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Influence of preparative procedures on the membrane viscoelasticity of human red cell ghosts

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The effects of systematic variations in the preparative procedures on the membrane viscoelastic properties of resealed human red blood cell ghosts have been investigated. Ghosts, prepared by hypotonic lysis at 0°C and resealing at 37°C, were subjected to: (1) measurement of the time constant for extensional recovery (t_c); (2) measurement of the membrane shear elastic modulus (μ) via three separate techniques; (3) determination of the membrane viscosity (η_m) via a cone-plate Rheoscope. Membrane viscosity was also determined as $\eta_m = \mu \cdot t_c$. Compared to intact cells, ghosts had shorter t_c, regardless of their residual hemoglobin concentration (up to 21.6 g/dl). However, prolonged exposure to hypotonic media did increase their recovery time toward the intact cell value. The shear elastic modulus, as judged by micropipette aspiration of membrane tongues (μ_p), was similar for all ghosts and intact cells. This result, taken with the t_c data, indicates that ghosts have reduced membrane viscosity. Rheoscopic analysis also showed that η_m was reduced for ghosts, with the degree of reduction (approx. 50%) agreeing well with that estimated by the product $\mu_p \cdot t_c$. However, flow channel and pipette elongation estimates indicated that the ghost membrane elastic modulus was somewhat elevated compared to intact cells. We conclude that: (1) ghosts have reduced membrane viscosity; (2) ghosts have membrane rigidities close to intact cells, except possibly when the membrane is subjected to very large strains; (3) the reduction in η_m is not directly related to the loss of hemoglobin; (4) prolonged exposure of ghosts to low-ionic strength media increases the membrane viscosity toward its initial cellular level. These data indicate that the mechanical characteristics of ghost membranes can be varied by changing the methods of preparation and thus have potential application to further studies of the structural determinants of red cell membrane viscoelasticity.

Introduction

The ability of red blood cells to deform and enter small capillaries is an essential requirement for their passage through the circulation. This deformability is dependent on the cell geometry (surface area, volume and shape), the membrane viscoelasticity and the cytoplasmic viscosity [1]. Although the viscoelastic properties of the membrane are thought to be determined by its underlying cytoskeletal protein network [2], it is not known how alterations of this protein structure affect the membrane mechanical characteristics [3]. Nor is it certain what role interactions between membrane

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and cytoplasmic proteins play in regulating these characteristics [4]. Insight into the factors which determine cellular deformability and into the structural bases of membrane mechanical behavior should be possible via studies of red cell membrane ghosts: (1) variations in ghost preparative procedure might be used to modify membrane structure, and the effects of these modifications on the membrane viscoelasticity could be evaluated; (2) by varying the composition of the ghost interior, e.g., hemoglobin (Hb) concentration, the role of interactions between the membrane and cytoplasmic contents could be examined.

Relatively few measurements of ghost deformability and membrane viscoelasticity have been made, and variations in ghost preparative procedures make it difficult to compare results, since it is not known how membrane mechanical properties are affected by the processes of lysis and resealing. Usami and Chien [5] have measured the viscosities of suspensions of resealed ghosts, and found them to be lower than the viscosities of intact cell suspensions at comparable hematocrits. This finding was attributed to the lower internal viscosity of the ghosts, with the proviso that the membrane flexibility might also have been altered by hemolysis. We have previously determined the membrane shear elastic modulus (μ, measured using a flow channel) and the time constant for extensional shape recovery (t_c, measured via micropipette manipulation) for ghosts with low residual Hb content [6]. Compared to intact cells, the ghosts exhibited decreased deformation in the flow channel and had shorter t_c. It was thus concluded that the ghost membrane was more rigid (i.e., elevated μ), but that the membrane viscosity η_m calculated as the product of μ times t_c [7], was nearly unaltered. On the other hand, using micropipette aspiration, Heusinkveld et al. [8] found that the membrane elasticity was essentially the same for ghosts and intact cells. The deformability of ghosts has been tested by Heath et al. [9], using a Couette device to induce shear deformation; for a specific suspending phase viscosity, the ghost deformation response closely followed that of intact cells. This finding was interpreted as indicating that the preparative procedures had not altered the physical properties of the ghost membrane. However, the ghosts used in that study [9] had volumes one half that of intact red cells and much lower internal viscosities, thus possibly vitiating the membrane-specific interpretation of their data.

The present study was designed to provide measurements fo the membrane viscoelasticity of resealed ghosts prepared via hypotonic lysis under varying conditions. Ghosts were exposed to hypotonic media for different periods of time and were resealed so as to provide a range of intracellular hemoglobin concentrations. The membrane shear elastic modulus was tested via three different techniques: two using micropipettes [10,11] and one using a flow channel [12]. The time constant for extensional recovery was also measured, so that the membrane viscosity could be calculated [7]. In addition, the shear deformation and membrane 'tank-tread frequency' of selected ghost types was measured using a cone-plate Rheoscope, thus enabling an independent estimate of the membrane viscosity [14].

Methods

Ghost preparation

Ghosts were prepared by hypotonic lysis, using methods based on those previously described [15]. Blood was obtained from healthy laboratory personnel by venipuncture into heparin (5 I.U./ml) and was used immediately. The red cells were washed three times, via $2000 \times g$ centrifugation, in phosphate-buffered saline (0.122 M NaCl, 0.030 M $KH_2PO_4 + Na_2HPO_4$, 2 mg/ml glucose (pH 7.44), 300 ± 5 mosmol/kg), and the buffy coat was discarded after each centrifugation. Finally, the packed cells were suspended at 12.5% (v/v) in phosphate buffer and cooled to 0°C. Concentrated phosphate buffer was prepared using five times the above NaCl, KH_2PO_4, Na_2HPO_4 and glucose concentrations.

The lysing medium was dilute phosphate buffer (0.007 M $KH_2PO_4 + Na_2HPO_4$ (pH 7.4)) containing 0.004 M $MgSO_4$, 0.5 mg/ml adenosine triphosphate and penicillin and streptomycin (100 U/ml each), cooled to 0°C. For preparation of 'standard' ghosts, one volume of the 12.5% cell suspension was added to thirty volumes of lysing medium (final lysate was 40 ideal mosmol/kg (pH 7.0)). After a variable delay time (t_d, from 30 s to 60 min) at 0°C, the lysate was returned to 300

mosmol/kg by addition of 5-times concentrated buffer. After a further 5 min at 0°C, the ghosts were resealed by incubation at 37°C for 1 h. The ghosts were then harvested by centrifugation at $20000 \times g$ for 10 min, washed once in a large excess of phosphate-buffered saline and finally suspended in buffer containing 0.2 g% human serum albumin.

The above method for preparation of standard ghosts (i.e., red cell suspension volume/lysing medium volume = 1/30, cell dilution in the lysate = 1/248 (v/v), resealing medium 300 mosmol/kg) was varied for some experiments.

Method A. To obtain ghosts with higher residual Hb content using a fixed t_d, concentrated red cell suspensions of nearly 100% cells (i.e., centrifugally packed at $2000 \times g$) were added to the lysing medium and the ratio of cell suspension to lysing medium volume was altered to either 1:10 or 1:2.5. 5 min after lysis at 0°C, the lysate was returned to 300 mosmol/kg for resealing for 1 h at 37°C.

Method B. Ghosts were subjected to prolonged exposure to hypotonic media by carrying out resealing at 40 to 100 mosmol/kg; either no concentrated buffer or a reduced volume was added to the standard lysate (after a t_d of 5 min) before incubation at 37°C for 1 h. These ghosts were washed and suspended in phosphate buffers with osmolalities equal to the two resealing media.

Method C. Ghosts were prepared with very high Hb content by using packed red cell suspensions and a ratio of cell suspension to lysing medium of 1:2, and by osmotically shrinking the ghosts after resealing at 37°C for 1 h. Two procedures were used: (1) ghosts were resealed and washed at 100 mosmol/kg and then suspended at 300 mosmol/kg; (2) ghosts were resealed and washed at 300 mosmol/kg and then suspended at osmolalities ranging from 450 to 800 mosmol/kg. In this way, ghosts could be prepared with high Hb content, but with or without prolonged exposure to hypotonic media.

All the preparative techniques yielded ghosts with smooth, regular outlines and morphology close to that of intact red cells. Subsequently they are referred to as resealed ghosts, meaning that they underwent incubation for 1 h at 37°C. Our previous studies [15] have indicated that the different ghost types were indeed resealed in that: (1) ghosts remained at the top of sucrose cushions after centrifugation on them; (2) they acted nearly as perfect osmometers with respect to volume changes induced by variations in suspending medium osmolality.

For the standard ghosts, the quantity of residual Hb was analyzed by the pyridine hemochromagen technique of Dodge et al. [16]. Calibration was carried out using red cells whose Hb had been independently determined by the cyanmethemoglobin method. The latter method was used directly to determine the Hb content of ghosts made using concentrated red cell suspensions.

Measurement and adjustment of ghost volumes

Mean ghost volume (MGV) was measured using a computerized Electrozone Celloscope orifice system (Particle Data, Inc. Elmhurst, IL, model 112 LA/ADCW), as recently described [15]. In this earlier study [15], we found that the mean ghost volume varied depending on the preparative procedure employed. Since both the flow channel and Rheoscope measurements (see below) are critically affected by changes in cell volume, the MGV was adjusted to approximately 90 μm^3 (i.e., comparable to the mean cell volume of intact cells) when these techniques were used. Initially, suspensions were diluted to approximately 50000 ghosts/ml using media with osmolalities equal to the resealing media. After volume measurement in these media, ghosts were suspended in buffers whose osmolalities were adjusted so that a mean ghost volume of approximately 90 μm^3 was obtained. This volume adjustment procedure was not employed for ghosts prepared by Method C, where extra shrinkage was used to maximize residual hemoglobin concentration. Osmolality was altered by adding either concentrated buffer or water to the original phosphate buffer as required, and was measured using a freezing point osmometer (Model 2007, Precision Systems, Inc., Sudbury, MA). Volume measurements were possible in the various media because the Celloscope has an adjustable normalizer which compensates for changes in suspending medium conductivity. The osmolalities utilized and the final MGV obtained for the different ghost preparations are shown in the Results.

Measurement of membrane viscoelasticity

The membrane shear elastic modulus (μ) was measured using three separate techniques: the two different micropipette techniques of Evans and LaCelle [10] and of Hochmuth and Hampel [11], and a flow channel technique based on that of Hochmuth and Mohandas [12]. The time constant for extensional shape recovery (t_c) was measured and analyzed using the methods of Hochmuth et al. [7]. These techniques were used to test the properties of intact red cells in isotonic media as well as those of resealed ghosts. The micropipette, flow channel and microscope/video systems used were as recently described (6); all measurements were carried out at room temperature ($23 \pm 1°C$). Note that for ghosts with low Hb content (Hb concentration less than 3% of intact red cells), all video observations were carried out using phase-contrast microscopy. For ghosts with intermediate Hb levels (between 3% and 10% of intact cells), visualization of membrane tongues in pipettes was made possible with bright field optics by use of a video contour synthesizer (model IV-530, For-A Co., Los Angeles, CA); phase contrast optics were used when these ghosts were analyzed using the other techniques. In general, if the ghost Hb concentration was greater than 10% of the red cell levels, bright field microscopy yielded the better image for all deformation techniques employed.

In order to measure t_c, ghosts were suspended in phosphate buffers containing 0.2 g% human serum albumin, with osmolalities adjusted as noted earlier. The ghosts were allowed to settle and attach to the surface of a rectangular glass chamber. The chamber was then flushed with buffer of the appropriate osmolality containing 9% v/v autologous plasma, which helps prevent further sticking of ghosts to the glass. A micropipette (internal diameter, $D_p \approx 1.2$–1.5 μm) was used to aspirate a membrane portion from the rim of point-attached ghosts. The ghosts were elongated and released by slowly withdrawing the micropipette. Video recordings of shape recovery after release were analyzed as previously described [6], in order to determine t_c.

For some ghost samples, the membrane shear elastic modulus was measured by the micropipette elongation method of Hochmuth and Hampel [11] at the same time as measuring t_c. Ghost length (L) and width (W) were measured immediately prior to the moment the ghost was released from the pipette, i.e., just prior to shape recovery. This measurement was repeated for three or four, equally spaced, increasing aspiration pressures, P, such that the maximum L/W obtained was approximately 2. Ghost deformation was thus measured as a function of the applied force, $F = P \cdot (\pi \cdot D_p^2/4)$. This deformation response was analyzed as described by Hochmuth and Hampel [11], in order to calculate the membrane elastic modulus (subsequently referred to as μ_1).

Membrane shear elastic modulus was also measured using the micropipette method of Evans and LaCelle [10], in which small membrane tongues were aspirated into the micropipette from flat portions of the ghost surfaces. For each ghost, tongue length was measured at several increasing pressures, P. The rate of change of tongue length as a function of P was analyzed [10] to obtain the shear elastic modulus (hereafter called μ_p). Note that the shear elastic modulus could not be measured by this method for the low-Hb ghosts (less than 3% of intact cells), since the membrane tongues were not sufficiently visible within the micropipettes.

For the flow-channel analysis of the elastic modulus (termed μ_f), ghosts were suspended in phosphate buffers with adjusted osmolalities and were allowed to settle and attach to the surface of a rectangular channel. Subsequently, buffer of equal osmolality containing 9% v/v autologous plasma was flowed through the channel at controlled, variable rates. The length, L, of deformed, point-attached ghosts was measured as a function of increasing wall shear stress, calculated from the known flow rates, fluid viscosity and channel dimensions. Three or four stresses were applied, up to a level of approximately 1 dyn/cm^2; this maximum stress range caused a 15 to 20% increase in ghost length (i.e., $dL/L = 0.15$ to 0.2). The elastic modulus (μ_f) was calculated from the data for deformation versus shear stress, as previously described [6].

Rheoscope determination of membrane viscosity

The methods used for Rheoscope analysis were as previously described [14,17]; only ghosts prepared using Method A were tested with this de-

vice. To enable measurement of the 'tank-tread' motion of the ghost membranes, 1 μm diameter polystyrene beads (Interfacial Dynamics Co., Portland, OR) were attached to the ghosts [14]. The ghosts were then suspended at about 2% hematocrit in phosphate-buffered dextran solutions [17]; for each ghost type studied, the dextran solution osmolality equalled that which had been found to give an MGV of about 90 μm (see Results). Dextran solutions were prepared with viscosities of 0.20 and 0.40 poise. The viscosities of these dextran solutions were measured at 25°C using a cone-plate viscometer (model 1/2 RVT-200, Brookfield Engineering Labs., Stoughton, MA).

The ghost suspensions were placed between the transparent, counter-rotating cone and plate of the Rheoscope and sheared at fixed rates up to 125 s^{-1} (maximum shear stress 50 dyn/cm^2). Ghost deformation was observed using bright field video-microscopy, and the length (L) and width (W) of the ghosts were measured at each shear rate. The deformed ghosts underwent membrane tank treading, i.e., the membrane continuously rotated about the ghost interior while the shape and orientation of the ghost remained stable. The tank-tread frequency (TTF) of this motion was measured as previously described [14], by observing the motion of the beads attached to the ghost surfaces. The membrane viscosity of the ghosts was calculated as recently published [14], using: (1) the measured ghost volume, L, W and TTF values; (2) a value for the membrane surface area of 135 μm^2 [18]; (3) internal viscosity values calculated for the measured Hb concentration [19].

Results

Values for the viscoelastic properties of standard ghosts and of intact red blood cells are shown in Table I; data are given for ghosts prepared using delay times (t_d) from 30 s to 60 min. Although the time constant for extensional recovery (t_c) did increase with increasing t_d, t_c was shorter for all ghost preparations than for intact cells. As we have previously observed for heat treated and control red cells [20], the absolute values of the elastic modulus (μ) varied systematically between the three methods used for its measurement (i.e., $\mu_p > \mu_l > \mu_f$, see Discussion). In addition, the elasticity values for ghosts relative to intact cells varied depending on the measurement technique; based on micropipette aspiration of membrane tongues (μ_p), the shear elastic modulus was not significantly different for ghosts and red cells, whereas both the flow channel (μ_f) and micropipette elongation (μ_l) methods indicated a higher elastic modulus for ghosts (i.e., stiffer membrane). On average, for standard ghosts prepared at the various t_d, μ_f was 41% and μ_l was 57% higher than for intact cells. Note that none of the

TABLE I

PROPERTIES OF STANDARD GHOSTS PREPARED USING VARIOUS DELAY TIMES

Data are means ± S.D. of means from (n) samples; on average, the viscoelastic properties of seven ghosts or red cells measured in each sample. Residual ghost hemoglobin concentration decreased from about 3 g/dl to less than 0.3 g/dl for t_d increasing from 0.5 to 60 min [15].

t_d for ghost preparation	Measurement osmolality (mosmol/kg)	Mean ghost volume (MGV, μm^3)	Time constant (t_c, s)	Membrane shear elastic modulus (10^{-3} dyn/cm)		
				μ_p	μ_f	μ_l
0.5 min	423 ± 27 (6)	94.8 ± 3.9 (6)	0.053 ± 0.006 (4)	6.53 ± 0.66 (2)	2.38 ± 0.38 (3)	5.11 ± 0.41 (2)
1 min	383 ± 24 (4)	90.7 ± 3.0 (4)	0.064 ± 0.011 (3)	5.27 ± 0.04 (2)	2.91 ± 0.35 (2)	–
2 min	300 ± 5 (11)	91.0 ± 4.0 (11)	0.067 ± 0.010 (8)	6.38 ± 0.50 (3)	2.71 ± 0.14 (6)	5.15 ± 0.16 (2)
5 min	221 ± 9 (6)	95.0 ± 2.6 (6)	0.072 ± 0.009 (2)	–	3.00 ± 0.21 (6)	–
60 min [a]	300	100	0.083 ± 0.022	–	–	–
	150	43	0.084 ± 0.019	–	–	–
Intact red cells	300 ± 5 (10)	88.2 ± 5.9 (10)	0.140 ± 0.019 (6)	5.84 ± 0.34 (6)	1.96 ± 0.15 (6)	3.27 ± 0.23 (5)

[a] One sample measured at 300 and one at 150 mosmol/kg; t_c values are means ± S.D. of eight ghosts.

μ values appeared to vary with t_d, and that the pipette aspiration method (μ_p) could not be used for $t_d > 2$ min, since residual Hb levels were too low to allow visualization of membrane tongues.

Table II presents data for the viscoelastic properties of ghosts prepared via Method A, i.e., using concentrated cell suspensions, fixed t_d (5 min) and volume ratios of cell suspension: lysing medium of either 1 : 10 or 1 : 2.5. These ghosts contained sufficient hemoglobin to allow all three elasticity methods to be used. Again, t_c values were much shorter for ghosts than for intact cells, micropipette aspiration values for the ghost membrane elastic modulus (μ_p) did not differ from those for intact cells, and the absolute elastic moduli values varied depending on the measurement technique employed. However, the differences between ghost and intact cell μ_f and μ_l values were less than those noted above (μ_f 30% greater and μ_l 26% greater on average, for ghosts compared to intact cells). Note that neither t_c nor any of the μ values were significantly influenced by the dilution ratio employed when preparing the ghosts. In overview, the combined data in Tables I and II indicate that ghost recovery times vary with the length of exposure to the hypotonic lysing medium, but are not affected by variation of residual hemoglobin concentration up to a level of 12.4 g/dl (ghosts II, Table II).

To specifically test the effects of low-ionic strength media on the viscoelastic parameters, ghosts were resealed at 37°C in hypotonic buffers (Method B). These low-hemoglobin ghosts were resealed at either 40 or 100 mosmol/kg and measured at 75 or 150 mosmol/kg in order to make their final shapes more closely resemble those of intact cells. The recovery times for these ghosts, shown in Table III, indicate that the rate of extensional recovery was slower for the ghosts resealed at 50 than at 100 mosmol/kg, and that both preparation procedures yielded longer t_c values than for ghosts resealed at 300 mosmol/ kg after comparable t_d (Table I).

In order to obtain ghosts with very high hemoglobin concentrations and to further test the effects of hypotonic media, centrifugally packed cells were lysed by a 1 : 2 dilution in lysing medium (Method C). These ghosts were resealed at 37°C either in the lysate (approx. 100 mosmol/kg) or at 300 mosmol/kg; subsequent micropipette manipulations were carried out at higher osmolalities (see Table IV) in order to cause shrinkage and elevation of residual hemoglobin concentration. Regardless of the measurement osmolality and thus the final Hb concentration, ghosts resealed at 300 mosmol/kg exhibited time constants (Table

TABLE III

PROPERTIES OF GHOSTS PREPARED WITH DILUTE CELL SUSPENSIONS AND RESEALED AT LOW TONICITY (METHOD B)

Data are means ± S.D. of means from (n) samples; time constants were measured for an average of seven ghosts in each sample.

Ghost type	Mean ghost volume (MGV, μm^3)	Time constant (t_c, s)
Resealed at 40 mosmol/kg, measured at 75 mosmol/kg	84.8 ± 10.3	0.116 ± 0.018
Resealed at 100 mosmol/kg, measured at 150 mosmol/kg	90.6 ± 7.8	0.094 ± 0.004

TABLE II

PROPERTIES OF GHOSTS PREPARED USING CONCENTRATED CELL SUSPENSIONS AND FIXED DELAY TIME (METHOD A)

Data are means ± S.D. of means from (n) samples; on average, the viscoelastic properties of seven ghosts were measured in each sample. Residual ghost hemoglobin concentration averaged (I) 3.3 g/dl and (II) 12.4 g/dl ($n = 3$ in both cases).

Cell suspension, dilution in lysing medium	Measurement osmolality (mosmol/kg)	Mean ghost volume (MGV, μm^3)	Time constant (t_c, s)	Membrane shear elastic modulus (10^{-3} dyn/cm)		
				μ_p	μ_f	μ_l
(I) 1 : 10	300 ± 5 (7)	91.4 ± 9.1 (7)	0.063 ± 0.005 (4)	6.57 ± 0.64 (3)	2.70 ± 0.18 (3)	4.36 ± 0.76 (2)
(II) 1 : 2.5	402 ± 25 (4)	95.2 ± 5.4 (4)	0.068 ± 0.010 (3)	6.02 ± 0.13 (2)	2.38 ± 0.36 (2)	3.85 ± 0.50 (2)

TABLE IV

PROPERTIES OF GHOSTS PREPARED WITH HIGH HEMOGLOBIN CONTENT, WITH OR WITHOUT PROLONGED EXPOSURE TO HYPOTONIC MEDIA (METHOD C)

Ghosts resealed at 100 mosmol/kg were exposed to hypotonic media during lysis, resealing and washing (total time 100 min), whereas those resealed at 300 mosmol/kg were in hypotonic medium for only 5 min (i.e., $t_d = 5$ min). Data are means ± S.D. of sample means from (n) samples, where n is given; otherwise, data are means from one sample. On average, the viscoelastic properties of seven ghosts were measured in each sample.

Resealing osmolality (mosmol/kg)	Measurement osmolality (mosmol/kg)	Mean ghost volume (MGV, μm^3)	Hemoglobin concentration (g/dl)	Time constant (t_c, s)	Shear elastic modulus (μ_p, 10^{-3} dyn/cm)
100	300 ± 5 (4)	58.5 ± 8.4 (4)	19.6 ± 1.4 (4)	0.089 ± 0.020 (4)	6.32 ± 0.43 (3)
300	450	90.0	13.5	0.060	5.46
	600	75.2	16.0	0.052	5.88
	730	61.1	18.0	0.051	6.10
	800 ± 10 (2)	55.8 ± 1.1 (2)	21.6 ± 1.5 (2)	0.056 ± 0.005 (2)	5.59 ± 0.15 (2)

IV) less than or approximately equal to those measured for standard ghosts with low residual Hb (Table I). However, high hemoglobin ghosts resealed at 100 mosmol/kg had t_c which were longer than those for high Hb ghosts resealed at 300 mosmol/kg (Table IV), but which were comparable to those for low hemoglobin ghosts resealed at 100 mosmol/kg (Table III). Moreover, all high hemoglobin ghosts had membrane elasticity (μ_p) indistinguishable from other ghost types or intact cells. Thus, varying the residual ghost hemoglobin concentration up to 21.6 g/dl does not appear to affect ghost membrane viscoelasticity, whereas prolonged exposure to hypotonic media increases t_c.

Membrane viscosities (η_m) were calculated for the ghosts studied in the Rheoscope using the ghost length and width data and the measured tank-tread frequencies (TTF); values for η_m and TTF are shown in Table V. TTF increased linearly with shear rate ($\dot{\gamma}$), and for each sample, a TTF value was calculated for $\dot{\gamma} = 100\ s^{-1}$ by linear regression and interpolation; this procedure allowed comparison of ghost TTF data with that

TABLE V

PROPERTIES OF GHOSTS SUBJECTED TO RHEOSCOPE ANALYSIS

Ghost data are means ± S.D. of values from (n) experiments. Ghosts were prepared via Method A and were measured in dextran media with osmolalities of (I) 300 ± 5 mosmol/kg and (II) 450 ± 5 mosmol/kg. For each suspending phase viscosity, TTF was measured for five ghosts at 5–7 shear rates ($\dot{\gamma}$) between 12.5 and 125 s^{-1}. The TTF at 100 s^{-1} was calculated by interpolation of the linear regression fitted to the data for TTF vs. $\dot{\gamma}$. For each suspending phase viscosity. η_m was calculated for five ghosts at four shear rates between 20 to 125 s^{-1}. Since η_m was not found to correlate significantly with $\dot{\gamma}$, η_m values obtained at the various $\dot{\gamma}$ were averaged. Data for intact cells are from Tran-Son-Tay et al. [14]. TTF at 100 s^{-1} was calculated by interpolation of the linear regression fitted to the data for TTF vs. $\dot{\gamma}$; η_m was calculated by averaging values obtained for $\dot{\gamma}$ between 29 to 114 s^{-1}.

Suspension, dilution in lysing medium	Mean ghost volume (MGV, μm^3)	Hemoglobin concentration (g/dl)	Tank-tread frequency at $\dot{\gamma} = 100\ s^{-1}$ (TTF, radians/s), suspending phase viscosity (η_0)		Membrane viscosity (η_m, 10^{-4} dyn·s/cm), suspending phase viscosity (η_0)	
			0.20 poise	0.40 poise	0.20 poise	0.40 poise
(I) 1:10 ($n = 3$)	94.2 ± 2.6	3.2 ± 0.3	29.3 ± 4.3	31.1 ± 5.9	0.30 ± 0.05	0.31 ± 0.10
(II) 1:2.5 ($n = 2$)	92.8 ± 5.2	13.2 ± 0.1	23.7 ± 0.2	25.0 ± 0.4	0.36 ± 0.07	0.46 ± 0.08
Intact red cells	–	–	–	20.0	–	0.81

obtained for intact cells by Tran-Son-Tay et al. [14]. The data presented in Table V indicates that both ghost types had higher TTF than intact cells, and that TTF was greater for the ghosts with the lower hemoglobin concentration. Calculated membrane viscosity values did not correlate significantly with shear rate over the range tested, although previous studies have shown that over a wider range, η_m decreases with increasing shear rate for intact cells [14]. Thus, for each ghost sample, η_m values were averaged over the four shear rates used (Table V). The membrane viscosity values obtained for the ghosts were considerably lower than those previously measured for red cells [14] (ratio of ghost/intact cell $\eta_m = 0.48$ on average). External viscosity did not consistently influence membrane viscosity, but η_m did appear to be slightly higher for the ghosts with higher Hb content (Table V). However, it should be noted that, compared to intact red cells, optical limitations make it more difficult to make accurate measurements of ghost dimensions and their TTF in the Rheoscope. Thus, small disparities in the measured parameters and in the calculated membrane viscosities may not be reliable reflections of true differences.

Discussion

Regardless of the preparative procedure employed, all ghosts had shorter time constants (t_c) for extensional recovery than intact red cells. Since t_c is equal to the ratio of the membrane viscosity divided by the membrane elastic modulus (i.e., $t_c = \eta_m/\mu$, See Ref. 7), this finding indicates that the ghost membrane elastic modulus is increased, or that the membrane viscosity is decreased, or that both factors have changed so as to reduce the ratio η_m/μ. Note that the reduced internal viscosity (η_i) of the ghosts consequent to their lower Hb content does not affect this interpretation, since reductions in η_i have negligible effects on shape recovery rates [7]. However, a problem does arise in interpreting the ghost t_c data in terms of changes in μ or η_m, since the different methods for measuring the membrane elasticity yielded disparate results when comparing ghosts and intact cells. The absolute μ values obtained via the three techniques differed, most likely because the theoretical models employed do not exactly reflect the true cell shape and deformation in the flow channel and pipette elongation methods [20]. In addition, for the flow-channel analysis, the shear forces acting on the cells are underestimated [6]. However, these inaccuracies should apply equally for intact cells and ghosts, so that it was anticipated that relative values (i.e., the ratio of ghost/intact cell μ values) would be similar for each method; such similarity was not found in the present study.

We have previously suggested that the micropipette aspiration value (μ_p) is the best estimate of the membrane elasticiy [20]. Using this technique, ghosts had membrane rigidities very close to intact cells (e.g., Table I). Given this equality of shear moduli, the t_c data indicate that ghosts have greatly reduced membrane viscosity, e.g., judging from Table II, ghost η_m calculated as $\mu_p \cdot t_c$ is 50% less than intact cells. If the μ_f and μ_l values are used to calculate membrane viscosities, the data in Tables I and II indicate smaller reductions for η_m (i.e., average decrease of 37%), and that ghosts act as if their membrane is stiffer than intact cells. It should be noted that the flow channel and pipette elongation procedures both induce large extension ratios in the membrane near its point of attachment to the glass substrate. It is thus possible that the ghost membrane is stiffened at these high strains, but not at the lower strains induced during micropipette aspiration of membrane tongues. Such a variation in the ghost membrane response to the different deformations could be the basis for the discrepancies in the relative μ values obtained by the three methods.

An independent estimate of the membrane viscosity was made by measuring the shear deformation and membrane tank-tread frequency of ghosts in a Rheoscope. Compared to Rheoscopic data for red cells obtained by Tran-Son-Tay et al. [14], this method indicated that ghosts had η_m reduced by 52% on average (Table V). This reduction in η_m is in close agreement with that noted above when the membrane viscosity was calculated using μ_p values for the same types of ghosts (Table II). We thus conclude that ghost preparation most likely causes little or no stiffening of the membrane, but does cause significant reduction in the membrane viscosity. Note that this conclusion

is contrary to our earlier findings, based on flow-channel estimates of membrane rigidity for ghosts with low hemoglobin content [6]. Results of that study, in which ghosts were not measurable via micropipette aspiration, showed μ_f to be increased and t_c reduced, leading us to believe that the ghost's membrane was stiffened but not more viscous.

The viscoelastic characteristics of the red cell membrane are mainly derived from the protein structure associated with its cytoplasmic surface [2]. Specifically, spectrin, actin and protein band 4.1 form a protein network which is linked to the trans-membrane protein band 3 by ankyrin [21]. Alterations in this protein assembly might affect the mechanical properties of the membrane. We have previously found that the extensional recovery time increases if the Hb concentration within intact cells is osmotically elevated [6]. This increase of t_c was attributed to a reversible, concentration-dependent, Hb/membrane interaction which caused the membrane viscosity to rise. Thus, for ghosts, it could be postulated that removal of most of the Hb could directly cause reduction of the membrane viscosity. However, when ghosts were resealed at 300 mosmol/kg with relatively high residual Hb concentrations, time constants were still much shorter than the values obtained for intact red cells (Table II and IV). Thus the reduced membrane viscosity of ghosts does not appear to be due to loss of Hb. Although it is possible that a concentration-dependent change in η occurs between Hb levels of 21.6 g/dl (i.e., the highest ghost level tested) and 33.5 g/dl (the intact cell level), we consider it more likely that η_m is only affected by Hb/membrane interaction when Hb concentrations are raised above the physiologically normal range (e.g., by osmotic shrinkage of intact cells). We also note that cytoplasmic constituents other than Hb (e.g., enzymes and 2,3-bisphosphoglycerate) are considered to play a role in the maintenance of membrane structure [4,22], and their removal during lysis could cause inequalities between intact cell and ghost membrane mechanical properties.

Our data suggest that the process of hypotonic lysis causes alteration of the red cell membrane viscosity. In addition, further changes occur when ghosts are exposed to hypotonic media for prolonged periods of times: (1) if ghosts were left in the lysing medium (approx. 40 mosmol/kg) at 0°C for longer times (t_d) before resealing, their recovery times tended to be increased (Table I); (2) if resealing at 37°C was carried out at 40 or 100 mosmol/kg rather than 300 mosmol/kg, t_c values were again longer, with resealing at 40 mosmol/kg yielding the largest t_c values (Table III and IV). Since these variations in procedure did not measurably affect the membrane elasticity, extended exposure to hypotonic media must have increased the membrane viscosity. It is of interest to note that two types of membrane structural changes have been previously related to such exposure to low ionic strengths: (1) spectrin is gradually removed from the membrane of ghosts [23]; (2) spectrin tends to change from a tetrameric to a dimeric form of association [3]. Such removal or rearrangement of cytoskeletal protein could give rise to changes in membrane mechanical behavior, though it is presently unclear why an increase, rather than a decrease, in membrane viscosity would occur as a result of this loss or rearrangement of spectrin. However, it is evident that the mechanical characteristics of ghost membranes can be varied by changing their method of preparation, and thus our data provide a basis for further studies of the structural determinants of membrane viscoelasticity.

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