Separation and Collection of Leukocytes

EMIL J. FREIREICH, GEORGE JUDSON, AND ROBERT H. LEVIN[1]

National Cancer Institute, Bethesda, Maryland, and the International Business Machines Corporation, Endicott, New York

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 aggregation. 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 $1 \times g$ 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 γ-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 10^{11} 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 γ-globulin (Table 2). The paired studies shown are separate units from the same donor studied at the same time. Twenty ml of γ-globulin (16.5%) were added to each unit (500 ml) of blood. The recovery was regularly increased. Unfortunately, ad-

[1] Deceased.

TABLE 1

SEPARATION OF LEUKOCYTES FROM DONORS WITH CHRONIC MYELOCYTIC LEUKEMIA

No. of units	Centrifugation speed (rpm)	Time (min)	Average plasma volume	WBC/cu mm plasma	WBC	
					Total ($\times 10^{10}$)	Yield (%)
2	3,200	3	200	1,700	—[a]	
1	1,500	10	150	17,000	—[a]	
6	600	30	200	150,000	3.00	21
4	600	20	180	170,000	3.08	20
6	400	30	190	234,000	3.19	23
18	400	20	140	258,000	3.50	27
6	Dextran	30			7.42	89

[a] 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

Donor	Yield (%)	
	No γ-globulin	With γ-globulin
1	48	87
	32	85
2	40	87
	36	67
	26	65
	27	74
	55	83
	—	—
Median	33	83

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[2] 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 3

RECOVERY OF LEUKOCYTES FROM SEPARATE DONORS

Donor	Recovery (%)
B.	100, 100, 100, 100, *90*,[a] 80, 80, 80, 67
P.	72, *69*, *69*, 64
W.	86, *65*, 64
S.	72, 67, 64, 60, *55*, *55*, 48, 40, 37
W.	55, 40, *36*, 27, 26

[a] Values in italics are medians.

[2] I. Djerassi, Acute Leukemia Task Force, November 18, 1963.

TABLE 4

SEPARATION OF WHOLE BLOOD IN THE CONTINUOUS FLOW CENTRIFUGE

PORT	VOLUME		WBC		POLYS AND BANDS		LYMPHS AND MONOS		HEMOGLOBIN		PLATELETS	
	ml	%	$\times 10^9$	%	$\times 10^9$	%	$\times 10^9$	%	gm	%	$\times 10^{11}$	%
Red cell	2960	66	9.2	40	6.0	35	3.5	45	537	98	10.6	64
Plasma	1500	33	0.5	2	0	0	0.5	10	0	0	4.5	28
Leukocyte	55	1	13.5	58	9.8	56	3.5	45	5	1	1.0	0.6
Total	4500	100	23.3	100	17.3	100	7.8	100	538	100	16.4	100

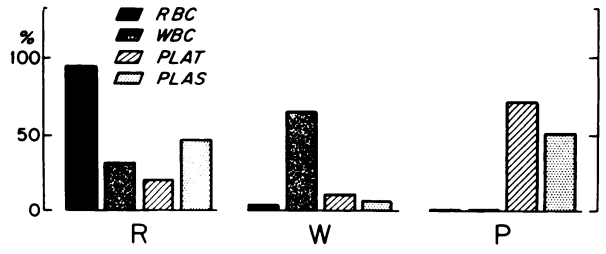

CHART 1.—Separation of whole blood by continuous flow centrifuge. Red cell port (*R*) contains outflow from outer wall of centrifuge. Plasma port (*P*) is outflow from inner wall. White cell port (*W*) is the buffy coat collection. The fraction of red cells (*RBC*), leukocytes (*WBC*), platelets (*PLAT*), and plasma (*PLAS*) which appears in each port is shown in %.

TABLE 5

SEPARATION OF LEUKOCYTES

Sample	Total WBC $\times 10^3$	Lymphs and monos (%)	Polys and bands (%)	Hemoglobin (gm/100 ml)
1	7.7	92	7	0
2	16.2	91	6	0
3	12.5	45	44	0.8
4	13.4	17	76	1.4
5	13.1	22	72	1.5
6	6.7	15	77	1.4
7	3.1	32	65	3.1
8	2.1	23	75	2.9
9	1.7	32	62	3.3

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-free atmosphere, and lack of hemolysis of red cells, we turned to *in vivo* study. This required design of a pump for metering of anti-coagulant, acid-citrate-dextrose A (ACD-A), in the proper proportion during bleeding. This was accomplished by an occlusive roller pump rolling on 2 tubes simultaneously. The ratio of the cross-sectional volumes was selected to deliver the proper amount of anti-coagulant to blood on a continuous basis. Mixing occurs near the phlebotomy needle and further mixing occurs during passage through the pump. The centrifuge accepts blood on a continuous basis, and the outflows from the red cell and plasma pumps are combined and reinjected intravenously on a continuous basis through a 2nd needle. Thus most of the red cells, plasma, and platelets are returned to the donor. Because the donation of blood is from an antecubital vein, a pressure cuff and muscular forearm work are required to ensure adequate flow rates. Thus, the bleeding must be intermittent to permit rest periods. This collation of intermittent bleeding with continuous flow through the centrifuge was accomplished by design of a buffer system. It operates automatically and is designed to limit the amount of blood away from the donor at any time to less than 500 ml. It operates as follows: The donor bleeds at a faster rate than the flow into the centrifuge. The excess blood is collected in a buffer bag. When the bag fills so that the maximum amount of blood is obtained, the instrument senses this, stops the bleeding pump, deflates the blood pressure cuff, and initiates a needle rinse to rinse the noncitrated blood out of the tube, maintaining a slow drip of saline to keep the donor needle open. This is the rest period. The centrifuge continuously draws blood from the buffer bag, processes it, and reinjects it into the donor. When the bag is nearly depleted, the instrument senses this and initiates the bleeding. The needle rinse is stopped, the pressure cuff is inflated, and the bleeding pump begins. This phase of the instrumentation is efficient and effective.

Initial clinical trial was begun in patients with chronic leukemia. The first experiment involved processing of 250 ml of whole blood, and this quantity was progressively increased in subsequent studies to a maximum of 11,000 ml of whole blood processed at 1 time in a single donor. To date 44 experiments have been performed in 9 patients, ranging from 15 min to over 6 hr in duration. In 1 donor with chronic myelocytic leukemia 72 liters of blood were processed over a 23-day period; 59 liters were processed in 11 days of this period, being the most intensive leukapheresis to date. This donor had 1.6×10^{12} leukocytes removed in this 11-day period. His white count at the start of this

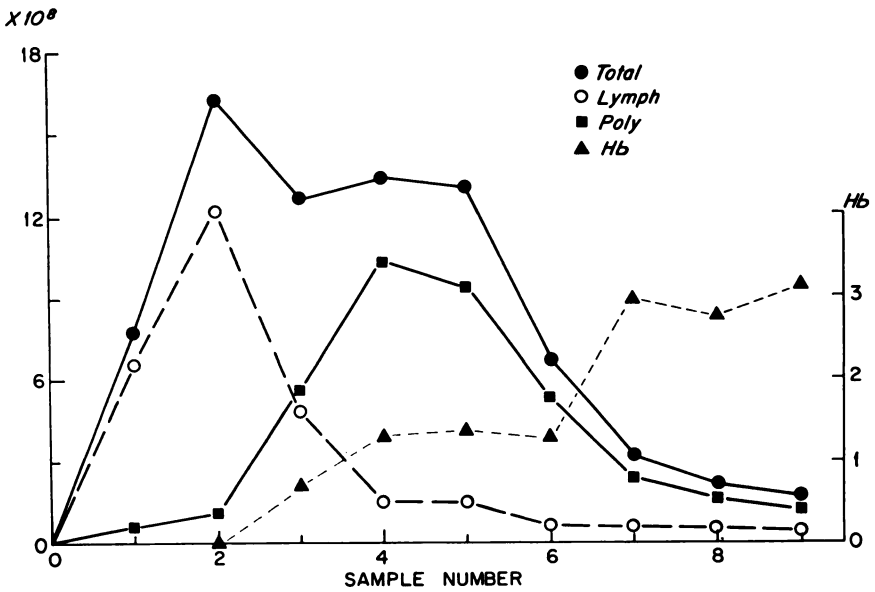

CHART 2.—Separation of lymphocytes and granulocytes in the centrifuge. The absolute numbers of total leukocytes (*Total*), lymphocytes (*Lymph*), and granulocytes (*Poly*) are shown on the left vertical axis in units of 10^8 cells. The hemoglobin is shown in gm on the right vertical axis. Samples were removed from the plasma side of the buffy coat (Sample 0) out toward the packed red cell layer (Sample 9).

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, recalification 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.

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