Effects of Hydrocortisone on the Yield and Bactericidal Function of Granulocytes Collected by Continuous-flow Centrifugation

By Mamoru Shoji and William R. Vogler

The usefulness of granulocyte transfusions is in part dependent upon the number of granulocytes transfused. The invention of the continuous-flow cell separator has made it possible to obtain granulocytes from normal donors. Efforts to improve the yield are under study. This controlled study was undertaken to determine the effect of a single dose of hydrocortisone on granulocyte yield from volunteer donors and on granulocyte bactericidal function. Twenty-two normal volunteers were randomized between no therapy or a single intravenous injection of 120 mg/sq m of hydrocortisone 2 hr prior to initiation of a 4-hr leukapheresis using the Aminco cell separator operated at 750 rpm and a flow rate of 41 ml/min. Significant increases in granulocyte yield and reductions in lymphocyte and monocyte yields were obtained in the hydrocortisone-treated group. Granulocytes from each group were equally effective in the phagocytosis of yeast particles and in vitro bactericidal activity.

HIGH MORTALITY RATES of 60%-90% for patients with gram-negative septicemia in association with leukemia have been reported. Morse et al. have demonstrated clinical benefit in patients with severe neutropenia receiving on the order of $7 \times 10^{10}$ granulocytes obtained from patients with chronic myelocytic leukemia. The invention of the continuous-flow leukocyte separator (CFLS) has made it possible to collect $1.5 \times 10^9$ granulocytes per hour from a single donor. Graw et al. have reported that 11 of 37 patients in a concurrent control group survived septicemia due to gram-negative bacteria compared to 18 of 39 survivors in a group receiving granulocyte transfusions collected from normal donors. However, all 12 patients who received four or more transfusions survived. Thus, it would seem that the efficacy of granulocyte transfusions would depend in part upon giving adequate numbers of granulocytes. Yields from 4 hr of leukapheresis would equal approximately $6 \times 10^9$ granulocytes, ten times less than the number required. Therefore, efforts to increase granulocyte yields seem in order. Bishop et al. have shown that 3–5 hr following the intravenous injection of a single dose of 200 mg of hydrocortisone, the blood granulocyte pool increased from 79 to $138 \times 10^9$ cells/kg body weight. Other agents known to increase the circulating granulocyte pool are etiocholanolone, exotoxin, and exercise. However, these other substances seem to have more side effects, and exercise is not practical. The first part of our study was designed to determine if the number of granulocytes harvested by means of the CFLS could...
be increased by giving a single intravenous injection of hydrocortisone 2 hr prior to centrifugation.

However, it is also known that hydrocortisone seriously blunts the inflammatory response, interferes with mobilization of granulocytes into areas of tissue damage, suppresses phagocytosis, and diminishes lysosomal release. Thus, the second part of our study was done to determine whether a single intravenous hydrocortisone injection interfered with the bactericidal capacity of the harvested granulocytes.

**MATERIALS AND METHODS**

**Continuous-flow Leukocyte Separation (CFLS)**

**Donors.** Normal adults, after informed consent, were randomized to receive either no medication or hydrocortisone given as a single intravenous injection (120 mg/sq m) 2-3 hr prior to beginning the leukocyte collection. Some donors served both in control and hydrocortisone groups. In these instances there was a lapse of at least 1 wk between experiments.

**Equipment.** The equipment utilized in these studies was an Aminco Celltrifuge from American Instrument Co. (Silver Springs, Md.). Sixteen-gauge scalp vein needles were used.

**Anticoagulation.** Anticoagulation of the donors and extracorporeal blood system was performed with a mixture of heparin and acid-citrate-dextrose (USP-NIH formula A) anticoagulants as described by Graw et al. Following each procedure protamine sulfate (25-30 mg) was slowly infused intravenously to correct the donor's Lee-White clotting time to normal. Leukocytes were collected in a 300-ml plastic bag transfer unit (Fenwal Laboratory, Morton Grove, Ill.) containing 15 ml of ACD solution.

**Centrifuge speed, flow rate, and centrifuge period.** Centrifugation speed of the bowl was kept at 750 rpm in all experiments with a constant flow rate through the bowl of 41 ml/mm. Collections of leukocytes were made at a flow rate of 1.0 ml/min throughout all of the experiments. Centrifugation periods were 4 hr in all experiments.

**Bactericidal Capacity of the Granulocytes**

A modified method of Solberg et al. was used. Leukocytes collected with the CFLS were further concentrated in 3% dextran-isotonic saline (1:1 volume). The cells were allowed to sediment for 30 min at room temperature; then the plasma, buffy coat layer, and a few millimeters of RBC were expressed through a No. 21 scalp vein needle and washed once in Hanks' balanced salt solution (BSS) (North American Biological Inc., North Miami, Fla.) and centrifuged at 120 g for 10 min at room temperature. Viability count of leukocytes by trypan blue dye exclusion was performed. The leukocytes were suspended in 10% autologous serum in Medium 199 (North American Biological Inc., North Miami, Fla.) in a concentration of 10⁵ neutrophils/ml.

**Bacteria.** Escherichia coli obtained from the hospital Bacteriology Laboratory and kept at 4°C were incubated in a trypticase-soybean broth at 37°C overnight prior to each experiment. The bacteria were suspended in sterile isotonic saline to an optical density of 0.6 at 620 nm in a Beckman spectrophotometer against a standard of trypticase-soybean broth. This suspension was diluted in Hanks' BSS to a ratio of 1:1 which contained approximately 2.5 x 10⁴ colony-forming bacteria (CFU) (1.5-5.0 x 10⁵) per 0.1 ml.

**Serum.** Serum was collected from each donor before the injection of hydrocortisone.

**Nutrient broth-agar.** One and a half grams of Bacto-agar (Bacto) and 0.8 g of nutrient broth (Bacto, Difco Co., Detroit, Mich.) were mixed in 100 ml of distilled water and autoclaved at 20 lb pressure (121°C) for 20 min and kept at 4°C. Upon use the agar was melted and kept at 47°C-48°C.

**Leukocyte-bacteria suspension.** Four milliliters of leukocyte suspension and 0.4 ml of E. coli suspension were mixed in a 50-ml Falcon plastic conical graduated centrifuge tube with a cap (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif. 90045). This provided about two to three E. coli per neutrophil. This suspension was further equally divided into two tubes after taking samples (0.1 ml) at 0 min. One tube was used to measure the “total” viable
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number of bacteria and another the "intracellular" viable number of bacteria. As a control, 2 ml of 10^6, serum-M 199 and 0.2 ml of E. coli suspension were mixed. The tubes were incubated in a moist incubator at 37°C with 95% air and 5% carbon dioxide for 2 hr. Samples were taken at 30, 60, 90, and 120 min.

The total number of viable bacteria was determined by adding 0.1 ml of leukocyte bacteria suspension to 1 ml of sterile distilled water to facilitate osmotic disruption of the leukocytes. Viable bacteria were counted by diluting this suspension (1:10^3 dilution), mixing with approximately 10 ml of nutrient-broth-agar, and using a standard pour-plate technique. The plates were incubated overnight at 37°C, and one CFB was counted as a bacterium.

The number of viable intracellular bacteria was determined by washing 0.1 ml of leukocyte bacteria suspension in 2 ml of Hanks' BSS five times at 900 g for 2 min at 4°C. Then 1 ml of sterile distilled water was added to a cell pellet for disruption of leukocytes. The rest of the technique was identical to the total viable bacterial assay.

Phagocytosis

Yeast. Baking yeast (Fleishman) was mixed with hot (but not boiling) water and diluted in sterile isotonic saline to yield a suspension of approximately 100-200 particles per high power field. Granulocyte suspensions, prepared as above, and yeast suspensions were mixed and incubated in a moist incubator at 37°C for 2 hr. An aliquot was examined under a phase microscope for phagocytic ability.

Statistical Methods

The Student's t test was used to determine differences between sample means on the hypothesis that no difference existed. A p value of 0.05 or less was considered significant.

RESULTS

Effect of Hydrocortisone on Granulocyte Yield

Thirty-six centrifugations were attempted in 22 donors without significant problems. Thirteen donors served both in the control and hydrocortisone groups. In addition, T.S. served a second time in the hydrocortisone group. Table 1 shows the absolute yields of leukocytes, granulocytes, lymphocytes, and monocytes per bag for each individual donor and the mean following 4 hr of centrifugation. In 13 donors who served in both control and hydrocortisone groups, all but three showed greater yields of total granulocytes than lymphocytes when they received hydrocortisone. T.S. had a much greater granulocyte yield on the second hydrocortisone study. The first experiment was complicated by platelet plugging of pores in the ceramic seal. The mean numbers of leukocytes collected were not significantly different in the two groups (p > 0.10). However, the total yield of granulocytes was significantly greater in the hydrocortisone group (p < 0.05). The yield of lymphocytes and monocytes was significantly less in the hydrocortisone group (p < 0.01).

The means and ranges of hemograms on the leukocyte-rich blood from both groups are shown in Table 2. As can be seen at similar volumes and hematocrit and platelet counts, significant differences were observed not only in the yield of granulocytes and lymphocytes, but hydrocortisone significantly reduced the number of monocytes collected.

Viability tested by trypan blue dye exclusion was found to be 100%, in the bags in all instances.
<table>
<thead>
<tr>
<th>Don.</th>
<th>WBC</th>
<th>PMN</th>
<th>LC</th>
<th>Mon.</th>
<th>Don.</th>
<th>WBC</th>
<th>PMN</th>
<th>LC</th>
<th>Mon.</th>
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<td>0.3</td>
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<td>5.4</td>
<td>1.4</td>
<td>0.9</td>
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<td>1.3</td>
<td>1.6</td>
<td>1.2</td>
<td>T.S.</td>
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<td>B.R.</td>
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<td>6.3</td>
<td>1.0</td>
<td>B.R.</td>
<td>7.8</td>
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<td>1.6</td>
<td>0.2</td>
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<td>M.R.</td>
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<td>3.6</td>
<td>3.3</td>
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<td>M.R.</td>
<td>3.9</td>
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<td>0.3</td>
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<td>6.2</td>
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<td>J.S.</td>
<td>7.5</td>
<td>6.4</td>
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<td>0.6</td>
<td>0.4</td>
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<td>1.7</td>
<td>0.9</td>
<td>0.2</td>
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<td>5.7</td>
<td>2.2</td>
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<td>4.0</td>
<td>2.7</td>
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<td>3.9</td>
<td>0.8</td>
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<td>2.1</td>
<td>0.3</td>
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Collection Efficiency

The collection efficiency was calculated by dividing the total number of cells collected in the bag (corrected for differential counts) by the initial WBC per milliliter times the volume of blood processed during the 4-hr period (41/ml min x 240 min). Although a lower percentage of WBC ($p < 0.05$) and lymphocytes ($p < 0.05$) were collected in the hydrocortisone group, no differences in the efficiency of granulocyte collection were observed between the hydrocortisone (8.4% ± 4.3%) and control groups (7.8% ± 4.8%).

Table 2. CBC in WBC-rich Bag

<table>
<thead>
<tr>
<th>Blood Volume in bag (ml)</th>
<th>HCT (%)</th>
<th>WBC/cu mm (x 10^3)</th>
<th>PMN (%)</th>
<th>Lymph (%)</th>
<th>Mono (%)</th>
<th>Eos + Baso (%)</th>
<th>Plot (%)</th>
</tr>
</thead>
<tbody>
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<td>Control (18 collections)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>302 ± 40* (230–380)†</td>
<td>20 ± 5</td>
<td>24.6 ± 10.9</td>
<td>40 ± 18</td>
<td>43 ± 16</td>
<td>14 ± 8</td>
<td>2 ± 2</td>
<td>229 ± 63 (132–398)</td>
</tr>
<tr>
<td>Hydrocortisone (18 collections)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>293 ± 33 (230–360)†</td>
<td>20 ± 4</td>
<td>19.4 ± 9.2</td>
<td>73 ± 12</td>
<td>22 ± 10</td>
<td>5 ± 3</td>
<td>1 ± 1</td>
<td>240 ± 63 (157–366)</td>
</tr>
</tbody>
</table>

*p Mean ± SD.
†Range.
NS Not significant.
Effects of Hydrocortisone on the Peripheral Blood Counts

The means of the peripheral counts before, during, and after the continuous-flow leukocyte separation are shown in Table 3. There was no significant increment of granulocytes, lymphocytes, and monocytes from 7:30 a.m. to 2 p.m. in the control group. However, a significant increase in percentage as well as absolute number of granulocytes and significant decreases in the absolute number of lymphocytes and monocytes in the hydrocortisone group were noted. Thus, hydrocortisone increased granulocytes and decreased lymphocytes and monocytes in the peripheral counts. Hydrocortisone did not affect hemoglobin, hematocrit, platelet, or reticulocyte counts.

Bactericidal Function

Bactericidal capacity of granulocytes. The effect of hydrocortisone on the bactericidal capability of granulocytes was compared to the controls in five experiments in each group. As shown in Fig. 1, there was a logarithmic growth of bacteria without granulocytes. The addition of granulocytes significantly reduced the total number and the intracellular number of bacteria in each group. As shown in Fig. 2 comparing the bactericidal capacity of the granulocytes between the control and hydrocortisone groups at each time interval resulted in no significant differences during incubation. Thus, hydrocortisone treatment did not induce a gross impairment in the bactericidal capability of granulocytes.

Viability of leukocytes at the end of the experiments described above was 85%-95%.

Phagocytosis

Yeast. No differences in phagocytic capacity between groups were observed. Granulocytes made clumps around yeast particles and engulfed them. One granulocyte contained an average of three yeast cells.
**DISCUSSION**

In attempting to develop methods of increasing the yield of granulocytes, we chose hydrocortisone because we felt it would produce less discomfort for the donor than etiocholanolane which is pyrogenic and reaches its maximum effect 12 hr after injection.\(^{16,17}\) In contrast, hydrocortisone produces no fever and in our hands produced its maximum effect between 2 and 6 hr after injection.

Koza et al.\(^ {18} \) demonstrated that a single intravenous dose of dexamethasone increased the yield of the granulocytes through the NCI-IBM Blood Cell Separator. However, only three donors received dexamethasone in contrast to 36 donors who received none. Graw et al.\(^ {4} \) reported that 13 donors received...
Bactericidal Function of PMN's

\[
\left( \frac{\text{Bacterial Count with PMN's}}{\text{Bacterial Count without PMN's}} \right) \times 100
\]

Fig. 2. Bactericidal capacity of the granulocytes was calculated according to the following formula:

\[
\frac{\text{Bacterial count after incubation with granulocytes}}{\text{Bacterial count after incubation without granulocytes}} \times 100\%
\]

Each bar represents a mean of the five experiments. The empty bar represents the control and the shaded bar the hydrocortisone group. There are no significant differences between the two groups during incubation (p > 0.1).

etiocholanolone or cortisone and gave an increased yield of granulocytes by 100\% (range, 29\%-272\%). However, no data were described in detail.

In these studies we have presented observations that confirm the findings that corticosteroids will increase the granulocyte yield using the leukocyte separator. Furthermore, we found that a single dose of intravenous hydrocortisone not only increased the yield of granulocytes but also decreased the
yields of lymphocytes and monocytes. This reduction in the number of lymphocytes might reduce the risk of graft-versus-host reaction (secondary syndrome) which has been reported in recipients of granulocyte transfusions.19

The mean yields of granulocytes in our hands were less than those reported by Clift et al.20 Because they wished to do repeated donations from a single individual, they inserted an arteriovenous shunt and were able to obtain a mean of \(10.8 \pm 3.6 \times 10^9\) granulocytes over a 4-hr period using a centrifugation speed of 500 rpm and a flow rate of 50 ml/min. It is not clear whether the shunt or collection methods were responsible for their better yields. Graw et al.4 reported a median granulocyte yield of \(4.86 \times 10^9\) and, in a later publication,5 \(5.6 \times 10^9\) without hydrocortisone, similar to our results with hydrocortisone. They used an IBM cell separator with a centrifugation speed of 500 rpm and a flow rate of 40 ml/min. Preliminary studies in our hands using the Aminco leukocyte separator gave higher yields at 750 rpm than at 500 rpm, and we decided to use the former for the purposes of this study. Obviously, methods to improve granulocyte yields are needed. A full explanation for the variability in yields among donors is lacking, and further investigation is necessary. The usefulness and safety of hydroxyethyl starch is under study and may prove superior to other methods.21

Graw et al.5 have described that the granulocytes collected by the CFLS had normal phagocytic and bactericidal capacity, but no data were shown. To our knowledge, no experimental data of the bactericidal capacity of the granulocytes collected by the CFLS have been reported in humans. Epstein et al.22 demonstrated good functional activity of dog granulocytes collected by means of the leukocyte separator. In our studies the granulocytes from the hydrocortisone-treated donors were shown to have grossly intact bactericidal capacity equal to those from the control group of donors.

The total number of killed bacteria was not as high as found by Solberg et al.24-26 Our methods differed from theirs in that an end-over-end rotation with 0.1% gelatin in the cell suspension medium was not used. In our opinion, fewer contacts between the granulocytes and the bacteria resulted in the reduced killing of the bacteria. This postulate is supported by our early experiments. Using a ratio of granulocytes and \(E. coli\) of 18-30 to 1 resulted in almost no viable bacteria after 60 min of incubation.

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