Evaluation of a New Single-Gradient, Ficoll-Hypaque with Hypotonic Lysis Method for Leukocyte Separation

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Objective: Separation techniques for radiolabeled leukocytes possess inherent problems: procedure length, leukocyte numbers and contaminates (e.g., platelets and erythrocytes). A new leukocyte separation technique is compared to current methods.

Methods: These four leukocyte separation methods were compared: volex sedimentation (VS), volex sedimentation with hypotonic lysis (VL), Ficoll-Hypaque two density gradients (FH) and a new single density gradient Ficoll-Hypaque with hypotonic lysis (FL). CBC data was used to determine the cellular composition of each method. Time to perform each method was also documented. In vitro viability of the leukocytes separated by the FL method was compared to the FH separation method using chemotaxis and trypan blue staining.

Results: The VS and VL methods demonstrated the largest number of leukocytes and platelets. The FH and FL techniques yielded reduced numbers of platelets and erythrocytes with a high percentage of neutrophils present (average ~94%). FH required the most time (3 hr) for the separation completion, whereas VL required the least time (90 min). FH and FL methods yielded viable leukocyte populations by chemotaxis and trypan blue staining evaluation.

Conclusions: The new FL separation method compares favorably to other leukocyte separation techniques. The reduction in the number of cell contaminants, increased percentage of neutrophils present, a viable leukocyte population, and the time required to perform the method are all factors which make the FL technique attractive.

Key Words: Ficoll-Hypaque gradient densities I and II; hypotonic lysis; leukocyte in vitro viability; leukocyte separation techniques

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Indium-111-oxine (*1–3*) and ^{99m}Tc-HMPAO-labeled leukocytes (*4,5*) are currently used for the detection of infectious and inflammatory lesions. The leukocyte separation technique should retain the highest possible number of neutrophils and as few as possible cellular contaminants (*6,7*). It is also important that the neutrophils retain their viability during the in vitro separation, so that they can function in vivo for infection and inflammation localization. Only viable neutrophils will retain chemotaxic ability and thereby concentrate in infectious and inflammatory sites (*8*).

For the first part of our study, we evaluated the cellular compositions of the leukocyte preparations from three different types of leukocyte separation techniques that are commonly used in clinical settings: volex sedimentation (VS), volex sedimentation with hypotonic lysis (VL) and Ficoll-Hypaque using two density gradients (FH) (*9–13*). These three methods were compared with a new leukocyte separation method, Ficoll-Hypaque using a single-density gradient with hypotonic lysis (FL). The amounts of other cellular contaminants were studied using complete blood count (CBC) and leukocyte differential data.

Since the FL method uses a hypotonic lysis, the question of neutrophil viability remains. Therefore, the second part of the study addressed this question and involved the determination of the in vitro viability of neutrophil preparations from FH and FL separation methods. The in vitro viability of the FL-separated neutrophils was compared with the neutrophils isolated by FH method because both the FL and FH techniques yielded leukocyte preparations which were predominantly neutrophils (>93%). The in vitro viability of the neutrophils was determined by using chemotaxis measurement and trypan blue staining.

MATERIALS AND METHODS

Collection and Sedimentation of Whole Blood

Donor blood samples of 40 ml were drawn into each of two 60-ml syringes containing 10 ml of anticoagulant citrate dextrose solution (ACD), USP, Solution A. A total volume of 100 ml was drawn for each study (Fig. 1, step 1).

The following procedure was performed using aseptic technique in a vertical laminar flow hood. Twenty-five ml of anticoagulated blood was then placed into four 50-ml conical tubes, each containing 10 ml hydroxyethyl starch (HES; 6%

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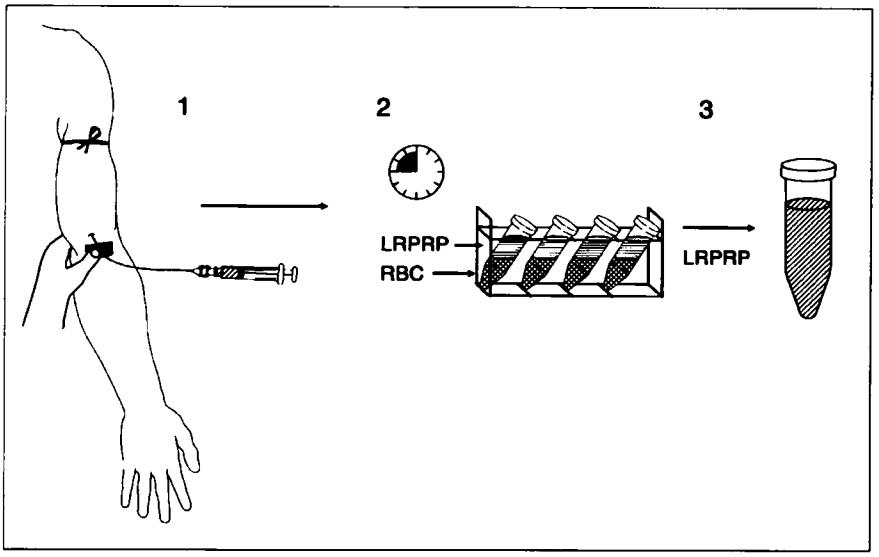

FIGURE 1. Schematic diagram of blood collection and gravity sedimentation of whole blood using hetastarch. LRPRP = leukocyte-rich platelet-rich plasma; RBC = red blood cells.

hetastarch in 0.9% NaCl solution) (Volex®, American Critical Care, McGaw Park, IL; Hespan®, DuPont Pharmaceuticals, Wilmington, DE). These tubes were placed in a rack at a 45° angle and allowed to gravity sediment at room temperature for 30–45 min (Fig. 1, step 2). This requires 10–15 min for VL and FL methods.

After sedimentation, the leukocyte-rich platelet-rich plasma (LRPRP) supernatant was removed and placed into two 50-ml conical tubes (Fig. 1, step 3). The collection and sedimentation of whole blood up to this point was identical among the four techniques of leukocyte separation used for this study.

Separation of Leukocytes

Volex Sedimentation (VS). The LRPRP supernatant tubes were centrifuged at 100 g for 5 min to remove additional erythrocytes (Fig. 2, step 2). After centrifugation, the supernatant was again removed and placed into two additional 50-ml conical tubes. These tubes were then centrifuged for 10 min at 160 g to create platelet-rich plasma (PRP) and a button of mixed cells including the leukocytes (Fig. 2, step 3). Twelve milliliters of the supernatant were pipetted into two 15-ml tubes. The tubes were centrifuged at 3,200 g for 10 min to remove all cellular matter and create the platelet-poor plasma (PPP) (Fig. 2, step 4). PPP was used for the final resuspension of the separated leukocytes. The remainder of the supernatant was discarded, and 5 ml of 12.6% ACD/NS (ACD mixed with 0.9% NaCl, v/v) were added to the leukocyte button. This suspension was centrifuged at 100 g for 5 min to remove additional platelets which remained in the supernatant (Fig. 2, step 5). After this centrifugation the supernatant was removed and discarded. The bottom pellet was resuspended in 2 ml of PPP (Fig. 2, step 6).

Volex Sedimentation with Hypotonic Lysis (VL). The two LRPRP tubes were centrifuged at 160 g for 10 min (Fig. 3, step 1). After centrifugation, 12 ml of the PRP were pipetted into

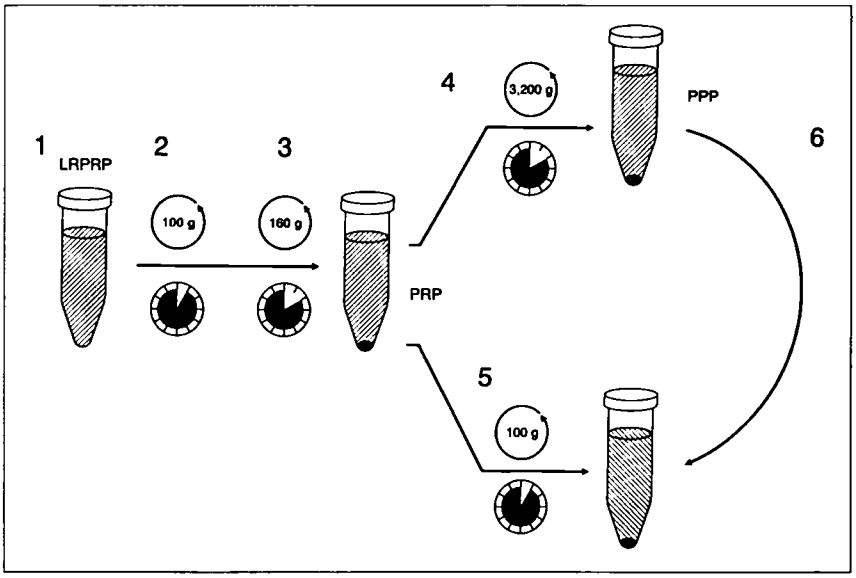

FIGURE 2. Schematic diagram of various steps of the VS leukocyte separation technique. PRP = platelet-rich plasma; PPP = platelet-poor plasma.

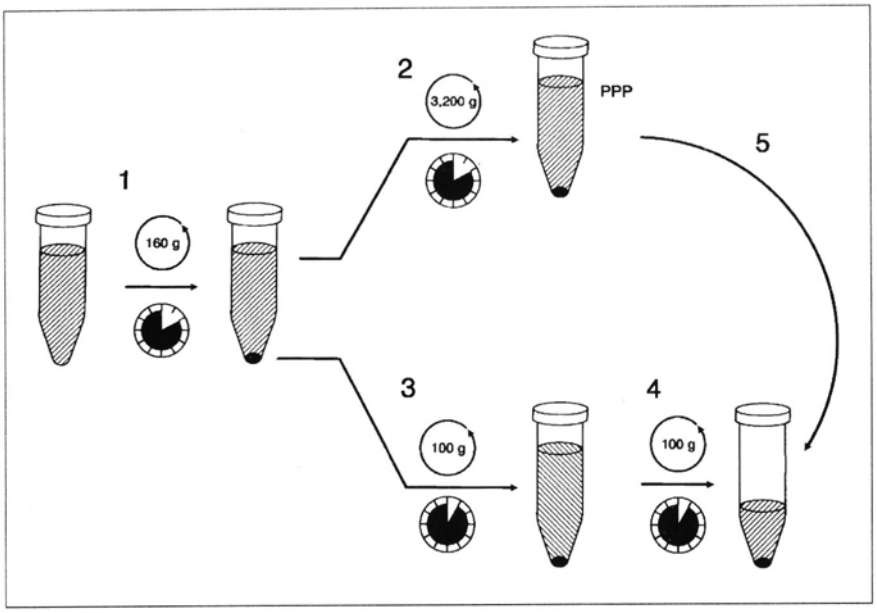

FIGURE 3. Diagrammatic illustration of the VL leukocyte separation method.

two 15-ml conical tubes and centrifuged for 10 min at 3,200 g to create the PPP (Fig. 3, step 2). The remainder of the supernatant was discarded, and the cellular pellets were pooled into one 50-ml conical tube. Ten milliliters sterile water were added to the 50-ml conical tube to lyse the erythrocytes present. The sterile water was allowed to interact with the cells for 15 sec (*14*). Immediately after the 15-sec exposure, 2 ml of 5% NaCl and 10 ml of 12.6% ACD/NS were added to restore isotonicity. This lysed suspension was centrifuged at 100 g for 5 min (Fig. 3, step 3). After centrifugation, the supernatant was discarded and 8 ml of 12.6% ACD/NS were added to the bottom button. After resuspension, the tube was again centrifuged at 100 g for 5 min (Fig. 3, step 4). The supernatant was again removed and the leukocyte button resuspended in 2–3 ml of PPP (Fig. 3, step 5).

Ficoll-Hypaque Two Density Gradients (FH). Three to four milliliters of LRPRP supernatant were pipetted into each of twelve 15-ml conical tubes for each study (Fig. 4, step 1). Three milliliters of Ficoll-Hypaque gradient I were very carefully added to the bottom of each tube containing the LRPRP, using a 20-g spinal needle attached to a 12-ml syringe containing 12 ml of Ficoll-Hypaque gradient I (specific gravity = 1.08 g/ml). Once all tubes contained the plasma and Ficoll-Hypaque gradient I, 3 ml of Ficoll-Hypaque gradient II (specific gravity = 1.13 g/ml) were carefully added through the spinal needle to the bottom of each tube (Fig. 4, step 1). The end result was a series of 12 tubes which contained three distinct layers.

The tubes were then centrifuged for 13 min at 1,650 g. During the centrifugation process, the platelets and lymphocytes localized in the plasma and Ficoll-Hypaque gradient I interface. The neutrophils were concentrated at the Ficoll-Hypaque gradient I and Ficoll-Hypaque gradient II interfaces, and the erythrocytes were deposited at the bottom of the tube (Fig. 4, step 1). After centrifuging, a total of 12 ml of the supernatant plasma were removed from 6 of the 12 tubes with a pipette and saved in two 15-ml conical tubes. These 15-ml

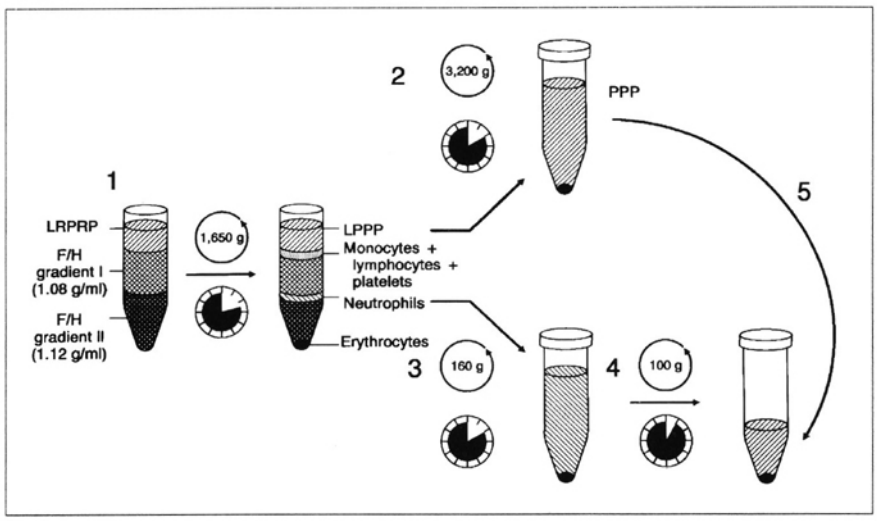

FIGURE 4. Schematic diagram of various steps of the FH leukocyte separation technique.

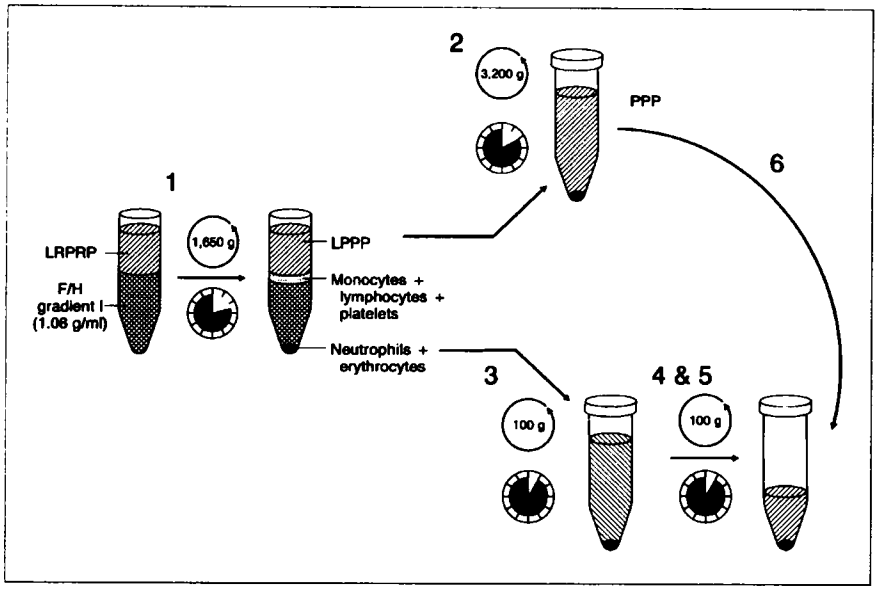

FIGURE 5. Diagrammatic representation of the FL leukocyte separation method.

tubes were centrifuged at 3,200 g for 10 min to remove all cellular components from the plasma (Fig. 4, step 2). The supernatant PPP was saved for the final resuspension of the separated neutrophil preparation. The remainder of the plasma and FH gradient I layers were very carefully removed and discarded. The neutrophil layers were removed with another pipette and pooled into two 50-ml conical tubes. An equivalent volume of 12.6% ACD/NS was added to each of the tubes. The tubes were centrifuged for 10 min at 160 g (Fig. 4, step 3). After spinning, the supernatant was removed and the neutrophil pellet resuspended in 8 ml of 12.6% ACD/NS. The suspension was recentrifuged for 5 min at 100 g (Fig. 4, step 4). Again, the supernatant was removed and the leukocytes resuspended in 2–3 ml of PPP (Fig. 4, step 5). This suspension was used for the studies.

Ficoll-Hypaque Single-Density Gradient with Hypotonic Lysis (FL). After sedimentation, 3–4 ml of LRPRP were pipetted into twelve 15-ml conical tubes (Fig. 5, step 1). Three milliliters of Ficoll-Hypaque gradient I were very carefully added to the bottom of each plasma tube, using a 20-g spinal needle attached to a 12-ml syringe containing 12 ml of Ficoll-Hypaque gradient I. These tubes were centrifuged at 1,650 g for 13 min (Fig. 5, step 1). After centrifuging, 12 ml of the plasma from six tubes was removed and saved in two 15-ml conical tubes. These two tubes were centrifuged at 3,200 g for 10 min to collect the PPP (Fig. 5, step 2). The remainder of the plasma and Ficoll-Hypaque gradient I were removed with a pipette and discarded. The bottom pellet was carefully removed from each tube and pooled together in a 50-ml conical tube. An equal volume of 12.6% ACD/NS was added to the pellet, and the tube was centrifuged at 100 g for 5 min (Fig. 5, step 3). After centrifuging, the leukocyte pellet was exposed to sterile water to lyse the erythrocytes. The lysis was done the same as the VL method using sterile water and restoring isotonicity with 5% NaCl and 12.6% ACD/NS (Fig. 5, step 4) (*14*). The tube was centrifuged for 5 min at 100 g. The supernatant was discarded, and the leukocytes were resuspended again in 8-ml of 12.6% ACD/NS and centrifuged for 5 min at 100 g (Fig. 5, step 5). Finally, the supernatant was removed and the pellet was resuspended in 2–3 ml PPP (Fig. 5, step 6).

CBC

All leukocyte aliquots were analyzed by the Coulter STKS® (Coulter Corp., Hialeah, FL). The STKS is an automated hematology analyzer which yields a full blood count report that includes erythrocytes, platelets, leukocytes and a five-population leukocyte differential (neutrophils, lymphocytes, monocytes, eosinophils and basophils). This instrument measures volume, conductivity and light scatter simultaneously for each cell. The STKS computer system provides a precise analysis of a multicellular blood sample (*15–17*).

An aliquot of each leukocyte suspension was diluted and directed to a flow cell in the STKS in order to perform the blood cell count. The results were displayed in a three-dimensional analysis called a scatterplot (Fig. 6). The scatterplot displays patient information, leukocyte scatterplots, erythrocyte histogram, platelet histogram and five-part leukocyte differential. Calculations for the leukocyte suspension cellular composition were based upon these parameters and the total volume of the analyzed sample.

In Vitro Viability Tests

Chemotaxis. Chemotaxis is a method used to evaluate the viability of neutrophils by their ability to detect and move toward an attractant (i.e., bacteria). This simulates the capabilities of neutrophils in the human body.

The agarose plate preparation was done by the method of Nelson, et al (*18*). The FH or FL leukocyte suspension, E. coli and buffer aliquots were pipetted into holes on the agarose plates. To demonstrate the viability of the neutrophil suspension, these cells should migrate along the plate surface directed toward the E. coli. The greater the distance the neutrophils migrate towards the bacteria, the more chemotaxic abilities the

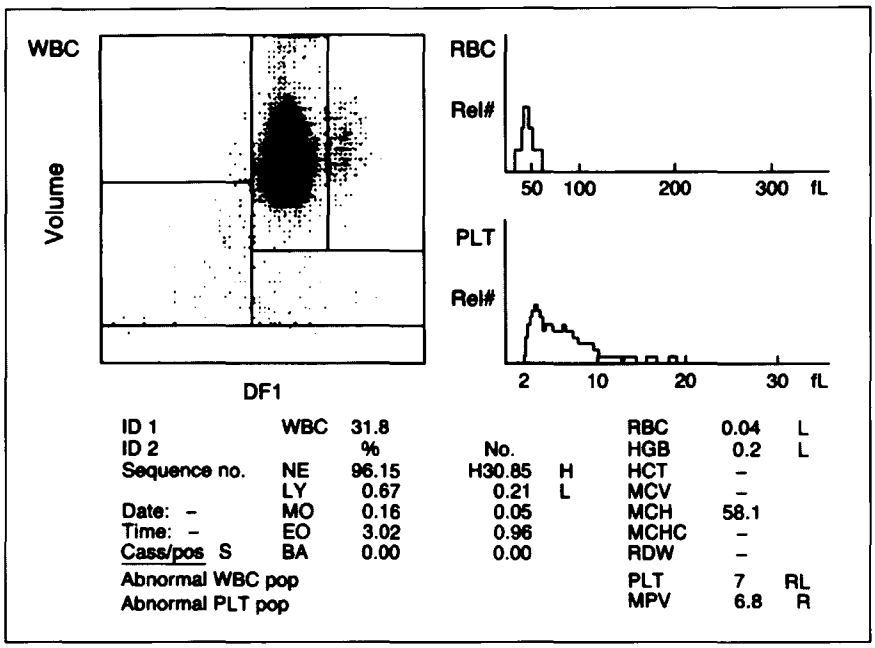

FIGURE 6. Representative display scatterplot of the FH separated leukocyte suspension on a Coulter STKS instrument. WBC = white blood cells; RBC = red blood cells; PLT = platelets; NE = neutrophils; LY = lymphocytes; MO = monocytes; EO = eosinophils; BA = basophils.

cells possess (*18,19*). The migration pattern towards the bacteria is called directed migration (Fig. 7). The migration patterns at the end and sides of the neutrophil hole are patterns of the cells diffusing into the agar and buffer. The migration pattern is called random migration (Fig. 7). The migration patterns were measured using a magnified light reader and a ruler. The calculation of chemotaxis results was done as ratios by dividing the distances of directed migration (mm) by the distances of random migration (mm) (Fig. 7). The ratios were compared to a normal value range of 1.5–2.8.

Trypan Blue Staining. The FH and FL leukocyte suspensions used were stained with trypan blue (Newcomber Stain, Chicago, IL) (*20,21*) to evaluate their viability. Twenty-five microliters of trypan blue were added to a test tube and an equal volume of leukocyte suspension was added to the tube. The mixture was gently mixed and a drop of this solution placed on a hemocytometer. The hemocytometer was placed under a light microscope and the neutrophils were observed for any uptake of trypan blue, indicating that the cells were nonviable.

RESULTS

CBC

CBC was performed on leukocyte samples obtained from the four leukocyte separation techniques (Table 1). The VS and VL suspensions contained the larger number of the leukocytes, with the VL having the greatest number of

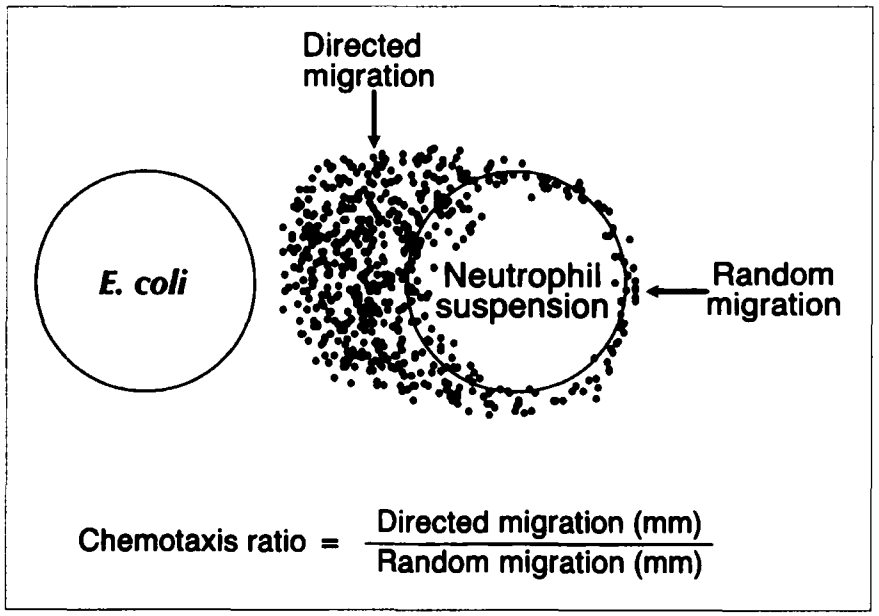

FIGURE 7. Graphic representation of chemotaxic migration of neutrophils towards an attractant (E. coli) and formula for calculation of the chemotaxis ratio.

TABLE 1
Comparison of CBC from Four Leukocyte Separation Methods*

Blood component	VS (n = 6)	VL (n = 6)	FH (n = 5)	FL (n = 5)
Leukocytes ($\times 10^6$)	53.9 ± 25.5	90.3 ± 27.9	32.5 ± 12.7	43.2 ± 15.1
Neutrophils (%)	64.9 ± 13.0	68.2 ± 9.9	93.7 ± 3.1	93.6 ± 2.9
Erythrocytes ($\times 10^6$)	99.2 ± 43.2	54.4 ± 35.6	2.2 ± 2.4	3.3 ± 4.5
Platelets ($\times 10^6$)	251.3 ± 184.5	348.8 ± 193.8	8.4 ± 13.4	9.3 ± 1.5

*All values are expressed in mean ± s.d.

leukocytes present. The density gradient separations (i.e., FH and FL) contained fewer total leukocytes, but the percentages of neutrophils were higher than with the VS and VL methods (Table 1). The amounts of erythrocytes and platelets present were higher in the VS and VL techniques, and reductions of both cell types were noted with the FH and FL methods.

Preparation Time. In comparing the length of time to perform each of the four separation methods, a significant time difference was noted. The VL method required 90 min, the least amount of time. The VS and FL needed 2 hr and the FH method required a minimum of 3 hr to complete the separation.

In Vitro Viability Tests

Chemotaxis. The FH chemotaxis ratios were 2.2 ± 0.3%, and the FL ratios were 2.2 ± 0.5%. The data from both methods yielded values which were in the normal range for chemotaxis (i.e., 1.5–2.8). These results indicated that the neutrophils obtained from the FH and FL leukocyte separation techniques retained their viability in vitro by the E. coli chemotaxis assay.

Trypan Blue Staining. The FH and FL suspensions examined by this technique yielded neutrophils which did not concentrate the dye, indicating viable neutrophils.

DISCUSSION

The use of radiolabeled leukocytes is important to the diagnostic evaluation associated with infectious and inflammatory processes in patients (*2–9*). The neutrophils are the cells responsible for fighting infectious processes in the human body. The techniques for leukocyte separation are done in vitro and may involve damage to the leukocytes and contain some contaminants (erythrocytes and platelets), which may interfere with the imaging (*10*). It is important to have a population of viable and functioning leukocytes with as few contaminants as possible. Questions remain about the viability and function of leukocytes, especially leukocytes following exposure to a hypotonic solution (*2,8*).

The first part of our study evaluated the four methods of leukocyte separation for cellular contaminants. Comparing the four separation methods, we observed that any technique using hypotonic lysis demonstrated a reduction in erythrocytes. Also density gradient separation techniques (FH and FL) removed most of the platelets and greatly reduced the total number of erythrocytes. Overall, the VL method demonstrated the greatest number of leukocytes present, but the platelet number was the highest. The FL technique exhibited a high number and percentage of neutrophils with low platelet and erythrocyte contaminants.

The FL method is attractive for clinical use due to the ease with which the blood contaminants may be removed. This technique removed the platelets and lymphocytes in Ficoll-Hypaque gradient I, and the hypotonic lysis removed the erythrocytes. In our experience with the FH method, erythrocytes may be present in the neutrophil layer, adding to the number of contaminants present.

The FH method was the most technically demanding due to the addition and removal of the two density gradients. This is a time-consuming process which requires a minimum of 3 hr to complete the leukocyte separation and radiolabeling. The density gradient solutions used for FH were prepared in- house and required several days to equilibrate. The FL method only uses one density gradient and requires 2 hr to finish the leukocyte separation and labeling.

The work of Chowdhury, et al. (*14*) has previously demonstrated that leukocyte suspensions from VS, FH and VL maintain their in vitro viability with these separation techniques. For our study, we compared a new method, FL, separated neutrophils to FH neutrophil suspensions. The comparison was made due to the similarity of both techniques using density gradients and high percentage (>90%) of neutrophils extracted (Table 1). The in vitro chemotaxis assay using E. coli as a chemoattractant demonstrated that the FH and FL neutrophil suspensions retained their chemotaxic ability and migrated towards the E. coli. This indicated a viable and functioning neutrophil population in vitro. Staining the leukocyte suspensions with trypan blue showed that the dye was not concentrated by any of the FH and FL leukocytes. This was another indicator of the viability of the leukocyte preparation used for these studies. Therefore, chemotaxis and trypan blue staining results determined that all of the FH and FL samples contained viable cells.

CONCLUSION

The use of ^{111}In-oxine- and ^{99m}Tc-HMPAO-labeled leukocytes for imaging infection and inflammation remains of clinical importance. For optimal results, the radiolabeled leukocyte preparation should contain as few cellular contaminants as possible, a viable population of leukocytes and the greatest number of neutrophils extractable. Our study indicated that

the method of choice for leukocyte separation was FL. It has a low number of erythrocytes and platelets present, with >90% neutrophils present (Table 1). It also contains viable leukocytes as demonstrated by the in vitro viability tests and can be performed in a reasonable amount of time. We believe this method should be considered for routine clinical use.

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