# The Function, Physiology, and Isolation of Granulocytes

Radiolabeled granulocyte scintigraphy has become a well established method for the detection of occult infectious and inflammatory processes (*1,2*). Those involved with the labeling of granulocytes, as well those interpreting the biodistribution following injection, should be familiar with normal function and physiology of granulocytes as well as consideration of the separation and quality control techniques.

Various aspects of granulocyte function, physiology, and isolation will be discussed in this review and are applicable independent of which radiopharmaceutical is used for the actual labeling process. Excellent review papers may be consulted for details and considerations of the labeling processes (*1–5*).

## GRANULOCYTE FUNCTION AND PHYSIOLOGY

### White Blood Cell Types

White blood cells (WBCs) or leukocytes constitute a major defense system within the body and are divisible into three major classes: polymorphonuclear leukocytes (or granulocytes), lymphocytes, and monocytes. Figure 1 illustrates their appearance, size, and gives their relative abundance in the circulation.

***Polymorphonuclear Leukocytes (PMNL).*** PMNLs are called as such because the nuclei of the cells in the circulation are segmented to form two or more lobes. PMNLs are also called granulocytes since they have numerous granules in their cytoplasm. The coloration of these granules after reaction with Wright's stain determines the further subdivision of PMNLs into three classes: neutrophilic PMNL or neutrophils have fine granules which are stained pale yellow-pink or almost colorless, eosinophilic PMNLs or eosinophils have large bright orange-red granules, and basophilic PMNLs or basophils contain purplish-black granules. Neutrophils are the most abundant so that in some cases the terms granulocyte or PMNL are used as synonyms for neutrophil, and other granulocytes are termed "eosinophil" or "basophil." Granulocytes cannot divide, and once in the tissues they do not return to the blood. They, therefore, must be replaced continuously by new granulocytes from the bone marrow.

***Lymphocytes.*** These cells are generally smaller than neutrophils (unless activated), lack granules, and have a relatively round dense nucleus. The two primary functional classes of lymphocytes are T- and B-lymphocytes. T-lymphocytes typically make up ~ 70% of the lymphocytes in the blood and are involved in cellular immune processes and in the regulation of antibody synthesis (*6*). B-lymphocytes make up 10%–20% of the circulating lymphocytes. B-cells participate in humoral immune processes and are the precursors of the principal antibody-forming cell of the body, the plasma cell. T-cells (and to a lesser extent B-cells) move back and forth between the tissues and the blood and, thereby, continuously monitor the antigens throughout the body. The average life span of circulating lymphocytes as immunocompetent quiescent cells has been estimated to be 4.4 yr but for some it may exceed 20 yr (*7*). When exposed to an immunologic determinant to which it is programmed, a quiescent lymphocyte undergoes activation and divides into a clone of lymphocytes responsive to that immunologic determinant.

***Monocytes.*** These are large cells with an oval, indented nucleus, which circulate for ~ 12 hr before entering tissues and differentiating into macrophages. Included among the tissue macrophages are: lung macrophages, Kupffer cells of the liver, microglial cells of the brain, macrophages of the spleen and lymph nodes, and (possibly) the osteoclast. These phagocytes have many functions including defending against intracellular parasites and microbial invaders, removing cells that are damaged or dying, and aiding in the remodeling and repair of tissues, including bone (*7*).

### Neutrophil Production

The production and maturation of neutrophils occurs in the bone marrow where they may be considered to be in two divisions: mitotic and maturation-storage compartments. The development stages are depicted in figure 2.

***Mitotic (Proliferative) Compartment.*** This consists of the progressive division and development from myeloblasts through to promyelocytes and then to myelocytes. Each of these cells is capable of replication. It is estimated that 4–5 cell divisions occur from the myeloblast to myelocyte so that 16–32 myelocytes are derived from each myeloblast (*8*).

***Maturation-storage Compartment.*** This consists of metamyelocytes, band neutrophils, and mature PMNLs. The cells do not divide in this compartment. The mature neutrophils act as a reservoir that typically contains many more neutrophils than are contained in the circulation. The myelocyte-to-blood transit time is estimated to be 5–7 days (*8*). During maturation the granulocyte acquires the ability to undergo

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MAJOR LEUKOCYTE CELL TYPES

| | subclasses | average size | % circulating leukocytes (normal adults) |
|---|---|---|---|
| polymorphonuclear leukocytes | neutrophil | 9–15μm | 55–67 % |
| | eosinophil | 9–15μm | 2–3% |
| | basophil | 9–15μm | 0.5–0.7% |
| lymphocytes | T & B lymphocytes | 7–8μm* | 23–38 % |
| monocytes | monocytes and tissue macrophages | 12–20μm* | 4–6 % |

* Size increases up to two fold when cells are activated

**Fig. 1.** Major leukocyte cell types. The values listed for percentage of circulating leukocytes are for normal healthy adults (*12*). Other publications and institutions may use a broader range for acceptable normal ranges of each cell type.

| | Stage of Maturation | Percent of Total Nucleated Bone Marrow Cells |
|---|---|---|
| Precursor cells | Commited stem cell | ? |
| Mitotic Pool | Myeloblast | 0.2 - 1.5 |
| | Promyelocyte | 2 - 4 |
| | Myelocyte | 8 - 16 |
| Maturation Storage Pool | Metamyelocyte | 10–25 |
| | Band form PMNL | 10–15 |
| | Polymorphonuclear leukocyte | 6–12 |

**Fig. 2.** Maturation stages of neutrophils and their relative abundance in bone marrow. [Adapted with permission (*9*)].

chemotaxis, ingest particulate matter, and kill microorganisms (*9*). Compartment turnover studies indicate that the normal production rate is $0.62 - 4.0 \times 10^9$ cells/kg per day (*7*) (40–280 billion cells/day for a 70-kg standard man). The stimulus for proliferation is thought to be from a protein called colony stimulating factor (CSF) (and granulopoietin) released from activated mononuclear cells, tissue macrophages, and lymphocytes. During stress and infection, the production rate may increase several fold by: decreasing the maturation time, skipping divisions, and prematurely releasing immature cells into the circulation (*7,9*).

## Granulocyte Turnover and Kinetics

Neutrophils leave the marrow storage compartment (Fig. 3) and enter the blood without significant reentry into the marrow. The total blood neutrophil pool consists of all neutrophils in the vascular spaces (*8*). Half the neutrophils circulate (circulating granulocyte, pool, CGP) and are readily sampled by venipuncture. The remainder of the PMNLs (marginated granulocyte pool, MGP) are reversibly marginated along the walls of small blood vessels such as postcapillary venules in various body tissues (e.g., liver and spleen) (*7*). The maximum intravascular recovery after an injection or transfusion of neutrophils is 40%–50% (*10*). With $^{111}$In oxine-labeled neutrophils, the recovery of cells in the CGP is slightly lower (30% in man) than calculated by other methods (*11*). Marginated cells are thought to be functionally more mature than circulating neutrophils as assessed by levels of granulocyte alkaline phosphatase, a marker of cell maturity (*12*). The CGP and MGP each contain ~ 20–25 billion cells in a 70-kg man (Fig. 3) and readily exchange between both compartments (*7*). Cells shift from the MGP to the CGP with exercise, fever, epinephrine injection, or stress.

Neutrophils are end-stage, highly differentiated cells that do not divide. Once they leave the bloodstream, they do not reenter it. Exit from the blood has been thought to be random, rather than age-related with an estimated half-life of 6 to 7 hr (transit time of 12 hr) (*7*). The circulation time of the neutrophil is very short when compared with red blood cells (120 days), lymphocytes (4 yr), and platelets (7 days) (*9*). The granulocyte turnover rate averages 0.62–4 billion neutrophils/kg a day (*7*). Upon leaving the vascular space, the neutrophils migrate into tissues where they perform their phagocytic

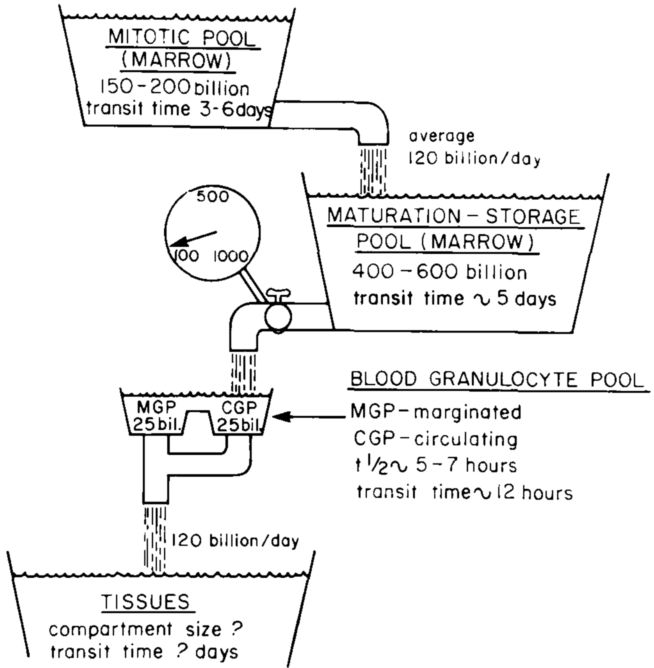



**Fig. 3.** Normal neutrophil turnover in a 70-kg standard healthy person is schematically represented as a series of pools that drain into one another at an average rate of 120 billion cells per day. The turnover can be increased ten fold on demand. Average transit times and cell numbers are shown in each compartment. The blood granulocyte pool is composed of two fractions, the marginated and circulating granulocyte pools, with ready exchange between them. (Adapted from Refs. *7, 9, and 12*).

functions and eventually die or are excreted. Neutrophils normally migrate primarily to the spleen, liver, lung, and to a lesser extent to the oral cavity, and gastrointestinal tract (*8*). Three to nine percent of $^{111}$In-labeled PMNLs also localizes in the bone marrow (*10*). Four hours following the injection of $^{111}$In oxine-labeled granulocytes, 29% of the administered radioactivity was in the spleen, and 21% was in the liver (*10*).

Many (up to 10%) of circulating neutrophils in rats (*13*) migrate to sites of inflammation. Neutrophils live another 2–5 days in normal tissue, but their survival may be much shorter in sites of infection and inflammation (*12*).

## Neutrophil Function and Physiology

The major function of neutrophilic granulocytes is to prevent or retard the intrusion of infectious agents and other foreign material into the host environment. This is accomplished by phagocytosis and digestion of the material. Neutrophils also have a secretory function as they release various substances into their environment (*7*). The importance of neutrophils is amply demonstrated by the frequency of serious bacterial infections with high mortality rates in patients with agranulocytosis (circulating blood levels of $< 500$ per $\mu$l). Phagocytosis is a complex series of physiologic events involving the following phases as shown (Fig. 4): (a) chemotaxis (b) recognition, (c) engulfment, and (d) killing of microorganisms.

***Chemotaxis.*** Chemotaxis refers to the directed movement of a phagocyte along a concentration gradient toward an offending particle (*12*). Neutrophils, eosinophils, basophils, and mononuclear phagocytes are all able to undergo chemotaxis. A concentration gradient (gradually increasing concentration) is necessary so that directional rather than random migration occurs (*7*).

Bacterial invasion or tissue necrosis initiates the production of chemoattractants from a wide variety of systems such as complement system, kinin generating system, and the clotting system as well as from lymphocytes, mononuclear phagocytes, and neutrophils. A few chemotactic agents are listed in Table 1 and more detailed information may be found elsewhere (*8*).

At a local site of tissue damage or infection, neutrophils adhere to endothelial cells of the vessel wall and are able to migrate into tissue within minutes (*7*). The adherence of the neutrophils to the endothelial surface is probably enhanced by certain chemotactic agents (*14*). After the initial sticking, neutrophils undergo diapedesis and project pseudopods between endothelial cells and force a passage through the en-

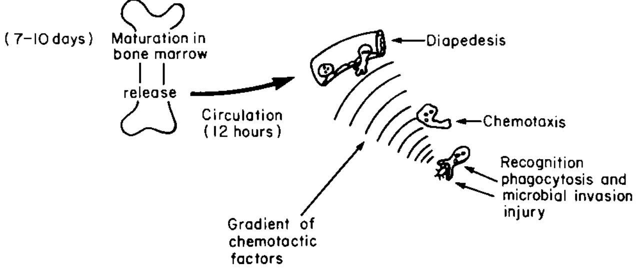



**Fig. 4.** Fate and function of neutrophils. (Adapted from *Ref.* 9 with permission.)

**TABLE 1. Chemotactic Factors**

1. **Activated complement components:** C3a and C5a generated by the antigen-antibody reaction on the surface of an organism or by nonspecific proteases (*12*). C567 complement reaction products are also chemotactic.
2. **Proteolytic and coagulation system proteins:** Kallikrein produced from prekallikrein following the activation of the clotting cascade factor XII (Hageman factor) by bacterial endotoxins (*12*).
3. **Fibrin split products:** e.g., fibrinopeptide B (*8*).
4. **Neutrophil secretions and lymphocyte secretions (lymphokines)** such as leukotriene B4 which is an oxidation product of arachidonic acid formed in activated neutrophils (*8*).
5. **Bacterial and mitochondrial protein breakdown products** such as N-formyl-methionyl-oligopeptides (*6*).
6. **Platelet factors**: Platelet factor 4 and platelet derived growth factor from platelet alpha granules.

dothelium. The PMNLs then move between the endothelium and basement membrane until a passage into surrounding connective tissue is found (*7*).

It has been postulated that the chemotactic agents occupy specific receptor sites on the outer surface membrane of the PMNLs that signals the cell's motility system (*7*). Once exposed to chemotactic agents, the neutrophil loses its ability to respond a second time (*8*). It is, therefore, very important that separating and labeling techniques do not expose the neutrophils to chemotactic agents such as bacterial endotoxins and lipopolysaccharides (*15*).

The PMNLs migrate in an ameboid manner through the tissue by adhering and crawling along tissue fibers by mechanisms involving complement, $Mg^{++}$, contractile proteins, actin, microtubules, microfilaments, and ATPase activity (*9, 12,16*).

***Recognition.*** The granulocytes leaving the circulation encounter microorganisms or damaged cells, which will be ingested if they are appropriately recognized as foreign entities or damaged tissue. An important mechanism for rendering microbes and particles recognizable is a process whereby serum components coat the microbes. The molecules that coat organisms and enhance their ingestion are called opsonins (from the greek meaning, "to prepare for dining") (*9*). These molecules are normal constituents of plasma. The major plasma opsonins are specific IgG antibodies and complement fragment C3b which become deposited on the microorganisms (*9*). Polymorphonuclear leukocytes contain cell surface membrane receptors, which recognize the complement (C3b) and IgG (Fc fragment) opsonins coating the particle. Without opsonins, phagocytosis can occur but generally much more slowly (*12*) by nonspecific interactions that may or may not lead to recognition (*16*).

***Engulfment.*** Once attachment occurs, the phagocytic cell membrane invaginates and pseudopodia extend to envelop the opsonized objects (Fig. 5). Fusion of the pseudopodia on the opposite side of the particle encloses the foreign material in a ring of invaginated cell membrane (*12*). This phagocytic vacuole or phagosome becomes detached and is now the container for the digestion of the ingested particle by the contents of neutrophil granules (*12*).

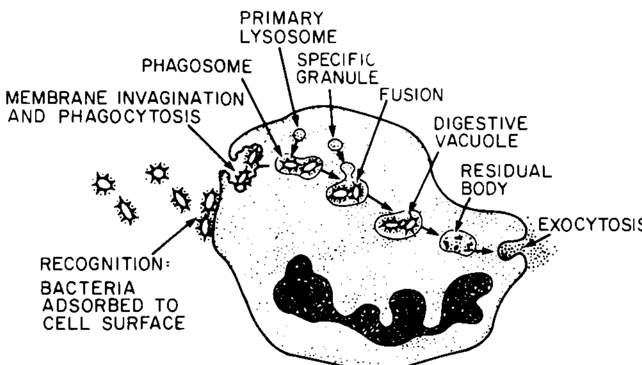



**Fig. 5.** Schematic representation of neutrophil recognition, engulfment and digestion of bacteria. Bacteria coated with opsonins undergo phagocytosis. The internalized phagosome fuses with cytoplasmic granules that kill and digest the bacteria so that only the remnants are expelled by exocytosis. (Adapted from Ref. 7 with permission.)

The segregation of the phagocytic vacuole away from the cytoplasm of the PMNLs helps prevent damage to the phagocyte itself as degradative enzymes and reactive metabolites are released into the phagosome to attack the microbes.

The process of ingestion, like chemotaxis, is driven by the actin system of fibers beneath the cell membrane and requires myosin, energy, and ATP (*12,16*).

***Killing and Digestion of Microorganisms.*** The vital step of killing the ingested organisms is accomplished by the fusion of the cytoplasmic granules (lysosomes) with the phagocytic vacuole (phagosome), followed by discharge of the contents of the granules into the vacuole (*7*). A dramatic increase in oxygen consumption occurs in the stimulated neutrophil (the respiratory burst), which is due to a series of metabolic events that include: a sharp increase in cellular oxygen uptake, oxidation of NADPH, stimulation of glycolysis and lactate production, increased use of the hexose-monophosphate shunt, and a drop in cellular pH (*12*).

Killing of ingested organisms involves several systems, most of which are dependent on oxygen (*12*). Certain highly unstable and reactive oxygen metabolites such as hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^-$), hydroxyl radicals, and singlet oxygen (*7,12*) are produced in the phagosome and have potent bactericidal capabilities (*9,12*). An enzyme, myeloperoxidase, from the primary granules forms a very potent antimicrobial system in combination with hydrogen peroxide and halide cofactors (*7*). The oxygen-independent antimicrobial systems of the neutrophil include acid, lysosome, lactoferrin, and granular cationic proteins. Release of the various bactericidal agents into surrounding tissue during phagocytosis is believed to damage normal tissue so that they are thought to be important mediators of inflammation (*9*). Details of the interactions of the various systems involved in bacterial killing may be found in recent review papers (*7,12, 16*).

### Physiologic and Pharmacologic Variations in Neutrophil Numbers

The normal level of circulating leukocytes is 4,000–11,000/$\mu$l of blood (4–11 $\times$ $10^9$ per litre) (*12*) and usually 55%–67% of them are neutrophils. A large number of physiologic conditions or pharmacologic agents are known to alter the number of neutrophils present in the circulating blood.

***Infection, Inflammation, and Necrosis.*** The presence of microbes or damaged tissues releases chemotactic factors, which causes release of neutrophils into the circulating pool from the marginal granulocyte pool and the storage compartment of the bone marrow. A persisting stimulus results in an increased production rate of neutrophils and neutrophilia (increased circulating level of neutrophils) (*9*). A strong stimulus may raise the level of circulating neutrophils as much as 10 fold and immature forms may appear in the blood. Pain, nausea, vomiting, and emotional stress can also lead to increased circulating neutrophils (*9,12*). Some viral infections may actually result in a decreased neutrophil count due to a direct toxic effect of the virus infection on the production of phagocytes in the bone marrow (*9*).

***Exercise.*** Marked elevation of WBC peripheral count occurs following strenuous exercise. Counts as high as 35 $\times$ $10^9$/l have been recorded for a runner after sprinting (*7*). Blood levels usually recede back to normal within an hour. The source of the temporary increase is primarily a shift of the neutrophils from the marginal to circulating pool (*7*).

***Epinephrine.*** Injection of epinephrine normally results in the mobilization of granulocytes into the circulation from the marginal pool causing neutrophilia within 5–10 min that lasts $<$ 20 min. Subcutaneous injection of epinephrine 4 hr after reinjection of $^{111}$In oxine-labeled granulocytes resulted in a 65% increase of cell associated radioactivity and circulating granulocyte count within 20 min (*10*). Epinephrine can, therefore, be used to assess abnormalities of cell distribution in these pools (*10*) and may be a useful quality control measure of damage of neutrophils from new labeling and cell separation techniques.

***Endotoxins.*** Endotoxins and fever result in an initial decrease in the circulating granulocyte pool due to increased cell margination and sequestration (*8*). This is followed by an increased neutrophil blood count as more neutrophils are released from the bone marrow (*17*).

***Steroids.*** Glucocorticoids such as cortisol or prednisone increase the blood neutrophil count by increasing the influx from the marrow and marginal granulocyte pool and also decreasing the egress from the circulation. The t-1/2 in blood increases from 6 to 10 hr (*8*). The accumulation of the neutrophils at induced sites of skin inflammation is decreased (*8*). Dexamethasone has opposite effects. It increases the mobilization of neutrophils from the circulation and increases accumulation at inflammatory sites (*8*).

## GRANULOCYTE SEPARATION TECHNIQUES

### Requirements of Separation Techniques

Most radioactive agents for cell labeling are nonselective and will label all cell types (erythrocytes, granulocytes, lymphocytes, monocytes, and platelets) to some extent in a blood sample. It is, therefore, generally necessary to isolate the leukocytes or neutrophils from whole blood before labeling.

The requirements of an ideal separation technique of leukocytes or granulocytes are:

1. Aseptic technique is maintained throughout.
2. Mechanical trauma from centrifugation and pipetting techniques are minimized.
3. Cells are maintained in a relatively physiologic environment.
4. Cells are reinjected as soon as possible (Many separation techniques require a considerable proportion of the normal circulating t-1/2 of a neutrophil [5–7 hr].).
5. A relatively pure population of cells is isolated without contamination from other cell types (For example, in an average blood sample, the red blood cells (RBCs) typically outnumber WBCs 700–1000:1.).
6. Neutrophil losses throughout the procedure are minimized (labeling efficiency generally increases with higher cell numbers and concentrations).
7. A viable population of neutrophils is obtained which functions normally.

Not all of the requirements can necessarily be met concurrently to their full extent with the separation techniques available today. For instance, low centrifugal speeds for short periods help to minimize the platelet contamination in crude white cell pellets (see 5 above) and are also probably less mechanically traumatizing to the cells (see 2 above), but the number of the neutrophils recovered may not be as high as compared to a longer faster centrifugation speed (see 6 above).

There is no standard method of isolating the leukocytes and granulocytes, and many variables exist between laboratories in the steps and reagents used. The advantages and disadvantages of various steps and reagents will be discussed but details of specific protocols are best obtained from the original articles.

## Collection of Blood

Blood is collected into syringes containing either acid-citrate-dextrose (ACD, NIH formula A) or heparin as anticoagulants. ACD may be preferred since there is less adherence of the neutrophils to plastic tubes and pipettes (*2,18*), but heparin is still widely used (*19*). ACD is typically used at 15% of final anticoagulant-blood volume (*19*). Heparin is used in the range of 250–1000 IU heparin/ml blood (*18*).

## Erythrocyte Sedimentation

When anticoagulated whole blood is allowed to stand for a period of time, the RBCs settle out from the plasma. The rate at which this occurs is termed the erythrocyte sedimentation rate (ESR), and a number of factors affect it (*20*). An elevated ESR is frequently a nonspecific response to tissue damage that accompanies acute and chronic infections, tumors, and degenerative diseases (*20*).

If the ESR of the blood sample is normal, an erythrocyte aggregating agent is typically added to increase the rate of sedimentation of the RBCs. The erythrocyte aggregating agents are generally polysaccharides (such as hydroxyethyl starch, methylcellulose, and dextran) that change the erythrocyte surface so the cells no longer repel one another. The resulting aggregated red cells have an increased mass and consequently settle faster (*20,21*).

Hydroxyethyl starch* is generally the most preferred erythrocyte sedimentation agent since it is approved by the FDA for administration to humans (primarily for use as a plasma expander) (*2*). For erythrocyte sedimentation, final concentrations of hydoxyethyl starch in anticoagulated blood are typically 0.6%–1.0% (Hespan is supplied as 6% suspension) (*2,21*). Many patients with infectious disease will already have an elevated sedimentation rate so that an erythrocyte aggregating agent is not required (*19*).

The blood is allowed to settle for 30–60 min (shorter for patients with increased ESR) at either room temperature or 37 degrees celsius (*21*). It is advisable to remove any bubbles present as they will retard the settling of the RBCs. Wider tubes and a 30-degree incline may increase the sedimentation rate since the aggregated erythrocytes can pass through a shorter column of cells before contacting the side and rolling along it to the lower portion of the tube.

## Separation of Crude Leukocytes

The upper layer of the sedimented blood contains about 50%–70% of the leukocytes (lymphocytes, monocytes, and PMNL) obtained in the original blood sample (*18*) and has been termed the leukocyte-rich platelet-rich plasma (LRPRP) (*21*). This fraction also contains plasma, platelets and some remaining RBCs. A syringe or sterile plastic pasteur pipette is used to decant the LRPRP to sterile tube(s) for centrifugation (Fig. 6). A centrifugation speed is chosen so as to create leukocyte pellet but leave the platelets in suspension. Some workers centrifuge the cell suspension at forces up to 500 g for 10 min but a slower spin of 100–150 g for 5–8 min may be preferred since the leukocyte pellet is softer, there is less mechanical trauma for the cells and less platelet contamination occurs (*18,21*).

***Minimal Volume Technique.*** McAfee, et al. described a minimal volume technique (*2*) in which cell transfers and the

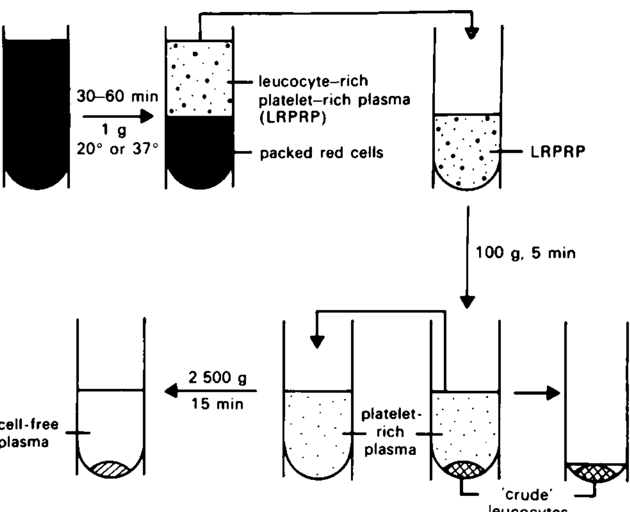
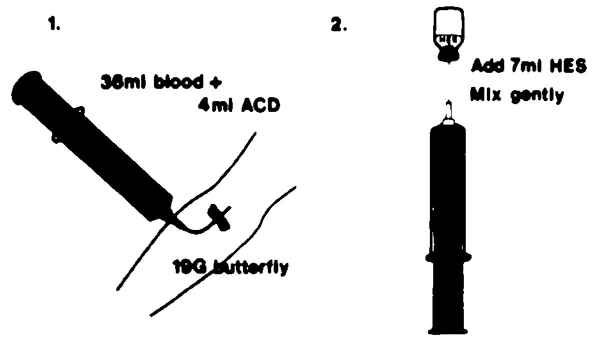
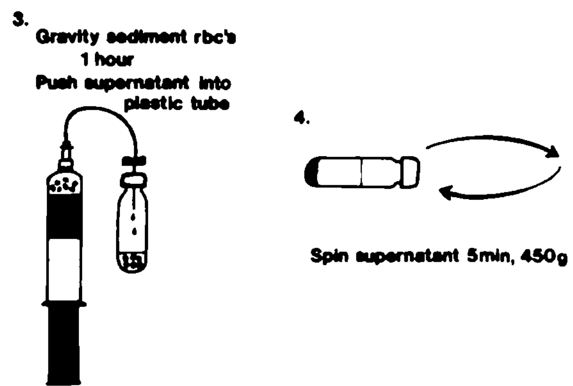



**Fig. 6.** Separation of mixed leukocytes from whole blood. [Reproduced with permission (*21*)].

use of multiple sterile tubes are minimized (Fig. 7). Gravity sedimentation is performed directly in an inverted plastic syringe with a bent needle so that the supernatant can be expelled after sedimentation simply by using the syringe plunger.

***Purity of Mixed Leukocyte Preparations.*** Since the isolation of mixed WBCs is relatively straightforward, many laboratories use the mixed cells for radiolabeling with agents such as $^{111}$In oxine (*18*), tropolone (*22*), acetylacetone (*23*), mercaptopyridine (*24*), or $^{99m}$Tc HMPAO (*25*). The lipophilic complexes used for radiolabeling are nonselective and will label all the cells in a mixture to varying degrees. Therefore, the biodistribution of all cell types will be followed. As little as 22 ± 14% of the radioactivity may be associated with the granulocytes and lymphocytes (*26*).



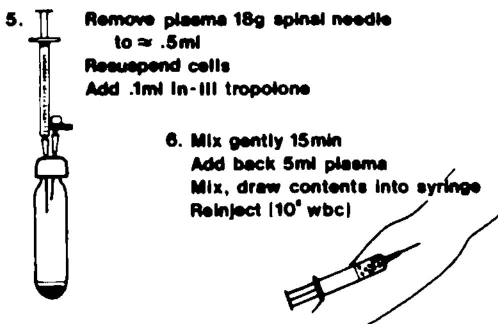




**Fig. 7.** Minimal volume technique for separating and labeling mixed white blood cells with $^{111}$In tropolone. [Reproduced with permission (2)].

Lymphocytes normally compose ~ 23%–38% of the circulating leukocytes so that the nonspecific labeling processes will result in a considerable fraction of the radioactivity being associated with the lymphocytes in a radiolabeled mixed leukocyte preparation (*22*). A much longer circulating half-life for the reinjected lymphocytes (average of 4.4 yr) as compared to the granulocytes would result in an increased blood pool (*7*). Lymphocytes are also very radiosensitive long-lived cells with the capacity to divide. Therefore, the possibility exists that some radiolabeled cells may undergo a mutagenic change. A small increase in abnormal chromosomes has not been thought to be significant by some investigators (*19*).

Frequently, it is assumed that lymphocytes are the only contaminating cells in the fraction obtained, but many mixed leukocyte pellets will contain 15 times as many platelets and 4 times as many RBCs as granulocytes (*22*). Following labeling with $^{111}$In tropolone or $^{111}$In acetylacetone, averages of 29%–34% of the radioactivity of the mixed white cell pellets have been associated with the contaminating platelets and 30% with the red cells (*22,27*). Even by optimizing conditions to minimize platelet and RBC contamination in a mixed leukocyte preparation, 8% of radioactivity was associated with RBCs and 30% with the platelets and lymphocytes (*28*). Since the contaminating RBCs and platelets have blood circulating half-lives of 20–300 times those seen for granulocytes, the blood pool would be increased and there would be an opportunity for false-positive studies due to platelet or RBC localization.

Schauwecker, et al. (*29*) reported that in acute infections there was no significant diagnostic difference between radiolabeled mixed leukocytes and purified granulocytes although they did find a higher average visual score for the purified granulocytes. The method utilized to prepare their "mixed" leukocyte preparation involved an extra density gradient purification step which would remove contaminating platelets and RBCs (*29*). Therefore, the results are not easily extrapolated to mixed white cells typically used clinically.

The use of pure granulocytes has the advantage that labeled cells can be used for quantitative studies such as the measurement of cell kinetics (*10,11*) or measurement of the loss of granulocytes in the bowel for patients with inflammatory bowel disease (*22*).

Experienced technologists are able to prepare radiolabeled granulocytes in a time period comparable (e.g., 75–120 min) to those required to label mixed WBCs. Density gradients for granulocyte isolation (see below) can be prepared while waiting for the RBCs to settle so only one additional centrifugation is needed before radiolabeling. A shortened RBC settling time may also be utilized since contaminating RBCs will be further removed during the density gradient centrifugation step.

***Red Blood Cell Lysis.*** Contaminating red blood cells can be eliminated in mixed leukocyte preparation by hypotonic lysis with distilled water, 0.2% saline or ammonium chloride. Although this is a valid technique for many in vitro techniques, it can adversely affect the cell viability, morphology, and chemotaxis (*1,30*) and is best avoided.

## Separation of "Pure" Granulocytes

***Density Gradient Centrifugation.*** This method depends on Stoke's Law where the rate of sedimentation is zero when the cell encounters a medium of identical density. Preparing layers of different density and allowing cells to settle through the layers by gravity or by applying centrifugal force allows the cells to move until they reach a density that is either equal or greater than their own (*14*). Human formed blood elements have an increasing density in the order of: platelets < lymphocytes < granulocytes < erythrocytes (Fig. 8). Use of discontinuous or continuous gradients in the density range corresponding to the blood cells allows the isolation of the various fractions.

Boyum (*31*) was the first to take advantage of this principle for the isolation of lymphocytes from whole blood. Several other investigators later modified the technique to separate the PMNLs from the erythrocytes (*32,33*). Ficoll-Hypaque was the density gradient media utilized. Ficoll is a synthetic high molecular weight (M.W. 400,000) polymer of sucrose (*19*). Ficoll alone becomes too viscous at the densities needed for granulocyte separations so a radio-opaque contrast media such as Hypaque (sodium and meglumine diatrizoate) is added to increase the density.

Single density mixtures of 1.095 g/ml (*32*) and discontinuous density gradients with layers of 1.076 and 1.120 specific gravity (*33*) have both been used successfully for isolation of PMNLs. Other water soluble iodinated contrast media have been used in place of Hypaque, including nonionic metrizamide (Amipaque) (*2*).

Although Ficoll-Hypaque gradients are popular, they may not be the most ideal agent for density gradient centrifugation. The medium is usually hyperosmolar compared to plasma so that water is pulled from the granulocytes and their density increases (*31*). The metabolism of the leukocytes may be adversely affected following Ficoll-Hypaque (*34*). Neutrophils were found to demonstrate spontaneous changes in cell shape, reduced chemotaxis, and enhanced release of bactericidal agents following isolation on Ficoll-Hypaque (*15*). These same changes could be induced by lipopolysaccharides or endotoxins added to media (*15*).

The introduction of a colloidal media, Percoll,[†] made up of 15–30 nm silica particles with a polyvinylpyrrolidone coating[†] provided some advantages over Ficoll (Fig. 9). This medium has a low viscosity and osmolarity and its initial density of 1.13 is easily varied over a wide range by adding plasma, balanced salt solutions, or sucrose solutions (*21*). It is provided as a sterile solution that is easily made iso-osmotic by mixing nine parts Percoll with one part 9% saline.

Danpure, et al. (*21*) used cell-free plasma from patients to prepare 65, 60, and 50 percent Percoll suspensions. A discontinuous density gradient (Fig. 10) can be prepared by carefully layering decreasing densities above one another with a sterile plastic pipette or syringe. It is sometimes easier to add layers of increasing densities below one another (using a 3.5-in. spinal needle to get to the bottom of the tube) to avoid disturbance of the gradient interfaces. The mixed white cell pellet in plasma is carefully layered on top. Following centrifugation, the band of neutrophils are found at either the 50%–

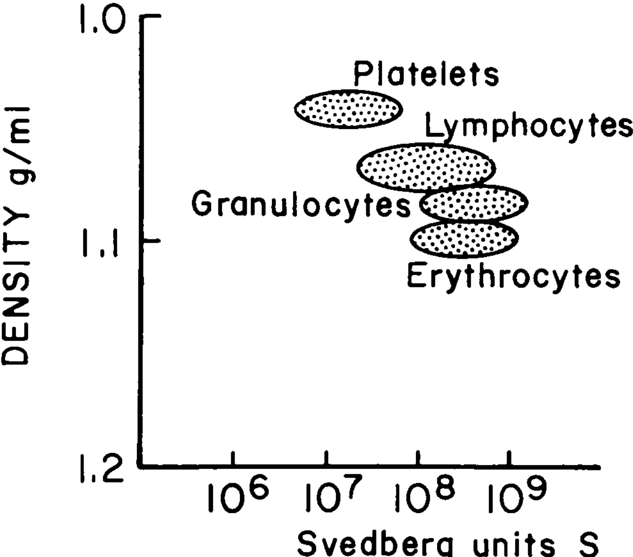



**Fig. 8.** Approximate densities and sedimentation rates of human blood cells. The erythrocytes have the highest density and platelets have the lowest density. [Adapted with permission (*48*)].

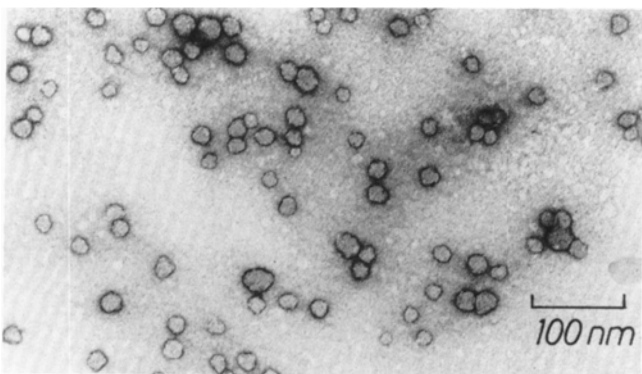



**Fig. 9.** Electron microscopy of Percoll particles. The colloidal media has a density of 1.13 and is composed of silica particles of an average size of 15–30 nm, coated with polyvinylpyrrolidone. For size comparison, an average sulfur colloid preparation contains particles that are 10–100 nm; an average macroaggregated albumin particle is 20,000–50,000 nm. [Reproduced with permission (*49*)].

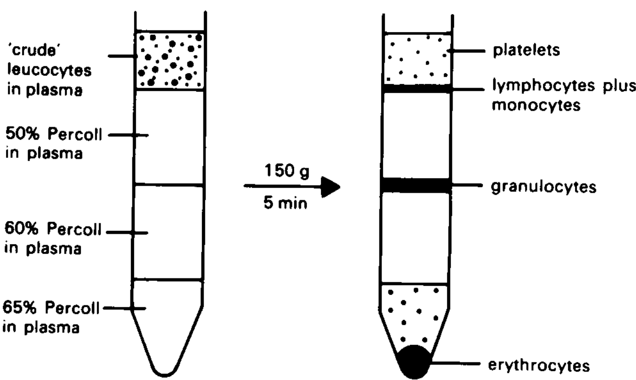



**Fig. 10.** Percoll-plasma gradients used to isolate granulocytes from mixed leukocytes. [Reproduced with permission (*21*)].

60% interface and sometimes throughout the 60% layer and are sampled for labeling (*21*). If the labeling method can also be done in the presence of plasma, the neutrophils will go through the entire separation and labeling processes within its own plasma environment. Granulocytes which remain in plasma are thought to have a faster migration through the lungs and to localize at earlier time points in inflammatory lesions (*35*).

***Other Methods of Granulocyte Separation.*** Density gradient centrifugation is by far the most popular method used today for clinical studies of neutrophil in vivo kinetics and localization. A few other methods of granulocyte separation are mentioned only briefly here since they are quite technically demanding and generally require purchase of sophisticated equipment.

***Centrifugal Elutriation or Counterstream Centrifugation.*** In this process, cells are exposed to two opposing forces—an outward centrifugal force and an inward centripetal flow (*2*) that allows cells to be separated on the basis of size as well as density (*14*). A special separation chamber (elutriator rotor) and centrifuge are used for this sophisticated technique in which many variables need to be controlled (*2, 18*). The technique is faster than some other methods and can process a larger number of cells without exposing them to sedimenting agents (*1*). The isolated cells are in suspension rather than in a centrifuged pellet (*14*). Details on the technique are reviewed elsewhere (*36*).

***Flow Cytometry.*** This technique separates cells according to differences in light scatter of fluorescence as cells pass individually through a laser beam (*2*). The technique requires sophisticated expensive equipment, which is not routinely available to most nuclear medicine departments. A brief description (*2*) and a review article (*37*) can be consulted for more details.

***Surface Adherence.*** The different adherent properties of leukocytes may be used to separate them on columns of glass beads (*38*) or glass or nylon wool (*39*). Although the yields are relatively high for granulocytes, contamination with lymphocytes and monocytes is 10%, and the older PMNLs, being more adherent, are concentrated in higher percentages (*1*).

**TABLE 2. Quality Control Tests for Neutrophils**

| Category | Function or morphology | Test | Reference |
|---|---|---|---|
| Morphologic assessment | Cytoplasmic membrane integrity | Ultrastructure and electromicroscopy of control, labeled, and chemotactic-stimulated PMNL | 43 |
| | Number of lysosomal granules | | |
| | Presence cytoplasmic granules | | |
| | Appearance and integrity nucleus | | |
| | Presence of inclusion bodies | | |
| In vitro functonal assessment | Adherence | Adherence on nylon, wool, or glass beads | 1, 41, 43 |
| | Motility | | |
| | Random (spontaneous or activated) | On glass slides | 1, 42, 43 |
| | Directional (chemotaxis) | Under agarose (Cutler-Nelson technique) | 30, 43 |
| | | Modified Boyden chamber | 44, 45, 46 |
| | | Zigmond chamber | 43, 44 |
| | | Radioassay chamber | 45 |
| | Phagocytosis | Uptake lipopolysaccharide-paraffin | 45 |
| | | Uptake heat-killed yeast or particles | 1, 41 |
| | Bactericidal activity and metabolism | Nitroblue tetrazolium reduc'n test (NBT) (spontaneous, stimulated, and quantitative) | 1, 43, 45, 46 |
| | | Yeast-Fungus killing (candidacidal) | 45 |
| | | Beta-glucuronidase (degranulation) | 46 |
| In vivo kinetics and function | Blood kinetics | Intravascular recovery and t-½ | 10 |
| | | Epinephrine stimulation test | 10 |
| | Biodistribution | Lung transit times | 21 |
| | | Liver/Spleen ratios | 47 |
| | In vivo chemotaxis | Skin window technique (Rebuck) | 45 |
| | | Plastic skin chamber technique (Senn) | 45 |
| | Abscess localization | Abscess bearing dogs | 30 |

## QUALITY CONTROL TESTS

Following the various manipulations that leukocytes and neutrophils undergo during separation and radiolabeling procedures, one must be able to assess that the cells are not adversely affected.

Many of the quality control tests that assess the functional and structural integrity of the labeled neutrophils require a substantial amount of time and normally can not be completed before patient administration. There are three major categories for assessment of the neutrophil viability and function: morphologic, in vitro functional, and in vivo functional-kinetic. Table 2 lists some of the tests performed on granulocytes from the three categories.

Most of the tests listed in Table 2 are based on specific function and morphology of the neutrophil and are therefore best performed on purified neutrophils and not on mixed white cell preparations. Since many of the assessments are relatively technically demanding, they are usually not done routinely and are only performed on neutrophils when a new technique of separating or labeling neutrophils is being developed.

Tests which should be done in each institute include:

1. Perform retrospective sterility or pyrogenicity testing (especially when new personnel are learning the technique of separation and labeling) (*40*).
2. Observe cell purity, structural integrity, and morphology (make a smear of the cells, stain it with Wright's stain and examine it under a light microscope) (*41*).
3. Analyze contamination from other cell types (this is especially important for mixed white cell preparations).
4. For radiolabeled cells, assess the percentage of radioactivity associated with each cell type (this is important for the physician to know during interpretation of the scans).

All four tests should be done when a new technique is first started in an institute and when new personnel learn the technique. The first two tests should be done on a more regular basis (ideally, retrospectively on each sample or at least monthly for everyone performing the procedure). Many authors use trypan blue exclusion test to assess cell viability but this test is not thought to be very sensitive or totally reliable (*42*). Only the dead cells will make up the trypan blue and no distinction is made between normal and nonfunctioning granulocytes (*1*). Most investigators who use neutrophils or leukocytes for abscess localization appear to rely upon the ultimate in vivo results (*1*).

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## NOTES

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