Morphometry of Human Leukocytes

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In order to establish quantitative models of leukocyte functions, several morphometric parameters on individual white cells are needed. These include the diameter, volume, and membrane area of the cells and their nuclei in the undeformed state. A stereologic method was used to obtain these quantities from transmission electron microscopy of random sections through human white blood cells (neutrophils, lymphocytes, monocytes, and eosinophils). In order to estimate possible artifacts due to preparation of the cells for transmission electron microscopy, a detailed comparison with light microscopy was made. The results show that undeformed white cells in isotonic solution are spherical with many membrane foldings and have a significantly smaller diameter than that measured on blood smears. A method of chemical fixation was employed so that the shrinkage due to fixation of the cells is below the resolution of light microscopic measurements. Further, it was shown that all leukocytes, including lymphocytes, have much more membrane area than is needed to cover their volumes, and this membrane area remains constant when the cell is hypotonically swollen.

STUDIES ON THE morphology of white blood cells (WBCs) are the first step towards development of quantitative models describing leukocyte dynamics in the microcirculation and in the extravascular space. Hematology textbooks provide a general description of human leukocytes, but very little quantitative information about their morphology. Usually the dimensions of the WBCs, as seen on blood smears, are reported to fall between 10 and 20 μm, despite the fact that these dimensions are incompatible with a normal flow of WBCs through capillaries with diameters smaller than 8 μm. On a blood smear, many leukocytes are severely deformed into a pancake shape, whereas in vivo WBCs are spherical and their diameters in homologous plasma are considerably less than 10 μm.

The velocity of single WBCs in capillaries is different from that of red blood cells (RBCs). This leads to hydrodynamic collisions between WBCs and RBCs and the attachment of WBCs to the endothelium of postcapillary vessels; these interactions are critically dependent on the dimensions of the WBCs. In addition, for the analyses of WBC chemotaxis and phagocytosis, measurements of the cell membrane area, the volume and membrane area of the cell nucleus and granules, and the concentration of granules are needed. These quantities are important determinants of the deformability of the white cells.

Because WBCs have numerous fine membrane foldings that cannot be resolved with light microscopy (LM), we studied transmission electron microscopy (TEM) of thin cell sections with the aid of the principles of stereology. We report the volume and membrane area of different types of WBCs and their nuclei. The diameter distribution of human WBCs as determined by morphometric analysis of TEM data has been compared with the results of LM measurements, which serve as a standard. Another method frequently used to demonstrate the numerous membrane foldings on the WBC and other tissue cell surfaces is scanning electron microscopy (SEM). Conflicting results, however, have been reported regarding the presence or absence of these membrane foldings on various types of lymphocytes. During preparation of WBCs for TEM and SEM, i.e., during chemical fixation, alcohol dehydration, and embedding in resin, or drying the cell volume may change. In the present study, the SEM results were compared with the data obtained from LM and TEM, and the possible shrinkage was evaluated for each method employed.

The present study demonstrates that the diameters of all leukocytes in isotonic solution are less than 10 μm and therefore significantly smaller than measured on a blood smear. All leukocytes, their nuclei and granules have more membrane area than needed to cover their volume (i.e., in a sphere), and these membrane areas remain constant during hypertonic shrinking and hypotonic swelling of the cell. That is, the cells swell by unfolding the membrane without area change. In the limit of a sphere, the membrane opposes further swelling. In order to document these important features of the WBC membranes, morphometric quantitations were also performed on WBCs in different solution osmolalities.

MATERIALS AND METHODS

Fresh venous blood samples were obtained from two healthy human subjects (age 27 and 31 yr) using EDTA as anticoagulant. The RBCs were allowed to sediment at room temperature for 25-40 min. The supernatant plasma layer containing WBCs, platelets, and...
a few RBCs were collected about every 10 min with a Pasteur pipette and suspended in 50 ml NaCl solution, which contained 0.1 g/100 ml EDTA and with pH adjusted to 7.4. To prevent sedimentation of WBCs, this solution was gently agitated. The NaCl concentrations were adjusted to cover a range of solution osmolalities from 50 to 625 mosmole (310 mosmole). The osmolality of each solution was measured by the freezing point depression method (Fiske Osmometer, Fiske Assoc., Bethel, Conn.).

Light Microscopy (LM)

A small aliquot of the suspension of WBCs in NaCl solution was transferred to an objective carrier and immediately covered with a cover glass and sealed along its edge to prevent water evaporation while they were freely suspended (as evidenced by the Brownian motion of the whole cell), and measurements were made from prints at about 3500× final magnification. Each photograph was accompanied by a length calibration (2-mm micrometer, with 0.010-mm divisions, American Optical Co., Southbridge, Mass.).

Scanning Electron Microscopy (SEM)

The cells in the NaCl solution were fixed by adding 2% glutaraldehyde solution at the same NaCl concentration as the cell suspending medium (adjusted to pH 7.4 with HCl) at a rate of about 1 drop/10 sec with continuous stirring. The final concentration was 1% glutaraldehyde. This procedure usually required about 1 hr, after which the cells were fixed for another hour in fresh 2% glutaraldehyde solution. The cells were washed in NaCl solution and then postfixed for 1 hr with 1% OsO₄ dissolved in NaCl solution at the same osmolality as the cell suspending medium and adjusted to pH 7.4 with HCl. The postfixed cells were washed in cacodylate buffer (0.1 M cacodylate, Fisher Scientific Co., Springfield, NJ., adjusted to pH 7.4 and filtered) and then fixed in distilled water. Thereafter, the cells were rinsed in ethanol (80%, 90%, 95%, and 3 times in 100%). Some specimens were transferred into small bags of micro-filter paper (3 μm pore size) and dried in a Sorvall critical point dryer filters. The WBCs were photographed on 35-mm film at about 3500× final magnification. Each photograph was accompanied by a length calibration (2-mm micrometer, with 0.010-mm divisions, American Optical Co., Southbridge, Mass.).

Transmission Electron Microscopy (TEM)

Fixation and Embedding of Cells

After fixation in glutaraldehyde, postfixation in OsO₄ and washing in cacodylate buffer and distilled water as described for SEM, the cells were stained in tannic acid and rinsed in distilled water. In some samples ruthenium red (0.05 g/liter, Alfa Corp., Ventron, Mass.) was added to the tannic acid to outline the WBC membrane. The cells were then stained with 2% uranyl acetate (Polysciences, Warrington, Pa., 2 g/100 ml distilled water, pH 3.5–3.8) and 100 mg ethanol gallate (Tridom/Fluka, Hauppauge, N.Y.). After rinsing in distilled water the cells were dehydrated in ethanol in steps of 10% from 10% to 100%, rinsed three times quickly in 100% propylene oxide (Eastman Kodak, Rochester, N.Y.), infiltrated with 50% epon in propylene oxide for 24 hr, embedded in 100% epon, and hardened at 60°C.

Sectioning and Photography

The embedded specimen was sectioned with a diamond knife on an automatic microtome (DuPont/Sorvall MT2-B) at approximately 80–100 nm thickness (silver section). In order to insure that a larger sample of different WBCs in the epon block was sectioned, every 2 or 3 thin sections were followed by a thick section (about 5–10 μm) and then followed again by thin sections. The sections were carried on a copper mesh (G 300 grid, Polysciences), stained with 6% uranyl magnesium acetate (Polysciences; pH 5) and lead citrate (Eastman Kodak), and examined and photographed on a Zeiss transmission electron microscope (EM992S) at magnifications between 5000 and 7000×. On each day of investigation a length caliper (54,000 lines/inch; Polysciences) was photographed and printed at the same magnification as the WBC section photographs. Print size was 8 × 10 inch at a final magnification of about 25,000×.

Stereologic Principles

The three-dimensional geometry of individual WBCs was calculated with the aid of stereology from TEM thin sections at random radial positions of the WBCs. All WBCs studied show an overall spherical shape and have numerous membrane foldings in isotonic or hypertonic solutions. Studies were performed on neutrophils, lymphocytes, monocytes, and eosinophils; an insufficient number of basophils was obtained for this analysis. On each section a quadratic test line system with a grid size 0.3 or 0.4 cm was superimposed for stereologic counts.

Diameter Distribution and Average Volume

Consider the WBCs to be a polydisperse system of spheres. The sphere diameters Dᵢ may be grouped in K intervals with step size so that Dᵢ - 1 < Dᵢ - Dᵢ-1 < d < Dᵢ, until Dᵢ = Dₓₖ - k d. There are (Nᵢ, k) numbers of WBCs per unit control volume with sphere diameter Dᵢ. On random sections, the WBC yield section diameter dᵢ (which is to be distinguished from Dᵢ), with (Nᵢ, k) number of cell sections per section area through the control volume. The dᵢ and (Nᵢ, k) are measured from the TEM photographs with an areal count,11 and the diameters dᵢ are also grouped in k intervals with stepsize Δ. (Nᵢ) is then computed according to the equation:

\[
(Nᵢ) = \frac{1}{\Delta} \sum_{i=1}^{k} \left( \alpha_i (Nᵢ) - \alpha_{i-1} (Nᵢ) \right) = \cdots + \alpha_k (Nᵢ) \Delta_i
\]

(1)

The coefficients αᵢ arise because a planar section of diameter dᵢ can come from the major diameter of a cell with diameter Dᵢ or from any section of a larger cell with diameter Dᵢ, . . . , or Dᵢ. The coefficients αᵢ have been listed assuming random sections for j = 1, . . . , 15, and they have been recomputed for j = 1, . . . , 30.

\[
\bar{V}_c = \frac{1}{6} \sum_{j=1}^{30} \frac{(Nᵢ) (Dᵢ)^6}{\sum_{j=1}^{30} (Nᵢ)}
\]

(2)

Equations (1) and (2) can be applied to all WBC types and to the nucleus of the lymphocytes whose overall shape closely approximates a sphere.

Volume Ratios

Consider random sections through a population of WBCs. The average volume ratio (Vᵧ)ᵢ, (Vᵧ)ᵢ, and (Vᵧ)ᵢ, which the nuclei,
granules, and mitochondria, respectively, occupy with respect to the cell volume, can be obtained by the point counting procedure. The average volumes occupied by the nucleus $V_N$, granules $V_G$, and mitochondria $V_M$ are then:

$$V_N = (V_N)/V_C; \quad V_G = (V_G)/V_C; \quad V_M = (V_M)/V_C \quad (3a, b, c)$$

**Surface Volume Ratio**

Let $S_C$ be the average cell membrane area in a population of WBCs with average volume $V_C$. The area can be measured from random sections according to the equation:

$$\frac{S_C}{V_C} = \frac{2P_l}{P_p} \quad (4)$$

$P_l$ is the sum of all intersections between the test line system and the cell membrane divided by the length of the test lines; $P_p$ is the number of test points inside all cell sections divided by the number of test points on the grid. The same relation can be applied to derive the surface/volume ratio of the cell nucleus $(S_N/V_N)$ and granules $(S_G/V_G)$.

**RESULTS**

**Light Microscopy (LM)**

WBCs suspended in EDTA-NaCl solution have an overall spherical shape and their diameter is a function of the solution osmolality. Figure 1 (A, B, and C) shows LM views of a group of human WBCs in 525 mosmole, 305 mosmole and 100 mosmole. At 305 mosmole, we can distinguish two populations: the larger granular leukocytes and the smaller essentially nongranular leukocytes. An exact measurement of the cell diameter from these photographs is not possible because the cell dimension is comparable to the wavelength of the light. Sharp focus on the outer edge of the cell shows a dark interference ring with two adjacent concentric light rings. The geometric boundary of the cell is expected to fall between the inner and outer edges of the dark ring whose thickness is typically 0.35 μm.

Figure 2 shows the histograms of WBC diameter as measured on the inner edge of the dark ring at 643 mosmole, 310 mosmole and 100 mosmole. Diameter measurements made on the outer edge of the dark ring yield histograms with the same shapes, but shifted by about 0.7 μm to the right. In isotonic and hypertonic solutions, the diameter histograms have two peaks, whereas in hypotonic solution it appears to have only one peak.

Figure 3 shows the average diameter values measured on the inner and outer edges of the dark ring as a function of the tonicity of the suspending medium. The cell population swelled markedly in solutions below 250 mosmole. Below 100 mosmole, the diameter values rose even higher, but many WBC burst and cell fragments were visible. Because the average diameter
values in such media with very low tonicity were no longer taken over the entire WBC population, these data are not included.

Scanning Electron Microscopy (SEM)

Figure 4 (A, B, C, and D) shows human WBCs fixed in media at different osmolalities. Cells in Figs. 4A, B, and C were air dried, and those in Fig. 4D were critical point dried. WBCs at 600 mosmole (Fig. 4A) and 300 mosmole (Fig. 4B) have membrane foldings. The cells fixed in 100 mosmole do not exhibit surface foldings, whether they were air dried (Fig. 4C) or critical point dried (Fig. 4D); it is noteworthy that their diameter is almost identical to that of cells fixed at higher osmolalities (Fig. 3). Figure 2B shows diameter histograms obtained from SEM examination of WBCs at different osmolalities. After the cells have been dried for SEM, their diameters are small and almost independent of the osmolality in which they were fixed. The cells that were fixed in 100 mosmole and suspended in aqueous or alcohol medium without drying have the same average diameter (swollen) as the unfixed cells in 100 mosmole, and hence the reduction of cell diameter occurs following drying.

Figure 5 shows a SEM photograph of a blood smear. The WBCs are deformed into pancake shapes with thicknesses of about 1 μm.
Transmission Electron Microscopy (TEM)

The WBCs are classified according to their cytoplasmic content in accordance with earlier investigators.28,29 Figure 6 shows single sections through neutrophils, eosinophils, lymphocytes, and monocytes fixed in media at 310 mosmole (left) and 105 mosmole (right). The general appearance of the cells in hypertonic solutions is similar to that in isotonic medium. Without any exception, all WBCs investigated (approximately 5000 cells) have a folded membrane in isotonic or hypertonic media, whereas in hypotonic NaCl solution, the membrane becomes unfolded and the cell approximates the shape of a smooth sphere. A similar trend is seen for the cell nucleus and lymphocytes. The nuclei of the other leukocytes are swollen, but still segmented, as seen on many sections.

Figure 2C shows reconstructed diameter distributions determined by stereology according to equation (1) at 520, 310, and 105 mosmole. Their average values $D$ lumped for all cells are shown in Fig. 3 by the crosses (X). Figure 7 shows the diameter distribution for each WBC population separately. The average diameters of the neutrophils, monocytes, and eosinophils are similar, whereas lymphocytes are about 0.8 μm smaller. Therefore, in a combination of all cell populations, the lymphocytes tend to form a separate peak in isotonic and hypertonic media.

Figure 8 shows the average volumes of cell and nucleus, the ratio of nuclear and cell volumes, the average membrane areas and fractional excess membrane areas $\sigma$ of the cell, and the nucleus as a function of the solution osmolality for (A) neutrophils, (B) lymphocytes, and (C) monocytes. When the cells are swollen in hypotonic media, note that the membrane areas of the cell and the nucleus remain constant within experimental errors. The nucleus–cell-volume ratio also remains essentially constant during swelling of leukocytes. One exception is noted: during swelling of lymphocytes their nucleus soon becomes a sphere, whereas their cell membrane is still partially folded (see Fig. 6B, right side); as a result,
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Fig. 7. Diameter histograms of human WBCs (fixed in 310 mosmole) from three-dimensional reconstruction of thin section transmission electron microscopy. The histograms are based on 346 sections for lymphocytes, 246 for neutrophils, 266 for monocytes, and 85 for eosinophils.

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concentration of 2% (additional osmolality ~180 mosmole) show that the shrinkage during fixation averages 0.45 μm. Such shrinkage can be avoided when a 2% glutaraldehyde solution is slowly titrated into a cell suspension with equal salt concentration to a final glutaraldehyde concentration of 1%, as has been done in the present study. The Brownian motion of the WBC granules ceased when the glutaraldehyde concentrations reached a level of the order of 0.01%, and progressive increases of the concentration of glutaraldehyde did not change the WBC diameter within the error of LM measurements. In addition, the postfixation did not alter the cell diameter. This indicates that the cell becomes osmotically inactive at relatively low glutaraldehyde concentrations.

### Table 1. Eosinophil Morphometry*

<table>
<thead>
<tr>
<th></th>
<th>Volume (cu μm)</th>
<th>Membrane Area (sq μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>206</td>
<td>324</td>
</tr>
<tr>
<td>Nucleus</td>
<td>37</td>
<td>99</td>
</tr>
</tbody>
</table>

*At 310 mosmole.

#### Scanning Electron Microscopy

Preparation of cells for SEM requires a drying procedure (critical point or air drying), which causes the evaporation of the free ethanol in the cell. LM observations have shown that shrinking of the hypo-

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**Fig. 8.** The volumes ($V_{c}$, $V_{n}$), volume ratio ($V_{c}/V_{n}$), membrane areas ($S_{c}$, $S_{n}$), and fractional excess membrane area ($e$) of the cell and the nucleus as a function of the NaCl solution osmolality in which the cells were fixed. Each point represents the mean value for more than 220 sections, and the vertical bar indicates the measurement error (see Appendix). The results are shown for (A) neutrophils, (B) lymphocytes, and (C) monocytes. The data for neutrophils and monocytes at 523 and 625 mosmole were lumped together and plotted at 575 mosmole, because in this way the assumption of having random section is better approximated and in these hypertonic media the cell diameter does not change significantly (see Fig. 3).

**Fig. 9.** The volume ratio of granules ($V_{g}$) (A and B) and mitochondria ($V_{m}$) (C and D) as a function of the NaCl solution osmolality in which the cells were fixed. The vertical bars indicate the measurement errors (see Appendix). Each point represents the mean from at least 50 sections. Note the differences in ordinate scales from A to D.
SEM in contrast to B lymphocytes, which have a smooth, unfolded membrane in isotonic solution, we should have observed a few of these cells, but none has been seen. According to our experience, such filtration and subsequent washout of cells at high pressure causes cell damage, and the filtrate contains many cells that are partially or fully swollen. All WBCs that have been swollen, either as a result of mechanical damage or in a hypotonic medium, have a smooth surface, and under SEM they also have a similar diameter as the undamaged cell. In the course of the TEM investigation, we have observed and photographed more than 2000 human lymphocytes in thin sections. All of the lymphocytes fixed in 310 mosmole had a folded membrane (Fig. 6). Even if only a small fraction of lymphocytes in the venous blood had a smooth membrane in isotonic solution, we should have observed a few of these cells, but none has been seen. Smooth cell membranes were seen only in lymphocytes fixed at 105 mosmole.

**Table 2. Leukocyte Volume Distribution**

<table>
<thead>
<tr>
<th>Leukocyte Type</th>
<th>Nucleus (%)</th>
<th>Granules (%)</th>
<th>Mitochondria (%)</th>
<th>Cytoplasm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte</td>
<td>44.4</td>
<td>2.6</td>
<td>3.5</td>
<td>51.6</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>21.3</td>
<td>15.4</td>
<td>0.6</td>
<td>62.7</td>
</tr>
<tr>
<td>Monocyte</td>
<td>25.9</td>
<td>2.6</td>
<td>3.5</td>
<td>68.1</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>17.9</td>
<td>23.0</td>
<td>0.7</td>
<td>58.4</td>
</tr>
</tbody>
</table>

*At 310 mosmole.

An interesting SEM finding is that membrane foldings, which are observed in all WBCs fixed in isotonic and hypertonic media, are not seen in WBCs fixed in a swollen state in an hypotonic medium (Fig. 4 C and D). This loss of membrane folding in hypotonic medium is also demonstrated in TEM (Fig. 6). An increase in WBC diameter in hypotonic medium, however, is found by TEM but not by SEM (Fig. 3). These results can be explained in the following manner. When a WBC (or any other soft tissue) is fixed with glutaraldehyde, its normal viscoelastic interior is chemically converted into an elastic gel containing different amounts of free water, which is then replaced by ethanol. When the cell is dried during preparation for TEM examination, the ethanol evaporates and the cell shrinks isotropically, i.e., the cell shape is preserved with a uniform change in volume at every point. Therefore, the drying process, by removing the ethanol that replaced the free water, eliminates the variation in cell size with solution tonicity without affecting the surface characteristics (presence or absence of membrane foldings) that were determined at the time of fixation.

It has been claimed that human thymus-derived T lymphocytes have a smooth, unfolded membrane in SEM in contrast to B lymphocytes, which have a membrane with many microvilli. There are several reports that support this finding, but there are also studies reporting conversely that B lymphocytes have a smoother membrane than T lymphocytes. Furthermore, several investigators find that both B and T lymphocytes have a villous membrane, but no satisfactory explanation has been given for the smooth surface on some of these lymphocytes. Our findings may provide an explanation for these observations. Many investigators obtained the T lymphocytes by filtering minced tissues through a wire mesh, and in addition, the cells were filtered through nylon fibers. According to our experience, such filtration and subsequent washout of cells at high pressure causes cell damage, and the filtrate contains many cells that are partially or fully swollen. All WBCs that have been swollen, either as a result of mechanical damage or in a hypotonic medium, have a smooth surface, and under SEM they also have a similar diameter as the undamaged cell. In the course of the TEM investigation, we have observed and photographed more than 2000 human lymphocytes in thin sections. All of the lymphocytes fixed in 310 mosmole had a folded membrane (Fig. 6). Even if only a small fraction of lymphocytes in the venous blood had a smooth membrane in isotonic solution, we should have observed a few of these cells, but none has been seen. Smooth cell membranes were seen only in lymphocytes fixed at 105 mosmole.

**Transmission Electron Microscopy and Stereology**

In preparing cells for TEM, the ethanol in the cell is replaced by epon rather than being evaporated. Epon changes its volume only insignificantly after hardening. Although we no not exclude the possibility of a small degree of cell shrinkage, the agreement between mean diameters from LM and TEM (Fig. 3) suggests that the reduction of cell diameter during preparation for TEM is less than the resolution with LM.

Two populations of lymphocytes (small and large lymphocytes) have been described on the basis of size. This claim was based on individual TEM sections without knowledge of the radial position on the cell where the section was made. Stereologic analysis of lymphocyte dimensions yields a diameter distribution with a mean value of 6.2 μm in isotonic solution (Fig.
but there is no indication of the existence of two
classes of lymphocyte diameters unless their average
diameter is less than about 0.5 μm apart. Studying T
lymphocytes in the mouse thymus, Ito and Abe did
find a difference in lymphocyte dimensions between
the cortex and medulla of the thymus, and between old
and young mice. Although they did not present any
data on peripheral lymphocytes, the average diameters
of the mouse thymus lymphocytes agree in general
with our data on human lymphocytes in peripheral
blood (Fig. 7).

Our results indicate that the membrane areas of the
WBCs and nuclei (Fig. 8), as well as the granules
(Fig. 10), remain essentially constant during swelling.
In an isotonic medium, the average excess membrane
areas for the cells and for the nuclei (Fig. 8) are,
respectively, 84% and 98% for the neutrophils, 130%
and 32% for the lymphocytes, 137% and 73% for the
monocytes, and 92% and 84% for the eosinophils.
When the cells swell, their membrane unfolds and in
the limit assumes a spherical shape. At 105 mosmole,
the granules and nuclei of the lymphocytes are spheri-
cal, and their excess membrane area is zero. The other
membranes are not yet unfolded at 105 mosmole.
The granules have a relatively small excess membrane
area in comparison to the cell or nuclear membrane.
The granules swell in equal proportion to the
whole cell between 600 mosmole and 300
mosmole, but as their shape becomes spherical, further
swelling is restricted by the membrane. Therefore, we
find that at low tonicities, their volume ratio \( V_r \) is
reduced in all leukocytes (Fig. 9A and B).

A reduction of the volume ratio in hypotonic
medium is also observed in the mitochondria (Fig. 9C
and D). However, the majority of the mitochondria
are not spherical at 105 mosmole. Mitochondria
contain internal membranes that may prevent these
structures from swelling to the same extent as the cell.
At 105 mosmole, many mitochondria in the monocytes
and lymphocytes appear to have ruptured internal
membranes, whereas the membrane surrounding the
mitochondria is generally intact.

The finding that WBC membranes preserve the
surface areas within narrow limits is supported by the
results of our current micropipette experiments. The
ability of the WBC to spread or to stretch out during
deformation is therefore limited by the available
membrane area, and this is of importance in the
understanding of WBC functions. For example, when
a WBC invaginates a particle during phagocytosis,
part of the cell membrane is incorporated, leaving the
WBC with reduced excess membrane. This may limit
the number of particles that the cell can phagocytose
at a given time.

The major shortcoming of this study is the relatively
small number of sections for TEM studied. One would
like to increase the number of sections by at least 5–10
times so that the assumption of randomness underly-
ing the stereologic relationships is truly satisfied, the
shape of the reconstructed histograms becomes more
definitive, and basophils can also be included in the
analysis. These objectives may be attained with the
availability of semiautomatic morphometric counting
devices.

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APPENDIX: ESTIMATES OF MEASUREMENT ERROR

Errors in stereologic measurements from TEM may result from (A) alterations occurring during fixation, dehydration, and embedding, (B) experimental departure from the assumptions inherent in the stereologic analysis, and (C) the finite section thickness that cannot be neglected.

With respect to (A), the diameters established from stereologic analysis of TEM micrographs at various osmolalities are about equal to the LM value for the inner edge of the dark ring (Fig. 3). The TEM diameter was based on that of a smooth sphere with a mass equal to that of the actual cell, whereas the LM image was obtained on cells with surface foldings. Since the average height of these foldings at 305 mosmole is about 0.35 µm (vertical bars in Fig. 3), the TEM value indicates that the average diameter at the outer edge of the membrane folding would be 6.72 + (2 x 0.35) = 7.42 µm, which agrees with the LM value for the outer edge of the dark ring. This agreement between TEM and LM indicates that any possible change of diameter during preparation for TEM is below the resolution of our LM. In contrast, the distortion of the cell during preparation for SEM is significant (Fig. 3), and SEM images cannot be used for quantitative morphometric measurements except when the cells are dehydrated in a medium with high osmolality (>400 mosmole).

With respect to (B), in order to satisfy the assumption that random sections through the cells have been obtained, all WBC sections encountered on the grid must be photographed and analyzed, and a relatively large number of sections is needed. When the average cell volume \( V \) was computed for increasing number of cumulative sections (100, 120, 140, etc.), we found that above 200, the values vary from the final mean by less than 10%, which is considered to be acceptable. However, this number is not sufficient to obtain a constant histogram, as individual frequency values within the histograms may vary by more than 10%. The measurement of volume ratio \( V \) contains an error due to the finite number of point counts (see ref. 30, Fig. 13). All values of \( V \) are based on more than 10,000 counts, so that the relative error is less than 5%. The values of \( V \) for neutrophils, monocytes, and eosinophils and \( V \) for lymphocytes and monocytes are based on more than 4000 counts, so that the relative error is less than 6%. \( V \) for lymphocytes and \( V \) for neutrophils and eosinophils, which have small volume ratios, may contain an error of 15%. The determination of surface-volume ratios is based on the assumption that the direction of the normal vector on the corresponding membrane surface is random in space.

Due to the overall spherical shape of the cell and the presence of many surface foldings, this assumption is expected to be well satisfied. Indeed, computations of the surface-volume ratio with increasing number of cumulative sections showed that the variations of the ratio became less than 10% when the number of sections was more than 100.

With respect to (C), the fact that the diameter of the granules is comparable to the section thickness leads to an overestimation of the volume ratio \( V \). For a typical section thickness of 0.08 µm, the \( V \) for lymphocytes in Table 2 is overestimated maximally by 44%, monocytes by ~29%, neutrophils by ~28%, and eosinophils by ~19%. Smaller errors are expected for \( V \) of mitochondria. The error in the \( V \) measurement is negligible, because the section thickness is small in comparison to nuclear dimensions.

Measurement errors due to uncertainty in the magnification and errors in the counting procedures with the test grid were estimated by repetitive determinations and found to be less than 2% of the mean values.
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