Granulocyte Transfusion Therapy of Experimental Pseudomonas Septicemia: Study of Cell Dose and Collection Technique

By Frederick R. Appelbaum, Charles A. Bowles, Robert W. Makuch, and Albert B. Deisseroth

To determine the contributions of cell dose and collection technique to the success of granulocyte transfusion therapy, we developed a canine model of granulocytopenia with gram-negative septicemia. Beagles were made granulocytopenic with a single intravenous (i.v.) injection of cyclophosphamide (40 mg/kg); 5 days later, when all animals were granulocytopenic, graded doses of Pseudomonas aeruginosa were injected i.v., followed in 4 hr by antibiotics with or without granulocyte transfusions. Survival of animals not treated with granulocytes was related to the dose of Pseudomonas injected, with $1 \times 10^8$ bacteria/kg being lethal in greater than 95% of animals. Survival of animals treated with daily granulocyte transfusions after $1 \times 10^8$ bacteria/kg was critically dependent on the dose of cells transfused and the collection technique employed. When continuous-flow centrifugation was the method employed, none of five animals receiving $1 \times 10^8$ granulocytes (PMN)/kg daily survived the infectious episode, while four of five receiving $1.5 \times 10^8$ and five of five receiving $2.0 \times 10^8$ PMN/kg survived. When cells collected by filtration leukapheresis were used, 2.5 to 3.0 times more PMN were necessary to provide the same protection: none of five survived at $2.0 \times 10^8$ PMN/kg, one of five survived at $3.0 \times 10^8$, three of five survived at $4.0 \times 10^8$, and five of five survived at $5.0 \times 10^8$ PMN/kg. Pretreatment of the recipient of filtration leukapheresis PMN with 25 mg hydrocortisone did not alter the ability of these cells to provide protection. These findings show that the success of PMN transfusion therapy for sepsis is critically dependent on the dose of PMN transfused and the collection technique employed.

Granulocyte transfusion therapy has been shown to be an effective complement to appropriate antibiotic treatment of gram-negative septicemia in granulocytopenic animals and man. There remain, however, many unanswered questions regarding appropriate dose, collection technique, pharmacologic pretreatment of the donor and recipient, histocompatibility, and scheduling. Many of these questions cannot be easily answered by techniques in vitro or by clinical trials in man.

In order to study such questions we developed a canine model of granulocytopenia with Pseudomonas aeruginosa septicemia that closely parallels the situation often confronted in the clinic. Using this model, which is a modification of earlier work by Epstein and co-workers, we were able to show that the success of granulocyte (PMN) transfusion therapy is critically dependent on the dose of PMN transfused and on the collection technique employed. We were also able to investigate the effect of pretreatment of the recipient with steroids prior to PMN transfusions.
MATERIALS AND METHODS

Dogs. English-American foxhounds were chosen as leukocyte and platelet donors because their large size and docile temperament allow for repeated leukapheresis and plateletpheresis. The dogs infected were male previously untransfused 6-12-mo-old beagles weighing 7-12 kg chosen because of our previous experience with the effects of cyclophosphamide on such dogs and because their size and temperament allow for easy handling. Prior to use, all animals were individually caged and given uniform feeding and access ad libitum to water. At the time of infection, animals were moved to individual cages in a second, isolated room.

Induction of granulocytopenia. Granulocytopenia was produced by a single intravenous (i.v.) injection of cyclophosphamide 40 mg/kg. This dose was previously shown to reliably produce profound granulocytopenia after 5 days. Previous studies also established that over 95% of animals will recover from this dose of cyclophosphamide if the dogs are adequately supported with fluids and platelets, given ampicillin and gentamicin in the schedule described below, and not inoculated with exogenous P. aeruginosa.8,9

Infection. P. aeruginosa, strain Fisher Type 2, was isolated from a clinical surveillance culture on tryptic soy agar (TSA). The isolate was cultured through three subcultures prior to placing it under 5 ml sterile mineral oil on TSA slants and storing it at room temperature. The culture was identified for type using standard techniques.10 Antibiotic susceptibility patterns were determined every 2 wk throughout the course of the experiment using both disc sensitivity and routine serial dilution testing to establish the minimal inhibitory concentration (MIC).11

Prior to each inoculation, a sample of the bacteria was transferred to fresh TSA and grown overnight at 37°C. The morning of its use, it was washed and centrifuged twice in sterile water; the washed pellet was resuspended to a concentration of 1 x 10^6 bacterial/ml as determined by spectrophotometry (OD at 620 nm). The total number of viable organisms in the final cell suspension was confirmed by quantitative agar pour plating.

The actual septicemia was produced by a single i.v. injection over 2-5 min of the Pseudomonas inoculum; the tubing and needle were then flushed with sterile saline.

Experimental design. To establish the minimal lethal dose of Pseudomonas, a series of animals was given graded doses of Pseudomonas on day 5 after cyclophosphamide followed in 4 hr by antibiotics and fluid support without granulocyte transfusions. Once the minimal dose of Pseudomonas that caused death in greater than 95% of animals was established, the effects of granulocyte transfusions were studied in a controlled randomized trial. Groups of four dogs were simultaneously given cyclophosphamide, infected 5 days later, and identically handled until 4 hr after the injection. At this time, the animals were randomly assigned to receive either antibiotics and fluid support alone or such support plus daily transfusions of granulocytes until the animal died or recovered to a peripheral blood granulocyte count of greater than 1000 PMN/mm^3 at least 20 hr after the previous PMN transfusion. Each set of four animals generally consisted of one control animal (no PMN transfusion) and three transfused animals receiving different doses of granulocytes.

In a series of experiments designed to test the effect of steroid pretreatment on the granulocyte recipient, three animals were studied at a time: one control (no PMN), one transfused with granulocytes, and one transfused with an identical number of granulocytes 30 min after pretreatment with 25 mg hydrocortisone i.v.

Fluids. Beginning the fifth day after cyclophosphamide (or sooner if clinically warranted), the dogs received 500 ml Ringer's lactate solution (Abbott, North Chicago, Ill.) daily by subcutaneous clysis.

Antibiotics. Beginning 4 hr after the i.v. injection of Pseudomonas all animals received intramuscular injections of gentamicin (5 mg/kg) and ampicillin (50 mg/kg) every 12 hr until death or hematologic recovery. Gentamicin levels 1, 6, and 12 hr after receiving the antibiotic were determined on selected animals by radioimmunoassay.12

Blood products. Platelets were obtained from fresh acid-citrate-dextrose (ACD) anticoagulated type A negative blood from the foxhound donors. All dogs were given 50-100 ml platelet concentrate daily to keep peripheral platelet counts above 20,000/mm^3.

Granulocytes were obtained by either continuous-flow centrifugation (CFC) or by filtration leukapheresis (FL) as previously described.13,14 For both methods, donors had indwelling arteriovenous shunts inserted 24 hr prior to the leukapheresis procedure. To collect PMN by
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CFC, animals were leukapheresed for 3 hr using a cell separator (NCI-IBM, Endicott, N.Y.), obtaining 250 ml leukocyte-rich anticogulated suspension. When FL was used, the peristaltic pumps of the cell separator were used to pump blood at 80 ml/min over two nylon-wool filters (Leukapak, Fenwal, Morton Grove, Ill.). After 2 hr, elution was accomplished using 20% ACD NIH formula A plasma in saline with gentle tapping. The number of PMN contained in each collection was measured and the volume adjusted to contain the dose of PMN required for that experiment. The suspensions were kept at room temperature and transfused within 2 hr of collection. No donors were used for more than three leukaphereses.

Clinical observations. Animals were examined at least every 8 hr to assess their general condition, administer fluid or antibiotics, and record deaths. Rectal temperatures were measured every morning.

Laboratory tests. Complete blood counts and differentials were performed daily and 1 hr after each leukocyte transfusion. Platelet counts were done by phase-contrast microscopy; leukocyte counts of above 2000 cells/mm³ were performed by an electronic particle counter (Coulter Electronics), while lower counts were done by direct visual counting with a hemocytometer. Differential counts were made on air-dried Wright-stained smears.

Blood cultures were obtained prior to and 4 hr after infection and daily thereafter. At autopsy, cultures of heart blood were obtained. Pseudomonas antibody titers were measured by the serum slide agglutination test on leukocyte donors and on all test animals after 2 min agitation was established as the antibody titer. Donors or recipients with titers greater than 1:16 were excluded from the study.

Blood chemistries were obtained twice weekly on all test animals.

Statistical methods. Length of survival after Pseudomonas injections in groups of control animals was compared by Gehan's generalized Wilcoxon test. Overall survival in animals treated with differing doses of granulocytes collected by FL and CFC was compared using the Mantel-Haenszel test statistic. Comparisons of posttransfusion increments were by analysis of covariance on data transformed by the procedure of Box and Cox.

RESULTS

Development of septicemia model. The single i.v. dose of cyclophosphamide (40 mg/kg) reliably produced neutropenia (<500 PMN/mm³) in 30 of 32 control animals by the fifth day (the two control animals and any later animals whose count did not fall below 500 PMN/mm³ by the fifth day were excluded from the study. Total WBC on day 5 was 169 ± 124 (mean ± SD), and the total PMN count on day 5 was 135 ± 110. Graded doses of Pseudomonas were given to the 30 dogs in an effort to determine the minimal dose that was repeatedly lethal. The results are shown in Table 1. In this model, 1 x 10⁸ bacteria/kg was the lowest dose lethal with greater than 95% reliability. Length of survival after the dose of bacteria was also compared; animals receiving doses of 5 x 10⁸ bacteria/kg died more quickly than those receiving 1 x 10⁸ bacteria/kg (Gehan's test, p < 0.01). Thus both the ultimate lethality and the rapidity of

<table>
<thead>
<tr>
<th>Bacteria/kg</th>
<th>Overall Survival</th>
<th>Time to Death* (hr)</th>
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<tbody>
<tr>
<td>1 x 10⁴</td>
<td>2/2</td>
<td>—</td>
</tr>
<tr>
<td>1 x 10⁵</td>
<td>2/2</td>
<td>—</td>
</tr>
<tr>
<td>1 x 10⁶</td>
<td>3/4</td>
<td>96</td>
</tr>
<tr>
<td>1 x 10⁹</td>
<td>1/16</td>
<td>72 (24-96)</td>
</tr>
<tr>
<td>5 x 10⁹</td>
<td>0/6</td>
<td>24 (4-48)</td>
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*Mean, range in parentheses.
death were dependent on the dose of bacteria administered. For the remainder of the study, a dose of $1 \times 10^8$ bacteria/kg was employed.

Results of postmortem heart blood cultures of those animals dying at any dose of *Pseudomonas* revealed the presence of the same strain of *Pseudomonas* used for infecting the animals; in 8 of 22 cases other bacteria were recovered as well as *Pseudomonas*. These included group D streptococcus in three cases, *Escherichia coli* in three cases, and *staphylococcus aureus* in two cases.

Repeated testing of the *Pseudomonas* strain used for infecting the animals showed the MIC of gentamicin to be 3.0 μg/ml with no more than one tube dilution (i.e., 1.5–6.0 μg/ml) variance from week to week throughout the study. Gentamicin levels determined on selected animals revealed peak levels of 6–9 μg/ml 1 hr after injection, with levels above the MIC for 4–5 hrs.

**Effect of CFC dose on survival.** Twenty animals were given graded doses of PMN collected by CFC to determine what effect dose had on ultimate survival. As illustrated in Fig. 1, survival was critically dependent on the dose of PMN transfused. None of the five animals receiving $1 \times 10^8$ PMN/kg survived the infectious episode, while all five of those receiving $2 \times 10^8$ PMN/kg survived. An additional five animals receiving $3 \times 10^8$ PMN/kg all survived as well. Thus $2 \times 10^8$ PMN/kg collected by CFC appears to assure protection from an otherwise lethal *Pseudomonas* infection in this model. Every animal failing to survive had *Pseudomonas* recovered from postmortem heart blood cultures; in 2 cases group D streptococci were also present.

**Effect of FL PMN dose on survival.** Twenty animals were given graded doses of PMN collected by FL to determine what effect dose had on survival. As illustrated in Fig. 1, survival was again critically dependent on the dose of PMN transfused. However, 2.5–3.0 times more FL granulocytes were needed to provide the same degree of protection offered by CFC-collected granulocytes. None of the five animals receiving $2.0 \times 10^8$ FL PMN/kg survived (a dose 100% protective using CFC PMN); a dose of $5.0 \times 10^8$ FL PMN/kg was needed to assure survival after an otherwise lethal *Pseudomonas* septicemia. Based on Mantel-Haenszel test statistic with the small sample variance and correction for continuity, a significantly higher proportion of animals given doses of PMN
collected by FL died when compared to those treated with equivalent doses of PMN collected by CFC ($p < 0.001$). This test was formulated on the assumption that all animals given doses of PMN above $3 \times 10^8$/kg collected by CFC will live and all animals receiving doses of PMN below $2 \times 10^8$/kg collected by FL will die. *Pseudomonas* was recovered at autopsy from all but one animal in this group (whose heart blood was sterile), and two animals had polymicrobial sepsis.

In an effort to abort transfusion reactions, many clinicians pretreat PMN recipients with steroids. Because of the known inhibitory effect of steroids on granulocyte migration through vessel walls, we wished to examine if steroid pretreatment might impair the protective capacity of transfused granulocytes. Twelve dogs were made granulocytopenic and septic and then treated with either $4 \times 10^8$ FL PMN/kg alone or with such transfusions plus pretreatment with 25 mg hydrocortisone 30 min before each granulocyte transfusion. While four of six animals survived after granulocyte transfusions alone, five of six pretreated with steroids survived. Thus we observed no adverse effects of steroid pretreatment of FL recipients in this particular model of *Pseudomonas* septicemia ($\chi^2$ corrected for continuity, $p > 0.50$).

**Preinfection leukocyte counts.** We examined the preinfection leukocyte counts of dogs in every group to determine if variances in these counts might explain differing survivals among the groups. The results presented in Table 2

<table>
<thead>
<tr>
<th>No. of Dogs</th>
<th>Leukocyte Count</th>
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<tbody>
<tr>
<td>Controls</td>
<td>30</td>
</tr>
<tr>
<td>Recipients of CFC PMN</td>
<td>20</td>
</tr>
<tr>
<td>Recipients of FL PMN</td>
<td>26</td>
</tr>
<tr>
<td>Pretreated FL recipients</td>
<td>6</td>
</tr>
</tbody>
</table>

**Table 2. Leukocyte Count on Day of Infection (Mean ± SEM)**

Fig. 2. Posttransfusion (1 hr) granulocyte increments provided by different doses and types of granulocytes. Each point, mean increment in series of granulocyte transfusions given to five animals at each dose; bars, SEM at each point. Curves differ significantly at $3 \times 10^8$ PMN/kg.
would suggest that differences in survival among groups were not caused by the different preinfection white blood cell counts.

Posttransfusion PMN increments. The increments in peripheral blood granulocyte counts determined 1 hr after white cell transfusion are presented in Fig. 2 and Table 3. An analysis of covariance was performed to determine if the average increment in peripheral blood granulocyte counts differed significantly between the collection techniques employed. The data were transformed using the Box and Cox procedure to ensure that the statistical assumptions associated with this method were more closely satisfied. While it appears that at any given dose level the transfusion of CFC PMN provided greater posttransfusion increments, this difference was significant only at the dose of $3.0 \times 10^9$ PMN/kg. This is due, in part, to the high degree of variability associated with the increments.

We further wished to examine whether or not decreased posttransfusion increments were solely responsible for the decreased survival in the group receiving FL PMN. In Fig. 3 we show survival as a function of posttransfusion increment. It appears that even at the same level of posttransfusion increments,
granulocytes collected by centrifugation allowed for a greater percentage of animals to recover from sepsis.

Finally, we compared the posttransfusion increments in recipients of 4.0 × 10^8 FL PMN/kg with and without steroid pretreatment. Those treated with granulocytes alone had 1-hr posttransfusion increments of 496 ± 118; those pretreated with steroids before the transfusion had posttransfusion increments of 490 ± 144. These numbers are not significantly different (Gehan’s test, p > 0.50).

DISCUSSION

The transfusion of granulocytes collected from normal donors to neutropenic septic patients has been shown to be of benefit in several prospectively randomized clinical trials, and the use of such transfusions is becoming increasingly widespread. There is, however, little agreement about dosage, collection techniques, scheduling, pharmacologic manipulations of donor and recipient, and the importance of histocompatibility from center to center. Attempts to settle these questions with clinical protocols would be difficult; the enormous patient-to-patient variability with respect to underlying disease, concurrent treatment, type and degree of sepsis, and other inherent biologic differences would make it nearly impossible to segregate out single, subtle factors without resorting to extremely large and lengthy clinical studies.

In an attempt to resolve some of these problems, we developed a preclinical canine model of neutropenia with gram-negative septicemia. The data presented here established in this model that the effectiveness of granulocyte transfusion therapy of gram-negative sepsis is critically dose dependent. The dose of granulocytes/kg collected by CFC necessary to protect a dog from a “just lethal” septicemia is 2 × 10^8/kg, which when extrapolated to man is at the upper limit of what is normally collected and transfused; two to three times more PMN are required to provide the same degree of protection when FL is used to collect PMN. Our data indicated that the pretreatment of recipients of FL PMN with hydrocortisone does not appear to interfere with the protective function of these cells.

The quantitative relationship between the level of circulating granulocytes and the incidence and fatality of septic episodes has been well demonstrated in the past. Thus it is not surprising that the quantity of granulocytes transfused to neutropenic subjects would also affect the outcome of septic episodes. The steepness of the curve, however, is impressive and emphasizes the fact that the success or failure of any trial of granulocyte transfusion therapy may depend on the dose of cells employed rather than the overall utility of the approach. This study also points out the need for improved technology that would allow for even greater numbers of granulocytes to be collected from normal donors.

The difference observed here in the protective effect of granulocytes collected by CFC and FL emphasizes the fact that FL granulocytes are functionally deficient. In general, it requires two to three times more cells collected by FL to provide the same protection given by CFC PMN. Our experience, and that of others, generally indicates that two to three times more cells can be collected by
FL, so that this apparent superiority of PMN collected by CFC may be offset by the decreased yields of this procedure.\textsuperscript{21,22}

Assays in vitro of FL PMN, especially chemotaxis and bactericidal killing, have suggested that these cells are deficient. Cell damage may occur during the adhesion of PMN to nylon fiber, as suggested by the observations of Fehr et al., who described complement-mediated degranulation during adhesion.\textsuperscript{23} Klock and Bainton found decreased bactericidal power in vitro of granulocytes collected by FL, which they also related to active degranulation during PMN adhesion to nylon.\textsuperscript{24} Wright et al.\textsuperscript{25} showed that it is the small peroxidase-negative specific granule that is preferentially lost. Schiffer et al. attempted to reverse the deficiencies in vitro of FL PMN by eluting PMNs from fibers in the presence of tetracaine;\textsuperscript{26} Weight et al. explored donor pretreatment with colchicine.\textsuperscript{27} The overall clinical impact of the functional deficiency in vitro of FL PMN had not been examined prior to this study; similarly, the benefit of pharmacologic manipulations of these cells remains to be clinically evaluated.

Examination of the posttransfusion increments in this study illustrates that higher doses of PMN collected by either method result in higher posttransfusion increments and agrees with the results of Herzig et al.,\textsuperscript{22} who showed that cell for cell PMN collected by FL result in lower posttransfusion increments than those collected by CFC. We also examined the relative survival of animals treated with FL- and CFC-collected PMN as a function of the posttransfusion increments obtained. This analysis illustrates that even when similar posttransfusion increments are obtained, the animals receiving CFC-collected PMN had a better survival rate.

Many institutions pretreat recipients of FL PMN with steroids in an effort to inhibit or abort febrile transfusion reactions. Steroids are known to decrease the egress of PMN from the circulation, and it has been a concern of some that steroid pretreatment might interfere with the protective capacity of FL-collected PMN. This was not seen in our model.

Extrapolation of laboratory data to the clinic is always unsure; humans do not become septic by receiving an i.v. injection of \textit{Pseudomonas}; they may receive different antibiotics than those used in our study; dog and human granulocytes may differ in the damage FL inflicts in each; and the effects of steroids may differ in the two species. Nonetheless, our model of \textit{Pseudomonas} sepsis in leukopenic dogs would appear to be at least internally consistent and was developed in order to study the physiology of granulocyte transfusion therapy in an orderly, controlled fashion. These studies suggest a critical dose dependence of granulocytes on the outcome of \textit{Pseudomonas} sepsisemia and suggest that every attempt should be made to maximize PMN doses in the treatment of gram-negative sepsisemia in man.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Kathleen McCormick, Jim Edgbert, Robert Corbitt, Donna Purvis, and Dail Deitrick.

REFERENCES

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