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Simple Model for the Study of *Pseudomonas aeruginosa* Infections in Leukopenic Mice

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A simple, reproducible model of fatal *Pseudomonas aeruginosa* sepsis in mice during immunosuppression was developed. Mice were rendered leukopenic (≤800 leukocytes per mm^3 of blood) for a period of 5 days by multiple injections of cyclophosphamide. Mice were challenged at the onset of leukopenia by instilling the bacteria onto a 0.5-mm incision made into the back. The mean lethal dose (LD_{50}) for *P. aeruginosa* PA220 and M-2 was less than 20 bacteria. The mean time to death for these strains ranged from 46 to 59 h. Leukopenic mice were comparatively resistant when challenged with *Klebsiella pneumoniae* ($LD_{50} = 1.5 \times 10^6$) or *Staphylococcus aureus* ($LD_{50} > 10^6$). Infection with *P. aeruginosa* was characterized by rapid bacterial multiplication in the skin at the site of infection, producing ecthyma gangrenosum. Bacteremia and colonization of the liver were pronounced 21 h postinfection. This model should prove to be a useful tool for studying the pathogenesis of *P. aeruginosa* infections under immunosuppressed conditions.

Patients who are rendered neutropenic as the result of either underlying malignancy or treatment with immunosuppressive agents are highly susceptible to *Pseudomonas aeruginosa* infections (14, 15, 17). The use of antibiotic therapy has met with only moderate success in controlling *P. aeruginosa* infections in this patient population. Studies utilizing immunotherapeutic means to control *P. aeruginosa* infections in cancer patients have yielded equivocal results (4, 11, 21).

Given the large number of factors which may contribute to the virulence of *P. aeruginosa* (9, 20), relevant animal models are needed, not only to study the pathogenesis of infections under leukopenic conditions, but also to identify the protective antigens. Current models are hampered by one or more of the following factors: (i) the use of large animals (dogs) (2); (ii) a large challenge inoculum (≥10^7 bacteria) is necessary to produce a high mortality rate (2, 3, 22); (iii) the route by which the challenge inoculum is administered (intravenous or intraperitoneal bolus) bears little resemblance to the route(s) by which infection is believed to occur in humans (2, 3, 18); and (iv) the course of experimental infection proceeds under conditions of transient leukopenia (7) or when the leukocyte count is unknown (3).

In the present report, we describe a simple, reproducible model of fatal *P. aeruginosa* sepsis in leukopenic mice. By using a regimen of multiple cyclophosphamide injections, mice were rendered neutropenic throughout the entire course of infection. The challenge was instilled onto a traumatized area of the back and resulted in an ecthyma gangrenosum-type lesion within 48 h. These features make the model suitable for studying the pathogenesis of *P. aeruginosa* infections under immunosuppressed conditions and for the evaluation of therapeutic agents.

(All animal studies conformed to all requirements listed in the Swiss Federal Government Guidelines for Protection of Animals, May 27, 1981.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* PA220 was kindly provided by B. Wretlind, Karolinska Institute, Stockholm, Sweden. *P. aeruginosa* M-2 was a gift of I. A. Holder, Shriners Burns Institute, Cincinnati, Ohio. Various characteristics of these strains have been described elsewhere (10, 16). Both strains produce toxin A and elastase. *Klebsiella pneumoniae* BL-1151 and *Staphylococcus aureus* BL-631 were human bacteremic isolates provided by H. Wetzstein, University of Berne, Institute of Hygiene, Berne, Switzerland. Cultures were maintained lyophilized and routinely grown on the TSBD medium of Bjorn et al. (1) at 37°C with rigorous aeration, except for *S. aureus* BL-631, which was cultured on brain heart infusion broth (Difco Laboratories, Detroit, Mich.).

Leukocyte quantitation. Blood samples were collected directly into a leukocyte pipette from the tail vein and immediately mixed with 9 volumes of Türk solution (E. Merck and Co., Darmstadt, West Germany) for 15 min before counting. Values are expressed as

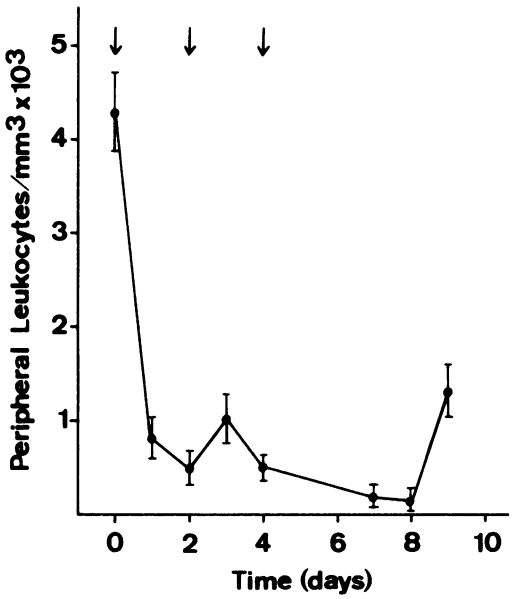

FIG. 1. Effect of cyclophosphamide administration on the circulating leukocyte count. Each time point represents the mean ± standard error of the mean of three mice. Arrows indicate times of cyclophosphamide administration.

the number of leukocytes per cubic millimeter of blood.

Experimental infection. Outbred male Swiss Webster white mice, 20 to 22 g, were used. Leukopenia was induced by an intraperitoneal injection of cyclophosphamide (Aldrich Chemical Co., Milwaukee, Wis.) in 0.25 ml of phosphate-buffered saline (PBS) (pH 7.4) on days 0, 2, and 4. The dose of cyclophosphamide routinely used was 150 μg/g of mouse weight.

The bacterial challenge was prepared as follows. A mid-log culture (absorbance at 540 nm ≈ 0.7 measured against water) was harvested by centrifugation and washed in 10 ml of PBS and resuspended in PBS. The culture was appropriately diluted in PBS and kept on ice until used (within 1 h). Viable counts were determined from each challenge inoculum.

Mice were challenged on day 4 of the cyclophosphamide administration schedule. The backs were shaved, and mice were anesthetized by an intraperitoneal injection of 100 μg of Valium (Hoffmann La Roche, Basel, Switzerland), followed by a subcutaneous injection of 0.2 ml of Hypnorm (Duphar, B. V., Amsterdam, The Netherlands) containing 10 mg of fluanisol and 0.2 mg of fentamyl base per ml. An incision (approximately 0.5 cm in length) was made with a surgical scalpel. The challenge inoculum, in 5 μl, was applied directly onto the wound with a sterile-tipped micropipette. Animals were then returned to their cages and given food and water. Mice routinely recovered from the anesthesia with 2 h. The mortality due to the anesthesia was less than 5%. For each experiment, the leukocyte count from three to five mice was determined to confirm a state of leukopenia (≤800 leukocytes per mm^3 of blood).

Histology. Full-thickness specimens were taken from the site of infection and fixed in Bouin solution (75 ml of picric acid, 25 ml of formaldehyde, and 5 ml of acetic acid) for 4 h. Specimens were then dehydrated in a stepwise ethanol gradient (70 to 100%) and treated for 8 h in benzyl benzoate. Samples were treated in benzol for 16 h, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Quantitation of *P. aeruginosa* in the blood and tissue. Groups of three mice were sacrificed at various times postchallenge, and the number of *P. aeruginosa* organisms in the blood, liver, and skin at the site of infection was determined. Blood samples were collect-

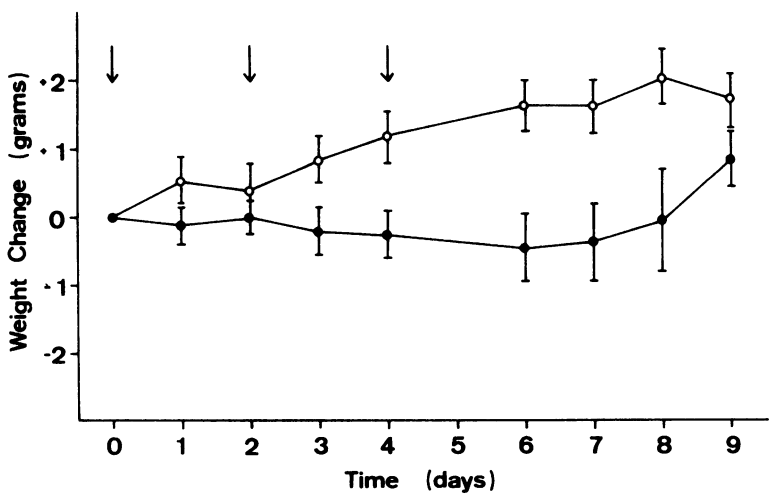

FIG. 2. Weight gain curves for mice receiving cyclophosphamide (●) or PBS (○). Each time point represents the mean ± standard error of the mean for groups of 10 mice. The mean weight at day 0 was 20.75 g. Arrows indicate times of cyclophosphamide administration.

ed in sodium citrate-treated plastic syringes. A full-thickness skin specimen (3 to 500 mg [wet weight]) was obtained from the challenge site, weighed, and homogenized in 10 ml of PBS for 2 min in a Sorvall Omnimixer (DuPont Co., Newtown, Conn.). Livers were aseptically removed and homogenized in 10 ml of PBS. Appropriate dilutions made in PBS were plated on Trypticase soy agar plates (BBL Microbiology Systems, Cockeysville, Md.).

Statistical analysis. The mean lethal dose (LD_{50}) was calculated by the method of Reed and Muench (13).

RESULTS

It was our desire to develop experimental conditions under which the course of *P. aeruginosa* infections could be studied against a background of persistent leukopenia. Prior results from other laboratories (7) and our initial studies (data not shown) demonstrated that a single dose of cyclophosphamide induced only a transient state of leukopenia lasting approximately 2 days. Therefore, multiple injection regimens were tested. The intraperitoneal injection of cyclophosphamide on days 0, 2, and 4 at a dose corresponding to 150 μg/g of mouse weight was found to be optimal. This regimen resulted in a rapid drop in the peripheral leukocyte count and induced a state of leukopenia (≤800 leukocytes per mm^3 of blood) lasting for approximately 5 days (Fig. 1). Bone marrow recovery was evident by day 9. Cyclophosphamide administered in such a manner was only rarely lethal (mortality rate, less than 5%). Mice routinely survived for 14 days after the initial injection.

The toxicity of cyclophosphamide, given as described above, was gauged by comparing the weight gain curves for mice receiving cyclophosphamide to curves for those which received only PBS (Fig. 2). A slight weight loss extending from day 3 through 7 was noted in the cyclophosphamide-treated group. However, mice did begin to gain weight normally by day 8. Therefore, although the described cyclophosphamide regimen did induce a protracted state of leukopenia, it was not unacceptably toxic for mice.

Minor trauma to a surface colonized with *P. aeruginosa* is believed to be a primary route of infection in neutropenic patients (22). We therefore chose to initiate the infection in our experimental model by instilling the challenge organisms onto a small incision on the backs of leukopenic mice. We found this procedure to be both simple and rapid. Ecthyma gangrenosum, a skin lesion commonly associated with *P. aeruginosa* infections, developed within 48 h after challenge with fewer than 15 *P. aeruginosa* PA220 organisms. Leukopenic mice sham-injected with PBS routinely survived. The incision usually healed within 3 to 4 days with no overt sign of infection. Microscopic sections of the area immediately surrounding the incision 48 h postinfection showed necrosis and marked dilation of blood vessels. No inflammatory reaction was noted, and few granulocytes were observed.

Normal mice were resistant to challenge with greater than 10^5 *P. aeruginosa* PA220 or M-2 organisms (Table 1). However, leukopenic mice were exquisitely sensitive to infection with these two strains. Less than 15 organisms of either strain was sufficient to kill greater than 85% of the mice.

Next, the susceptibility of leukopenic mice to challenge with *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* was studied (Table 2). Mice were found to be far more susceptible to infection with *P. aeruginosa* than with either *K. pneumoniae* or *S. aureus*. The LD_{50} for both *P. aeruginosa* challenge strains was less than 15 organisms. Even at such low challenge doses, death occurred within 46 to 58 h. In contrast, leukopenic mice were comparatively resistant to challenge with either *K. pneumoniae* or *S. aureus* (LD_{50}, 10^6 bacteria).

To better characterize the experimental model described, normal and leukopenic mice were challenged with *P. aeruginosa* PA220, and the course of the infection was studied by monitoring the multiplication and dissemination of the bacteria (Fig. 3). In normal mice, the bacterial challenge remained localized at the site of infection, with little increase in number. By 48 h postinfection, 66% of skin specimens possessed undetectable amounts of bacteria (<100 per g of skin). No evidence of bacteremia or liver colonization was noted. In contrast, rapid bacterial multiplication occurred in the skin of leukopenic mice after a delay of approximately 4 h. Bacter-

TABLE 1. Effect of cyclophosphamide on the susceptibility of mice to *P. aeruginosa* infections

Challenge strain	Challenge dose[a]	Cyclophosphamide administered	Mortality rate[b] (%)
P. aeruginosa PA220	1.2×10^1	Yes	100
P. aeruginosa PA220	1.75×10^5	No	0
P. aeruginosa M-2	0.8×10^1	Yes	83
P. aeruginosa M-2	1.6×10^5	No	0

[a] Number of viable organisms.
[b] Groups of six mice were used.

TABLE 2. LD_{50} and mean time to death for *P. aeruginosa* PA220 and M-2, *K. pneumoniae*, and *S. aureus* BL-631 in leukopenic mice

Challenge strain	Determination 1		Determination 2	
	LD_{50}[a]	Mean time to death (h ± SD)	LD_{50}[a]	Mean time to death (h ± SD)
P. aeruginosa PA220	0.63×10^1	45.8 ± 4.2	1.2×10^1	58.6 ± 14
P. aeruginosa M-2	0.83×10^1	49.3 ± 4.5	0.8×10^1	48 ± 0.5
K. pneumoniae BL-1151	1.5×10^6	58.4 ± 8.7	ND[b]	ND
S. aureus BL-631	10^{6c}		ND	ND

[a] Number of viable bacteria. Groups of six mice were used.
[b] ND, Not determined.
[c] Highest challenge dose tested; 90% of animals survived.

emia and colonization of the liver was noted 21 h postinfection. In leukopenic mice sham-infected with PBS, the blood was usually sterile (≤10 bacteria per ml). However, approximately 30% of livers and skin specimens contained detectable, but low, numbers of *Escherichia coli* and nonhemolytic streptococci.

DISCUSSION

The management of *P. aeruginosa* infections in immunosuppressed patients continues to be a persistent problem, even with the introduction of improved antibiotic regimens and supportive therapy (14, 15, 17, 19). Antibodies to several *P. aeruginosa* antigens have been shown to confer protection in several experimental animal models (8, 10, 12); however, evaluation under immunosuppressed conditions has been limited (5, 6). Although a large number of putative *P. aeruginosa* virulence factors have been identified (9, 20), their role in the pathogenesis of infections during leukopenia has not been studied in depth (22). To facilitate such studies, relevant animal models are needed.

The experimental model described in the present report possesses several clinically relevant features which would make it suitable for studying various aspects of *P. aeruginosa* infections under immunosuppressed conditions. An important feature of this model is that a fatal infection can be established locally with a very low challenge dose ($LD_{50} < 20$ *P. aeruginosa* organisms). The lesion observed (ecthyma gangrenosum) at the site of traumatization 48 h postinfection is commonly seen in human patients suffering from *P. aeruginosa* infections. Additionally, local proliferation of the challenge is followed by invasion of the blood stream and dissemination to the liver. This is in contrast to several other leukopenic models in which either a high dose of challenge organisms (10^7 to 10^8 bacteria) (2, 3, 22) or a systemic challenge (2, 3, 18) is used.

Our findings are consistent with those of Ziegler and Douglas (22) in that leukopenic animals appear to be rendered susceptible only to infection with *P. aeruginosa*. Although we could establish lethal infections with *K. pneumoniae*, the LD_{50} value was approximately 5 orders of magnitude greater than that for *P. aeruginosa*. Which specific attributes of *P. aeruginosa* allow for its increased virulence in leukopenic animals is unknown.

We feel that the model described in the present report will prove to be a useful tool in studying *P. aeruginosa* infections under immunosuppressed conditions. Its simplicity and reproducibility, together with the fact that small experimental animals are used, should also facilitate studies aimed at developing immunotherapeutic agents.

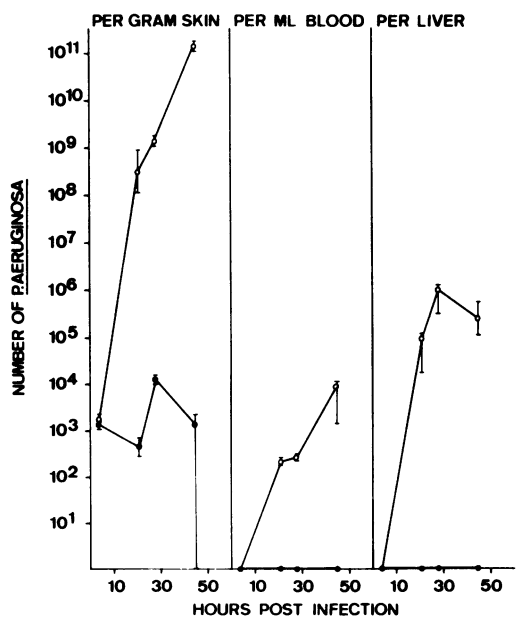

FIG. 3. Isolation of *P. aeruginosa* PA220 from the skin, blood, and liver after challenge with 10^3 organisms. Each point represents the mean ± standard error of the mean of three mice. Symbols: ○, mice that received cyclophosphamide; ●, mice that received PBS before infection.

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