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Abstract

COMPARISON OF TWO TECHNIQUES FOR ENUMERATION
OF HUMAN B AND T LYMPHOCYTES

by

William F. Forsythe, Jr.

The assay of Bursa-derived and Thymus-derived lymphocytes (B and T cells) in man has become increasingly useful during the past few years in normal and in clinical conditions. There has been quite a range reported in the literature in the percent of B and T cells in normal subjects by different investigators.

The purpose of this study was to see if monocyte contamination was one cause of non-specific rosette formation. Twenty-one subjects were used to compare the technique involving trypsin and neuraminidase treatment of erythrocytes and the technique of glass wool removal of monocytes. A peroxidase stain was used to confirm monocytes on ten of the twenty-one subjects and a fluorescent antibody stain was done on four subjects to confirm the EAC rosettes as B cells.

There was a definite decrease in the number of monocytes after glass wool column separation. This led to a slight increase in the number of T cells and a slight decrease of B cells.

A method is described which will help eliminate more of the monocytes and give a more accurate value of B and T cells.

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COMPARISON OF TWO TECHNIQUES FOR ENUMERATION OF HUMAN B AND T LYMPHOCYTES

by

William F. Forsythe, Jr.

A Thesis in Partial Fulfillment of the Requirements for the Degree Master of Science in the Field of Microbiology

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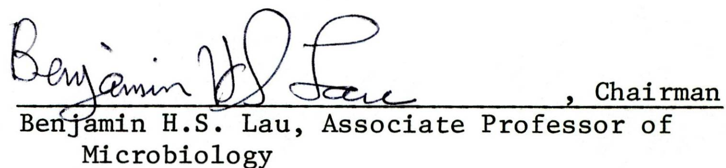
June 1976

Each person whose signature appears below certifies that this thesis in his opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.



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INTRODUCTION

The assay of Bursa-derived and Thymus-derived lymphocytes (B and T cells) in man has become increasingly useful during the past few years in normal and in clinical conditions. By differentiating and enumerating the B and T cells, it provides considerable help in differentiating malignant from benign lymphoproliferative diseases, in documenting certain immunodeficiency disorders, and in differentiating benign monoclonal gammopathy from multiple myeloma (14).

The technique to differentiate B and T cells has not been standardized yet. Several methods have been used by various workers. Using erythrocyte rosette forming cell (E-RFC) technique, Fröland reported 19.1% T cells and 13% B cells (5), Jondal, Holm, and Wigzell showed 67.4% T cells and 30.8% B cells (10), while Stjernswärd et al. found 60% T cells and 32% B cells in normal subjects (13). Bobrove et al. used the cytotoxicity method and found 65% T cells and 21% B cells in normal subjects (2). Williams et al. used immunofluorescence technique and showed 75% T cells and 21.6% B cells in normal subjects (16). There is quite a range in the percent ratio of T and B cells between the different methods and even between the same methods performed by different investigators.

Some of the reasons for this variation may be the binding of rosettes due to non-specific receptors on the lymphocyte cell surface. The lymphocytes purified on the Ficoll-Hypaque gradient (14) are contami-

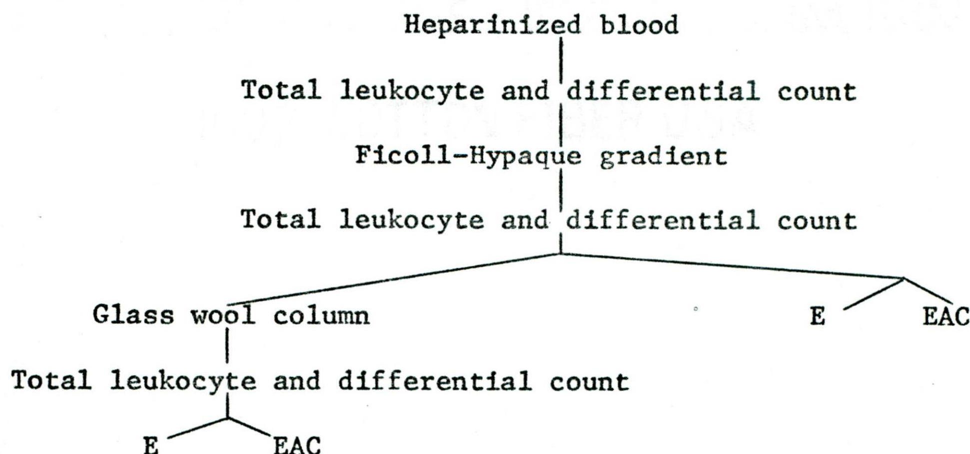
nated by a variable number of monocytes which may give a non-specific rosette formation and give a falsely high value for B cells.

The purpose of this study was to compare two techniques to determine E-RFC, namely, the technique of Weiner, Bianco, and Nussenzweig involving trypsin and neuraminidase treatment of erythrocytes (15) as compared to the technique of glass wool column removal of monocytes by adsorption (9).

EXPERIMENTAL DESIGN

Heparinized blood from twenty-one normal subjects was used to compare two techniques to quantitate T and B cells. Total leukocyte and differential counts were done on whole blood, after Ficoll-Hypaque separation, and after monocyte separation to obtain the total leukocyte count and lymphocyte and monocyte counts.

The whole blood was diluted in saline, carefully layered onto Ficoll-Hypaque gradient and centrifuged. Lymphocytes from the cell suspension were divided into equal portions. One ml was used to determine erythrocyte (E) and erythrocyte-antibody-complement (EAC) rosettes while one ml was first passed through glass wool and then E and EAC rosettes were determined. The procedure was done according to the scheme below:



In addition to the above techniques a peroxidase stain to confirm monocytes was done on ten of the twenty-one subjects and a fluorescent antibody stain was done on four subjects to confirm the EAC rosettes as B cells.

MATERIALS AND METHODS

Subjects

Thirteen male and eight female normal subjects, ranging in age from 22 to 55 years old were used in this study.

Total Leukocyte and Differential Count

The total leukocyte count was obtained by using a MK-2S/400 Haema-count (General Science Corp., Bridgeport, Conn.). A differential count was performed on smears stained with Hemal-stain (Danbury, Conn.).

Separation of Lymphocytes

Two ml heparinized blood was mixed with 6 ml of normal saline (0.85%). The diluted blood was layered onto 3 ml of Ficoll-Hypaque (3) (24 parts of 9% Ficoll, Sigma Chem., St. Louis, Mo., to 10 parts of 33.9% Hypaque, Winthrop Lab., N.Y.). The mixture was centrifuged at 400 x g for 40 minutes at room temperature. The lymphocytes were aspirated from the interface, washed three times in normal saline at 200 x g for 10 minutes and adjusted to a final concentration of 10^6 cells/ml in saline. Trypan blue was used to assess viability.

Enumeration of T and B Lymphocytes

T and B cells were quantitated by the rosette technique of Weiner, Bianco, and Nussenzweig using neuraminidase-treated sheep erythrocytes (SRBC) to enhance T cell rosettes and trypsin-treated SRBC to prevent spontaneous B cell rosettes (15). T cells have the capacity to form

erythrocyte (E) rosettes spontaneously when mixed with sheep red blood cells (SRBC). Since B cells have receptors for complement on their cell surface, they form erythrocyte-antibody-complement (EAC) rosettes when mixed with EAC indicator cells.

E rosettes. Neuraminidase (*Vibrio cholerae*, Calbiochem, LaJolla, Calif.) one unit/ml, pH 6.5 in 0.2 ml was added to each ml of 5% SRBC in Hank's Balanced Salt Solution (HBSS) and incubated at 37 C for one hour. The SRBC were then washed twice with HBSS and adjusted to a final concentration of 1% in HBSS. One-fourth ml of neuraminidase-treated SRBC and an equal volume of lymphocytes were mixed and incubated 15 minutes at 37 C. The mixture was then centrifuged at 200 x g for 10 minutes and incubated at 4 C for 30 minutes. Two hundred lymphocytes were counted and the number of lymphocytes with three or more SRBC attached were recorded.

EAC rosettes. To prepare the EAC indicator system, an equal volume of 5% SRBC in HBSS and trypsin (Calbiochem, LaJolla, Calif.) 2 mg/ml in HBSS were incubated at 37 C for 1 hour. The cells were then washed twice with normal saline and reconstituted to a final concentration of 5% in saline. An equal volume of sheep cell hemolysin, 1:2000 in saline (Hyland, Costa Mesa, Calif.) was then added, and the mixture was allowed to incubate 30 minutes at 37 C. The sensitized SRBC were washed three times with saline and adjusted to 5% in saline. An equal volume of guinea pig complement (Hyland, Costa Mesa, Calif.), 1:100 in saline was added. The mixture was incubated 30 minutes at

37 C, washed three times with saline and adjusted to a final concentration of 1% in saline. One-fourth ml of EAC indicator cells and 0.25 ml of lymphocytes were mixed, centrifuged 2 minutes at 200 x g and incubated 30 minutes at 37 C. After the cells were gently resuspended, 200 lymphocytes were counted and the number of lymphocytes with three or more SRBC were recorded.

Removal of Monocytes by Glass Wool Column

After Ficoll-Hypaque separation, monocytes were removed from the cell suspension by glass wool column (1,9). The glass wool column was prepared by packing 50 mg of glass wool (Pyrex brand wool, Corning Glass Works, Corning, N.Y.) in an 8 cm glass tube with an inside diameter of 4 mm. The cell suspension and the glass wool column were first warmed up for 30 minutes at 37 C incubation. One ml of the suspension was passed through the column. The column was rinsed with 10 ml of saline. The washings were centrifuged and resuspended in 2 ml of saline.

Peroxidase Stain for Monocytes

The peroxidase stain of Kaplow (11) was used to differentiate monocytes from lymphocytes. Benzidine dihydrochloride is used as the substrate for detecting intracellular peroxidase activity. Monocytes gave a positive reaction and showed blue granules in the cytoplasm while lymphocytes remained unstained.

Fluorescent Antibody Stain for B Cell Rosettes

Slides made from the pellet of the tube with EAC rosettes were

fixed in absolute methanol for 2 minutes. One-tenth ml of fluorescein conjugated polyvalent anti-human-globulin (anti-IgG, anti-IgA and anti-IgM, Microbiological Associates, Bethesda, Md.) was added to each slide. After incubation for 30 minutes in a moist chamber at room temperature, the slides were rinsed in phosphate buffered saline, then distilled water, and air-dried. They were then mounted with FA buffered glycerol and coverslipped. The slides were examined using a fluorescent microscope.

RESULTS

Total Leukocyte Counts

The mean total leukocyte counts at the three steps in the study are shown in Figure 1 and Table 1. The mean total leukocyte counts of the heparinized whole blood of 21 subjects was $5176/mm^3$ which is within normal limits. After separation by Ficoll-Hypaque gradient there was a significant decrease in the total leukocyte count ($3018/mm^3$) due to the elimination of granulocytes which left only lymphocytes and some monocytes. After passing through the glass wool column, there was a further decrease of the count ($665/mm^3$).

Lymphocyte and Monocyte Counts

The mean absolute lymphocyte and monocyte counts of the three types of specimens also are shown in Figure 1 and Table 1. There is a decrease in both lymphocytes and monocytes after Ficoll-Hypaque separation and a further decrease after passage through the glass wool.

The mean percentage of lymphocytes and monocytes on the three types of specimens are shown in Figure 2 and Table 1. The lymphocytes showed a definite increase after Ficoll-Hypaque separation due to the concentration. After passage through glass wool a further increase of lymphocytes occurred while the monocyte count was decreased.

T and B Lymphocytes

The mean percentage of T and B cells of the two types of specimens are shown in Figure 2 and Table 2. After glass wool separation the percentage of B cells decreased while the T cells showed an increase.

Figure 1. Mean Absolute Leukocyte, Lymphocyte, and Monocyte Counts of 21 Subjects

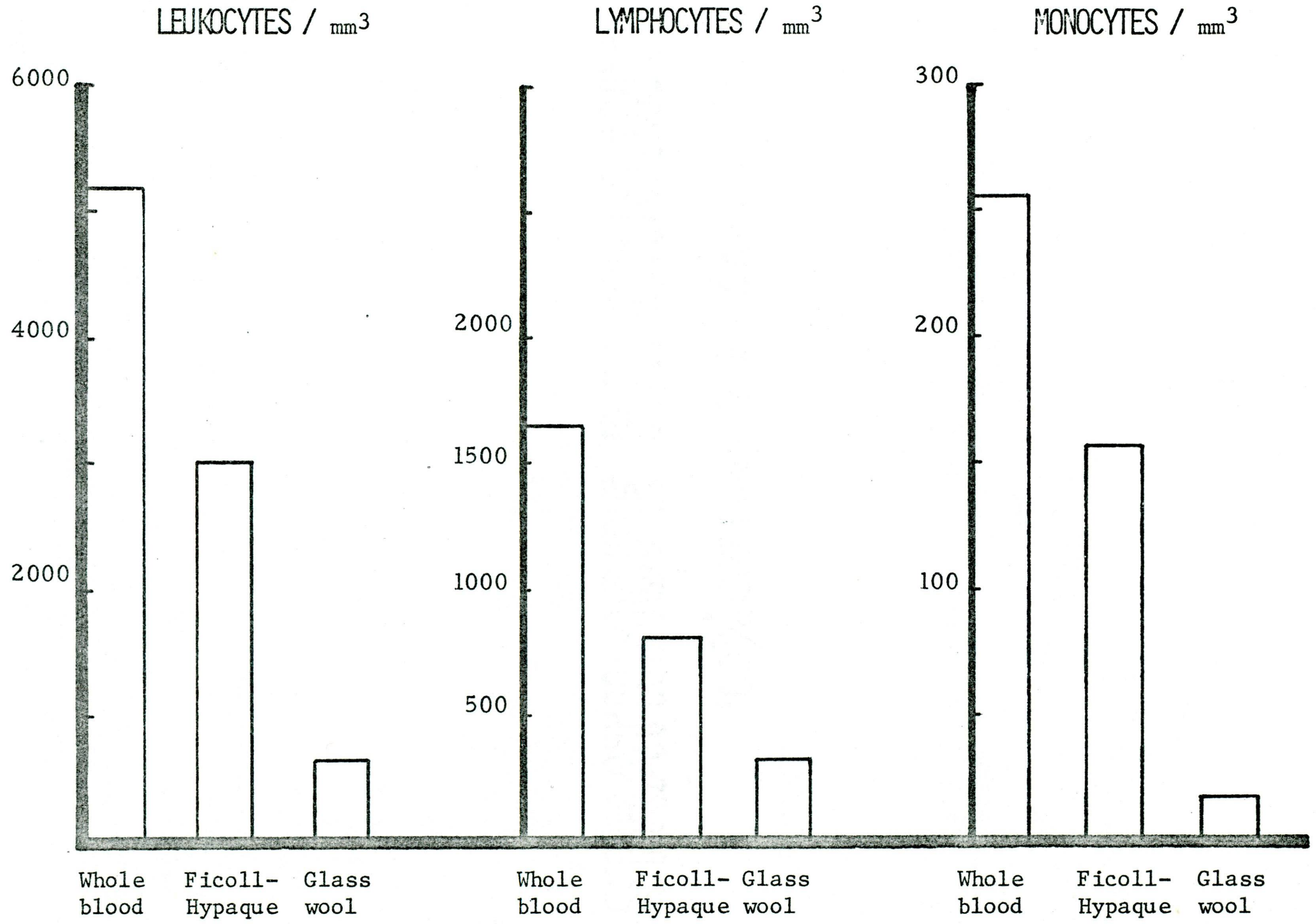

LEUKOCYTES / mm^3
6000
4000
2000
Whole blood
Ficoll-Hypaque
Glass wool
LYMPHOCYTES / mm^3
2000
1500
1000
500
Whole blood
Ficoll-Hypaque
Glass wool
MONOCYTES / mm^3
300
200
100
Whole blood
Ficoll-Hypaque
Glass wool

Table 1

Mean Total Leukocytes, Percent and Absolute Lymphocytes and Monocytes of 21 Subjects

Specimen[a]	Total Leukocytes /mm^3	% Lymphocytes	Lymphocytes /mm^3	% Monocytes	Monocytes /mm^3
Whole Blood	5176	32	1656	5	260
	(3100-8300)[b]	(16-52)	(578-2800)	(1-11)	(55-615)
Ficoll-Hypaque Separation	3018	82	809	17	158
	(1400-7400)	(61-90)	(396-2168)	(9-39)	(51-312)
Glass Wool	665	94	327	6	19
	(150-1850)	(87-98)	(94-1183)	(2-12)	(5-49)

a. Three types of specimens were compared: whole blood from subjects, cell suspension after Ficoll-Hypaque separation, and cell suspension after glass wool separation.

b. Range of values.

Figure 2. Comparison of the Percentage of Lymphocytes from Hemal-stain and of T and B Lymphocytes After Ficoll-Hypaque Separation and After Glass Wool Separation

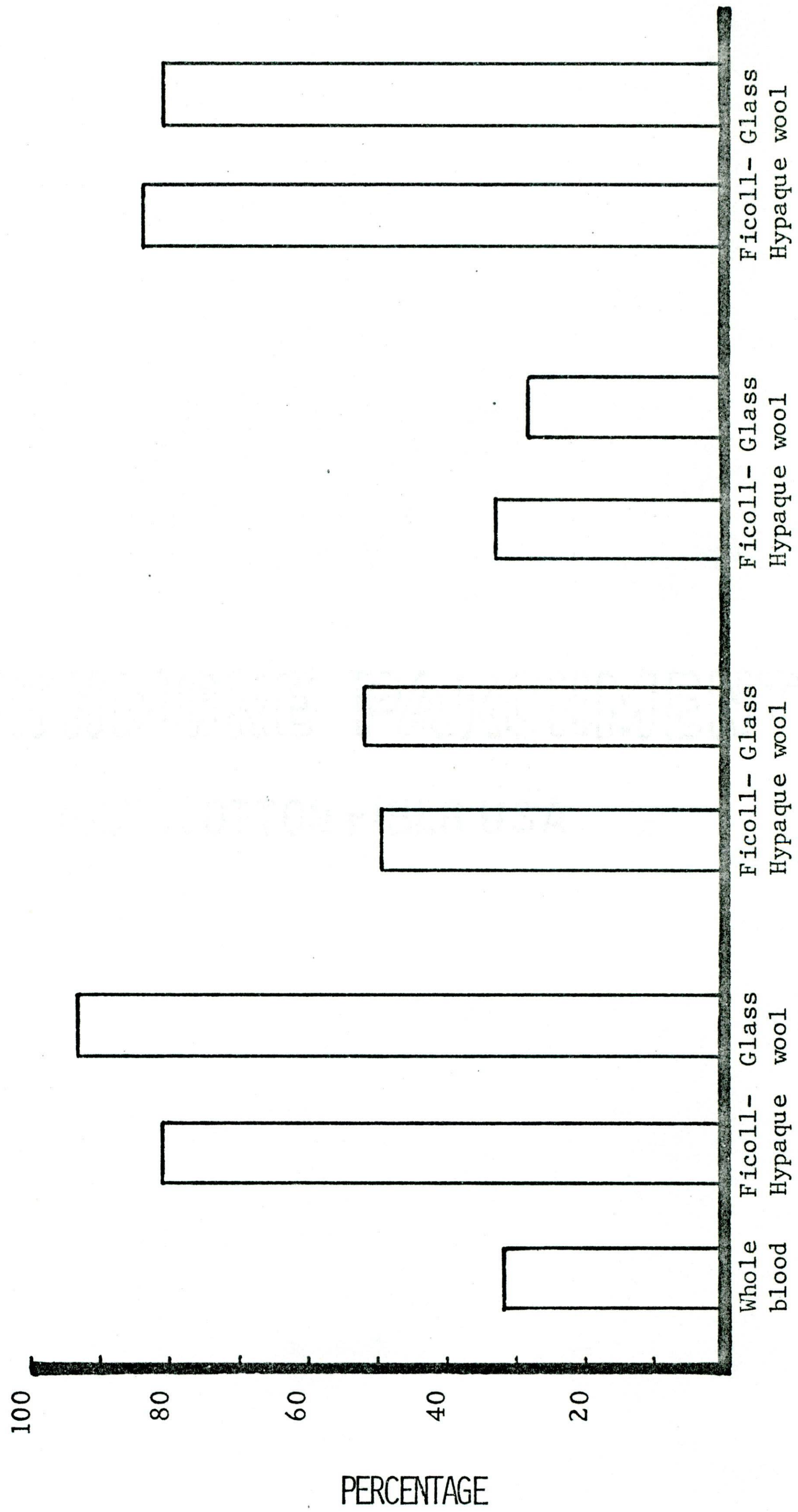
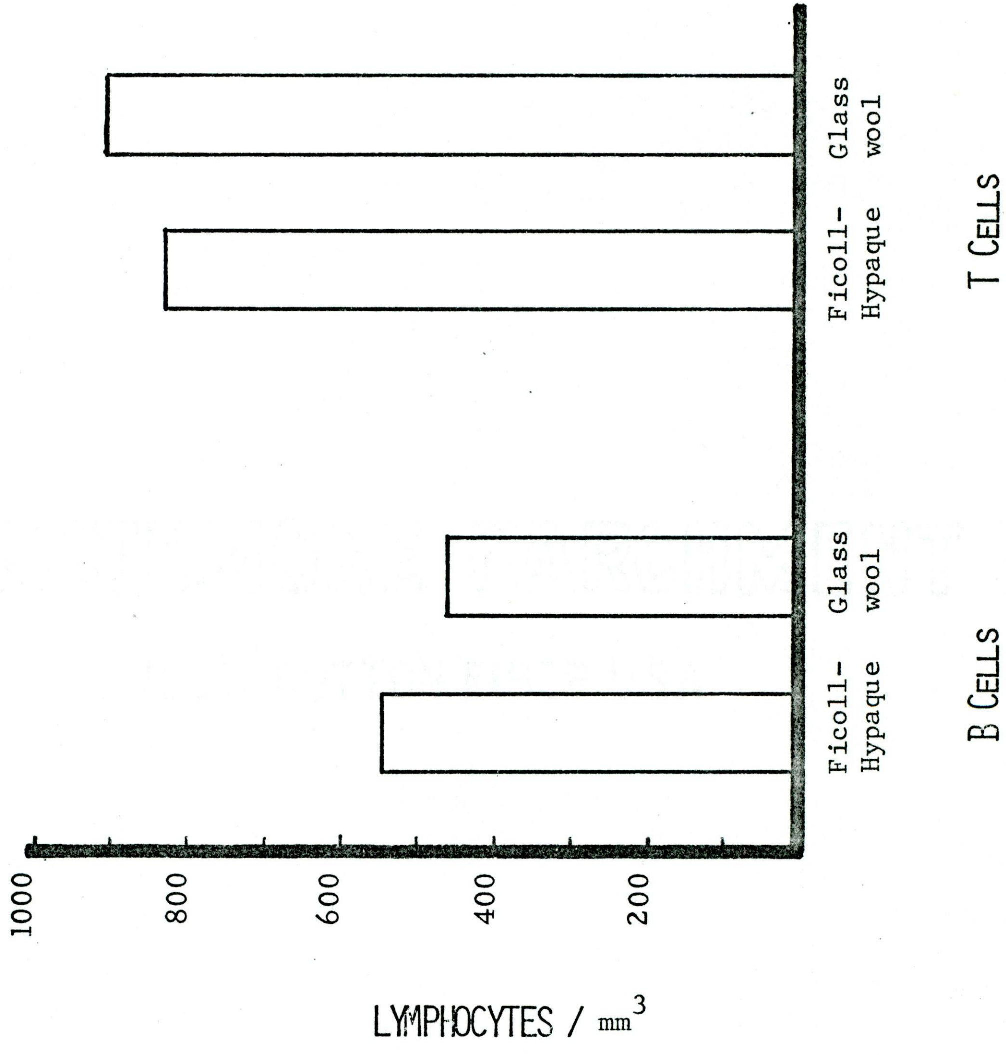

PERCENTAGE
100
80
60
40
20
Whole blood
Ficoll- Hypaque
Glass wool
LYMPHOCYTES IN HEMAL-STAIN
Ficoll- Hypaque
Glass wool
T CELLS
Ficoll- Hypaque
Glass wool
B CELLS
Ficoll- Hypaque
Glass wool
SUM OF T AND B CELLS

Figure 3. Mean Absolute T and B Lymphocytes After Ficoll-Hypaque Separation and After Glass Wool Separation



LYMPHOCYTES / mm3
1000
800
600
400
200
Ficoll-
Hypaque
Glass
wool
B CELLS
Ficoll-
Hypaque
Glass
wool
T CELLS

Table 2

Percentage and Absolute T and B Lymphocyte Counts of 21 Subjects

Specimen[a]	% T Cell	% B Cell	T Cell/mm^3	B cell/mm^3	Sum of % T + % B	Sum of Absolute T and B
Ficoll-Hypaque Separation	50[b] (15-65)	34 (14-60)	826 (153-1681)	549 (119-901)	85 (42-112)	1376 (428-2184)
Glass Wool	53 (34-80)	29 (12-56)	908 (213-2204)	460 (112-965)	82 (63-100)	1366 (450-2755)

a. Two types of specimens were compared: cell suspension after Ficoll-Hypaque separation, and cell suspension after glass wool separation.

b. Figures represent mean of 21 subjects: numbers in parenthesis give the ranges.

The absolute value of each agreed with the percentage respectively. The sum of the percent T and B cells was slightly higher after Ficoll-Hypaque separation than after passage through glass wool.

Peroxidase Stain for Monocytes

The mean percentage of monocytes after Ficoll-Hypaque separation and after glass wool separation on ten subjects stained by two different methods is shown in Table 3. After Ficoll-Hypaque separation the mean percent of monocytes using the Peroxidase stain was 19% while the Hemal-stain showed 15%. There was a significant decrease in the percent of monocytes after glass wool column separation with the two stains showing 6% and 5% respectively.

Fluorescent Antibody Stain for B Cells

The mean percentage of B cells on four subjects using two different methods is shown in Table 4. The mean percent of B cells by the fluorescent antibody method was 26% while the EAC rosette method gave 30%.

Table 3

Comparison of % Monocytes using Peroxidase Stain and Hemal-stain on Ten Subjects

Specimen[a]	Peroxidase stain	Hemal-stain
Ficoll-Hypaque Separation	19[b] (12-30)	15 (10-22)
Glass Wool	6 (4-10)	5 (3-8)

a. Two types of specimens were compared: cell suspension after Ficoll-Hypaque separation, and cell suspension after glass wool separation.

b. Figures represent mean of ten subjects: numbers in parenthesis give the ranges.

Table 4

Comparison of % B Cells on Four Subjects Using FA Technique and EAC Rosette Technique

FA	EAC
26[a]	30
(23-30)	(27-38)

a. Figures represent mean of four subjects: numbers in parenthesis give the ranges.

DISCUSSION

The mean total leukocyte count from whole blood was $5176/mm^3$. After Ficoll-Hypaque separation, the count decreased due to elimination of granulocytes from the cell suspension. This left lymphocytes and some monocytes in the cell suspension. After Ficoll-Hypaque separation the percentage of lymphocytes increased from 32% to 82% due to the concentration process. The percentage of monocytes also increased due to the fact that only lymphocytes and monocytes were left. After passage through glass wool the total leukocyte count was further decreased, but the percentage of lymphocytes increased slightly due to a decrease of monocytes during the procedure. The purpose was to eliminate monocytes and Table 1 shows that after Ficoll-Hypaque separation there were 17% monocytes which decreased to 6% after glass wool passage. There were still some monocytes left, but the number was greatly reduced.

The method of Weiner, Bianco, and Nussenzweig (15) was used in this study to determine E-RFC because by using neuraminidase-treated SRBC to enhance T cell rosettes and trypsin-treated SRBC to prevent spontaneous B cell rosettes the method was more specific.

After Ficoll-Hypaque separation the E-RFC showed 34% B cells and 50% T cells (Table 2). After glass wool separation the B cells decreased to 29% and the T cells increased to 53%. The higher percentage of B cells after Ficoll-Hypaque separation was due to monocyte rosettes. Other investigators have also found this to be true (6,7,8). After elimination of monocytes the percentage of B cell rosettes decreased due to fewer monocytes. The percentage of T cells increased after glass wool

separation. With fewer monocytes in the cell suspension, more cells could be counted to give a more accurate value.

The peroxidase stain of Kaplow (11) was used as a confirmatory stain for monocytes. Ten subjects were tested with both Hemal-stain and the peroxidase stain as shown in Table 3. The peroxidase stain showed a slightly higher percentage of monocytes than did the Hemal-stain and confirmed the cells as indeed being monocytes. Some of the small monocytes must have been counted as lymphocytes on the Hemal-stain.

The fluorescent antibody stain was done on four subjects to confirm the EAC rosettes as B cells (4,6,12). Table 4 shows a mean of 26% B cells using fluorescein conjugated polyvalent anti-human-globulin and 30% B cells with EAC method. The FA stain did confirm the EAC rosettes as B cells. No fluorescence was seen with rosettes on a slide made from the pellet from T cell rosettes.

In conclusion the glass wool column separation is a very useful technique and might be used whenever the monocyte count is at all elevated. A person with a great increase of monocytes will give a falsely high B cell value. However, both T cell and B cell absolute counts are reduced as well as the monocyte count. The glass wool technique is not specific for monocytes. Other methods are available to separate monocytes, but some of these are very costly. The glass wool column can be used in any laboratory. One disadvantage with the glass wool column is that many lymphocytes are lost during the procedure as well as monocytes. More work needs to be done to standardize the procedure to make it even more efficient.

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