Experimental Pseudomonas Pneumonia in Leukopenic Dogs: Comparison of Therapy With Antibiotics and Granulocyte Transfusions

By David C. Dale, Herbert Y. Reynolds, James E. Pennington, Ronald J. Elin, and Geoffrey P. Herzig

Pseudomonas aeruginosa pneumonia was produced in dogs with radiation-induced leukopenia to study the comparative efficacy of several different therapies. In a randomized control trial, five treatment regimens were compared: no antibiotics or granulocytes (controls), gentamicin (5 mg/kg/day), carbenicillin (500 mg/kg/day), gentamicin and carbenicillin (same dosages), and daily granulocyte transfusions (minimum 5 x 10^6 cells/day) plus gentamicin (5 mg/kg/day). The most effective therapy was gentamicin plus granulocyte transfusions. Gentamicin alone was not significantly better than no specific therapy. Carbenicillin with or without gentamicin gave intermediate results. This study further supports the utility of granulocyte replacement therapy of infections in severely granulocytopenic subjects. The results also indicate that the relative value of granulocyte transfusions depends upon the specific antibiotic regimen with which these transfusions are compared.

The treatment of infections caused by Pseudomonas aeruginosa is particularly difficult in patients with severe granulocytopenia. Recent clinical studies suggest that carbenicillin therapy with or without gentamicin has substantially increased the number of granulocytopenic patients who survive P. aeruginosa infections. Gentamicin alone has been judged ineffective for severe P. aeruginosa infections in this clinical setting by some investigators, although it remains in wide use because studies both in vitro and in vivo indicate gentamicin and carbenicillin have synergistic effects against many strains of P. aeruginosa.

Since the blood granulocyte count is such a critical factor in determining outcome in severe bacterial infections, another therapeutic approach to this problem is to replace granulocytes by transfusion therapy. We have recently developed an animal model of severe P. aeruginosa pneumonia in leukopenic dogs to evaluate the efficacy of granulocyte replacement as an adjunct to antibiotics. This controlled trial has established that therapy with granulocyte transfusions plus gentamicin is a more effective treatment than gentamicin alone as measured by longer survival and better clearance of inoculated organisms. To investigate further the treatment of P. aeruginosa infections in the leukopenic host, we have conducted another controlled trial in this canine model comparing the following therapies: (1) controls—no antibiotics or gran-
ulocyte transfusions; (2) carbenicillin; (3) gentamicin; (4) carbenicillin and gentamicin; and (5) granulocyte transfusions plus gentamicin.

MATERIALS AND METHODS

Model

The experimental model has been described previously. In brief, 6-12-mo-old male beagle dogs, weighing 7-12 kg, were given 350 rads of total body irradiation. This radiation dose produced a gradual leukopenia with total leukocyte counts falling to less than 100/cu mm after about 10 days. At 6 days postirradiation, when leukocyte counts were about 2000/cu mm (neutrophils were 1500/cu mm), the dogs were lightly anesthetized and intubated with a sterile cuffed endobronchial tube (Metras Catheter, Rusch, Inc., New York). Then $5 \times 10^8$ Pseudomonas aeruginosa (Fisher immunotype 2 [No. 05142], obtained from Parke, Davis and Co., Detroit, Mich.) were inoculated into the distal portion of one lower lobe of the lung through a polyethylene catheter. In previous studies it was established that this challenge reproducibly produced pneumonia in leukopenic dogs but not in normals. The organism used was susceptible to gentamicin at 3 $\mu$g/ml and carbenicillin at 50-100 $\mu$g/ml.

A controlled therapy trial was performed. Groups of five dogs were similarly housed, fed, irradiated, and inoculated with $P$. aeruginosa. At 24 hr after inoculation of bacteria, dogs were randomized to five treatment groups: (1) control—no antibiotics or leukocyte transfusions, (2) carbenicillin alone, (3) gentamicin alone, (4) both carbenicillin and gentamicin, and (5) daily granulocyte transfusions plus gentamicin. In the event that a dog died before 24 hr, therapies were randomized among the remaining dogs. The doses and administration of antibiotics and supportive care are discussed below.

Supportive Care

All dogs were given 500 ml of Ringer’s lactate solution (Abbott Laboratories, North Chicago, Ill.) daily by subcutaneous clysis beginning at the time of irradiation and continuing until death or complete hematologic recovery. Platelet concentrates were obtained by centrifugation of fresh, ACD anticoagulated, type A negative blood of English-American foxhound donors. Daily platelet transfusions were given beginning 24-48 hr after infection in order to maintain platelet counts above 25,000/cu mm.

Antibiotic Therapy

Disodium carbenicillin (supplied by the Roerig Division, Pfizer Pharmaceuticals, New York, N.Y.) (500 mg/kg/day) was administered intravenously as a bolus injection five times per day (7 am., 11 a.m., 3 p.m., 7 p.m., 11 p.m.). Gentamicin sulfate (Schering Corp., Bloomfield, N.J.) (5 mg/kg/day) was given intramuscularly every 8 hr. For combination therapy with carbenicillin and gentamicin, both drugs were given at the same doses and dose intervals as above. All antibiotics were begun 24 hr after infection and continued until death or hematologic recovery. Previous studies in this laboratory established that in dogs these antibiotic dosages gave serum gentamicin levels of 9.2-10.0 $\mu$g/ml 30 min after injection and carbenicillin levels of 250-550 $\mu$g/ml 30 min after injection.

Granulocyte Transfusions

Granulocytes were collected from a colony of English-American foxhounds, maintained as blood donors. Indwelling carotid-jugular shunts were inserted, and the dogs were leukapheresed daily to obtain at least $5 \times 10^8$ granulocytes by the filter adherence technique (Leukopak, Fenwal Laboratories, Morton Grove, Ill.).

To procure granulocytes, heparinized blood was withdrawn from the arterial line, passed by a peristaltic pump over one or two nylon wool filters at a flow rate of 40-50 ml/min per filter and returned via the venous line in a continuous flow procedure. After 90-120 min, the filter was disconnected and flushed with saline to remove erythrocytes. Then the adherent leukocytes (predominantly neutrophils and monocytes) were removed by elution with 500 ml of a solution prepared by mixing 75 ml acid-citrate-dextrose (ACD— NIH formula A), 200 ml ACD-
coagulated dog plasma, 250 ml normal saline, 500 ml one-half normal saline and 15 ml of a 40\% solution of sodium citrate. The leukocytes were concentrated by centrifugation and resuspended in approximately 100 ml of ACD-plasma. Before transfusion, the leukocytes were irradiated with 2500 rads from a 137Cs source (Gammator M, Kewaunee Scientific Equipment Corp., Adrian, Mich.) to prevent the possibility of graft-versus-host disease caused by the transfused cells. The granulocyte recipient received daily transfusions beginning 24 hr after infection and continued until death or hematologic recovery.

Clinical Observations
Rectal temperatures were measured before and 4 hr after infection and at least daily thereafter. Chest x-rays were made before infection and at 2-3 day intervals thereafter. Animals were examined at least five times per day to assess their general condition and give antibiotics.

Blood Counts
Total and differential leukocyte counts and platelet counts were measured as previously described. Counts were made before irradiation and daily thereafter and also 1 hr after each granulocyte transfusion.

Blood Cultures, Limulus Tests, and Autopsies
Blood cultures were obtained before and 4 hr after infection and daily thereafter. Three milliliters of blood were inoculated into an anaerobic blood culture bottle (Difco Labs, Detroit, Mich.). After 2 days, the bottles were subcultured anaerobically. Then they were vented with air and observed for 3 additional days for aerobic growth and subcultured aerobically. Isolated bacteria were identified by standard laboratory methods. The P. aeruginosa isolates were identified with type specific rabbit antisera.

Limulus tests were performed on plasma samples collected simultaneously with the blood cultures. At autopsy, cultures of heart blood, lungs, and tracheal fluid were obtained, and the isolated organisms were identified as mentioned above. Tissues of all major organs were examined at autopsy.

Statistical Methods
The study was designed chiefly to compare the effects of the various treatment regimens on survival of the dogs. The duration of survival with the various treatments was compared by Wilcoxon test. Other comparisons were made using Student's t test.

RESULTS
Survival
The comparative survival data for the five treatment regimens are illustrated in Fig. 1 and statistically compared in Table 1. It can be seen that granulocyte transfusions plus gentamicin gave the longest survival, significantly longer than the controls or gentamicin therapy. Carbenicillin plus gentamicin was significantly better than the controls and probably better than gentamicin alone (p = 0.06). Results with carbenicillin, with or without gentamicin, were rather similar. Gentamicin alone was not significantly better than controls (p = 0.16). The survival rate for the granulocyte transfused group was not significantly better than either group given carbenicillin. There were no significant differ-

*Antiimmunotype P. aeruginosa antisera were kindly supplied by Dr. Henry B. Devlin, Research and Development Division, Parke, Davis & Co. P. aeruginosa immunotyping was kindly performed by Dr. C. H. Zierdt and Mr. Willard Williams, Microbiology Section, Department of Clinical Pathology, National Institutes of Health, Bethesda, Md.
Fig. 1. Survival data for dogs with experimental Pseudomonas pneumonia treated with various therapies. Numbers in parentheses indicate number of animals in each group.

ences in total leukocyte, neutrophil or platelet counts at the time of infection or randomization to account for these results (Table 2). The differential counts showed a mean of $61\% \pm 13\%$ (SD) neutrophils before infection and $38\% \pm 6\%$ neutrophils at 24 hr after infection as in the previous study. The granulocyte-transfused dogs had the highest preinfection leukocyte counts, although not significantly higher than any other group ($p = 0.1$). The controls had the highest average leukocyte counts at 24 hr after infection, although not significantly higher than any other group. Similarly, rectal temperatures were the same before and after infection for the various groups (Table 2).

**Blood and Autopsy Cultures**

Bacteremia was a relatively infrequent event in these infected leukopenic dogs, as observed previously. Only 14% of 495 blood cultures grew any orga-

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**Table 1. Comparison of the Duration of Survival for the Five Treatment Regimens**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Gentamicin</th>
<th>Carbenicillin</th>
<th>Carbenicillin and Gentamicin</th>
<th>Granulocytes and Gentamicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.16*</td>
<td>0.014</td>
<td>0.006</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.12</td>
<td></td>
<td>0.06</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Carbenicillin</td>
<td></td>
<td>0.9</td>
<td></td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Carbenicillin and gentamicin</td>
<td></td>
<td></td>
<td></td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>No. of long-term survivors</td>
<td>0/9</td>
<td>1/10</td>
<td>3/10</td>
<td>1/10</td>
<td>4/7</td>
</tr>
<tr>
<td>Median survival (days)</td>
<td>2</td>
<td>4</td>
<td>11</td>
<td>13</td>
<td>&gt; 25†</td>
</tr>
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</table>

*p value for Wilcoxon test (two-tailed test).†Hematopoietic recovery had occurred by 25 days in the four long-term survivors of this group.
nisms. *P. aeruginosa* was isolated from the blood of less than half of the dogs at any time during their infection (Table 3), most of these positive cultures occurring within the last 2 days of life in all of the treatment groups. Positive cultures for *P. aeruginosa* were somewhat less frequent in the carbenicillin plus gentamicin and granulocyte plus gentamicin groups (Table 3). From autopsy cultures of lung and/or heart blood, the inoculated *P. aeruginosa* immunotype was isolated from all dogs in the control group and from other groups of dogs with only slightly lesser frequency (Table 3). One of the three dogs which died in the group given granulocytes and gentamicin had received only one transfusion by the time of his death. Another granulocyte-transfused dog from which *P. aeruginosa* was isolated had a lung abscess, the only animal in the study to die with abscess formation.

**Limulus Tests**

Before infection all dogs had negative Limulus tests. Positive Limulus tests occurred slightly more frequently than positive blood cultures; 84 of 470 (17%) tests were positive. The per cent of positive tests ranged from 11% to 24% in the

<table>
<thead>
<tr>
<th>Table 3. Comparison of Blood and Autopsy Cultures, Limulus Tests, and Chest X-Ray Results for the Five Treatment Regimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>No. of dogs with blood cultures positive for <em>Pseudomonas</em></td>
</tr>
<tr>
<td>No. of dogs with autopsy cultures positive for <em>Pseudomonas</em></td>
</tr>
<tr>
<td>No. of dogs with positive Limulus test</td>
</tr>
<tr>
<td>No. of Limulus tests positive</td>
</tr>
<tr>
<td>No. of dogs with x-ray improvement on therapy†</td>
</tr>
</tbody>
</table>

* Autopsy cultures were not obtained in a few animals.
† A few dogs in each group lacked x-ray evidence of pneumonia.
various groups (Table 3). There was a highly significant correlation of positive *Limulus* tests and blood cultures positive for gram-negative bacteria ($p < 0.005$, chi-square test). There were, however, numerous instances when the two tests were not simultaneously positive or negative. In the therapy groups with the longest survival, more animals eventually developed positive tests. Most of these positive *Limulus* tests occurred late in the course of therapy in all treatment groups.

Chest X-rays

There was roentgenographic evidence of pneumonia in 33 of the 45 dogs by 48 hr after infection. Reduction of the pulmonary infiltrate occurred in three of the granulocyte-transfused dogs and in two of the dogs given carbenicillin with or without gentamicin before hematopoietic recovery.

Granulocyte Transfusions

Eighty-two granulocyte transfusions were given to the seven dogs; an average of 12.1 ± 0.6 x 10^9 (mean ± 1 SEM) leukocytes (9.5 ± 0.5 x 10^9 granulocytes) per transfusion was given. The mean increase of the recipient blood leukocyte count at 1 hr posttransfusion was 400 ± 50 cells/cu mm. An average of 2.5% of the total leukocytes transfused, and 2.4% of the granulocytes transfused were present in the circulation 1 hr after their injection.

DISCUSSION

The problem of treating infections, particularly *P. aeruginosa* infections, in granulocytopenic patients is well known.\(^1^5\) Since the introduction of carbenicillin and gentamicin to replace tetracycline, colistin, and polymyxin, most authorities agree that the therapy of *P. aeruginosa* infections has been substantially improved (3–5,15). In vitro and in vivo studies have indicated that carbenicillin and gentamicin act synergistically against many strains of *P. aeruginosa*.\(^16^,17\) In clinical trials, the combination of these agents has usually been more effective than either alone.\(^3^,16–18\) From clinical observations on therapy of infections in granulocytopenic patients, the efficacy of gentamicin without carbenicillin has been questioned by some investigators.\(^6\) These observations indicate that it is probably carbenicillin which has substantially lowered mortality from *P. aeruginosa* infections over the last few years.\(^15\)

Granulocyte transfusion techniques have developed during a period of changing antibiotic therapy. Several investigations indicate that granulocyte replacement is beneficial.\(^3^,16–23\) Critical evaluation of some of these studies is difficult because the antibiotics used were not specified or might not now be regarded as optimal. For instance, at the time of a study at the National Cancer Institute which reported benefit from granulocyte transfusions, the mortality from gram-negative septicemia was 70% in granulocytopenic patients treated with “appropriate” antibiotics.\(^19\) Since that study, carbenicillin has been used more commonly and at an earlier phase of infection. In a recent controlled trial with both transfused and nontransfused patients receiving carbenicillin, the control group mortality was 40%. With this lower control group mortality, the benefit from granulocyte transfusions was apparent only for certain patients with persisting granulocytopenia.\(^14\)
The present study was undertaken to compare the efficacy of several therapeutic regimens for treating experimental *P. aeruginosa* pneumonia. Our objective was to accumulate firm evidence on the comparative benefit of granulocyte transfusions and the relative efficacy of different antibiotics. A previous study in monkeys with leukopenia and *P. aeruginosa* infections had shown no significant difference in outcome for various antibiotic therapies.\(^2\)\(^5\) It seemed entirely possible that we had found granulocyte transfusions to be beneficial previously principally because our comparison group received only gentamicin, a relatively ineffective agent.\(^9\) We observed, in fact, that gentamicin gave results not significantly different from controls. On the other hand, therapy with granulocytes plus gentamicin gave the highest number of survivors. Thus, this study again demonstrates the potentially important role for granulocyte transfusions in some severe infections. However, it is noteworthy that results for the granulocyte-transfused group and the carbenicillin-treated groups were not significantly different. Although a trend toward better survival with granulocyte transfusions emerged, a substantially larger study would have been required to establish if there were statistically significant differences between these therapies. Several recent clinical trials similarly indicate that, when carbenicillin is given to both the granulocyte-transfused and the control groups, the benefit of granulocytes is not readily demonstrable.\(^26\)\(^28\)

Several additional observations in this study may be noteworthy. Compared to our studies using granulocytes procured by continuous flow centrifugation,\(^9\) the 1 hr posttransfusion granulocyte increments were substantially less for the filter adherence collected cells, but the overall survival data were comparable. This observation indicated that transfused granulocytes may function to limit the course of an infection without necessarily being detected in the circulation, as others have also noted.\(^22\) The frequency of positive *Limulus* tests was also much higher in these dogs receiving filter collected cells than when centrifuge collected cells were used;\(^9\) the reason for this difference is not yet known, but could relate to the frequent febrile reactions with filter collected cells.\(^26\) Finally, the reasons why gentamicin may not work alone but may be effective when granulocytes are present remains a puzzling finding.

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