Volume and Osmotic Properties of Human Neutrophils

By H. Ping Ting-Beall, David Needham, and Robert M. Hochmuth

Quantitative models describing the dynamics of human neutrophils in the microcirculation require accurate morphometric parameters such as volume and surface membrane area. Using both a micropipette technique and video light microscopy (LM) to measure the diameters of the spherical cells, we have accurately determined the volume of the human neutrophil. Our value, 299 ± 32 μm³, is in good agreement with our earlier results, but 55% larger than that reported by Schmid-Schönbein et al (Blood 56: 866, 1980). However, the measurements of Schmid-Schönbein et al were based on the actual mass of the cells derived from transmission electron microscopic (TEM) images. The membrane surface area, at lysis, was calculated to be 2.6 times its initial projected area. After lysis, the cells do not reduce their size, indicative of the possibility of a F-actin network formation that would stiffen the structure. Further, we show that neutrophils behave as ideal osmometers when exposed to anisotonic solutions at 21°C, as predicted by the Boyle-Van’t Hoff relationship. The calculated Ponder’s value, R, is 0.77, which corresponds to 77% of the cell volume being osmotically active under isotonic conditions. However, at 37°C, the cells are able to regulate their volumes toward the original volumes after an osmotic stress.

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Materials and Methods

Cell Preparation

Human neutrophils were isolated from the peripheral blood of three healthy volunteers in a sterile environment as described previously. Approval was obtained from the Institutional Review Board for these studies. Volunteers were informed that blood samples were obtained for research purposes, and that their privacy would be protected. Whole blood drawn into an EDTA (K3) vacutainer (Becton Dickinson, Rutherford, NJ), was centrifuged at 300g for 25 minutes at 25°C to remove the majority of erythrocytes. The plasma and buffy coat were diluted to 30% with modified endotoxin-free Hanks balanced salt solution (HBSS; Sigma, St Louis, MO; no Ca2+ and Mg2+) and carefully layered over Ficoll-Hypaque gradients (Sigma Histopaque-1077 and -1119) having densities of 1.077 and 1.119, respectively, at 25°C. After a 20-minute centrifugation at 800g and 25°C, the neutrophils at the 1077/1119 interface were collected, and washed twice with 10X volume HBSS, and finally resuspended in 50% autologous plasma/HBSS to prevent adhesion to glass surfaces. The cells were 99% viable as determined by trypan blue exclusion test, and 90% passive as determined visually by the spherical shape and absence of pseudopods.

The osmotic pressures of all the solutions were measured using a freezing-point depression osmometer (Advanced Instruments, Inc, Needham Heights, MA). Increase and decrease of plasma solution osmolality was done by addition of NaCl and distilled water, respectively, to the isotonic solution (300 mOsm).

Volume Measurement of Spherical Cells by Light Microscopy

The cells were placed in a microchamber on the microscope stage and examined with a Leitz Diavert bright-field microscope (Rockleigh, NJ) equipped with two 10X eyepieces and a vertical 25X eyepiece on a trinocular head and a 40X, 0.65-numerical aperture (NA) long working distance Nikon objective (Garden City, NY). For comparison, cell images were also checked with a 100X oil immersion objective that had a NA of 1.25. Illumination was provided by a 200-W mercury lamp with a 4,358-nm monochromatic filter. Experiments were recorded on videotape with a video camera (Dage-MTI, Michigan City, IN) mounted above the vertical eyepiece. Geometric measurements were performed using a position analyzer (Vista Electronics, La Mesa, CA) via a contour synthesizer (Model IV-53; For-A, West Newton, MA). The diameter of the cell was the average value of the mean cell volume of 150 neutrophils from five individuals is 280 μm³, is in actin (bin). In contrast to the RBC, 77% of the volume of neutrophils is osmotically active water. We also show that the ability of cells to regulate their volume after osmotic stress is temperature-dependent.

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pulled to a fine point and broken by quick fracture to the desired tip dimension. Only the pipettes with constant diameters within 25 μm of the tip were used. The pipette diameter was measured optically by inserting an ink-filled pipette into the immersion oil (Fig 2b), and by scanning electron microscopy (Fig 2c) of the same pipette. Both techniques gave the same pipette diameter. The pipette was filled with NaCl solution that was equiosmotic with the equilibrium cell bathing solution (ie, 150, 300, or 450 mOsm), and was connected to a manometer via water-filled tubing. Cells were aspirated into the pipette into a sausage shape as shown in Fig 1, either by mouth suction or by a simple displacement of a syringe. Experiments were video-recorded for subsequent volume analyses. For temperature-dependent studies of volume regulation, temperature-controlled double chambers were used. Cells in a 300-mOsm solution chamber were transferred with a micropipette to the chamber containing a 100-mOsm solution.

horizontal and vertical dimensions of the cell. Sharp focus of the outer edge of the cell shows a thick 0.4-μm dark ring (Figs 1 and 2). The contour synthesizer enhances the image, and this thick diffraction ring is reduced to a sharp line that falls between the inner and outer edges of the ring. Because of the small size (0.2 μm) and uncertainty as to where to place the calipers to give the absolute measurement of cell diameter, we calibrated our video caliper system by measuring a sample of polystyrene latex beads of known diameter (9.6 ± 0.08 μm; Polyscience, Warrington, PA). Our measurement of the beads gave a value of 9.5 ± 0.4 for the diameter, which is 1% smaller than that provided by the manufacturer, who used TEM to measure the bead size. To check further the relationship between the geometric border and the diffraction pattern of a cell, we measured the epifluorescent and phase-contrast images of individual RBCs and RBC ghosts stained with a lipophilic carbocyanine membrane probe, Dil-C18:3 (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; Molecular Probes, Eugene, OR). The two white lines in Fig 1a represent the geometric border of all the cells we measured.

Micropipette Manipulation

Micropipettes were formed from 1-mm outer diameter and 0.75-mm inner diameter glass capillary tubing (A-M System, Everett, MA)
The geometry of a neutrophil in the pipette is illustrated in Fig 1c. The geometry depicts a cylinder capped by two hemispherical segments. Thus, the total volume of the cell can be calculated from the equation $V = \pi R^2 (L - 2R) + (4/3)\pi R^3$.

Scanning Electron Microscopy

Cells were prepared by first depositing them on 12-mm glass disks previously treated with Cell-Tak (Collaborative Biomedical Research, Bedford, MA). After 1 minute, the cells were fixed with 0.1% glutaraldehyde for 1 hour at room temperature followed by another hour in 1% glutaraldehyde. After washing the cells with buffer, the cells were postfixed in 1% OsO$_4$ for 1 hour in veronal acetate buffer at pH 7.4. Dehydration of the cells was performed in a graded series of acetone and then critical-point-dried using liquid CO$_2$ in a Ladd critical-point dryer (Burlington, VT). Disks with monolayers of cells were mounted on aluminum stubs using a conductive paint, and coated with a thin layer (~20 nm) of gold-palladium using a Hummer V sputterer (Anatech, Ltd, Alexandria, VA). Samples were stored under vacuum until they were examined and imaged in a Philips 501 scanning electron microscope (Mahwah, NJ) at 15 kV.

RESULTS

Cell Response in Anisotonic Solutions

Spherical cell measurements by light and electron microscopy. Table 1 shows the average volume of human neutrophils of our studies, as well as those reported earlier by other laboratories. The average cell volumes from our study at 300 mOsm were 294 ± 36 μm$^3$ and 304 ± 27 μm$^3$, which is much larger than the value reported by Schmid-Schönbein et al.$^2$ who used both light microscopy (LM) and TEM-stereology to obtain a value of 190 μm$^3$. However, our value is in good agreement with our earlier report,$^1$ as well as that of Evans and Yeung.$^6$ The average cell diameter obtained by Evans and Yeung via LM (sphere) was 8.5 μm, which corresponds to a volume of 322 μm$^3$ at 290 mOsm. At all osmolalities, the cells that were fixed, dehydrated, and critical-point-dried were generally obtained with LM. At 60 mOsm, the cells were fixed, dehydrated, and critical-point-dried were generally smaller. It has been shown that the greatest cell shrinkage actually occurs during critical-point drying.$^2,3$ The cells fixed in low osmolality (Fig 3e) did not exhibit the same type of microvilli or foldings as the ones fixed in higher osmolalities (Fig 3f through h). However, the surfaces were not perfectly smooth; they were either undulating or had small foldings.

Cell volume by micropipette measurement. Because of defined geometry (Fig 1c), the total aspiration of a single cell into a micropipette allows a more accurate measurement of cell volume than simply measuring the diameter of the spherical cell. Typically, a ±0.2-μm error in cell radius would lead to a 15% error in cell volume, whereas the same error in the length of the cell in the micropipette gives an error of 1%. Thus, we have also used this technique to measure cell volumes in isotonic and anisotonic solutions. The results of these studies are shown in Fig 4. In this figure, we also plotted the data from spherical cells using LM and SEM. It is interesting to note that the volume measurements from undeformed spherical cells and micropipette-deformed cells are close. Thus, careful studies by measuring the size of the spherical cells with appropriate calibration of the optical systems can give accurate volume measurements. In comparison, SEM results show that the cell volumes were smaller at all osmolalities.

If we assume that the number of osmotically active particles (mainly inorganic ions) within a neutrophil remains constant as the cell swells and that only a fraction, R, of the isotonic volume is osmotically active, then the relationship between the volume, V, of the cell relative to the isotonic volume and the osmolality, P, of the suspending medium relative to the isotonic osmolality (300 mOsm) can be derived:$^3$ $V = (R/P) + (1 - R)$. Then the plot of V versus 1/P will be a straight line if the cell behaves as an “ideal” osmometer. Figure 5 shows that at 21°C the neutrophil volume varies linearly with the inverse of the osmolality over a wide range. The slope, R, is also referred to as Ponder’s value and is 0.77 in this case. Thus, we find that 77% of cell volume under isotonic condition is osmotically active. This value is consistent with the published values that range from 0.8 and 0.9 for human lymphocytes$^8$ to 0.6 for granulocytes.$^9$

Cell surface area estimation. At 60 mOsm, the cells swelled and lysed within 30 minutes. The volume just before lysis was estimated to be approximately 1,200 μm$^3$ and the final area was calculated to be 2.6 times the initial area (Table 2). However, when the cells were suspended in distilled water (0 osmolality) they lysed in 2 minutes and the projected surface area at lysis was only 2.1 times the initial area. This number is consistent with the value reported by Evans and Yeung,$^6$ who used the micropipette to estimate the surface area. After lysis in distilled water, the membrane appeared to be resealed and returned almost to its spherical shape with a much smaller diameter (Fig 6). The cell, lysed in a 60-mOsm solution, did not reduce in size after lysis. Figure 7 shows that most of the granules appeared to adhere to the plasma membranes.

<table>
<thead>
<tr>
<th>Source</th>
<th>Osmolality (mOsm)</th>
<th>Volume (μm$^3$)</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schmid-Schönbein et al.$^2$</td>
<td>310</td>
<td>190</td>
<td>TEM-stereology</td>
</tr>
<tr>
<td>Bagge and Bränenmark$^4$</td>
<td>290</td>
<td>382</td>
<td>LM (sphere, in vivo)</td>
</tr>
<tr>
<td>Evans and Yeung$^6$</td>
<td>290</td>
<td>322</td>
<td>LM (sphere)</td>
</tr>
<tr>
<td>Needham and</td>
<td>300</td>
<td>281 ± 30</td>
<td>LM (micropipette)</td>
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<td>Hochmuth$^1$</td>
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<tr>
<td>Present study</td>
<td>300</td>
<td>294 ± 36</td>
<td>LM (sphere)</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>304 ± 27</td>
<td>LM (micropipette)</td>
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</tbody>
</table>

Abbreviations: LM, light microscope, with the cell either in free solution and shaped like a sphere or in a micropipette and shaped like a sausage; TEM, transmission electron microscope.
Fig 3. Video micrographs of passive human neutrophils at four different osmolalities: (a) 70 mOsm, (b) 150 mOsm, (c) 300 mOsm, and (d) 450 mOsm. At these resolutions, the membrane foldings are not visible. Scanning electron micrographs of cells suspended in equivalent osmolalities are shown in e through h. On the whole, the cells were much smaller in size, and at very low osmolality (e), the membrane had lost its foldings, although they were not perfectly smooth.
Volume Regulation in Neutrophils

Regulatory volume decrease (RVD) following application of hypoosmotic condition has been described in many cell types including leukocytes. Neutrophils also showed a volume adjustment after osmotic shock. The typical result shown in Fig 8 is for a neutrophil transferred from an isotonic solution to a chamber containing a 100-mOsm solution at 37°C. The cell swelled rapidly (within 1 minute) and then slowly decreased in volume, but did not reach its original volume. For comparison, the cell response at 21°C is also included in Fig 8. Here, the cell did not show any RVD.

DISCUSSION

Quantitative models describing the deformation and flow of human neutrophils in the microcirculation require accurate morphometric parameters such as volume and surface area. Thus, the precise measurement of neutrophil volume has been of interest. Using LM and TEM-stereology, Schmid-Schönbein et al. reported a value of 190 \( \mu \text{m}^3 \), whereas using micropipette techniques, we recently obtained a value for cell volume that is 47% larger. The average cell volume for 150 cells from five individuals, measured during slow aspiration, is 281 ± 30 \( \mu \text{m}^3 \). In this study, using both undeformed spherical cells and micropipette-deformed cells, we again obtained a value of 294 \( \mu \text{m}^3 \) ± 36 and 304 ± 27 \( \mu \text{m}^3 \) for the neutrophil volume at 300 mOsm. In our micropipette measurements, the microvilli contributed negligibly to volume measurements. Since our values for cell volumes agreed in both optical and pipette measurements, it is reasonable to assume that the microvilli did not enhance the apparent cell diameter, as seen under a light microscope. Our results are more compatible with the measurement of Evans and Yeung, who obtained a value of 322 \( \mu \text{m}^3 \) at 290 mOsm, and also with in vivo measurements in humans by Bagge and Brämark. The discrepancy between the results of Schmid-Schönbein et al. and those presented here and elsewhere could be that the cells shrunk during the preparation procedures for TEM. The measurements of Schmid-Schönbein et al. were based on the actual mass of the cell derived from TEM images. Due to the unavoidable drying shrinkage associated with SEM images, neutrophils shown in Fig 3e through h are not the true representation of neutrophil dimensions. In agreement with Schmid-Schönbein et al., we also observed the loss of membrane foldings for the cells fixed in a swollen state in hypotonic solutions (Fig 3e). This could be due to an isotropic shrinkage during critical-point drying. The cell shape and surface characteristics are preserved with a uniform change in volume at every point.

Neutrophils and other leukocytes (in contrast to RBC) have a large excess surface area because of the membrane foldings or villi. During swelling under hypotonic conditions, the volume of the cell increases, with a decrease in the number and

| Table 2. Neutrophil Surface Areas at Lysis |
|---------------------|---------------------|---------------------|
|                     | Initial \( \mu \text{m}^2 \) | Slow Swelling \( \mu \text{m}^2 \) | Fast Swelling \( \mu \text{m}^2 \) |
| Area                | 214 \( \mu \text{m}^2 \)    | 546 \( \mu \text{m}^2 \)    | 486 \( \mu \text{m}^2 \)    |
| Area ratio          | 1                       | 2.6                   | 2.1                   |
NEUTROPHIL VOLUME AND OSMOTIC PROPERTIES

Fig 6. A neutrophil was transferred from an isotonic suspending medium to distilled water. The cell dimensions were monitored before and after the cell lysed.

In animal cells, the major determinant of cell volume is water content, which constitutes approximately 70% of the total cellular mass. Cell volume is determined by the content of osmotically active solutes and the osmolarity of the extracellular fluid. The largest component of the active solutes has been determined to be inorganic ions: 140-mmol/L K⁺, 10- to 20-mmol/L Na⁺, and 30- to 50-mmol/L Cl⁻. On this basis, control of cell volume must depend, to a large extent, on control of ion content, particularly of K⁺, Na⁺, and Cl⁻. Permeability to these ions under physiologic conditions is generally much lower than the water permeability. It has been shown that in Ehrlich ascites tumor cells, Na⁺, K⁺, and Cl⁻ permeability are all 5 orders of magnitude lower than the water permeability. Thus, the membranes of animal cells can be regarded as semipermeable. Many cell types, including leukocytes, allow the flow of water from one side to the other. They behave as perfect osmometers, as predicted by the Boyle Van't-Hoff relationship, when subjected to changes in the osmolality of their surrounding medium. In our studies with neutrophils at 21°C, we showed that human neutrophils indeed behaved as ideal osmometers (Fig 5). RVD was not observed over a period of 1 hour. RVD modulated by temperature has also been reported by Deutsch and Lee, who showed that quiescent mouse T-cell clone (L2) did not exhibit RVD at 17 or 25°C, but show limited RVD at 37°C. However, Grinstein et al reported RVD for human lymphocytes at 24°C. At 37°C, human neutrophils also showed RVD, as shown in Fig 8.

The underlying mechanisms in the RVD of cells is at present unclear. It is generally accepted that this regulation is
mediated by independent K⁺ and Cl⁻ efflux, which osmotically withdraws water from the cells. However, the signals that trigger K⁺ and Cl⁻ channels during hypotonic stress are yet to be identified. Many hypotheses have been proposed. These include Ca²⁺-activated K⁺ channels and stretched-induced Cl⁻ channels. However, accumulating evidence suggests that intracellular Ca²⁺ can induce RVD, but this does not imply that swelling-induced K⁺ permeability is Ca²⁺-activated. In lymphocytes, neither Ca²⁺-activated K⁺ channels in RVD nor increase in intracellular Ca²⁺ has been observed. Our results also indicate that neutrophils showed RVD at 37°C despite the absence of Ca²⁺ in the isolation medium and prolonged exposure to Ca²⁺-free medium (Fig 8). More sensitive Ca²⁺ fluorescent probes, which allow accurate minute quantitation of the intracellular free Ca²⁺ level during RVD, should give us some insight into the role of Ca²⁺.

REFERENCES


Fig 8. Volume regulation of human neutrophils at (©) 21°C and (©) 37°C. The cells were transferred from an isotonic suspending medium to a 100-mOsm hypoosmotic suspending medium and allowed to equilibrate for 25 minutes.
Volume and osmotic properties of human neutrophils

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