Ficoll Flotation for the Separation of Blood Leukocyte Types

By P. B. Noble, J. Harry Cutts and K. K. Carroll

It should be possible to separate cells of different types by utilizing differences in their specific gravities. Centrifugation of whole blood against high density fluids has been successful, at least in part, in separating leucocytes from peripheral blood. Gum acacia and bovine albumin have been used in this manner, but the method has been criticized because of the cost and labor involved in preparing the media, and doubts have been expressed as to the viability of the cells isolated. We have reported previously the use of Ficoll as a density fluid for the isolation of leucocytes from peripheral blood. It is inexpensive, stable and appears to be nontoxic to cells. The present paper describes further experiences with, and some modifications of the method, which illustrate the usefulness of Ficoll for separation of cells by the flotation method.

Methods and Observations

Ficoll, an inert, high molecular weight polysucrose was dialysed to remove the NaCl present, and lyophilized. For dialysis, the Ficoll was made up as a 50 per cent aqueous solution: this was reduced to approximately 20 per cent during dialysis. Concentrations much greater than this did not lyophilize well, and in place of a light fluffy material, the Ficoll was recovered as a solid "glassy" substance which appeared to contain water. The recovered Ficoll was stored in tightly stoppered glass bottles to prevent absorption of water. A 10 per cent solution of Ficoll in Seligmann's balanced salt solution (SBSS), has a specific gravity of 1.039. Provided the specific gravity of the lyophilized material was checked, no detectable variations have been noted in the different batches of Ficoll.

Appropriate concentrations of Ficoll were made up as needed by dissolving the Ficoll in SBSS. The solution was filtered through a Millipore filter of 0.45 μ pore size, and distributed.

*Ficoll obtained from Pharmacia (Canada) Ltd., 100 Place Cremazie, Montreal 11, Quebec, Canada.

†SBSS—7.65 gm NaCl, 0.20 gm KCl, 1.50 gm NaCO₃, 0.05 gm NaH₂PO₄, 0.10 gm KH₂PO₄, 0.70 gm NaHCO₃, 1.0 gm glucose, 0.003 gm ascorbic acid; twice distilled water 1000 ml. pH of the solution = 7.3.

Cellulose nitrate tubes 1 in. diam. x 3 in. Spinco Division, Beckman Instruments Inc., Palo Alto, California.

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This work was supported by the National Cancer Institute of Canada.

First submitted May 3, 1967; accepted for publication July 6, 1967.

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in cellulose nitrate tubes of one inch diameter. In all cases, 9 ml. of Ficoll was used to facilitate recovery of the cell layer. Quantities less than this resulted in the cell layers being formed too near the bottom of the tube to permit convenient recovery.

The methods described below are those employed for isolating leukocytes from chicken blood, but the method is applicable to other species. We have used it to separate leukocytes from human and from rat peripheral blood. Modifications of the original method have been made and are described below and outlined in Figs. 1 and 2.

Stage I: Separation of Leukocytes from Erythrocytes

Initially, heparin was used as the anticoagulant, and possibly as a result of this, there was loose clumping of the leukocytes. To overcome clumping, 5 ml. of blood were collected directly into 5 ml. of Seligmann's solution containing 5 mg. of EDTA as the anticoagulant. The blood was gently mixed and layered over 9 ml. of 35 per cent Ficoll, and centrifuged at 10800 g for 30 min. The entire procedure, including withdrawal of the blood sample, was carried out at 4 C. All reagents and equipment used had been chilled previously to the same temperature.

As can be seen in Fig. 1, a grayish-white band formed just below the Ficoll: plasma interface, clearly separated from the underlying erythrocytes. This layer of discretely dispersed cells was recovered by puncturing the side of the tube just below the buffy layer, using an 18 gauge needle and syringe. The cells were washed, resuspended in Seligmann's solution and examined by phase contrast. The cells of this layer, shown in Figs. 2 and 3 of Plate 1, consisted of lymphocytes, monocytes and thrombocytes with only an occasional erythrocyte. The granulocytes remained intimately mixed with the red cells of the upper portion of the erythrocyte layer, and it was not possible to separate these two cell types further by Ficoll flotation.

The above method successfully separated lymphocytes and monocytes in a high degree of purity. Because of our failure to recover granulocytes, clumping of all leukocyte types was promoted by withdrawing 5 ml. of blood into 1 ml. of Hank's balanced salt solution (HBSS) which contained 100 units of heparin. The blood was layered over 9 ml. of 35 per cent Ficoll in SBSS and centrifuged at 1000 g for 30 minutes at 4 C.

The leukocytes separated as a cohesive sheet just below the Ficoll: plasma interface as shown in the upper portion of Fig. 2. The cells could be removed by gentle suction, using a syringe and a large bore needle having a long bevel. This layer contained all the leukocyte types mixed with thrombocytes and a few erythrocytes.

Stage II: Separation of Individual Leukocyte Types

The lower portion of Fig. 2 shows the various Ficoll preparations used to bring about further separation of the leukocyte types. The cells were washed and redispersed in SBSS and layered over a single-step preparation consisting of 9 mls. of 28 per cent Ficoll in SBSS (Tube 1, Fig. 2). The tubes were centrifuged at 300 g for 15–20 min. at 4 C. Lymphocytes, monocytes and thrombocytes formed at the Ficoll interface (Tube 1, a). Granulocytes and the few contaminating erythrocytes and thrombocytes clumped at and just below the interface and could not be separated. Although some of the granulocytes, contaminated with a few red cells and thrombocytes, were present in the lower portions of the tube, additional centrifugation served only to throw all cell types to the bottom of the tube.

In order to avoid the abrupt interface between the Ficoll and the overlying cell suspension, further modifications were tried. Three ml. of Seligmann's solution were layered over 9 ml. of 28 per cent Ficoll and allowed to equilibrate for 4 hr. or for 24 hr. at 4 C. These are shown as tubes 2 and 3 respectively, in Fig. 2. Nonlinear continuous gradients were prepared also, by layering 5 cc portions each of 30 per cent, 25 per cent and 20 per cent Ficoll in successive layers, and allowing them to equilibrate for 48 hrs. at 4 C. (Tube 4, Fig. 2). The cells were layered over the gradients and centrifuged at 300 g for 15 min. at 4 C.

Good separation of lymphocytes and monocytes from granulocytes and erythrocytes was achieved by any of the latter three preparations. The cells so separated are shown in Figs.
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Blood taken into SBSS containing 5 mg EDTA. Layered on 9 mls Ficoll, centrifuged at 1000 g for 30 min. at 4°C

HETEROPHILS and RBC, LYMPHOCYTES, MONOCYTES and THROMBOCYTES

Fig. 1.—Leukocyte separation using Seligmann's balanced salt solution.

10 and 11, Plate 2. Discontinuous Ficoll gradients separated an additional distinct band of lighter erythrocytes, not previously seen. This band contained a number of granulocytes discretely dispersed throughout the red cells. The remainder of the granulocytes settled with the heavier erythrocytes to the bottom of the tube, and were present in coarse, loose clumps. The granulocytes could be freed from the few contaminating red cells by "paradoxical sedimentation" as shown in Fig. 12, Plate 2.

Although evaluation of the morphological appearances and the results of viability studies are not yet completed, it can be stated that the leukocytes separated by flotation over Ficoll retain morphologic integrity, both on stained smears and in living cells examined by phase microscopy. Staining with Trypan blue has shown an excess of 80 per cent of the cells to exclude the dye. When warmed to 37°C on a warm stage, the cells showed good ameboid activity, and phagocytosis of thrombocyte particles by monocytes were seen. Such a phagocytic monocyte is seen in Plate 1, Fig. 6.

DISCUSSION

Ficoll appears to offer a medium suitable for the separation of morphologically intact, viable leukocytes from whole blood by cell flotation. In our early studies, it was thought that separation of the individual leukocyte type could be achieved only by preventing the nonspecific clumping of leukocytes which appears to be so excessive with flotation methods. The problem of clumping was surmounted by collecting the initial blood sample directly into Seligmann's balanced salt solution and avoiding heparin. Leukocyte separation was then
Blood taken into HBSS containing 100 units of heparin. Layered on 9 mls. Ficoll, centrifuged at 1000 g for 30 min. at 4°C.

Lymphocytes, heterophils, monocytes, thrombocytes. Dispersed in SBSS layered on 9 mls. Ficoll, centrifuged at 300 g for 15 min. at 4°C.

a-lymphocytes and monocytes
b-light erythrocytes and heterophils
c-heavy erythrocytes and heterophils

Fig. 2.—Leukocyte separation using Hank’s BSS and Heparin.

achieved by Ficoll flotation, but only the lymphocyte-monocyte fraction could be recovered. Since some of the erythrocytes and granulocytes appeared to have the same specific gravities, no distinct separation of the granulocytes was obtained.

When clumping of leukocytes occurred, the white cells separated as aggregates of mixed leukocyte types, in which were trapped a few red cells and thrombocytes. No distinct separation of the various leukocyte entities occurred, but nearly all of the applied cells could be recovered as a single band. These cells were easily dispersed in Seligmann’s solution. In further trials of cell separation, heparin was used to encourage clumping, and additional Ficoll flotation was used to separate the various leukocyte types.
Plate 1.—Peripheral blood from the chicken showing lymphocytes heterophils and thrombocytes. (1) Phase contrast $\times$ 125. Chicken heterophils contain refractile rod-shaped granules which made the cell difficult to photograph. (2 & 3) Leukocytes isolated from stage I Ficoll flotation using Seligmann's solution. Note the absence of clumping and the loss of granulocytes and erythrocytes. Phase contrast $\times$125. (4) A clump of leucocytes isolated from stage I Ficoll flotation using SBSS and heparin. Phase contrast $\times$1125. (5) The same cells as in (4), showing the clumped nature of the cells. Note that granulocytes (heterophils) are present in large numbers, along with lymphocytes, monocytes and a few erythrocytes. Phase contrast $\times$125. (6–8) Morphological features of leukocytes isolated by stage I Ficoll flotation. (6) Monocytes. One cell is shown engulfing a thrombocyte (arrow). Phase contrast $\times$1125. (7) Large monocyte from stage I Ficoll using HBSS. Phase contrast $\times$1125. (8) Heterophils. These cells show typical refractile rod-shaped granules. Phase contrast $\times$1125. (9) Human granulocytes isolated by stage I Ficoll flotation using HBSS. Phase contrast $\times$1125.
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In order to show cell detail more clearly, the preparations were crushed slightly prior to photography. The legend is the same for all illustrations. M = monocyte, L = lymphocyte, H = heterophil, Th = thrombocyte.

On the initial 35 per cent Ficoll, the cells separated as a band a few mm. below the Ficoll; serum interface but subsequent separation could be achieved on a lower concentration of Ficoll. It would appear that as the erythrocytes passed through the interface, small amounts of serum were carried along also, thus modifying the original interface. The Ficoll became diluted in a narrow
zone extending below the interface, and had become in fact, a short continuous gradient extending a few mm. from the junction of the two liquids.

At the second stage of Ficoll flotation, single-step Ficoll flotation failed to achieve separation sufficient to permit recovery of leukocyte types. Lymphocytes and monocytes separated as a band at the Ficoll interface, immediately above and in contact with a layer of red cells and granulocytes. The absence of large numbers of erythrocytes prevented the establishment of a short linear gradient at the interface, so that a high density interface remained at the Ficoll: medium junction. As the cells met this interface, they tended to collect and form clumps of mixed types of leukocytes. High density interfaces could be avoided, either by allowing the Ficoll to equilibrate with SBSS, or by making discontinuous gradients. This type of preparation separated, in addition to a band of leukocytes, a band of lighter red cells. Ferrebee, using dense solutions to plasma to obtain differential flotation of erythrocytes parasitized by malaria, found the parasites in a layer of lighter red cells, and presented evidence to show that these lighter cells were young forms of erythrocytes. Vallee, Hughes and Gibson have noted the separation of lighter erythrocytes from the blood of patients with anemia, after flotation on albumen. We have noted light erythrocytes separating from rat and human blood, and these contain a high proportion of polychromatic cells. Preliminary studies on the separation of cells from bone marrow, suggest that reticulocytes do form a layer of less dense erythrocytes, clearly separated from the more mature cells. The presence of lighter erythrocytes explains our inability to separate granulocytes from erythrocytes obtained by SBSS at stage I.

Throughout the various stages of cell separation, contamination of the different cell fractions by small numbers of thrombocytes (platelets) was a problem. Thrombocytes constantly altered their specific gravity as they formed small clumps, or broke down and shed their cytoplasm. Thrombocyte contamination was not great, however, and in rat and human blood, did not pose the problem that it did in chicken blood. In the latter, thrombocytes are present as nucleated elements closely approximating lymphocytes in size. The specific gravities of chicken monocytes, lymphocytes and thrombocytes appear to be very close, making separation of these cell types more difficult. Mammalian blood with much lighter platelets, does not present the same problem.

Summary

A method for the separation of leukocytes from peripheral blood, using flotation over Ficoll, is described. The method is amenable to considerable variation, and the leukocytes isolated show good preservation of their morphologic integrity, are motile and capable of phagocytosis.

Summario in interlingua

Es describite un metodo pro le separation de leucocytos ab sanguine peripheric con le utilisation de flottation supra Ficoll. Le metodo se distingue per un alte grado de adaptabilitate. Le leucocytos isolate manifesta un bon preservation de lor integritate morphologic. Illos es motil e capace de phagocytosis.
ACKNOWLEDGMENTS

Thanks are extended to Miss Tia Brinkman for excellent technical assistance and to Mr. Barry Forbes for photography and art work.

REFERENCES

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