Lymphocyte Depletion and Immunosuppression With Repeated Leukapheresis by Continuous Flow Centrifugation

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Leukapheresis by continuous flow centrifugation (CFC) was studied in normal volunteers and patients with rheumatoid arthritis to determine whether this procedure, when carried out repeatedly, can cause lymphocytopenia and immunosuppression similar to that produced by thoracic duct drainage. The removal of lymphocytes from blood by CFC was maximal in our hands at centrifuge speeds of 900-1300 rpm and could be related directly to blood flow rates. In addition, it was found that the proportional removal of different lymphocyte subpopulations is affected by the position within the plasma-blood cell interface at which lymphocytes are removed during CFC. Repeated leukapheresis, with techniques designed to favor the removal of T lymphocytes, was carried out in 8 patients with rheumatoid arthritis at a rate of 2-3 CFC/wk for periods of 5-7 wk (total CFC were 13-18/patient). Up to 18.6 x 10^9 lymphocytes were removed from the patients (mean 13.0 x 10^9 lymphocytes) at an average rate of 3.5 x 10^9 lymphocytes/day, resulting in significant lymphocytopenia in each patient (mean decrease of blood lymphocyte counts, 72%). As has been observed with thoracic duct drainage, in similar patients, decreases in lymphocyte counts occurred most rapidly during the first 10 days of repeated leukapheresis. Lymphocytopenia reflected predominately a loss of circulating T lymphocytes, and in vitro lymphocyte responses to T-cell mitogens were reduced. Lymphocytopenia (lymphocyte counts <50% preleukapheresis values) persisted for up to 12 mo following repeated leukapheresis. A consistent fall in circulating IgM was also observed with lymphocyte depletion. An analysis of lymphocyte counts in normal volunteers who were repeat leukapheresis donors indicated that a minimum rate of <10^9 lymphocytes removed/day by CFC is necessary for there to be measurable declines in blood lymphocyte counts. These studies demonstrate that repeated leukapheresis by CFC can produce lymphocyte depletion and immunologic changes analogous to those observed with thoracic duct drainage.

Immunosuppressive therapy has assumed an important role in clinical medicine. It is responsible for successful allogeneic organ transplantation and for significant advances in the treatment of autoimmune inflammatory diseases. In general, therapeutic immunosuppression is accomplished with drug and radiation regimens designed to kill immune cells or to inhibit their function. However, the effects of immunosuppressive drugs and radiation are not selective for immune cells, nor are all their effects therapeutically pertinent and safe. An alternate and perhaps more selective approach for achieving immunosuppression is the physical removal of circulating immunocompetent cells from patients. It has been recognized for some time that lymphocyte depletion by thoracic duct drainage is immunosuppressive. In studies with animals and human subjects, it has been observed that thoracic duct drainage, and reinfusion of cell-free lymph, can produce profound and prolonged lymphocytopenia, atrophy of peripheral lymphoid tissues, suppression of delayed hypersensitivity, inhibition of allograft rejection, and impairment of primary antibody responses. These observations have led to the experimental use of thoracic duct drainage both in renal graft recipients and in patients with rheumatoid arthritis. However, thoracic duct drainage in patients is technically complex, difficult to manage, and hazardous, such that the practicality of thoracic duct drainage as a clinical tool is questionable even if therapeutic benefits were to be proven. Modern techniques of leukapheresis with continuous flow centrifugation cell separators provide another means of removing circulating lymphocytes from a patient. Continuous flow centrifugation (CFC) leukapheresis is relatively simple and safe, and there is extensive experience with its use in normal volunteers to obtain leukocytes for transfusion. We evaluated the potential of repeated leukapheresis by CFC to reproduce the effects of thoracic duct drainage in studies with normal volunteers and patients with rheumatoid arthritis. In these studies, optimum conditions of CFC for removing lymphocytes from blood and the efficiency of CFC in removing lymphocyte subpopulations were investigated. Repeated leukapheresis of patients, with techniques that promoted the removal of T lymphocytes, was found to produce both a persistent lymphocytopenia and immunosuppression similar to that which has been shown to follow thoracic duct drainage.
in studies of repeated leukapheresis on circulating numbers of lymphocytes in these patients were compared with those observed in normal volunteers who were repeat leukapheresis donors.

MATERIALS AND METHODS

Normal Subjects and Patients

Studies designed to evaluate technical variables of leukapheresis were carried out with healthy individuals (males and females, ages 26-34 yr) who had volunteered to be blood component donors for platelet and leukocyte support of patients at the National Cancer Institute. These donors were determined to be hematologically normal prior to leukapheresis and were not receiving medications at the time of study.

Twelve patients with active, erosive, seropositive rheumatoid arthritis were also studied. These patients were drawn from the clinic service of the Arthritis Branch of the National Institute of Arthritis, Metabolism and Digestive Diseases. Each had failed to respond adequately to antiinflammatory medications and to at least two slow-acting antirheumatic drugs (e.g., gold salts, antimalarials, penicillamine). Concurrent antiinflammatory medications, which consisted of salicylates and low-dose prednisone (<7.5 mg/day), remained constant throughout the month preceding leukapheresis, during the course of repeated leukapheresis, and during succeeding months. Four patients were part of an open clinical trial of leukapheresis in rheumatoid arthritis, and eight were part of an ongoing, randomized clinical trial designed to compare leukapheresed patients with patients who underwent similar control apheresis procedures in which no leukocytes were removed. Four of these 8 patients underwent repeated leukapheresis, and 4 underwent repeated control, or "sham," procedures.

All studies of normal volunteers and patients were done with informed consent and in accordance with protocols approved by the Clinical Research Committee at the Clinical Center, National Institutes of Health.

Leukapheresis Procedures

Leukapheresis was achieved by continuous flow centrifugation techniques using an Aminco Blood Cell Separator. Access to the circulation of normal donors for outlet and return lines was achieved by cannulation of peripheral veins with 16 gauge needles. Access to the circulation of patients was achieved via forearm arteriovenous fistulae, constructed when patients were selected for study. Systemic anticoagulation was accomplished with heparin (2500 U) throughout the procedure (30 U/min). Total extracorporeal blood volume during the procedures was 500 ml. The design of the cell separator used in our studies (Aminco Celltrifuge) was derived from the original NCI-IBM continuous flow centrifuge. This instrument, like newer cell separators of somewhat different design, achieves blood component separation according to principles that have been described in detail elsewhere. The continuous flow cell separator permits independent adjustment of centrifuge speed, total blood flow rate through the instrument, and the position within the leukocyte-rich interface created by blood centrifugation at which leukocytes are removed. The effects of each of these variables upon lymphocyte removal were studied in normal volunteers. All leukapheresis procedures with patients were done at a blood centrifuge speed of 1000 rpm with blood flow rates of 50-100 ml/min. In all studies, the leukocyte-rich blood fraction was removed at 2 ml/min. Complete blood counts and leukocyte differentials were done in all cases before and after each leukapheresis procedure. The leukocyte, erythrocyte, platelet, and plasma content of blood fractions removed by leukapheresis was determined after each procedure. The duration of all leukapheresis procedures was 4 hr.

Studies of Blood Lymphocytes

The proportions of T and B lymphocytes were determined in circulating blood of study subjects and in blood fractions removed by leukapheresis using standard surface markers; lymphocytes that lacked T- or B-cell markers were referred to as "null" cells. T lymphocytes were further characterized as to relative proportions of T
d or T
cells using methods described previously. Lymphocytes were separated from the blood of patients before and after courses of leukapheresis using Hypaque-Ficoll gradient separation techniques. Lymphocyte suspensions (10⁷/ml) were then tested for mitogenic responses to phytohemagglutinin (2 µg/ml). Lymphocyte mitogenic responses were expressed as counts per minute (cpm) of stimulated 3-day lymphocyte cultures after 4-hr exposure to ³H-thymidine minus cpm of unstimulated or control lymphocyte cultures.

Evaluation of Patients

In addition to the assessment of blood cell counts in patients during and following courses of repeated leukapheresis, serum immunoglobulin levels, rheumatoid factor titers, total serum protein, and albumin levels were also determined sequentially. Rheumatoid disease activity in the patients was assessed weekly using the Ritchie-Camp articular index, which was derived from the objective assessment of joint pain at rest, joint tenderness, and joint swelling.

RESULTS

Leukapheresis Variables That Affect Lymphocyte Removal During Continuous Flow Centrifugation

The leukapheresis variables of total blood flow rate, centrifuge speed, and interface position were each found to influence lymphocyte collection, in general confirming previously reported observations. The importance of interface position is illustrated in Figs. 1 and 2. At the leukocyte-rich plasma–blood-cell interface, erythrocytes are mixed with leukocytes throughout but are present in lower concentrations near the plasma side of the interface than near the packed blood cell side. By determining hemoglobin concentrations, or hematocrit determinations, in the blood fraction removed from the interface, one may obtain a measurement that reflects the region of the interface from which leukocytes are being removed. Figures 1 and 2 illustrate studies with normal volunteers in which multiple samples were taken from the leukocyte ("WBC") line as the leukocyte-rich interface was moved across the aperture for the "WBC" line, with the blood flow rate and centrifuge speed held constant. Under the conditions used for the study shown in Fig. 1, neutrophils were largely centrifuged into the packed red cell layer, although there was some concentration of these cells at the packed red cell side of the
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Fig. 1. Distribution of leukocytes at the plasma–blood-cell interface. Multiple samples were taken from the interface at different interface positions during leukapheresis of a normal volunteer. Total blood flow rate and the rate at which the leukocyte-rich blood fraction was removed were held constant.

interface. However, there was a clear concentration of monocytes at this side of the interface, and lymphocytes were concentrated throughout the interface. Furthermore, when T and B lymphocytes were identified in these samples, it was apparent that these lymphocyte subpopulations did not position themselves equally at the plasma–blood-cell interface. T lymphocytes tending to be concentrated at the plasma side of the interface. Replicate studies in normal volunteers indicated that maximal T-cell concentration occurred at an interface position characterized by a hemoglobin concentration of 2.0–3.0 g/dl. Related studies shown in Fig. 2 demonstrate the effect of centrifuge speed both on the concentration and on the distribution of leukocytes at the plasma–blood-cell interface. These studies consistently showed that at optimum centrifuge speeds for lymphocyte removal there was a biphasic distribution of lymphocytes at the leukocyte-rich interface. Assessment of the morphology of lymphocytes concentrated in the two distribution peaks (Fig. 2, 1000 rpm) showed that small lymphocytes were preferentially concentrated near the plasma side of the interface, while large lymphocytes were concentrated in the peak at the packed red cell side. The efficiency of lymphocyte collection (that is, the number of lymphocytes removed per minute at a given total blood flow rate through the cell separator) increased with increasing centrifuge speeds up to 900–1000 rpm, but began to decline at speeds greater than 1300 rpm. It was also observed that the efficiency of lymphocyte collection increased as the rate of blood flow through the cell separator was increased, other variables being constant; collection of lymphocytes per unit time was 3 times as great at a blood flow rate of 70 ml/min than it was at 30 ml/min. However, the use of peripheral veins for blood access and the size of

Fig. 2. Distribution of leukocytes at the plasma–blood cell interface. Samples were taken at different interface positions and at different centrifuge speeds during leukapheresis of a normal volunteer. Total blood flow rate and the rate at which the leukocyte-rich blood fraction was removed were held constant.
tubing in the extracorporeal circuit did not permit blood flow rates higher than 100 ml/min.

Depletion of Lymphocytes From Patients by Repeated Leukapheresis

The effects of repeated leukapheresis on circulating lymphocyte pools were studied in 8 patients with chronically active rheumatoid arthritis. In these studies, each leukapheresis procedure was of 4 hr duration and attention was given to interface position to maximize the collection of small lymphocytes. Interface positions were monitored by hemoglobin measurements in the leukocyte-rich effluent and were set to coincide with the leukocyte concentration peak that could be identified near the plasma side of the leukocyte-rich interface (Figs. 1 and 2). In the patients, this leukocyte concentration peak was found to coincide with lower hemoglobin measurements (Hb = 0.8-2.0 g/dl) than were observed in the normal volunteer studies. Access to the patients' circulation was via arteriovenous fistulae in all but 2 patients, permitting higher blood flow rates (70-100 ml/min) than are usually possible with peripheral veins as access.

The cumulative removal of lymphocytes from these patients during 5–7-wk courses of repeated leukapheresis is summarized in Fig. 3. Each patient underwent leukapheresis 2–3 times per week for a total of 13–18 procedures. The mean rate of lymphocyte removal in these patients was approximately $3.5 \times 10^8$ lymphocytes/day or $2.45 \times 10^{10}$ lymphocytes/wk. As is also shown in Fig. 3, the yield of lymphocytes per leukapheresis procedure declined with time, as is consistent with a depletion of circulating lymphocytes.

The numbers of circulating lymphocytes decreased in each patient during repeated leukapheresis (mean decrease 72%) (Fig. 4), absolute numbers of circulating T lymphocytes decreasing in particular (mean decrease 80%). Lymphocytopenia induced by leukapheresis was prolonged. In 3 of the patients, lymphocyte counts rose above 50% of preleukapheresis levels by days 89, 187, and 192 after the last leukapheresis. In the other patients, lymphocyte counts remained below 50% of preleukapheresis levels throughout follow-up (112–366 days).

Immunologic Changes in Patients Following Repeated Leukapheresis

In addition to quantitative changes in circulating lymphocytes, there were also changes in the proportions of circulating lymphocyte subpopulations following repeated leukapheresis. The percentage T lymphocytes fell from a mean of 77% to a mean of 57% (Table 1). Proportions of B and null cells increased slightly (null > B). However, no consistent changes in the proportions of Ty, Tm, and T null lymphocytes were observed (Table 1).

Serum immunoglobulin levels declined in the patients as a consequence of repeated leukapheresis (Table 1). A decrease in IgM was most consistent. However, no consistent changes in rheumatoid factor titers were observed in the patients. Changes in circulating immunoglobulins could not be explained by protein loss from the removal of plasma during leukapheresis procedures. There was no concomitant decline in plasma albumin. Furthermore, the removal of plasma during the 5–7-wk courses of leukapheresis
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Depletion of Blood Components Other Than Lymphocytes, Side Effects, and Clinical Observations

In addition to lymphocytes and small volumes of plasma, patients also lost platelets, neutrophils, and erythrocytes during the courses of leukapheresis (59.1 ± 10.2 × 10¹¹ platelets, 11.7 ± 4.6 × 10¹⁰ neutrophils, and 141.2 ± 16.7 g of hemoglobin; mean losses ± SEM for 8 patients). However, these losses were less than the loss of lymphocytes when expressed in terms of the relative proportions of the different blood elements in whole blood. Relative platelet loss was <1/4, neutrophil loss was <1/5, and erythrocyte loss was <1/50 that of lymphocytes. While neutrophil and platelet counts tended to rise during the weeks of leukapheresis, blood hemoglobin levels gradually fell and 6 of the 8 patients required transfusion of packed RBC (mean 2 units) in order to maintain blood hemoglobin within 10% of preleukapheresis levels.

Three of 121 leukapheresis procedures were stopped prematurely because of adverse reactions, all in the same patient. Two procedures were stopped because the patient developed shaking chills and low grade fever, but tests for bacterial contamination in the extracorporeal circuit or for endotoxemia were nega-

Mitogenic responses to PHA by lymphocytes remaining in the circulation were significantly reduced following repeated leukapheresis (Table 1). Furthermore, the reduction of mitogenic responses in vitro correlated with the degree to which the proportion of T lymphocytes had decreased in individual patients. No changes in lymphocyte subpopulations, in immunoglobulin levels, or in the PHA responsiveness of circulating lymphocytes were observed in the four patients who underwent “sham” leukapheresis. The lack of any decrements in immunoglobulin levels in these “sham” patients provided further evidence that the removal of plasma (equivalent for the “sham” and lymphocyte-depleted patients) could not explain decreased immunoglobulin levels following repeated leukapheresis.

![Fig. 4. Changes in blood lymphocyte counts. Courses of repeated leukapheresis were divided into 3-day intervals for purposes of summarizing data. Lymphocyte counts are expressed as percent of preleukapheresis values. Data shown by closed circles (●) represent lymphocyte counts ± SEM for 8 patients who underwent repeated leukapheresis. Data shown by open circles (○) represent mean lymphocyte counts ± SEM for 4 comparable patients who underwent repeated sham leukapheresis procedures during which only plasma was removed.](image)

<table>
<thead>
<tr>
<th>Parameter</th>
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*Determinations before and after leukapheresis, means ± SEM, n = 8.
†Percentages of total T cells that were Tγ, Tδ, or T null.
‡Differences before and after leukapheresis are significant, p < 0.01, paired t test.
Lymphocyte Depletion in Normal Blood Donors Who Undergo Repeated Leukapheresis

It was clear from studies with the rheumatoid patients that removal of lymphocytes by leukapheresis at a rate of $3-4 \times 10^8$ lymphocytes/day causes a consistent and rapid fall in blood lymphocyte counts. However, these studies did not demonstrate the minimum rate of lymphocyte removal necessary to cause a measurable decline in blood lymphocyte counts. To make this determination, we reviewed data from normal blood component donors who underwent repeated leukapheresis for the sake of granulocyte transfusions* at the National Cancer Institute from 1976 to 1980. Twelve normal donors were identified who were leukapheresed multiple times during 4-wk periods. Data that reflected changes in blood lymphocyte counts and the rate of lymphocyte removal by leukapheresis were available for 20 periods of repeated leukapheresis (3 or more leukaphereses per month) in individual donors. There was no consistent change in blood lymphocyte counts when donors were repeatedly leukapheresed with overall rates of lymphocyte removal that were less than $10^8$ lymphocytes removed/day ($n = 15$; mean change in lymphocyte count $\pm$ SEM = $-12.2 \pm 147$ lymphocytes/cu mm). However, there was a consistent drop in lymphocyte counts for donors when the rate of lymphocyte removal exceeded $10^9$/day ($n = 5$; mean change in lymphocyte count $\pm$ SEM = $-780 \pm 159$ lymphocytes/cu mm).

DISCUSSION

These studies demonstrate that repeated leukapheresis by continuous flow centrifugation can be used to deplete recirculating lymphocyte pools, resulting in a preferential loss of T lymphocytes and a persistent lymphocytopenia. The changes in circulating lymphocytes caused by multiple leukaphereses were similar to those effected by thoracic duct drainage in patients very similar to our study subjects.10 Repeated leukapheresis also had immunologic consequences analogous to those of thoracic duct drainage: reduced mitogenic responsiveness and a decrease in the proportion of T cells in lymphocytes that remain in the circulation and decreased levels of circulating immunoglobulins (IgM in particular).

Several variables of continuous flow centrifugation technique were observed to have a significant influence on the efficiency of lymphocyte removal during leukapheresis: blood flow rate, centrifuge speed, and the position within the plasma–blood-cell interface at which lymphocytes are removed. The variable of interface position was found to be particularly relevant to lymphocyte depletion, not only because the distribution of lymphocytes at the plasma–blood-cell interface is different from that of monocytes or neutrophils but also because lymphocyte subpopulations are distributed differently at this interface. It is apparent that the efficiency and selectivity of lymphocyte removal may be improved greatly with technology for the precise, automatic control of interface position.26 Attention to technical variables relevant to lymphocyte removal permitted us to achieve much greater levels of lymphocyte depletion than has been reported by others who have studied repeated leukapheresis.27,28 The preferential depletion of T lymphocytes reflected techniques that favored the removal of these cells, but it also reflected the relative long life and slow turnover rate of this subpopulation of lymphocytes.29

Thoracic duct drainage, when maintained for 7–15 wk in patients like those studied here, has been found to cause lymphocyte depletion at a rate of $3-20 \times 10^8$ lymphocytes/day.10 Repeated leukapheresis permitted rates of lymphocyte depletion that were within this range. However, leukapheresis does not have the significant problems of volume and fluid management that complicate thoracic duct drainage, nor does it have the risks of infection that are associated with prolonged cannulation of a central vessel.8,30 Lymphocyte depletion by thoracic duct drainage has been shown clearly to prolong allograft survival in experi-

*Significant numbers of lymphocytes are removed during leukapheresis procedures designed to collect granulocytes.
mental animals,\textsuperscript{5} and this procedure has been used in renal transplant programs as a means of achieving immunosuppression.\textsuperscript{30,31} Renal graft recipients may represent ideal candidates for repeated leukapheresis, for they are usually accustomed to hemodialysis, a procedure that is similar to leukapheresis in many respects, and have AV fistulae in place for this purpose.

In many hematologic support programs, normal volunteers are used repeatedly as platelet or leukocyte donors. This practice has provoked concern that immunosuppression from lymphocyte depletion may constitute a risk for these donors.\textsuperscript{32} In repeat leukocyte donors, we observed that short-term lymphocyte depletion of \textit{i}0 or more lymphocytes per day leads to significant decrements in circulating lymphocyte counts. Donors who achieve this rate of lymphocyte depletion are unusual. Nonetheless, we must assume that such donors may experience some degree of immunologic change analogous to that seen in the patients who are purposely depleted of lymphocytes.

Rheumatoid arthritis became the focus of our studies because of reports that thoracic duct drainage could induce remissions of this disease,\textsuperscript{3,4} and as reported earlier,\textsuperscript{5} lymphocyte depletion by leukapheresis appears to have antirheumatic effects similar to those reported for thoracic duct drainage.\textsuperscript{6} However, the clinical effects of lymphocyte depletion by leukapheresis cannot be evaluated properly without randomized trials that control for all features of this procedure other than the removal of lymphocytes.\textsuperscript{6} Although the clinical applicability of repeated leukapheresis remains to be shown, our studies demonstrate unquestionably that this procedure can have distinct immunologic effects that are associated with a marked and prolonged depletion of circulating lymphocyte pools.

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