T AND B LYMPHOCYTE ENUMERATION IN PEDIATRIC LYMPHORETICULAR NEOPLASMS AND APPLICATION OF THE CYTOCENTRIFUGE TO THE ENUMERATION OF T LYMPHOCYTES

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Figure 16: Comparison of percent rosetted lymphocytes in cell suspension and on cytocentrifuge preparation on blood samples from 22 pulse chemotherapy patients. The mean cell suspension rosettes was 68.2±13.3% and on cytocentrifuge the mean rosetted lymphocytes was 64.1±16.2%. Differences in means $p<0.2$, correlation $r=0.35$, $p>0.05$.

Figure 17: Blood rosette cytocentrifuge preparation illustrating rosette-like monocytes.

INTRODUCTION

A. STATEMENT OF THE PROBLEM

The recognitiion of two distinct circulating lymphocyte populations has resulted in numerous studies of lymphocyte surface markers. In the early 1970's, work began on the cellular origin of lymphoreticular neoplasms using surface marker analysis. The results of these investigations have given new insights into the pathophysiology of these disorders and have stimulated new approaches to classification and treatment of malignant lymphoproliferative disorders.

The intent of this study was to perform T and B lymphocyte evaluations on three groups of pediatric patients. Group I patients had newly diagnosed, or relapsed, acute lymphocytic leukemia. Group II included patients having newly diagnosed non-Hodgkin's lymphomas. The T and B lymphocyte classification of the malignancy was determined on groups I and II for Children's Cancer Study Group protocols. Group III was patients on pulse chemotherapy who were tested to evaluate the effect of chemotherapeutic drugs on circulating T and B lymphocytes. Peripheral blood, bone marrow, and tissue specimens were examined.

In addition, the application of the Shandon cytocentrifuge was evaluated as a technique in the identification of leukocytes prepared for T and B lymphocyte testing and the enumeration of rosetted T lymphocytes. Cytocentrifuge preparations were Wright's-stained, and morphologic identification of leukocytes was performed by standard hematologic techniques (93). The percent of rosetted lymphoid cells was determined on cytocentrifuge slides and compared to the percent rosettes counted in cell suspension preparations.

The Children's Cancer Study Group (CCSG) is a voluntary, inter-institution organization established to nationally coordinate data collection and evaluate trial therapeutic regimens for pediatric lymphoreticular and other pediatric neoplasms. In order to admit a patient to certain CCSG studies, the participating institution must perform lymphocyte surface marker tests on the diseased tissue. According to CCSG protocols, the thymus-derived or T lymphocytes are determined by the attachment of sheep red blood cells (SRBC) to human lymphocytes. Bursa-equivalent or B lymphocytes are defined as lymphocytes bearing detectable surface immunoglobulins. For this study, T and B lymphocyte enumeration was performed using methods in the CCSG protocol CCG-161 for the treatment of acute lymphoblastic leukemia (42), and the World Health Organization/International Agency for Research on Cancer special technical report on human B and T cells (98).

Acute lymphocytic leukemia, the most common form of leukemia in children, is frequently considered a heterogenous disease. Although the malignant lymphoblasts from different patients may have morphologic dissimilarities, the morphologic pattern does not necessarily predict the clinical presentation, response to therapy, course, and prognosis (8,13,21,39,92). T and B lymphocyte surface marker analysis also reveals heterogenity among patients (21,22,53,80,85). The cell marker type is not clearly related to differences in lymphoblast morphology (14,21, 40). There is some evidence that surface markers may correlate with the clinical disease (13,20,21,24,39,80,85).

Non-Hodgkin's lymphomas are classified according to the histologic and cytologic characteristics of the involved lymph node. These classification systems are based upon the pattern of growth, degree of differentiation, and suspected origin of the malignant cells. Use of lymphocyte surface markers in non-Hodgkin's lymphomas suggests possibilities of classifying this group of diseases by the immunologic characteristics of the neoplastic cells (1,2,11,48,59,60,61,62,72,92). The results of these investigations may reveal the true origin of the malignant cell, and may have diagnostic and prognostic significance (11,31, 47,60,61,62,72,88,99).

T and B lymphocyte enumeration is usually performed on cells in suspension. Because preparations can have high degrees of contamination by other leukocytes, the accurate identification of lymphoid cells is essential for valid results (78,81,106). Since classification of lymphoreticular diseases depends on markers present on malignant cells, normal and abnormal leukocytes must be correctly identified (20,62,83,99).

The Shandon cytocentrifuge concentrates cell suspensions on glass slides which may be stained by a variety of techniques (7,33, 55,89,97). Cytocentrifuged rosette preparations are not recommended for enumeration of rosettes because the apparent proportion of SRBC attaching to cells may be altered (98). However, cytocentrifuge preparations are frequently used to identify rosette forming cells, and this method is an accepted means of confirming the number and type of rosette forming malignant cells (11,42,54,62,88,99). Rothbarth,

Tank, Gmelig-Meyling, Ballieux, and Stoop demonstrate close correspondence between normal human rosetted lymphocytes counted in cell suspension and rosetted lymphocytes by cytocentrifuge preparations (79).

This study demonstrates that T and B lymphocyte enumerations can be performed on blood, bone marrow, and tissue samples, but the significance of the result is dependent on accurate morphologic identification of the leukocytes tested. While the results of Rothbarth, et al., were confirmed using a different technique in normal donors, blood samples from patients treated with pulse chemotherapy did not demonstrate correlation between the number of rosetted lymphocytes by cytocentrifuge and the number of rosettes in cell suspensions. Furthermore, blood from these patients contained significantly more monocytes in the isolated leukocyte fraction than in preparations from normal blood samples. Although rosetted lymphocytes and blast cells can be identified on cytocentrifuge preparations, the percent of rosettes enumerated by cytocentrifuge preparation does not consistently correspond with cell suspension results when malignant cells are present in bone marrow or blood specimens.

B. BACKGROUND

1. Thymus-derived Lymphocytes (T Lymphocytes)

a. Definition of T Lymphocytes

In the early 1970's, it was noted that unsensitized SRBC adhered to human lymphocytes. This interaction is termed rosette formation, and the lymphocyte is sometimes known as an E rosette forming cell. A rosette is defined as a lymphocyte having three or more SRBC attached to its surface (6,7,18,32,48,56,65,100,101).

The E rosette is considered a thymus-derived lymphocyte. Rosette formation is higher in fetal thymic tissue than in fetal bone marrow, liver, and splenic tissue (100,101,102). Normal thymic tissue contains almost 100 percent E rosettes (48,56). Rosette formation is absent or markedly decreased in patients with impaired cellular immunity (41,100,101). Athymic patients have markedly reduced numbers of rosettes, and thymic transplants result in an increased rosette-forming capacity (45). Treatment of human lymphocytes with antilymphocyte serum (ALS) reduces peripheral rosette formation, and the decrease in rosettes is correlated with delayed skin graft rejection in lower primates(5).

b. Characteristics of Rosettes

Despite the general use of SRBC rosette formation as a marker for thymus derived lymphocytes, little is known about the nature of the SRBC receptor of lymphocytes. Rosette formation is considered a non-immune reaction (118,32,48,100). The number of rosettes is not correlated with the presence of antibodies to SRBC in the donor of the tested lymphocytes (18,56,100) or with donor blood type (5,100). Antihuman immunoglobulin serum does not inhibit rosette formation (9,19,32).

Rosette formation is enhanced by pretreatment of SRBC with 2-aminoethyisothiuronium bromide (50), neuramidase (10), and papain (94). It is inhibited by low concentrations of sodium azide, potassium cyanide, and sodium iodoacetate (9,18,19,32,48). The SRBC receptor on the lymphocyte is destroyed by trypsin and phospholipase A,

and is enhanced by papain treatment of lymphocytes (27). The receptor is shed from lymphocytes incubated at 37°C for 30 minutes. With further incubation at 37°C, rosette forming capacity returns (77). Thymocytes form rosettes that are stable at 37°C. Peripheral blood, splenic, and pleural fluid E rosettes rapidly lose bound SRBC at 37°C (14,64).

Owen and Franger report isolation of a water soluble glycoprotein capable of blocking the inhibition of rosette formation by antithymocyte globulin. The proposed receptor can be isolated from cell culture supernatants (66). Boldt and Armstrong report that glycopeptides from SRBC membranes also inhibit rosette formation (12).

c. Technical Aspects of Rosette Formation

The mechanics of rosette formation appear simple. Isolated lymphocytes and SRBC are mixed, centrifuged, incubated, resuspended, and the percent of rosetting cells determined. Technical details are very important, and variations in procedure may account for widely differing reported results (29,62,63,64,77,82).

Formation of rosettes requires viable lymphocytes (9,32,48) and calcium ions (48,68). The addition of protein such as fetal calf serum, bovine serum albumin, or human AB serum enhances rosette stability, and may increase the number of rosetting cells (9,19,82,98,102). The age of SRBC used in testing may (23), or may not (64), reduce the number of rosettes. With increasing concentrations of SRBC, more rosettes are formed, and the percent rosette formation reaches maximum values when the SRBC/lymphocyte ratio is greater than 30 (29). The lymphocyte-SRBC mixture is usually incubated at 37°C for 5-15 minutes. Different laboratories have reported that this preincubation step is either re-

quired (56), unnecessary (14,52), or reduces rosette formation (82). The mixture is then centrifuged for five minutes, and after centrifugation, the pellet is incubated at 4°C or room temperature. Maximum rosette formation is reported to occur in one to two hours (52,62, 86), but may take up to five hours, and remains stable up to 24 hours (104).

Rosettes may be easily broken apart during resuspension of the pelleted lymphocyte-SRBC mixture. The amount of mechanical force, rather than the duration of resuspension, has the greatest disruptive influence on rosettes (44). Extensive handling of rosette preparations also reduces the number of rosetting cells. Therefore, care is required to minimize and standardize manipulation of rosette preparations in order to decrease variation (6,19,48,62,65,82,104).

Most rosettes have numerous bound SRBC, and visualization of the central leukocyte is not possible. Clumps of cells may be present in the preparation. Enumeration of leukocyte clumps may be excluded, or attempted, depending upon the laboratory (63). Non-specific binding of neutrophils and SRBC may occur at levels of less than five percent (35,86). Hsu and Fell report rosette formation by neutrophils and eosinophils from normal donors and from patients with X-linked agammaglobulinemia (46). Rosette formation occurs in a few blast cells, promyelocytes, and eosinophils in chronic myelocytic leukemia (37). Occasional macrophages in hairy cell leukemia, leukemic reticuloendotheliosis, are also reported to form rosettes with SRBC (67).

2. Bursa-Equivalent Lymphocytes (B Lymphocytes)

a. Surface Immunoglobulin Bearing Lymphocytes

In birds, the bursa of Fabricius is the site of lymphocyte differentiation into immunoglobulin-producing cells, but no equivalent site has been found in humans. Thus, immunoglobulin-producing cells are referred to as bursa-equivalent, or B lymphocytes (90,100). These lymphocytes have surface immunoglobulins which are detected by fluorescein-conjugated antibodies to human immunoglobulins (38,69). By definition, B lymphocytes possess readily detectable surface immunoglobulins which are synthesized by the cell and are attached to the cell surface (69,98). The number of surface immunoglobulin-positive cells is decreased in Bruton's agammaglobulinemia, and is increased in chronic lymphocytic leukemia (41,38,69).

b. Technical Aspects of B Lymphocyte Testing

Purified fluorochrome conjugated antihuman immunoglobulin serum, which may be purchased commercially, is incubated with cell suspension and, after washing, the number of fluorescent staining cells is determined. However, there are technical problems making surface immunoglobulin testing difficult and exacting (62,74).

If the fluorescein-conjugated antiserum is not optimally diluted, non-specific staining or decreased staining may result. Thus, the antiserum should be diluted and tested against fixed cell concentrations. Four times the highest dilution giving a constant number of positive cells is used for testing (98).

B lymphocytes, non- B lymphocytes, and other leukocytes have Fc receptors for IgG immunoglobulins (20,42,57,81). Since these leukocytes can be positive for aggregated IgG, the antiserum must be ultracentrifuged, or centrifuged at high speed in a microcentrifuge, to remove aggregates. The diluted antiserum can be frozen, but thawed only once to avoid aggregate formation (74,98). Whole antihuman immunoglobulin serum may also be used to detect the Fc receptor on leukocytes causing an apparent increase in the number of surface immunoglobulin positive cells (74,95,96,98). Because the Fc fragment of immunoglobulin can be removed by pepsin digestion, use of $F(ab')_2$ fragment antiserum may increase specificity (95,96). Whole molecule antiserum prepared in goats is reported to bind Fc receptors less avidly than antiserum prepared in rabbits (3,40).

Lymphocytes may passively absorb plasma antibodies resulting in falsely elevated results. When IgG immunoglobulin bound to lymphocytes by the Fc portion is released by incubation at $37^{o}C$, and removed by washing the cells, the number of surface immunoglobulin bearing cells is reduced (20,57,74). Furthermore, lymphocytes may bind immune complexes or autoantibodies from patient's plasma and may result in an increase of fluorescent cells. Thus, surface immunoglobulins may be removed from lymphocytes by incubation at $37^{o}C$, extensive washing, or proteolytic enzymes. The culturing of immunoglobulin stripped cells to allow resynthesis of immunoglobulin and retesting is recommended to prove the detected surface immunoglobulins are synthesized by the lymphocyte (20,74,98).

Redistribution and pinocytosis of antibody may occur. Sodium azide added to the reagent used to wash excess antiserum from the leukocyte suspension may prevent both (58).

Incident, or epi-illumination, is superior to transmitted illumination for fluorescent microscopy (51, 98). The amount of fluorescence defining a positive cell is subjective (83). Positive cells usually have bright fluorescence around the lymphocyte membrane, but the fluorescent pattern may be patches, aggregates, or fluorescent-shaped crescents at the cell periphery. Speckled patches on the cell surface have also been considered positive. Dead cells fluoresce with a homogenous pattern (41,74,87,95).

3. Preparation of Samples for Surface Marker Testing

a. Isolation of Cell from Samples

Buoyant density centrifugation is the most commonly employed technique for leukocyte isolation from peripheral blood (16,17). Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, New Jersey) is a commercially prepared solution of Ficoll-diatrizoate with a specific gravity of 1.077 gm/ml. The manufacturer reports 95±5% mononuclear cells will be present in the fraction of isolated leukocytes and 50±15% of the lymphocytes from the original sample are recovered (70). Brown and Greaves find that Ficoll-Paque isolation of blood results in selective loss of T lymphocytes, and they suggest 70% recovery of the original sample is essential to reflect in vivo populations (23). Non-selective loss of T or B lymphocytes is also reported (82,91).

Isolation and recovery of purified lymphocytes and blast cells from normal bone marrow is more difficult than recovery from peripheral blood. Unsuccessful enrichment of normal bone marrow lymphoid elements by Ficoll-diatrizoate has been reported (87). Cells from bone marrow involved with a single mononuclear cell population can be prepared by direct lysis of erythrocytes (42).

Isolation of lymphocytes from tissue is performed by teasing the sample apart and passing it through nylon or steel mesh. Alternatively, the tissue may be finely minced with scissors and aspirated through syringe needles of decreasing bore (99). The whole tissue sample may not be involved in disease, and recovery of malignant cells may not be complete due to adhesion of cells to tissue fibers, or differences in resistance of neoplastic cells to manipulation (62, 99).

b. The Problem of Contaminating Non-Lymphoid Cells

Although the majority of granulocytes are removed from blood by Ficoll-diatrizoate buoyant centrifugation, peripheral blood monocyte contamination can be more than 50% of the isolated cells (81, 106). Bone marrow and tissue may also contain contaminating granulocytes, monocytes, and other nucleated cells. Since T and B lymphocytes are reported as percent of leukocytes counted, the presence of non-lymphoid nuclear cells will distort results.

Monocytes and granulocytes possess adherent and phagocytic properties which may be utilized to remove these leukocytes from samples (23,26,74). However utilization of special techniques to remove conta-

minating non-lymphoid cells increases cell loss, and may result in selective loss of B lymphocytes (20,98). Phagocytic cells may be identified by incubating cell suspensions with latex particles. The leukocytes with ingested particles can be identified in suspension (30).

Light, phase contrast, or darkfield microscopy may permit distinction of lymphocytes from non-lymphoid cells by cell size, nuclear morphology, and the presence or absence of cytoplasmic granules (87). Blast cells may also be identified by similar criteria. However, identification of leukocytes in cell suspension is complicated by overlapping cells sizes and similar nuclear configuration in different cell lines. Therefore, identification of leukocytes in cell suspension is not considered valid by some investigators (20,81,83). Cytochemical stains for monocytes and granulocytes can be used for identification of these leukocytes in fixed smears (103).

Since SRBC often obscure the central cell, it is difficult to identify the cell involved in rosetting by size, nuclear morphology, or ingested latex particles (20,63). Fluorescent stains are reported to permit accurate identification of the central cells in rosettes (5, 49,79). Supravital stains such as toluidine blue and crystal violet may aid in cell identification (49).

4. Application of the Cytocentrifuge to Lymphocyte Surface Marker Testing

Hansen, Bender, and Shelton have reported the use of the Shandon cytocentrifuge in cerebrospinal fluid cytology (43). Specially de-

signed cuvettes spread cells on glass slides by centrifugal force. A filter paper strip between the cuvette and the slide absorbs the suspending fluid, thus leaving the leukocytes concentrated in a small area on the slide. The dried slides may then be fixed and stained as desired (43). With cytocentrifuge preparations, there is a reported loss of cells ranging from 69 to 89 precent, but whether this loss is selective for certain types of cells was not reported (7,33,89). The cytocentrifuge produces some distortion of leukocyte morphology, but can be used to identify malignant cells in cerebrospinal fluids (7,33,55,97).

The cytocentrifuge is used in T and B lymphocyte testing to identify isolated cells or the central cell in rosetted preparations. Since rosettes are fragile, SRBC may be stripped off during centrifugation. On the other hand, SRBC may become attached to, or be randomly deposited near, the lymphocytes increasing the apparent number of rosettes (79). Ranki, Tötterman, and Häyry describe quantitation of mouse T and B lymphocytes on cytocentrifuge preparations,and report no disruption of rosetted leukocytes (76). Jaffe, Braylan, Frank, Green, and Berard report that pseudorosette formation is not observed on cytocentrifuge preparations of human blast cells and IgM immunoglobulin coated erythrocytes (47). Rothbarth, et al. describe cytocentrifuge preparation of rosetted human leukocytes. The percent of SRBC in cell suspension closely corresponds with the fluorescent methyl green pyrionin-STS stained cytocentrifuge slide results. Because cells are flattened, the central SRBC bound cells can be clearly visualized. Since glutaraldehyde-fixed SRBC do not form spontaneous rosettes, cytocentrifugation does not induce artifical rosette formation (79).

5. Lymphoreticular Neoplasms

a. Acute Lymphocytic Leukemia (ALL)

In most cases of childhood acute lymphocytic leukemia (ALL), the lymphoblasts do not have detectable surface immunoglobulins, or form rosettes. These are the non-T, non-B, or null cell leukemias. Between 17 and 35% of the ALLs have malignant cells bearing T lymphocyte markers (14,20,21,22,39,53,54,85), and occasional cases of B lymphocytic ALLs have been reported (20,21,22,39).

b. Non-Hodgkin's Lymphomas

Malignant lymphomas are divided into Hodgkin's disease and non-Hodgkin's lymphomas. Traditional classification systems for non-Hodgkin's lymphomas were developed in the adult. Studies of lymphocyte markers reveal heterogeneity of T and B lymphocyte malignant cells within the categories (11,24,47,60,61,62,92,99). The standard classification systems are less useful in children where different categories predominate (25).

c. Surface Markers on Malignant Lymphoid Cells

The application of surface marker analysis, cytochemical testing, and ultrastructural analysis to lymphoreticular neoplasms has led to possibilities of classifying these disorders on functional and morphological grounds, rather than the traditional histologic/cytologic classification systems (1,2,11,48,59,60,61,62,72,92,99). Lukes and Collins are major advocates for reclassification of lymphoreticular neoplasms using cellular morphology and lymphocyte surface markers. Their fundamental premise is that normal lymphocytes undergo morphologic and functional changes during the cell cycle, and malignant cells

are bizarre copies of these changes. Neoplastic cells bear surface markers which may have diagnostic and predictive clinical importance (60,61,62).

Brown and Greaves summarized the theoretical problems of applying T and B lymphocyte analysis to neoplastic leukocytes (24): (1) the marker may not have the same pattern of expression when cells are activated and in cycle; (2) leukemic cells may express both T and B lymphocyte markers; (3) immature lymphocytes may not express normal T and B lymphocyte markers; and (4) the cell surface type may not be the same as its normal counterpart (e.g., markers may be lost or hidden, or markers normally absent may be expressed on malignant cells).

Malignant cells may express surface markers differently than normal lymphocytes. Neoplastic T lymphocytic cells may form rosettes with fewer SRBC than normal T lymphocytes (20), or form large multilayered rosettes on cytocentrifuge preparations (88). Neoplastic rosettes may be stable at 37°C, while normal rosettes are unstable at 37°C (14). B lymphocytic cells in chronic lymphocytic leukemia and diffuse well-differentiated lymphoma stain more faintly than normal B lymphocytes. In poorly-differentiated lymphocytic lymphoma and lymphosarcoma cell leukemia, lymphoid cells stain more intensely than normal lymphocytes (1,2).

METHODS

A. NORMAL CONTROLS

Normal adults were selected from the hospital staff, and control blood was tested simultaneously with patient specimens. The control subjects were screened for absence of colds or influenza viral infections and were in good physical health. A total of 16 controls were tested, and several donors were tested on multiple days.

Pediatric controls were obtained from hematologically normal patients during cardiac catherization and from patients examined for non-hematologic reasons. These controls were not necessarily tested simultaneously with patient samples.

Patient evaluation and parental consent was obtained by Dr. D. Ridgway.

B. PATIENT SELECTION

Patients were evaluated and followed by the pediatric hematology staff. The cases were suspected, newly diagnosed, or relapsed acute lymphocytic leukemia (group I), and non-Hodgkin's lymphomas (group II), and were candidates for treatment under Children's Cancer Study Group protocols. Single tests and multiple sequential studies were also performed on selected patients during pulse chemotherapy, (group III patients). Patients and times for testing were selected by the physician.

In all cases, parental consent was obtained by the physician.

C. ISOLATION OF BLOOD CELLS

Between five to ten ml blood was collected in ten ml vacuum tubes containing 143 units heparin (Venoject, Kimble-Terumo, Elkton

MD, or Vacutainer, Becton-Dickson, Rutherford, NJ). A blood smear was made from the heparinized specimen, and duplicate manual leukocyte count and 200 cell differentials were performed, or the results obtained from the hematology laboratory.

The heparinized blood was diluted with 2 volumes phosphate-buffered saline (PBS) in a 50 ml plastic centrifuge tube and gently mixed. Bloods with leukocyte counts over 50,000/cu mm were diluted with PBS to a count of 20,000 leukocytes/cu mm.

Leukocytes were isolated by centrifugation over a Ficoll-Paque gradient (16). Using a plastic pipet, 5 ml diluted blood was layered over 3 ml Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). The tubes were placed in the centrifuge and centrifuged at 400 x g (force at the interface) for 30 minutes at room temperature,average temperature 23^{o}C. Immediately after centrifugation, the tubes were gently removed from the centrifuge. The upper layer of the diluted plasma was aspirated to within 1 ml of the interface between the diluted plasma and Ficoll-Paque. The interface was removed using a Pasteur pipet and transferred to a 50 ml plastic centrifuge tube. Attempts were made to remove all cells and minimal amounts of Ficoll-Paque and diluted plasma.

PBS was used to fill the tube containing isolated cells, and the tube was gently inverted several times. The cells were centrifuged for 10 minutes at 400 x g. The supernatant was discarded, and the cells transferred to a 15 ml plastic centrifuge tube. The leukocytes were suspended in 3 to 5 ml phosphate-buffered saline with 1% bovine serum albumin (PBS-P) and centrifuged at 200 x g. The

sample was washed again and resuspended in 0.5 ml RPMI-1640 with 10% fetal bovine serum (FBS). Duplicate leukocyte counts were performed. The volume of diluent was adjusted to a leukocyte count of 20×10^6 cells/ml by centrifugation and removal of diluent or by the addition of more RPMI-1640 with 10% FBS.

Occasionally, after the initial wash, the pellet contained sufficient erythrocytes to produce a visible pink or red color. In these cases, the cells were transferred to a 15 ml centrifuge tube, and 15 ml 0.14M TrisNH_4Cl was added. The tube was allowed to mix on an aliquot mixer (Lab-Tech Instruments Company, Westmont, IL) for 10 minutes at room temperature. The cells were centrifuged and washed 3 times in PBS-P as outlined above.

D. ISOLATION OF BONE MARROW CELLS

Bone marrow aspirates were collected in plastic syringes containing preservative-free heparin. The attending physician coated the syringe with heparin (Vitarine Company, Inc., N.Y., NY) and 2 to 4 ml bone marrow was aspirated from the iliac crest after the specimen for hematologic studies had been obtained. A smear of the bone marrow sample was prepared. The sample was placed in a 15 ml plastic centrifuge tube and the tube filled with PBS. The well-mixed tube was centrifuged for 10 minutes at 200 x g. The packed cell volume was noted, and the supernatant was removed. The cells were transferred to a 50 ml centrifuge tube, and 20 to 30 ml 0.14M TrisNH_4Cl was added to every 0.5 ml packed cells. The suspension was mixed for 10 minutes at room temperature and centrifuged for 10 minutes at 200 x g. The supernatant was removed.

The isolated cells were transferred to a 15 ml centrifuge tube and washed three times with PBS-P as described for bloods. After the final wash, leukocytes were resuspended in one ml RPMI-1640 with 10% FBS. Duplicate cell counts were performed, and the volume of diluent adjusted to a nucleated cell count of 20×10^6 cells/ml.

When erythrocytes were not completely lysed, treatment with 10 to 20 ml 0.14 M TrisNH_4Cl was repeated. If erythrocyte lysis still did not occur, 1 to 3 ml distilled-deionized water was added to the pellet. The cells were quickly resuspended, and within 30 to 60 seconds, 30 to 50 ml PBS-P was added. The leukocytes were then centrifuged again at 200 x g for 10 minutes.

E. ISOLATION OF CELLS FROM TISSUE SAMPLES AND BONE MARROW BIOPSIES

Tissue specimens and bone marrow biopsies were obtained by the physician and placed in sterile preservative-free saline. Soft tissue biopsies were cut into 2 mm pieces in a 100 x 75 mm plastic petri dish with sterile fine scissors and forceps. The tissue was kept moist with three to five ml PBS. The pieces and the PBS were transferred to either a 5 ml glass centrifuge tube or a 15 ml plastic centrifuge tube. A 1 ml plastic syringe without needle was used to aspirate and expell the PBS and tissue pieces 3 to 5 times. An 18 gauge needle was attached to the syringe and aspiration-expulsion was repeated 3-5 more times. Bone marrow biopsies and hard tissue pieces were placed directly in a 5 ml glass centrifuge tube with 3 ml PBS, and a 1 ml syringe without needle was used to free the cells.

After separation of leukocytes, the sample was allowed to settle for one to two minutes. The supernatant was transferred to a 15 ml centrifuge tube which was then filled with PBS-P. The leukocytes were centrifuged for ten minutes at 200 x g, and washed three times with PBS-P as described for blood samples. If the sample contained numerous erythrocytes, cell lysis was accomplished using 0.14M TrisNH_4Cl followed by three washes. The cells were resuspended to 0.5 ml RPMI-1640 with 10% FBS. Duplicate cell counts were performed, and the nucleated cell concentration was adjusted to 20 x 10^6/ml.

F. CELL VIABILITY

A microcapillary tube was partially filled with the isolated cell suspension and an equal amount of 0.4% trypan blue. The tube was inverted until the stain was uniformly mixed. The suspension was discharged on a glass slide and coverslipped. A minimum of 200 nucleated cells was counted, and viable cells were recorded as percent nucleated cells excluding the trypan blue.

G. T LYMPHOCYTE TESTING

A 10 x 75 mm plastic culture tube was filled with 0.4 ml RPMI-1640 with 10% FBS, and 0.1 ml of the 20 x 10^6 nucleated cell/ml suspension was added to the tube. The final cell concentration was 4 x 10^6 nucleated cells/ml. To another 10 x 75 mm plastic culture tube, 0.25 ml of the 4 x 10^6 nucleated cells/ml suspension was combined with 0.25 ml 0.05% SRBC preparation. The tube was mixed, and incubated in a 37°C dry incubator for 7 minutes. The tube was removed

and centrifuged for 5 minutes at 200 x g. The pelleted cells were covered with Parafilm, and incubated at 2 to 3°C.

After 2 to 20 hours incubation (most testing was done after 16 to 18 hours incubation), the tube was removed from 2 to 3°C refrigeration and placed on an aliquot mixer until the fluid contacted the bottom of the tube 5 times. A plain microcapillary tube was filled 2/3 full with the suspension. One side of a hemocytometer chamber was loaded. An equal volume of 0.4% trypan blue was added to the fluid remaining in the microcapillary tube, and the tube was inverted 2 to 4 times. The second half of the hemocytometer was charged with the mixture.

The cell suspension was examined microscopically at 43x magnification. On the unstained side, 500 nucleated cells were counted, 100 cells in each of the 4 large corner squares and in the center square. Occasionally, part of the other squares had to be counted to attain 500 cells. Rosetting was defined as the attachement of at least 3 SRBC to nucleated cells. Clumps of less than 5 cells were counted, attempting to visualize the presence of attached SRBC. Larger clumps were excluded from enumeration. When the central cell was not visible, the cell was considered a rosette if the configuration of the SRBC was discretely organized. The percent of rosetting cells was determined.

The trypan blue preparation was counted for non-viable and rosetted leukocytes. Working diagonally across the chamber's large squares, 200 cells were counted. The percent of cells excluding

trypan blue was reported, and the percent of rosetted leukocytes was also determined.

H. B LYMPHOCYTE TESTING

For titration of the antiserum, heparinized blood was collected from 2 donors in 3 10 ml heparinized vacuum tubes. The isolation of leukocytes was performed as previously described.

The reconstituted fluorescein antiserum was centrifuged in a Model 152 Microfuge (Beckman Industries, Fullerton, CA) for 5 minutes. The antiserum was serially diluted to a 1:128 dilution the initial tube, containing 0.2 ml undiluted antiserum, was diluted with 0.2 ml PBS-P. All procedures were performed in a darkened room.

For each donor, 7 10 x 75 mm plastic culture tubes were labelled starting with 1:2 through 1:128. In each tube, 0.1 ml cell suspension was mixed with 0.1 ml serially diluted antiserum in the appropriately labelled tube. The tubes were incubated in a covered ice-bath for 45 minutes. At the end of incubation, the culture tubes were filled with 3 ml cold PBS-P with 0.1% sodium azide and centrifuged in the Serofuge (Clay-Adams Co., Pisappany, NJ) for 3 minutes. The supernatant was decanted, and 2 more washes were performed. After the third wash, the supernatant was thoroughly decanted, and the cells suspended in the residual medium. The tubes were returned to the ice-bath. A microcapillary tube was filled with the suspension and discharged on a glass slide. The slide was coverslipped. After the cells settled, a total of 500 nucleated cells were counted. The fluorescent microscope used in this study permitted some discrimination between polynuclear and mononuclear cells. Leukocytes with clear-

ly visible nuclear segmentation were not counted. A positive cell was defined as having small specks uniformly distributed in a ring or in patches at the cell periphery. The percent positive cells was recorded.

The highest dilution of antiserum giving the maximum positive cells was determined. The working dilution was defined as 1/4 of that dilution. Once the working dilution was established, 3 to 5 vials of antiserum were reconstituted and diluted to that concentration. The reagent was aliquoted into 250 µl microcentrifuge tubes (Cole Scientific, Calabasas, CA) and stored in a light-excluding container at -20°C for no more than three months.

For B lymphocyte testing, the 20 x 10^6 nucleated cells/ml test suspension was delivered in 0.1 ml volume to a 10 x 75 mm culture tube, and the tube placed in an ice-bath. A microcentrifuge tube of diluted antiserum was thawed and centrifuged in the Microfuge for 5 minutes. A 0.1 ml aliquot of the antiserum was added to the cell suspension. The cells were incubated for 45 minutes as previously described. After incubation, the cells were washed 3 times using 1 to 2 ml PBS with 0.1% sodium azide, and centrifuged in the Serofuge for 30 seconds. Microscopic analysis was performed as described previously, except cells were mounted, coverslipped, and sealed with diluted coverslip-mounting medium. The slides were placed in a covered box and placed on ice until examined.

I. CYTOCENTRIFUGE PREPARATION OF CELLS

Isolated cells were diluted to a concentration of 70 to 100 nucleated cells/cu mm by adding 1 microcapillary tube of suspension

to 0.5 ml PBS-P. The cells were mixed and counted on a hemocytometer. The volume was adjusted to produce the appropriate cell concentration.

A microcapillary tube was filled with the rosetted cell suspension, and 1 drop added to 0.5 ml PBS-P. The nucleated cells were counted on a hemocytometer, and adjusted to 10 to 20 cells/cu mm.

For preparation of cytocentrifuge slides, Hematology Division Procedure #537 was followed. In summary, the cytocentrifuge cuvette was assembled as illustrated in Figure 1 and inserted in the cytocentrifuge. To each 0.5 ml sample, 1 drop of 22% bovine albumin was added and mixed. The cuvette was filled with the preparation and capped. The samples were centrifuged at 600 rpm for 10 minutes. After centrifugation, the slides were carefully removed and dried for 5 minutes. The slides were stained on the Gam-Rad hemomatic slide-stainer (Gam-Rad, Detroit, Michigan) and coverslipped with Protex.

On each cell suspension slide, a 200 cell differential was performed using standard criteria for morphologic identification of leukocytes (93). Lymphocyte purity was defined as the percent lymphocytes on the cytocentrifuged cell suspension. The preparations with blast cells, tissue specimens, and bone marrow cells were evaluated by Dr. Ridgway and this investigator. Selected slides were also reviewed by Dr. Sheth.

The area selected for rosette enumeration is demonstrated in Figure 2. It is defined as having no clumps of more than 10 nucleated cells and no sheets or clumps of SRBC under 10x magnification. Areas

unsatisfactory for enumeration are illustrated in Figures 3 and 4. The illustrated fields have clumps of leukocytes, or clumps and sheets of SRBC.

A rosette is defined as a lymphocyte or blast cell having 3 or more SRBC attached, or in very close proximity. Estimated by comparison with 0.8 μ latex particles suggested the lymphocyte/SRBC separation was 2 μ or less. Clumps of fewer than 5 nucleated cells were enumerated if the cells were arranged in a manner permitting visualization of SRBC between the nucleated cells. A 200 leukocyte differential was performed, and rosettes were expressed as percent of leukocytes of lymphocytes counted, and/or blast cells.

J. CALCULATIONS

1. Leukocyte Counts

Absolute cell counts were determined by the following:

Total Leukocytes/cu mm X (% Lymphocytes in Differential) =
Absolute Lymphocyte Count/cu mm

2. Recovery of Leukocytes

The recovery of cells was calculated by the following formulas:

a | Absolute Lymphocyte Count/cu mm X Volume of Sample $X10^3$ =
Total Lymphocyte Count/ml

b | $\frac{\text{Total Number of Cells Recovered/ml}}{\text{Total Number of Lymphocytes in the Original Sample/ml}}$
X 100 = % Recovery of Cells

The percent lymphocyte recovery was calculated as follows:

$$\frac{\text{Total Number of Lymphocytes Recovered/ml}}{\text{Total Number of Lymphocytes in the Original Sample/ml}} \times 100 = \% \text{ Lymphocyte Recovery}$$

3. Statistical Tests

Statistical tests, such as mean, standard deviation, correlation (r), linear regression, group t-test, and paired t-test were performed using a Texas Instrument TI-55 calculator and standard statistical methods (71).

Where applicable, results were expressed as mean $\pm$ one standard deviation.

RESULTS

A. BASIC STUDIES: ISOLATION AND PURIFICATION OF CELLS

1. Isolation of Leukocytes from Peripheral Blood

Preliminary studies were performed to determine the optimal blood dilutions for lymphocyte isolation. Three normal bloods diluted with 2 volumes PBS had a mean lymphocyte purity 10.7% lower than 3 bloods diluted with equal volumes PBS. However, the mean cell recovery was higher (19.0% and 3.3%, respectively) in bloods diluted with more PBS. Based on this small sample, it was decided to dilute blood in at least 2 volumes PBS.

Since the procedures for T and B lymphocyte enumeration required 3 washes after Ficoll-Paque leukocyte isolation, significant cell losses were possible during separation or washing of the cells. In one experiment, there was a 31.4% leukocyte loss after Ficoll-Paque separation of blood cells, and a 12.0% cell loss during washing. Total leukocyte recovery was 56.6% of the original number of lymphocytes. Because the greatest cell loss occured during the isolation procedure, maximum care was always taken to remove all the cells at the interface.

2. Purification of Bone Marrows

Isolated cells from 3 bone marrows with greater than 70% non-lymphoid leukocytes were diluted with PBS to approximately 20×10^6 nucleated cells/cu mm and layered over Ficoll-Paque as was done for blood. Differentials were performed on the original sample or cytocentrifuged isolated cell preparation and the cytocentrifuged preparation of Ficoll-Paque-treated cells. Table 1 shows that there was no significant increase in the percent of lymphocytes after Ficoll-Paque treat-

ment of isolated bone marrow cells, mean and one standard deviation difference 5.0±2.0%.

B. BASIC STUDIES: CYTOCENTRIFUGE PREPARATIONS

1. Cytocentrifuge Preparation of Cell Suspensions

Initially, cell suspensions were examined by placing a drop of suspension on a glass slide. A large drop was also placed on a slide and allowed to run part way down the slide. When these slides were allowed to dry and were Wright's-stained, the leukocytes appeared much smaller than on peripheral blood smears. Furthermore, the stain tended to be dark, and nuclear detail was not clearly visible. Destaining with methanol or Wright's stain did not enhance morphologic detail. Films were also made by the "wedge" method as used for peripheral blood smears. Many leukocytes were disrupted, and similar problems with cell identification occured. Figure 5 is an example of malignant cells prepared by air-drying a drop of the cell suspension. Accurate identification of blast cells was not possible. Therefore, it was decided to utilize the cytocentrifuge.

Cell suspension cytocentrifuge preparations contained significant leukocyte disruption, up to 50 smudge cells/100 leukocytes counted on some preparations. Attempts were made to reduce cell disruption by adding 2 drops of 22% bovine albumin instead of the usual 1 drop/0.5 ml of cell suspension. No reduction of smudge cells was noted.

Cellular identification was not difficult on cytocentrifuge preparations. Occasional specimens contained 1-5% small "monocytoid lymphocytes". These cells were classified according to the predominant mononuclear cell type present. Blast cells were readily identified by standard criteria used in hematology (93).

2. Cytocentrifuge Preparations of Rosetted Cells

Rosetted cell preparations were originally diluted to the same leukocyte concentration as the isolated cell suspensions. However, the preparations often had large areas where leukocytes and red cells were pooled in masses. The leukocyte concentration was decreased to 10-20 nucleated cells/cu mm. Sufficient cells were present for rosette enumeration. Because the central cell was clearly visible, enumeration of rosetted leukocytes was simplified. The area selected from counting is described in the methods section.

C. BASIC STUDIES: B LYMPHOCYTE TESTING

Titration of fluorescein isothiocyanate-conjugated antihuman immunoglobulin serum produced a maximum titier of 1:8. The working dilution was 1:2. When additional antiserum was reconstituted, it was titered with 1 control. The same titer resulted.

Using the washing technique described for titration of antiserum resulted in 10-20% cells with bright fluorescent rims which appeared under the cell membrane. The areas were associated with an outward bulge in the cell contour. Washing with PBS-P containing 0.1% sodium azide did not eliminate this fluorescence. Cells washed with 1-2 ml PBS with 0.1% sodium azide and 30 second Serofuge centrifugation reduced the number of these cells, thus, these conditions were chosen for B lymphocyte testing.

D. NORMAL CONTROLS

The patient population ranged from 7 months to 17 years of age. Therefore, to assess the T and B lymphocyte results, the control group was divided into an adult group, 23-45 years old, and a pediatric group,

1-13 years old. Of the 31 adults used as controls, the results of 1 were not included because of a possible viral infection. As summarized in Table 2, the absolute lymphocyte mean and 1 standard deviation for adults was 2,500±800/cu mm with a range of 1,160-4,280 lymphocytes/cu mm. Children had a mean of 3,700±2,030 lymphocytes/cu mm, and a range of 1,420-7,100 lymphocytes/cu mm.

The adult T lymphocytes were 69.8±7.2%, range 58-84%, and the children were 68.5±9.6%, range 49-81% T lymphocytes. The absolute T lymphocytes in adults was 1,740±630/cu mm (range 700 to 3,040/cu mm),and in children 2,460±360/cu mm (range 950 to 4,900 cu mm). B lymphocytes ranged from 5-17% mean 9.4±3.0% in adults, and 7-16% mean 10.7±2.5% in children. The pediatric normal samples had 380±200 B lymphocytes/cu mm, and a range of 170-500 B lymphocytes/cu mm was found in adults. No statistical difference was found between adult and children T and B lymphocyte percents, $p<0.05$.

The leukocyte recovery results for 36 controls are summarized in Table 3. Three samples had T and B lymphocyte enumeration without cytocentrifuge preparations. The cell recovery was 50.1±24.5%, with a range of 19 to 121%. Lymphocyte purity ranged from 45 to 98%, with a mean of 79.7±11.9%, and lymphocyte recovery ranged from 21 to 80%, with a mean of 39.2±16.3%. In cell suspensions, the percent of monocytes present was 15.3%±10.5% (range 1-53%), and granulocytic cells 5.3±6.9% (range 0-36%). As indicated in Figure 6, the lymphocyte recovery was less than 35% in 56.3% of the samples, and lymphocyte recovery greater than 70% was attained in 8.5% of the controls.

In 18.8% of the controls tested, the percent of rosetted lymphocytes determined in cell suspension was higher than the lymphocyte purity. The difference in percent ranged between 1-36.

Before T and B lymphocyte testing, all control cell suspensions had leukocyte viabilities 95% or greater. Leukocyte viability in rosetted preparations was 95% or greater in all samples except one where viability was 86%. Mixing of trypan blue and cell suspension may disrupt rosettes. As shown in Figure 7, the correlation between rosettes with and without trypan blue was significant ($r = 0.71$, $p<0.001$) and the mean rosettes similar, ($p>0.7$).

The results of percent rosettes by cell suspension and by cytocentrifuge on 32 normal samples are compared in Figure 8. The mean rosettes by cytocentrifuge was $56.6 \pm 16.6\%$. By the paired t-test, the means were significantly different ($p<0.001$), and the correlation between the two testing methods was significant ($r = 0.56$, $p<0.001$).

The percent rosetted lymphocytes per number of lymphocytes obtained by cytocentrifugation was also calculated and compared with the lymphocytes rosetted by cell suspension (Figure 9). The mean and standard deviation by this method ($67.9 \pm 13.0\%$) was significantly similar to the cell suspension rosette ($p>0.5$) and the correlation was significant ($r = 0.60$, $p<0.001$).

Because 200 leukocytes were counted on cytocentrifuge preparations of isolated leukocytes, 200 cell differentials were performed on rosette cytocentrifuge preparations. Comparison of differentials on lymphocyte-enriched cell suspensions and rosette slides resulted in a mean difference of $4.9 \pm 6.5\%$.

E. NON-MALIGNANT BONE MARROWS

Six bone marrows judged uninvolved with malignant disease by Dr. Ridgway were tested. This was a heterogenous group of patients whose bone marrows were not part of any CCSG study. The results are reported for comparison with malignant bone marrow. Since rosettes were based on all nucleated cells in the cell suspension, the percent rosetted lymphocytes per 100 nucleated cells and rosetted lymphocytes were studied. The results shown in Table 4, columns 5 and 6, indicate that the cell suspension results were closer to the percent rosettes (column 4) results on cytocentrifuge slides ($r=0.79$), and the percent lymphocytes ($r= 0.48$).

F. GROUPS I AND II: PATIENTS WITH MALIGNANT LEUKOCYTES

The samples in group I consisted of 6 bone marrows from newly diagnosed cases of acute lymphocytic leukemia and 2 bone marrows from 1 patient in relapse. Of the 8 bone marrow samples, peripheral blood was simultaneously tested on 5 patients. Three solid tissues from newly diagnosed non-Hodgkin's lymphoma patients were also examined (group II samples).

Isolation of leukocytes from patient bone marrows and blood with malignant cells was more difficult than with normal samples. Bone marrow cells tended to form large aggregates of cells which were difficult to disrupt, and on occasion had to be removed from the sample with an applicator stick. Although leukocytes for tissues and some of the blood did not significantly aggregate, most of the blood samples tended to clump more than normal samples. Most leukocyte clumps were disrupted

by aspiration through a Pasteur pipet. Nucleated cell viability by trypan blue was 90% or greater on all samples, except one tissue which showed a cell viability of 86%.

After overnight incubation at 2 to 3°C, resuspension of the malignant leukocyte-SRBC pellet was often incomplete, containing more large aggregates of leukocytes and SRBC than was noted with normal cells. The blast cells and lymphocyte rosettes could not be distinguished with regularity. By trypan blue exclusion, leukocyte viability in rosetted preparations was the same as in initially isolated cells, except one bone marrow when viability had decreased to 86%, and the tissue with initial cell viability of 86% had decreased to 70%.

The results of T and B lymphocyte testing are shown in Table 5. All of the bone marrows had blast cells 69% or greater, and 2 patients, K.St. and A.R., had T lymphocyte markers on the majority of the bone marrow cells. The rosettes on A.R. were more fragile than normal, and disrupted after 15 minutes at room temperature. B lymphocytes were decreased in all bone marrow samples. The peripheral blood T lymphocytes were significantly decreased when the bone marrow rosette leukocytes were decreased, except in 2 cases, B.C. (1) and J.Hi., where the original blast count was 10 and 0 percent, respectively. The bone marrows with increased T lymphocyte markers contained normal or slightly decreased peripheral blood T lymphocyte marker percents. B lymphocytes ranged from 0-9% in the blood samples. Two of the tissues had high percent T lymphocyte markers and numerous blast cells. The third tissue, D.A., had 37% T lymphocytic markers and 4% B lymphocyte markers.

Rosetted cytocentrifuge preparations on patients with lymphoreticular neoplasms were more difficult to interpret than normal bloods and non-malignant bone marrows. Although nucleated cell concentrations were adjusted as was done for controls, the slides contained more cells. Rosette preparations were evaluated by strictly adhering to the defined area for cell enumeration, and only single cells with touching SRBC were counted. The results shown in Table 6 include the differential of lymphocytes and blast cells. The percent total rosetted lymphocytes and blasts are reported as percent of total leukocytes counted. As shown in Table 6, the percent total rosetted lymphocytes and blast cells did not always correspond with the rosettes in cell suspension. However, in most cases, the total rosetted lymphocytes and blast cells is higher than the cytocentrifuge results. As demonstrated in Figures 10 and 11, rosetted lymphocytes and rosetted blast cells were readily identified, and the percent rosetted lymphocytes and percent rosetted blast cells are included on Table 6, columns 3 and 5.

The cytocentrifuge differentials on the isolated cell suspensions and rosetted preparations were compared. The lymphocyte purity was not significantly different on the 2 preparations, mean difference $0.7 \pm 6.4\%$. Also, the percent of blast cells on the 2 preparations was not different, mean difference $0.8 \pm 7.5\%$.

G. GROUP III: PATIENTS ON PULSE CHEMOTHERAPY

T and B lymphocyte enumerations were performed on blood from 8 patients undergoing pulse chemotherapy. Four of the patients were tested before pulse therapy was initiated, and on successive days prior

to additional therapy. There were 23 samples tested.

The percent B lymphocytes was markedly reduced in all cases, except M.M., and R.I., where B lymphocytes were 15% and 13%, respectively. As indicated in Figure 12, the percent T lymphocytes decreased on the fourth day after initiation of chemotherapy in S.J. and C.L., and increased over 7-10 days as therapy continued. In patient R.C., there was a slight increase in the percent T lymphocytes between day 0 and day 7 when chemotherapy was interrupted after the first treatment. When chemotherapy was reinstated, a small decrease in percent T lymphocytes was noted on day 9. The percent T lymphocytes was lowest on day 7, and subsequently increased to almost the day 0 value on S.T.

In all samples, the percent T lymphocytes in cell suspension exceeded lymphocyte purity 48.5% of the time. The range of difference was between 4 and 33%.

Leukocyte viability, in all cases, was 90% or greater when tested after cell isolation, and when rosettes were enumerated. As indicated in Figure 13, the mean percent rosettes in cell suspension with and without trypan blue was not significantly different, $p>0.6$, and the correlation was significant, $r= 0.90$, $p<0.001$.

The cell recoveries of 23 samples are summarized in Table 7. The mean cell recovery was 99.1$\pm$42.3%, range 39-243%. The range of lymphocyte purity was 25-96% (mean 70$\pm$20.1), and the range of lymphocyte recovery was 31-142% (mean 62.8$\pm$27.3%). Monocytes ranged from 4-74% with a mean of 25.8$\pm$17.8%. The mean for granulocytic cells was 4.2$\pm$2.1% and range was 0-17%. As shown in Figure 14, 21.6% of the samples had

lymphocyte recoveries greater than 70%, and 8.7% of the samples had lymphocyte recoveries less than 35%.

Since monocyte contamination appeared higher in the isolated leukocytes from patients on pulse chemotherapy, the absolute number of monocytes in the original samples was compared with the absolute monocytes in the original samples of the 9 pediatric controls. No monocyte difference in the unseparated blood could be demonstrated.

Of 23 samples tested, one rosette cytocentrifuge preparation contained insufficient leukocytes for evaluation. The results of percent rosettes by cell suspension and cytocentrifuge are compared in Figure 15. The correlation between the 2 tests was significant ($r= 0.60$, $p<0.001$), and the means were ($68.3\pm13.3\%$ by cell suspension and $44.9\%\pm18.2\%$ by cytocentrifuge) significantly different, $p<0.001$.

Figure 16 demonstrates a failure of correlation between percent rosetted lymphocytes on cytocentrifuge preparation, $r= 0.35$, $p>0.05$. Since monocytes were the next largest population in these samples, the rosette-like monocytes were specifically analyzed. SRBC binding to monocytes was similar to lymphocyte rosettes; however, formation of tightly adherent SRBC rosette-like monocytes was observed less often than in lymphocytes. As indicated in Table 8, no relationship could be determined between rosette-like monocytes and the difference between cell suspension rosetted lymphocytes on cytocentrifuge slides. On the samples where the difference between percent rosetted lymphocytes on cytocentrifuge slides was 13% or less (13% was the standard deviation for cytocentrifuge rosetted lymphocytes) than the cell suspension rosetted, 40% contained less than 5 rosette-like monocytes. Also

shown on Table 8, 54.5% of the blood rosette cytocentrifuge preparations contained more monocytes than the mean plus one standard deviation of monocytes found on the separated cell suspension preparations from pediatric control samples (14.9+9.0%=23.9%).

The presence of increased monocytes in treated patient samples may have influenced the percent rosetted lymphocytes on cytocentrifuge preparation. To further investigate this possibility, 8 normal blood slides with greater than 15% monocytes in the isolated leukocyte suspensions were treated in the same manner. As shown in Table 9, monocytes with 3 or more attached SRBC was 2.5 to 10 per 100 leukocytes. The percent rosettes in cell suspension and percent rosetted lymphocytes on cytocentrifuge preparations was significantly different, $r= 0.30$, $p>0.05$. However, since the mean and standard deviation of this sample differed by less than 5% from all of the 32 control samples, the difference between cell suspension and cytocentrifuge results may not be significant, due to the small sample size. In samples where the difference between percent rosetted lymphocytes on cytocentrifuge slides was 13% or less than the rosettes in cell suspension, 75% contained less than 5 rosette-like monocytes, and 25% contained greater than 5 rosette-like monocytes. Because these results are the reverse of the patient group, the presence of monocytes apparently does not affect the percent rosetted lymphocytes in normal controls.

To check the reproducibility of the percent rosetted lymphocytes on cytocentrifuge preparations, the results shown on Table 6 were compared with the results originally obtained and illustrated in Figure

15. The difference in results was 5.6±12.3%. The difference in lymphocyte purity between the isolated leukocyte preparations and the original differentials on rosetted cytocentrifuge slides was 4.9±14.6%.

DISCUSSION

Spontaneous sheep red blood cell rosette formation and detection of surface immunoglobulins are established tests for thymus-derived, or T, and bursa-equivalent, or B, lymphocytes, respectively. These are functional tests for identifying normal lymphocyte populations which are morphologically homogenous. Although testing principles are simple, a variety of technical factors may influence the results (29,62,63,64,74,82). Thus, extensive application of surface marker analysis must consider both the technical aspects, and the validity of applying T and B lymphocyte testing to diseases.

The lymphoreticular neoplasms are a heterogenous group of malignancies involving the lymphoid system. The patients in group I were newly diagnosed or relapsed acute lymphocytic leukemias, and group II were newly diagnosed non-Hodgkin's lymphomas. T and B lymphocyte enumerations were performed to provide data for a national study group, The Children's Cancer Study Group. Testing was based upon procedures given in the CCG-161 protocol for acute lymphocytic leukemia (42), and the World Health Organization/International Agency for Research on Cancer special technical report on human T and B lymphocytes (98). Additional sequential studies were planned on peripheral blood from patients undergoing pulse chemotherapy (group III patients).

In the beginning of this study, preliminary work was performed to establish optimal blood dilution for isolation of leukocytes from peripheral blood, study cell loss during isolation and washing of Ficoll-Paque isolated leukocytes, and techniques of preparing slides

for morphologic identification of cells in suspension. From these studies, it was decided to dilute blood in at least 2 volumes of PBS. Since cell loss was greatest during the isolation procedure, maximum care was taken to remove all of the cells at the interface. It was obvious that accurate identification of leukocytes in suspension was a major problem since lymphocyte preparations were not as pure as expected. Cytocentrifuge preparations provided a superior means for identification of cells, and since it is frequently used to identify malignant rosetted cells, the feasibility of using the cytocentrifuge for rosette enumeration was evaluated.

When T and B lymphocyte percents were compared in normal adult and pediatric blood samples, no statistical difference could be demonstrated ($p < 0.05$). According to Augner, Cohen, Reuter, and Brittinger (4), the percent of peripheral blood T lymphocytes are the same in adults and children, similar to the controls used in this study, but they report that the percent of lymphocyte levels (assuming the standard deviations for both groups in this study remained the same) would require analysis of over 40 normal pediatric blood samples. Because differences in adult and pediatric peripheral lymphocyte levels have been reported, it would have been preferable to include additional normal control children in this study.

Trypan blue was used in this study to measure cell viability, but it also increased the color contrast between non-rosetted cells and the background of the hemocytometer chamber. Since trypan blue did not affect rosette formation (Figures 7 and 13), gentle mixing of trypan

blue and rosetted cell suspension preparations in a microcapillary tube may be used for simultaneous enumeration of rosettes and cell viability testing. Because visualization of non-rosetted nucleated cells is enhanced, the accuracy and ease of cell enumeration is increased. This is a technical advance which will save a great deal of time.

During the period of this study, 6 cases of newly diagnosed acute lymphocytic (ALL), 1 case of ALL in relapse, and 3 cases of non-Hodgkin's lymphoma were studied for T and B lymphocyte markers. None of the patients showed significant B lymphocyte markers in the tissue predominantly involved with disease. Two cases of leukemia and 2 patients with non-Hodgkin's lymphoma had predominant T lymphocytic markers on their blast cells. Five patients with ALL had no T or B lymphocytic markers on leukemic cells. Although the sample size is small, it is consistent with the reported incidence of T lymphocytic markers in ALL (14,20,21,22,39,53,54,85).

In addition to studies of T and B lymphocytic markers on these cases of lymphoreticular neoplasms, other observations were also made. T and B lymphocyte enumerations can be performed on bone marrow, tissue, and blood samples. However, the significance of the results depends upon the number of blast cells present in the cell suspension tested, e.g., the T lymphocytic marker was 66% in the blood of J.Hi., but none of the bone marrow cells rosetted. The original blood sample contained no blast cells. Some investigators do not test blood samples unless the sample contains greater than 70% blast cells (20,21). Based upon the experience in this study, this is probably advisable. Bone marrow samples with large numbers of non-lymphoid cells were treated with

Ficoll-Paque, and as indicated in Table 1, no enrichment of lymphocytes occured. Thus, bone marrows with low numbers of blast cells should not be tested for surface marker identification of malignant cells. For acceptance of data by CCSG, presence of malignant cells 50% or greater in the tissue examined was required (42).

T and B lymphocyte enumerations were performed on blood from 8 patients undergoing pulse chemotherapy. Four of the patients were tested before pulse therapy was initiated and on successive days prior to additional therapy. The intent of this study was to determine the immune status of the patients undergoing chemotherapy by evaluating the T and B lymphocyte levels. Due to the limited number of cases in this study, and inadequate size samples in all cases, it is difficult to derive any definite conclusion from this investigation. However, 2 of the cases demonstrate a decrease in T lymphocyte levels on day 4, and the values gradually increased over a 7 to 10 day period as therapy continued. This study should be extended before any definite conclusions can be drawn.

Although Ficoll-Paque is widely used for lymphocyte isolation of peripheral blood, decreased lymphocyte recoveries and contamination of preparations with other leukocytes pose theoretical and technical problems. It has been proposed that lymphocyte recoveries of less than 70% may not reflect in vivo cell populations due to selective loss of T lymphocytes, but most investigators fail to report lymphocyte recoveries (23). In this study, recovery of lymphocytes was greater than 70% in only 8.3% of the normal blood samples (Figure 6) and 21.6% of

the pulse chemotherapy patient blood samples had lymphocyte recoveries greater than 70% (Figure 14). Since others report that Ficoll-Paque does not result in selective loss of T or B lymphocytes (82,91), the lymphocyte recovery results in this study were accepted as properties of Ficoll-Paque separated populations and the techniques used in this study.

Although no difference in the original blood absolute monocyte counts could be demonstrated between the chemotherapy group and the pediatric control group ($p>0.5$), the isolated blood leukocytes from pulse chemotherapy patients contained more monocytes than the mean and one standard deviation of the isolated leukocytes in the pediatric controls in 54.5% of the samples. Increased monocytes in isolated leukocyte fractions of blood have also been reported in lepromatous leprosy (81) and in untreated Hodgkin's disease (36). These findings suggest a disproportionate percent of monocytes in Ficoll-Paque separated populations occur in the pathological state, and is an important laboratory artifact which should be considered when lymphocytes are isolated by buoyant density centrifugation.

In 43.8% of the pulse chemotherapy patients and 18.3% of the controls, the percent of rosetted leukocytes in cell suspension was higher than the lymphocyte purity. This may be due to incomplete resuspension of the pelleted cells, or rosette formation by non-lymphoid cells. Since rosettes are considered fragile (6,19,48,62,65,82,104), longer mixing of pellets was not attempted. All of the control and patient pellets were resuspended in the same manner, suggesting that

there may be differences in properties of rosetted cells in chemotherapy patients. Others have reported that rosetting of non-lymphoid cells (35,37,46,67,86), and rosetted non-lymphoid leukocytes may account for the greater number of rosettes than the lymphocyte purity. This underlies the importance of the previous finding. If monocytes are present in abnormally high levels in the Ficoll-Paque separated population and they rosette, inaccurate high levels of rosetted cells will be reported.

Even if a population of patients does not have circulating malignant cells, it cannot be assumed that blood leukocyte isolation characteristics of T lymphocyte enumerations will be comparable with the results from normal peripheral blood samples. It has been demonstrated that peripheral blood lymphocyte recoveries were different, and generally higher, in pulse chemotherapy treated patients with lymphoreticular neoplasms. Monocyte contamination was higher in these Ficoll-Paque purified preparations;therefore, monocytes must be depleted, if possible, or the cytocentrifuge should be used to differentiate between lymphocytes and monocytes present in samples. Finally, patients may have lymphocyte rosettes or rosetting non-lymphoid cells in greater numbers than in normal samples.

Cytocentrifuge preparations were made on isolated cell suspensions and rosetted cell preparations. The advantage of cytocentrifuge preparations is the preservation of cell morphology so that slides may be stained and cell identification can be performed. The slides can be kept as permanent records. Several reports in the literature suggest different techniques for preparing smears of rosetted cells

(63,81), but as shown in Figure 5, the preservation of cell morphology is poor, especially for identification of malignant cells. The cytocentrifuge slide gives a superior quality preparation with well preserved cellular morphology.

Leukocyte loss during cytocentrifugation may be selective for certain cell populations, but close similarities of differentials on initial cell suspensions and rosette preparations suggest cell loss is non-specific (mean difference in lymphocyte purity 4.9±6.5% for normal blood samples, 0.7±6.4% for groups I and II samples, and 4.9±14.6% for group III samples). In the hematology laboratory, a difference of ±10 polymorphonuclear neutrophils is allowable in duplicate 100 leukocyte differentials on peripheral blood smears. These leukocytes are at approximately the same concentration as lymphocytes on Ficoll-Paque purified cytocentrifuge preparations. Disintegration of cells was probably related to cell damage by centrifugal force applied during slide preparation. The cellular damage resulting from manipulation of cells during isolation may have been sufficient to rupture the cells on cytocentrifugation. Although cell viability was almost always greater than 90% when tested during rosette enumeration, trypan blue is excluded by slightly damaged cells (84).

Cytocentrifuge slides of rosetted cells were easily prepared. The SRBC and nucleated cell concentration used in this study were important to obtain uniform distribution of leukocytes and SRBC on the slide.

Rothbarth, et al. report close correlation and similar means of percent rosettes by cell suspensions and by cytocentrifuge (79). In this study, when rosetted cytocentrifuge preparations of normal samples were enumerated, the percent rosettes correlated with the cell suspension results ($r= 0.56$, $p<0.001$). However, the means were significantly different ($p>0.05$). Since rosetted lymphocytes define T lymphocytes, the percent of rosetted lymphocytes was calculated from the number of lymphocytes counted in the differential, rather than the total number of leukocytes enumerated. By this method, the similar means (cell suspension rosettes $69.4\pm8.3\%$ and by cytocentrifuge rosetted lymphocytes $69.0\pm13.1\%$, $p>0.5$) and significant correlation ($r= 0.60$, $p<0.001$) found in normal controls is in agreement with Rothbarth, et al. In this study, there are several factors which may account for the differences in techniques required to obtain results comparable to the study of Rothbarth, et al. In 18.6% of the normal blood samples, cell suspension rosettes were greater than the lymphocyte purity. It was also demonstrated that rosette-like monocytes were present on cytocentrifuge preparations, but the presence of monocytes in the samples did not affect the results. Rothbarth, et al. did not report their lymphocyte purity, and they report no other cell types rosetting on cytocentrifuge preparations. Since accurate morphological differentiation of nucleated cells is not possible in cell suspension preparations, rosetting non-lymphoid cells would give results that would not correlate with cytocentrifuge results where the non-lymphoid rosettes would not be counted.

On the cytocentrifuge preparations of patients with lymphoreticular neoplasms, it was expected that the number of rosetted cells in suspension would correspond to the percent rosetted lymphocytes and rosetted blast cells based on the number of nucleated leukocytes. In the non-malignant bone marrow samples (Table 4), rosetted lymphocytes per 100 nucleated cells on cytocentrifuge preparations and rosettes in cell suspension correlated ($r = 0.79$). As indicated in Table 6, the results of total cytocentrifuge rosetted lymphocytes and blast cells did not always correspond with the cell suspension rosettes.

This study demonstrates the usefulness of a cytocentrifuge slide in identifying the rosetted cell (Figures 10 and 11), and this capability permits separate determinations of percent rosetted lymphocytes, and rosetted blast cells. Using the suspension method, the percent of rosetted blast cells can only be inferred by comparison of the number of blast cells in the suspension and the number of rosettes in cell suspensions, while the cytocentrifuge method allows separate determination of rosetted leukocytes. Identification of surface markers (T or B) on leukemic blast cells is greatly facilitated by the cytocentrifuge. In peripheral blood, bone marrow, and tissues, it is possible to identify and enumerate the percent of T, B, or null blast cells. Total rosette determinations have limited value only since the blast linage is of clinical value. However, the cell suspension rosette results of bone marrows on all but one of the presumed null cell leukemias have greater than 25% rosetted blast cells on cytocentrifuge preparations, and, according to CCSG (42), these cases would be classified as T lymphocytic leukemias. Since all of the bone marrow samples had greater

than 69% blast cells, the clinical usefulness of the cytocentrifuge results cannot be assessed until consistent agreement between cytocentrifuge and cell suspension rosetted leukocytes can be attained, or further correlations between the patient's initial clinical presentation, response to therapy, and prognosis, and the cytocentrifuge rosetted blast cells are conducted.

As indicated in Figure 15, the chemotherapy-treated patients demonstrated correlation between percent rosettes in cell suspension and on cytocentrifuge preparations ($r = 0.60$ $p < 0.001$). However, like the normal samples similarly evaluated, the means were significantly different (p 0.001). As indicated in Figure 16, no correlation was demonstrated between percent rosettes in cell suspension and percent rosetted lymphocytes when the number of enumerated lymphocytes was used to determine the percent on cytocentrifuge slides ($r = 0.35$, $p > 0.05$), although the differences in means was not significant ($p > 0.2$). The failure of correlation did not appear to be related to the presence of more monocytes than in normal samples, and the reason for this difference from the normal samples is unknown, but it may be related to general differences in patients with pathological states discussed previously. When rosetted lymphocyte enumeration was repeated on these samples, the difference was within the allowable range permitted in duplicate differentials on peripheral blood smears, attesting to the reproducibility of the cytocentrifuge techniques.

A close working relationship between the physician, the pathologist, and the laboratory personnel to assure that sample sizes and quality are adequate for analysis is mandatory for successful completion of this type of testing. Lymphocyte surface marker

analysis is time-consuming, and since specimens such as bone marrows and tissues are difficult to obtain, cooperation is required to insure sufficient time is available for careful and accurate analysis.

SUMMARY AND CONCLUSION

T and B lymphocyte surface determinates in the peripheral blood, bone marrow, and tissue of acute lymphocytic leukemia and non-Hodgkin's lymphoma patients were enumerated. The pediatric patients were newly diagnosed or relapsed acute lymphocytic leukemias, newly diagnosed non-Hodgkin's lymphomas, and pulse chemotherapy treated patients undergoing a variety of different therapeutic regimens. Sheep red blood cell rosette formation and surface immunoglobulin detection were used for T and B lymphocyte enumeration, respectively. Isolated cells were morphologically analyzed on Wright's-stained cytocentrifuged slides. Rosettes were enumerated in suspension and on cytocentrifuge slide preparations to evaluate the clinical application of the cytocentrifuge.

It was determined that T and B lymphocyte enumeration on bone marrow, blood, and tissue samples are feasible, but isolation of lymphocytes from peripheral blood results in variable lymphocyte recoveries which cannot be predicted by the original leukocyte count or differential in both normal donors and patients with pathological conditions. Monocyte contamination of isolated blood leukocyte samples can also be significant, and more so in pathological states. Since separation techniques currently available do not significantly increase the number of lymphocytes and blast cells from a bone marrow sample, bone marrow samples should be examined without attempts to enrich for lymphocytes or blast cells.

The value of results obtained from T and B lymphocyte testing is dependent on accurate identification of the leukocytes tested. The cyto-

centrifuge permits rapid morphologic identification of cells isolated from samples. Since mature lymphocyte have surface markers, samples tested for malignant cell surface marker identification should contain large proportions of neoplastic cells so the normal lymphocyte contribution to the results is minimized.

When normal blood samples are tested, the means and correlation of the number of rosetted lymphocytes from cytocentrifuge preparations are significant when compared with the number of rosettes obtained from cell suspensions. Cytocentrifuge rosette results from samples containing malignant cells and on blood samples from patients on pulse chemotherapy do not correspond as well to the result obtained from cell suspension preparations. Because morphologic identification of the rosetted leukocyte is possible, the cytocentrifuge has been widely used to identify malignant cells. The results of this study suggest that the cytocentrifuge and the cell suspension rosetted leukocyte results should be similar before clinical usefulness can be established from the cytocentrifuge results.

Although T and B lymphocyte enumerations have been widely used for ten years, numerous controversies concerning basic testing procedures remain. This study demonstrates that normal donors and patients on pulse chemotherapy show differences in leukocyte recoveries and in rosetting properties that must be considered during testing. Lymphocyte surface marker analysis is time-consuming and technically complex, but is of clinical value. Before T and B lymphocyte testing is routinely used as a clinical test, the laboratory must consider both the technical aspects and the types of patients to be analyzed.

APPENDIX A

REAGENTS

Leukocyte Counts

Unopette test 5855 for WBC/platelet determination (Beckton-Dickson, Rutherford, NY)

Phosphate-Buffered Saline, pH 7.4 (PBS) (28).

Stock Solution A. 0.2M sodium phosphate, monobasic 13.8 g sodium phosphate, monobasic, monohydrate (J.T. Baker Chemical Company, Phillipsburg, NJ)
500 ml distilled water

Stock Solution B. 0.2M sodium phosphate, dibasic 28.4 g sodium phosphate, dibasic, anhydrous (Mallinckrodt Chemical Works, St. Louis, MO)
1000 ml distilled water

Working Solution
9.5 ml solution A
40.5 ml solution B
8.5 g sodium chloride (J.T. Baker Chemical Company, Phillipsburg, NY)
Add distilled water and adjust pH to 7.4 before dilution to 1000 ml.

Phosphate-Buffered Saline with 1% Bovine Serum Albumin

5 g bovine albumin powder, fraction V from bovine plasma (Rehis Chemical Co., Phoenix, AZ)
Filter in 0.45μ Nalgene filter unit (Nalgene, Co., Rochester, NY). Store 2-4^{o}C in sterile bottle

REAGENTS (Continued)

RPMI-1640 with 10% Fetal Bovine Serum (FBS)

11.1 sterile, heat-inactivated FBS (Grand Island Biologicals Co., Grand Island, NY) absorbed at 37°C with washed sheep red blood cells (Prepared Media, Tualitan, OR)
100 ml RPMI-1640 (Grand Island Biological Company, Grand Island, NY)
Store 2-4°C

Tris-Ammonium Chloride Erythrocyte Lysing Reagent (15, 105)

7.5 g ammonium chloride (JT Baker Chemical Co., Phillipsburg, NJ)
2.05 g Tris-hydroxymethylaminomethane (THAM) (Sigma Chemical Co., St. Louis, MO)
1000 ml distilled/deionized water
Adjust pH to 7.2 with 1 N hydrochloric acid before final volume adjustment.

Trypan Blue (34, 84)

0.2 g trypan blue (Allied Chemical Corp., NY, NY)
50 ml PBS

0.5% Sheep Red Blood Cells (SRBC)

0.5 ml SRBC in Alseaver's Solution (Prepared Media, Tualitan, OR), washed three times in PBS- (five to fifteen days old when used).
10 ml RPMI with 10% FBS

Fluorescein Conjugated Antiserum

Lyopholized fluorescein conjugated isothiocyanate-conjugated antibody globulin human immunoglobulin (IgG, IgA, IgM) (Heavy and light chain specific). (Behring Diagnostics, Sommerville, NY).

REAGENTS (continued)

Phosphate-Buffered Saline with 0.1% Sodium Azide

0.5 g sodium azide (JT Baker Chemical Co., Phillipsburg, NY)

500 ml PBS

APPENDIX B

EQUIPMENT

FLUORESCENT MICROSCOPE

Ortholux microscope (Leitz, Inc., Raleigh, NY) supplied with a lamp housing 250 and superpressure mercury lamp and K6 1 heat filter, KP 490 FITC excitation filter, and K510 supression filter

CYTOCENTRIFUGE

Shandon Cytospin, model SCA-0031, cuvettes, caps, and filter paper strips were from Shandon, Southern Instruments, Inc., Sewickley, Penn.

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TABLE 1

CELLULAR COMPOSITION ON FICOLL-PAQUE ISOLATED NUCLEATED NON-MALIGNANT BONE MARROW SAMPLES

	% Non-Lymphoid Leukocytes	% Lymphocytes*	% Blast Cells
Patient 1			
Original Sample	28	72	0
Isolated Sample	36	65	0
Patient 2			
Original Sample	69	28	3
Isolated Sample	75	25	0
Patient 3			
Original Sample	82	15	3
Isolated Sample	87	10	3

* Comparison of % lymphocytes in original samples and Ficoll-Paque purified sample mean $\pm$ one standard deviation difference $5.2 \pm 2.0\%$

TABLE 2

T AND B LYMPHOCYTE LEVELS IN BLOOD OF NORMAL CONTROLS

	Adults		Children	
Number of Samples	30		9	
Age (years old)	23-45		1-13	
	Mean±1 S.D.	Range	Mean±1 S.D.	Range
Absolute Lymphocytes/ cu mm	2,500 ± 800	1,160-4,280	3,740 ± 2,030	1,420-7,100
% T Lymphocytes	69.8 ± 7.2	58-84	65.8 ± 9.6	49-81
Absolute T Lymphocytes/ cu mm	1,740 ± 630	700-3,040	2,460 ± 1,360	950-4,900
% B Lymphocytes	9.4 ± 3.0	6-17	10.7 ± 2.5	7-16
Absolute B Lymphocytes/ cu mm	230 ± 100	100-490	380 ± 200	170-500

TABLE 3

RECOVERY OF BLOOD LYMPHOCYTES SEPARATED BY FICOLL-PAQUE
NORMAL CONTROL SAMPLES

	Mean ± 1 S.D.	Range
% Cell Recovery	50.4 ± 24.5	19-121
% Lymphocyte Purity	79.7 ± 11.9	45-98
% Monocytes in Separated Cell Suspensions	15.3 ± 10.5	1-53
% Granulocytic Cells in Separated Cell Suspensions	5.3 ± 6.9	0-36

TABLE 4

SRBC ROSETTING CELLS IN NON-MALIGNANT BONE MARROWS*
DETERMINED IN SUSPENSION AND BY CYTOCENTRIFUGATION

	Differential on Cytocentrifuge Preparations			% Rosettes in Cytocentrifuge Preparations**		% Rosettes in Cell Suspensions
	% Non-Lymphoid Leukocytes	% Lymphocytes	% Blast Cells	Rosetted Lymphocytes (per number of Lymphocytes	Rosetted Lymphocytes (per 100 Leukocytes)	
Columns	1	2	3	4	5	6
Patient						
1	30	70	0	49	34	47
2	66	34	0	12	4	12
3	80	20	0	18	3	15
4	77	23	0	34	8	8
5	66	32	2	56	18	11
6	82	15	3	13	14	7

* Bone marrows prepared by Tris-ammonium chloride lysis of erythrocytes

** Correlation between % rosetted lymphoctes/100 leukocytes in cytocentrifuge preparations and % rosettes in cell suspension, r=0.79.

Correlation between % rosetted lymphocytes/number lymphocytes in cytocentrifuge preparations and % rosettes in cell suspension, r=0.48.

TABLE 5

T AND B LYMPHOCYTE LEVELS IN BONE MARROWS, PERIPHERAL BLOOD, AND TISSUE OF PATIENTS WITH LYMPHORETICULAR NEOPLASMS (GROUPS I AND II)

Patient	Specimen*	Differential on Original Sample: Leukocytes (x 10^3/cu mm)	% Lymphocytes	% Blast Cells	% T Lymphocytic Markers	% B Lymphocytic Markers	Presumptive Clinical Diagnosis**
K.St.	BM		7	75	86	1	T Lymphocytic ALL,
	PB	217.0	5	92	70	1	Untreated
A.Ro.	BM		5	87	54	1	T Lymphocytic ALL,
	PB	15.9	82	3	52	9	Untreated
H.G.	BM		5	92	5	<1	Null Cell ALL, Untreated
J.Hi.	BM		4	93	10	0	Null Cell ALL,
	PB	2.2	94	0	66	2	Untreated
J.Ho.	BM		7	89	0	0	Null Cell ALL,
	PB	10.2	56	42	26	60	Untreated
K.M.	BM		1	88	12	3	Null Cell, ALL,
	PB	10.6	63	31	24	6	Untreated
B.C. (1)	BM		3	97	<1	0	Null Cell, ALL,
	PB		58	10	56	2	Relapse
B.C. (2)	BM		13	79	2	0	Null Cell, ALL, Relapse
K.Su.	Tis.		7	93	77	<1	T-Lymphocytic Lymphoblastic Lymphoma
S.C.	Tis.		8	90	97	0	T-Lymphocytic Lymphoblastic Lymphoma
D.A.	Tis.		85	7	37	4	Histiocytic Lymphoma

* B.M.= Bone marrow, P.B.=Peripheral Blood, Tis.=Tissue

** When bone marrow T lymphocytic markers were >25%, patient was classified as T lymphocytic ALL or lymphoma.

TABLE 6

SRBC ROSETTING CELLS IN BONE MARROW, PERIPHERAL BLOOD, AND TISSUE OF PATIENTS WITH LYMPHORETICULAR NEOPLASMS (GROUPS I AND II)

		Cytocentrifuge Preparations					% Rosettes in Cell Suspension
	Specimen*	% Lymphocytes	% Rosetted Lymphocytes (per number of lymphocytes)	% Blast Cells	% Rosetted Blast Cells (per number of blast cells)	% Rosetted Lymphocytes and Blast Cells (per 100 leukocytes	
Columns	1	2	3	4	5	6	7
Patient							
K.St.	BM	4	5	8	36	34	86
	PB	1	100	96	98	84	70
A.Ro.	BM	12	75	85	74	92	54
	PB	74	79	20	25	9	52
H.G.	BM	3	33	96	26	26	5
J.Hi.	BM	12	25	81	32	29	10
	PB	80	79	8	25	65	66
J.Ho.	BM	15	67	83	86	42	0
	PB	14	57	85	28	32	26
K.M.	BM	12	67	76	34	34	12
	PB	5	40	93	32	31	24
B.C.(1)	BM	10	20	86	9	10	2
	PB	20	30	83	10	14	56
B.C.(2)	BM	12	33	69	30	25	2
K.su.	Tis.	1	100	99	96	96	77
S.C.	Tis.	6	100	93	99	98	97
D.A.	Tis.	94	40	3	33	59	37

* B.M.= Bone Marrow, P.B.=Peripheral Blood, Tis.=Tissue

TABLE 7

RECOVERY OF PERIPHERAL BLOOD LYMPHOCYTES SEPARATED BY FICOLL-PAQUE FROM PATIENTS BEFORE OR DURING PULSE CHEMOTHERAPY (GROUP III)*

	Mean ± 1 S.D.	Range
%Cell Recovery	99.1 ± 42.3	39-243
% Lymphocyte Purity	70.0 ± 20.1	25-96
% Lymphocyte Recovery	62.8 ± 27.3	31-142
% Monocytes in Separated Cell Suspension	25.8 ± 17.8	4-74
% Granulocytic Leukocytes in Separate Cell Suspensions	4.2 ± 2.1	0-17

TABLE 8

SRBC ROSETTING CELLS IN PERIPHERAL BLOOD FROM GROUP III PATIENTS UNDERGOING PULSE CHEMOTHERAPY DETERMINED BY CELL SUSPENSION AND CYTOCENTRIFUGATION

	Cytocentrifuge Preparations					% Rosetted Cells in Cell Suspensions
	% Lymphocytes	% Monocytes	Number of Rosette-like Monocytes	% Rosetted Lymphocytes	Any Leukocytes Binding 3 or more SRBC (per 100 Leukocytes)	
RI Day 0	53	32*	9.0	62	48	73**
MM Day 0	82	14	4.0	60	54	83
CM Day 0	82	18	1.0	62	52	87
ARe Day 0	85	14	4.5	69	64	73**
ARe Day 14	45	50*	9.5	52	34	60**
ST Day 0	96	2	0	54	52	82
ST Day 4	98	1	0	26	26	72
ST Day 7	97	1	0	24	24	60
ST Day 12	73	20	0	88	73	75**
SJ Day 0	44	55*	42.5	49	66	83
SJ Day 2	36	52*	36	57	59	73
SJ Day 4	54	24*	8.5	71	49	58**
SJ Day 9	96	2	0	57	55	76
SJ Day 15	95	4	0	29	28	64
RC Day 0	60	28*	18.5	54	52	49**
RC Day 7	46	52*	3	57	32	66**
RC Day 9	80	15	3	57	44	58**
RC Day 11	72	25*	1.5	85	62	71
RC Day 24	69	31*	5.5	68	53	71**
CL Day 0	36	59*	43.0	64	68	82
CL Day 2	21	78*	22.5	64	36	58**
CL Day 4	26	50*	15.0	82	40	29

* Cytocentrifuge preparation contained >23.9% monocytes (mean + 1 SD of the control pediatric cell suspension monocytes.

** Difference between % rosetted lymphocytes on cytocentrifuge was .13% of % rosetted cells in cell suspensions. In these samples, 40% contained less than 5 rosette-like monocytes, and 60% contained greater than five rosette-like monocytes.

TABLE 9

SRBC ROSETTING CELLS IN BLOOD OF NORMAL CONTROLS WITH GREATER THAN 15% MONOCYTE CONTAMINATION

	Cytocentrifuge Preparations					% Rosetted Cells in Cell Suspension
	% Lymphocytes	% Monocytes	Number of Rosette-like Monocytes	% Rosetted Lymphocytes	% Any Leukocytes binding 3 or more SRBC (per 100 Leukocytes)	
Control						
1	61	34	10.0	59	46	71
2	79	19	5.0	66	30	77*
3	72	22	2.5	43	33	65
4	76	23	4.0	67	55	72*
5	76	21	10.0	74	68	84*
6	71	28	2.5	40	31	58
7	72	26	8	71	55	55
8	81	18	4	62	54	67*

* Difference between % rosetted lymphocytes on cytocentrifuge was >13% of the rosetted cells in cell suspensions. In these samples, 75% contained less than five rosette-like monocytes, and 25% contained greater than five rosette-like monocytes.

FIGURE 1: Cytocentrifuge cuvette assembly
1) cytocentrifuge cuvette, 2) sample well,
3) exit port, 4) double thin filter paper
5) glass slide, 6) cap for cytocentrifuge

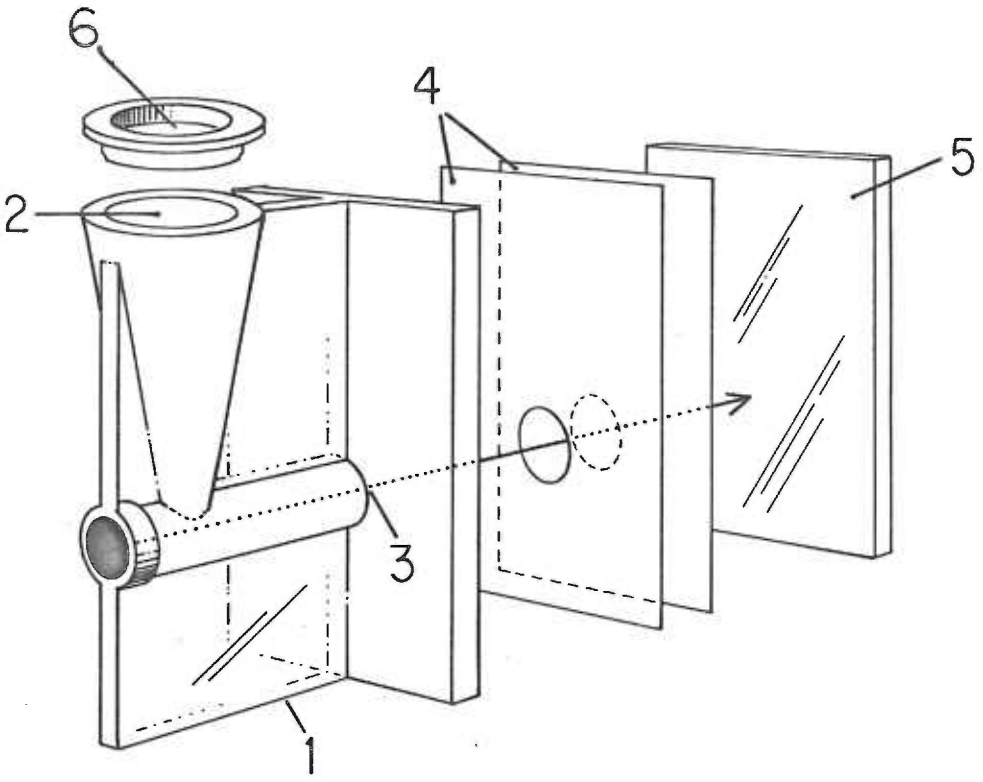
6
4
5
2
3
1

FIGURE 2: Rosetted cytocentrifuge preparation demonstrating an area suitable for enumeration of rosettes. The field has no clumps of more than 10 nucleated cells and no sheets or clumps of SRBC

FIGURE 3: Rosetted cytocentrifuge preparation demonstrating an area unsuitable for enumeration of rosettes. The SRBC in this field are in clumps and sheets.

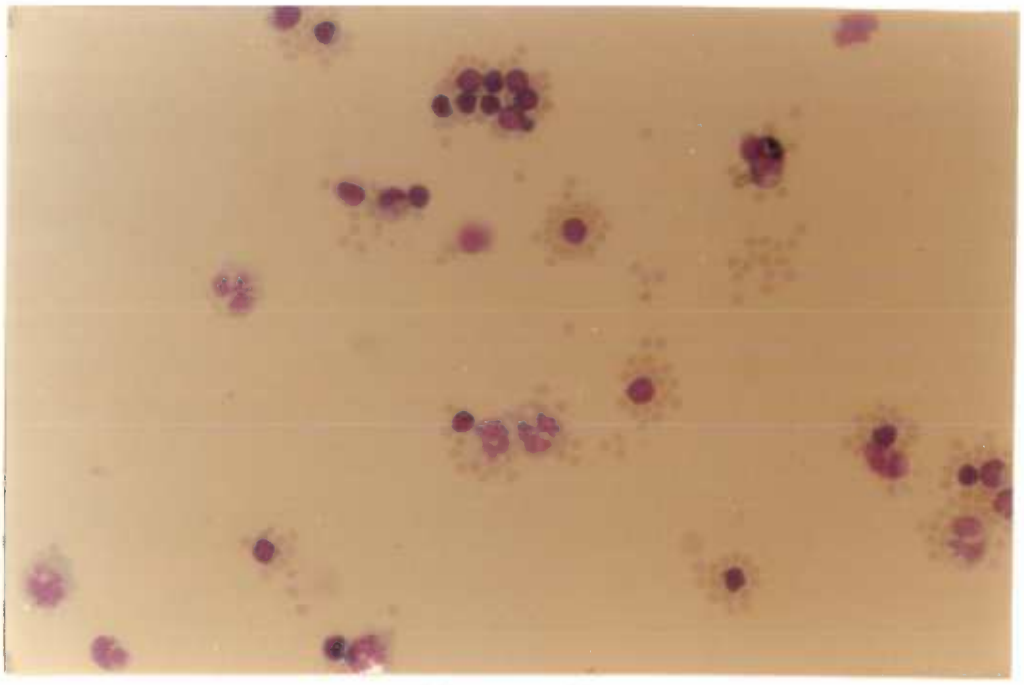

figure 2

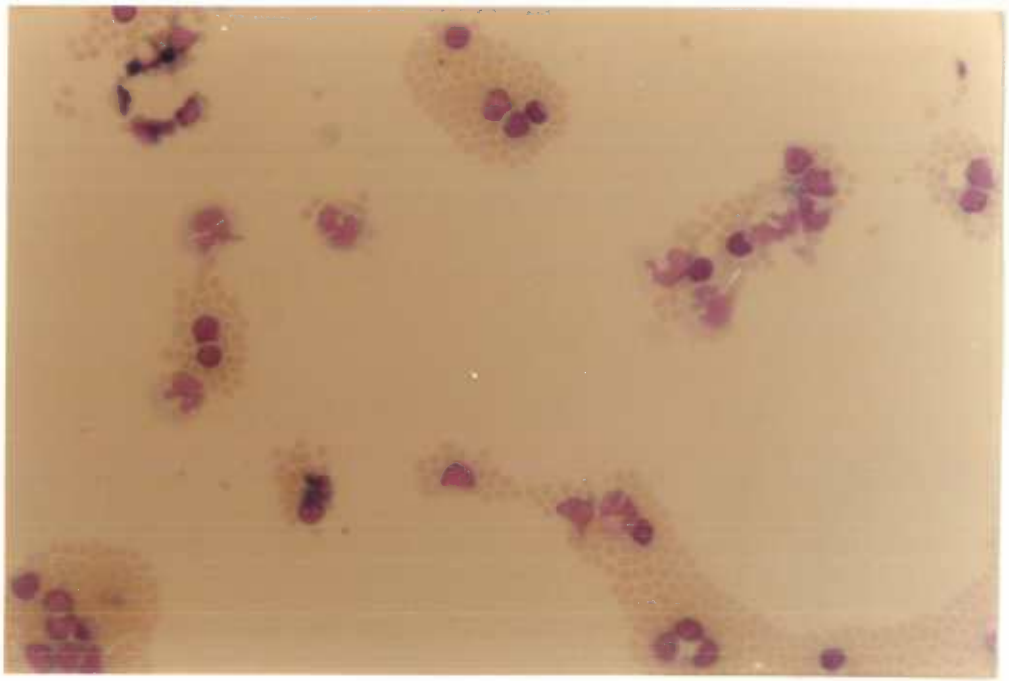

figure 3

FIGURE 4: Rosetted cytocentrifuge preparation demonstrating an area unsuitable for enumeration of rosettes. The SRBC are in sheets and leukocytes are arranged in a manner that does not permit visualization of SRBC between the nucleated cells.

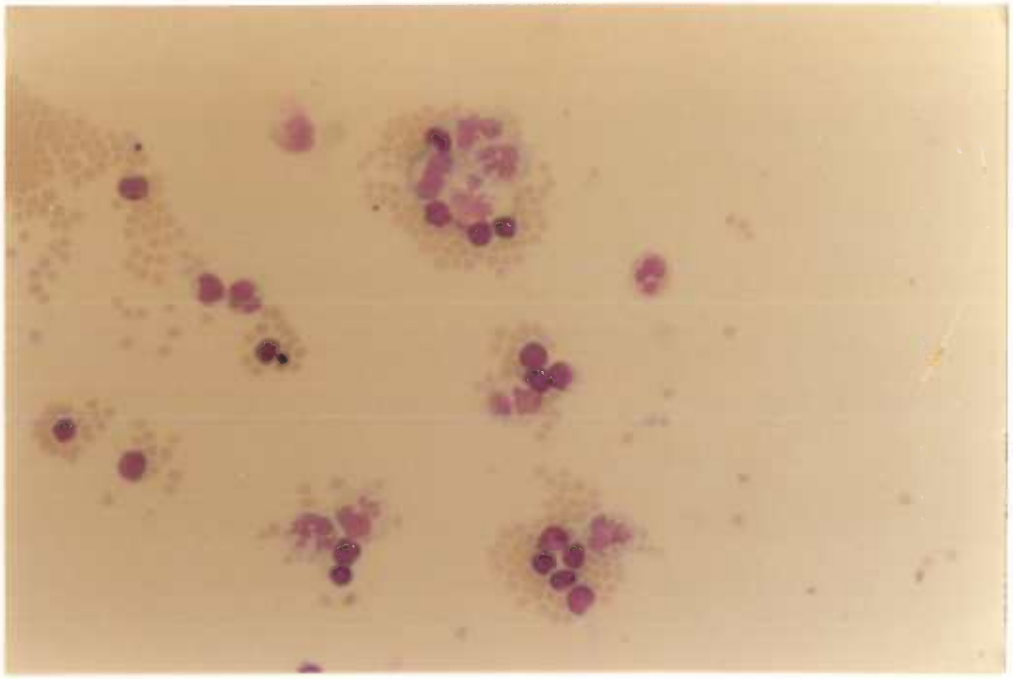

figure 4

FIGURE 5: Wright's-stained preparation of malignant cells prepared by air-drying a drop of cell suspension. Morphologic identification of cells is not possible.

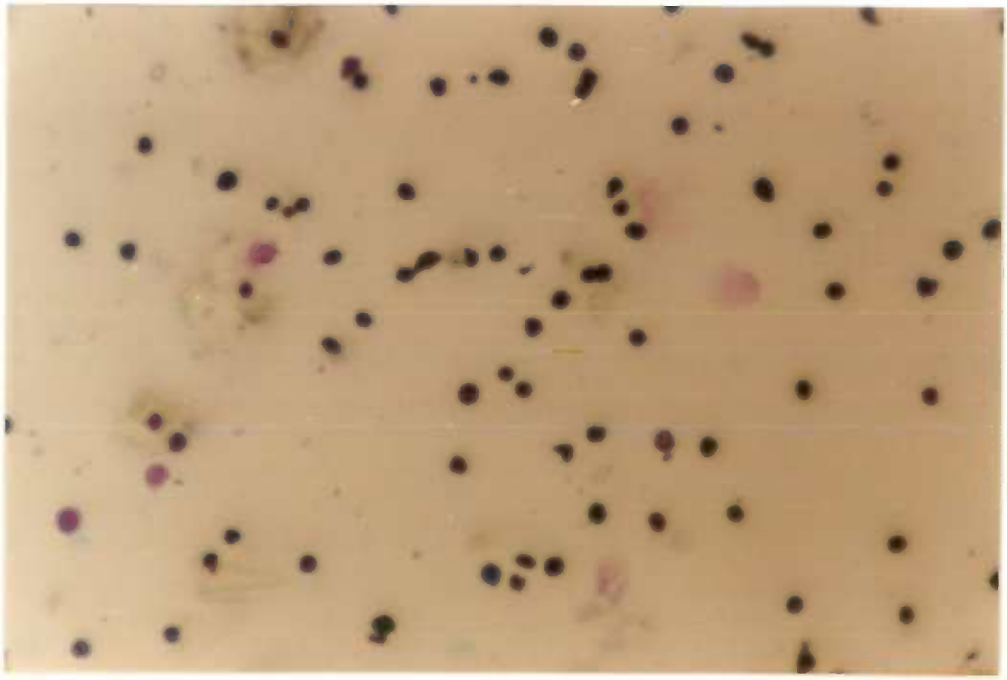

figure 5

FIGURE 6: Histogram illustrating the lymphocyte recovery distribution of 36 normal blood samples. The majority of lymphocyte recoveries (56.3%) were less than 35%.

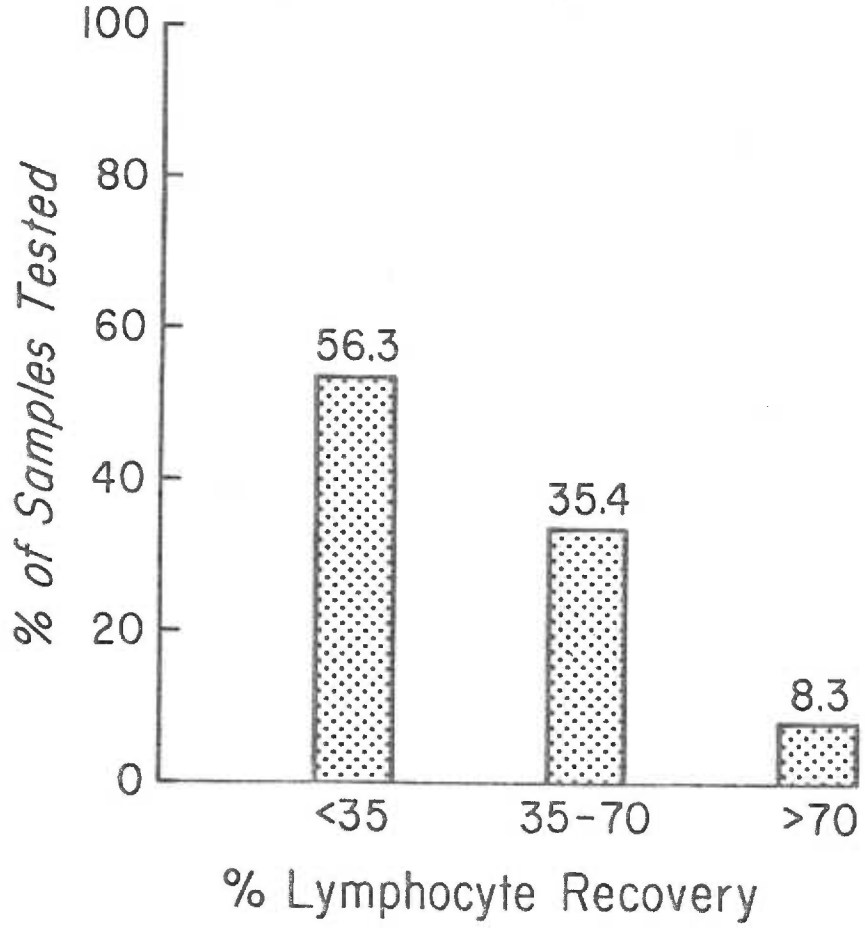
100
80
60
40
20
0
% of Samples Tested
56.3
35.4
8.3
<35
35-70
>70
% Lymphocyte Recovery

FIGURE 7: Comparison of percent rosettes in cell suspension with and without trypan blue on 39 normal blood samples. The mean cell suspension rosettes with trypan blue was 68.6+9.9%, and without trypan blue the mean was 68.9+7.9%. Differences in means $p>0.7$, correlation, $r=0.71$, $p<0.001$.

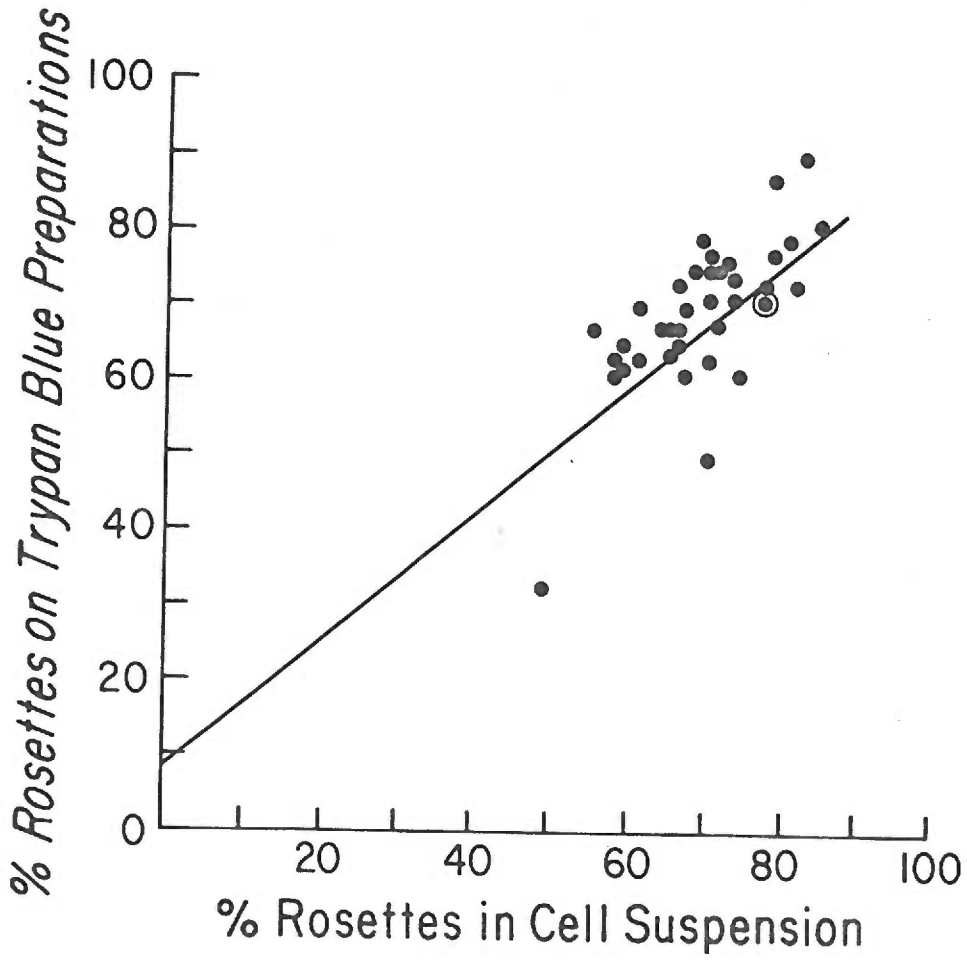
% Rosettes on Trypan Blue Preparations
100
80
60
40
20
0
20
40
60
80
100
% Rosettes in Cell Suspension

FIGURE 8: Comparison of percent rosettes in cell suspension and on cytocentrifuge preparations on 32 normal bloods. The mean cell suspension rosettes was 69.1±8.1% and on cytocentrifuge the mean was 56.6±16.6%. Differences in means $p<0.001$, correlation $r=0.56$, $p<0.001$.

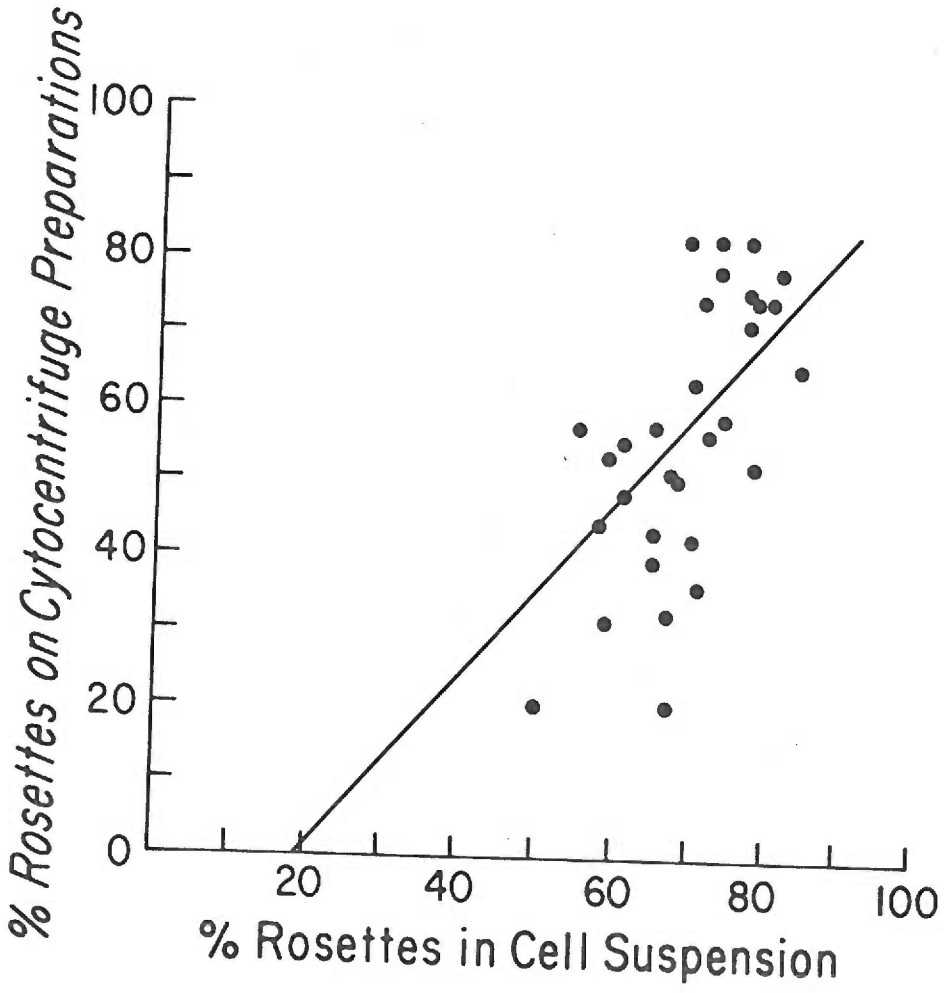
% Rosettes on Cytocentrifuge Preparations
100
80
60
40
20
0
20
40
60
80
100
% Rosettes in Cell Suspension

FIGURE 9: Comparison of percent rosetted lymphocytes in cell suspension and on cytocentrifuge preparations on 32 normal bloods. The mean cell suspension rosettes was 69.1+8.1% and on cytocentrifuge the mean rosetted lymphocytes was 67.94+13.0%. Difference in means $p > 0.5$, correlation $r=0.60$, $p < 0.001$.

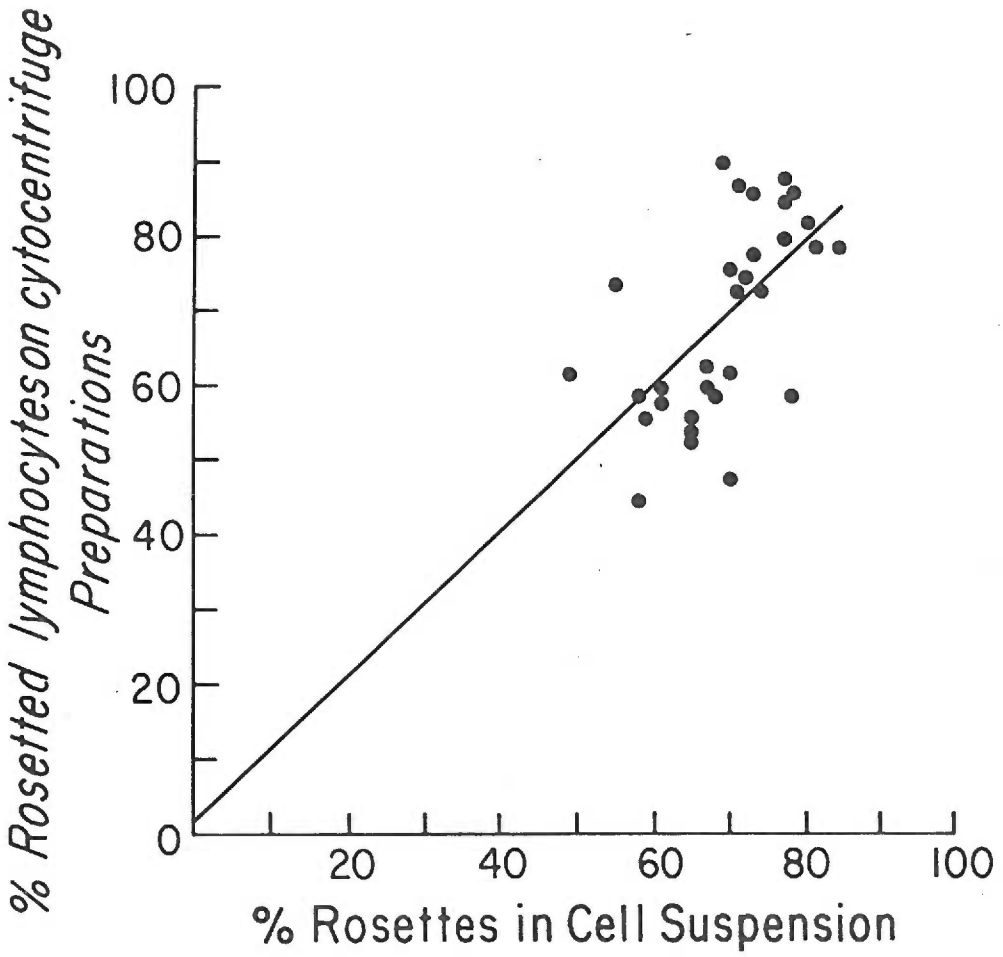
% Rosetted lymphocytes on cytocentrifuge Preparations
100
80
60
40
20
0
20
40
60
80
100
% Rosettes in Cell Suspension

FIGURE 10: Rosette cytocentrifuge preparation illustrating four rosetted mature lymphocytes.

FIGURE 11: Rosette cytocentrifuge preparation illustrating a rosetted malignant cell.

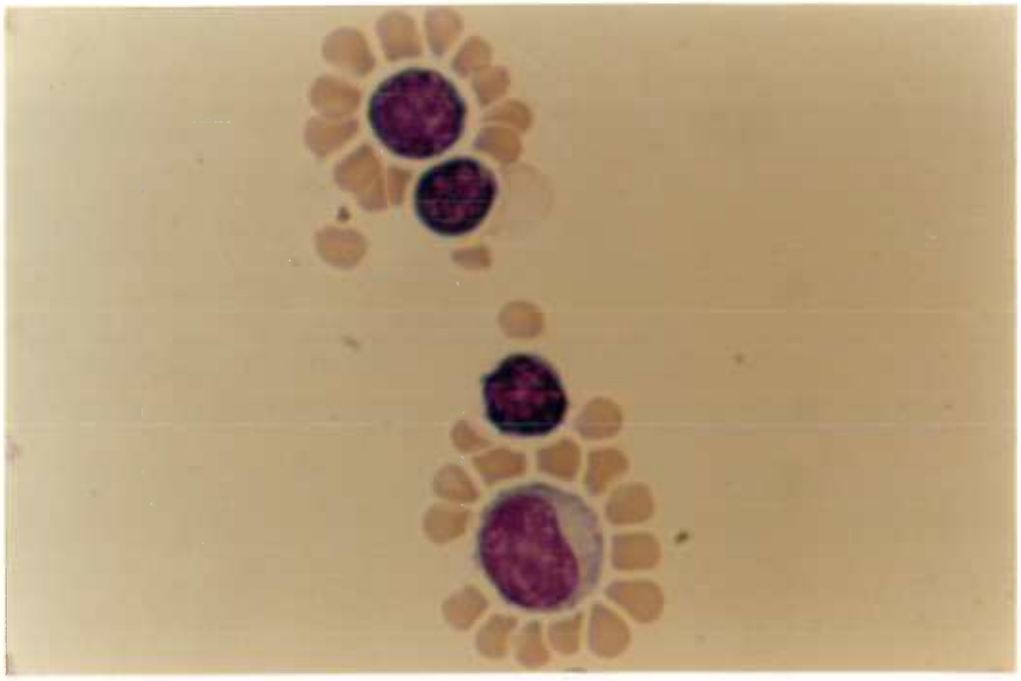

figure 10

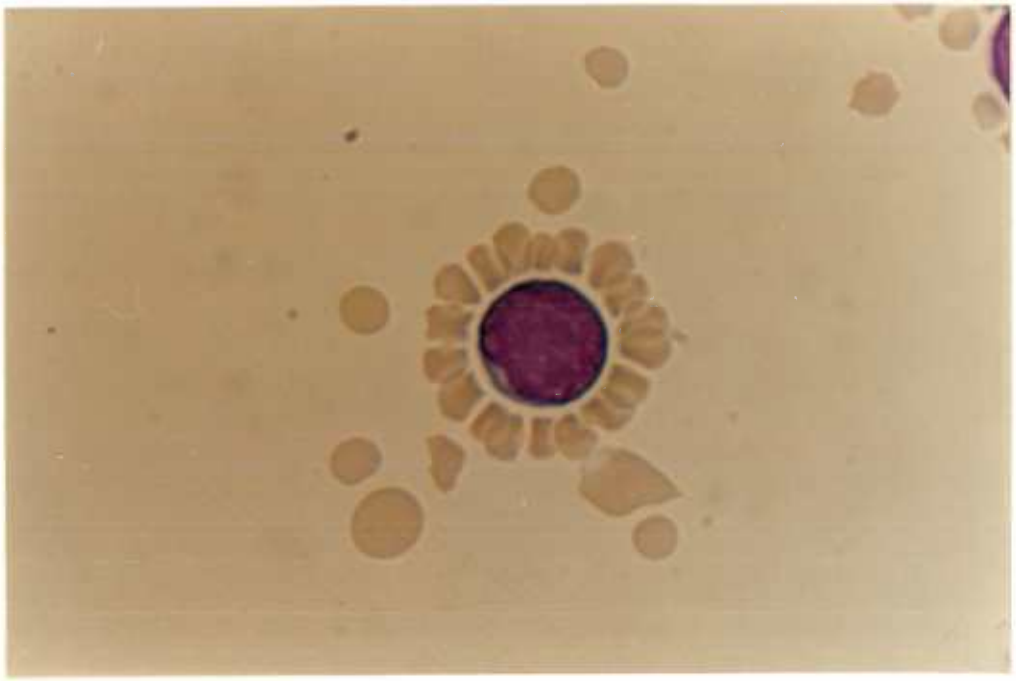

figure 11

FIGURE 12: The percent T lymphocytes by rosetting our four patients studied sequentially during pulse chemotherapy. Day 0 sample was before initiation of chemotherapy. Drugs were administered on Day 0.

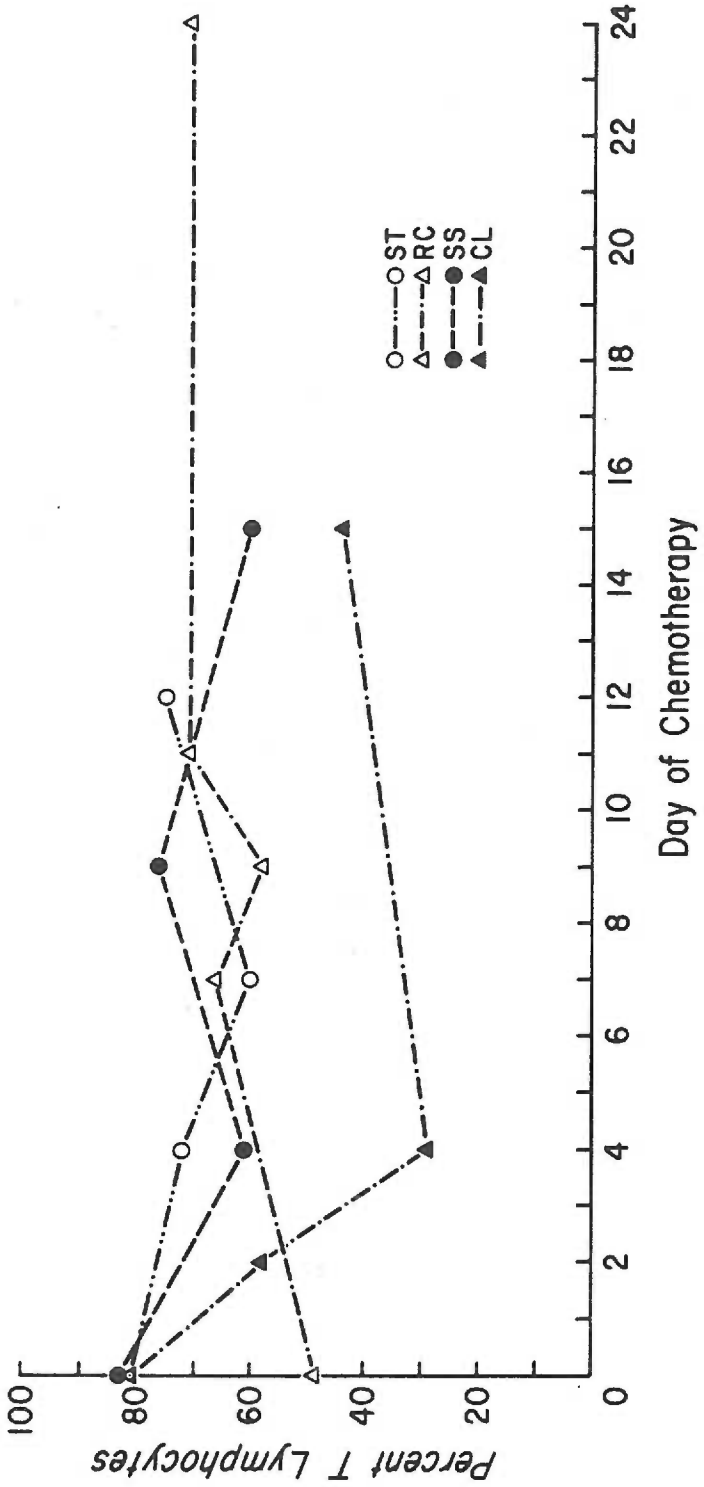
Percent T Lymphocytes
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
22
24
Day of Chemotherapy
ST
RC
SS
CL

FIGURE 13: Comparison of percent rosettes in cell suspensions with and without trypan blue on 23 pulse chemotherapy patients. The mean cell suspension rosettes with trypan blue was 69.0±13.4%, and without trypan blue the mean was 68.1±13.0%. Difference in means, $p>0.6$. Correlation $r=0.90$, $p<0.001$.

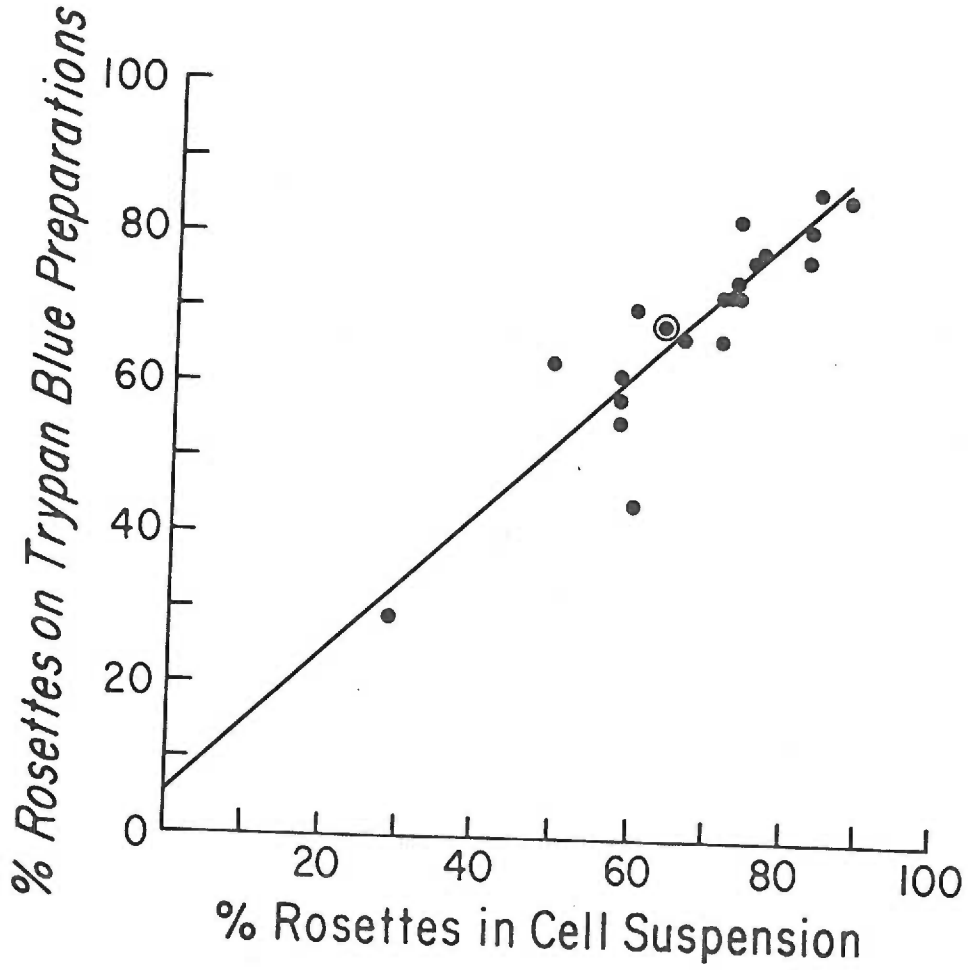
% Rosettes on Trypan Blue Preparations
100
80
60
40
20
0
20
40
60
80
100
% Rosettes in Cell Suspension

FIGURE 14: Histogram illustrating the lymphocyte recovery distribution of 22 pulse chemotherapy patient blood samples. The majority of lymphocyte recoveries (69.7%) were between 35 and 70%.

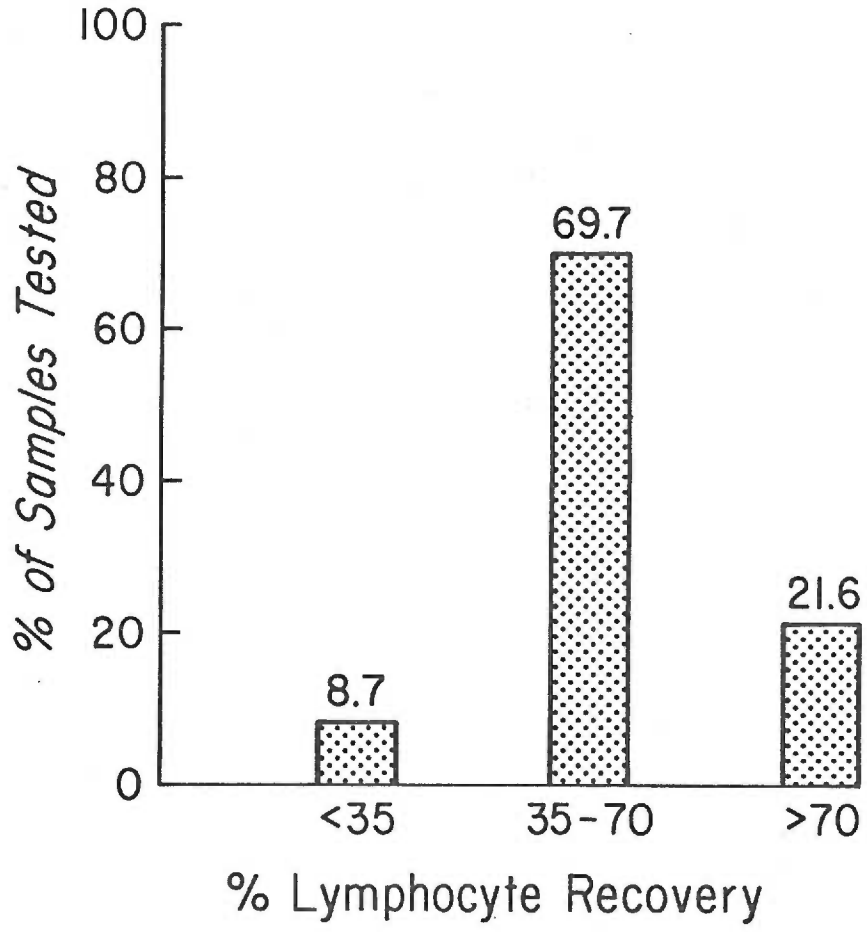
100
80
60
40
20
0
% of Samples Tested
69.7
21.6
8.7
<35
35-70
>70
% Lymphocyte Recovery

FIGURE 15: Comparison of percent rosettes in cell suspension and on cytocentrifuge preparations on blood from 22 pulse chemotherapy patients. The mean rosettes in cell suspension was 68.3±13.3%, and on cytocentrifuge preparations 44.9±18.2%. Differences in means $p<0.001$, correlation, $r=0.60$, $p<0.001$.

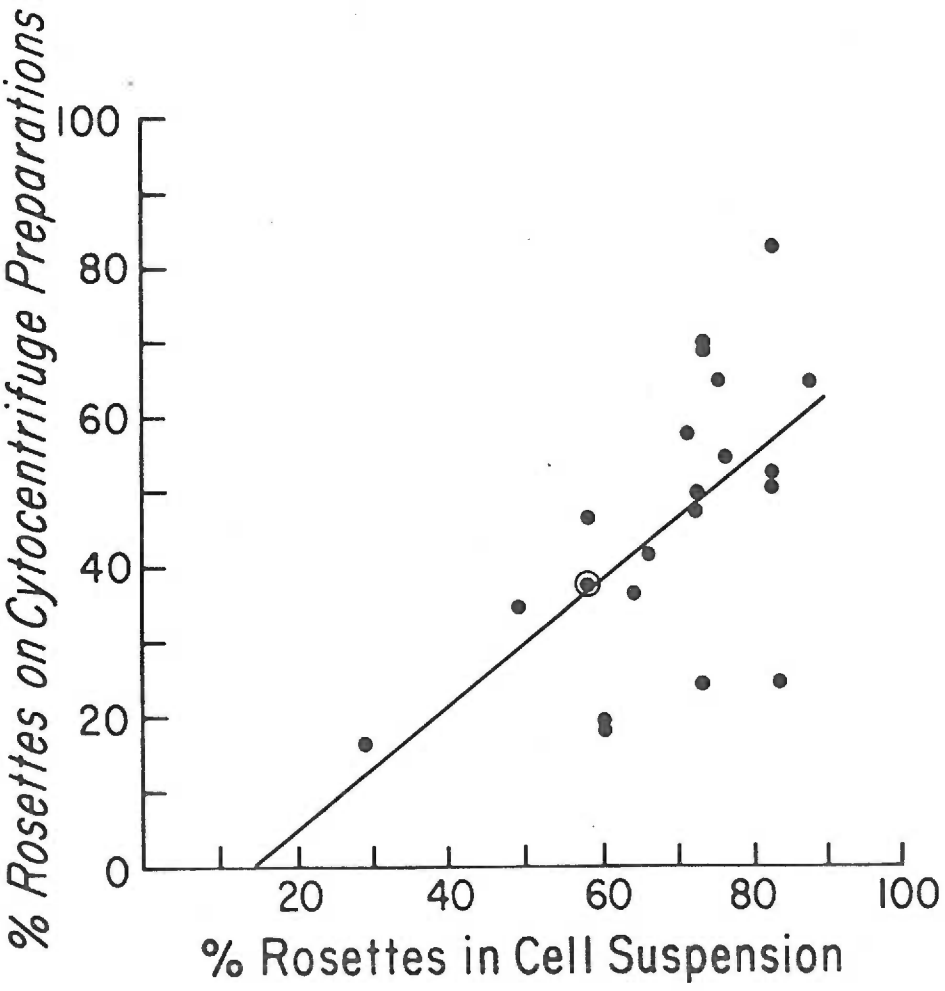
% Rosettes on Cytocentrifuge Preparations
100
80
60
40
20
0
20
40
60
80
100
% Rosettes in Cell Suspension

FIGURE 16: Comparison of percent rosetted lymphocytes in cell suspension and on cytocentrifuge preparations on blood samples from 22 pulse chemotherapy patients. The mean cell suspension rosettes was 68.2±13.3% and on cytocentrifuge the mean rosetted lymphocytes was 64.1±16.2%. Differences in means $p > 0.2$, correlation $r = 0.35$, $p < 0.05$.

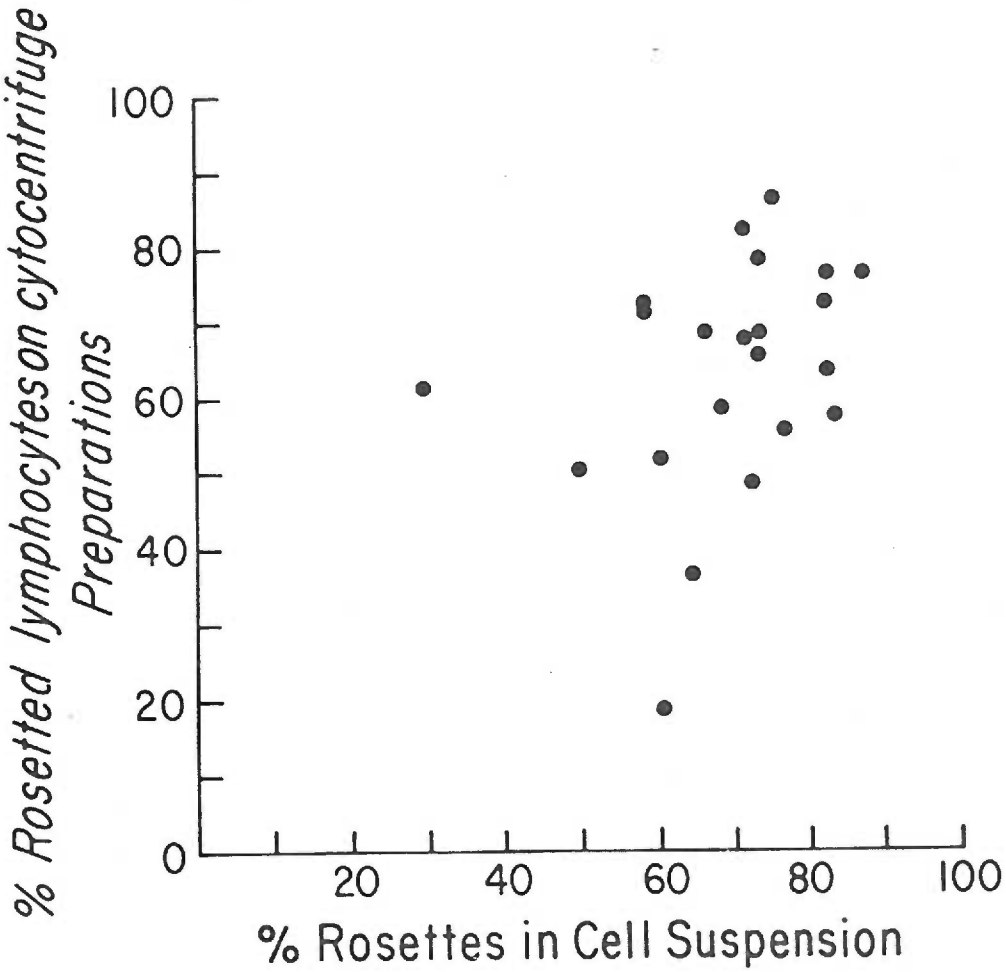
% Rosetted lymphocytes on cytocentrifuge Preparations
100
80
60
40
20
0
20
40
60
80
100
% Rosettes in Cell Suspension

FIGURE 17: Blood rosette cytocentrifuge preparation illustrating rosette-like monocytes.

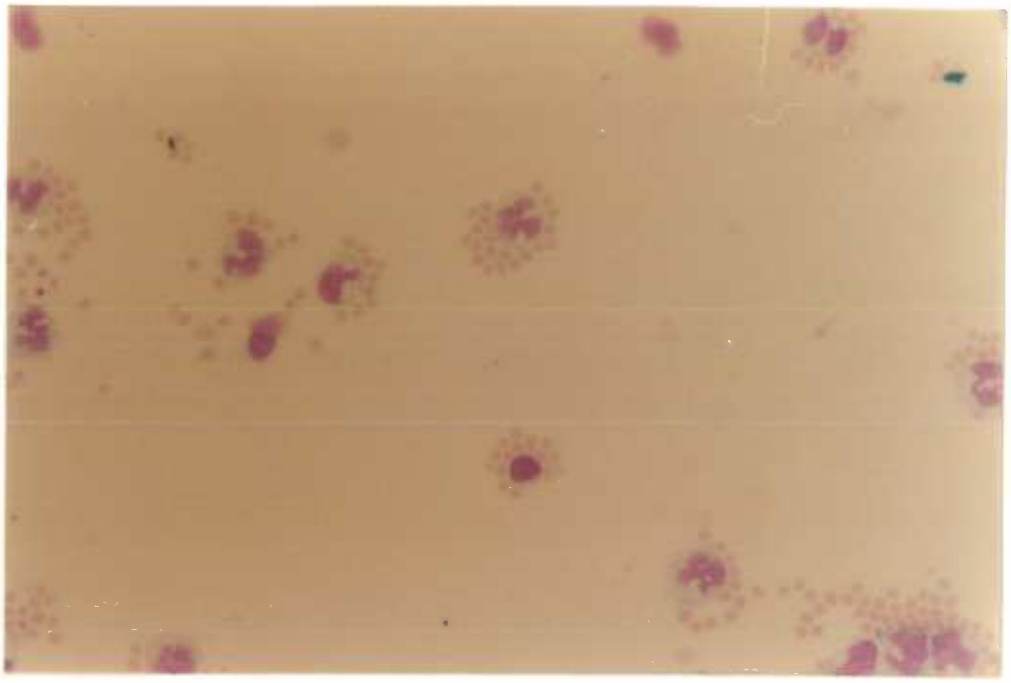

figure 17