed to colonize any of the mice ($n = 12$), whereas the complemented counterpart [PAO1 $\Delta galU$ (pCD204)] colonized the mice at a high level (average, 2.61×10^8 CFU/g of feces; $n = 12$; standard error [SE], 1.37×10^7 CFU/g of feces) (Fig. 6), which was comparable to that of the parental PAO1 strain (Fig. 1). The drinking water was tested intermittently to ensure that PAO1 $\Delta galU$ could survive in this environment, and bacterial levels in the drinking water were found to be comparable to those of the complemented strain (data not shown). When these mice were given Cy at a dose of 125 mg/kg, all 12 of the mice given PAO1 $\Delta galU$ survived, whereas none of the mice colonized with the complemented strain survived (0 of 12; $P < 0.001$, Fisher's exact test). All deceased mice had evidence of PAO1 $\Delta galU$ (pCD204) dissemination, as determined by splenic homogenates yielding oxidase-positive colonies on cefrimide agar with both tetracycline and gentamicin.

Effect of mutation in the *aroA* gene on *P. aeruginosa* colonization and dissemination. PAO1 $\Delta aroA$ is a strain we have produced as a prototype of live, attenuated vaccines. This strain requires exogenous aromatic amino acids for growth and does not grow on minimal media lacking these factors. Strain PAO1 $\Delta aroA$ is highly attenuated in neutropenic mice challenged by the respiratory route, wherein 10^9 CFU of this strain per mouse is without significant effect, whereas <50 CFU of the parental PAO1 strain in similarly neutropenic mice is lethal to 100% of the challenged animals (42). To study the GI colonization and dissemination capabilities of this strain, both PAO1 *aroA*(pUCP19) (*aroA* deletion mutant with empty plasmid pUCP19) and its complemented counterpart containing the *aroA* gene in pUCP19 [PAO1 $\Delta aroA$ (pMB1)] were made streptomycin resistant. To verify the identity of the unmarked PAO1 $\Delta aroA$ (pUCP19), we documented that there was a lack of growth on minimal salts medium agar containing 0.5% glucose. PAO1 $\Delta aroA$ (pUCP19) did establish GI colonization, with an average of 5.44×10^4 CFU/g of feces ($n = 8$; SE, 1.81×10^4 CFU/g of feces), albeit at a significantly lower level ($P < 0.01$, Mann-Whitney test; median, 1.22×10^8 CFU/g of feces;

25th and 75th percentiles, 3.84×10^4 and 5.94×10^8 CFU/g of feces) than the complemented counterpart (average, 5.66×10^8 CFU/g of feces; $n = 12$; SE, 6.95×10^7 CFU/g of feces). After a Cy dose of 125 mg/kg was administered to mice colonized with PAO1 $\Delta aroA$ (pUCP19), no mice died of *P. aeruginosa* infection, although one did die from undefined causes, and the remaining survivors did not exhibit any signs of being ill or moribund. In contrast, all mice that were colonized with the complemented strain died after being given a Cy dose of 125 mg/kg. To confirm persistence of GI colonization in the surviving mice that were initially colonized with PAO1 $\Delta aroA$ (pUCP19), feces were collected from the surviving mice 6 days after the last Cy dose, and the average level of GI colonization had increased to 2.41×10^8 CFU/g of feces ($n = 7$; SE, 4.25×10^7 CFU/g of feces) (Fig. 6). Thus, the PAO1 $\Delta aroA$ (pUCP19) strain could obtain sufficient aromatic amino acids in the GI tract of the mouse to survive but was unable to disseminate from this tissue during severe neutropenia.

DISCUSSION

Finding appropriate settings in laboratory animals to study bacterial pathogenesis is a consistent challenge due to differences in the susceptibilities of animals to typical human pathogens and the lack of comparability of animal systems to those of humans at risk for infections from nosocomial pathogens like *P. aeruginosa*. As both mucosal colonization and dissemination in the face of neutropenia are features of human susceptibility to *P. aeruginosa* bloodstream infection, we made changes in protocols from previously reported animal models evaluating these aspects of infection to define parameters for a murine model that leads to reproducible GI colonization with numerous strains of *P. aeruginosa*. Interestingly, the levels of GI colonization were substantially higher than those achieved with a prior model of chronic mucosal colonization by *P. aeruginosa* developed in this laboratory (38). This higher rate of colonization is most likely due to the addition of penicillin to the water during the GI microbial flora depletion phase of the

experiments, leading to the elimination of endogenous anaerobic flora and subsequent greater intestinal overgrowth with *P. aeruginosa* (54). In order to assess whether transit through an acidic environment (the stomach) could affect survival and thus the resultant GI colonization levels, we tested numerous *P. aeruginosa* strains for pH sensitivity. Not surprisingly, none of the strains tested could survive in a pH of <3.0 (data not shown). Nonetheless, transit time through the stomach does not appear to be long enough to affect eventual colonization of the small intestine and large colon.

Two neutropenic rodent models of GI-derived *P. aeruginosa* bacteremia and sepsis have been reported previously (31, 36). Both models used antibiotics to disrupt the intestinal flora (intramuscular cefamandole [36] or i.p. ampicillin [31]) followed by ingestion of *P. aeruginosa* in the drinking water (10^6 to 10^7 CFU/ml). Neither model verified endogenous GI clearance nor quantified GI colonization levels with *P. aeruginosa*. Neutropenia was induced with i.p. Cy injection (150 to 200 mg/kg/dose for one or two doses). Dissemination was confirmed by culturing *P. aeruginosa* from the blood or homogenates of specific organs (lungs or liver). Both of these rodent models were used to evaluate therapeutic modalities rather than study pathogenesis.

In the murine model reported here, we employed three primary treatments that have been used to promote bacterial translocation in animal models: (i) disruption of the ecologic GI equilibrium, allowing intestinal bacterial overgrowth (e.g., use of antibiotic decontamination); (ii) increased permeability of the intestinal mucosal barrier (e.g., Cy-induced mucosal damage); and (iii) deficiencies in the host immune defenses (Cy-induced neutropenia and lymphopenia or RB6-8C5-induced neutropenia) (4).

The translocation studies showed that Cy alone does not cause morbidity and/or mortality. Mice that were fed *P. aeruginosa* water but that did not receive Cy showed evidence of sporadic but not sustained bacteremia and no substantial extraintestinal colonization of MLN. Interestingly, in prior studies investigating GI translocation of *E. coli*, it has been found that *E. coli* can translocate to the MLN by merely eliminating the anaerobic flora, and the bacteria can survive in MLN for days after elimination of the cecal flora (53). Thus, it is conceivable that the *P. aeruginosa* organisms were able to translocate from the GI tract to the MLN and then blood, even with the lack of immunosuppression. That the mice in these groups did not develop fatal septicemia is probably due to a combination of low bacterial burden in the blood (data not shown) and a competent immune system which was able to clear this low level of bacteremia. The lungs were not an additional site of *P. aeruginosa* colonization, which was a theoretical concern given that the mice could have aspirated the *P. aeruginosa* water and the bronchial mucosa could have thus been colonized. Widespread dissemination tended to occur on the sixth day after the first Cy dose (days 6, 7, and 8). Most importantly, this study confirmed that *P. aeruginosa* that colonizes the GI tract can systemically disseminate after Cy administration.

As might have been anticipated, the depth and duration of neutropenia were directly proportional to the dose of Cy. The ALC followed the same general trends as the ANC, which was not surprising given Cy's ability to suppress all hematopoietic lineages of cells. Profound and prolonged lymphopenia has

also been seen in human cancer patients receiving systemic chemotherapy (30), but the contribution of the lymphopenia to susceptibility to *P. aeruginosa* dissemination is not known.

It is abundantly clear that neutrophils play an important role in host defense against *P. aeruginosa* infections, including GI-derived bacteremia. It is unclear, however, whether it is only the neutropenia associated with the use of cytotoxic drugs or neutropenia plus other effects of the multitude of drugs used in cancer patients that so dramatically increases the susceptibility to *P. aeruginosa* infection. In an attempt to show the critical contribution of neutrophils to innate immune defense against *P. aeruginosa* after initial GI colonization, we tried to eliminate any potential Cy-induced GI mucosal damage and lymphopenia by using an alternative means of neutropenia induction. Neutrophil depletion by monoclonal antibody treatment (RB6-8C5 MAb) has been used in number of animal models of infection (7, 9, 12, 43, 51). RB6-8C5 MAb reacts with a common epitope on Ly-6G and Ly-6C, previously known as the myeloid differentiation antigen Gr-1 (19). In bone marrow, the level of antigen expression is directly correlated with granulocyte differentiation and maturation. Ly-6G and Ly-6C are also expressed on monocyte lineages in bone marrow but not on erythroid cells (25). In the periphery, RB6-8C5 MAb recognizes granulocytes (neutrophils and eosinophils) and monocytes (10, 29, 52). A single intraperitoneal 0.2-mg dose of RB6-8C5 induced 5 days of severe neutropenia. Interestingly, the ALC did decrease somewhat during this time period but was generally over 1,000. RB6-8C5 MAb is not known to target lymphocytes, although a recent study reported that the RB6-8C5 MAb caused almost complete elimination of a subset of Ly-6C⁺ memory-type CD8⁺ T cells as well as Ly-6G⁺ granulocytes (32). In any case, the degree of lymphopenia seen with RB6-8C5 MAb is not as profound as that seen with Cy doses of 75, 100, 125, and 150 mg/kg. We have also found that recombinase-activating gene-deficient mice are not any more susceptible to *P. aeruginosa* infection than are wild-type mice (A. Y. Koh, G. P. Priebe, and G. B. Pier, unpublished observation).

When we substituted RB6-8C5 MAb for the Cy doses in our murine model, we found that *P. aeruginosa* was consistently able to disseminate from the GI tract to the spleen. RB6-8C5 administration was without observable effects when given alone. In fact, the overall mortality with the MAb was comparable to that achieved when we used Cy. When we tried to assess whether the administration of various pharmacologic agents administered to the mice (oral antibiotics, Cy, or RB6-8C5 MAb) caused any degree of GI mucosal damage, we found that there was no significant histopathologic damage caused by any of the agents. This finding suggests that in our model neutropenia is sufficient to allow *P. aeruginosa* to translocate from the GI tract.

Interestingly, when we have used a similar murine model with *Candida albicans* (strain SC5314), we found that *C. albicans* was able to translocate from the GI tract when either Cy or methotrexate was given but not when RB6-8C5 was administered (A. Y. Koh and G. B. Pier, unpublished observation). In this case, it appears that *C. albicans* requires both neutropenia and chemotherapy-induced GI mucosal damage and/or chemotherapy-induced lymphopenia in order to translocate from the GI tract. This finding implies that different pathogens

may require different deficits in the host immune system in order to translocate from the GI tract.

Since we were unable to titrate finely the level of *P. aeruginosa* GI tract colonization in our model, we conducted Cy dose-response experiments with the goal of finding the minimal Cy dose needed to induce lethal bacteremia with a given *P. aeruginosa* strain. Given comparable GI colonization levels (e.g., inoculum), those strains inducing greater mortality at a lower Cy dose (e.g., less depth and shorter duration of neutropenia) would be considered more virulent. We evaluated this hypothesis using *P. aeruginosa* strains containing the *exoU* gene, which would be expected to be more virulent in animals (2). In general, the experiments suggested that the use of differing doses of Cy could be a means to differentiate virulence among strains of *P. aeruginosa*. The introduction of the *exoU* gene into *P. aeruginosa* strain PAO1 clearly increased its virulence, while the expression of ExoU by strain 15921 had a modest effect on its virulence. Immunoblot analysis suggests that the ExoU⁺ recombinant strain 15921 may only express low levels of ExoU, which could account for its not being quite as virulent as the other ExoU⁺ strains. Interestingly, there was no difference in the virulences of strains PAK and PAK ExoU⁺, with both strains causing dissemination and death at a dose of 75 mg of Cy/kg given three times. Immunoblot analysis confirmed the expected absence or presence of ExoU in these two strains. It appears that strain PAK has other factors that make it somewhat more virulent in our murine model than strains PAO1 and 15921, which do not express ExoU. Two other ExoU⁺ strains, PA14 and PA103, also demonstrated the highest virulence in the GI colonization-dissemination model, as defined by the low dose of Cy needed to effect mortality in the mice. This result occurred with strain PA14 in spite of the low levels of ExoU protein produced in vitro. Overall, for the most virulent *P. aeruginosa* strains (those producing ExoU protein), as well as for strain PAK, there was a threshold dose of 75 mg of Cy/kg that resulted in consistent dissemination and death, whereas for non-ExoU-producing strains, except PAK, a higher dose of Cy was needed for these effects. Thus, Cy dosing appears to be a general means to compare and contrast the virulence of *P. aeruginosa* strains and mutants in this model.

Further validation of the use of this model to measure other aspects of *P. aeruginosa* virulence was obtained with studies of other mutants made in the PAO1 background. The *galU* gene is critical for the production of UDP-glucose, one of the precursors for LPS core oligosaccharide biosynthesis, and *galU* mutants produce a truncated LPS core and concomitant absence of an O antigen (14). The $\Delta galU$ strain was unable to colonize or disseminate from the GI tract, whereas the transcomplemented strain had parental levels of colonization and virulence. The inability of the $\Delta galU$ mutant to establish GI colonization is consistent with a previously published work in which it was shown that levels of GI colonization were reduced when immunity to homologous O antigens was elicited (37). Together, these findings suggest that the production of unencumbered LPS O side chains is needed for GI colonization. LPS and O-antigen synthesis have also found to be critical in establishing GI colonization with *Yersinia pseudotuberculosis* (33) and *Yersinia enterocolitica* (48). More recently, an *E. coli* LPS deep-rough core mutant was shown to be unable

to colonize the mouse large intestine (35). However, it is not clear whether the complete LPS is needed for survival for anchoring the organisms to tissues or mucosal factors in the GI tract to establish colonization or is needed to resist the effects of antimicrobial factors, such as defensins and bile salts, which tend to be more active on LPS rough gram-negative bacteria (5).

Mutations in the *aroA* gene, which encodes an enzyme essential for the synthesis of aromatic amino acids, have been utilized with other pathogens, notably a *Salmonella* sp. (49) and *Aeromonas hydrophila* (24), for the production of live, attenuated vaccine strains. Aromatic amino acids are generally not available in host tissues and thus restrict the ability of $\Delta aroA$ strains to grow in mammals. However, we did find that the PAO1 $\Delta aroA$ strain could establish GI colonization, albeit at a significantly lower level than the complemented strain. This result indicates that the aromatic amino acids the organism must obtain exogenously are available in the murine GI tract. Following Cy treatment, there was no dissemination of the PAO1 $\Delta aroA$ strain, indicating insufficient levels of aromatic amino acids outside the GI tract for sustained growth of this mutant strain. Interestingly, the level of GI colonization with the PAO1 $\Delta aroA$ strain increased over 4,000-fold after Cy treatment and achieved a level comparable to that of parental strain PAO1, indicating that it was not the lower colonization level that prevented the PAO1 $\Delta aroA$ strain from disseminating. The results with both the $\Delta galU$ and $\Delta aroA$ strains provide further insight into how this model of GI colonization and dissemination in the face of severe neutropenia can be used to study various aspects of *P. aeruginosa* pathogenesis.

In conclusion, we have developed a reproducible murine model of *P. aeruginosa* GI colonization and systemic spread during neutropenia. The duration and intensity of neutropenia, related to the Cy dose, was found to be a means to compare the virulences of different *P. aeruginosa* strains, as defined primarily by comparisons of strains lacking or producing the virulence-enhancing ExoU cytotoxin. However, at least one strain lacking ExoU production, PAK, was as virulent as the ExoU-producing strains, indicating that other virulence factors could provide this added edge to some strains. In general, a dose of 75 mg of Cy/kg given three times appeared to allow the most virulent strains of *P. aeruginosa* to disseminate from the GI tract, whereas higher doses of 100 or 125 mg of Cy/kg given three times were needed for apparently less virulent strains. The LPS outer core polysaccharide and O side chains were critical in establishing GI colonization, and *P. aeruginosa* mutants lacking the *aroA* gene necessary for synthesizing aromatic amino acids were able to establish GI colonization but unable to disseminate. Thus, both the colonization and dissemination phases of *P. aeruginosa* pathogenesis can be studied in this model, which should prove useful not only for evaluating pathogenesis but for evaluating therapies and associated means to control *P. aeruginosa* nosocomial infections.

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