Separation and Collection of Leukocytes

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**Summary**

Existing methods for separation and collection of leukocytes permit collection of only limited quantities of leukocytes. An instrument has been designed to process large volumes of blood on a continuous flow basis. The instrument collects venous blood, separates leukocytes in a centrifuge, and reinjects the red cells, plasma, and platelets. The separation process is 30–60% efficient in vitro at flow rates up to 50 ml/min. In vivo tests demonstrated safety, sterility, lack of hemolysis, and adequate anti-coagulation. However, leukocyte recovery in vivo is low (under 20%). If this poor in vivo recovery can be overcome, the instrument should prove useful for collection of large quantities of leukocytes.

Because acute leukemia is a disease which involves primarily the leukocytes, collection of leukocytes for study is of great importance. Unfortunately, these cells are the least accessible component of the blood. Red cells can be obtained in pure form and in large quantity with ease because of their high specific gravity and their tendency to aggregate. The platelets are also relatively easy to collect because of their small size and lower specific gravity. Even the plasma portion of the blood can be readily obtained in pure form. The leukocyte, unfortunately, is intermediate and almost all technics of collection face the problems of contamination with red cells and platelets. This limitation is aggravated by the fact that in normal individuals and in most patients with acute leukemia the blood contains over 500 red cells and over 20 platelets for every leukocyte. Finally, the leukocytes are a heterogeneous population of various myeloid leukocytes and lymphoid leukocytes. Witness the usual differential count, which almost always distinguishes 5 different types of leukocytes with clearly different functions. These facts present a major obstacle to the solution of the leukemic problem. An understanding of the physiology, biology, and biochemistry of the normal and leukemic leukocyte will require effective technics of leukocyte collection.

For experimental purposes there are a wide variety of technics for leukocyte separation (9, 19). These include separation from a source of pure cells such as peritoneal exudates (14), lymph nodes, and thoracic duct lymph (3, 6); filtration through fritted glass (4) or glass bead columns (5) to remove granulocytes; selective lysis of red cells (15); or flotation on density gradients (18).

By far the most commonly used methods utilize sedimentation of red cells at 1 × g or at higher forces in a centrifuge. In 1948 the addition of high molecular weight substances was introduced, fibrinogen being the first substance studied (12). These materials cause aggregation of red cells and greatly increase their rate of sedimentation. In addition to fibrinogen, dextran, phytohemagglutinin, polybrene, and y-globulin have been used effectively (7, 8, 16, 17).

While most of these technics are useful for processing small quantities of blood, only the sedimentation technics have proven practical for processing whole blood in relatively large quantities, i.e., over 500 ml. The technic of Maupin (10, 11) is among the best for collection of leukocytes without additives. Collection of buffy coat usually gives yields of 30–60% of the leukocytes in whole blood. Addition of red cell-aggregating agents usually increases these yields to over 80%.

For supportive therapy of patients with leukemia, replacement transfusion with both granulocytic and lymphocytic leukocytes offers an attractive approach. By using as donors patients with chronic myelocytic leukemia (CML) who have leukocyte counts 30 or more times normal, it has been possible to show that transfusion of 10¹¹ granulocytes per sq m of the body surface area of the recipient produces increments in circulating leukocytes of over 1000/cu mm (13). For collection of the cells from such donors, centrifugation at slow speeds has generally produced the best separation (Table 1). Addition of dextran greatly increases the yield of leukocytes. Unfortunately, because a plasmapheresis technic is used, some of the dextran is reinjected into the donor as well as into the recipient. Frequent dextran injection can result in immunization and possible anaphylaxis, and prolonged storage of this material by the recipient also limits its usefulness. The use of fibrinogen for this purpose is limited by the potential contamination with hepatitis virus. For this reason we have studied y-globulin (Table 2). The paired studies shown are separate units from the same donor studied at the same time. Twenty ml of y-globulin (16.5%) were added to each unit (500 ml) of blood. The recovery was regularly increased. Unfortunately, ad-

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1 Deceased.
ministration of γ-globulin intravenously can occasionally produce severe transitory hypotension. Also to be emphasized is the extreme variability in separation efficiency from donor to donor (Table 3).

To collect comparable quantities of leukocytes (10^11) from normal donors, would require the cells from 20 to 40 units of blood if leukocyte concentrations were 5,000-10,000/cu mm. With the less than optimal recovery from separation and with the fact that only 50-70% of these cells are granulocytes, 2-4 times this quantity of blood might have to be processed. Djerassi has reported that collection of leukocytes from a large number of units of normal blood can increase the circulating leukocytes in a recipient. To process quantities of blood of this magnitude a leukapheresis technic would be desirable. Bierman et al. (1, 2) have described leukapheresis of normal persons and leukemic patients with the use of the ADL Cohn fractionator.

RESULTS AND DISCUSSION

We set out to design an instrument which would process large quantities of whole blood from a donor, without any additive, utilizing a leukapheresis technic. The objectives of the instrument design were as follows: (a) Leukocytes should be separated from whole blood at a reasonable efficiency by sedimentation in a centrifuge. (b) Operation should be conducted on a continuous flow basis to allow processing of large quantities of blood at optimal speed and efficiency. (c) A vein-to-vein procedure should be used to avoid arterial puncture. (d) An anti-coagulant that does not require anti-coagulation of the donor, with its associated risks, should be employed. (e) The loss of platelets, red cells, and plasma should be minimal to allow processing of large volumes of blood in a single donor. (f) The system should be completely closed, needle-to-needle, without any air-blood interface to obviate the danger of air injection or bacterial contamination. (g) The entire system should contain a volume of blood under 500 ml at all times. (h) The system should be easily cleaned, mostly disposable, and sufficiently automated to be operated by a single nonprofessional operator.

After completion of 3 model designs, we are currently investigating a centrifuge which subjects the blood to 42 g of horizontal sedimenting force (850 rpm), in a vertical cylinder through which the blood passes continuously in the vertical direction. When the column of blood reaches the bottom of chamber it has separated into red cells, buffy coat, and plasma. There are 3 collecting ports: one at the outer wall which continuously removes from the bottom of the packed cell layer (red cell port), one at the inner wall which collects from the top of the plasma layer (plasma port), and one in the center (leukocyte port) which can collect buffy coat after enough has accumulated in the centrifuge. Each collecting port has its outflow controlled by a separate peristaltic pump which permits the operator to vary the collection from each independently. The capacity of the centrifuge is 190 ml. All of the design and developmental work was performed on freshly drawn units of whole blood from normal blood donors, which were pooled prior to use and studied within 8 hr of collection. The results of processing whole blood at a through flow of 50-60 ml/min are shown in Table 4. Of the 4500 ml of blood run through the instrument, only 55 ml (1%) were removed through the leukocyte port, which contained 58% of the total leukocytes. This is a 50-fold increase in leukocyte concentration. This buffy coat contained only 1% of the total red cells and 0.6% of the platelets of the original blood. These data are shown diagrammatically in Chart 1. The red cell output contains virtually all of the red cells and 50-60% of the plasma. It contains most of the leukocytes which would be lost during collection. The plasma pump contains 30-50% of the plasma and 30-50% of the platelets with little leukocyte contamination. The

### Table 1

**Separation of Leukocytes from Donors with Chronic Myelocytic Leukemia**

<table>
<thead>
<tr>
<th>No. of Units</th>
<th>Centrifugation Speed (rpm)</th>
<th>Time (min)</th>
<th>Average Plasma Volume</th>
<th>WBC/cu mm Plasma</th>
<th>WBC Total (x10^6)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3,200</td>
<td>3</td>
<td>200</td>
<td>1,700</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>1,500</td>
<td>10</td>
<td>150</td>
<td>17,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>600</td>
<td>30</td>
<td>200</td>
<td>150,000</td>
<td>3.00</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
<td>20</td>
<td>180</td>
<td>170,000</td>
<td>3.08</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>400</td>
<td>30</td>
<td>190</td>
<td>254,000</td>
<td>3.19</td>
<td>23</td>
</tr>
<tr>
<td>18</td>
<td>400</td>
<td>20</td>
<td>140</td>
<td>288,000</td>
<td>3.50</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>Dextran</td>
<td>30</td>
<td>7.42</td>
<td>89</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Marked contamination of buffy layer with red blood cells.

### Table 2

**Effect of γ-Globulin on Recovery of Leukocytes in Patients with Chronic Myelocytic Leukemia**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No γ-globulin</td>
</tr>
<tr>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>Median</td>
<td>33</td>
</tr>
</tbody>
</table>

* Values in italics are medians.

### Table 3

**Recovery of Leukocytes from Separate Donors**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.</td>
<td>100, 100, 100, 100, 90, 80, 80, 80, 67</td>
</tr>
<tr>
<td>P.</td>
<td>72, 69, 69, 64</td>
</tr>
<tr>
<td>W.</td>
<td>86, 65, 64</td>
</tr>
<tr>
<td>S.</td>
<td>72, 67, 64, 60, 65, 55, 48, 40, 37</td>
</tr>
<tr>
<td>W.</td>
<td>55, 40, 36, 27, 26</td>
</tr>
</tbody>
</table>

* Values in italics are medians.
buffy coat or white cell pump has 30–60% of the leukocytes with some red cell and platelet contamination.

Also of interest is that some degree of separation of lymphocytes and granulocytes is also observed (Table 5). If a large buffy coat is allowed to accumulate, and is then removed from the plasma side out to the packed red cell layer, the 1st samples have almost pure lymphocytes and the latter samples contain predominantly granulocytes. In the experiment shown, the peak of lymphocytes is seen in Tube 2, containing over 90% lymphocytes, while the granulocyte peak is observed in Tube 4, where 76% of cells are granulocytic. These data are shown graphically in Chart 2. The peak leukocyte concentration is in Sample 2, before measurable red cell contamination is observed. The red cells increase in quantity whereas leukocytes decrease after Sample 6. The peak concentration of lymphocytes precedes the peak for granulocytes, the latter being contaminated with red cells.

After in vitro studies had established sterility, pyrogen-
period was 188,000 and remained at this level, ending with a count of 208,000. In addition his splenomegaly increased during the procedure. Platelet count was not significantly decreased by this procedure.

These preliminary studies were designed primarily to give information on safety and operating effectiveness of the system. The potential toxicity of citrate injection has been studied. EKG monitoring is employed, and Q–T interval is observed during the procedure. At rates of injection below 50 ml of blood per min, no effects are observed. At higher rates, paresthesia about the face and fingers has been noted even though Q–T intervals are normal. Plasma levels of citrate increase to 25–35 mg/100 ml at the time Q–T interval prolongation is seen. Decreasing the rate of injection results in prompt reversal of these signs. Citrate levels have returned to under 5 mg/100 ml within 1 hr after cessation of injection in all 7 studies to date.

Blood coagulation is not altered, and measurement of plasma prothrombin, recalcification time, and fibrinogen levels shows no significant change. Plasma hemoglobin levels have remained in the normal range for all studies save 1, when a hemolytic episode occurred because of personnel failure. The step in question has since been automated to avoid recurrence. No evidence of clinical pyrogen or bacterial contamination has been seen in 39 of the studies. Patients have been afebrile and asymptomatic. In our early trials, 1 major fever-chill reaction and 4 low-grade febrile episodes were observed immediately following the procedure; each lasted less than 6 hr. We have incorporated a water bath at 38°C to warm the blood prior to reinjection, which has cooled toward room temperature. Since then, no febrile reactions have been observed. These studies have established the safety of the instrument.

While the instrument has proven safe for in vivo leukapheresis, the efficiency of leukocyte separation in vitro could not be confirmed in vivo. As already emphasized, initial studies were conducted in patients with chronic leukemia. The 2 patients with chronic lymphocytic leukemia had circulating white counts of 17,000 and 190,000/cu mm. Recovery ranged from 25 to 60% of the leukocytes passing through the instrument and consisted almost entirely of small lymphocytes, as did the peripheral blood. Five patients with chronic myelogenous leukemia were studied. White counts ranged from 10,000 to 250,000. Leukocyte recovery ranged between 2% and 22% (median, 12%). In these patients recovery consisted almost entirely of immature myeloid cells. Because we felt that the poor leukocyte recovery could result from the very abnormal leukocyte-erythrocyte ratios in these patients, we studied 3 patients with normal peripheral blood, 1 patient with CML in remission, and 2 patients with malignant disease. In 7 experiments leukocyte recoveries have ranged from 1 to 17% (median, 9%), and those leukocytes collected are almost all lymphocytes. Thus, the in vivo experiments have given very poor leukocyte recoveries to date.

The cause of the difference between in vitro and in vivo experiments remains enigmatic at present. We have investigated the importance of temperature. Experiments were run with an ice bath, at room temperature, and with a 37°C bath. Blood was equilibrated at these temperatures before in vitro experiments. Leukocyte recoveries were lower in the cold (20%), but room temperature (20–22°C) and 37°C gave comparable separation efficiency, 58 and 44%, respectively. Moreover, both granulocytes and lymphocytes were collected. The effect of hematocrit was investigated by preparing plasma rich in white blood cells and preparing samples from 6 to 17 gm/100 ml of hemoglobin. Again no major difference was observed; recoveries ranged from 30 to 80% with median recovery of 50%, independent of hemoglobin concentration.
To date we have not been able to duplicate the low in vivo recoveries in in vitro experiments. We are left with two possibilities: the first is that blood 4—8 hr old differs from fresh blood in some parameter; the second is that pooling several units of blood causes some alteration in physical properties. Common to both is the possibility that some red cell aggregation has taken place in vitro and aided in separation. These possibilities are being investigated.

Even if the problem of in vivo separation is overcome, other problems still remain. The present laboratory model uses hand-assembled components including 64 ft of plastic tubing, 50 nylon friction fittings, 10 three-way stopcocks, and 11 intravenous solution sets and bottles. These are all cleaned and assembled by hand, representing 3 hr of work. Cleaning and assembling the centrifuge requires 1 hr: 30 min for assembling the apparatus and 2 hours for tear-down procedure. The seal assembly is nondisposable, hand-tooled, and expensive. Evidently, we have much development ahead to make the instrument easy to use and mostly disposable.

If these problems can be solved, we hope that an instrument will result which will make leukocyte collection in quantity a reality. This tool could be enormously useful for overcoming the obstacle of leukocyte collection and serve as a biopsy technic for study of these important organ systems, the leukocytes.

REFERENCES

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