Granulocyte Transfusions in Leukopenic Dogs: In Vivo and In Vitro Function of Granulocytes Obtained by Continuous-Flow Filtration Leukopheresis

By Kim M. Debelok, Robert B. Epstein, and Burton R. Andersen

The present studies were carried out to (1) evaluate a leukoadhesive technique for obtaining granulocytes for transfusion, (2) assess the granulocytes by in vitro techniques, and (3) determine the efficacy of granulocyte transfusion in preventing sepsis in leukopenic dogs. Dogs were rendered transiently leukopenic (<500 per cu mm) by intravenous cyclophosphamide, 40 mg/kg. Quantitative and qualitative blood cultures were obtained from all animals until death or hematologic recovery. Granulocytes were obtained on nylon filters by a continuous flow system and eluted with an ACD plasma saline solution. Granulocyte function was studied in vitro by chemotaxis, phagocytosis, intracellular killing, and electron microscopy. In vivo studies consisted of the measurement of granulocyte increments in transfused leukopenic dogs, T of infused granulocytes, and protection of transfused dogs from septicemic episodes. Eluted granulocytes, when compared to normal controls, showed reduction in in vitro functions. These functions improved in granulocytes isolated post-transfusion from recipient dogs. An average of $3 \times 10^{10}$ granulocytes could be obtained during a 1-hr leukopheresis of normal donors. Increments in recipient dogs averaged 2590 per cu mm. Five nontransfused leukopenic dogs developed septicemia and died within 7 days. Six dogs were treated with infusions of granulocytes. Three recovered completely, and three died of thrombocytopenic hemorrhage with negative blood cultures. One dog showed a transiently positive blood culture that became negative following transfusion. Septic episodes were significantly reduced in granulocyte transfused dogs ($p < 0.01$). It was concluded that continuous-flow leukofiltration yielded granulocytes in sufficient number and with adequate functional capabilities to provide significant protection against septic death in the leukopenic host.

PREVENTION AND THERAPY of intercurrent infection remains a major problem in the management of patients with disease-associated or iatrogenically induced severe granulocytopenia.$^{1-3}$ In addition to sophisticated patient isolation procedures and intensive use of antibiotics, granulocyte transfusions have been increasingly employed in attempts to support the neutropenic patient.$^{4,5}$

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Essentially, two techniques are utilized at the present time to secure large numbers of granulocytes from normal donors for transfusion. Granulocytes in quantity can be obtained by a system of continuous-flow centrifugation, based on density characteristics of blood cellular components. More recently, the principle of granulocyte adhesion to nylon-fiber columns has been utilized for large-scale granulocyte procurement. Elution of these cells from the columns with ACD plasma solutions and clinical transfusion procedures have been carried out. The leukoadhesive technique may offer advantages of practicality and economy important for wide-spread application, but in vivo functional studies have been difficult to evaluate. One of the major problems in assessing the efficacy of leukocyte transfusion has been the complex clinical circumstances under which such attempts have been undertaken. This is particularly true in severely ill patients receiving concomitant antibiotic therapy. Clinical studies to date, however, have been encouraging.

The present studies in the dog were undertaken to (1) assess the leukoadhesive technique as a means for obtaining large numbers of granulocytes from normal donor animals, (2) evaluate the in vitro functions of these granulocytes following elution and transfusion, (3) obtain kinetic data subsequent to transfusion, and (4) evaluate the in vivo effectiveness of infused granulocytes in preventing spontaneous infection in leukopenic animals.

**MATERIALS AND METHODS**

**Granulocytopenic Dogs**

Random mongrel dogs weighing between 10 and 25 kg, immunized against hepatitis and distemper, and dewormed, were observed for a period of 3 wk prior to use. Granulocytopenia was induced by an intravenous administration of cyclophosphamide (Mead Johnson Laboratories, Evansville, Ind.), 40 mg per kg body weight. In all dogs, granulocytes reached levels of less than 200 per cu mm by day 5. Parenteral fluids were routinely administered during periods of anorexia and vomiting.

Daily hematocrits, white blood cell, and platelet counts were obtained. Blood cultures and body temperatures were monitored twice daily. Qualitative and quantitative blood cultures were performed in triplicate on all specimens using the pour plate method at dilutions of 1:10 and 1:100. Recovery was considered complete when hematologic values returned to normal, no further fluids support was necessary, and animals were afebrile. Gross and microscopic postmortem examinations were conducted on animals dying during the study.

**Granulocyte Isolation**

On the day of the initial transfusion, donor animals were anesthetized with phenobarbital, and a carotid artery to jugular vein shunt was inserted. Donor dogs were anticoagulated with 5000 U of intravenous heparin prior to leukopheresis and at 30 min. Leukopheresis was carried out by interposing a nylon column (Leukopak, Fenwal Laboratories, Morton Grove, Ill.) between the arterial and the venous side of the shunt. Flow rates through the filter averaged approximately 100 ml/min. The procedure was carried out for 1 hr with a change of filter at 30 min.

**Elution Procedure**

Preliminary studies were carried out with several eluting solutions. These included ACD plasma (formula A) diluted 1:5 with physiologic saline and adjusted to a pH of 6.5 by additional ACD; 9.2% EDTA diluted 1:5 with 20%, heparinized plasma saline solution and adjusted to pH of 6.5 with 1 N sodium hydroxide; ACD solution diluted 1:5 with Aminosol (Abbott Laboratories, North Chicago, Ill.) and adjusted to pH 6.5. In the separation procedure for clinical transfusion experiments, an eluting solution was employed, consisting of 20% ACD plasma in saline adjusted
to pH between 6.4 and 6.5. One thousand milliliters of this solution was allowed to flow through
each filter at a rate of 30–40 ml/min. The granulocyte-rich eluate was collected in 600-ml plastic
blood bags and concentrated by centrifugation at 400 g for 10 min. Granulocytes were infused in
100-200-ml volumes.

In Vitro Assessment of Granulocyte Function

Studies of in vitro function were carried out on granulocytes obtained from donor dogs prior to
leukopheresis, following elution, and recovered from leukopenic dogs 1–2 hr posttransfusion.
Leukocytes were obtained fromuffy coats following sedimentation in plasmagel and suspended in
Hanks balanced salt solution (HBSS) with 2 mg of glucose per ml (HBSS-G) and adjusted to the
cell concentration required. On some occasions, granulocytes were washed in HBSS-G to which
magnesium chloride (MgCl₂) had been added to give a final concentration of 1 mM. Cell viability
was determined by trypan blue dye exclusion.¹²

Chemoattraxis

The chemotactic technique of Boyden, as modified by DeMeo and Andersen,¹³ was used. The
chemotactic agent was casein-saturated HBSS solution. Values were expressed as the mean number
of granulocytes migrating through a 3-μ-Millipore filter and per cent of control values.

Phagocytosis of Yeast

The phagocytic capabilities of granulocytes were determined by a modification of the technique
described by Maaløe.¹⁴ Equal volumes of solutions containing 3 x 10⁶ granulocytes per ml
suspended in a 40%, autologous serum HBSS-G solution, and bakers yeast particles at a concentra-
tion of 4 x 10⁷, were mixed and incubated for 30 min at 38°C in a shaker water bath. Smears
of pelleted cells were stained with Wright’s Giesma. Two hundred granulocytes were counted and
the number containing yeast particles recorded. Values were expressed as the per cent of granu-
locytes with phagocytized particles.

Intracellular Killing of Candida Albicans

The method of Lehrer and Cline¹⁵ was used, except that leukocytes were suspended in HBSS-G.
Two hundred C. albicans cells were counted, and the per cent of stained cells was determined.
These cells were assumed to be nonviable.

Intracellular Killing of Pseudomonas Aeruginosa

The method was identical to the C. albicans assay with the following modifications. P. aeruginosa
was prepared from an exponential growth culture and adjusted to a concentration of 1.5 x 10⁷
organisms per ml. After incubation, the number of viable organisms remaining was determined by
the pour plate technique.¹⁶ Values were expressed as the percentage of viable organisms remaining
at 0, 30, 60, and 90 min.

Light and Electron Microscopy

Coverslip preparations were made for light microscopy and stained with Wright’s Giemsa. For
electron microscopy, granulocyte preparations were processed by routine techniques.¹⁷

In Vivo Studies

Cell yields were expressed as the number of granulocytes obtained after elution. Increments
in transfused animals were determined from the difference between the pretransfusion count and
counts taken at hourly intervals up to 6 hr. The T₁/₂ of transfused cells was determined from
the slope of the granulocyte disappearance.

Transfused and Nontransfused Leukopenic Dogs

Dogs were assigned randomly before cyclophosphamide administration to one of the following
groups: (1) six dogs were treated with two granulocyte transfusions given on days 5 and 6 following
cyclophosphamide administration; (2) five dogs served as nontransfused granulocytopenic con-
RESULTS

Preliminary Studies With Eluting Solutions

In preliminary studies, granulocytes were isolated from nylon columns with solutions consisting of ACD plasma, ACD Aminosol, or EDTA. The eluted cells were washed and resuspended in Hanks' solution containing 2 mg/ml of glucose with or without additional 1 mM magnesium chloride. Table 1 shows the chemotaxis and phagocytic capacities of the various granulocyte eluates as compared to granulocytes not passed over the column. A considerable reduction in chemotaxis and moderate reduction in phagocytosis was noted in eluates from which magnesium was absent. The addition of magnesium to the final preparation of granulocytes improved chemotaxis and phagocytosis. ACD plasma or ACD Aminosol appeared to be superior to the EDTA solutions. For in vivo transfusion studies, the ACD plasma solution was employed without addition of magnesium.

In Vitro Studies on Eluted Granulocytes Used in Transfusion Experiments

The average viability of granulocytes obtained from seven consecutive elution procedures, as measured by trypan blue exclusion, was 95% (92%–97%). Table 2 summarizes eight chemotactic, seven candidacidal, and seven phagocytic studies on granulocytes obtained prior to leukopheresis, following elution from the column, and isolated at peak granulocyte increments. Significant differences from control values were noted in both eluted and recovered cells, chemotaxis and candidacidal (p < 0.01) and phagocytosis (p < 0.025). However, recovered cells showed a return towards normal function in all three parameters when compared to the eluted preparations (p < 0.01). Figure 1 shows the curves for intracellular killing of *P. aeruginosa* by granulocytes isolated from donor blood and by granulocytes obtained from nylon columns after elution. Eluted cells maintain almost normal killing capacity for pseudomonas.

Comparison of Wright's Giesma-stained smears and electron micrographs
Table 2. Chemotactic Candidacidal and Phagocytic Capabilities of Granulocytes Prior to Leukapheresis, Following Elution, and Following Transfusion*

<table>
<thead>
<tr>
<th>Cells Before Leukapheresis</th>
<th>Eluted Cells</th>
<th>Recovered Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemotaxis (mean count/HPF) (n = 8)</td>
<td>181 ± 19</td>
<td>74 ± 27</td>
</tr>
<tr>
<td>Candida killed (%) (n = 7)</td>
<td>29 ± 1.8</td>
<td>16 ± 4.7</td>
</tr>
<tr>
<td>Phagocytosis (%) (n = 7)</td>
<td>63 ± 2.12</td>
<td>50 ± 3.5</td>
</tr>
</tbody>
</table>

*Results are expressed as average ± SD.

were made from granulocytes obtained from donor dogs before leukapheresis, following elution, and isolated from recipient animals 1 hr posttransfusion. No significant differences were observed between eluted granulocytes, those isolated posttransfusion, and controls.

**In Vivo Studies**

Table 3 summarizes the data on granulocyte procurement and posttransfusion kinetics in six donor-recipient combinations involving 12 granulocyte transfusions. Granulocyte yields for a 1-hr period averaged 33.3 x 10^6. Absolute granulocyte counts in recipient animals varied between 0 and 361 per cu mm at the time of transfusion. All animals showed significant responses in granulocyte counts averaging 2590 per cu mm, with a peak between 1 and 4 hr.

![Fig. 1. Per cent viable P. aeruginosa remaining after incubation with granulocytes obtained prior to leukapheresis and after elution (pretransfusion).](image-url)
Granulocyte survival posttransfusion showed a $T_{1/2}$ averaging 3.8 hr. Figure 2 illustrates the posttransfusion granulocyte changes noted in three recipient animals.

**Survival and Bacteriologic Findings**

Table 4 shows the survival time and premortem culture data for five non-transfused control animals and six granulocyte-transfused dogs. Death occurred in 5–7 days following the administration of cyclophosphamide in the nontransfused group. Platelet counts taken within 24 hr of death were above 68,000 per cu mm, and evidence of hemorrhage was minimal or absent at postmortem examination. A septic terminal episode was documented in each case by positive qualitative and quantitative blood cultures prior to death. Figure 3A illustrates the granulocyte, temperature, and culture data on dog No. 51. As the granulocyte level fell below 1000 cu per mm, a progressive temperature eleva-
Table 4. Survival of Nontransfused and Transfused Leukopenic Dogs

<table>
<thead>
<tr>
<th></th>
<th>Survival (days)</th>
<th>Premortem Cultures*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nontransfused</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>7</td>
<td>K. Aerogenes</td>
</tr>
<tr>
<td>2029</td>
<td>5</td>
<td>S. Aureus</td>
</tr>
<tr>
<td>2035</td>
<td>6</td>
<td>E. Coli</td>
</tr>
<tr>
<td>52</td>
<td>5</td>
<td>E. Coli</td>
</tr>
<tr>
<td>51</td>
<td>6</td>
<td>K. Aerogenes</td>
</tr>
<tr>
<td><strong>Transfused</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>recovery</td>
<td>negative</td>
</tr>
<tr>
<td>45</td>
<td>recovery</td>
<td>negative</td>
</tr>
<tr>
<td>47</td>
<td>recovery</td>
<td>negative</td>
</tr>
<tr>
<td>48</td>
<td>recovery</td>
<td>negative</td>
</tr>
<tr>
<td>49</td>
<td>7</td>
<td>negative</td>
</tr>
<tr>
<td>50</td>
<td>9</td>
<td>negative</td>
</tr>
</tbody>
</table>

*Quantitative (> 10 organisms per ml) blood cultures became positive 12–24 hr prior to death.

tion was noted. On the afternoon prior to and the morning of death, blood cultures were positive for *Escherichia coli*. In contrast to nontransfused dogs, Table 4 shows the survival and premortem culture data on dogs receiving granulocyte transfusions on days 5 and 6 following cyclophosphamide administration. Three dogs recovered completely and remained in good health for a period of 3–4 wk of observation. Three dogs died between days 7 and 10 with negative blood cultures and rising white counts. At this time, platelet levels were below 10,000 per cu mm. Postmortem examination revealed widespread visceral hemorrhage involving the gastrointestinal tract, lungs, and, in dog No. 50, the kidney. Death in these animals was therefore attributed to thrombocytopenic bleeding. Figures 3B and 3C illustrate granulocyte, temperature, and cultural data on dog No. 45 and dog No. 50. Dog No. 45 experienced no septic episodes and recovered completely. Dog No. 50 had a positive culture for *E. coli* prior to granulocyte transfusion on day 5, which became negative following granulocyte transfusion.

**DISCUSSION**

The canine model has proven to be useful in evaluating the effect of granulocyte transfusion under controlled experimental conditions. Epstein et al. demonstrated the effectiveness of granulocytes for transfusion obtained by use of the continuous-flow centrifuge. In these experiments, dogs were rendered leukopenic by lethal whole-body irradiation and challenged with an intravenous inoculum of *E. coli*. Although all animals died, prolongation of life and delay of terminal sepsis occurred. More recently, granulocytes obtained by the continuous-flow leukofiltration technique were shown to be effective in reducing blood bacterial levels in dogs with ongoing pseudomonas septicemia. The present studies clearly indicate the value of granulocytes obtained from normal donors by continuous-flow leukofiltration in preventing spontaneous infection in dogs subject to transient periods of leukopenia. Of six dogs transfused during the nadir of the granulocyte curve, spontaneous bacteremia failed to occur in five, and a single positive blood culture reverted to negative in the remaining
Fig. 3. Temperature, granulocyte, and blood culture results following cyclophosphamide administration in (A) dog No. 51 (nontransfused), (B) dog No. 45 (transfused), and (C) dog No. 50 (transfused). In dog No. 50 (C), a blood culture of E. coli (<10 organisms per ml) was positive prior to granulocyte transfusion. Negative cultures were obtained following transfusion.
dog. In no instance did death appear to be attributable to septicemia, and in contrast to previous studies, three of the six dogs recovered completely.

Transfused granulocytes produced significant increments in circulating granulocyte counts and disappeared at normal or near normal rates. This contrasts with reports by Herzig et al. of poor transfusion increments and shortened survival measured by DF$^{32}$P and $^{51}$Cr labeling techniques in the dog.

Mild to moderate reductions of in vitro granulocyte function were noted in the transfused cells. Chemotaxis seemed to be the most sensitive method for detection of granulocyte abnormality, but this function was shown to return toward normal following circulation in recipients. Improvement of in vitro function following transfusion could be explained by either rapid disappearance of defective granulocytes or by exposure to a normal in vivo environment of magnesium and other factors necessary for granulocyte function. Herzig noted reduced bactericidal and phagocytic function in granulocytes obtained by leukoadhesion. Magnesium was not added to the granulocytes obtained prior to testing, which might be the reason for the reduction in function. Preliminary studies indicate that in vitro granulocyte function with respect to chemotaxis and to phagocytosis was significantly improved when magnesium was added to the solutions in which the granulocytes were tested. The mechanism of chemotaxis has yet to be defined, but is thought to involve a membrane-bound esterase. It is possible that, in the process of adhesion to nylon columns, the membrane esterase is damaged, resulting in an impairment of granulocyte ability to respond to a chemotactic stimulus.

The use of plasma in eluting solutions imposes limitations on multiple plasma donations from the same donor. Furthermore, the use of plasma is almost always associated with risks of hepatitis. For these reasons, a plasma substitute, Aminosol, was investigated, using ACD Aminosol as an eluting solution. Although slightly less efficient than ACD plasma, ACD Aminosol-eluted granulocytes showed functional capabilities equivalent to ACD plasma-eluted granulocytes. The poor results obtained with EDTA might be due to the absence of plasma from this eluting solution. Various plasma, Aminosol, EDTA, and ACD combinations are currently being used in hopes of finding the optimal eluting solution in terms of efficiency, preservation of viability, and functional capabilities.

The present studies support recent clinical reports suggesting that granulocytes obtained by the continuous-flow leukoadhesive technique may be of benefit to patients in the prophylaxis or treatment of infectious complications of leukopenia. The canine model provides a basis for studying both induced and spontaneous infection in the leukopenic host. Further questions involve the use of combinations of antibiotic and granulocyte transfusion support. The recent clarification of a DL-A histocompatibility system in the dog, analogous to the human HL-A, also provides a basis for further studies of presensitization to granulocyte transfusions carried out on a long-term basis.

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