brief communication

A sensitive measure of surface stress in the resting neutrophil

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ABSTRACT The simplest parameterized model of the "passive" or "resting receptive" neutrophil views the cell as being composed of an outer cortex surrounding an essentially liquid-like highly viscous cytoplasm. This cortex has been measured to maintain a small persistent tension of ~0.035 dyn/cm (Evans and Yeung. 1989. *Biophys. J.* 56:151–160) and is responsible for recovering the spherical shape of the cell after large deformation. The origin of the cortical tension is at present unknown, but speculations are that it may be an active process related to the sensitivity of a given cell to external stimulation and the "passive–active" transition. In order to characterize further this feature of the neutrophil we have used a new micropipet manipulation method to give a sensitive measure of the surface stress as a function of the surface area dilation of the highly ruffled cellular membrane. In the experiment, a single cell is driven down a tapered pipet in a series equilibrium deformation positions. Each equilibrium position represents a balance between the stress in the membrane and the pressure drop across the cell. For most cells that seemed to be "passive," as judged by their spherical appearance and lack of pseudopod activity, area dilations of ~30% were accompanied by only a small increase in the membrane tension, indicative of a very small apparent elastic area expansion modulus (~0.04 dyn/cm). Extrapolations back to zero area dilation gave a value for the tension in the resting membrane of 0.024 ± 0.003 dyn/cm, in close agreement with earlier measures. A few cells showed virtually no change in cortical tension and fit the persistent cortical tension model of Evans and Yeung (1989. *Biophys. J.* 56:151–160). However, other cells that also appeared "passive," as judged by their spherical appearance, had membrane tensions that increased as the apparent surface area was increased. Thus, the postulated, persistent "cortical tension" does not appear to be a unique and constant parameter for all cells as the membrane area is dilated. This measurement of membrane tension could represent a sensitive indication of the first stages of cell activation and the "passive–active" transition.

INTRODUCTION

The neutrophil can exist in three states: a resting receptive, or passive state; a primed state; and an active state. From a cellular physiology standpoint these states are characterized via changes in receptor expression and chemical responses to stimulation. Physically, the transformation from passive to active states involves gross changes in morphology, shape, and material properties. The spherical cell undergoes rapid changes associated with the formation of F-actin rich, solid-like projections during pseudopod activity and cell "activation."

In order to characterize the neutrophil in more detail, (local) composition, structure and mechanical properties must eventually be correlated with the biochemical and physiological states of the cell. In this paper we are concerned with the in vitro mechanical properties of the ostensibly unprimed, unstimulated "passive" state as a prelude to studies on the other two states.

A nonadherent, passive neutrophil has a spherical shape as long as it is not subjected to an external force. As shown in Fig. 1, the surface membrane of the cell is highly ruffled, creating an excess of membrane surface area that is about double the projected surface area of the spherical cell (1). Thus, for equilibrium deformations from spherical geometry, the cell can be significantly deformed and elongated without its membrane being pulled taut. After being deformed, the cell recovers its spherical shape in about 1 min (2). This behavior can be explained in two different ways. On the one hand, the cell can be considered as a viscoelastic solid (3, 4, 5) with an internal, possibly homogeneous, elastic structure embedded in an aqueous liquid. The elastic structure "remembers" its natural reference state. For such an elastic solid, the force of deformation, at equilibrium, must increase with the extent of deformation according to an elastic constitutive equation like Hooke's Law. On the other hand, the cell can be viewed as a viscoelastic liquid with a persistent cortical (surface) tension (6) that pulls the cell into a spherical shape.

Strong evidence for the view that the cell behaves as a liquid drop has been given by Evans and Yeung (1), and Needham and Hochmuth (7). They showed that a resting neutrophil will flow continuously into a small capillary tube or "micropipet" as long as the aspiration

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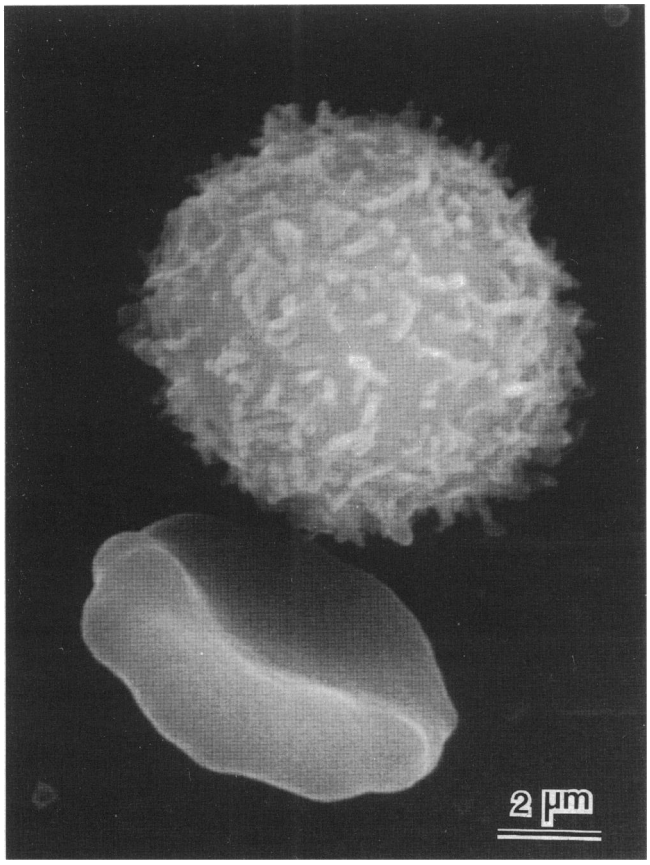

FIGURE 1 Scanning electron micrograph of a "passive" neutrophil and an erythrocyte. For the neutrophil, the large amount of excess lipid membrane is clearly seen to be in the form of microvilli and folds that can unravel when the cell is made to deform from its natural spherical geometry.

pressure exceeds a "critical pressure." This critical pressure, ΔP_{cr}, which is the pressure required to form a static, hemispherical projection in the micropipet, is related to the cortical surface tension, $\overline{T}$, by the "law of Laplace" for a liquid drop:

$$\Delta P_{cr} = 2\overline{T}\left(\frac{1}{R_p} - \frac{1}{R_c}\right), \quad (1)$$

where R_p is the radius of the pipet and R_c is the radius of the cell external to the pipet. For a cell with a radius of 4 μm, from which a hemispherical projection is formed in a 2-μm radius pipet, a critical pressure of 150 dyn/cm^2 gives a value for the cortical tension of 0.03 dyn/cm.

The continuous flow of a neutrophil into a pipet caused by an aspiration pressure in excess of the critical pressure is indicative of a liquid-like behavior. This liquid-like behavior can occur even if the cortex behaves as an elastic solid and the cortical tension increases in value as the surface area is expanded. In this case, if the increase in cortical tension is large enough and the suction pressure in excess of the cortical pressure is small enough, the cell could halt at a new point of metastable equilibrium beyond the point where the hemispherical projection is formed. Alternatively, the cell could retract from the pipet when the aspiration pressure is decreased to the value of the critical pressure. In some cases, Evans and Yeung (1) observed a small amount of retraction.

The origin of the cortical tension is at present unknown, but indications are that it may be related to the sensitivity of a given cell to external stimulation and the passive–active transition. In this study we have undertaken an investigation of the cortical tension. We wish to obtain a precise measure of the cortical tension and to see if it changes as the cell is deformed. This is accomplished by driving the neutrophil down a slightly tapered tube with a small pressure ($\sim$ 10–100 dyn/cm^2) until it comes to rest in the taper. The apparent surface area of the cell expands as it moves down the taper and the cortical tension is calculated at any equilibrium position with a form of the law of Laplace for a tapered tube. Eq. 1 is a specific application of the law of Laplace for a uniform tube.

METHODS

Cell preparation

Methods for the preparation of cells have been described in detail elsewhere (8) and so here we will only outline the main features. The cell preparation procedure was designed to give a pure sample of neutrophils in as physiological an environment as possible, while minimizing the cells' active response to the glass surfaces of the microchamber and the pipet. Human neutrophils were isolated according to the procedure of English and Andersen (9). Whole venous blood, anticoagulated with EDTA, was diluted to 50% (vol/vol) with Ca^{2+} and Mg^{2+}-free modified Hanks' balanced salt solution (HBSS, pH 7.4, 300 mosm; Sigma Chemical Co., St. Louis, MO) containing EDTA and carefully layered over Ficoll-Hypaque gradients (Sigma Histopaque-1077 and -1119) having densities of 1.077 g/cm^3 and 1.119 g/cm^3, respectively, at 25°C. After 20 min centrifugation at 800 *g* at 25°C, the cells at the 1077/1119 interface were collected and washed twice with 10× volume HBSS. The final suspension of neutrophils was in 50% autologous plasma/HBSS solution to prevent adhesion to glass surfaces (6). The purity and viability of sample cells were determined by Wright stain and trypan blue exclusion tests. Cells were prepared from three different donors on different days.

Micropipet manipulation

Micropipets were made from 1-mm capillary glass tubing pulled to a fine point with a gradual taper, and broken by quick fracture to the desired tip diameter of 4.0 μm. Pipets were filled with equiosmotic buffer and were connected to a manometer via water filled tubing. Sensitive measures of pipet pressures in the range from 2 to 100 dyn/cm^2 were obtained by using a micrometer driven displacement of a water-filled reservoir. Cells were manipulated at room temperature in a microchamber on the microscope stage by a micropipet that has

been flushed previously with 50% autologous plasma solution in order to prevent adhesion of the cell to the glass surface. Experiments were viewed with an interference contrast video microscope and recorded on video tape for subsequent geometric analysis using video calipers.

As shown in Fig. 2, a single "passive" cell was selected and aspirated into the tapered pipet and allowed to recover to its resting spherical shape in the larger lumen of the pipet. Initial choice of the cell was made on the basis of it appearing spherical with an absence of pseudopod activity. Once in the pipet, only those cells that maintained this spherical, passive appearance were tested. An extremely small positive pipet pressure (10 dyn/cm^2) was then applied, and the cell was very gently deformed into a bolus shape as it was driven down the pipet and came to rest in the taper. The pipet pressure was then made to gently oscillate about this particular driving pressure in order to ease the cell into as true an equilibrium position as possible and to detect for any tendency of the cell to stick to the pipet wall. The cell was allowed to reach this equilibrium position over a period of 30 s or so. A step increase in pipet pressure then drove the cell to a new equilibrium position. Several such equilibrium measurements were made on a given cell. In some instances the process was reversed, in which case the cell was gently moved back up the pipet, and a new reduced pressure was applied. Each equilibrium position represents a balance between the cortical tension in the membrane and the driving pressure that is imposed across the cell, scaled by the difference in the radii of curvature at each end of the bolus. Membrane tension was calculated at each pressure as a function of the area dilation of the cell bolus.

Analysis

A schematic diagram of a neutrophil in a tapered pipet is shown in Fig. 3. The spherical caps at the leading and trailing ends of the cell have radii of r_a and r_b, respectively. We assume a liquid filled cell with a uniform membrane tension $\overline{T}$. Then, the law of Laplace gives,

$$\Delta P = \overline{T}\left(\frac{1}{r_a} - \frac{1}{r_b}\right), \tag{2}$$

where $\Delta P = P_b - P_a$. With the use of basic trigonometric and geometric relations, we calculate r_a and r_b from our measurements of L_p, D, and the radius of the pipet opening r_p:

$$r_a = \frac{L_p \cdot \tan\theta + r_p}{\cos\theta - \tan\theta(1 - \sin\theta)} \tag{3a}$$

$$r_b = \frac{D \cdot \sin\theta + r_a(1 - \sin\theta)}{1 + \sin\theta}. \tag{3b}$$

At any position, we calculate the volume of the cell, which should remain constant (and does), from

$$V = \frac{\pi}{3}(C_2R_2^3 - C_1R_1^3), \tag{4}$$

where

$$R_1 = r_a/\tan\theta$$

$$R_2 = r_b/\tan\theta$$

$$C_1 = \sin^2\theta\cos\theta - \tan^3\theta(1 - \sin\theta)^2(2 + \sin\theta)$$

$$C_2 = \sin^2\theta\cos\theta + \tan^3\theta(1 + \sin\theta)^2(2 - \sin\theta).$$

The radius of an equivalent sphere is calculated from the volume, $V = (4/3)\pi r_0^3$, and thus, the surface area of the sphere is calculated from $A_0 = 4\pi r_0^2$. The surface area of the deformed cell as shown in Fig. 3 is calculated from

$$\frac{A}{2\pi} = r_a^2(1 - \sin\theta) + \frac{(r_b^2 - r_a^2)\cos^2\theta}{2\sin\theta} + r_b^2(1 + \sin\theta). \tag{5}$$

Now, the fractional area change, $(A - A_0)/A_0$ is readily calculated at any location in the tube and compared with the value for the cortical tension at that location as given by Eq. 2.

RESULTS

We observed a range of behaviors for cells taken from the same sample, and from sample to sample and donor to donor.

Average behavior

For most of the apparently passive cells studied, the dilation of membrane area by as much as 32% resulted in only a slight increase in membrane tension from the resting state. Fig. 4 shows typical results from seven different cells. The slopes from these plots of surface stress versus membrane area dilation represent an apparent elastic area expansion modulus associated with the cortical region of the passive cell. As the figure shows, this elastic modulus is extremely small (0.039 ± 0.008 dyn/cm), and it would appear that the area dilation is merely smoothing out the excess membrane of the highly microvilliated surface. This process does not involve direct dilation of the lipid bilayer membrane per se because the area expansion modulus for the lipid membrane is likely to be on the order of 500 dyn/cm (10).

One cell selected from the same sample showed an apparent elastic modulus of zero. Perhaps this zero modulus represents a truly "passive" cell. By extrapolating these plots back to the tension axis, a membrane tension of 0.024 ± 0.003 dyn/cm is obtained for the undilated resting cell. This value represents the threshold tension that exists in the spherical undeformed cell. It is slightly smaller than the value of 0.035 dyn/cm measured by Evans and Yeung (1), and slightly higher than the value of 0.013 dyn/cm measured by Evans and Kukan (6). In their experiments, part of the cell was aspirated into an untapered pipet and the suction pressure required to maintain a constant cell projection in the pipet at a length in excess of one pipet radius was measured. Typical area dilation in their experiments was ~10%, which is at the lower end of our measurements (Fig. 4). They report that the presence of EDTA in the medium caused an increase in the cortical tension. In our preparation we included EDTA in order to reduce

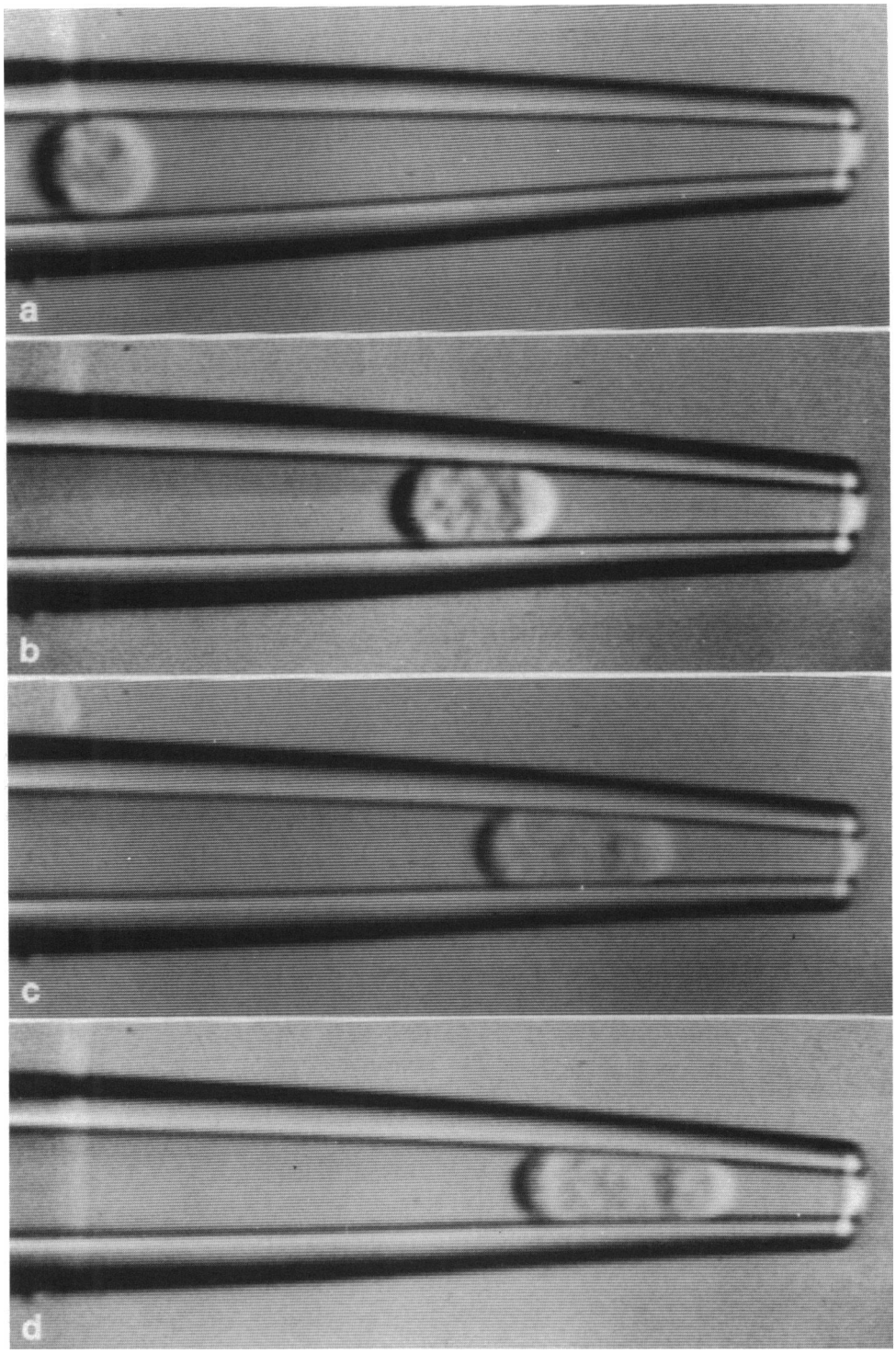

FIGURE 2 Videomicrograph of experimental measurement. Radius of pipet opening = 4 μm. (*a*) ΔP = 0 dyn/cm^2: a single "passive" cell was selected and aspirated into the tapered pipet and allowed to recover to its resting spherical shape in the larger lumen of the pipet. Once in the pipet, only those cells that maintained this spherical, "passive" appearance were tested. (*b*) ΔP = 25 dyn/cm^2: an extremely small positive pipet pressure was then applied and the cell was very gently deformed into a bolus shape as it was driven down the pipet; it then came to rest in the taper. The pipet pressure was then made to gently oscillate about this particular driving pressure in order to ease the cell into as true an equilibrium position as possible and to detect for any tendency of the cell to stick to the pipet wall. The cell was allowed to reach this equilibrium position over a period of 30 s or so. (*c*) ΔP = 50 dyn/cm^2 and (*d*) ΔP = 75 dyn/cm^2: step increases in pipet pressure then drove the cell to new equilibrium positions.

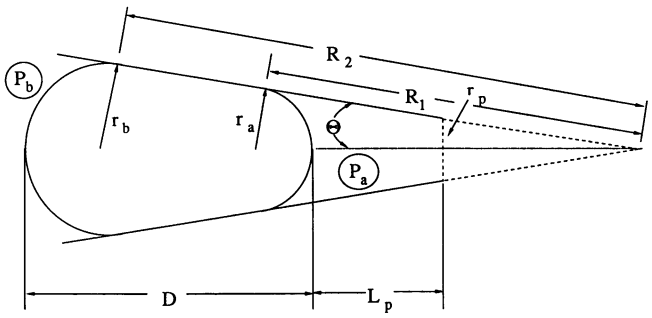

FIGURE 3 Line drawing of a cell in a tapered pipet with a half angle θ. r_a and r_b are the radii of the spherical end caps of the cell. R_1 and R_2 are the distances from the vertex of the conical pipet for the points where the spheres and cone intersect. r_p is the radius of the pipet opening, L_p is the distance from the opening to the leading edge of the cell, and D is the overall length of the cell.

the amount of calcium in the bathing medium and eliminate its potential stimulation of the cells.

Extreme behavior

On a different day, cells were prepared in exactly the same way but were taken from a different donor. Cells were again chosen on the basis of apparent passivity as indicated by their spherical appearance. In tests on nine cells, greater ranges of membrane tensions and apparent elastic moduli were observed for area dilations of up to 35%. Fig. 5 shows three cells that represent the range of behavior. Membrane tensions ranged from 0.01 dyn/cm to (in excess of) 0.08 dyn/cm, and apparent area moduli ranged from 0.015 dyn/cm to 1.751 dyn/cm. The plot with the steepest slope was for a cell that seemed to be activating during the measurement; its appearance became unsymmetrical and darker regions in the cytoplasm were observed. This indicates that activation may cause the whole cortex to change character and become much stiffer than the cortex of the resting cell. For this cell, it is probably better to think of the measurement in Fig. 5 as an "apparent stiffness" rather than a cortical tension. In our experiments we did not stimulate the cells with any recognized chemical stimulant, and the "spontaneous" activation appeared to be in response to the glass surface or the deformation during entry into the measuring pipet. Curiously, not all cells reacted in this way.

Reversibility and hysteresis

The results of experiments that attempted to assess the reversibility of the deformation response are shown in Fig. 6. During unloading we always observed hysteresis that returned on the "top side" of the loading curve. Thus, as the membrane area was allowed to contract upon unloading, the membrane tension remained on the high side of the loading curve, indicating that the stress

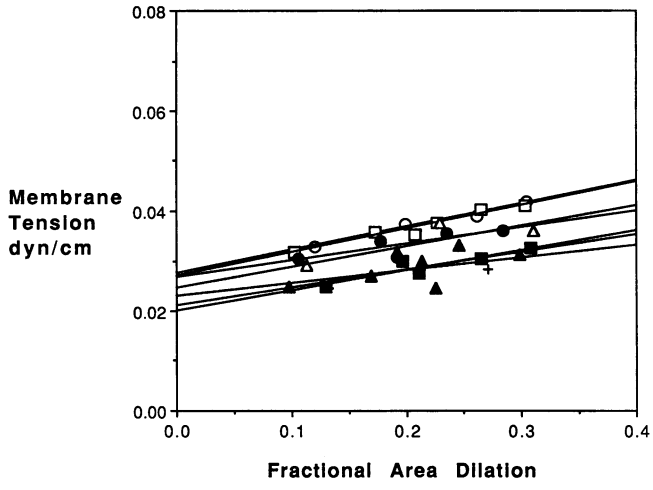

FIGURE 4 Membrane tension as a function of fractional membrane area change for several neutrophils selected from the same sample. The mean apparent elastic area expansion modulus K_{app} was obtained from the slope of this stress versus strain plot and was 0.039 ± 0.008 dyn/cm. The mean threshold cortical tension for the undeformed cell was obtained by extrapolating back the deformation response to the tension axis at zero area dilation and was 0.024 ± 0.003 dyn/cm.

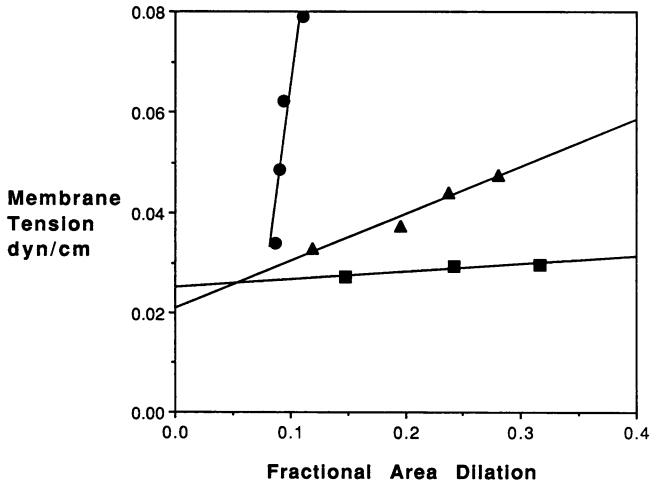

FIGURE 5 Membrane tension versus fractional membrane area dilation showing intermediate (▲) and extremes (■) (●) of behavior for three cells taken from different donors but prepared in exactly the same way. Cells showed behaviors ranging from an almost zero elastic area expansion modulus (K_{app} = 0.015 dyn/cm) (passive?) to a very stiff (K_{app} = 1.76 dyn/cm) (active?) resistance to area dilation. The stiff cell showed some signs of activation in that it eventually developed a small protrusion at one end of the bolus during the measurement.

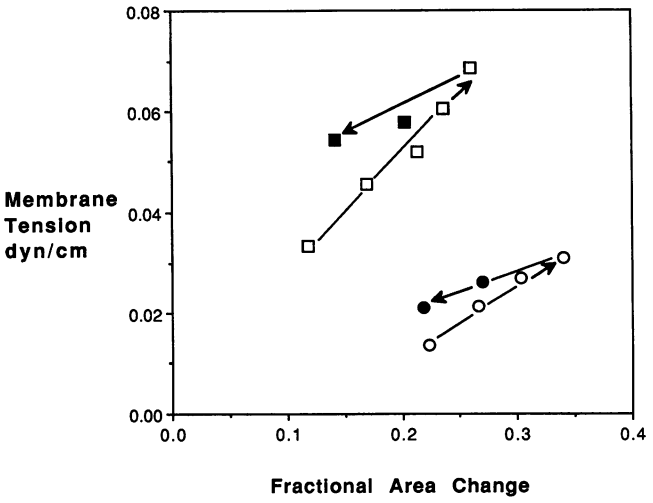

FIGURE 6 Membrane tension versus fractional membrane area dilation showing reversibility and hysteresis effects for two different cells. Cell 1: loading (○), unloading (●). Cell 2: loading (□), unloading (■). Hysteresis was seen with cells that had either low or high elastic moduli. The hysteresis took the form of a "high-side" return with respect to the loading curve upon unloading and would appear to rule out an interpretation based on a purely inanimate-like elastic or creep deformation. Rather, the cell maintains an elevated membrane tension during area dilation and could indicate the first signs of activation in response to either close proximity with the pipet surface or large deformation.

developed during loading was being "actively" maintained. This may be a quantitative indication of the beginnings of an active response. Fig. 6 also makes it clear that this kind of hysteresis occurs for cells independent of whether they have a low or a high apparent modulus. The cells in Fig. 6 do not undergo a "hysteresis loop"; i.e., they do not exhibit the geometric reversibility characteristic of a (passive) dissipative process. Rather, they may have undergone a more permanent change in structure caused by activation.

DISCUSSION

In this paper we deal with the passive state of the neutrophil only to the extent that priming and activation are eliminated by preparative procedures and individual cell selection. If the cell was truly passive, it would appear to have a low threshold tension of ~0.02 dyn/cm that remains at this low level when the membrane area is dilated. This behavior is analogous to the persistent cortical tension in the liquid drop model (1, 6) and would seem to represent the baseline behavior. Other cells in the same sample, under the same conditions, exhibited some stiffness in response to membrane area dilation. These undeformed cells, however, also have an initial cortical tension of ~0.02 dyn/cm but then show an increase in cortical tension with increasing area dilation. Note that we never saw constant cortical tension behavior at elevated levels of tension. Also, at one extreme, is the very stiff cell (Fig. 5) that was clearly activating, and so we may associate activation with stiffness and interpret the apparent elastic moduli and "high-side" hysteresis (Fig. 6) in terms of an active process. Obviously, more loading and unloading experiments need to be carried out to fully characterize this behavior.

What is the origin of the cortical tension? Possibly, it is an active process in which metabolic energy is used to keep the cell surface in a state of isometric contraction. F-actin is usually thought to be responsible for this. Studies in which fluorescent phalloidin is bound to cells indicate that polymerized actin does exist in resting cells. Furthermore, the localization of the phalloidin fluorescence indicates that essentially all the polymerized actin is present in a thin cortical submembranous layer (11, 12). However, other studies have shown a diffuse cytoplasmic fluorescence and seem to indicate that F-actin in the resting neutrophil is no more concentrated at the surface than it is in the interior (13). These apparently disparate observations must be rationalized if we are to relate mechanical properties to structure.

One possible source of inconsistency lies in the response of the cell itself to its preparation history and immediate environment. It has only recently been demonstrated in population studies that in in vitro preparations, isolated neutrophils are highly responsive to trace amounts of lipopolysaccharide (LPS) impurities (14). Lipopolysaccharide is known to prime cells such that subsequent stimulation results in a more activated response. Although priming is not associated with a rise in F-actin content, any subsequent stimulation can lead to the onset of an activated state that is associated with actin polymerization. Inconsistencies in fluorescence studies may reflect differences in the presence of trace amounts of LPS. In our own experiments, although we used sterile HBSS and prepared cells with sterile technique, we did not control for LPS, which could have been present in trace amounts in the small volumes of solutions that were used to make pH and osmolarity adjustments. By examining individual cells with the micropipet, we have some control over cell selection and chose only those cells that "appeared" passive. We, therefore, have confidence in our result that passive cells show a small cortical tension and deform elastically, albeit with a small modulus. It is, however, necessary to definitively establish the properties of the resting neutrophil by combining assays for receptor expression (that indicate priming) and fluorescence assays for F-actin

(that indicate activation) with mechanical measurements, all made on the same individual cells.

At this stage, our present conclusion is that the spherical appearance of the resting neutrophil indicates the presence of a cortical tension, that this tension is on the order of 0.024 dyn/cm, and that the elastic response to deformation from the spherical geometry is exquisitely sensitive to the state of the cell. The exact nature of the spectrum of states of the cell has yet to be clearly defined by a combination of chemical, structural, and mechanical assays.

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