A New Technic for Separation of Human Leukocytes

By Parviz Lalezari

Increasing interest in the study of various biological functions of leukocytes has stimulated efforts to prepare these cells by various technics. Maupin has recently reviewed the literature and compared the results of most available methods.

The technic reported here is simple: red cells are agglutinated by hexadimethrine bromide, and leukocytes, not affected by the compound, are separated by differential sedimentation.

Materials and Methods

Siliconized glassware was used throughout. Fasting blood was collected into 100 ml. cylinders containing 0.1 ml. of 10 per cent disodium ethylenediamine-tetra-acetate (EDTA) for each 10 ml. of blood. Fifteen ml. aliquots of the anticoagulated blood were transferred to 125 x 25 mm. test tubes. To each 5 ml. of blood, 0.1 ml. of 1 per cent Polybrene (hexadimethrine bromide) was added and the tubes were inverted 10 times. Droplets of blood remaining on the tube wall above the blood level were carefully removed by a cotton applicator. The tubes were then allowed to stand at room temperature for about 20–30 minutes. At this point, when most of the agglutinated red cells were sedimented down, the tubes were centrifuged in a No. 213 swinging head of a Model CL International clinical centrifuge, at exactly 300 rpm. After five minutes of initial centrifugation, the tubes were rotated 180 degrees on the long axis in the centrifuge cups and recentrifuged for an additional five minutes. The purpose of this rotation was to prevent sedimentation of the red cell agglutinates on the tube wall. The leukocyte-rich supernates were then decanted.

For the purpose of counting, the red cell clumps were dispersed by further addition of one volume of 10 per cent EDTA to 24 volumes of supernate. Counts in all samples were done in duplicate and differentials were determined by counting 300 cells on slides stained by Wright's stain. Direct platelet counts were performed by phase contrast microscopy.

To prepare platelet-poor leukocyte suspensions, platelets were removed by differential centrifugation prior to addition of Polybrene. Tubes containing 15 ml. of anticoagulated blood were centrifuged at 1500 rpm for 15 minutes. The platelet-rich, leukocyte-poor supernates were separated and recentrifuged at 3000 rpm for 10 minutes. The platelet-poor supernates were then returned to the original blood samples, mixed and processed for separation of leukocytes, as described above. Defibrination technic by glass beads likewise was applied to remove the platelets prior to separation procedure.

Supravital staining of isolated leukocytes was performed with Trypan Blue as described by Stähelin et al.

Hemolytic anti-red-cell antibodies were used to produce erythrophagocytosis. A red cells and anti-A iso-antibody with a hemolytic titer of 1:4 was used in these experiments. In order to remove both Polybrene and EDTA from the medium, platelet-poor leukocyte suspensions were diluted in 10 volumes of isotonic saline and centrifuged at 1200 rpm for five minutes. The sedimented cells were resuspended in donor's own fresh serum to

From the Department of Hematology, Laboratory Division, Montefiore Hospital, New York, N. Y.

Supported by the Health Research Council of the City of New York under Contract No. U-1024.


produce a final leukocyte concentration of about 10,000/cu.mm. To 0.1 ml. of the cell suspension, 0.05 ml. of 2 per cent donor's own red cells in fresh serum, and then 0.1 ml. of anti-A serum were added. The mixtures were incubated at 37 C. for about 10 minutes and the details of the reaction were studied by phase microscopy as described elsewhere.7

Oxygen uptake determinations were done by the direct Warburg method. Two-tenths ml. of 10 per cent KOH and small filter papers were placed in the center wells of 20 ml. flasks; the fluid phase was 2.2 ml. and gas phase was room air. The flasks were subjected to continuous shaking at 120 oscillations per minute and 37 C. Since high concentrations of leukocytes are needed for metabolic studies, and concentrating procedures such as centrifugation depress leukocyte respiration,8-9 leukemic blood with high leukocyte count was used. Leukocyte suspensions were prepared by spontaneous sedimentation of the red cells. The leukocyte counts were adjusted to about 5 x 10^7/cu.mm., and the number of platelets was about the same. To 1.5 ml. of cell suspensions used in each flask, 0.5 ml. of various dilutions of Polybrene in isotonic NaCl were added and 30 minutes after mixing, oxygen uptake was measured at 10 minute intervals and compared to controls with isotonic saline instead of Polybrene.

RESULTS

Table I demonstrates the results of leukocyte separation in 15 normal blood samples separated at 22 C. The platelet contamination could not be evaluated by platelet counts because of irreversible platelet agglutination by Polybrene;4 grossly, however, it appeared that more than half of the platelets were removed during separation.

Employing identical procedures, the results of leukocyte separation varied for different blood donors; the required incubation time primarily appeared to depend upon the ratio between fluid and cellular phase10 in the sample. An attempt to increase the fluid phase by addition of saline was not satisfactory in that such treatment resulted in increased red cell contamination. The data indicate that the average number of recovered leukocytes per cu.mm. in leukocyte-rich supernatants is about 80 per cent of that in the original blood sample. It also appears that a relatively fixed number of red cells remains, giving a predictable purity of the yield which increases by use of blood samples with high leukocyte counts.

Table 2 shows the results of leukocyte separation following preliminary removal of platelets. It may be seen that by preliminary differential centrifugation, close to 90 per cent of platelets were removed; further removal was accomplished during the separation procedure.

Table 1.—Leukocyte Separation from 15 Normal Donors

<table>
<thead>
<tr>
<th></th>
<th>Average</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte count in original blood sample</td>
<td>7613</td>
<td>5000—12900</td>
</tr>
<tr>
<td>Hematocrit in original blood sample</td>
<td>45%</td>
<td>49%—40%</td>
</tr>
<tr>
<td>Leukocyte count in cell rich supernate</td>
<td>6073</td>
<td>4000—10500</td>
</tr>
<tr>
<td>Erythrocyte count in cell rich supernate</td>
<td>1280</td>
<td>550—2000</td>
</tr>
<tr>
<td>Leukocyte-erythrocyte ratio in cell rich supernate</td>
<td>4.8</td>
<td>2.5—9</td>
</tr>
<tr>
<td>Recovery of total leukocytes</td>
<td>40%</td>
<td>31%—55%</td>
</tr>
<tr>
<td>Recovery of polymorphonuclears</td>
<td>51.5%</td>
<td>39%—70%</td>
</tr>
</tbody>
</table>
A NEW TECHNIC FOR SEPARATING LEUKOCYTES

Table 2.—Leukocyte Separation Following Preliminary Platelet Removal by Differential Centrifugation

<table>
<thead>
<tr>
<th></th>
<th>Average of 3 Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte count in original sample</td>
<td>5780</td>
</tr>
<tr>
<td>Platelet count in original blood sample</td>
<td>248000</td>
</tr>
<tr>
<td>Platelet count following differential centrifugation</td>
<td>28000</td>
</tr>
<tr>
<td>Leukocyte count in cell rich supernate</td>
<td>4666</td>
</tr>
<tr>
<td>Erythrocyte count in cell rich supernate</td>
<td>1033</td>
</tr>
</tbody>
</table>

Table 3.—Average Differential Count on 15 Normal Blood Samples

<table>
<thead>
<tr>
<th>Polymorphonuclears</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
<th>Basophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before separation</td>
<td>60%</td>
<td>32</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>After separation</td>
<td>79%</td>
<td>13</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

The differences between leukocytes and lymphocytes were found to be statistically significant with $P < 0.001$.

Almost complete platelet removal could also be accomplished by the defibrination technic, but this procedure usually decreased the leukocyte yield.

Effects of temperature: Leukocyte separation was carried out at room temperature, 37 C. or 4 C. with essentially similar results.

Effects on differential count: Total and differential counts were done on 15 blood samples before and after separation, and, as is shown in table 3, lymphocytes were partially removed during the separation procedure. Lymphocytes of normal blood samples and especially samples obtained from patients with chronic lymphatic leukemia were agglutinated by Polybrene.

Properties of Separated Leukocytes

Phase microscopy: The cells appeared normal and highly mobile when warmed to 37 C. and examined at 400X magnification.

Supravital staining: Nuclei of fewer than 3 per cent of the cells were stained by Trypan Blue.

Effects on the cells by washing: The separated leukocytes did not undergo spontaneous clumping even when kept at room temperature for over a week. However, following washing in isotonic saline, strong clumping occurred. The clumps were found to be mixed aggregates of leukocytes and platelets. The degree of clumping was proportionate to platelet contamination and no clumping occurred if platelets were completely removed. Aggregation was likewise inhibited by addition of 0.1 per cent EDTA to the washing fluid.

Phagocytosis: Highly active erythrophagocytosis was produced in almost all phagocytic cells. This is illustrated in figure 1. Phagocytosis could be best evaluated if platelet-poor preparations were used, to avoid clumping of the phagocytes following the washing procedure.

Oxygen uptake: Figure 2 demonstrates the oxygen uptake of a representative blood sample obtained from a patient with chronic myelogenous leukemia. It may be seen that there was no significant difference in oxygen uptake fol-
lowing addition of 1 or even 5 mg. of Polybrene to 1.5 ml. of cell suspension prepared from anticoagulated blood.

**DISCUSSION**

Qualitative studies of leukocytes prepared by the present method reveal intact viability and no evidence of any harmful effect caused by Polybrene, as measured by supravital staining, study of phagocytosis and cell mobility. In the presence of large amounts of Polybrene, the oxygen uptake of leukemic cells was not altered. The present technic by its speed, simplicity and quality of the preparation, may be considered a significant improvement in the technics available for the separation of human leukocytes. It provides a greater degree of purity and a satisfactory yield.

The described procedure is standardized for the blood volume and the test tube size used. It might have to be modified if these conditions are changed. Slow centrifugation is necessary to sediment down the remainder of floating red cell clumps. The application of proper centrifugal force is critical in that by inadequate centrifugation, although fewer of the leukocytes...
A NEW TECHNIC FOR SEPARATING LEUKOCYTES

Fig. 2.—Oxygen uptake of leukemic cells in presence of varying amounts of polybrene.

may be lost, larger number of red cells remain in the supernate. By the combination of this method with the procedure of "osmotic shock," in which red cells are lysed by 30 seconds exposure to distilled water,12 we were able to accomplish further removal of the contaminating red cells.

The curious observation that mixed leukocyte-platelet agglutination occurs following removal of plasma from the leukocyte-rich supernates must be considered if preparations of non-agglutinable cells are desired.

The separated cells are mostly polymorphonuclears, lymphocytic contamination being reduced because of agglutinating effects of Polybrene on lymphocytes. This suggests that lymphocytes, as compared to granulocytes, may have a greater degree of net electronegative charge. Similar to lymphocytes, a weak agglutination was observed in cells obtained from patients with acute leukemias and immature cells of bone marrow.

The technic described here is not applicable to rabbit leukocytes because rabbit red cells fail to agglutinate with Polybrene.4 However, in other laboratory animals such as rats, mice or dogs which have red cells with higher electronegative charge,11 red cell agglutination may be induced by Polybrene; accordingly, leukocyte separation by this technic might be applicable.

SUMMARY

A simple technic for separation of human leukocytes is described. It utilizes differential sedimentation following red cell and platelet agglutination induced by Polybrene. In this technic, lymphocytes are partially removed and over 50 per cent of polymorphonuclears are recovered. The leukocyte/red cell ratio is about 5, if blood samples with normal leukocyte counts are used. By use
of blood samples containing higher white cell counts this ratio may be increased. The separated cells proved to be highly viable as tested by phase microscopy, supravital staining and phagocytosis. There was no significant change in oxygen uptake of leukemic cells in the presence of a relatively large Polybrene concentration.

**SUMMARIO IN INTERLINGUA**

Es describite un simple technica pro le separation de leucocytos human. Illo utilisa le principio del sedimentation differential post agglutination erythrocytic e plachettal inducite per Polybreno. In iste technica le lymphocytos es partialmente eliminate e plus que 50% del polymorphonucleares es recovrate. Le proportion leuco-erythrocytic es circa 5:1 si le specimen usate es caracterisate per un normal numeration leucocytic. Per le uso de specimenes de sanguine con plus alte numerationes leucocytic on pote augmentar ille proportion. Le separate cellulas se mostrava altemente viabile secundo tests a microscopia de phase, a tincturation suprivial, e a phagocytosis. Esseva constatate nulle significative alteration del accpetation de oxygeno in le presentia de relativemente alte concentrationes de Polybreno.

**ACKNOWLEDGMENT**

The author wishes to thank Dr. Theodore H. Spaet for his helpful suggestions in the preparation of this manuscript.

**REFERENCES**

A New Technic for Separation of Human Leukocytes

PARVIZ LALEZARI

Updated information and services can be found at:
http://www.bloodjournal.org/content/19/1/109.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml