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Cell-Membrane and Rheological Mechanisms in Dynamic Osmotic Hemolysis and Repair of Erythrocytes and Ghosts

by

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Abstract. Normal human erythrocytes undergoing abrupt osmotic hemolysis display a single, transient, localized "blowout" over 15 to 20% of their total surface area. An immediate and striking drop in apparent volume for such cells, as measured by electronic volume spectroscopy (resistive pulse spectroscopy, RPS), is ascribed primarily to a greatly increased, flow-induced, cell-membrane deformation and associated expulsion of "ghost" contents rather than to an intrinsic property of hemolysis per se. The time and flow-rate dependence of the RPS spectra measure the osmotic and mechanical repair of the hemolytic lesion in the membrane. Restored ghosts rehemolyze at a critical volume and lesion size similar to those of the original lysing cells. Membrane rather than cytoplasmic (internal viscosity) properties dominate deformability measured from RPS spectral shape.
Mechanisms involved in the failure of the red blood cell membrane under osmotic stress, and in its recovery, are of interest in a variety of pure and applied biomedical areas. Although such osmotic hemolysis has long been studied (1), many aspects of the phenomenon have remained unclear. The technique of resistive pulse spectroscopy (RPS), recently developed for assaying cell-membrane deformability and for improving resolution of cell-size distributions (2), offers new opportunities to study hemolytic mechanisms and membrane properties per se (3). We report herein new results on the following: (i) the nature and size of the lesion in abrupt osmotic hemolysis; (ii) the kinetics of formation of ghosts from intact cells swollen to critical volume; (iii) the dynamics of ghost-membrane lesion recovery and repair; and (iv) the osmotic, rheological, and rehemolyzing properties of isotonically restored ghosts. In all of this work, glutaraldehyde (glut.) responses of both intact cells and ghosts play an important role.

Cells subjected to osmotic hemolysis have been variously reported to leak selectively (4,5,6), to burst suddenly (7,8), or to do both (9). The different experimental conditions used by different observers render difficult any simple generalization of mechanism. In a recent review, Seeman leans toward a local-lesion interpretation but indicated that the question is still not settled (10). Baker and Gillis (11) used glutaraldehyde treatment to trap up to 80% of cells in a state of local hemolysis. Although their microscopic pictures could be taken as evidence of a local-lesion event occurring during normal osmotic hemolysis, these investigators believed instead that these observations represent a glutaraldehyde-chemical artifact.
From our initial studies on the interactions of intact and hemolyzing red blood cells (RBC) with glutaraldehyde, we have been able to select simple conditions to effectively arrest cell populations in predictable stages of swelling and lysis, including up to 100% undergoing hemolysis. Cells caught in the act of hemolysis show a volcano-like eruption through a single, circular, localized site (Fig. 1). Measurements made on 33 of these "neonate" ghosts (cells lysed in hypotonic solutions) indicate that the lunar surface areas corresponding to the hemolytic lesion occupy about 19% of the total surface area, i.e., a lesion area of $27\mu^2 \pm 6$ (s.d.), based on an initial total surface estimated at $140\mu^2$ (12).

The RPS analysis technique is based on the extension of conventional Coulter-type counting-sizing principles (13). Using this technique, along with additional microscopic observations and experiments clarifying the role of glutaraldehyde, we have acquired evidence that the large local lesions seen in Fig. 1 are indeed representative of a normally transitory phase of the osmotic hemolytic mechanism rather than of an artifact. Specifically, our experiments show the following.

1) The RPS spectra of neonate ghosts have characteristics consistent with the existence of a large local lesion. A dramatic and nearly instantaneously induced (apparent) size drop of neonate ghosts, compared to critically swollen intact cells, is clearly seen in Fig. 2A(a). The observed response can be likened to the reaction of an uncapped tube of toothpaste when squeezed. Considerable "forces" are known theoretically and experimentally to be exerted on intact cells flowing through
a small aperture (2, 14, 15, 16, 17). This interpretation is contrary to the "intrinsic size reduction and reswelling" concept previously hypothesized by others, based on the limited data obtainable from the sizing techniques then available (18).

2) **Cells exposed to prehemolytic, hypotonic solutions** (i.e., between 150 and 300 mosm in buffered saline) containing 0.25% glut. show no evidence of adverse reaction to glut. That is, they display no microscopically observable lesion or other evidence of hemolysis, and no significant differences in swelling or surface morphology from cells not treated with glut.

3) **In the process of being "captured in the act" by glut., cells swell to a volume equal to the maximal critical volume achieved by native cells, prior to hemolysis as demonstrated by RPS (19), as well as phase contrast microscopy (using the methods given in Figs. 1 and 2A). That is, the membranes of cells hemolyzing in the presence of glut. have not been ostensibly weakened, and the cells' normal influx of osmotically driven water has not been impeded.

4) **Restored ghosts treated with 0.25% glut. and analyzed by the RPS methods shown in Fig. 2B retain the ability to respond osmotically, and remain highly permeable to water and relatively impermeable to sodium ions.** (Restored ghosts are the spontaneously self-repaired membranes derived from red blood cells that have lost most of their hemoglobin during hemolysis and have been subsequently returned to isotonicity (20).) This behavior
further supports the supposition that 0.25% glut. is a mild agent - not chemically corrosive - with respect to the cell membrane.

Ghost Properties. When the neonate ghosts are treated with glut., the "squeezing" forces encountered while flowing through the device aperture become, as expected, much less effective in deformation and volume expulsion of intracellular contents (compare solid and dashed curves, Fig. 2A(b)). For the unfixed, neonate ghosts, if the stressing forces are themselves reduced, there is also a proportionate reduction in deformation. This is illustrated by a comparison of the curves in Fig. 2A (a and a') for which the ratio of flow rates is 5:1.

The opening of the neonate ghost is transient; that is, with increasing time, the lesion effectively repairs itself. This is evidenced in RPS by the reduced deformability of ghosts responding to the challenge of flow stress, as they recover (see Fig. 2A(a-d)). The fraction of lysed cells (ghosts) in the whole population is determined from the relative number of counts ("cells") in the two RPS subpopulations. This fraction is dependent upon the time elapsed since exposure to the lytic solution (e.g., compare Fig. 2A (a) with 2A (b)) as well as on the condition of the cells themselves. This kind of subpopulation analysis allows us to detect small differences in rates of hemolysis between different cell samples.

It is reasonable to interpret the increasing apparent ghost volume as a measure of the effective rate and strength
of the mechanical repair of the membrane lesion. This repair appears to commence no later than 20 to 30 sec after the lytic event. The "mechanical strength" then increases in a nearly linear fashion until it reaches a relative plateau of recovery. With a reduction in external stress (flow rate), an increase in the rate of progression to the plateau state is found. For example, at 20% of the normal flow rate, about 150 sec are required to plateau as opposed to 400 sec at the normal flow (0.008 ml/sec). In sum, the effective mechanical strength of the repairing lesion is flow stress and time dependent.

Although the repairing lesion is mechanically vulnerable for at least 400 sec, the effective osmotic barrier of the neonate ghosts re-establishes much more quickly, i.e., within 10 to 60 sec following the lytic event. This is demonstrated by a comparison of the volume response of the ghosts exposed at any given time to changes in external salt concentration. Both young ghosts (10 to 60 sec) and older ghosts (10 min) behave as nearly ideal osmometers (as determined by later RPS volume measurements) as long as they are not mechanically stressed until late in the recovery process.

The RPS spectra of ghosts allowed to repair for 10 min and then restored to an isotonic medium are seen in Fig. 2B. The dependences of the apparent volume distribution of the restored ghosts on changes in flow rate and on glutaraldehyde fixation are very similar to those of isotonically suspended
intact cells. In particular, with normal flow, unfixed ghosts (Fig. 2B(a)) and native RBCs show comparable bimodal distributions (21). Furthermore, this distribution becomes unimodal with reduced flow rate or upon rigidification with glut. (We have previously related the RPS bimodality characteristics to deformability of intact RBCs (2). This is to be distinguished from the "volume expulsion-deformation" behavior discussed above and illustrated in Fig. 2A(a)). At first this seems remarkable, considering that an intact cell containing about 34% hemoglobin has an internal viscosity of about 7 centipoise (cp) while that of a restored ghost is about 1 cp (22). Despite this approximately seven-fold difference in internal viscosity, the "bimodality-deformation" of the restored ghosts is nearly the same as that of intact cells, as indicated by the similarity of the spectra (23). It has been reported elsewhere that for measured RBC deformability and deformation inferred from viscometry, the internal viscosity is the dominant factor (24). Our results suggest that for the short-time (about 15 μsec), high-stress (2000 to 4000 dynes/cm²) challenge, it is the intrinsic membrane deformability that dominates the RPS bimodality response.

In further investigations of the posthemolytic repair processes, we have used RPS to follow the response of isotonically restored ghosts to subsequent re-exposure to hypotonic solutions. We find that as the external salt concentration is reduced below the original lytic osmotic composition, the repaired ghosts reach a critical volume very nearly the same as the original intact-cell critical volume. The ensuing "neonate II" ghosts have a flow-induced, volume drop measured by RPS very similar
to that of the initial event seen in Fig. 2A(a). These findings suggest that the process of hemolysis and the repair of the initial lesion have occurred without significant change in total membrane area compared to that of the original intact cell. They also indicate that both the effective mechanism and the size of the lesion in the rehemolysis are similar to those in the original lytic process (25).

The above types of membrane-hemolysis investigations are not limited to studies on normal cells or to RBCs alone. Valuable information can be obtained in this manner, simply and rapidly, relevant to a variety of other types of cell investigations. These include the effects of aging, drugs and other agents, and studies on pathologies involving cell-membrane integrity and repair mechanisms.

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References and Notes


2. H. C. Mel and J. P. Yee, Blood Cells 1, 391 (1975).


12. Based on our microscopic measurements and in agreement with values reported by P. B. Canham and A. C. Burton, Cir. Res. 22, 405 (1968); and E. A. Evans and Y. C. Fung, Microvas. Res. 4, 335 (1972).

13. As an individual cell in a dilute suspension traverses a current limiting aperture-transducer, a single "resistive" pulse is generated. The height or magnitude of the pulse is commonly taken as proportional to the volume of the (non-conductive) cell, and hence is used as a measure of cell size (W.H. Coulter, Proc. Nat. Electron. Conf.).
12, 1034 (1956); G. Brecher, E.F. Jakobek, M. A. Schneiderman, G. Z. Williams, P. J. Schmidt, *Ann. N.Y. Acad. Sci.* 99, 242 (1962). The pulse height is also dependent, in a complex way, on cell deformability and shape - factors which influence the preferred orientation and trajectory of each cell as it flows through the aperture. This is crucial to the pulse form generated, given the irregularity of electrical field and hydrodynamic flow associated with relatively short apertures (16, 17, 21). RPS spectra as well as Coulter "volume spectra" are cumulative pulse height distributions obtained from the passage of thousands of cells over controlled volume or time intervals. Unlike conventional Coulter sizing, however, RPS analysis utilizes controlled variations in flow rate and in glutaraldehyde rigidification to permit evaluation of deformability as well as optimal resolution of actual cell size distribution (2).


18. Weed and Bowdler proposed than an intrinsic volume loss occurred with hemolysis, followed by a subsequent reswell- ing resulting from colloidal osmotic effects of residual

19. \[ V_a = \gamma V_t \] where \( V_a \) is the apparent volume (peak channel), \( V_t \) is the true volume, and \( \gamma \) is the theoretical shape factor. Generally, the shape factor is taken as 1.5 for a sphere, 1.2 for a biconcave disc, and 1.0 for an elongated prolate ellipsoid having its major axis oriented in the direction of the electric field (17, 18; E.C. Gregg and K.D. Steidley, Biophys. J. 5, 393 (1965)). From our RPS measurements, \( V_a \) for the critically swollen cell is 2.3 times as large as \( V_a \) for the initial, isotonic, native cell. Using these shape-factor corrections, we estimate \( V_t \) (critically swollen) to be 1.6 to 1.7 times \( V_t \) (isotonic). This is consistent with our optically measured critical volume of \( 150 \mu^3 \) compared with a normal RBC volume of \( 90 \mu^3 \), which gives a comparable ratio, 1.67. We calculate \( V_a \) (neonate ghost)/ \( V_a \) (critically swollen cell) to be about 0.60 (based on subpopulation peak channels, such as those seen in Fig. 2B(a)). A substantial volume expulsion-deformation effect must underlie such a large (40%) apparent volume decrease, given the requirement of such an expulsion to effect a shape change for a sphere bounded by an inextensible membrane (12).

21. We (2) and others (e.g., Shank et al.) have discussed the apparent bimodal size distributions observed for intact normal RBCs in short apertures (approximately 50μm) of approximately 50μm diameter (B.B. Shank, R.B. Adams, K.D. Steidley, J.R. Murphy, Lab. Clin. Med. 74, 63 (1969)).


23. Oscilloscope observations of individual resistive pulse forms for restored ghosts indicate that these forms (and hence the ghost trajectories) are about the same as those for intact cells.


25. The rate of repair of the rehemolyzed ghosts is, however, observed to be slower than that of the primary event.


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Figure Legends

Fig. 1. Views of hemolyzing red blood cells (RBC). Human RBCs were added to a glutaraldehyde-containing, hypotonic, phosphate-buffered saline (PBS) (26) (0.1 ml packed cells mixed with 10 ml of solution containing 9 ml of 107-mosm PBS, pH 7.3, and 1 ml of 2.5% glutaraldehyde, E.M. grade, Polysciences, Inc.). The 0.25% final concentration of glutaraldehyde was chosen because it sufficiently cross-linked the hemoglobin so that a "cast-molding" of the blowout lesion could be seen yet it was sufficiently mild for the membrane to remain osmotically responsive. (If the glut. fixative is added subsequent to exposure to the hemolytic solution, even as little as 1 sec later, a smooth symmetrical shape without visible lesion is observed.)

(a) Phase-contrast photomicrographs (about X 1850), composite of segments from three fields of hemolyzing RBCs, chosen to display different aspects of the hemolytic lesion. A single, localized, circular rupture is seen for all hemolyzing cells. Essentially all cells in the fields observed were in the erupting condition. The RPS-measured volume distribution of these lysing cells is the same as that for maximally swollen intact cells; their mean volume estimated from optical measurements of diameters is about 150μm³.

(b) Composite of three SEM fields taken from similar samples, selected to include some intact cells. Considerable shrinkage occurs during the dehydration and critical-point
drying, but the localized rupture is clearly preserved. The "wrinkled skin" appearance of the hemolyzing cells can be contrasted with the "taut skin" appearance of the critically swollen intact cells, attributable to the greater (artifactual) shrinkage of the ghosts.

**Fig. 2A.** Kinetics of the hemolytic process. Human RBCs mixed at time 0 with hypotonic PBS (140 mosm, pH 7.3) (26) were followed by RPS in a continuous sequence of 5-sec measurement intervals under automatic program control (5 x 10^5 cells/ml; 200 μAmp current; 48-micron-diameter orifice, Particle Data, Inc., Elmhurst, Ill; Digital Equipment Corp. PDP 8-I computer). The dynamic behavior of the cell-ghost subpopulations that occurs at a normal flow rate (0.008 ml/sec, about 4 m/sec mean velocity) is shown in selected spectra (a - d). The end-interval times (in seconds) and peak positions are indicated for each of the 64 channel spectra. The strong dependence of the spectra on flow stress is evidenced by comparison with a repeat preparation (a' - d') using reduced sample flow (0.0024 ml/sec). In separate experiments, the intrinsic cell-membrane deformability was greatly reduced at t = 60 sec by rapid addition of glutaraldehyde (final concentration 0.25%). Dashed curves in b and b' represent fixed cells corresponding to the native cells (solid curves in b and b'). Microscopic counts on these sample suspensions and on others prepared with compositions ranging from 0 to 100% in ghosts confirm that the "smaller" of the two (unfixed) subpopulations, as measured by RPS, corresponds to ghosts (most
distinct in a - c). The ghost population is seen to blend upward into the intact cell population with increasing time (e.g., series a - d), reduced flow rate (i.e., reduced shear stress: compare a - a', b - b', etc), or reduced deformability from glutaraldehyde treatment (i.e., b vs. dashed b). These and other results strongly suggest that the initial "blowout" lesion in the membrane renders the cell highly deformable, especially for early times before appreciable repair has occurred (see text). (Accordingly, it is the dominant role played by the increased deformability of the "young" or "neonate" ghost that makes its size unnaturally small.) The rate of "growth" of the ghosts toward the intact cell peak is interpreted as the rate of repair of the hemolytic lesion (e.g., compare peak channels in series a - d).

Also to be noted here and in Fig. 2B is the intrinsic flow-rate dependence of apparent size (peak position) for relatively less deformable objects (e.g., 2A: d - d', dashed b - dashed b') as previously described (2).

Fig. 2B. Rheological properties of restored ghosts. Normal human RBCs were added to 120 mosm PBS (26). After 10 min at 22°C, the resultant suspension of ghosts was adjusted to 300 mosm by adding concentrated PBS, and the four basic RPS spectra were run as shown (2) (200 μamp current; gain relative to spectra of Fig. 2A: 1.67x). The peak and mean channel values are indicated for each of the 64-channel spectra. These ghosts behave as intact native cells with respect to the following characteristics: (i) the cumulative RPS spectral distri-
bution is bimodal at normal flow but transforms to unimodal at slow flow (i.e., a and a', respectively); (ii) the individual pulse forms for ghosts (not shown) are very similar in rise time and overall shape and duration to those of intact native cells (23); (iii) fixation of the ghosts (0.25% glutaraldehyde) converts the bimodal distribution (a) to a unimodal distribution (a'). The above phenomena have previously been associated with the deformability and deformation of native intact cells (2). (One notable difference is that fixed ghosts, unlike fixed RBCs, retain osmotic activity.) The present results demonstrate that restored ghosts have rheological responses in flowing through small capillaries which are in some ways very similar to those of intact cells. The similar RPS bimodality behavior allows us to conclude that it is the membrane rather than the internal viscosity of the cell which dominates this type of deformability property of RBCs.
Fig. 2A

Normal flow

Critically swollen intact cell peak

Ghost peak

Relative number of cells

Channel No.

Slow flow

Fig. 2A
Normal flow

Native

(a)

16.9

\( \bar{v} = 21.2 \)

Fixed

(b)

22.8

\( \bar{v} = 26.3 \)

Slow flow

(a')

22.8

\( \bar{v} = 25.1 \)

(b')

28.5

\( \bar{v} = 31.6 \)

Fig. 2B
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