Viscosity of passive human neutrophils undergoing small deformations

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ABSTRACT At issue is the type of constitutive equation that can be used to describe all possible types of deformation of the neutrophil. Here a neutrophil undergoing small deformations is studied by aspirating it into a glass pipet with a diameter that is only slightly smaller than the diameter of the spherically shaped cell. After being held in the pipet for at least seven seconds, the cell is rapidly expelled and allowed to recover its undeformed, spherical shape. The recovery takes ~15 s. An analysis of the recovery process that treats the cell as a simple Newtonian liquid drop with a constant cortical (surface) tension gives a value of 3.3×10^{-5} cm/s for the ratio of the cortical tension to cytoplasmic viscosity. This value is about twice as large as a previously published value obtained with the same model from studies of large deformations of neutrophils. This discrepancy indicates that the cytoplasmic viscosity decreases as the amount of deformation decreases. An extrapolated value for the cytoplasmic viscosity at zero deformation is ~600 poise when a value for the cortical tension of 0.024 dyn/cm is assumed. Clearly the neutrophil does not behave like a simple Newtonian liquid drop in that small deformations are inherently different from large deformations. More complex models consisting either of two or more fluids or multiple shells must be developed. The complex structure inside the neutrophil is shown in scanning electron micrographs of osmotically burst cells and cells whose membrane has been dissolved away.

INTRODUCTION

Spherically shaped passive neutrophils must undergo a significant amount of deformation as they squeeze through the smallest vessels of the circulation and the small openings between vessels and tissue. This ability to deform in response to external pressures and shear forces and then return to an undeformed spherical shape shows that the neutrophil exhibits characterizable material behavior. That is, its deformation can be described by constitutive equations relating material stresses to material strains and strain rates through the appropriate viscous and elastic parameters. The major issue, then, is what kind of material constitutive equation should be used to describe the deformation behavior of the neutrophil *when it undergoes all possible types of deformation?*

Usually materials can be described as either solid-like or liquid-like. Solid-like materials remember their undeformed state and have an increase in their resistance to deformation as they are deformed. This seems like an obvious way to describe the deformation of a neutrophil because the unstressed neutrophil readily returns, within about one minute, to its spherical shape after it is deformed. Bagge et al. (1) were the first to propose that the neutrophil behaved as a solid when deformed in a tapered pipet or a capillary. This solid-like model received its ultimate refinement in work by Schmid-Schönbein et al. (2). Their so-called "standard solid model" has been used in many other studies of cell deformation (3–5). However, these studies involved only small deformations of small portions of neutrophils as they were aspirated into small pipets. Although the standard solid model, which involves two elastic parameters and one viscous parameter, would fit the experimental data, the experiments did not adequately test the model. For a solid model to be tested, the cell must be subjected to large deformations as well to see if the resistance to deformation continues to increase. A solid model means that it would become harder and harder to deform cells as they are squeezed through smaller and smaller openings.

By studying relatively large deformations of neutrophils as they were aspirated into small pipets, Evans and Kukan (6) and Evans and Yeung (7) showed that the neutrophil behaves more like a liquid than a solid. The cell assumes a spherical shape when in a "stress-free" state because of a persistent "cortical" tension acting over the surface of the cell. Thus, the cell could have a liquid interior and still "remember" its stress-free spherical shape just as a solid would. Evans and Yeung modeled the interior of the cell as a Newtonian liquid and obtained a value for the viscosity of the cytoplasm of ~2,000 poise (200 Pa · s) over the range of deformation of their experiments and to within a fairly large standard deviation of ±50%. Needham and Hochmuth (8) used a result from Yeung and Evans (9) to analyze the very rapid and complete flow of neutrophils into pipets and obtained a value for the viscosity of 1,350 ± 540 poise. In the work of Needham and Hochmuth there was some indication of "shear thinning"; i.e., the viscosity decreased in some cases as the rate of deformation was increased. Thus, the interior liquid or cytoplasm behaves as a liquid but not a Newtonian liquid. In any case, the values for the cytoplasmic viscosity for cells undergoing large deformations (6–8) and analyzed according to a liquid drop model (9) are about a factor of ten larger than those values obtained for cells undergoing small deformations and analyzed according to either a "standard solid model" (2–5) or a Maxwell liquid model (10). Although a true comparison can only be made by using the same model when analyzing cellular deforma-

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tion, it is still surprising that the measured values are in such disagreement. This leads us to suggest, and to show in this work, that small deformations of the human neutrophil are inherently different from large deformations and will give different values for an "apparent" viscosity.

We use the technique and analysis of Tran-Son-Tay et al. (11) to determine the viscosity of a neutrophil when it is modeled as a Newtonian liquid drop (6–9). Here a neutrophil is aspirated into a pipet and then allowed to recover its undeformed spherical shape. This yields a value for the ratio of the cortical tension to the cytoplasmic viscosity. Then, the value for the viscosity is readily calculated using an accurate measurement for the cortical tension over the same range of deformation (12). Tran-Son-Tay et al. studied only the large deformation recovery of neutrophils; here we study the small deformation recovery. We find in fact that the value for the small-deformation viscosity is significantly less than that for large deformations.

MATERIALS AND METHODS

Cell preparation

Human neutrophils are isolated (in sterile EDTA (K_2) vacutainers (Becton Dickinson)) from the peripheral blood of healthy volunteers as described previously (13, 14). The blood is centrifuged at 300 *g* for 25 min at 25°C to remove the majority of erythrocytes. The plasma and buffy coat are diluted to 50% with modified endotoxin-free Hanks' balanced salt solution (HBSS, no Ca^{+2} or Mg^{+2}) and carefully layered over Ficoll-Hypaque gradients (Sigma Histopaque −1077 and −1119) having densities (g/cm^3) of 1.077 and 1.119 at 25°C. After 20 min centrifugation at 800 *g* and 25°C, the neutrophils at the 1.077/1.119 interface are collected and washed twice with 10× volume HBSS and finally resuspended in 50% autologous plasma/HBSS to prevent adhesion to glass surfaces. This procedure yields cells that are 99% viable as determined by trypan blue exclusion test and 90% passive as determined visually by a spherical shape and an absence of pseudopods.

Scanning electron microscopy of cytoplasm

Cells in modified HBSS are first deposited on 12 mm glass disks previously treated with Cell-Tak (Collaborative Biomedical Research, Bedford, MA). For osmotically lysed cells, the glass disks with cells are placed in distilled water for 15 min whereas for detergent extraction, cells are placed in 0.25% Triton X-100 in HBSS for 30 s at 21°C.

After osmotic lysis and detergent treatment, the cells are fixed in 0.1% glutaraldehyde for 1 h at 21°C followed by another hour in 1% glutaraldehyde. After washing with HBSS, the cells are postfixed in 1% OsO_4 in veronal acetate buffer, pH 7.4 for 1 h. Dehydration of the cells is carried out in a graded series of acetone and then critical-point dried using liquid CO_2 in a Ladd critical point drier (Burlington, VT). Disks with monolayers of cells are mounted on aluminum stubs using a conductive paint and coated with a thin layer (~20 nm) of gold-palladium using a Hummer V sputter. Samples are stored under vacuum until they are examined and imaged in a Philips 501 scanning electron microscope at 15 kV.

Cell Micromanipulation

Pipets are made from 1-mm capillary glass tubing pulled to a fine point and broken by quick fracture to the desired tip diameter of either 6.5 or 7.2 μm. Cells at room temperature (~22°C) are rapidly drawn by small suction pressures into these relatively large pipets and held there for at least seven seconds before they are quickly expelled from the pipet. These cells show consistent recovery times whereas cells held for a shorter time period recover more quickly. Tran-Son-Tay et al. (11) observed similar behavior in their study of the recovery of neutrophils following large deformation. Cellular recovery is shown in Fig. 1. Recovery is almost complete after 15 s. As the cell recovers, the length and width of the cell are measured at intervals ranging from 0.25 s at the beginning of the recovery process to 10 s at the end of the process. Typically ~15 length-width pairs are measured. A few cells recover in a highly irregular way with periods of stasis followed by rapid recovery. These cells are probably activating and are not included in the data.

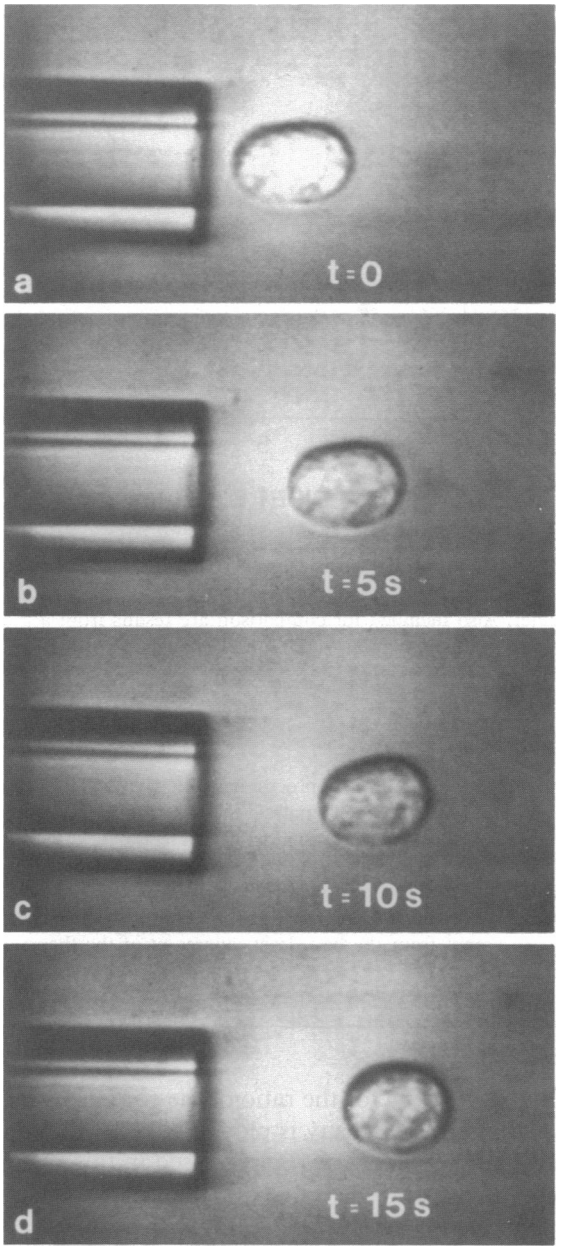

FIGURE 1 Video micrographs of neutrophil recovery as a function of time after a small deformation (pipet diameter = 7.2 μm). (*a*) The cell is shown immediately after expulsion from the pipet at $t = 0$, (*b*) $t = 5$ s, (*c*) $t = 10$ s, and (*d*) the cell is shown almost recovered to its initial spherical shape at $t = 15$ s.

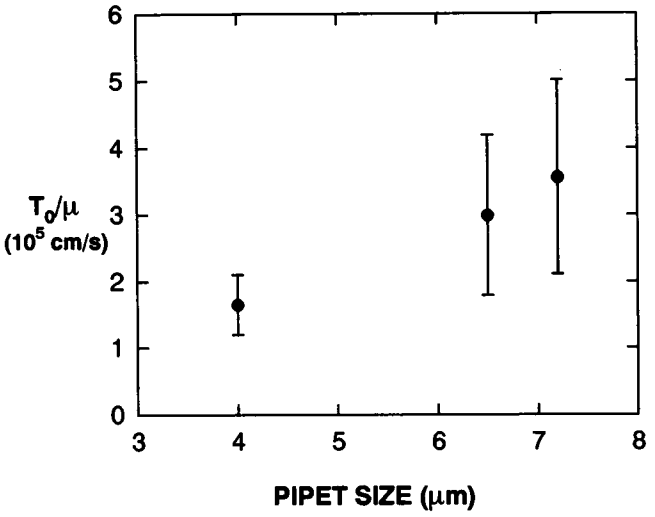

FIGURE 2 The ratio of the cortical tension T_0 to the cytoplasmic viscosity μ from the present study with pipets that are 6.5 and 7.2 μm in diameter. Also included for comparison are results from a previous study (11) with a pipet that was 4 μm in diameter.

Analysis

The exponential-like recovery process has been analyzed by Tran-Son-Tay et al. (11). From their analysis they were able to describe the recovery process with a polynomial series that relates the dimensionless length or width (length or width divided by the undeformed, spherical diameter of the cell) to the dimensionless time (time divided by T_0/μ, the ratio of the cortical tension to the cytoplasmic viscosity). The value for T_0/μ comes from the best, least-squares fit of the theory to the experimental data.

RESULTS

In Fig. 2 the value for the ratio of the cortical tension to the cytoplasmic viscosity is plotted as a function to the pipet diameter for the two pipets used in this study (diameter = 6.5 and 7.2 μm) and the pipet used in our previous study (diameter = 4 μm) of the shape recovery of neutrophils following large deformations (11). It is apparent that T_0/μ increases as the pipet diameter increases. The result from this study with either of the two larger pipets is significantly different ($P < 0.001$) from the result of our previous study with the smaller pipet.

Fig. 3 shows a plot of T_0/μ as a function of the initial deformation in the pipet, as given by L_p/D_0 (the length of the cell in the pipet before ejection, L_p, relative to the spherical diameter of the cell in the undeformed state, D_0). In all, 36 cells were studied here while 34 cells were studied previously (11). Even for a given pipet diameter (Fig. 2), the data show a range in values for L_p/D_0 because of a variation in cell size (volume). It can be seen in Fig. 3 that the value for T_0/μ tends to decrease (and, thus, the viscosity tends to increase for a given value for the cortical tension T_0) as the initial state of deformation increases. This led us to analyze the original results of Tran-Son-Tay et al. (11) as a function of L_p/D_0 (this was not done in the original paper) and plot these results as the filled circles in Fig. 3. As can be seen, the values for T_0/μ for cells undergoing large deformations are considerably less than the values for small deformations. In the experiments reported here, the average value for T_0/μ is $3.3 \cdot 10^{-5}$ cm/s. This is about twice as large as the average value of $1.7 \cdot 10^{-5}$ cm/s given by Tran-Son-Tay et al. (11) in their study of the recovery of neutrophils following large deformations. For a given value for the cortical tension of 0.024 dyn/cm (12), the two results give an average value for the viscosity of 730 poise for "small" deformations and 1400 poise for "large" deformations.

A linear regression line to the data in Fig. 3 gives an R^2 value of 0.40. One datum point was dropped as a statistical outlier (15). The P value of this regression line is significant at a level of $P < 0.001$ (15). The extrapolated value for T_0/μ at zero deformation ($L_p/D_0 = 1.0$) is $3.9 \cdot 10^{-5}$ cm/s. This gives a limiting value for the viscosity of about 600 poise when $T_0 = 0.024$ dyn/cm.

Values for the cytoplasmic viscosity μ are readily calculated from the data shown in Fig. 3 if the value for the cortical tension T_0 is known. The results of such a calculation are shown in Fig. 4 for $T_0 = 0.024$ dyn/cm (12). Here the R^2 value is 0.55. This figure clearly illustrates how the cytoplasmic viscosity increases as the initial deformation of the cell increases.

Each independent set of data in Fig. 4 (and Fig. 3) gives a similar trend in that the apparent viscosity increases as the initial state of deformation increases. The slope of the line for the results for the smaller pipet

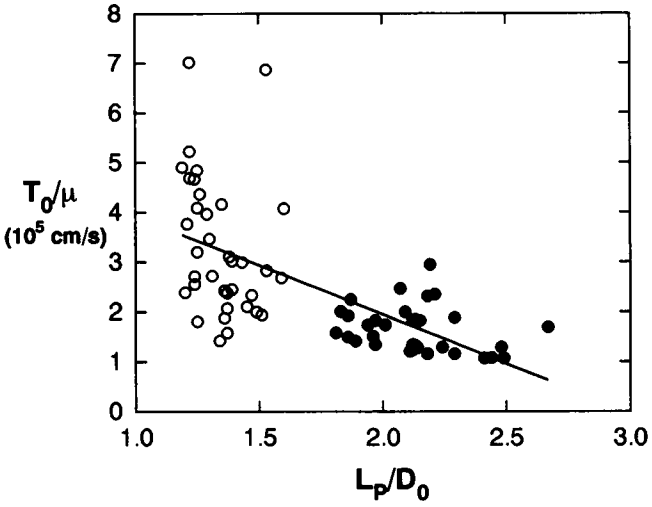

FIGURE 3 The ratio of the cortical tension to the cytoplasmic viscosity T_0/μ (Fig. 2) plotted as a function of the initial deformation in the pipet as given by L_p/D_0, the length of the cell in the pipet before ejection relative to the spherical diameter of the cell in its undeformed, spherical state. The open circles represent the 36 cells in this study while the closed circles are for 34 cells from a previous study (11). Even for a given pipet diameter (Fig. 2), the data show a range in values for L_p/D_0 because of a variation in cell size (volume). The linear regression line shown in the figure is significant at a level of $P < 0.001$ (15).

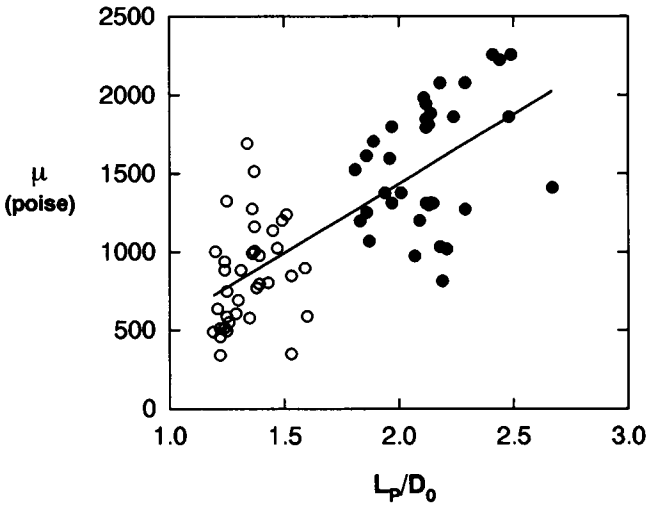

FIGURE 4 Calculation of an "apparent" viscosity from the data shown in Fig. 3 by using a constant, fixed value for the cortical tension of T_0 = 0.024 dyn/cm (12). The open and closed circles are as given in the caption of Fig. 3.

(*closed circles*) is virtually the same as that for the two larger pipets (*open circles*). At an intermediate point where $L_p/D_0 = 1.7$, the straight line fit to the data from the smaller pipet predicts an apparent viscosity of 1250 poise while that for the data from the two larger pipets predicts a value of 1170 poise. Thus the extrapolated values from each set of data agree closely and the trends from the two sets of data when combined as shown in Figs. 3 and 4 strongly reinforce each other.

The complexity of the cytoplasm within the neutrophil is shown in Fig. 5. Here scanning electron micrographs (SEM) of an osmotically swollen and lysed cell (Fig. 5 *a*) reveal the particulate (granular) nature of the cytoplasm, while an SEM of a detergent extracted cell (Fig. 5 *b*) reveals the filamentous structure of the cytoplasm at and near the inner membrane surface. These cellular components are embedded in an aqueous environment and are responsible for the large values of viscosity reported here and elsewhere. Clearly, treating what appears to be a highly structured interior of a cell as a continuum described by a single viscous coefficient results in a loss of resolution. More complex models must be developed.

DISCUSSION

We observe that the apparent viscosity of the cellular cytoplasm decreases as the cell undergoes smaller deformations (Fig. 4). The limiting value for the apparent viscosity as the degree of deformation approaches zero is ~600 poise. However, this value is still two to three times larger than the values obtained by Sung et al. (5) and Dong et al. (10) in their studies of the recovery of neutrophils following small deformations in a pipet. In the first case (5), a three parameter "standard solid model" (2) was used to analyze the date, while in the second case (10) a three-parameter Maxwell liquid drop model was used. In both of these studies, three-parameter, least-squares fits are used to determine the elastic and viscous coefficients, whereas in our study only a single parameter (T_0/μ) is varied during the least-squares fit. A lack of sensitivity of the least-squares fit to the values for the three parameters could lead to the discrepancy between our results and those of Dong et al. (10) for the Maxwell liquid drop model. Also, in the studies of Dong et al. (10), it appears that only one or two cells

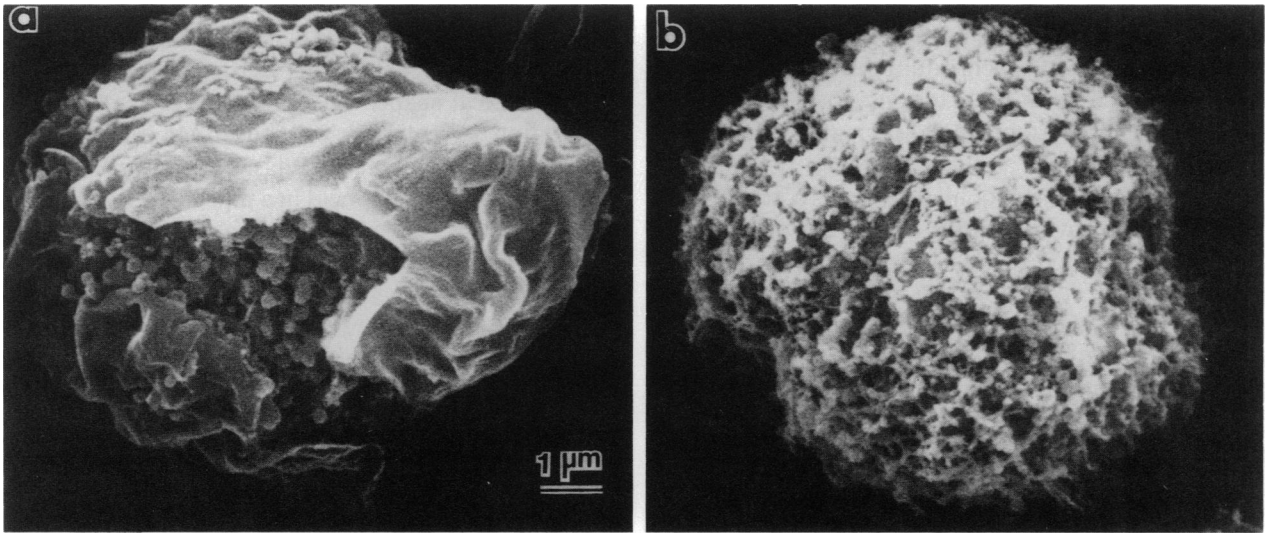

FIGURE 5 Scanning electron micrographs of an osmotically lysed (*a*) and a detergent solubilized (*b*) neutrophil. By bursting a hole in the plasma membrane or by dissolving away the whole membrane the complex filamentous and granular nature of the cytoplasm is revealed. These micrographs lend credence to a two-fluid model of the cytoplasm.

were analyzed. As can be easily seen from the results shown in Fig. 4, individual cells differ greatly in their values for the apparent viscosity. In any case, it is clear that the apparent viscosity of the cytoplasm depends on the extent of deformation. Thus, the cytoplasm can not be modeled as a simple Newtonian liquid or even as a simple Maxwell liquid (16).

At this point, it will be worthwhile to summarize all observations about the material behavior of passive neutrophils. The neutrophil behaves as a non-Newtonian liquid drop with a constant, or nearly constant, cortical tension (12). When undergoing large deformations, the cellular cytoplasm is very viscous, with a viscosity on the order of 1000–2000 poise (7, 8). At higher rates of deformation, some evidence of shear-thinning behavior is observed (8). If a force is applied rapidly to a spherical cell and removed suddenly (within 0.3 s), the cell responds like an elastic body in that it returns quickly (within 0.3 s) to a nearly spherical shape. (These unpublished observations were made by D. Needham when an initially spherical cell was rapidly forced down and out of a tapered tube with an opening that was about half of the undeformed diameter of the cell.) When undergoing small deformations, the cytoplasm is less viscous, with a limiting viscosity at zero deformation of 600 poise (this study) or 100–300 poise (2, 3, 10). Cells held in a pipet have a fading elastic memory that lasts ~5 (11) to 7 s (this study). Neutrophils rapidly aspirated into a pipet with a diameter that is smaller than the cell diameter enter at an initial velocity that is much faster than the "steady-state" velocity (8, 16). This can be explained by a smaller apparent viscosity at smaller deformations and larger rates of deformation. If the initial phase of entry in the studies of Needham and Hochmuth (8) is analyzed in this way, we calculate a viscosity of 300 poise (unpublished result). Dong et al. (16) used a finite element analysis and attempted to explain the initial phase of entry into a pipet with a Maxwell liquid-drop model. However, they could only fit the experimental data to their Maxwell model by systematically increasing the elastic and viscous coefficients as the cell moved further into the pipet. Clearly the neutrophil behaves as a liquid drop (6–12), although not as a simple Newtonian or Maxwell liquid drop. Other models must be formulated to explain our observations.

How can we describe the behavior of the neutrophil as it undergoes small and large deformations at slow and fast rates? Dong et al. (1991) have suggested that the neutrophil be modeled as a three-layered structure. Tözeren et al. (17) have suggested a similar model for other cell types such as T-lymphocytes. The outer layer in this three-layer model consists of a thin cortical shell with a persistent isotropic surface tension. The next layer is a thick shell of cytoplasm that is modeled as either a Newtonian or Maxwell liquid with a relatively small viscosity. The inner layer is a core region that consists of the segmented nucleus and surrounding cytoskeleton. The core material is modeled as a Newtonian or Maxwell liquid with a relatively large viscosity. The peripheral cytoplasm with its smaller viscosity could act as a lubricating layer around the more viscous nucleus. Such a model would predict a smaller viscosity for small deformations in a larger pipet (in which the more viscous nuclear core would not deform) and a larger apparent viscosity for larger deformations that involve the nucleus. Also, such a model could exhibit shear-thinning behavior in that rapid deformations could preferentially involve the less viscous periphery while slower deformations could involve both the less viscous periphery and the more viscous core. Finally, such a model would have several material coefficients (two viscous coefficients, possibly one or two elastic moduli and one cortical tension) and two geometric parameters (the radius of the core and the thickness of the inner shell).

An alternative to a model based on several layers is a two-fluid model proposed by Dembo (personal communication). In this model the cytoplasm consists of a less viscous liquid intermixed with a much more viscous liquid that is rich in granules and cytoskeleton. Fig. 5 *a* illustrates the rich granular structure of the cytoplasm, while Fig. 5 *b* shows a complex filamentous and granular structure at and near the inner membrane surface of the cell. Imagine, then, a less viscous liquid flowing through a highly viscous liquid-like structure. The less viscous liquid flows preferentially during small deformations and during the initial phases of rapid deformation. Osmotic stresses are created when the two liquids are separated. (Note that a cortical tension of 0.024 dyn/cm gives a pressure in a 4 μm radius cell of 120 dyn/cm^2 and such a pressure can be osmotically created by a difference in the concentration of, say, large proteins of only 5×10^{-6} gmol/liter.) These colloid osmotic stresses will tend to drive the fluids back to an equilibrium state just as the small cortical tension (12) causes the cell to return to a spherical shape. An osmotic stress created by the separation of two liquids will cause a Maxwell-like behavior that explains the fading elastic memory exhibited by a neutrophil when it is held in a pipet for a few seconds or longer.

At this point it is difficult to chose between a model based on separate layers and one based on two intermixed fluids, especially since the analytical solutions to these models have not been reported. However, the experiments reported here and elsewhere speak for themselves. The model that is the most consistent with the morphology of cell and cytoplasm, and that best explains the experimental observations using the fewest geometric and material (elastic, viscous) parameters, will be the most appropriate model.

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