Separation and Functional Characterization of Human Neutrophil Subpopulations

By Mark S. Klempern and John I. Gallin

Human neutrophils have been considered to be a functionally homogeneous population of cells. We have developed a density sedimentation technique for separation of neutrophils into two populations based on their ability to form rosettes with IgG-coated human erythrocytes (7SEA). Under the experimental conditions 80% ± 4.3% of normal human peripheral blood neutrophils form rosettes. Functionally, rosette-forming neutrophils are more adherent to nylon wool, able to phagocytize more 11C-labeled Staphylococcus aureus, more efficient in killing S. aureus, and more responsive to endotoxin-activated human serum in a 51Cr chemotaxis assay than the non-rosette forming neutrophils. However, there is no difference among neutrophil subpopulations' ability to phagocytize latex particles. Paired samples of exudate neutrophils from cutaneous abscess fluid and peripheral neutrophils from three patients were investigated for their ability to form 7SEA rosettes. In each case exudate neutrophils contained greater than 96% rosette-forming neutrophils, whereas peripheral blood contained the normal 80% (p < 0.01). Thus, we show that peripheral blood contains at least two distinct populations of neutrophils. However, an essentially homogeneous neutrophil population is present in cutaneous exudate fluid.

Neutrophils have long been considered to be a homogenous population of cells. Despite this assumption, recent studies on neutrophil membrane receptors have consistently shown that a fraction of neutrophils lacks certain receptors. For example, in studies of human neutrophil Ig receptors by Messner and Jelinek using erythrocytes passively sensitized with Staphylococcus aureus antigen and then coated with anti-Staphylococcus IgG only 43.9% of neutrophils formed rosettes. More recently Wong and Wilson investigated the optimal conditions for IgG-coated sheep erythrocyte (7SEA) and IgM- and complement-coated erythrocyte (19SEA) rosette formation by human neutrophils. With technical refinements they found that only 80% of neutrophils form 7SEA rosettes.

As part of our studies on neutrophil function, we have developed a method for separating rosetting and nonrosetting neutrophils. We show that in addition to having different rosetting characteristics these two neutrophil populations differ functionally.

Materials and Methods

Preparation of Neutrophils

Heparinized venous blood was obtained from normal volunteers, and neutrophils were isolated by Ficoll-Hypaque density centrifugation followed by dextran sedimentation. Residual erythro-
Erythrocytes were lysed with two cycles of hypotonic saline. Cells were then washed twice in a balanced salts solution (RPMI-1640, Grand Island Biological, Grand Island, N.Y.) and finally suspended in RPMI-1640 at 1 × 10^7 cells/ml. Neutrophils obtained in this manner were greater than 99% pure.

Exudate neutrophils were obtained by needle aspiration into syringes containing heparinized (20 U/ml) 6.7 mM phosphate-buffered 0.85%, NaCl (PBS), pH 7.4 (NIH, Media Unit) from three patients with acute (less than 72 hr old) bacterial subcutaneous abscesses. Peripheral blood samples were obtained at the same time. Neutrophils from both sources were isolated by Ficoll-Hypaque density centrifugation followed by dextran sedimentation.

Preparation of 7SEA

Erythrocytes obtained from a single AB donor and stored up to 4 days in veronal-buffered saline were used to form 7SEA. Red cells (RBC) were washed three times in PBS and adjusted to 1 × 10^9 RBC/ml in RPMI-1640.

Rabbit anti-human RBC IgG was obtained commercially (Cappel Laboratories, Cochranville, Pa.). The agglutinating titer of this IgG preparation as determined in microtiter plates was 1:100. Equal volumes of RBC (1 × 10^9/ml in RPMI-1640) and IgG preparation (1:150 in RPMI-1640) were mixed and incubated at 37°C for 30 min. Following incubation, 7SEA were washed twice in cold RPMI-1640 and adjusted to 4 × 10^9/ml in fresh RPMI-1640.

Preparation of EA Rosettes

Neutrophil EA rosettes were prepared by a modification of methods previously described. Neutrophil preparations were allowed to come to room temperature. Sixteen milliliters of neutrophil suspension, 4 ml of salt-poor human albumin (25%), and 20 ml of E or EA were placed into a 40-ml round-bottom glass tube. The cell suspension was pelleted by centrifugation at 60 g at 20°C for 10 min. The pellet was allowed to stand for 15 min at 20°C and then gently resuspended. A small aliquot was removed for enumerating the percentage of rosette-forming neutrophils. At least 200 cells were counted to determine the number of rosetted neutrophils. Neutrophils with three or more attached RBC were considered rosettes.

Separation of Rosetting and Nonrosetting Neutrophils

Following resuspension of neutrophil-EA or neutrophil-E pellets, 8-ml aliquots of the suspension were placed into 15-ml conical tubes (Corning, Corning, N.Y.) over a Ficoll-Conray discontinuous-density gradient. The gradients were prepared as previously described. Solutions used contained 11.2 g Ficoll (solution I) or 7.2 g Ficoll (solution II) in 100 ml distilled water plus 20 ml Conray (meglumine iothalamate 600 mg/ml, Mallinkrodt, St. Louis, Mo.). Using a spinal needle, 3 ml Ficoll solution I was layered beneath 3 ml solution II. Cell suspensions were then layered over solution II and centrifuged at 1500 g for 25 minutes at 4°C.

Red cell ghosts and contaminating monocytes sedimented at the cell suspension–solution II interface. The non–rosette-forming neutrophils (non-RFN) were at the interface between the two Ficoll-Conray solutions, and the more dense rosette-forming neutrophils (RFN) were in the cell pellet (Fig. 1). Each fraction was collected by aspiration and washed twice in RPMI-1640. Because of the different sedimentation characteristics of non-RFN and neutrophils that had formed rosettes, non-RFN were not exposed to solution I during the usual separation procedure. Therefore in some experiments non-RFN were collected from the gradient and incubated in solution I for 15 min at 4°C. The suspension was then diluted with PBS and centrifuged at 200 g for 10 min to recover the non-RFN pellet. These cells were then washed and resuspended in the usual manner.

EA were removed from rosette-forming neutrophils by either hypotonic lysis or incubation at 37°C for 60 min. Before functional assays, only hypotonic lysis was used to remove EA. Although there were virtually no erythrocytes in the non-RFN fraction, in initial experiments these neutrophils were also exposed to hypotonic saline lysis. Control cell suspensions containing erythrocytes (without antibody) and neutrophils were similarly placed over the Ficoll-Conray gradient and the unfractionated neutrophils sedimented to the interface between solutions I and II with the erythrocytes sedimenting as a pellet.
Functional Assays

Adherence to nylon wool. Adherence was measured using a modification of a previously described technique. Tuberculin syringes were packed with 50 mg washed nylon wool and fitted with a 27-gauge needle. RFN, non-RFN, and control unfractionated neutrophils exposed only to E were adjusted to $5 \times 10^8$/ml in RPMI-1640 plus 10% human AB serum. Cell suspensions were placed over the nylon wool column and allowed to incubate at room temperature for 10 min. Flow through the nylon wool column was impeded by this small-gauge needle, but by 10 min the fluid had completely traversed the column. Effluents of each sample were collected and counted. Each experiment was run in triplicate. In some experiments unfractionated neutrophils were placed over the nylon wool column and effluent cells were collected and exposed to EA to determine whether passage over nylon wool caused depletion or enrichment of a neutrophil subpopulation.

Bactericidal assay. A previously described technique using *S. aureus* (NIH stock culture) was employed. Unfractionated cells, non-RFN, and RFN were adjusted to $5 \times 10^8$/ml in Hank’s balanced salt solution (HBSS) plus 10% AB serum and incubated for various times with bacteria.
from an overnight culture at a bacteria to neutrophil ratio of 4:1. Aliquots of the incubation mixture were removed at intervals, diluted with sterile water to lyse the leukocytes, and transferred to pour plates that were counted for bacterial colonies 24 hr later. Killing of bacteria was expressed as percentage of inoculated bacteria surviving at each interval.

Phagocytic capacity. Uptake of $^{14}$C-radiolabeled heat-killed *S. aureus* was measured as previously described. RFN, non-RFN, and unfractonated neutrophils suspended in HBSS plus $10^{-5}$ AB serum were incubated with bacteria at a ratio of 5:10 bacteria per neutrophil. After specified incubation intervals, phagocytosis was stopped by adding iced buffer, and the suspension was centrifuged at low speed (75 g) for 10 min. Supernatants were aspirated to remove non-cell-associated bacteria, the cells were resuspended, and the wash procedure was repeated three times.

A histogram of frequency of cells as a function of their mean deformed length is shown. Cell population made from equal numbers of fresh cells (right-hand peak) and cells fixed in 0.0125% glutaraldehyde, before the cells were deformed. Total of 800 cells was sized.

RESULTS

Deformation of Partially Hardened Cells

Partial fixation of red cells decreased cell deformation by the centrifugal technique. This effect is seen in Fig. 3, where the mean length of the deformed cells is shown to decrease as the concentration of glutaraldehyde used to treat the cells increases. With the exception of the cells fixed in 0.0125% glutaraldehyde, the mean deformed length of each cell population is significantly ($p < 0.001$) less than the length of the cells treated with a lower concentration of glutaraldehyde. Determinations of cell density showed that the concentrations of glutaraldehyde used to partially fix the cells did not change their density.

The effect of glutaraldehyde and the capacity of the technique to detect differences in cell deformability are also shown in Fig. 4, a histogram of the frequency of deformed cell lengths from a cell suspension made by mixing equal numbers of unfixed cells and cells partially fixed with 0.0125% glutaraldehyde. The unfixed and glutaraldehyde-fixed cells were mixed after half the cells had been exposed to glutaraldehyde but before they were deformed. The two populations are clearly resolved in the histogram.

Deformation of Density-separated Cells

Figure 5 shows the mean deformed length of density fractionated cells as a function of the mean density of the various cell fractions for three individuals. In all cases, decreased with increasing cell density.

Effect of Tonicity

Figure 6 demonstrates the manner in which the relative deformed length of the cell changes as a function of relative cell volume. The relative volume of the cell refers to the ratio of the nondeformed cell's volume in a buffer of given tonicity to its volume under isotonic conditions. The relative deformed length of the cell is the ratio of in a given buffer to in isotonic conditions, $x$.

Cells deformed in isotonic PBS have by definition a relative cell volume and a relative deformed length of 1.0. When cells were placed in increasingly hypotonic buffers, the relative volume increased, and the relative deformed length decreased.

Statistical Analysis

The two-tailed Student's *t* test was used to test statistical significance throughout this report.

RESULTS

Conditions Affecting Percentage of Rosette-forming Neutrophils

Over the range of 5–20 EA/neutrophil, the number of rosettes increased proportionally with the number of EA (Fig. 2). From 20 to 200 EA/neutrophil, the percentage of rosette-forming neutrophils plateaued consistently at 80%. A ratio of 50 EA per neutrophil was used for further experiments. When EA were prepared with dilutions of rabbit anti-human erythrocyte IgG between 35%
and 80% of the agglutinating titer, a plateau of maximal rosetting was observed (Fig. 3). Hence 66% of the agglutinating titer was chosen as the standard for preparation of 7SEA. Under the conditions outlined, in 35 individuals 80% ± 4.3% (range 73%–87%) of human peripheral blood neutrophils formed rosettes.

In related studies peripheral blood neutrophils obtained from three patients were compared to neutrophils recovered from their abscess fluid. The peripheral blood neutrophils contained 78.3% ± 2.9% RFN, while abscess fluid neutrophils were 96%, 99%, and 100% positive, with a mean of 98.3% ± 1.2% RFN ($p < 0.01$, Student's $t$ test). To insure that only viable neutrophils were counted, trypan blue was added at the time of enumerating the percentage of RFN. Only those cells excluding trypan blue were counted.

**Neutrophil Subpopulation Separation**

_**Cell morphology.**_ Differential counts on cyt centrifuge preparations revealed that non-RFN contained 80%–90% mature appearing neutrophils, 5%–10% eosinophils, and 0%–5% monocytes, while the RFN preparation contained 90%–95% mature neutrophils, 3%–5% eosinophils, and 0%–2% monocytes. Each neutrophil fraction was also examined for neutrophils that had ingested EA or E during the rosetting procedure; in each case less than 2% of thecells contained phagocytized erythrocytes.

_**Purity and recovery of the subpopulations.**_ In four individual experiments aliquots from the nonrosetting and rosetting neutrophil fractions obtained by Ficoll-Conray density gradient centrifugation were also investigated for the ability to rosette with a second exposure to 7SEA. Non-RFN were washed...
twice in buffer, adjusted to \(1 \times 10^7\) cells/ml, and reexposed to EA in the usual manner. Under these conditions 10%–12% of the neutrophils formed rosettes. Preincubation of the non-RFN at 37°C for 30 min had no effect on subsequent rosette formation. RFN were harvested from the Ficoll-Conray gradient pellets and EA were removed by either hypotonic lysis or incubation at 37°C for 60 min. Following EA removal, these neutrophils were washed twice and resuspended at \(1 \times 10^7\) cells/ml. With a second exposure to EA, 94%–96% of these neutrophils formed rosettes. The method of EA removal (lysis or 37°C incubation) had no effect on the percentage of neutrophils reforming rosettes. Similarly, exposure of non-RFN to conditions of hypotonic lysis did not affect their rosetting characteristics.

To insure that the initial Ficoll-Hypaque and Dextran separation did not damage 20% of unfractionated neutrophils, RFN were reexposed to this separation procedure, washed, and rerosetted. Under these conditions 94% of RFN reforming rosettes. Following gradient separation both populations contained greater than 97% viable cells, based on trypan blue dye exclusion.

The efficiency of the separation procedure was determined by calculating the predicted number of neutrophils in each population based on the initial ratio of rosetting and nonrosetting neutrophils and determining the actual number of cells recovered from each fraction. When unfractionated neutrophils were mixed with EA, 82% of the cells were recovered, representing recovery of 85% of RFN and 61% of non-RFN. Ninety-six percent of control neutrophils exposed only to E were recovered from the gradient.

**Functional Assays of Neutrophil Subpopulations**

**Adherence.** As shown in Table 1, 86% \(\pm 3\%\) of RFN adhered to the nylon wool, while only 65% \(\pm 1\%\) of non-RFN were adherent. Unfractionated (E-exposed) neutrophils gave an intermediate result, with 75% \(\pm 1\%\) adherent to the column. RFN and non-RFN were each significantly different from unfractionated cells in this regard (\(p < 0.05\) and \(< 0.01\), respectively).

**Bactericidal activity** (Fig. 4). In three individual experiments the RFN population was more efficient in killing *S. aureus* than the non-RFN. The differences were significant at each time interval examined (\(p < 0.05\)). In addition to differences in absolute number of bacteria killed at all time intervals, the initial rate of killing was significantly greater for RFN than non-RFN (\(p < 0.01\)).

**Phagocytosis** (Fig. 5). At each time interval phagocytosis of \(^{14}\)C-labeled *S. aureus* by the RFN population was significantly greater than by the non-RFN population (\(p < 0.05\) at 10 and 20 min).

<table>
<thead>
<tr>
<th>Neutrophil Population</th>
<th>Adherent to Nylon Wool* (%)</th>
<th>(p \dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>75 (\pm 1)</td>
<td></td>
</tr>
<tr>
<td>Rosette-forming</td>
<td>86 (\pm 3)</td>
<td>(&lt; 0.05)</td>
</tr>
<tr>
<td>Non-rosette-forming</td>
<td>65 (\pm 1)</td>
<td>(&lt; 0.01)</td>
</tr>
</tbody>
</table>

*Mean \(\pm\) SEM, three experiments.

†Significance level of difference compared to unfractionated cells, Student's \(t\) test.
In related experiments each neutrophil fraction was exposed to latex particles in a serum-free system. In contrast to phagocytosis of S. aureus, there was no difference in the ability of each neutrophil fraction to ingest latex particles over a wide range of particle to neutrophil ratios (Table 2). Furthermore, no obvious differences in number of latex particles per cell were apparent.

**Locomotion.** As shown in Fig. 6, there was no difference between the spontaneous random migration of the two neutrophil populations (256 ± 4 cor CPM versus 268 ± 80 cor CPM for RFN and non-RFN, respectively). However, under conditions of directed locomotion (chemotaxis) the RFN population was more responsive to E. coli endotoxin-activated sera over a wide concentration of chemotactant ($p < 0.01$).
Table 2. Phagocytosis of Latex Particles by Neutrophil Subpopulations

<table>
<thead>
<tr>
<th>Neutrophil Population</th>
<th>Latex Particle: Neutrophil Ratio (Percentage of Cells Ingesting Latex Particles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4:1</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>6.5 ± 1.5 (2)†</td>
</tr>
<tr>
<td>RFN</td>
<td>9.5 ± 3.5 (2)</td>
</tr>
<tr>
<td>non-RFN</td>
<td>8.5 ± 0.5 (2)</td>
</tr>
</tbody>
</table>

Mean ± SEM; number of individual experiments in parentheses.

**Effect of Separation Procedure on Neutrophil Function**

During the separation procedure RFN and non-RFN were exposed to different conditions. Non-RFN sedimented above solution I, whereas RFN pelleted through this solution. Also, by definition, RFN have a more intimate contact with antigen-antibody complex (EA). Since either of these conditions could have induced the observed functional differences, the following experiments were performed: To control for the differential exposure of the subpopulations in the gradient, isolated non-RFN were incubated in solution I for 15 min at 4°C and then washed, and adherence and chemotaxis assays were performed as described. The percentage of adherent non-RFN with or without incubation in solution I was unchanged (64.1% ± 3%, with preincubation, 65.0% ± 1% without, two experiments). Similarly, in four experiments of locomotion there was no effect of exposing non-RFN to solution I prior to the assay.

In order to control for the effect of contact with EA as an “activator” of the RFN population, a different approach was taken. Unfractionated neutrophils were passed over a nylon wool column under the conditions previously described, and effluent cells not adhering to the column were collected. Unfractionated neutrophils were also permitted to migrate through a 5-µm polycarbonate filter in response to C5a, and cells migrating into the lower chamber compartment were collected as outlined in Materials and Methods. In each experiment the initial Ficoll-Hypaque unfractionated neutrophil preparation and neutrophils obtained from the nylon wool column effluent or lower compartment of the Boyden chamber were exposed to EA in the usual manner. As

![Fig. 6. Locomotory response of neutrophil subpopulations to buffer (random migration) or to varying concentrations of E. coli endotoxin-activated serum using the 51Cr radioassay (see Materials and Methods). Mean ± SEM of four separate experiments.](image-url)
shown in Fig. 7A, cells eluting from the nylon wool column were depleted of RFN (or conversely, enriched with non-RFN). Similarly (Fig. 7B), cells that migrated through the polycarbonate filter in response to C5a were enriched with RFN. Control unfractionated neutrophils incubated in the presence of C5 fragment without migration through a filter showed no change in the percentage rosetting.

DISCUSSION

The results of these studies demonstrate that human peripheral blood neutrophils contain at least two subpopulations that can be distinguished by their ability to form 7SEA rosettes. These neutrophil populations are separable by differential density centrifugation and are functionally distinguishable. In addition, the relative proportions of PMN subpopulations present in peripheral blood and cutaneous abscess fluid are different.

Attempts to demonstrate maximal 7SEA rosette formation by human neutrophils were initiated by Wong and Wilson. They clearly demonstrated that temperature was a crucial factor for neutrophil rosette formation. Whereas 80%-82% of polymorphonuclear leukocytes (PMN) formed 7SEA rosettes at 20°C and 0°C, only 4% did so at 37°C. Similarly, they showed that centrifugation of EA with the neutrophil and short-term incubation were essential to maximize the number of EA rosettes formed. We have extended these findings by demonstrating the optimal antibody concentration to prepare EA and by defining the best EA:neutrophil ratio for rosetting. Furthermore, in a series of 35 normal subjects we confirmed the relative proportions of rosette-forming and non-rosette-forming neutrophils in peripheral blood.

Rosette sedimentation techniques have become a standard method for leukocyte subpopulation separation. The most commonly used Ficoll-Hypaque gradient of Boyum is not suitable for neutrophil rosette separations, since both rosetted and nonrosetted neutrophils pellet. At the appropriate density, Ficoll-Conray obviates this problem, since neutrophils and erythrocytes sediment in
different layers, making separation of rosetted and nonrosetted fractions possible. In preliminary experiments employing sheep RBC for EA we found that separations were inconsistent. However, we were able to separate reproducibly the two populations by substituting human RBC for EA preparation (see Results).

Several technical problems remain. The separation scheme is a long, cumbersome procedure requiring considerable manipulation of the cells, and a simpler separation method requiring fewer steps would be desirable. Zighelboim et al. reported fractionation of Fe-receptor-bearing neutrophils on poly-l-lysine monolayers of sheep EA prepared in a manner similar to that described by Kedar et al. for lymphocyte separation. They did not describe the purity of separation which would be difficult to ascertain, since attempts to rerosette EA-adherent leukocytes from poly-l-lysine-prepared monolayers have met with difficulty. In our hands about 50% of monolayer nonadherent neutrophils formed rosettes (unpublished observations).

The functional assays performed on these neutrophil populations provided several interesting observations. We have as yet found no function that one population performed exclusively. On the other hand, latex particle ingestion was equivalent for the two populations. This finding was consistent with recent studies that demonstrated macrophage Fe-mediated and -independent phagocytosis to proceed by independent mechanisms. Because RFN seemed to be functionally more efficient, we considered one explanation to be “activation” of this population of neutrophils by intimate antigen-antibody complex (EA) contact. However, assaying unfractonated neutrophils for their ability to form rosettes after performing a function (adherence or chemotaxis) indicated that RFN were intrinsically more active. It was of particular interest that RFN consistently exceeded unfractonated neutrophils in their ability to perform a function. Similarly, non-RFN were always less active than unfractonated cells. Owing to differences in purity between RFN and non-RFN fractions, the net activity of unfractonated neutrophils was not a simple weighted arithmetic mean of the two populations.

A fundamental question raised by these studies is the origin of the subpopulations. Several possibilities exist, including differentiation into different lines from a pluripotential stem cell, peripheral processing that commits these cells to a given population similar to T and B cells, cellular environmental changes that induce interconversion between subpopulations, or the possibility that the less active non-rosette-forming neutrophil is a young or senescent cell. Based on current knowledge, the first two possibilities seem unlikely. The finding that in three subjects cutaneous exudate cells were an essentially homogeneous population of rosette-forming neutrophils could be the result of several possibilities. Since RFN are more responsive to endotoxin-activated serum chemotactic factor, differential mobilization to the inflammatory site could partially explain this observation. Nevertheless, the non-RFN respond to the chemotactrantant, and we would expect at least some non-RFN to be present in exudates. Another possibility is that a substance present in abscess fluid causes interconversion of non-RFN to RFN. To date, preincubation of non-RFN with S. aureus, latex particles, and synthetic peptide chemotactic factor (f-nor-leu-leu-phe, kindly provided by Dr. Elliot Schiffman) has not induced such a conversion.

Based on light and electron microscopy, both neutrophil subpopulations
such occurrence would seem required if indeed there is retention of water during the polymerization of hemoglobin. Second, is there an actual uptake of water under conditions that induce a very strong Bohr effect? If this is the case, then the membrane is normally permeable and there is a normal responsiveness of the sickled cells to changes in intracellular osmotic pressure. Third, does the sickling brought about by deoxygenation at normal pH and tonicity alter the water distribution between red cells and plasma? There has been disagreement on this point, and it would seem to be of considerable importance in understanding sickling in vivo.

The data presented indicated that the deoxygenation in vitro of HbSS blood with carbon dioxide elicited a normal response in regard to water distribution. There was no indication of sequestering of intracellular water, and indeed the deoxygenation with CO2 induced a transfer of water from the plasma into the cells, as was the case with normal blood. Furthermore, there was no significant loss or gain of water when sickling was induced by deaeration with 95% N2 and 5% CO2.

MATERIALS AND METHODS

Experimental Design

To determine changes in distribution of blood water that avoids the problem of trapped plasma is to measure the changes in concentration of a material restricted to the extracellular fluid. If the concentration of the material in the plasma increases, there is an influx of water from the plasma into the cells; if the concentration decreases, there is an efflux of water from the cells. Similarly, changes in the total water that is in osmotic equilibrium can be determined by measuring the plasma concentration of a material with unrestricted distribution.

Fortunately, materials having each of these distributions are present naturally in blood. Plasma protein is restricted to the extracellular medium, while highly invasive urea is distributed throughout the total water.

To test the effectiveness of plasma protein and plasma urea determinations in estimating extracellular and total blood water, analyses were made on undiluted blood as well as on blood diluted with isotonic saline containing 0.15 moles NaCl as well as 4.5 millimoles glucose per liter. The glucose was included in the diluent so as not to impair red cell metabolism by dilution of substrate. From thoroughly mixed blood containing ethylenediaminetetraacetate (EDTA) as anticoagulant, five 1.00-mI samples were transferred to test tubes. To the separate tubes the following volumes of isotonic diluent were added: 0, 0.10, 0.20, 0.30, and 0.50 ml. The tubes were stoppered and the bloods mixed for 30 mm at room temperature (about 25°C) on a slowly turning laboratory rotator. After centrifugation, urea was determined by the direct method of Wybenga et al.9 using 20-μl plasma samples and the protein by a microadaptation of the Weichselbaum10 method. The biuret reagent was diluted with an equal volume of 0.33 moles/liter NaOH solution just before use, and 50 μl of plasma was rinsed into 3 ml of the diluted reagent. The relationship between the original pool volume \( V_0 \), the initial plasma concentration \( C_0 \), and the concentration \( C_x \) after addition of a volume \( V_d \) is given by:

\[
C_x = \frac{C_0 V_0 + C_0 V_d}{V_0 + V_d}
\]

Plotting the ratio of the initial to the diluted concentration \( \frac{C_0}{C_x} \) against the final volume of added diluent \( V_d \) gave a straight line in which the negative intercept of the X axis was the graphic estimate of the original pool volume \( V_0 \).

ACKNOWLEDGMENT

We wish to thank Dr. Anthony S. Fauci for his advice throughout these studies.

REFERENCES

Separation and functional characterization of human neutrophil subpopulations

MS Klempner and JI Gallin