

## Murine Gastrointestinal Tract as a Portal of Entry in Experimental *Pseudomonas aeruginosa* Infections

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Peroral administration of viable *Pseudomonas aeruginosa* into the stomach of mice resulted in an acute systemic infection, with death occurring within 72 h. One strain, ATCC 19660, a non-encapsulated form of *P. aeruginosa*, had a median lethal dose of  $5.3 \times 10^6$  colony-forming units, whereas two encapsulated strains, ATCC 17933 and 17934, had median lethal dose values of  $5.0 \times 10^7$  and  $5.6 \times 10^7$  colony-forming units, respectively. Each strain required fewer organisms to establish a lethal infection via the stomach than by intravenous or intraperitoneal routes. The non-encapsulated strain, ATCC 19660, did not cause any diarrhea in the infected animals, whereas the two encapsulated strains, although less virulent, caused diarrhea when administered perorally. No signs of necrosis were noted within the gastrointestinal tract; however, hematogenous spread of the organism resulted in a vasculitis associated with the pulmonary vessels and bacterial invasion of the renal tissues. Treatment of animals with antineoplastic drugs 24 h before or simultaneously with peroral challenge resulted in an increased susceptibility to infection.

Over the past few years, the importance of *Pseudomonas aeruginosa* as a human pathogen in a variety of infections has increased dramatically (4). Of particular interest is its increasing incidence in nosocomial infection of individuals already debilitated by other diseases or by antineoplastic chemotherapy (10, 12). However, one area of interest that has been relatively neglected is the involvement of this organism in gastrointestinal infections that may lead to systemic spread. There have been sporadic clinical reports of *P. aeruginosa* causing diarrhea and gastrointestinal disturbances, and descriptions of these date back as far as the late nineteenth century (3, 16). In at least one well-documented report there was an extensive outbreak of nosocomial infection of infants in a nursery that resulted in several deaths due to ingestion of pasteurized milk subsequently contaminated with *P. aeruginosa* (11). Concomitantly, there were several hundred cases that occurred in children and adults in the surrounding community. The sequence of events that occurred was diarrhea, vomiting, dehydration, cyanosis, abdominal pain, and fever. The experimental feeding of live organisms to normal healthy humans (1) or mice (8) in an attempt to cause gastrointestinal disturbances or systemic infections has been unsuccessful. Nevertheless, current comparative median lethal dose (LD<sub>50</sub>) studies in our laboratory have

indicated that mice are more prone to infection by the peroral route than by parenteral routes, and that systemic infection leading to death occurs. Green et al. (7) have also recently shown that a significant decrease of *Pseudomonas* infections in burn patients occurs after the elimination of fresh vegetables harboring *Pseudomonas* from their diet. Consequently, the purpose of this manuscript is to present an experimental model that describes the response of normal mice and antineoplastic drug-treated mice infected with *P. aeruginosa* perorally into the stomach.

### MATERIALS AND METHODS

**Organisms.** Three ATCC strains were used throughout the entire study. One strain, ATCC 19660, was a non-encapsulated form of *P. aeruginosa*, whereas the other two strains, ATCC 17933 and ATCC 17934, were both encapsulated.

**Cultivation.** The organisms were cultivated by inoculating a 125-ml Erlenmeyer flask containing 50 ml of 5% peptone (Difco, Detroit, Mich.) and 0.25% Trypticase soy broth (BBL, Cockeysville, Md.). The cells were grown for 18 h at 37°C with constant aeration. The cultures were then centrifuged at  $48,000 \times g$  for 20 min, and the resulting pellet was resuspended in nonpyrogenic saline and adjusted to the desired cell concentration by using a predetermined curve relating viable counts to optical density at 440 nm with a spectrophotometer (Coleman Jr. II, model 6/20). To establish the number of viable cells

administered to each animal, serial dilutions were made and tryptose agar plates (Difco, Detroit, Mich.) were inoculated and read for growth after 24 h. Because the cells tended to undergo autolysis over extended periods of time or upon additional centrifugation, they were used immediately for the *in vivo* studies.

**In vivo studies.** Cell suspensions were administered to Swiss-Webster female mice (Spartan Research Animals, Inc., Haslett, Mich.) weighing between 18 and 22 g. The LD<sub>50</sub> determinations of colony-forming units (CFU) for animals receiving living or dead cells were done by using a 15-day end point. Approximately 1,250 mice were used in this study. The final LD<sub>50</sub> values were calculated by the method of Reed and Muench (13), using equally spaced 10-fold dilutions after a preliminary titration. At least 30 mice were challenged per LD<sub>50</sub> value, using six mice per dose at five different dilutions. The peroral administrations into the mouse stomach of the challenging dose were achieved by intraperitoneal (i.p.) administration to each animal of an anesthesia of 70 mg of sodium pentobarbital per kg (Veterinary Laboratories, Inc., Lenexa, Kan.) and 0.45 mg of atropine sulfate per kg (W. A. Butler Co., Columbus, Ohio). Both drugs were given simultaneously and a 15-min waiting period before challenge was used to allow optimal effect of the atropine sulfate to reduce gut motility and bronchial secretions. The cell inoculum was given in 0.01-ml doses by using a 0.01-ml graduated 1.0-ml Hamilton microsyringe no. 0010 (Hamilton Co., Whittier, Calif.), which was mounted on a ring stand. A screw-mediated turning device was used to steadily administer the inoculum, which was contained in a sterile, intramedic polyethylene tubing (Clay Adams, Parsippany, N.J.). The microsyringe was filled with Fluorinert Fc48, density 1.93 g/ml (Instrumentation Specialities, Co., Lincoln, Neb.), which forced the bacterial suspension through the tubing. A male adapter was attached to the tube, and a 3-inch cannula needle was connected to the adapter. Peroral administration of organisms was performed by gently guiding the cannula down the esophagus into the stomach. Gross and histological examination revealed no esophageal damage associated with this procedure. After infection, six mice were placed in each cage and a heating lamp was used to warm them in order to facilitate a faster recovery from the anesthesia. Control animals were administered sterile saline or heat-killed cells instead of viable organisms after receiving the anesthesia. For comparative purposes, animals were also injected with various dilutions of organisms i.p. or intravenously (i.v.) into the tail vein. Both the i.p. and i.v. injections were given in 0.5-ml volumes with the desired concentration of viable organisms. Other control animals were also injected i.p. or i.v. with 0.5 ml of saline or heat-killed cells. To test the effect of anesthesia on lethality, groups of mice were given anesthesia, saline perorally, and simultaneous i.v. or i.p. bacterial challenge.

**Antineoplastic agents used to predispose mice to infection.** Several antineoplastic drugs were used in an attempt to predispose the mice to peroral

administration of *P. aeruginosa*. The drugs were administered parenterally in the following concentrations: methotrexate (Lederle Laboratories, Pearl River, N.Y.), 160 mg/kg; vincristine sulfate (Oncovin, Eli Lilly and Co., Indianapolis, Ind.), 1.25 mg/kg; and cytosine arabinoside (Cytosar, The Upjohn Co., Kalamazoo, Mich.), 500 mg/kg. All drugs were diluted to the desired concentrations in non-pyrogenic saline and used immediately. The antineoplastic drugs were given in 0.5-ml volumes via the i.p. route either simultaneously or 24 h before the peroral bacterial challenge. Uninfected control animals were tested to determine the effect of the predisposing agents. The treated animals were then challenged with either nonpyrogenic saline or heat-killed cells.

**Histopathological studies.** After gross postmortem observations were recorded, the heart, lungs, liver, spleen, stomach, intestines, pancreas, kidneys, and portions of blood vessels were removed and fixed in phosphate-buffered formalin, pH 7.2. Sections were cut on a rotary microtome and stained with hematoxylin and eosin.

**Effect of antineoplastic agents on circulating leukocytes.** Phlebotomies were done on animals by puncture of the orbital plexus of the right eye to determine what effect the various chemical treatments had on the number of circulating leukocytes. A heparinized microhematocrit pipette (Clay Adams) was used to initiate bleeding, and the blood sample (20  $\mu$ l) was taken by using the Unopette System (BD and Co., Rutherford, N.J.). An improved Neubauer hemacytometer was used to make the leukocyte determinations, and differential counts were made by using Giemsa-stained slides.

## RESULTS

Initial experiments were designed to determine whether normal, undebilitated mice were susceptible to infection when organisms were administered perorally into the stomach. Using a 15-day holding period, it was established that normal mice were susceptible to infection by this route and that all three ATCC strains were able to produce lethal infections (Table 1). Of the three strains, the non-encapsulated strain (19660) appeared to be the most virulent on the basis of cell numbers needed to produce death

TABLE 1. Comparative summary of LD<sub>50</sub> values for viable *Pseudomonas aeruginosa* in normal mice

Route of administration	LD <sub>50</sub> values <sup>a</sup>		
	Strain 17933	Strain 17934	Strain 19660
Peroral (stomach) <sup>b</sup>	5.0 × 10 <sup>7</sup>	5.6 × 10 <sup>7</sup>	5.3 × 10 <sup>8</sup>
Intraperitoneal <sup>c</sup>	1.1 × 10 <sup>8</sup>	7.2 × 10 <sup>8</sup>	3.6 × 10 <sup>7</sup>
Intravenous <sup>c</sup>	3.6 × 10 <sup>8</sup>	6.4 × 10 <sup>8</sup>	3.3 × 10 <sup>7</sup>

<sup>a</sup> Average of duplicate determinations calculated by method of Reed and Muench (minimum of 30 animals/determination).

<sup>b</sup> A 0.01-ml inoculum.

<sup>c</sup> A 0.5-ml inoculum.

in the animals. The LD<sub>50</sub> values for strains 19660, 17933, and 17934 were  $5.3 \times 10^6$ ,  $5 \times 10^7$ , and  $5.6 \times 10^7$  CFU, respectively. All controls consisting of 53 animals receiving up to  $10^9$  viable organisms perorally into the lungs did not die, indicating that mice infected by the stomach route were not dying from aspiration of organisms into the lungs.

Although animals were observed for 15 days, all deaths were the result of acute infection and occurred within 72 h after the peroral challenge. Viable *P. aeruginosa* was routinely cultured from the heart, blood, kidney, and other internal organs at necropsy. Those animals that survived the first 72 h spontaneously recovered and showed no subsequent symptoms. Anesthetized (control) animals administered saline perorally and simultaneously injected with each of the three strains i.p. or i.v. yielded LD<sub>50</sub> values identical to unanesthetized animals challenged by the same routes. For comparative purposes, the mice were also infected by the i.v. and i.p. routes, and the LD<sub>50</sub> values of the three ATCC strains (Table 1) were obtained. In all cases, the i.v. or i.p. injections yielded LD<sub>50</sub> values for each respective strain that were greater than the peroral value, indicating that the normal mice were more susceptible to bacterial challenge via the stomach than by these parenteral routes. In addition, the non-encapsulated strain (19660) appeared to be more virulent than the encapsulated strains regardless of the route of infection. However, the non-encapsulated strain (19660) did not produce any diarrhea in the infected animals, whereas the two encapsulated strains (17933 and 17934), although less virulent than the non-encapsulated strain, produced diarrhea when administered perorally into the stomach. The diarrhea, however, was only observed in animals that eventually died and only in concentrations of organisms at or above the LD<sub>50</sub>

values of the encapsulated strains. The onset of the diarrhea was rapid and occurred within 24-h post-peroral challenge. No diarrhea was produced by these encapsulated strains when they were administered i.v. or i.p.

Peroral administration of heat-killed cells was also performed to determine whether death or diarrhea would result. However, none of the test strains produced death or a diarrheal condition when the challenge doses were as high as an equivalent dose of  $1 \times 10^8$  CFU.

Attempts to increase the susceptibility of mice to peroral infection by treatment with antineoplastic drugs with the non-encapsulated strain (ATCC 19660) and one of the encapsulated strains (ATCC 17933) resulted in the following observations. The effects of antineoplastic drugs on depressing the natural immunity of animals that were challenged with strain 17933 are shown in Table 2. The greatest increase in susceptibility to a lethal infection was produced in animals treated with methotrexate, since drug treatment 24 h before and simultaneously with the challenge produced LD<sub>50</sub> decreases of 208- and 588-fold, respectively. Reduced LD<sub>50</sub> values were also demonstrated in animals that received vincristine sulfate or cytosine arabinoside, but of a lower magnitude.

Antineoplastic drug treatment of animals challenged with the non-encapsulated strain 19660 produced results similar to those observed with the encapsulated strain, with methotrexate-treated animals showing the greatest decrease in LD<sub>50</sub> value but not as great as resulted in 17933-challenged animals. Also, little change was produced in animals treated with cytosine arabinoside or vincristine sulfate.

When antineoplastic drug-treated animals were challenged with heat-killed cells of either 17933 or 19660 (Table 3), only animals treated with methotrexate were susceptible. Treatment

TABLE 2. Effect of antineoplastic drugs on the lethality of viable *Pseudomonas aeruginosa* administered perorally into the stomachs of mice

Antineoplastic drug administered	Time of drug administration relative to bacterial challenge	Strain 17933		Strain 19660	
		LD <sub>50</sub> value <sup>a</sup>	Fold decrease in LD <sub>50</sub>	LD <sub>50</sub> value <sup>a</sup>	Fold decrease in LD <sub>50</sub>
None	None	$5.0 \times 10^7$	1.0	$5.3 \times 10^6$	1.0
Methotrexate (160 mg/kg)	24 h before	$2.4 \times 10^5$	208.0	$6.1 \times 10^5$	8.7
	Simultaneously	$8.5 \times 10^4$	588.0	$1.4 \times 10^5$	38.0
Vincristine sulfate (1.25 mg/kg)	24 h before	$4.7 \times 10^6$	10.6	$6.7 \times 10^5$	7.9
	Simultaneously	$3.4 \times 10^7$	1.5	$3.0 \times 10^5$	17.7
Cytosine arabinoside (500 mg/kg)	24 h before	$1.3 \times 10^7$	4.0	$1.9 \times 10^6$	3.0
	Simultaneously	$8.4 \times 10^6$	6.0	$3.4 \times 10^6$	1.6

<sup>a</sup> Calculated by method of Reed and Muench (minimum of 30 animals/determination).

TABLE 3. Effect of antineoplastic drugs on the lethality of heat-killed *Pseudomonas aeruginosa* administered perorally into the stomach of mice

Antineoplastic drug administration	Time of drug administration relative to bacterial challenge	Drug <sup>a</sup> alone (saline challenge)	Heat-killed cells <sup>b</sup>	
			Strain 17933 <sup>c</sup>	Strain 19660 <sup>c</sup>
None			0/6	0/6
Methotrexate (160 mg/kg)	24 h before	0/17	4/12	6/14
	Simultaneously	2/30	4/11	8/12 <sup>c</sup>
Vincristine sulfate (1.25 mg/kg)	24 h before	0/12	0/5	0/4
	Simultaneously	0/12	0/5	0/6
Cytosine arabinoside (500 mg/kg)	24 h before	0/17	0/5	1/14
	Simultaneously	0/17	0/5	0/6

<sup>a</sup> Expressed as (total number dead)/(total number challenged).

<sup>b</sup> The heat-killed cell challenge dose was  $1.0 \times 10^8$  CFU.

<sup>c</sup> The LD<sub>50</sub> value determined was  $6.61 \times 10^6$  CFU.

of animals with methotrexate and simultaneous challenge with heat-killed cells of strain 19660 resulted in an LD<sub>50</sub> value equivalent to  $6.61 \times 10^6$  CFU. Also, further studies showed that no deaths occurred in the methotrexate-treated animals when the challenging dose of heat-killed 17933 or 19660 was equal to the LD<sub>50</sub> value of viable cells. In control animals, lethality resulted in only 2 of 30 animals when drug treatment was followed by a saline challenge at the appropriate time.

Histopathological studies of the acutely ill animals that had received peroral administrations of viable cells with or without drug treatment revealed no apparent differences between the three strains in the type or magnitude of inflammation. All inflammatory responses were acute in nature, with toxic changes such as hepatocellular degeneration and glomerular hypercellularity noted in those animals receiving sublethal doses. Examination of the lung parenchyma of animals that had died within 72 h revealed the pathognomonic vasculitis associated with systemic *Pseudomonas* infections found around the small and medium size blood vessels. Cellular exudate consisted of both polymorphonuclear and mononuclear inflammatory cells, with the latter predominating (Fig. 1). Also present within the lungs was alveolar wall thickening with early parenchymal abscess formation. There was also invasion of renal tissue evident in animals that had died within 72 h. The kidneys not only had infiltration of the glomeruli and surrounding tissue by mixed inflammatory cells, but organisms could also be seen within the glomeruli. Examination of the stomach and intestines did not reveal any gross lesions in the gastrointestinal tract. In addition, histological sections of the intestines were void of any focal areas of inflammation or necrosis. There was, however, gross enlarge-

ment of the mesenteric lymph nodes, which, under microscopic examination, did not show any evidence of bacteria. No gross or histopathological changes were detected in animals challenged perorally into the lungs.

Phlebotomies were done to determine what effect antineoplastic drugs used to treat the animals had on the number and types of circulating leukocytes. All three of the drugs tested were able to produce a lymphopenia within 24 h after administration. It appears that although cytosine arabinoside (Fig. 2) caused the greatest depression on lymphocyte numbers after 24 h, the drug had no effect on the number of circulating neutrophils. Both vincristine sulfate (Fig. 3) and methotrexate (Fig. 4) were, however, able to reduce neutrophil numbers during the first 48 h, with methotrexate continuing to exert a depressive effect for another 48 h. All values given were adjusted for the effect of multiple bleedings on saline-treated animals.

## DISCUSSION

Although there are a number of organisms that are classically associated with gastrointestinal infections, the potential role of *P. aeruginosa* in this type of infection has been generally overlooked. However, the present studies indicate that *P. aeruginosa* can cause an acute systemic infection, leading to death of mice within 72 h when the organisms are administered perorally into the stomach. Conceivably, from this site they could then spread via the lymphatics and eventually hematogenously to all of the major organs of the body. The presence of lesions in the kidneys and lungs is consistent with previous findings in our laboratory using animals infected by various parenteral routes (2, 6). Hammond et al. (8) have demonstrated that the intestinal tract is the

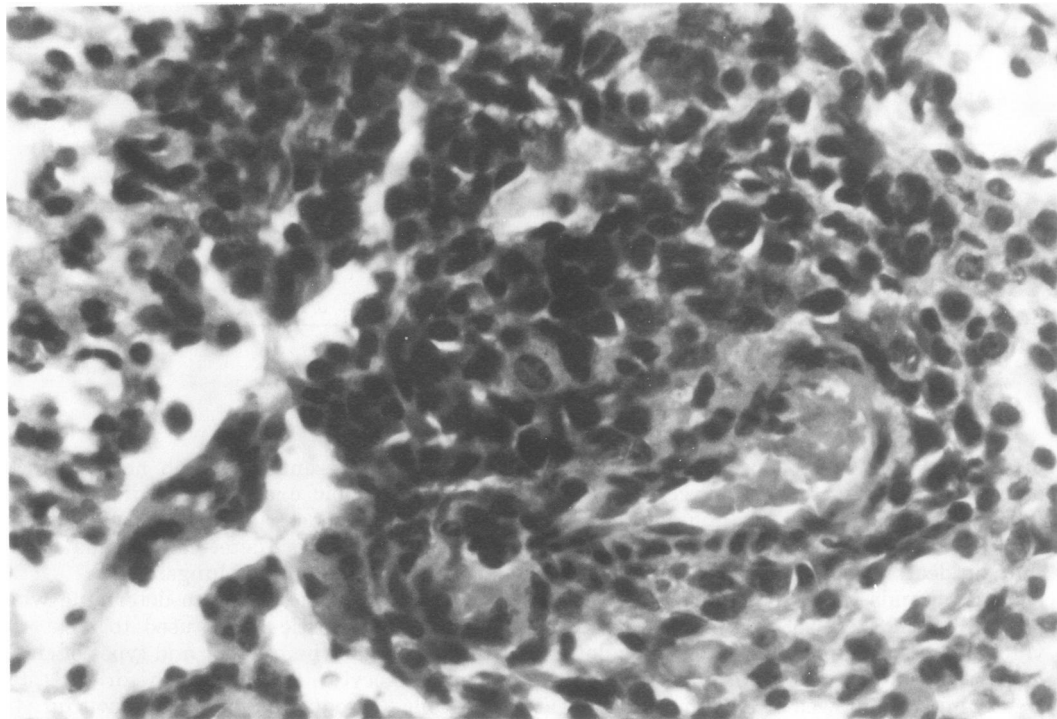


FIG. 1. Lung section from a mouse receiving *P. aeruginosa* perorally. This area shows consolidative changes with a plethora of round-cell infiltrates with occasional polymorphonuclear cells. Vascular infiltrates were also quite extensive.  $\times 310$ .

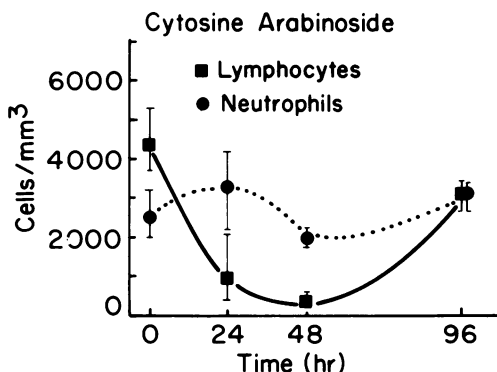


FIG. 2. Effect of cytosine arabinoside on circulating lymphocytes and neutrophils.

site of bacteremia, especially after X irradiation of experimental animals. The susceptibility of the kidneys and lungs in the present studies may be due to inherent physiological properties rather than any specific organotropism of the organisms, although the latter mechanism is also conceivable. On the other hand, there was an absence of histopathological damage to the murine gastrointestinal tract.

Of particular interest in our study is the observation that on the basis of comparative LD<sub>50</sub> determinations, the mouse appears to be more susceptible to infection by peroral administration of viable *Pseudomonas* into the stomach than by i.v., i.p., and peroral administration into the lungs. No apparent decrease in the resistance of the animals was associated with the administration of anesthesia or manipulation through gastric intubation. These results seem to be in direct contradiction to those of other investigators (8, 15), who noted that feeding of *P. aeruginosa* to normal mice via their drinking water was harmless, although irradiated mice were susceptible to infection. However, one of the inherent drawbacks in attempting to infect mice by seeding their drinking water is that one cannot control the numbers of organisms ingested. Howerton and Kolmen (9) were able to establish a bacteremia and bactichyla in burned rats receiving *P. aeruginosa* through gastric intubation, but the unburned rat was resistant to challenge via gastric intubation. Perhaps these contradictions may be explained by (i) our ability to better quantitate the actual numbers of organisms that are introduced into the mice by gastric intubation

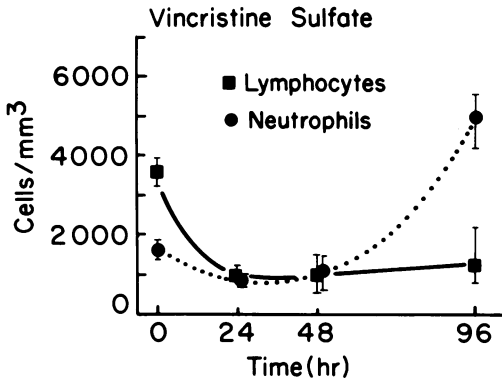


FIG. 3. Effect of vincristine sulfate on circulating lymphocytes and neutrophils.

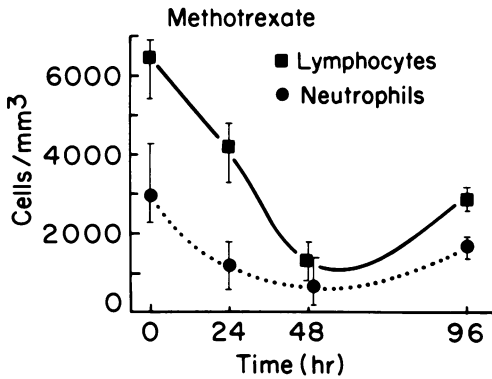


FIG. 4. Effect of methotrexate on circulating lymphocytes and neutrophils.

than by seeding of the animals' drinking water and (ii) inherent resistance differences between rats and mice. Another possible explanation may be that the particular strains of bacteria described herein are extremely virulent, although the large number of organisms required to parenterally kill the mice by the three strains suggested they are of relatively low virulence.

The treatment of mice with antineoplastic drugs showed that enhanced lethality could be produced, especially if methotrexate was administered. Although vincristine sulfate and cytosine arabinoside had a lesser effect than methotrexate, there appears to be a potentiation of the infection in animals receiving these drugs related to the effect of the drugs on the granulocyte counts. The potentiation of *P. aeruginosa* infections by antineoplastic and immunosuppressive agents is of significant clinical importance. Schimpff et al. (14) have concluded that the presence of *P. aeruginosa* indicates an ominous prognosis for the granulocytopenic patients with acute nonlymphocytic leu-

kemia. These infections occur more frequently in the granulocytopenic patient than in other types and are usually lethal if not treated promptly. Acquisition, they report, is probably via contaminated food and water. They conclude that the number of *Pseudomonas* infections could be reduced by the use of only cooked foods, sterile water, and sterile surroundings.

Our experimental results support their clinical observations that the gastrointestinal tract may serve as an important source of infection and that cytotoxic agents causing leukopenia, particularly neutropenia, do in fact render the animal more susceptible to *Pseudomonas* infections through the gastrointestinal tract. Ziegler et al. (17) were also able to establish a fatal bacteremia in rabbits by seeding their drinking water with *P. aeruginosa*, but only if the animals had been rendered agranulocytic with nitrogen mustard.

In conclusion, we believe the use of peroral introduction of *P. aeruginosa* into the stomach can be used in the further study of *Pseudomonas* infections in debilitated animals. This system also allows quantitation of the median inoculating dose leading to a systemic infection that is histologically similar to clinical findings in *Pseudomonas* sepsis (5).

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