EFFECTS OF INHERITED MEMBRANE ABNORMALITIES ON THE VISCOELASTIC PROPERTIES OF ERYTHROCYTE MEMBRANE

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ABSTRACT Several workers have identified molecular abnormalities associated with inherited blood disorders. The present work examines how these alterations in molecular structure affect the viscoelastic properties of the red blood cell membrane. Changes in the membrane shear modulus, the membrane viscosity, and the apparent membrane bending stiffness were observed in cells of eight patients having a variety of disorders: Two had reductions in the number of high-affinity ankyrin binding sites, two had abnormalities associated with the protein band 4.1, and six were known to be deficient in spectrin. The data suggest that the membrane shear modulus is proportional to the density of spectrin on the membrane and support the view that spectrin is primarily responsible for membrane shear elasticity. Although membranes having abnormalities associated with the function of ankyrin or band 4.1 exhibited reduced elasticity, the degree of mechanical dysfunction was quantitatively inconsistent with the extent of the molecular abnormality. This indicates that these skeletal components do not play a primary role in determining membrane shear elasticity. The membrane viscosity was reduced in seven of the eight patients studied. The reduction in viscosity was usually greater than the reduction in shear modulus, but the degree of reduction in viscosity was variable and did not correlate well with the degree of molecular abnormality.

INTRODUCTION

The erythrocyte membrane has served as a model system for the study of membrane mechanical properties. Viscoelastic stress–strain relationships have been developed that accurately describe and predict membrane behavior (1). These relationships contain material constants that characterize the intrinsic mechanical properties of the membrane. The range of values of the material constants for normal cells is known (2, 3), and the temperature dependence of the membrane material properties has been studied (4, 5). These observations have led to an understanding of the changes in free energy that occur during membrane deformation.

The molecular organization of the membrane has also been extensively characterized (6). The membrane consists of a phospholipid bilayer and a proteinaceous membrane skeleton attached to the intracellular surface of the bilayer. The bilayer contains the major integral proteins band 3 and glycophorin. The major component of the membrane skeleton is spectrin, which self-associates to form tetramers that are in turn coupled to short filaments of actin to form a network on the inner surface of the bilayer (7). The attachment between spectrin and actin is stabilized by the protein band 4.1 (8). The attachment between the skeleton and the bilayer is mediated by ankyrin, which connects spectrin to band 3 (9), and by band 4.1, which binds to glycophorin (10).

The contributions of the bilayer and the different skeletal components to the mechanical properties of the membrane have been the subject of considerable conjecture and surmise. Observations of lateral mobility of membrane lipids and integral membrane proteins indicate that the bilayer is essentially liquid-like in character (11) and is not likely to contribute to the membrane resistance to extensional (shear) deformation (12). However, substances such as dinitrophenol, chlorpromazine, and others interact with the bilayer to produce shape transformations in erythrocytes (13). This fact and the observation that bilayers have high resistance to changes in surface area (14) indicate that the bilayer makes an important contribution to the curvature elasticity of the membrane (12). The shear elasticity of the membrane is thought to be due to the presence of the membrane skeleton. Because of its predominance in the membrane skeleton composition and its highly flexible character, spectrin is thought to be primarily responsible for membrane shear rigidity. By implication, the proper interactions of spectrin with itself and with actin are also thought to be important for proper mechanical function.

In our laboratory we have pursued the structure–function relationship of the erythrocyte membrane skeleton by measuring viscoelastic membrane properties of cells having inherited structural abnormalities in the membrane

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skeleton. A previous report (15) showed a reduction in the membrane shear modulus and membrane stability[1] in cells from a patient having an inherited membrane abnormality. However, the biochemical nature of that abnormality is not known. In a more recent report (17), we observed a reduction in membrane stability in cells from several patients for whom skeletal abnormalities had been identified. (Some of those patients are also included in the present study.) The reduction in stability did not correlate well quantitatively with the biochemical defects as they had been characterized, suggesting that the identified biochemical abnormality was secondary in determining the extent of mechanical dysfunction.

In the present study we have looked for abnormalities in the membrane shear modulus, the membrane bending stiffness, and the membrane viscosity in eight patients for whom some sort of membrane structural abnormality has been found. The data indicate that there is a direct proportionality between the membrane shear modulus and the spectrin/band 3 ratio for several patients and confirms the importance of spectrin for membrane shear rigidity. Abnormalities in the function of band 4.1 did not correlate quantitatively with changes in the membrane viscoelastic properties. This result is consistent with the observations of Schanus et al. (18) that band 4.1 did not alter the storage modulus of spectrin–actin gels undergoing low amplitude oscillatory shear. These observations indicate that band 4.1 does not play a primary role in determining membrane shear elasticity. In contrast, Takakuwa et al. (19) have demonstrated an important role for band 4.1 in stabilizing membrane against fragmentation at high shear stresses. Thus, band 4.1 appears to be relatively unimportant in determining membrane mechanical behavior when it is subjected to relatively small forces, but does play an important role in stabilizing the membrane under severe mechanical stress.

MATERIALS AND METHODS

Abnormal blood samples were obtained from several cooperating laboratories. Biochemical data for all of the patients tested (except one) have appeared in the literature. Table I shows the literature reference and the literature patient designation corresponding to the patient number in the present study. Also given is the clinical diagnosis and a brief description of the biochemical abnormality.

Blood was obtained by venipuncture and suspended in acid-citrate-dextrose (ACD) for shipment from the cooperating laboratory. Blood was shipped on ice via overnight carrier, and experiments were performed within 36 h of phlebotomy. For mechanical measurements cells were diluted directly from the ACD-plasma into phosphate-buffered saline (PBS) (25 mM Na_2HPO_4, 6.2 mM KH_2PO_4, 120 mM NaCL, 10 μM sodium azide) containing 1.0–5.0 mg/ml bovine serum albumin (BSA).

Cells were placed in a thin chamber (10 × 20 × 1.5 mm) consisting of two glass coverslips separated by a U-shaped brass spacer and held together with vacuum grease. The coverslips were siliconized to reduce adhesion of the cells to the glass. The chamber was placed on the stage of an inverted (Nikon-M) microscope. A glass micropipette formed by breaking off the tip of a glass microneedle and filled with PBS was inserted into the chamber through the open side of the U. The pipette was connected to a water-filled reservoir, the height of which could be adjusted with a micrometer. The pressure inside the pipette was controlled by adjusting the height of the reservoir. Zero pressure (±25 dyn/cm^2) was determined by stopping the motion of cells or particles in the fluid at the pipette tip. The change in pressure relative to the zero point was set with the micrometer to an accuracy of ±3.0 dyn/cm^2. The pressure was monitored using a DP103-12 pressure transducer (Validyne Engineering Corp., Northridge, CA). The experiments were recorded on videotape along with the time and pressure data. The relevant data were obtained from the video recordings and processed by computer to obtain the material coefficients.

TABLE I
PATIENT DESIGNATIONS

No.	Clinical classification	Deficiencies	Reference
1.	Poikilocytosis	Ankyrin binding	(20), H.P.
2.	Poikilocytosis	Ankyrin binding	(20), D.W.
3.	Eliptocytosis	Glycophorin C, band 4.1	(21)
4.	Spherocytosis	Spectrin-band 4.1 binding	(22), 3
5.	Spherocytosis	Spectrin	None
6.	Spherocytosis	Spectrin	(23), 3
7.	Spherocytosis	Spectrin	(23), 10
8.	Spherocytosis	Spectrin	(23), 11

Shear Modulus Measurements

Pipettes with inside diameters between 1.0 and 1.2 μm were used to aspirate a single cell near the dimple region (24). An initial pressure of 200–300 dyn/cm^2 was applied, and the pressure was increased in several increments until the cell was observed to fold or buckle. Then the cell was expelled from the pipette and checked for residual deformation. If residual deformation was observed, the data for that cell were discounted.

From the recordings the length of the projection of the cell was measured as a function of the applied pressure, up to the pressure at which the cell surface was observed to form wrinkles. The shear modulus was calculated from the length–pressure data pairs by linear regression to the following equation (25):

$$\frac{\Delta P\, R_p}{\mu} = C_1\left(\frac{D}{R_p}\right) + C_2, \qquad (1)$$

where ΔP is the aspiration pressure, R_p is the pipette radius, D is the length of the projection in the pipette, and the constants are $C_1 = 2.45$ and $C_2 = -0.603$. The pressure at which the membrane forms wrinkles (creasing pressure) provides a measure of the membrane bending stiffness (26).

Membrane Viscosity Measurements

The membrane viscosity was determined using the whole-cell recovery technique developed by Hochmuth et al. (27). Cells were allowed to settle onto a glass surface and adhere. The adhesion depended critically on the concentration of BSA in the suspending buffer. This concentration was adjusted in the range 1.0–4.0 mg/ml to obtain the desired "stickiness" between the cells and the glass. Single point-attached cells were aspirated by micropipette at their free edge and extended to approximately twice their resting length by withdrawing the pipette. The cells were released suddenly and the time course of the recovery to their initial geometry was

[1]"Membrane stability" was assessed using the flow channel technique (16) to measure the force required to form tethers (membrane strands) from the cell surface.

observed. The length-to-width ratio of the cells decreases exponentially with time (27) and can be described by the following relationship:

$$\frac{(L/W) - (L/W)_\infty}{(L/W) + (L/W)_\infty} \cdot \frac{(L/W)_m + (L/W)_\infty}{(L/W)_m - (L/W)_\infty} = e^{-t/t_c}, \quad (2)$$

where $(L/W)_m$ is the largest ratio of length to width and $(L/W)_\infty$ represents the length to width ratio in the completely relaxed state. The time constant, t_c, is the ratio of the membrane viscosity coefficient to the membrane shear modulus: ($t_c = \eta/\mu$). The length and width of the cell were measured as functions of time, and the time constant was determined by nonlinear least squares regression with two free parameters, the time constant, t_c and the relaxed length-to-width ratio, $(L/W)_\infty$.

Statistics

The standard errors for the ratios given in Table V were estimated from the standard errors of the means given in Tables II–IV:

$$\left(\frac{\Delta(A/C)}{A/C}\right)^2 \simeq \left(\frac{\Delta A}{A}\right)^2 + \left(\frac{\Delta C}{C}\right)^2, \quad (3)$$

where Δ ( ) represents the standard error of the quantity, and A and C are the means for the abnormal and control populations. This equation is most accurate when the standard error is small compared with the mean. The standard errors of the mean, ΔA and ΔC, were calculated from the standard deviations given in Tables II–IV:

$$\Delta A = \frac{SD_A}{\sqrt{n-1}}, \quad (4)$$

where n is the number of cells measured.

RESULTS

Cellular Morphology

The cell populations of all of the patients studied showed some degree of morphological abnormality, as indicated by the clinical classification. Three of the samples (Nos. 1, 2, and 8 in Table I) showed such gross morphological abnormality that it was difficult to find cells suitable for making mechanical measurements. Less than 15% of the cells in those samples were suitable for measurement. The other five samples contained significant numbers of cells with more-or-less normal geometry as well as some cells with clearly abnormal geometry. More than 80% of the cells in those samples were suitable for measurement.

TABLE II
MEASURED VALUES OF THE MEMBRANE SHEAR MODULUS (DYN/CM)

Patient No.	Abnormal membrane			Control			*t* Test
	Avg.	SD	*n*	Avg.	SD	*n*	*P*
1	0.0060	0.0025	13	0.0083	0.0018	19	0.009
2	0.0046	0.0016	30	0.0058	0.0020	16	0.04
3	0.0065	0.0021	18	0.0079	0.0010	10	0.03
4	0.0055	0.0015	13	0.0066	0.0017	22	0.07
5	0.0049	0.0011	22	0.0062	0.0010	15	0.0015
6	0.0052	0.0007	14	0.0085*	0.0013	17	$<10^{-4}$
7	0.0066	0.0011	11	0.0085*	0.0013	17	0.0002
8	0.0053	0.0022	15	0.0085*	0.0013	17	$<10^{-4}$

*Patients 6, 7, and 8 were studied at the same time against a common control sample.

TABLE III
MEASURED VALUES FOR THE CREASING PRESSURE (DYN/CM²)

Patient No.	Abnormal membrane			Controls			*t* Test
	Avg.	SD	*n*	Avg.	SD	*n*	*P*
1	450	110	13	710	80	19	$<10^{-4}$
2	290	50	14	560	50	9	$<10^{-4}$
3	420	100	17	580	60	15	$<10^{-4}$
4	300	70	17	400	120	23	0.003
5	480	60	22	580	80	15	0.0003
6	400	20	14	610*	50	15	$<10^{-4}$
7	470	40	11	610*	50	15	$<10^{-4}$
8	390	60	17	610*	50	15	$<10^{-4}$

*Patients 6, 7, and 8 were studied at the same time against a common control sample.

Viscoelastic Coefficients

The results of the mechanical experiments are shown in Tables II–IV. The average, standard deviation, and number of cells tested are shown for both abnormal membrane and the matched controls. The rightmost column (P) gives the probability that the two means are equal as indicated by the Student's t test. It is important to compare the results of the measurements only with the corresponding control values. The creasing pressure and the calculation of the shear modulus (Tables II and III) depend on the size of the pipette used in the experiments. Because of possible error in measuring the pipette diameter, reliable comparisons can only be made between samples measured with the same pipette. The viscoelastic recovery time constant (Table IV) can also depend slightly on the conditions of the experiment, although the variability from experiment to experiment is generally less than that seen for the shear modulus measurements. The membrane shear modulus

TABLE IV
MEASURED VALUES OF THE VISCOELASTIC RECOVERY TIME CONSTANT (SECONDS)

Patient No.	Abnormal membrane			Controls			*t* Test
	Avg.	SD	*n*	Avg.	SD	*n*	*P*
1	0.085	0.037	20	0.080	0.025	14	0.65
2	0.079	0.025	18	0.065	0.022	20	0.07
3	0.041	0.022	17	0.061	0.015	17	0.003
4	0.085	0.042	9	0.114	0.048	13	0.07
5	0.106	0.030	20	0.101	0.020	17	0.58
6	0.074	0.014	18	0.097*	0.024	64	0.0003
7	0.082	0.017	20	0.097*	0.024	64	0.01
8	0.084	0.027	21	0.097*	0.024	64	0.04

*Patients 6, 7, and 8 were studied at the same time against a common control.

and the pressure at which the cells were observed to crease were smaller for all of the abnormal cells compared with the matched controls. The viscoelastic recovery time constant was either near to or slightly less than the control value for all samples except for patient 2.

Of the different biochemical abnormalities examined in the present study, the one that appears to affect the viscoelastic membrane properties most directly is a deficiency in spectrin. To illustrate this, the data are shown in Table V normalized with respect to the matched controls. As an estimate for the reduction in the density of spectrin on the abnormal membranes, the spectrin/band 3 ratios for the abnormal cells are shown normalized by the spectrin/band 3 ratios of their matched controls. These ratios were obtained from staining densities on SDS polyacrylamide gels in studies by Becker and Lux (22) (patient 4) and by Peter Agre and co-workers (23; personal communication). (Spectrin/band 3 ratios were not available for patients 2 and 3.) Assuming that the surface concentration of band 3 is normal for the abnormal cells, these values reflect the fractional reduction in the surface density of spectrin for the abnormal membranes relative to control. Note the similarity between the fractional reduction in spectrin/band 3 ratio and the fractional reductions in membrane shear modulus and creasing pressure for patients 1, 4, 6, 7, and 8. Based on Pearson's "r" value, the data for patients 1 and 4–8 show a probability, $P > 0.98$, of linear correlations between spectrin/band 3 ratio and shear modulus and between spectrin/band 3 ratio and creasing pressure. The data suggest a direct proportionality between spectrin density and membrane elasticity for most of the patients studied. Fig. 1 shows the shear modulus of the abnormal membranes, normalized with respect to the control, plotted as a function of the spectrin/band 3 ratio, normalized with respect to control. Vertical bars show the standard error of the ratio of the shear moduli. Horizontal bars show the empirical confidence intervals for the spectrin/band 3 ratio (Agre, P., personal communication). The solid line is the expected behavior if the shear modulus is directly proportional to spectrin/band 3 ratio.

In contrast to the similarity between the fractional reduction in spectrin density and the fractional reduction in membrane elasticity, the changes in viscoelastic properties for membranes with an abnormality associated with band 4.1 are not consistent with the extent of the molecular abnormality. Cells from patient 3 contain no band 4.1 (21). Cells from patient 4 contain a full complement of band 4.1, but these membranes contain an abnormal spectrin, 40% of which cannot bind to band 4.1 (22). Thus, membranes with a 40% deficiency in spectrin-4.1 binding have similar reductions in the apparent bending stiffness (P_{cr}) and shear modulus compared with cells that are completely lacking band 4.1 (see Table V). This result indicates that it is probably not the loss of band 4.1 or its function that is the direct cause of the reduction in the viscoelastic coefficients.

Patients 1 and 2 were originally identified as having an abnormally low number of high-affinity ankyrin binding sites (20). Although the number of sites for the two individuals was similar (42% of normal for patient 1 and 46% of normal for patient 2), patient 2 showed a greater

TABLE V
FRACTIONAL REDUCTION IN THE MATERIAL COEFFICIENTS AND THE APPARENT SURFACE DENSITY OF SPECTRIN

Patient	$[Sp]/[Sp]_0$*	μ/μ_0	P_{cr}/P_{cr0}	t_c/t_{c0}	η/η_0
		$\pm SE$	$\pm SE$	$\pm SE$	$\pm SE$
1	0.70	0.72	0.63	1.06	0.76
		(± 0.10)	(± 0.05)	(± 0.14)	(± 0.14)
2		0.79	0.52	1.22	0.96
		(± 0.09)	(± 0.03)	(± 0.13)	(± 0.15)
3		0.82	0.72	0.67	0.55
		(± 0.07)	(± 0.05)	(± 0.10)	(± 0.10)
4	0.80	0.83	0.75	0.75	0.62
		(± 0.08)	(± 0.07)	(± 0.16)	(± 0.15)
5	0.90	0.80	0.83	1.05	0.83
		(± 0.05)	(± 0.04)	(± 0.09)	(± 0.09)
6	0.65	0.61	0.66	0.76	0.46
		(± 0.03)	(± 0.02)	(± 0.04)	(± 0.04)
7	0.73	0.78	0.77	0.85	0.66
		(± 0.05)	(± 0.03)	(± 0.05)	(± 0.06)
8	0.55	0.62	0.64	0.87	0.54
		(± 0.07)	(± 0.03)	(± 0.07)	(± 0.08)

Subscript 0 indicates control. SE is the standard error of the ratio (see Materials and Methods).

*[Sp] is the ratio of spectrin to band 3 from staining densities on polyacrylamide gels, obtained from Becker and Lux (22) and Agre and co-workers (23; personal communication).

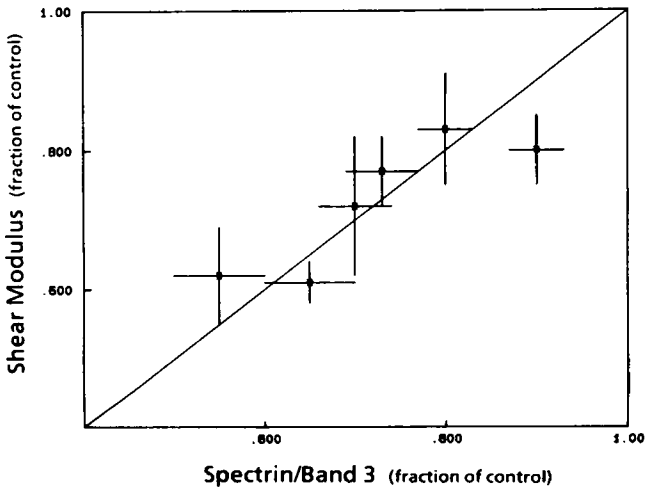

FIGURE 1 The ratio of the shear modulus of the spectrin-deficient membranes to the shear modulus of matched controls is plotted against the spectrin/band 3 ratio expressed relative to the spectrin/band 3 ratio of matched controls (*stars*). The slope of the solid line is one. Normal cells should fall at the point 1.0,1.0. If the shear modulus is directly proportional to the spectrin/band 3 ratio, the data would be expected to fall on the solid line. Vertical bars represented the standard error of the shear modulus ratios. Horizontal bars represent empirical confidence intervals for the spectrin/band 3 ratios (Agre, P., personal communication).

reduction in creasing pressure and a lesser reduction in shear modulus than did patient 1. Furthermore, the membrane viscosity for patient 1 is reduced significantly, but the viscosity for patient 2 is nearly normal. For patient 1, the degree of mechanical abnormality is much more consistent with the reduction in spectrin density than with the reduction in ankyrin binding sites. Unfortunately, the spectrin/band 3 ratio is not available for patient 2.

DISCUSSION

Importance of Geometric Abnormalities

It is important to consider the potential influence of cellular geometry on the present observations. The bending stiffness, the membrane shear modulus, and the membrane viscosity are all intrinsic parameters, that is, they depend on the nature of the membrane material and not its shape. However, to extract these parameters from the experimental data, assumptions are made about the natural and deformed geometries of the surface. Thus, differences in cellular geometry can affect the calculated values of the membrane material coefficients, in so much as the geometric approximations used in the calculations are more or less accurate for different cell shapes. Calculations of the membrane shear modulus and the membrane viscosity are relatively insensitive to the shape abnormalities found in the present study. The shear modulus is calculated assuming the membrane is an infinite flat plane. Because the membrane force resultants are inversely related to the square of the distance from the pipette (3), only a small region of approximately flat surface is required to obtain accurate results. The membrane viscosity is calculated assuming the cell is a rectangular strip. Hochmuth et al. (27) have shown that this model gives results that are only slightly different from modelling the cell as a circular disk.

The relationship between the creasing pressure and the bending stiffness, on the other hand, may be sensitive to shape abnormalities. This calculation (26) is based on an instability analysis, and irregularities at the cell periphery could significantly reduce the pressure required to form creases in the surface compared with the predicted creasing pressure. Thus, the reduction in creasing pressure observed for the cells of patients 1, 2, and 8 (which had extremely irregular geometry) and for the cells of patient 3 (which were elliptical) could be due in part to the abnormal geometry and may not be completely attributable to differences in membrane bending stiffness.

Shear Elasticity and Spectrin Density

One of the most interesting features of the present data is the similarity between the reduction in shear modulus and the reduction in spectrin density, as indicated by the spectrin/band 3 ratio. It has long been surmised that spectrin was responsible for the elastic character of the membrane because of its predominance in the membrane composition, its flexible character, and its organization into a network. However, the present observations are the first quantitative evidence of a relationship between spectrin density and membrane elasticity. Thermodynamically, these observations are consistent with the idea that the elastic work done to deform the membrane is stored by the spectrin molecules. Mechanical deformation of the membrane imposes changes in the arrangement of the spectrin molecules, disturbing them from their "natural" low energy state and increasing the average energy per molecule in the surface. When external forces are removed the molecules return to their natural arrangement, returning the membrane to its natural geometry.

The membrane shear modulus provides a measure of the free energy stored in deformation (3):

$$\Delta \tilde{F}_s = \frac{\mu}{2}(\lambda^2 + \lambda^{-2} - 2), \tag{5}$$

where $\Delta \tilde{F}_s$ is the change in free energy per unit area for surface shear deformation, and λ is the material extension ratio. For example, for a membrane shear modulus of 7.0×10^{-3} dyn/cm and an extension of the surface to 2.0 times its natural dimension, $\Delta \tilde{F}_s = 7.9 \times 10^{-3}$ erg/cm^2. Knowing the molecular density of spectrin, this can be interpreted as a change in free energy per mole of spectrin for the deformation. The normal density of spectrin on the membrane surface has been estimated (28) to be 1.0×10^{-7} g/cm^2. The molecular mass of a spectrin dimer is ~430 kD, indicating a molar density of 2.3×10^{-3} mol/cm^2. This corresponds to a free energy change of ~0.8 kcal/mol (dimer) for a material extension ratio of 2.0. This is a very small energy: it is on the order of thermal fluctuations. This result is consistent with the highly flexible nature of the spectrin molecules (29).

A useful analogy for understanding the molecular basis of the decreased shear modulus in the abnormal membranes is to think of the spectrin molecules as molecular springs. The data indicate that the spring constant of the molecules is not significantly affected by these disorders, but because there are fewer springs per unit area on the abnormal membrane it takes less energy to deform the abnormal surface. It is conceivable (even likely) that other disorders exist in which not only the density of springs is abnormal, but the "spring constant" as well. However, for five of the six cases examined here, the elastic resistance of the membrane appears to be directly proportional to the density of "springs" on the surface.

Correlation between Bending and Shear Rigidity

It is interesting that for the patients for whom the creasing pressures are most likely to give a reliable indication of changes in bending stiffness, there is close agreement between the fractional reduction in the bending stiffness

and the fractional reduction in shear modulus (see Table V, patients 4, 5, 6, and 7). This is surprising, because it suggests that there may be a common molecular basis for the bending stiffness and the shear modulus. Previously, it was thought that the shear rigidity of the surface was due solely to the spectrin network at the inner surface of the membrane (12), whereas the bending stiffness was due to the presence of the membrane bilayer (13) or the interaction between the bilayer and the membrane skeleton (3). One possible explanation for the present observations is that the absence of spectrin results in a change in bilayer composition, which subsequently reduces the membrane bending stiffness. At face value, however, they suggest an important (possibly predominant) contribution to the bending stiffness of the membrane from the membrane skeleton.

For the membranes that showed a difference in the fractional reduction in bending stiffness compared with the fractional reduction in shear modulus, the reduction in bending stiffness was always larger (Table V). It has already been suggested that this might have been due to the abnormal geometry of the cells. An alternative explanation is suggested by the observation that cells with fractionally greater reduction in bending stiffness all have an abnormality related to the attachments between the skeleton and the bilayer (ankyrin binding or band 4.1 deficiency). If the bending stiffness of the membrane is a consequence of a mechanical couple comprising the bilayer and the skeleton, deficiencies in the association between the skeleton and the bilayer would be expected to reduce the bending stiffness. Our observations are consistent with this hypothesis. However, it must be emphasized that it is not certain to what extent the relationship between creasing pressure and bending stiffness is affected by the types of geometric abnormalities seen in the present study, and it is possible that the increased reduction in creasing pressure may be due simply to differences in surface geometry.

The Membrane Viscosity

The present results indicate a more complicated relationship between the viscosity of the membrane and its molecular composition. Except for the patients with the ankyrin binding deficiency (1, 2), there was typically a larger fractional reduction in membrane viscosity than in membrane elasticity. This result is consistent with theories for the viscosity of polymers. These theories propose that the viscosity of polymeric suspension depends on the product of chain density times a coefficient representing the frequency of molecular entanglements (30). This coefficient also depends on the chain density, so that the viscosity is expected to depend on chain density raised to a power greater than one. It should also be recognized that the red cell membrane viscosity has been shown to depend on other factors (such as hemoglobin concentration) that may vary for the different patients studied. Thus, the variability in the fractional reduction in viscosity we have observed is not surprising.

Lack of a Correlation with Band 4.1 and Ankyrin Abnormalities

In contrast to the similarity between the reduction in spectrin density and the reduction in shear elasticity, there seems to be little correlation between the material properties we have studied and the function of band 4.1 or the number of high-affinity ankyrin binding sites on the membrane. Although all of these membranes showed reduced shear elasticity and bending stiffness there is no quantitative correlation between the severity of the molecular abnormality and the reduction in membrane elasticity. For example, the fractional reduction in the shear modulus is approximately the same for membranes in which 40% of the band 4.1 does not bind properly to spectrin as it is for membranes that have no band 4.1 at all. Our observations are consistent with the recent finding of Schanus et al. (18) who observed that the storage modulus of spectrin–actin gels undergoing small deformation is unaffected by the addition of band 4.1. These observations indicate that band 4.1 does not play a primary role in determining shear elasticity, and that band 4.1 is of secondary importance in determining the viscoelastic character of the membrane. This is in contrast to the important contribution that band 4.1 makes to membrane stability. Takakuwa et al. (19) have shown a direct relationship between the presence of band 4.1 on the membrane and the ability of cells to withstand high shear stresses without fragmentation. Taken together, these data indicate that band 4.1 is important for maintaining membrane stability, but proper spectrin density and function are the major determinants of membrane shear elasticity.

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REFERENCES

1. Evans, E. A., and R. M. Hochmuth. 1976. Membrane viscoelasticity. *Biophys. J.* 16:1–12.
2. Evans, E. A., and R. M. Hochmuth. 1978. Mechano-chemical properties of membrane. *Curr. Top. Membr. Transp.* X:1–64.
3. Evans, E. A., and R. Skalak. 1979. Mechanics and thermodynamics of biomembrane. *CRC Crit. Rev. Bioeng.* 3:181–330.
4. Waugh, R., and E. A. Evans. 1979. Thermoelasticity of red blood cell membrane. *Biophys. J.* 26:115–132.

5. Hochmuth, R. M., K. L. Buxbaum, and E. A. Evans. 1980. Temperature dependence of the viscoelastic recovery of red cell membrane. *Biophys. J.* 29:177–182.
6. Lux, S. E. 1979. Dissecting the red cell membrane skeleton. *Nature (Lond.)*. 281:426–429.
7. Byers, T. J., and D. Branton. 1985. Visualization of the protein associations in the erythrocyte membrane skeleton. *Proc. Natl. Acad. Sci. USA*. 82:6153–6157.
8. Ungewickell, E., P. M. Bennett, R. Calvert, V. Ohanian, and W. B. Gratzer. 1979. *In vitro* formation of a complex between cytoskeletal proteins of the human erythrocyte. *Nature (Lond.)*. 280:811–814.
9. Bennett, V., and P. J. Stenbuck. 1979. The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes. *Nature (Lond.)*. 280:468–473.
10. Mueller, T. J., and M. Morrison. 1981. Glyconnectin (PAS2), a membrane attachment site for the human erythrocyte cytoskeleton. *In* Erthrocyte Membranes 2: Recent Clinical and Experimental Advances. W. C. Kruckeberg, J. W. Eaton, and G. J. Brewer, editors. Alan R. Liss, Inc., New York. 95–112.
11. Singer, S. J., and G.L. Nicholson. 1977. The fluid mosaic model of the structure of cell membranes. *Science (Wash. DC)*. 175:720–731.
12. Evans, E. A., and R. M. Hochmuth. 1977. A solid-liquid composite model of the red cell membrane. *J. Membr. Biol.* 30:351–362.
13. Sheetz, M. P., and S. J. Singer. 1974. Biological membranes as bilayer couples. A mechanism of drug-erythrocyte interaction. *Proc. Natl. Acad. Sci. USA*. 71:4457–4461.
14. Kwok, R., and E. Evans. 1981. Thermoelasticity of large lecithin bilayer vesicles. *Biophys. J.* 35:637–652.
15. Waugh, R. E., and P. L. LaCelle. 1980. Abnormalities in the membrane material properties of hereditary spherocytes. *J. Biomech. Eng.* 102:240–246.
16. Hochmuth, R. M., E. A. Evans, and D. Colvard. 1976. Viscosity of human red cell membrane in plastic flow. *Microvasc. Res.* 11:155–159.
17. Waugh, R. E. 1983. Effects of abnormal cytoskeletal structure on erythrocyte membrane mechanical properties. *Cell Motil.* 3:609–622.
18. Schanus, E., S. Booth, B. Hallaway, and A. Rosenberg. 1985. The elasticity of spectrin-actin gels at high protein concentration. *J. Biol. Chem.* 260:3724–3730.
19. Takakuwa, Y., G. Tchernia, M. Rossi, M. Benabadji, and N. Mohandas. 1986. Restoration of normal membrane stability to unstable protein 4.1-deficient erythrocyte membranes by incorporation of purified protein 4.1. *J. Clin. Invest.*78:80–85.
20. Agre, P., E. P. Orringer, D. H. Chui, and V. Bennett. 1981. A molecular defect in two families with hemolytic poikilocytic anemia. Reduction of the high-affinity membrane binding sites for ankyrin. *J. Clin. Invest.* 68:1566–1576.
21. Mueller, T. J., J. Williams, W. C. Wang, and M. Morrison. 1981. Cytoskeletal alterations in hereditary elliptocytosis. *Blood.* 58:47a. (Abstr.)
22. Becker, P. S., and S. E. Lux. 1985. Hereditary spherocytosis and related disorders. *Clin. Hematol.* 14:15–43.
23. Agre, P., J. F. Casella, W. H. Zinkham, C. McMillan, and V. Bennett. 1985. Erythrocyte spectrin is partially deficient in hereditary spherocytosis. *Nature (Lond.)*. 314:380–383.
24. Evans, E. A., and P. L. LaCelle. 1975. Intrinsic material properties of the erythrocyte membrane indicated by mechanical analysis of deformation. *Blood.* 45:29–43.
25. Chien, S., K.-L. P. Sung, R. Skalak, and S. Usami. 1978. Theoretical and experimental studies on viscoelastic properties of erythrocyte membrane. *Biophys. J.* 24:463–487.
26. Evans, E. A. 1983. Bending elastic modulus of red blood cell membrane derived from buckling instability in micropipette aspiration tests. *Biophys. J.* 43:27–30.
27. Hochmuth, R. M., P. R. Worthy, and E. A. Evans. 1979. Red cell extensional recovery and the determination of membrane viscosity. *Biophys. J.* 26:101–114.
28. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.
29. Elgsaeter, A. 1978. Human spectrin I. Classical light scattering study. *Biochim. Biophys. Acta.* 536:235–244.
30. Mears, P. 1965. Polymers: Structure and Bulk Properties. Van Nostrand Reinhold Co., London. 352–359.