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PAPER IV

Ficoll-Isopaque Technique

Isolation of Mononuclear Cells and Granulocytes from Human Blood

Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g

A. BÖYUM*

When particles suspended in a liquid medium are subjected to a centrifugal field, their sedimentation rates can be calculated from the general sedimentation law (Paper I, p. 9) by substituting the centrifugal acceleration for the acceleration of gravity (de Duve & Berthet 1954, de Duve, Berthet & Beaufay 1959). During centrifugation at a constant speed the centrifugal acceleration and force increase linearly with the distance from the axis of rotation; in a 1 g gravity field the driving force remains constant. Otherwise, there is no principal difference between a centrifugal field and a 1 g gravity field. The general migration properties of blood cells can easily be studied at 1 g, thus providing general information which may be applied to centrifugal technique.

This paper describes a technique for isolation of mononuclear cells from whole blood by one centrifugation, and of granulocytes by a two-step procedure, combining centrifugation and sedimentation at 1 g. Previously (Paper III) the sedimentation characteristics of white blood cells were studied under the force of gravity alone, and these studies led to a technique for isolation of lymphocytes and granulocytes. In the present investigation, the sedimentation of blood cells in a centrifugal field correlated well with their sedimentation at 1 g. When anticoagulated blood was layered on top of a mixture of Isopaque and ficoll and centrifuged, the cellular elements were divided into two main fractions; granulocytes and erythrocytes sedimented to the bottom of the tube, while mononuclear cells, together with platelets, remained at the interface. The yield of mononuclear cells was almost 100 per cent, when the blood was diluted with saline before centrifugation. When mononuclear cells had been removed, the cell mass in the bottom of the tube was mixed with plasma and dextran, and the plasma layer containing the granulocytes was removed when erythrocytes had settled by sedimentation at 1 g.

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MATERIALS

All concentrations are given in grams (gm) of solute per 100 ml final solution.

Isopaque (Natrii N-methyl-3,5-diacetamido-2,4, 6-trijodbenzoas) was provided by Nyegaard & Co., Oslo, Norway. A stock solution of 33.9 per cent Isopaque (Ip), with density of 1.200 gm/ml, was used as described earlier (Paper I).

Ficoll a sucrose polymer was obtained from A.B. 'Pharmacia', Uppsala, Sweden, and aqueous solutions of 6, 7, 9 and 14 per cent were used.

Standard Ip-ficoll mixture. Ten parts Ip stock solution were mixed with 24 parts ficoll 9 per cent. This mixture was used throughout the study, except for the series presented in Tables V and VIII. The final ficoll concentration was 6.4 per cent. Relative viscosity = 3.85. Density = 1.077 gm/ml. Relative osmolarity = 1.12 plasma equivalents.

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Dextran 500 was obtained from A.B. 'Pharmacia', Uppsala, Sweden. Dextran, dissolved in 0.9 per cent NaCl, was used as a 4.5 per cent solution.

Ethylene-diamine-tetra-acetic-acid (*EDTA*) was used as 2.7, 4.5 and 10 per cent solutions, adjusted to pH 7.4 with NaOH. The relative osmolarities of these solutions were 1.0, 1.64 and 3.80 plasma equivalents, respectively.

Heparin was diluted with 0.9 per cent NaCl, to contain 200 I.U. per ml. A final heparin concentration of 10 I.U. per ml blood was used.

Staining fluid for white cell counting: Methylviolet 0.05 per cent, acetic acid 0.5 per cent, in distilled water.

Staining fluid for simultaneous white and red cell counting: Methylviolet 0.03 per cent in 0.9 per cent NaCl.

Siliconized glass tubes with inner diameter of 13 mm were used for centrifugation. Each tube contained 3 ml of the standard Ip-ficoll mixture.

Trypan blue was used as a 1 per cent solution in 0.9 per cent NaCl.

Particles for phagocytosis. One part yellow or black drawing ink ('Rotring', Riepe-Werk, Hamburg, Germany) was mixed with 9 parts 0.9 per cent NaCl.

METHODS

Density (gm/ml) was measured with a 10 ml pycnometer at room temperature (ca 22 °C).

Osmolarity was determined by the freezing point technique, using a Beckman thermometer and is expressed relative to the average osmolarity of plasma (or 0.9 per cent NaCl).

Relative viscosity was determined with an Ostwald viscosimeter at 20 °C and is expressed relative to the viscosity of distilled water.

Differential counting. Smears were stained with May-Grünwald and Giemsa solutions and 500 cells, or more, were counted in each smear.

Viability tests

Leucocytes were isolated from EDTA-blood. The supernatant was removed after centrifugation, and the cells were resuspended in heparin-plasma (20 I.U. per ml) when used for LE-tests or phagocytic tests, and in 0.9 per cent NaCl for trypan blue staining.

Trypan blue staining. A cell concentration of 3-5000 cells per c.mm was used. Three droplets of the cell suspension were mixed with one droplet of 1 per cent trypan blue, immediately after the cells had been suspended in saline. The number of stained cells, among 200 cells, were counted after 5 minutes.

Incubation with LE-serum. A volume of 0.2 ml of a cell suspension containing 3-5000 cells per c.mm was mixed with 0.5 ml of a positive LE-serum. After incubation for 40 minutes at 37 °C, the cells were spun down, smears were made and stained with May-Grünwald Giemsa. The percentage of cells that showed nuclear transformation to homogeneous LE-bodies was determined by counting 200 cells in each smear. Frozen cells (30 minutes at −34 °C) from the same individual were incubated similarly to serve as control, and more than 98 per cent of these cells showed nuclear changes.

Phagocytosis of LE-bodies by granulocytes. Mononuclear cells were frozen at −34 °C for 30 minutes, and were then used as antigenic material after thawing. The following procedure was used.

A volume of 0.4 ml of the mononuclear cells was incubated with 0.2 ml of a strongly positive LE-serum for 20 minutes at 37 °C. Thereafter either 0.1 or 0.05 ml of a suspension of autologous granulocytes was added. The average cell concentration in the mononuclear suspension was 3700 cells per c.mm, and the average ratio between mononuclear cells and granulocytes was 6 (4.5-7.0). After incubation for 40 minutes at 37 °C, the cells were spun down, smears were made and stained with May-Grünwald Giemsa. The percentage of granulocytes that had engulfed LE-bodies was determined by counting 200 granulocytes in each smear.

Phagocytosis of black or yellow ink particles. A volume of 0.3 ml of a suspension of granulocytes or mononuclear cells, with a cell concentration of about 5000 cells per c.mm was mixed with 3 droplets of black or yellow ink particles. After incubation for 40 minutes at 37 °C, the cells were

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minutes at 37 °C, the cells were spun down at 400 g for 5 minutes. The supernatant was removed, the cells resuspended in saline, and centrifuged once more. The cell button was finally suspended in heparin plasma, before smearing. The smears were stained with May-Grünwald Giemsa. Two hundred cells were counted in each smear to determine the percentage of cells that had engulfed ink particles. The yellow particles were very easily recognized within the cells. No attempt was made to determine the number of particles that had been ingested by each cell.

Centrifugation. A Christ-refrigerated centrifuge was used. The centrifuge was run for 40 minutes at 1550 rpm, giving 400 g at the interface. The temperature in the centrifuge rose from 18° to 21-22 °C during centrifugation. In one series (Table IV) the centrifuge was kept at 4 °C.

Presentation of results

Differential counts were made in smears from whole blood and from the top and bottom fractions after centrifugation. The contamination of erythrocytes among the mononuclear cells was calculated in per cent of the total number of white and red cells in the top fraction.

The lymphocyte yield is defined as the number of lymphocytes recovered from the top fraction, in per cent of the total number of lymphocytes in the tube. The monocyte yield is defined similarly. The granulocyte yield is defined as the number of granulocytes recovered from the bottom fraction, in per cent of the total number of granulocytes in the tube.

The yields of monocytes and lymphocytes were calculated by means of differential counts as described previously (Paper III). If granulocytes make up g_0 per cent and lymphocytes l_0 per cent of the leucocytes in whole blood, and the respective percentages of the same cell types are designated as g_1 and l_1 in the top fraction and as g_2 and l_2 in the bottom fraction, then the lymphocyte yield is given by the formula:

$$100 \cdot \frac{l_1}{l_0} \cdot \frac{g_2 - g_0}{g_2 - g_1}$$

The number of lymphocytes in the bottom fraction, in per cent of its total number in the tube, is derived from the formula:

$$100 \cdot \frac{l_2}{l_0} \cdot \frac{g_0 - g_1}{g_2 - g_1}$$

Similar expressions can be made for monocytes. When the recoveries of lymphocytes and monocytes are calculated for the top and the bottom fraction, the sum deviates more or less from one hundred, owing to errors in cell counting and for statistical reasons. The magnitude of the deviations is demonstrated in Table I, where the sum of recoveries from top and bottom fractions is presented uncorrected as total recovery. The deviations were smaller in all the other series, except in one where the total monocyte recovery was 122 per cent. The values were corrected finally, to make the sum equal to 100 per cent.

All Tables give average values of 10 separations, using blood from 10 persons.

Student's t-test was used for statistical calculations.

RESULTS

a) *Separation procedure. General observations*

The separation procedure is illustrated in Fig. 1. Diluted blood was applied on top of 3 ml Ip-ficoll mixture, keeping the pipette against the tube wall about 10 mm above the fluid meniscus. When the centrifugation was finished, the blood cells were separated into two fractions, a white layer consisting of mononuclear cells at the interface region and a bottom fraction containing erythrocytes and granulocytes. Some mixing and diffusion at the interface between plasma and Ip-ficoll took place during centrifugation, and the mononuclear cells often sedimented a few mm downwards into the Ip-ficoll mixture. The majority of platelets were found together with the mononuclear cells; a few sedimented downwards.

The plasma layer was clear and contained no cells. This layer was first removed down to about 5 mm above the white layer. Next, the white layer together with about half the volume of the Ip-ficoll

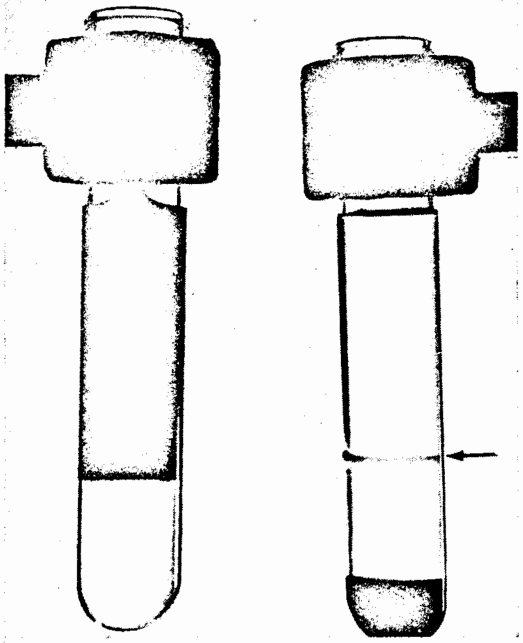

Fig. 1. Isolation of mononuclear cells by centrifugation at 20°C for 40 minutes at 400 g. A mixture of 2 ml EDTA-blood and 6 ml 0.9 per cent NaCl, was layered on top of 3 ml standard Ip-ficoll mixture (left), and centrifuged. After centrifugation, mononuclear cells and platelets were found on top of the Ip-ficoll mixture (right, see arrow); erythrocytes and granulocytes had sedimented to the bottom of the tube.

mixture was removed with a Pasteur pipette and centrifuged at 500 g for 5 minutes before making smears. Most of the mononuclear cells seemed to be located along the periphery of the tube, but to ensure a complete removal it was necessary to move the pipette over the whole cross area of the tube. Plasma was added to the bottom fraction, and smears were made.

b) *Varying the osmolarity of blood plasma*

Five ml blood were mixed with 1 ml EDTA 2.7 per cent in one tube and with 1 ml EDTA 4.5 per cent in another, giving osmolarities of 1.00 and 1.12, respectively. Two ml of each mixture were layered on top of the standard Ip-ficoll mixture and centrifuged simultaneously. The average differential counts and yields are given in Table I. There was no significant difference between the two series, except for a lower erythrocyte contamination ($0.02 < p < 0.05$) for the hyperosmotic blood-EDTA mixture (dilution B). The top fractions were almost completely devoid of granulocytes, and there was an overall tendency for lymphocytes, monocytes and basophils to move together. Monocytes were, however, withheld at the interface to a greater extent than lymphocytes. Fig. 2 shows mononuclear cells isolated by this technique.

c) *The effect of diluting blood with* 0.9 *per cent NaCl*

Previous experiments (Paper II) showed that, when erythrocytes in whole blood were clumped by dextran, some leucocytes were trapped in these clumps and therefore sedimented with the erythrocytes. This tendency was reduced by diluting the blood. If most of the erythrocytes were removed before the leucocytes sedimented through a gradient layer containing dextran (Paper III), the granulocytes tended to carry some lymphocytes downwards within aggregates of granulocytes, but this tendency was also reduced by diluting the blood. A similar mechanism could explain the rather high number of lymphocytes in the bottom fraction after centrifugation, and also why lymphocytes seemed to move faster than monocytes

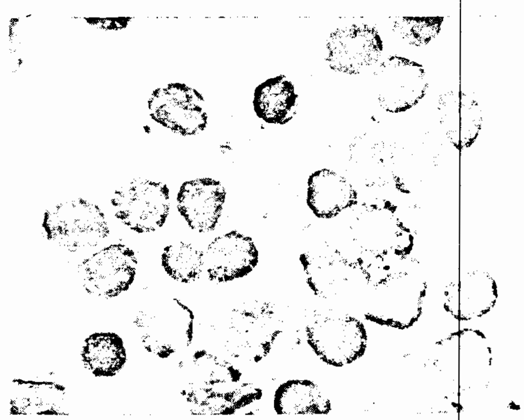

Fig. 2. Mononuclear cells isolated by centrifugation, as described in Fig. 1.

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Table I. *Isolation of mononuclear cells by centrifugation at 20°C for 40 minutes at 400 g: effect of varying the osmolarity of blood plasma*

	Dilution	Basophils	Granulocytes (Eo+N)	Lymphocytes	Monocytes	Erythrocytes
Top fraction	A	1.0	< 0.1	77.9	21.1	3.4
	B	0.7	0.0	77.4	21.9	1.8
Bottom fraction	A	0.2	88.5	10.6	0.7	
	B	0.2	90.2	9.0	0.6	

Lymphocyte yield: A 76.2, B 79.4 — Monocyte yield: A 93.3, B 94.6

Total lymphocyte recovery: A 105.3, B 104.1 — Total monocyte recovery A 85.1, B 91.2

Dilution A: 10 parts blood+2 parts EDTA 2.7 per cent; osmolarity = 1.00
Dilution B: 10 parts blood+2 parts EDTA 4.5 per cent; osmolarity = 1.12.
Each tube contained 2 ml blood and 3 ml standard Ip-ficoll mixture.
The Table gives the differential counts (per cent) of the top and bottom fractions; the erythrocyte contamination is given in per cent of the total number of white and red cells. The yields and total recoveries (per cent) of mononuclear cells are indicated below.

(Table I). It was possible, therefore, that the lymphocyte yield could be increased, simply by diluting the blood.

Equal volumes of EDTA-blood (EDTA 10 per cent/blood = 1/49) and 0.9 per cent NaCl were mixed. Four ml of this mixture were centrifuged in one tube and 6 ml in another, using the standard Ip-ficoll mixture. As seen from Table II, dilution with saline increased the lymphocyte yield to about 96 per cent, with no appreciable increase of the erythrocyte or granulocyte contamination in the top fraction. There was no difference whether 4 or 6 ml were centrifuged, demonstrating that efficient separation depends upon the degree of dilution and not upon the height of the blood cell column. This experiment indicates that lymphocytes are

Table II. *Isolation of mononuclear cells by centrifugation at 20°C: effect of dilution*

	Volume (ml)	Basophils	Granulocytes	Lymphocytes	Monocytes	Erythrocytes
Top fraction	4	0.8	0.1	79.6	19.5	4.1
	6	1.0	0.0	79.8	19.2	3.7
Bottom fraction	4	0.5	96.6	2.7	0.2	
	6	0.3	97.1	2.5	0.1	

80% Lymphs
20% Monocytes
1% gran

Lymphocyte yield: 4 ml 95.7, 6 ml 96.0 — Monocyte yield: 4 ml 99.2, 6 ml 99.3

One part EDTA-blood (EDTA 10 per cent/blood = 1/49) and 1 part 0.9 per cent NaCl were mixed, and two different volumes were centrifuged, using the standard Ip-ficoll mixture. The Table gives the erythrocyte contamination (per cent) in the top fraction, and the differential counts (per cent) of top and bottom fractions. Yields of mononuclear cells (per cent) are indicated below.

Table III. *Isolation of mononuclear cells by centrifugation at 20°C: effect of further dilution*

	Basophils	Granulocytes	Lymphocytes	Monocytes	Erythrocytes
Top fraction	1.2 (0.4-2.0)	0.1 (0.0-0.2)	80.2 (73.4-90.0)	18.5 (8.6-25.8)	6.2 (1.7-9.4)
Bottom fraction	0.2 (0.0-0.6)	98.3 (97.0-99.6)	1.4 (0.3-2.3)	0.1 (0.0-0.4)	

Lymphocyte yield: 97.9 (96.6-99.2) Monocyte yield: 98.7 (95.4-100.0)

Two ml EDTA-blood (EDTA 10 per cent/blood = 1/49) were mixed with 6 ml 0.9 per cent NaCl and centrifuged in a tube containing 3 ml of the standard Ip-ficoll mixture. The Table gives the differential counts (with ranges) of the top and the bottom fraction, and yields (with ranges) of mononuclear cells, all values in per cent.

transported downwards in aggregates of erythrocytes and granulocytes, a tendency which is reduced by dilution.

In the next series, a mixture of 2 ml blood and 6 ml 0.9 per cent NaCl was centrifuged in each tube (Fig. 1), using the standard Ip-ficoll mixture. One thousand cells were counted in each smear from the bottom fraction. Table III shows that the lymphocyte yield now increased to about 98 per cent. The blood used for the series presented in Tables II and III originated from the same 10 persons, and the increase of lymphocyte yield was demonstrated in every single case. There was a slight increase of the erythrocyte contamination in the top fraction; compare with Tables I and II.

The series presented in Table III can be considered as the routine method for isolating mononuclear cells from whole blood. It was demonstrated on several occasions, that equally good separations could be obtained with larger volumes of blood in tubes with larger diameters.

In some experiments, a suspension of leucocytes was used instead of whole blood. The leucocytes were first isolated by sedimentation at 1 g, using the Ip-dextran system (Paper II). A given volume was diluted with saline and centrifuged with the standard Ip-ficoll mixture. The lymphocyte yield then decreased, and 5-7 per cent lymphocytes were found among the granulocytes in the bottom fraction.

d) *Centrifugation at* 4°*C*

Table IV shows the effect of a lowered temperature. The centrifuge and the Ip-ficoll mixture were adjusted to 4°C at start of the centrifugation. Two ml blood were mixed with 6 ml saline

Table IV. *Isolation of mononuclear cells by centrifugation at 4°C for* 40 *minutes at 400 g*

	Basophils	Granulocytes	Lymphocytes	Monocytes	Erythrocytes
Top fraction	1.5 (0.4-4.8)	2.3 (0.0-13.5)	80.7 (70.6-85.1)	15.5 (13.0-20.4)	25.6 (6.4-67.1)
Bottom fraction	0.4 (0.0-1.6)	92.1 (88.4-97.8)	6.9 (2.0-13.2)	0.6 (0.0-1.2)	

Lymphocyte yield: 87.3 (80.4-95.0) Monocyte yield: 93.5 (82.9-100.0)

A mixture of 2 ml EDTA-blood and 6 ml 0.9 per cent NaCl was centrifuged in each tube. Standard Ip-ficoll mixture. The Table gives the differential counts (with ranges) of the top and the bottom fraction, and the yields ranges) of mononuclear cells, all values in per cent.

Table V. *Isolation of mononuclear cells by centrifugation at 20°C: effect of lowering the density and viscosity of the Ip-ficoll mixture*

	Ip-ficoll mixture	Basophils	Granulocytes	Lymphocytes	Monocytes	Erythrocytes
Top fraction	A	0.6	0.0	81.0	18.4	11.9
	B	1.3	0.3	81.7	16.7	35.2
Bottom fraction	A	0.3	91.6	6.7	1.4	
	B	0.2	93.4	5.8	0.6	

Lymphocyte yield: A 73.8
B 80.4

Monocyte yield: A 76.9
B 89.5

Mixture A: 10 parts Ip + 26 parts ficoll 7 per cent, with density of 1.069 gm/ml and relative viscosity of 3.05. Mixture B: 10 parts Ip + 26 parts ficoll 8 per cent, with density of 1.073 gm/ml and relative viscosity of 3.42. A mixture of 2 ml EDTA-blood (EDTA 10 per cent/blood = 1/49) and 6 ml 0.9 per cent NaCl was centrifuged in each tube. The Table gives differential counts (per cent) of top and bottom fractions, and yields of mononuclear cells.

of room temperature, and centrifuged immediately after withdrawal of the blood. The erythrocyte and granulocyte contamination in the top fraction now increased, as compared to centrifugation at 20°C (Table III). Most likely, this was due to the increased viscosity of the Ip-ficoll mixture (about 65 per cent increase) and to its reduced cell clumping ability at lower temperatures. On the other hand, the lymphocyte and monocyte admixture in the bottom fraction increased, in spite of a higher viscosity. This was probably related to a reduced tendency of erythrocytes to aggregate (see Discussion).

e) *Lowering the density and viscosity of the Ip-ficoll mixture*

Two Ip-ficoll mixtures were used. Ten parts Ip were mixed with 26 parts ficoll, either 7 or 8 per cent, giving final ficoll concentrations of 5.1 and 5.8 per cent and densities of 1.069 and 1.073 gm/ml, respectively. The osmolarities of these two mixtures were approximately the same as the average osmolarity of blood plasma. The results are given in Table V. The erythrocyte contamination in the top fraction was now slightly increased, compared to Table III, in spite of a lowered viscosity and density. This may be due to a reduced ficoll concentration, and hence a reduced cell clumping at the interface, and perhaps also to a slightly lowered osmolarity. This experiment, together with several additional separations in which the density and viscosity varied over a rather broad range, suggests a close relationship between the yield of mononuclear cells and the density of the Ip-ficoll mixture. A change of the viscosity seems less important. A density of 1.077 gm/ml, or more, ensures a high yield of mononuclear cells. Monocytes have possibly a slightly lower density than lymphocytes as judged by yields, but it is difficult to be certain, since the density depends upon the osmotic sensitivity of the cells (Paper III) and on the composition of the suspending medium.

f) *Comparing EDTA and heparin as antioagulants*

The following dilutions were used:

A: 0.5 ml heparin in 0.9 per cent NaCl + 9.5 ml blood

B: 0.5 ml EDTA 2.7 per cent + 9.5 ml blood.

Two ml of dilutions A and B were each mixed with 6 ml 0.9 per cent NaCl and centrifuged with the standard Ip-ficoll mixture. The results are given in Table VI. Heparin gave an increased contamination of erythrocytes in the top fraction ($0.02 < p < 0.05$) and a slight but not significant increase of the granulocyte admixture. The yields of mononuclear cells were satisfactory with both anticoagulants.

the tube. This intermediate layer contained almost no nucleated cells. The nucleated cells in the bottom fraction were almost exclusively granulocytes which could be further isolated by sedimentation at 1 g.

Both heparin and EDTA were used as anticoagulants and in the following concentrations:
A: 0.5 ml heparin + 9.5 ml blood
B: 0.5 ml EDTA 2.7 per cent + 9.5 ml blood.

the meniscus of the erythrocyte mass. One ml EDTA-plasma was added to one tube, and 1 ml heparin plasma to the other. Next, 0.4 ml dextran 4.5 per cent was added to each tube. The content was mixed with a Pasteur pipette, and transferred to small tubes with inner diameter of 8.5 mm. The blood cell column was then about 40 mm high. The erythrocytes were allowed to settle at 4 °C; after about 40 minutes the plasma layer was

Table VII. *Isolation of granulocytes: comparing EDTA and heparin as anticoagulants*

	Basophils	Granulocytes	Lymphocytes	Monocytes	Erythrocytes
EDTA	0.0	99.5 (98.4-100.0)	0.4 (0.0-1.0)	0.1 (0.0-0.6)	52.6 (37.0-71.4)

Granulocyte yield: Heparin 48.7 (38.4-60.8) EDTA 58.9 (42.8-71.3)

A mixture of 2 ml blood and 6 ml 0.9 per cent NaCl was centrifuged in each tube (at 20 °C for 40 minutes at 400 g). Standard Ip-ficoll mixture. The total supernatant, including mononuclear cells, was removed. One ml plasma and 0.4 ml dextran 4.5 per cent were mixed with the bottom fraction in each tube. This mixture was transferred to tubes with inner diameter of 8.5 mm, and the plasma layer was removed when the erythrocytes had sedimented at 1 g (at 4 °C). The Table gives differential counts (with ranges) of cells in the plasma layers, and yields (with ranges) of granulocytes.

transferred to graduated tubes and the cells were counted. Smears for differential counting were made after centrifugation. The average values and ranges of differential counts and yields are given in Table VII.

Granulocytes constituted about 99 per cent of the nucleated cells, and there was approximately one erythrocyte per leucocyte. EDTA gave a higher granulocyte yield than heparin ($0.02 < p < 0.05$). Fig. 3 shows granulocytes isolated by this procedure.

Fig. 3. Isolated granulocytes. Mononuclear cells were first removed by centrifugation, as described in Fig. 1. Plasma and dextran was added to the bottom fraction, and the plasma layer containing the granulocytes was removed when the erythrocytes had settled by sedimentation at 1 g.

h) *Centrifugation of blood with a mixture of Ip-ficoll and plasma*

In the Ip-ficoll mixture, the cells were subjected to a medium devoid of nutrient factors. In the present series, therefore, plasma was added in the following way: ten parts Ip were mixed with 22 parts ficoll 14 per cent, and 0.8 ml plasma was added to 3 ml of this mixture. The density of the final mixture was about 1.079 gm/ml, and the relative osmolarity was about 1.14. A mixture of 2 ml EDTA-blood (EDTA 2.7 per cent/blood = 1:19) and 6 ml 0.9 per cent NaCl was centrifuged with 3 ml of the mixture of Ip-ficoll and plasma. The top fraction was removed, and smears were made for cell counting. The rest of the Ip-ficoll mixture above the erythrocyte mass was removed; 1 ml plasma and 0.4 ml dextran 4.5 per cent were added to the bottom fraction. The plasma layer was removed when the erythrocytes had settled at 4 °C. The results are given in Table VIII. They were rather similar to those presented in Table III, but the yield of mononuclear cells was somewhat reduced, which was also reflected by a slightly larger admixture of mononuclear cells in the bottom fraction. The granulocyte yield was similar to that presented in Table VI.

Table VIII. *Isolation of mononuclear cells by centrifugation with a mixture of Ip-ficoll and plasma; isolation of granulocytes from the bottom fraction by sedimentation at 1 g*

	Basophils	Granulocytes	Lymphocytes	Monocytes	Erythrocytes
Top fraction	1.0 (0.2-3.2)	0.2 (0.0-1.2)	83.9 (75.8-91.8)	14.9 (7.8-23.6)	6.0 (2.0-16.1)
Plasma layer after sedimentation at 1 g	0.1 (0.0-0.4)	97.1 (95.8-98.4)	2.4 (1.4-3.8)	0.4 (0.0-2.2)	55.0 (32.4-88.1)

Lymphocyte yield: 93.7 (82.1-97.7) Monocyte yield: 92.5 (76.3-100.0) Granulocyte yield: 61.1 (45.5-75.0)

Two ml EDTA-blood and 6 ml 0.9 per cent NaCl were centrifuged at 20 °C for 40 minutes at 400 g. Ficoll concentration = 7.3 per cent. Plasma concentration = 21 per cent. Density = 1.079 gm/ml See Table VII for further explanation. The Table gives differential counts (with ranges) of top fraction and the plasma layer, and the yields (per cent) of mononuclear cells and granulocytes.

Fig. 4. LE-cells. Isolated granulocytes were incubated with an abundance of LE-serum and mononuclear cells which had been frozen for 30 minutes at −34 °C.

i) *Viability tests*

Leucocytes isolated from the blood of 10 different persons were used for viability tests. The separations were carried out as for the series presented in Tables III and VII.

Trypan blue staining. On the average, less than 1 per cent of mononuclear cells were stained by trypan blue, and never more than 2 per cent. About 1 per cent of the granulocytes were stained, and never more than 3 per cent.

Incubation with LE-serum. About 0.3 per cent of mononuclear cells showed nuclear transformation, and 2.5 per cent of the granulocytes (0.0-10.0).

Phagocytosis with LE-serum. On the average, 77.5 per cent (61.0-92.5) of the isolated granulocytes made completely developed LE-cells. Twenty per cent of the granulocytes were either in the process of phagocytosis, or had become attached to LE-bodies, and 2.5 per cent of the cells did not adhere to LE- bodies. Fig. 4 shows LE-cells from one of the smears.

Phagocytosis of ink particles. On the average, 85 per cent (64-92) of granulocytes had phagocytized black particles, and 81 per cent (64-98) contained yellow particles. Ninety-eight per cent of monocytes had engulfed yellow particles, and 0.3 per cent of the lymphocytes contained a few yellow particles.

DISCUSSION

The Ip-ficoll system divides the cellular components of whole blood into two main fractions; lymphocytes and monocytes in one group, and granulocytes and erythrocytes in the other. Basophilic granulocytes tend to follow the mononuclear cells. Promyelocytes and myelocytes, in fact all types of young nucleated cells, usually concentrated in the mononuclear fraction. The distribution of lymphocytes and monocytes in the top fraction depended upon the differential picture of whole blood; the ranges are given in Table III.

Several unsuccessful efforts were made to separate lymphocytes from monocytes by changing the density, viscosity and osmolarity of the Ip-ficoll mixture. Apparently lymphocytes and monocytes have similar densities. In the series presented in Table I, the monocyte yield was somewhat larger than the lymphocyte yield, which may suggest a slightly lower density of monocytes than of lymphocytes. However, this observation may also be explained as resulting from mutual interaction between mononuclear cells on one side, and granulocytes and erythrocytes on the other. It is possible that lymphocytes, rather than monocytes, are liable to be trapped within aggregates of granulocytes and erythrocytes.

When the osmolarity was increased, and hence the density of the cells, lymphocytes rather than monocytes, tended to sediment to the bottom of the tube. This indicates that lymphocytes, or part of them, are more sensitive to variations in osmolarity than are monocytes. It has not been possible, however, to utilize this to separate them from monocytes. To obtain lymphocyte suspensions with the lowest possible admixture of monocytes, it is thus necessary to use other techniques, such as the procedure described previously (Paper III), or the technique described by Lamvik (1966).

The mononuclear cells must have a density quite close to, or slightly less than 1.077 gm/ml. It can be calculated from the sedimentation law (Paper I, p. 9) that with a density of 1.079-1.080 gm/ml they would pass down to the bottom of the tube in the course of 40 minutes, but the mononuclear cells remained at the interface even when centri-

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fuged for 100 minutes at 400 g (2 occasions). The granulocytes did pass the 1.077 level of density in all experiments, but one cannot conclude from this observation that the initial density of granulocytes is higher than that of lymphocytes and monocytes. The density which the cells attain in the Ip-ficoll mixture is probably an important factor. Granulocytes are more sensitive to osmotic changes than are lymphocytes (Paper III), in the sense that the density of granulocytes increases more than the density of lymphocytes in hyperosmotic surroundings. The osmolarity of the standard Ip-ficoll mixture is 1.12 plasma equivalents, and the magnitude of the osmotic effect can be illustrated by the following example, calculating the density from the formula described previously (Paper I, p. 12). If the granu[illegible]ocytes have an initial density of 1.075 gm/ml, and their volume fraction of water is 0.7, then a 12 per cent increase of osmolarity will change the density to 1.081 gm/ml. Even if one starts with an Ip-ficoll mixture which is isosmotic with blood plasma, it may not remain isosmotic at the interface. If one considers, for the sake of simplicity, that the lower part contains Isopaque, and the upper part NaCl instead of plasma, a sort of Donnan effect is to be expected (Potts & Parry 1964). Both compounds are fully dissociated sodium salts, and there is initially no concentration gradient with regard to sodium ions. Chloride ions will diffuse into the Ip compartment and the Ip anion will diffuse into the NaCl compartment. The chloride ions, owing to their smaller size, diffuse faster than the Ip ions; the Ip compartment will therefore acquire a negative charge which will attract the sodium ions into the compartment as well, and an osmotic gradient is created. Osmotic equilibrium is not re-established, until a complete mixing has taken place.

The isolation of granulocytes by sedimentation at 1 g can be influenced in different ways. A rather low contamination of erythrocytes is obtained if the height of the blood cell column is diminished, either by reducing the volume or by enlarging the diameter of the tube. The granulocyte yield is thereby slightly reduced, since the part of plasma (1-2 mm) which is close to the erythrocyte mass cannot be removed, and the volume of this part depends upon the area of the erythrocyte meniscus which limits the plasma layer. The sedimentation of erythrocytes is more rapid if the dextran concentration is increased, for example by using a solution containing 6 per cent dextran instead of 4.5 per cent. However, the more rapid the sedimentation, the larger is the contamination of erythrocytes among granulocytes. If plasma and dextran are cooled to 4 °C before being added to the bottom fraction, the sedimentation process is prolonged, and the erythrocyte contamination is reduced. The granulocyte yield depends to some degree upon the duration of the sedimentation. Usually, the plasma layer was removed when its volume was about equal to the volume of the erythrocyte mass.

The present technique is standardized for centrifugal force, temperature, dilution of blood, and also for the properties of the Ip-ficoll mixture (volume, density, viscosity and osmolarity). The results are not appreciably affected by changing the volume of the cell suspension (Table II). The diameter of the tube can be enlarged, without changing the results. The centrifugal force can be reduced to about 300 g, without disturbing the separation appreciably; there is a slight increase of the erythrocyte contamination in the top fraction, rather than of the granulocyte admixture. If the centrifugation time is reduced to 30 minutes, there is a slight increase of the erythrocyte contamination, and also a few granulocytes appear in the top fraction. If the technique is used for preparing granulocyte suspension only, the time of centrifugation can be reduced to 30 minutes, or even less.

The lymphocyte yield is reduced if the temperature is lowered from 20 to 4 °C, and a still further dilution is required to obtain a good lymphocyte yield at 4 °C. The reduced yield at 4 °C can be explained by a mechanism which has been discussed previously (Paper I, p. 26 & II, p. 39). The erythrocytes in the plasma layer, or at the interface region, are more or less liable to form aggregates. When the temperature is raised this tendency increases, and by some mechanism the leucocytes move towards the periphery of the tube, where they sediment slower than the aggregates of erythrocytes in the middle of the tube. When the temperature is lowered, the tendency of erythrocytes (and granulocytes) to form aggregates is diminished, and the lymphocytes are less apt to move towards the periphery of the tube. A larger part of the lymphocytes now sediment in the middle of the tube along with the erythrocytes, and when they reach the interface level, they are trapped in the erythrocyte clumps and sediment to the bottom of the tube. When they sediment as single cells, granulocytes pass the interface level, probably owing to a high final density; lymphocytes and monocytes do not. An efficient isolation of lymphocytes thus depends upon the presence of a number of erythrocytes initially. In accordance with this principle, the separation was less efficient if the majority of erythrocytes was removed, by sedimentation at 1 g, before centrifugation. The lymphocyte yield then decreased, and the admixture of lymphocytes among granulocytes in the bottom fraction increased.

The separation is slightly better, using EDTA as anticoagulant instead of heparin.

Viability of the separated cells proved to be satisfactory, as judged by a persisting integrity of the cell membrane, and the ability to engulf solid particles.

It is probable that a nutrient broth can be added to the Ip-ficoll mixture instead of plasma. It is decisive, then, to have a final mixture with density of about 1.080 gm/ml and with an osmolarity similar to that of the Ip-ficoll standard mixture.

Small blood volumes (6 ml) can be applied on top of the Ip-ficoll mixture with a Pasteur pipette. Larger volumes can more conveniently be layered on the Ip-ficoll mixture through a thin plastic tube which is connected to a syringe or a byrette.

When rat blood was separated with an Ip-ficoll standard mixture (two occasions), a pure suspension of mononuclear cells was recovered from the interface region, but the bottom fraction contained more lymphocytes than with human blood.

Ficoll is a convenient compound for different techniques of cell separation (Uvnäs & Thon 1959), owing to its high solubility and relatively low viscosity. Dextran, instead of ficoll, can be used in combination with Ip, but since dextran is more viscous than ficoll, it is necessary to increase the centrifugal force considerably to achieve similar results.

SUMMARY

The present paper describes a technique for isolation of mononuclear cells and granulocytes from whole blood. When anticoagulated blood was layered on top of a mixture of Isopaque and ficoll and centrifuged at 20 °C, the cellular elements of whole blood were divided into two main groups; lymphocytes and monocytes together with the majority of basophilic granulocytes were found on the top of the Isopaque-ficoll mixture, while granulocytes and erythrocytes had sedimented to the bottom of the tube. The yield of mononuclear cells increased as the blood was diluted with NaCl, and an average yield of 98 per cent was obtained. The mononuclear fraction contained less than 0.2 per cent granulocytes and less than 10 per cent erythrocytes when EDTA was used as anticoagulant. When heparin was used, the erythrocyte contamination increased to about 15 per cent in average.

Granulocytes were isolated by adding dextran and plasma to the bottom fraction. The plasma layer containing the granulocytes was removed when the erythrocytes had settled at 4 °C by sedimentation at 1 g. About 99 per cent of the nucleated cells in plasma were granulocytes and 1 per cent were mononuclear cells. There was about 1 erythrocyte per leucocyte. The average yields of granulocytes were 49 per cent (heparin) and 59 per cent (EDTA).

The purity of both fractions decreased when the centrifugation was carried out at 4 °C.

STANDARD SEPARATION TECHNIQUE

Separation fluid: 10 parts Isopaque (33.9 per cent) with density of 1.200 gm/ml are mixed with 24 parts ficoll 9 per cent. Final density = 1.077 gm/ml.

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Blood-NaCl mixture: One part anticoagulated blood is mixed with 3 parts 0.9 per cent NaCl.

Eight ml (60 mm) of the blood-NaCl mixture is layered over 3 ml (22 mm) of the separation fluid in tubes with inner diameter of 13 mm by keeping the pipette with blood against the tube wall 10-15 mm above the fluid meniscus. The tube is then centrifuged at 20°C for 40 minutes at 400 g. The mononuclear cells at the top of the separation fluid are removed with a pipette. The rest of the separation fluid down to 1-2 mm above the erythrocyte mass is then removed. One ml plasma and 0.4 ml dextran 4.5 per cent dissolved in 0.9 per cent NaCl are added. After mixing, the content is transferred to another tube, and the plasma layer containing the granulocytes is removed when the erythrocytes have settled at 4°C.

Larger volumes can be separated in one procedure by increasing the diameter of the tube.

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