Beyond Hemoglobin Polymerization: The Red Blood Cell Membrane and Sickle Disease Pathophysiology

By Robert P. Hebbel

SICKLE CELL ANEMIA, a disease having both hemolytic and vasocclusive components, has been subjected to intense scrutiny at the clinical, cellular, biochemical, and molecular levels. Yet it remains a perplexing disorder for which exact interrelationships between clinical phenotype and cellular defects and presence of the mutant gene product (hemoglobin S [HbS], \( \beta^S \)) remain ill-defined and the subject of much speculation. In large part, this reflects a profoundly complex pathophysiology that is influenced not only by multiple genetic factors but also by the remarkably pleiotropic effects of the sickle gene itself. The latter ultimately must be explained by definable abnormal molecular behaviors of HbS, of which three are known. One of these, the effect of \( \beta \) chain charge on Hb tetramer assembly, helps determine the proportion of HbS in the nonhomozygous sickling states.\(^1\) Another behavior, the tendency of HbS to polymerize at low oxygen tension and cause the sickling phenomenon, has been assumed to be the dominant factor in disease pathophysiology.\(^4,5\) In addition, HbS is unstable, only modestly compared with the classical unstable hemoglobins, but sufficiently to impact on cellular physiology.\(^6\) This instability is hypothesized to explain, at least in part, the myriad abnormalities characteristic of the sickle red blood cell (RBC) membrane (Table 1). This review will discuss the current level of understanding of these membrane defects, including their potential pathophysiologic roles which, depending on defect, vary from unlikely to conceivable to virtually certain. The concluding sections will examine the broader perspective of overall pathophysiology and put forth the hypothesis that the true impact of sickle RBC membrane defects on clinical phenotype is to add a stochastic influence, thereby modulating (and obscuring the theoretical predictability of) the fundamental predisposition to disease severity conferred by genetic determinants of Hb polymerization. Unless specified otherwise, described data refer to RBC from patients homozygous for HbS.

CATION HOMEOSTASIS

Normal cation homeostasis maintains substantial gradients for monovalent cations, and an enormous gradient for calcium, across the RBC membrane. This extraordinarily complex property requires simultaneous participation of a permeability barrier, pumps for active ion transport, and various pathways for water or ion passage. That the latter are still defined only by their phenomenology is one reason why delineation of sickle RBC homeostatic defects has proven to be an elusive goal. However, most importantly, there is the confounding problem of RBC heterogeneity manifested as coexistence of multiple RBC subpopulations, differences among patients, and even temporal heterogeneity in behavior for single cells. Given this problem, the tendency to report average results can be misleading because it ignores the possibility that a single study may not require only a single answer. Also, most investigations have attempted to define abnormalities in terms of normal ion transport pathways, but it is possible that abnormal leak pathways exist in these cells. These caveats notwithstanding, a consensus picture of sickle cation homeostasis is beginning to emerge.

Abnormally Dense RBC

Unlike more uniform normal RBC, sickle RBC samples contain both increased reticulocytes and a subpopulation of abnormally dense cells, a fraction that varies in size among patients from less than 5% to greater than 50% of RBC.\(^7,13\) These cells are dehydrated, with diminished cell water, elevated mean cell hemoglobin concentration (MCHC), and altered monovalent cation content (low total Na + K due to slightly increased Na but very low K).\(^7,14,15\) Such cells are the most evident consequence of abnormal cation homeostasis (Fig 1). The irreversibly sickled cell (ISC) is an important constituent of this subpopulation and will be considered separately.

Calcium Homeostasis

Calcium content. The total cellular calcium content of sickle RBC is markedly abnormal, with average values of about 50 \( \mu \)mol/L RBC for unfractionated sickle RBC and two to four times this amount in most-dense cells,\(^16,20\) compared with about 5 \( \mu \)mol/L for normal RBC.\(^18,19\) In contrast, the ionized calcium in sickle cytosol is normal or near-normal at about 20 to 40 nmol/L RBC.\(^19,21,22\) The difference between ionized and total calcium for normal RBC is not fully explained, but the excess calcium in sickle RBC probably is sequestered in cytoplasmic inside-out vesicles that actively internalize calcium,\(^19,23\) that are more numerous in most-dense cells,\(^25\) and that may form by endocytosis during deoxygenation.\(^2,24\)

Calcium flux. Oxygenated sickle RBC have a modestly (about 50%) enhanced permeability to external calcium,\(^25\) and deoxygenation boosts calcium influx by about fivefold.\(^19,17,19,24,26\) and results in net calcium accumulation in vesicles.\(^10,17,19,22\) This influx occurs without a detectable increase in ionized calcium,\(^25\) but it appears that calcium influx is sufficient to activate calcium-dependent K channels for some cells\(^25,28\) (see below), which implies transient...
(and perhaps localized) cytosolic concentrations reaching 40 to 150 nmol/L. It is not known if sickling causes analogous release of any calcium from cytoplasmic vesicles.

**Ca**^{2+}-**ATPase.** Calmodulin-stimulated Ca^{2+}-ATPase activity of sickle RBC membranes is variously reported to be deficient, normal, and increased. Under optimized assay conditions it was found to be variable among patients, decreased. Of interest in view of the oxidant sensitivity of some sickle patients is improved by the reducing agent dithiothreitol (DTT). These observations notwithstanding, the prevailing opinion has been that any changes in Ca^{2+}ATPase activity are relatively minor and unlikely to significantly affect calcium-pumping capacity under physiologic conditions. This view is controverted by the activation of calcium-dependent K channels during which enhancement is a sickling effect that requires cell deformation.

**Monovalent Cations**

Oxygenated sickle RBC are found to be abnormally permeable to K in some but not all studies, and their permeability to Na may be variably increased. Studies of active pumping have yielded conflicting results, with Na'/K'ATPase activity of sickle membranes increased and that of intact cells reported to be deficient or fully intact. The latter study is perhaps most convincing and further identifies an increased rate of pump turnover for oxygenated most dense cells, consistent with compensation for ongoing leakiness to monovalent cation.

Deoxygenation of sickle RBC variably increases passive K efflux and Na influx threefold to fivefold, an effect on permeability that probably is cation selective and accompanied by stimulation of the Na'/K'ATPase. This leak enhancement is a sickness effect that requires cell deformation, so areas of membrane affected by spiculation are perhaps implicated as leak sites. The actual sufficiency of deformation for inducing monovalent cation leak has recently been documented, but given evidence for pathologic oxidative perturbation of the sickle membrane, it may be relevant that monovalent cation leak as a consequence of membrane deformation in model studies is greatly exaggerated for subtly peroxidized membranes.

**Relationship Between RBC Dehydration and Sickling**

The nature of sickling-induced monovalent cation leak has been embroiled in controversy ever since Tosteson et al first observed an unbalanced leak, with K loss substantially exceeding Na gain. A corresponding, albeit small, change in cell water content was not verified in follow-up studies, but compatible arterial-venous differences in RBC cation

![Fig 1. Density distribution for normal and sickle erythrocytes on discontinuous Spectran gradients. (Reprinted with permission.)](image-url)
contents were observed for some (but not all) sickle patients. Ever since, investigators using diverse techniques have struggled to determine whether sickling-induced cation leaks are imbalanced or not. In fact, it is probable that multiple leak pathways are involved during sickling and that activation of these can be either concurrent or variable depending on which RBC subpopulation, patient, or time frame is being examined. The following mechanisms have been proposed as possible routes to a dehydrated cell.

Passive flux and compensatory Na$^{+}$K$^{+}$ATPase activity. A number of studies performed on unfractionated RBC suggest that the passive component of sickling-induced leak ( + ouabain inhibition of Na$^{+}$K$^{+}$ATPase) causes balanced leak$^{39,49,51,52}$ (ie, directly dehydrating) or not. In fact, it is probable that multiple leak pathways are involved during sickling and that activation of these can be either concurrent or variable depending on which RBC subpopulation, patient, or time frame is being examined. The following mechanisms have been proposed as possible routes to a dehydrated cell.

Calcium-dependent K channels. There probably is an absence of ongoing activation of calcium-dependent K channels in oxygenated sickle RBC, and a role for these channels during deoxygenation may have been obscured in earlier studies by use of unfractionated RBC. However, recent investigations using separated least-dense fractions indicate that reticulocytes can respond to sickling with a calcium-dependent, directly dehydrating, unbalanced K leak.$^{39,56}$ Whether this occurs simply because only reticulocytes are permeabilized to calcium by deoxygenation or whether they also respond differently to calcium remains to be seen. These results raise an obvious interpretative problem for all those studies of cation homeostasis performed in absence of external calcium, and they emphasize the need for utilization of conditions that are at least well understood, if not actually physiologic. For example, because external divalent cations, including calcium, exert effects on both Na and K fluxes quite independent of any Gardos activity,$^{56}$ it becomes difficult to compare results obtained in differing media.

Of great interest, it has been suggested that there can be temporal heterogeneity in single-cell behavior so that a sickle cell that undergoes activation of calcium-dependent K channels during one deoxygenation period may not activate during the next deoxygenation period. If true, this might explain the failure to observe dense-cell formation during a single prolonged deoxygenation period in vitro despite the success of repeated oxy/deoxy cycles in forming dense cells.$^{37,58}$ Interestingly, there appears to be a high degree of interindividual variability in achievable $V_{max}$ of calcium-activated K channels for both normals and sickle patients.$^{39}$

K/Cl cotransport. A K/Cl cotransport pathway that responds to cell swelling or low pH is especially active in sickle reticulocytes and is somewhat active in more dense cells (Fig 3), probably because of the presence of some young cells in those fractions. Reticulocytes from HbA individuals show some activation of this pathway, but much less than is seen for sickle RBC. Hence, it is postulated that the abnormal interaction between HbS and
the RBC membrane might provide an unusual stimulating factor. In support of this, a similar pathway is evident in HbCC cells, and HbC is like HbS in manifesting avid interaction with the RBC membrane. That HbAC cells tend to be denser than HbAS cells lends plausibility to this theory, especially because HbAC cells also show activation of this pathway despite having normal reticulocyte counts.

Activation by lower pH is a likely physiologic stimulus and, in fact, is hypothesized to be a secondary and persistent consequence of calcium-dependent K channel activation. This pathway could exert an influence even without sickling because its activation by pH less than 7.4 has been shown to directly dehydrate oxygenated sickle RBC. In fact, K/Cl cotransport is actually inhibited significantly by deoxygenation due to the attendant rise in cytosolic Mg. Notably, this pathway may be related to the K/Cl cotransport pathway stimulated by the thiol perturbing N-ethylmaleimide (NEM), which is of particular interest given the thiol and lipid oxidation evident in sickle membranes, the stimulatory effect of various pharmacologic oxidants on selective K leak, and the fact that NEM-stimulated K/Cl cotransport activity is potentiated by membrane peroxidation. Activation of this transport pathway by NEM is widely variable among sickle samples (more so than among normals), perhaps consistent with acquired variability in pathway recruitment or sensitivity.

Consequences of Abnormal Cation Homeostasis

Regardless of specific mechanisms involved, the ensuing development of abnormally dehydrated RBC is directly responsible for several cellular defects including excessively elevated P.  Poor cellular deformability and abnormal membrane microrheology (see below), the very propensity for sickling due to the extraordinary concentration dependence of HbS polymerization, and perhaps even some of the tendency for sickle RBC to adhere to endothelial cells (see below). All these cellular features have been implicated in development of vasocclusion.

IRRREVERSIBLY SICKLED VERSUS MOST-DENSE RBC

ISC can constitute anywhere from a few percent to 50% of sickle RBC, yet to some extent our image of these unique cells is only assumed. Virtually all studies of ISC have been performed using the most-dense RBC subpopulation, and the degree of ISC enrichment has been highly variable and often less than 60%. Thus, it must be kept in mind that “most-dense” and “ISC” are not synonymous terms. Indeed, some ISC usually are found in least-dense fractions, while most-dense fractions contain not only ISC but also a sizable population of comparably dense discocytes, some reticulocytes, and even a small population of pseudovacuolated sequestrocytes. A distinction between constituents of the dense subpopulation has been made only in two cases so far: discocytes from the ISC-rich dense subpopulation may have less abnormal membrane microrheology than ISC themselves, and ISC show less endothelial adhesivity than other dense RBC because of their inflexibility. Of equal concern, any conclusions drawn from correlations with ISC counts must be tempered by the fact that the percent ISC observable after saturation with CO is decreased to less than half that of aerated blood, the preparation usually studied.

With these caveats in mind, it is best to describe reported characteristics as being of most-dense cells rather than of ISC per se, and these are apparent from the notations in Table 1. Because most sickle membrane abnormalities are exaggerated in this subpopulation, it is evident that correlative data can be of little value in assessing causal relationships. For example, cells of this subpopulation are shorter-lived so that attenuation of sickle RBC lifespan can appear to correlate with any number of membrane defects or cytoplasmic features. It is clear that accurate identification of interrelationships will require more critical analysis such as detailed examination of multiple density subpopulations. For example, even though membrane thiol oxidation in density-separated RBC is greater for bottom-half cells than for top-half cells, examination of smaller-density fractions indicates that thiol oxidation is increased in sickle reticulocytes as well (Rank and Hebbel, unpublished data). Another example is that ISC share with least-dense reticulocytes the feature of having lower HbF levels than mid-density sickle discocytes. Clearly, old notions of distribution of cellular characteristics among RBC subpopulations must be reexamined to help address a central question: Do the features of most-dense cells represent typical characteristics of those cells predestined to become ISC, or are they end-stage defects of highly damaged cells?

Formation of ISC: In Vitro Models

The most informative attempted simulations of ISC formation have used many hours of repetitive deoxygenation/oxygenation of metabolically replete RBC in buffered medium. Several caveats apply to interpretation of such results. First, CO or prolonged reoxygenation have not been used to establish that induced elongation of shape is truly irreversible. Also, results reflect methodology used because ISC formation is time-dependent. Moreover, effects are less evident if these studies use “rapid” deoxygenation, which is on the order of 2 minutes and thus occurs two orders of magnitude slower than physiologic deoxygenation. Thus, the degree to which these studies simulate physiology is uncertain, but they have been instructive in tentatively identifying certain principles.

It is likely that cellular dehydrogenation exerts a permissive effect on ISC formation, but it may not be absolutely necessary because repeated deoxy/oxy cycles also form new ISC that are in less-dense layers. The mechanism by which dehydrogenation influences cell shape is not known, but because ISC do not necessarily contain Hb polymer it seems likely that this involves the profound effect of HbS, especially in high concentration, on membrane microrheology (to be discussed below).

Regarding dense-cell formation, sickling in vitro probably causes direct dehydration of those least-dense sickle reticulocytes having lower HbF levels. This result presumably explains why the percent of very-dense cells in sickle samples correlates inversely with HbF level (although...
this relationship clearly is lost with concomitant α thalassemia. Thus, it is suggested that reticulocytes having lower HbF levels achieve extreme (ISC-like) density quickly after their release from the marrow, while reticulocytes having higher HbF levels perhaps take a different and slower route to modestly greater density. By analogy, it is possible that relatively different degrees of activation of various leak pathways are the reason that the density distribution of HbSC cells differs from that of HbSS cells in having fewer very-dense ISC and having RBC that are uniformly more dense than normal RBC. In aggregate, these data perhaps suggest that rapid dehydration is a consequence of calcium-activated K leak while slower, more uniform dehydration results from activation of K/Cl cotransport.

In any case, formation of ISC in vitro is generally prevented by an absence of external calcium or if calcium-activated K efflux is inhibited, as well as by calcium channel blockers and inhibitors of calmodulin-mediated processes. Although inhibition of ISC formation by zinc was not seen in vitro, others observed ISC counts to diminish in sickle patients given this calcium antagonist. The implied role for calmodulin is interesting given its interaction with the RBC cytoskeleton and the effects of calcium on certain protein/protein interactions. It has been reported that the dense cells that do form in the absence of external calcium are less rigid in the micropipet than those formed in presence of calcium, which is intriguing given observation of two ISC populations in sickle blood, “hard” and “soft,” based on membrane stiffness. Additional studies will be required to adequately discriminate between the mechanism of dehydration and mechanism of membrane shape change.

LIPID BILAYER

The lipid bilayer normally is arranged asymmetrically so that 75% to 80% of the phospholipids (PL) containing choline (phosphatidylcholine [PC] and sphingomyelin) are found in the outer monolayer, while the inner monolayer retains most of the aminophospholipid (~100% of the phosphatidylserine [PS] and 80% of the phosphatidyethanolamine [PE]). It is known that this state is maintained by at least two orientation enforcing mechanisms. Passive PL stabilization may involve interaction of PS with spectrin and band 4.1, and active enforcement of orientation is provided by an ATP-dependent PL translocating activity. Despite these constraints, PL can traffic between monolayers, so PL destabilization can take the form of enhanced transbilayer mobility and/or an actual, stable loss of asymmetry.

Bilayer Destabilization

Deoxygenation-induced sickling causes an alteration of PL organization, as evidenced by enhanced availability of PS/PE to digestion by external phospholipase over the time of enzyme exposure (Fig 4) and by abnormal labeling of aminoPL by a nonpenetrating amino group label, trinitrobenzenesulfonic acid (TNBS). (By these criteria, PL destabilization also has been observed in RBC from other disorders such as diabetes, chronic myelogenous leukemia, Plasmodium infection. More precisely, sickling results in elevated translocation rates for added PC or lysoPC. For reversibly sickled cells this destabilization is reversible, with reoxygenation allowing a return to near-normal PC translocation rates and near-normal PS availability to phospholipase. In contrast, the status of oxygenated sickle RBC is quite unclear. Indirect techniques suggest that oxygenated ISC manifest PL destabilization while oxygenated non-ISC are normal. Yet actual measurement of inward translocation rates of added PS analogues suggests that even oxygenated non-ISC have markedly deficient inward flip rates and that there is no significant difference between these and ISC. These discrepancies beg for clarification, as does the unknown status of sickle RBC compared with high-reticulocyte controls, so the true state of PL organization in oxygenated and sickle RBC remains to be defined.

The above observations are believed to reflect increased transbilayer mobility of PL without any stable loss of asymmetry because the latter has not yet been documented. The putative PL flip sites recruited by deoxygenation may well be provided by sickling-induced spicules, areas of membrane in which the constraining influence of the underlying cytoskeleton probably is dislodged. This notion is consistent with the fact that deoxygenation does not lead to PL destabilization if membrane deformation is prevented, and it is supported by the observation that the vesicles shed by repeated sickling have externally oriented PS while the deoxygenated remnant RBC retain normal asymmetry and have near-normal PC translocation rates. However, an isolated abnormality of neither membrane protein nor flipase is likely to destroy PL stabilization, so destabilization in these cells may reflect a compound defect. Several cellular abnormalities of sickle RBC could, in theory, contribute.
**RBC calcium.** Loading normal RBC with calcium, perhaps as low as 25 μmol/L, promotes PL destabilization,127,129,130 possibly by inducing lateral phase separation and assumption of a nonbilayer configuration.131-136 At micromolar concentrations, calcium displaces spectrin and band 4.1 protein from PS liposomes.104,105 Marked inhibition of normal RBC flipase activity for PE and PS transport occurs at 50 μmol/L and 0.2 μmol/L calcium, respectively,128 and the sickle RBC flipase appears to be abnormally susceptible to calcium-mediated inhibition.129

**Packing defect.** Depending on the lipid mixture examined, insertion of glycophorin into vesicles can cause accelerated PL translocation.137,138 Hence, the speculative concept that PL destabilization could be related to packing defects139 is worth noting, especially because certain transmembrane proteins are abnormally clumped in sickle membranes (see below).

**Oxidation.** The protein oxidation of sickle membranes (see below) could be implicated, because pharmacologic thiol oxidation of RBC membranes causes PL destabilization without any stable loss of asymmetry,140,141 an effect that may involve both cytoskeletal and noncytoskeletal thiols.140 In addition, lipid peroxidation and its byproduct, malondialdehyde (MDA), promote transbilayer movement of PL, perhaps by promoting assumption of a nonbilayer lipid configuration.143-146

**Lipid Peroxidation and Miscellaneous Lipid Defects**

PL in sickle membranes are abnormally peroxidized, as evidenced by the presence147,148 and excess spontaneous generation149 of malondialdehyde, as well as the presence of PS/PE adducts crosslinked by MDA.150 Sickle RBC also are abnormally susceptible to peroxidation stimulated by added peroxides.117,151,152 This result is partly due to membrane vitamin E deficiency153 and perhaps vitamin C deficiency,154 and it is partly due to abnormal iron deposits in the sickle membrane.152-155 That this susceptibility increases during deoxygenation17 or after calcium loading156 may imply that disruption of PL organization also contributes. Perhaps related to peroxidative change, sickle membranes manifest abnormally low lipid fluidity and increased microviscosity, a defect especially evident for ISC.157,158 Of unknown significance, sickling RBC manifest increased glycerol uptake (a measure of new synthesis by sickle reticulocytes)159 but diminished incorporation of palmitic acid (a measure of lipid renewal).117

**Consequences of PL Destabilization**

The abnormal exposure of PS on the RBC surface provides effective catalytic PL for promotion of coagulation during in vitro prothrombinase assay.116,160,161 Given the lack of evidence for stable loss of asymmetry, this result may imply trapping of translocated PS by coagulation proteins. In fact, studies on diamide-treated and ATP-depleted RBC suggest that procoagulant potential is of two kinds, one resulting from increased rate of PS translocation to the outer leaflet and a much greater effect if actual loss of asymmetry develops.116,144 Consistent with this, the spectrin-free spicules released due to sickling162 have lost normal asymmetry and are particularly effective promoters of clotting.152,153,161 It has been hypothesized that such activity could underlie the activation of coagulation evident in sickle patient plasma.153-166 Likewise, it has been suggested that loss of asymmetry could explain depletion of protein S167 and alternative pathway complement activation166 in sickle patients. The possible promotion of abnormal cell/cell interactions by PL destabilization will be considered below.

**MEMBRANE-ASSOCIATED IRON COMPARTMENTS**

Ghost membranes made from sickle RBC contain abnormal amounts of Hb,6 much of which is truly membrane-associated.6,155 This material probably exists predominantly as hemichrome, a denatured and oxidized Hb, and most dense cells contain increased amounts of it.155 At least some of this material takes the form of Heinz body-like aggregates, and much of it is associated with various membrane proteins (to some extent by disulfide bonding).6,155 In addition, there probably is a small amount of free heme and some ferritin-like iron in association with sickle membrane.152,155 Remarkably, there also is an unprecedented deposition of free iron in sickle membranes, perhaps associated with bilayer aminoPL, with no such compartment detected in normal RBC membranes.152 The accumulation of Hb presumably reflects the instability of Hbs,6 while the deposition of non-heme iron remains under study. Variability among sickle patients is several-fold for membrane Hb and many-fold for free iron,152,155 and the total amount of iron in sickle inside-out membranes averages nine times the amount in normal membranes.152

The probable importance of this membrane-associated iron (generic) is that it provides a mechanism by which the endogenous oxidant stress of sickle RBC (stemming from accelerated Hbs autoxidation)168 can be targeted directly to membrane structures. The considerable complexities of oxidative phenomena in RBC membrane pathobiology in general169 and of sickle RBC iron decompartmentalization and Hbs autoxidation in particular169 have been discussed recently and thoroughly and will not be reiterated here. The relative magnitude of oxidative risk in sickle RBC is summarized in Table 2.

**MEMBRANE PROTEINS**

**Membrane Topography**

**Glycophorin.** The distribution of glycophorin molecules on sickle membranes probably is abnormal, as detected by clustering of sites for binding of cationized ferritin,85 wheat germ agglutinin,160 and anti-glycophorin.161 In contrast, the quantity of glycophorin-borne sialic acid is probably close to normal85 despite a report to the contrary.174 Sickle RBC show increased susceptibility of sialate-containing oligosaccharides to release using endo-β-galactosidase.175 While Hb-dependent coagulation phenomena may underlie glycophorin clumping (see below), it may be noted that cellular dehydration and calcium loading both foster glycophorin clustering in vitro.82,176
Band 3. Similarly, band 3 molecules tend to be abnormally clustered.\textsuperscript{171,177} It is not known whether this is related to the increased expression of I and i antigens on sickle cells.\textsuperscript{175,178,179} The possibility that the latter might enhance the susceptibility of sickle patients to RBC lysis during appearance of cold agglutinins appears to have not been tested by clinical observation.

Glycolipid. Binding of Bandieraea simplicifolia lectin, which binds to a-galactose (a-gal) containing residues, is known to the increased expression of I and i antigens on sickle cells.\textsuperscript{\textsuperscript{\textsuperscript{175,178,179}}} The possibility that the latter might enhance the susceptibility of sickle patients to RBC lysis during appearance of cold agglutinins appears to have not been tested by clinical observation.

Abnormal deposits of IgG are detected on RBC from perhaps two thirds of sickle patients, even if untransfused.\textsuperscript{149,177,180,182,183} While averaging a several-fold increase over normal, the quantity of IgG is highly variable among patients and ranges, eg, to as high as 890 IgG molecules per cell (compared with less than 25 for normal RBC) when assayed by a complement fixing antibody consumption test.\textsuperscript{182} However, this perhaps underestimates the functional degree of opsonization, because this IgG actually is found only on a fraction of the RBC (ranging from 10\% to 60\%) from any one patient,\textsuperscript{177,180,183} and amounts are greater on most dense RBC.\textsuperscript{149,180,183} The antigenic specificity of this IgG remains unproven, because different studies support different candidate antigens including band 3 (but not its 65-Kd fragment),\textsuperscript{177} Galal\rightarrow\textsuperscript{3}Gal on a ceramide pentahexoside,\textsuperscript{180,183} and perhaps even MDA crosslinks.\textsuperscript{180} The observation of IgG on sickle RBC is reminiscent of similar findings for senescent normal,\textsuperscript{194} thalassemic,\textsuperscript{185} malaria-infected,\textsuperscript{186} and unstable hemoglobinopathic\textsuperscript{177} RBC. Peculiarly, RBC also were reported to be positive for IgG in 7 of 57 sickle trait patients.\textsuperscript{182}

Coclustering

Electron microscopic examination of sickle RBC shows subsurface heme-containing microbodies that tend to be associated with intramembranous particles,\textsuperscript{77} and recent immunologic probing has documented coclustering of band 3 protein with Heinz bodies and with surface IgG (Fig 5).\textsuperscript{171,177} The proximate cause of this coclustering is believed to be oxidative formation of hemichrome, which binds avidly to, and copolymerizes with, the cytoplasmic portion of band 3 in vitro with a stoichiometry of two hemichrome tetramers per band 3 subunit.\textsuperscript{187} Consistent with an etiologic role for hemichrome, similar coclustering is observed for thalassemic and unstable hemoglobinopathic RBC.\textsuperscript{177} IgG/band 3/Heinz body coclustering also is seen in oxidatively stressed RBC,\textsuperscript{188,189} although there can be doubts as to the validity of this model.\textsuperscript{189,190} However, from unmanipulated sickle RBC apparent confirmation is derived from successful isolation of membrane protein aggregates that comprise less than 1\% of membrane protein, that consist mostly of globin and band 3, and that have 75\% of the cell’s surface IgG.\textsuperscript{191} Thus, the IgG on sickle cells not only is concentrated on a subfraction of the cells but also is clustered in dense accumulations on single cells. It has been stated that thermodynamic considerations predict that intracellular events causing aggregation of band 3 molecules would make binding of any preexisting anti-band 3 antibody more favorable by several orders of magnitude.\textsuperscript{186} The interesting converse possibility, that anti-band 3 forms a nidus enhancing hemichrome accumulation, has not been tested.

Notably, study of three sickle patients revealed that coclustering is variable and shows an imperfect concurrence so that only 52\% to 84\% of Heinz bodies cocluster with band 3, 9\% to 33\% cocluster with glycolipid, and 28\% to 58\% cocluster with ankyrin.\textsuperscript{171} For the discrepancy between involvement of band 3 and ankyrin to suggest that hemichrome preferentially binds to that portion of band 3

![Fig 5. Coclustering phenomenon in sickle membranes.](http://www.bloodjournal.org)
that is unencumbered by attachment to ankyrin (or, alternatively, that hemichrome binding dislodges ankyrin) depends on the true size of the free-floating fraction of band 3, which is an unresolved issue. Nonetheless, the diffusional accumulation of band 3 molecules might explain the observation that RBC sickling in plasma doubles the amount of surface IgG (and the proportion of cells having IgG), but only after many hours of deoxygenation. That prolonged deoxygenation in buffer also leads to some increased antibody deposition on subsequent exposure to plasma could also reflect sickling-induced redistribution of band 3 molecules, but it is possible that sickling creates or enhances exposure of sites for antibody binding. The more relevant time dependence of IgG accumulation as a consequence of physiologic (ie, short) sickling/unsickling cycles has not been tested.

Cytoskeletal Dysfunction

Both the strength and the flexibility of the normal RBC derive from the protein component of its membrane, the essential feature of which is an underlying cytoskeleton (spectrins, actin, band 4.1, plus other minor components) connected by linking units (ankyrin and band 4.1) to proteins embedded in the lipid bilayer (band 3 and glycophorin). While of high affinity, these complex associations are not covalent, and opportunity exists for functional abnormalities involving the various subunits. The first hint of dysfunctional protein in the sickle membrane was the observation that ISC do not necessarily contain polymerized hemoglobin. The significance of this observation was dramatically clarified by the demonstration that ISC (but not reversibly sickled cells) tend to yield ISC-shaped ghost membranes, and that Triton extraction of these yields ISC-shaped cytoskeletons. Since then, a number of protein defects have been described.

Ankyrin. Sickle inside-out vesicles (IOV) have a variably diminished capacity for ankyrin-dependent binding of spectrin (Fig 6). This defect is more apparent for ISC-rich than reticulocyte-rich fractions, but both are markedly abnormal compared with high-reticulocyte control membranes. The same defect was seen for IOV from HbsA, HbSS with high HbF, and HbsA thal) normal spectrin-binding capacity, and the least symptomatic HbsS patient studied had the highest binding capacity for spectrin. Oddly, it was found that isolated sickle ankyrin binds normally to spectrin in solution. It is not known if this discrepancy is because the ankyrin molecule behaves differently in solution than in its membrane environment, or because the most abnormal ankyrin molecules are lost in the purification procedure, or because the DTT used in the ankyrin purification process normalizes these functions.

Band 4.1. Band 4.1 protein normally associates with both glycophorin and aminoPL, but isolated sickle band 4.1 is reported to have a markedly diminished capacity for binding to normal membrane vesicles depleted of 4.1. Pending confirmatory studies, it should be noted that the band 4.1 preparations used for these studies contained substantial amounts of aggregated protein not found in band 4.1 preparations obtained by others. Consistent with a binding abnormality, sickle IOV were found to have a slightly diminished quantity of band 4.1, although artifact due to protease action was not excluded.

Band 3. A preliminary report suggests that sickle band 3 has subnormal anion transport activity and a diminished number of high-affinity ankyrin binding sites and that these defects tend to normalize if patients are administered vitamin E. However, because the cells apparently were shipped before study, it is quite possible that results can be explained by oxidative deterioration during shipping. Work by others suggests anion transport capacity of sickle RBC is normal.

Hb/Spectrin Adduct

Careful analysis of sickle RBC membranes shows a small amount of abnormal spectrin/globin adduct. Although actual presence of heme in the adduct has not been documented, it is a reasonable assumption because presence of the adduct appears to reflect an oxidative process, possibly involving Hb radicals. The adduct involves spectrin (but not Hb) thiols, and its formation is simulated in vitro by admixture of Hb and spectrin and peroxide. Its formation is favored by dehydrated cytosol, and the amount of adduct increases along with MCHC for unmanipulated sickle RBC. This adduct also is seen in thalassemic, hereditary xerocytosis, and most-dense normal RBC. Its presence is implicated in altered membrane deformability (see below).

Protein Oxidation

Although sickle membranes do not contain the high-molecular-weight protein aggregates seen in some oxidative...
disorders, they do have a diminished number of titratable reduced thiols.\textsuperscript{73,146} This observation appears to reflect a generalized protein oxidation that is highly variable among patients and that includes spectrins, band 3, band 4.1, and ankyrin.\textsuperscript{73} Thiol oxidation is not detected in control RBC from non-oxidative high-reticulocyte states,\textsuperscript{73} and it seems to be greatest in both the most-dense,\textsuperscript{83,205} and the least-dense (Rank and Hebbel, unpublished data) sickle cells. It should be kept in mind that thiol oxidation has been measured as a convenient marker of protein damage and presumably coexists with oxidative damage to other amino acids, as found for band 4.1 isolated from sickle membranes.\textsuperscript{197}

Consistent with the notion that iron decompartmentalization provides a mechanism for targeting of oxidant damage to specific membrane components,\textsuperscript{6} observed amounts of membrane thiol oxidation do correlate with amount of heme associated with sickle inside-out membranes (IOM).\textsuperscript{110} On the other hand, despite ample precedent for believing that protein oxidation interferes with cytoskeletal protein function,\textsuperscript{6} it is not proven whether this process actually is responsible for described functional defects of the sickle membrane. The described coclustering of Heinz bodies with specific proteins certainly could focus oxidant damage to this area of the membrane.\textsuperscript{206} However, because the sickle membrane's spectrin-binding defect is not corrected by DTT,\textsuperscript{195} it would have to be caused by damage to a non-thiol amino acid, or it could reflect thiol damage not amenable to reduction with DTT. Indeed, only a portion of the thiol oxidation in sickle membrane proteins can be reversed with DTT,\textsuperscript{73} with band 4.1 being demonstrably resistant to such reduction.\textsuperscript{73,197}

Miscellaneous Protein Defects

The possible significance of altered patterns of sickle membrane protein methylation and phosphorylation cannot be assessed because there is no consensus as to whether abnormalities even exist.\textsuperscript{207,221} Increased amounts of GSH-transferase and catalase on sickle membranes could be footprints of prior calcium excess because deposition of both increases in response to calcium.\textsuperscript{212,213}

Consequences of Membrane Protein Defects

Fragility. The fragility of sickle RBC\textsuperscript{214} was dramatically illustrated by observation of RBC lysis in sickle patients undergoing vigorous exercise.\textsuperscript{215} Accompanying in vitro studies documented RBC shear sensitivity, with the most-dense cells being particularly susceptible to lytic damage. Notably, the shear sensitivity of intact sickle cells tended to diminish on cellular rehydration, although ISC themselves did not completely normalize.\textsuperscript{212} Consistent with this, ghost membranes made from most-dense (but not from least-dense) sickle RBC are mechanically fragile.\textsuperscript{214} It is reasonable to assume that membrane instability reflects some failure of protein/protein junctional associations.\textsuperscript{217} Thus, it may be relevant that free heme (which is found to excess in both sickle membranes\textsuperscript{155} and in sickle cytosol\textsuperscript{119}) profoundly destabilizes RBC cytoskeletons, as documented in studies of ghost membranes, intact RBC, and isolated proteins.\textsuperscript{169} It also is possible that protein oxidation would make the membrane more susceptible to the potentially disrupting influence of mechanical stress. Also, membrane stability is demonstrably decreased by greater than 1 \textmu mol/L Ca in presence of physiologic amounts of calmodulin.\textsuperscript{102} Whatever the proximate cause, RBC fragility may contribute to hemolysis.

Vesiculation. Sickle RBC have an abnormal tendency to vesiculate during thermal stress, a defect seen for all studied patients, although degree of susceptibility was found to be quite variable.\textsuperscript{80} This defect tends to be more evident for most-dense cells.\textsuperscript{88} It is assumed to reflect an oxidative protein defect because identical thermal sensitivity is evident in RBC having perturbed thiols or dysfunctional spectrin\textsuperscript{88,219} and because the sickle defect is significantly, albeit not fully, improved by reduction using DTT.\textsuperscript{88} Certain, other known causes of RBC vesiculation such as low pH, elevated calcium, or ATP depletion are unlikely to be causal for sickle RBC due to the discrepancy between the degree of perturbation that is required and that actually observed in sickle RBC. It is not known whether the biochemical defect leading to thermal sensitivity is related to release of microvesicles on reoxygenation of sickled RBC\textsuperscript{216,228} and the presence of procoagulant vesicular material in sickle plasma.\textsuperscript{463}

Coclustering and microrheology. The consequences of coclustering relate to RBC/macrophage interactions (see below), and it now seems likely that protein defects play a role in the abnormal microrheology of sickle cells.

MEMBRANE RHEOLOGY

Oxygenated sickle RBC are poorly deformable when studied by filtration,\textsuperscript{221,222} viscometry,\textsuperscript{222} ektacytometer,\textsuperscript{1,225} or micropipet.\textsuperscript{21,227} This abnormality is most characteristic of RBC in the most-dense, ISC-rich fraction. Because the ratio of membrane surface area to cell volume is not limiting in this fraction,\textsuperscript{8,88} poor deformability is explained by abnormalities of cytoplasmic viscosity and membrane microrheologic properties.

Cytoplasmic Viscosity

Deformability of normal RBC is optimal at their usual hydration state,\textsuperscript{225,228} so it is predictable that the severe dehydration of dense sickle RBC is harmful. This is evident in a dramatic improvement in ektacytometric deformability of unfractinated sickle RBC or ISC-rich fractions when cell hydration is normalized.\textsuperscript{4,229,230} These findings undoubtedly do reflect effects of abnormal internal viscosity, but hydration status also exerts an independent effect on membrane properties (see below). Furthermore, most-dense sickle RBC do not fully regain normal deformability even if their hydration is fully normalized or even if they are reconstituted with normal Hb,\textsuperscript{4,225,230} suggesting that factors in addition to hydration status contribute.

Membrane Microrheology

Studies of membrane stiffness have shown that sickle RBC have markedly abnormal viscoelastic proper-
ties, as exemplified by μ, the static extensional rigidity (stiffness of the membrane as it resists extension) quantitated by micropipet studies. Although μ does not change with MCHC for normal RBC, it increases markedly with MCHC of sickle RBC so that most-dense cells are strikingly abnormal, but highly variable, in this regard (Fig 7). That others report μ to be abnormal for ISC but not for dense discocytes from the same density subpopulation may or may not be because broader density fractions were used so that truly comparably dense cells were not examined. In addition, dynamic rigidity (a time-dependent property) of sickle RBC is abnormal. Most remarkably, sickle RBC exhibit plastic flow behavior so that residual bumps persist even after release of RBC from pipettes, in contrast to the prompt and complete (ie, elastic) recovery from deformation usually manifested by normal RBC. This recovery failure can be seen for normal RBC of extremely high MCHC, but it is seen for sickle RBC of any MCHC.

These observations identify a fascinating dependence of membrane properties on cell hydration status. Indeed, hydration of dense sickle cells allows a significant (but only partial) improvement in membrane rigidity and plastic flow behavior. That this effect is exerted uniquely by HbS (and not by HbA at comparable MCHC) further shows existence of an abnormal, dynamic interaction between the mutant Hb (which has both increased hydrophobicity and diminished negative charge) and the RBC membrane. Direct evidence for this is provided by the fact that loading normal RBC ghosts with HbS makes μ and plastic flow abnormal, while loading sickle ghosts with HbA improves μ and plastic behavior (although only partially for ghosts from most-dense cells). Various aspects of this dynamic HbS/membrane interaction have been reviewed recently. Perhaps the most crucial of these observations is that Hb concentration at the membrane/cytosol boundary of oxygenated sickle RBC is abnormally high and, unlike the case for normal RBC, is very MCHC-dependent, reaching perhaps 39 mmol/L for most-dense cells. It is not clear how much of this Hb is soluble and how much is irreversibly associated with the membrane, but deoxygenation does tend to deplete Hb from this area.

It is probable that membrane rigidity reflects alteration of normal folding/unfolding ability or creation of abnormal protein/protein associations, and effects of oxidative perturbation on either might help explain those aspects of normal microrheology that fail to improve with either hydration or HbA substitution. Indeed, abnormal μ and plastic flow behavior are reproduced in normal RBC by stimulation of excess superoxide generation and in parallel with membrane thiol oxidation. Likewise, the rigidity of sickle ghost membranes in the ektacytometer is simulated by the same manipulation. It is not known if this effect is related to Hb/spectrin adduct formation (discussed above), but there seems to be a relationship between presence of such adducts and RBC deformability as evidenced by a significant correlation between MCHC and amount of adduct and the rigidity of resealed (and, therefore, hydrated) sickle ghost membranes. Yet another contributing factor may be provided by increased amounts of polyamines in sickle RBC because these organic cations can interact with membrane proteins and at physiologic concentrations exert an adverse effect on membrane microrheology.

**Deoxygenation and Sickling**

Not surprisingly, the plummeting deformability of deoxygenating sickle RBC is highly dominated by development of Hb polymer. Even before morphologic sickling takes place, RBC filterability deteriorates as pO₂ decreases, with the required depression of pO₂ being substantially less for most-dense cells. Interestingly, these cells actually require less polymer formation for loss of filterability than is required by less dense cells, so this measure of poor deformability probably reflects the additive effects of cytoplasmic viscosity and polymer formation. In this regard, it has been argued that the osmotic effect of polymer formation would cause MCHC to increase to a much greater extent for most-dense cells than for less-dense cells. Regarding the membrane, micropipet measurements of static extensional rigidity for evaluable sickle RBC (those that remained as discocytes at low pO₂) were interpreted as being normal, but results were quite variable and may even hint at a slight trend toward improvement of μ as pO₂ was lowered. Unfortunately, the informative experiment of monitoring single cells at varying pO₂ was not done to determine if the adverse effect of HbS on membrane microrheologic properties decreases as HbS withdraws from the membrane during deoxygenation.

![Fig 7. Abnormal microrheology of sickle RBC membranes. This abnormality is dramatically illustrated by micropipet measurement of static extensional rigidity (μ), which here is plotted relative to that measured for normal red cells in the 32 g/dL density fraction (μo). Mean values of this ratio for normal RBC as a function of MCHC. For sickle RBC (△), μ is sensitive to MCHC and increases dramatically for the most-dense cells. (Reprinted with permission.)](www.bloodjournal.org)
Consequences of Abnormal Microrheology

It may be anticipated that abnormal microrheologic properties would participate in vasocclusion. Increased static rigidity would contribute to mechanical trapping of RBC during their attempted passage through the microvasculature, while dynamic abnormalities might well affect the rate of RBC entry into capillaries of limiting diameter and the flow velocity of cells therein. In addition, the plastic flow abnormality of sickle membranes may explain development of ISC shape.

CELL/CELL INTERACTIONS

In recent years it has been appreciated that, regardless of proximate intracellular events, clinical phenotype (anemia and vasocclusion) must reflect interaction between the sickle RBC and its environment. Most data pertain to abnormal attachment of sickle RBC to mononuclear phagocytes or vascular endothelial cells, interactions of suspected relevance to hemolytic anemia and vasocclusive disease, respectively.

Monocytes and Macrophages

Some early descriptions of sickle cell anemia included erythropagocytosis in marrow specimens as a feature of the disease, and at autopsy tissue macrophages contain ingested RBC. Not surprisingly, sickle RBC have been found to be abnormally adherent to normal marrow (or splenic or alveolar) macrophages and to normal peripheral blood monocytes, with most-dense cells having increased tendency for adherence. After attachment, sickle RBC are phagocyted by macrophages more readily than are normal RBC, with both adherence and erythropagocytosis being highly variable (over a sevenfold range) among patients. Several mechanisms have been proposed and supported.

The IgG on sickle RBC is implicated in that macrophage adherence and phagocytosis are inhibited by FC receptor blockade or elution of Ig from the RBC. Alternatively, the reported ability of externalized PS to promote RBC adherence to macrophages in vitro has been implicated because adherence of sickle RBC is enhanced by deoxygenation and is impeded by preincubation of macrophages with PS liposomes. In addition, exposure of normal RBC to peroxide increases their phagocytosis, as does treatment with the peroxidation byproduct malondialdehyde. Because MDA appears to be unique among small aldehydes in this regard, the mechanism probably is not simply altered hydrophobicity. Rather, it is suggested that an interrelationship exists between immunologic and oxidative mechanisms because either peroxide treatment or MDA treatment enhances erythropagocytosis by promoting RBC opsonization with Ig.

Whether abnormal interactions with phagocytic cells actually participate in sickle hemolysis has been addressed only indirectly. The number of IgG molecules per RBC does not correlate with hematologic parameters indirectly reflecting hemolytic rate, which is not surprising because this ignores the variability in proportion of positive cells and coating density described above. However, the presence of IgG does correspond to erythropagocytosis of sickle RBC in vitro, and adherence to macrophages was found to correlate significantly with hemolytic index and with [Hb]. Interestingly, erythropagocytosis apparently is evident in peripheral blood in about 40% of sickle patients, with these having lower mean hematocrit than the remainder of patients not showing erythropagocytosis.

Vascular Endothelial Cells

Sickle RBC are abnormally adherent to vascular endothelial cells derived from human umbilical vein, bovine aorta, and rat microcirculation. This observation has been documented in various experimental systems, including those using gravity sedimentation, mechanical apposition, and perfusion of umbilical cords or rat mesocecum, or endothelialized flow chambers. Endothelial adherence is observed with sickle RBC suspended in balanced salt solution, buffer/albumin, serum, or culture medium. This notion is partially supported in a static/plasma system by observation that the less-dense subpopulation tends to adhere using only small areas of contact while more-dense cells use multiple areas of contact. This difference could explain less-efficient removal of dense cells by endothelial monolayer washing, and it supports the notion that observations will reflect extant subpopulations, which may vary in size or composition. Conversely, adherence studies performed under conditions of flow in the presence of plasma proteins indicate that adhesivity is most exaggerated for the least-dense reticulocyte-rich population. Indeed, even normal reticulocytes are quite adherent in this model. In other static/plasma studies, it was found that regardless of RBC density, the irregularly shaped non-ISC are more adherent than discocytes. Regardless of assay system, an important principle is that adherence probably depends on intimacy of contact, eg, as evidenced by diminished adhesivity after RBC have become spiculated by deoxygenation. Thus, it could be that most-dense cells are quite adherent, but the requirement for intimacy of contact dominates in flowing assay systems and perhaps in vivo.

Tenacity of adherence. Tenacity of sickle RBC adherence to endothelium is variable, as evidenced by progressive detachment of adhering RBC as a function of increasing shear. Actual comparative measurement of adhesive forces showed sickle RBC to have an average 40% increase.
SICKLE RBC MEMBRANE

for a single attachment site. However, unlike normal RBC, sickle RBC adhere using multiple attachment sites, so it was estimated that the relative adhesivity of sickle RBC may be increased as much as 100-fold under physiologic conditions where detaching forces would be tangential to the endothelial surface (rather than normal to it as during force measurements). Of interest, observation of even the “non-adherent” sickle RBC under flow conditions has shown sequential endothelial detachment/reattachment so they exhibit an apparent rolling behavior along the endothelial surface, perhaps partly explaining their deficient flow velocity during microcirculatory flow.

Location of adherence. In endothelialized capillary tubes sickle RBC do not adhere under laminar flow conditions, but addition of bends to add turbulence promotes adherence. In perfused rat mesocecum, sickle RBC adhere to venular endothelium especially at junctions and bends, but also in straight segments without junctions (Fig 8). This result led to the suggestion that endothelial adherence is a phenomenon most relevant to low shear locations such as the immediate postcapillary circulation. However, few data address the likelihood that physiologic relevance will reflect a complex interplay between adhesive potential, microrheologic factors, and the additional constraining influence of limiting capillary diameter. A single study found that adherence increases dramatically as venular diameter decreases, with maximal adhesive retention in venules of 7 to 10 μm in diameter (the smallest vessels examined).

Mechanisms underlying endothelial adherence. Endothelial adherence by sickle RBC reflects both membrane and environmental factors. Regarding the former, studies using static buffer conditions implicate the topographical abnormality of sialic acid bearing residues (i.e., glycoporphin clumping) and suggest that this abnormality and adhesivity may be promoted by calcium loading and by RBC dehydration. Notably, dehydration also makes normally nonadherent sickle trait RBC adhere to endothelium. Consistent with this finding, the development of endothelial adhesivity by normal RBC subjected to oxidative stress imposed by phenazine methosulfate is mediated by ensuing RBC dehydration. Alternatively, it has been suggested that PS externalization might promote increased adhesivity. Whether the adhesogenic potential of sickle RBC themselves, independent of plasma proteins, changes in association with acute crisis is not settled.

Regarding environmental factors, the presence of protein may or may not be absolutely necessary for endothelial adhesivity. In any case, albumin/buffer clearly supports relatively weak adherence compared with that evident in plasma and it is likely that actual mechanism of adherence is different in these two systems. Several plasma constituents have been studied as potential adhesogens. Fibrinogen and fibronectin have been variably implicated in adherence. Thrombospondin, which mediates cytoadherence of malaria-infected RBC to venular endothelium in vitro, has not been studied in relation to sickle RBC adherence, but its influence might help explain the reported adherence-promoting effect of platelets. Although plasma von Willebrand factor (vWF) is a poor promoter of adhesion, the high-molecular-weight vWF multimers secreted by endothelial cells markedly facilitate adhesion of sickle reticulocytes under flowing conditions. A mechanism involving molecular bridging between integrin family receptors on RBC and/or endothelium is supported by blocking studies using RGDS peptide or antibodies to GpIb or GPIb/IIIa. Specific receptor molecules on reticulocytes have not been directly identified, although indirect evidence for fibronectin receptors has been offered. However, adherence mediated by other proteins may involve different mechanisms; e.g., specific receptors are not required for macromolecular bridging of cells by fibrinogen.

The noted effect of vWF is of particular interest given the association between pain crisis and clinical dehydration, a stimulant of vasopressin and, therefore, vWF release. Certainly, pharmacologic stimulation of vWF release in a model microvascular system is associated with increased trapping/adherence of sickle RBC. Thus, changes in levels of certain plasma proteins due to acute crisis possibly underlie the observed increase in adhesogenic potential of sickle plasma obtained during acute pain crisis. Unfortunately, it is not yet known.

---

**Fig 8. Adhesion of sickle RBC to venular endothelium in the rat mesocecum.** The top panel shows adherent sickle discocytes tethered to the endothelial wall of a venule during flow in the direction of the arrow. The bottom panel shows increased adhesion of sickle cells at venular bending and at junctions of smaller diameter post-capillary venules, which in this case are totally blocked (small arrows). (Reprinted with permission.)
whether these changes actually preceed or only accompany vasocclusive crisis.

Role of the endothelial cell. Sickle adherence in plasma was noted to be abolished by the presence of EDTA, but it was not determined whether this inhibition was due to the removal of calcium per se or to the documented ability of EDTA to irreversibly dissociate (and render non-functional) certain integrin structures. An additional aspect is that of endothelial injury, because sickle RBC are more adherent to endothelial cells injured by activated neutrophils in vitro. Another example is provided by the unique case of viral infection of endothelium causing surface expression of viral glycoprotein having Fc receptor capability, in which case the IgG on sickle RBC potentiates their adherence. Conversely, adherence of sickle RBC may adversely affect the endothelial cell, as evidenced by induction of prostacyclin production, the levels of which are increased in sickle patient plasma. Possibly relevant are observations of circulating endothelial cells during vasocclusive crisis, the fact that sickle adherence may inhibit endothelial DNA synthesis, and an inconstantly observed diminution in ability of sickle patients to release tPA. Anatomical study has provided evidence of vascular injury in patient material. With the exception of a single preliminary report suggesting that sickle RBC adherence is increased by interleukin-1 (IL-1), the effect of cytokines on sickle RBC endothelial cell surface.

Variability among patients. In both static/buffer and flowing/plasma systems, adherence propensity has been found to vary markedly among patients (over a 20- to 30-fold range) so that some have RBC barely distinguishable from normal while others have RBC that exhibit a much greater adherence. The extent to which these results reflect patient-to-patient differences in adherence tenacity versus variation in size of adherent subpopulations has not been established. In any case, for HbSS patients a correlation has been observed between adherence propensity and a score of vasocclusive severity. Moreover, RBC from the clinically less severe sickling disorders are less adherent to endothelium, and sickle trait RBC are indistinguishable from normal RBC.

PATHOPHYSIOLOGIC ROLE OF MEMBRANE ABNORMALITIES

Considerations of sickle disease pathophysiology generally have been dominated by an emphasis on HbS polymerization. However, it now is recognized widely that this disease is exceedingly complex, so attempts to neatly explain it on the basis of a single feature are artifical and belie the intriguing heterogeneity among patients. On the other hand, it clearly is not yet possible to identify the actual relative importance of all relevant factors potentially influencing pathobiology. The foregoing discussion of sickle membrane abnormalities has identified ways in which specific defects might impact on physiology. The following will attempt to place these defects in the perspective of current concepts of sickle disease pathophysiology.

Factors Contributing to Hemolysis

Severity of anemia for sickle patients correlates directly with the magnitude of the very-dense cell population, consistent with data indicating that ISC are short-lived cells. This conclusion is strengthened by the fact that both dense-cell formation and anemia are ameliorated by concurrent α-thalassemia and in patients with HbSC disease. This relationship then accounts for the described correlation between patient Hb levels and amount of calculated RBC polymer content, which is simply derived from MCHC, so it remains to be seen whether sickling per se is tied directly to anemia. The actual mechanism of RBC survival attenuation could involve several of the membrane characteristics reviewed here, including abnormal cellular fragility as well as pathologic deposition of surface IgG coclustered with band 3 protein. The former might be related to that portion of hemolysis which appears to be intravascular, while the latter is one (but probably not the only) reason for abnormal interaction between sickle RBC and mononuclear phagocytic cells. Specific data relating to the possibility that such abnormal cell/cell interactions actually participate in hemolysis have already been discussed.

Factors Contributing to Vasocclusion

HbS polymerization. Two theories address the role of HbS polymerization in vasocclusive events. One argues that Hb gelation occurs so readily that clinical expression of polymer formation is dominated by thermodynamics and the equilibrium polymer concentration, with supporting evidence in the form of detectable polymer even at oxygen saturations typical of the arterial circulation. The second theory argues that the system is never in equilibrium and that sickling is dominated by kinetics, so that clinical effects depend critically on the relationship between microcirculatory transit time (short) and the unavoidable (and relatively longer) delay time for onset of Hb polymerization for most sickle RBC. The relative merits of these two views have been argued elsewhere, but it may be noted that each requires coordinate participation of the RBC membrane. Both necessarily implicate those membrane features promoting sickle RBC dehydration because polymer formation is so extraordinarily dependent on Hb concentration. The former theory, in fact, demands a critical role for the most-dense cells (ISC and/or unsickleable discs). The kinetic theory further implicates other factors that could promote sickling by delaying transit time. One of these, of course, is the abnormal interaction of sickle RBC with endothelium, and a number of observations reviewed above successfully relate features of the adhesive process to known aspects of vasocclusion physiology.

Membrane as template. It is controversial whether or not the RBC membrane directly influences polymerization. That anisotropic polymer formation is promoted by open, but not by resealed, sickle ghosts does suggest a membrane effect, which might even explain other data arguing for preexisting nucleation sites in most sickle RBC. On the other hand, the absence of a membrane influence has been
argued based on a lack of significant effect of normal RBC membranes on HbS gelation in vitro.\textsuperscript{290} However, certain peculiarities of the sickle membrane such as the abnormal clustering of Hb binding sites (in the form of band 3) argue that this issue cannot be resolved until similar gelation studies are performed using sickle membranes themselves.

**Dense cells versus least-dense cells.** The most-dense cell fraction contains those RBC that not only are most likely to form polymer but also are predicted to have the greatest difficulty negotiating the microcirculation.\textsuperscript{198,214} Of course, all those membrane defects contributing to the rheologic incompetence of this cell population (because of increased internal viscosity, abnormal membrane micro rheology, ISC shape, enhanced tendency to form polymer, and so on) are relevant to its participation in vasocclusion. Yet, recent data perhaps even argue against a role for dense cells in initiation of this event. For example, the occurrence of pain crisis actually correlates inversely with percent dense cells and percent ISC and, conversely, correlates positively with adequate RBC deformability (a feature of the least-dense, very adhesive cells).\textsuperscript{15,286} Yet it should not be assumed that dense cells are irrelevant. Their numbers do diminish during vasocclusive crisis, possibly because of sequestration,\textsuperscript{11} and microcirculatory models provide compelling evidence that dense cells do play an important role in vasocclusion. However, this may be as a propagating factor rather than as an initiating factor, with trapping of dense cells occurring secondary to initial adherence of less-dense cells to the vascular endothelium.\textsuperscript{261,281,294,295} These issues have been reviewed recently.\textsuperscript{296}

**Vascular factors.** It also is possible that sickle pathophysiology includes a role for vascular dynamics, the complexities of which have been reviewed.\textsuperscript{397} Pertinent observations from sickle patients include inconstantly observed oscillatory microvascular flow\textsuperscript{298} and similar flow intermittency during hyperemia.\textsuperscript{299} An improvement in certain ocular findings achieved using nifedipine was interpreted as being due to improved vascular dynamics,\textsuperscript{300} but those defects are felt to be due to presence of dense cells\textsuperscript{296} and nifedipine does inhibit ISC formation in vitro.\textsuperscript{301} The role of endothelial cell injury has been discussed already. An unexplored area is the potential role of endothelium-dependent vascular responses that are either vasodilatory or vasoconstricting.

**Environmental factors.** Preceding discussion has already identified potential roles for various blood factors, including plasma proteins (as modulators of RBC adhesivity), osmolality (as a determinant of RBC hydration), blood pH (as a stimulus for cation depletion, as well as Hb polymerization), and zinc levels (as a calcium antagonist). Also in this category, it is apparent that regional influences peculiar to certain vascular beds can be important, as exemplified by the undoubted impact of the hyperosmolar/hypoxic renal medulla. Insofar as cellular pathobiology is influenced by oxidative phenomena, it even is possible that an effect could be exerted by dietary factors (eg, access to selenium, riboflavin, and vitamins E and C) in some cases, although little investigation has been applied to this aspect.\textsuperscript{9}

### Genetic Factors Influencing Phenotypic Diversity

An alternative to summarizing the vasocclusive process descriptively (as above) is to approach the problem by identifying determinants of clinical phenotype. Such a compilation will show some factors for which a genetic basis has not been identified (eg, most of those described above in the “vascular” and “environmental” categories), but data are most developed for several genetic factors.

**Other globin mutants.** Concurrent presence of other β globins has great influence and defines the various sickling syndromes.\textsuperscript{301} Some of these (eg, presence of HbA or HbF) exert their effect by inhibiting polymer formation, while others readily participate in gelation (eg, HbD and HbO\textsuperscript{486}). Coexistent β' that allows some HbA expression. In contrast, Hbc is like HbA in its inability to participate in polymerization, but it seems to exert a pathologic effect by affecting the membrane to promote cellular dehydration.\textsuperscript{16,67} The presence of some α globin mutants may exert an effect on phenotype.\textsuperscript{302}

**α Globin gene number.** Concurrent α thalassemia lowers the number of very-dense cells.\textsuperscript{12,13,67,91,95} but, if anything, it confers a somewhat increased tendency toward vasocclusive events.\textsuperscript{11,92,302} This result is believed to reflect a concomitant adverse rheologic impact of increased blood viscosity\textsuperscript{13,92,288,302} because Hb level is an independent predictor of pain crisis frequency.\textsuperscript{297,303}

**Beta cluster haplotype.** Sickle patients vary in having beta cluster haplotypes typical of the mutation origin.\textsuperscript{303,305} These translate into some differences in severity for undefined reasons. Haplotype influences on mean HbF level have been emphasized, but it must be noted that HbF levels vary more widely within any given haplotype than between haplotypes.

**HbF levels.** HbF levels vary enormously among sickle patients\textsuperscript{305} and probably are determined largely by heritable factors that influence number of F cells and amount of HbF per F cell.\textsuperscript{306-308} The ameliorating role of HbF on RBC survival\textsuperscript{309} and on vasocclusive severity\textsuperscript{310} is felt to reflect its role as an inhibitor of HbS polymerization. Other potential influences have been recognized in theory but remain virtually unexplored.\textsuperscript{3} In this regard, an exciting new observation is that the molecular behavior of HbF is variable depending on whether it is composed of the 0γ type or the 5γ type.\textsuperscript{311,312} The hypothesis that Hb instability in the cellular pathobiology of sickle RBC\textsuperscript{4} is of interest that the 0γ type is more hydrophobic and unstable than the 5γ type and that levels of the former are higher in the patients with β cluster haplotype predictive of more severe disease.\textsuperscript{300}

**Other genetic disorders.** The role of non-globin genetic disorders is relatively unexplored, but the principle is exemplified by concurrent G6PD deficiency. It appears to have no major influence on severity if large numbers of patients are surveyed,\textsuperscript{313} but this does not preclude some pathogenetic influence for some patients. Indeed, both deleterious and beneficial effects deserve further study because both have been observed anecdotally and both can be hypothesized to fit within the complex biology of
oxidative phenomena as they impact upon the RBC membrane.

Membrane features. It is clear that the degree of influence exerted by various membrane factors is both variable among patients and relevant to clinical phenotype. By way of illustration, variability in stimulatability of K/Cl cotransport or Ca-activated K channels could have an impact on clinical phenotype by influencing the degree or rate of dense-cell formation. In some instances, such variability may be found to reflect the influence of as yet unrecognized genetic factors. However, just as the very existence of a great variety of membrane defects undoubtedly reflects the pleiotropic effect of the sickle gene, it also is probable that the great heterogeneity among patients in expression of these defects reflects a molecular behavior of the mutant gene product. It is not apparent how these defects can be explained by the polymerization behavior of HbS, but they readily can be postulated to be consequences of the instability of HbS, as expressed by decompartmentalization of cellular iron and excess endogenous oxidant stress. Regarding this notion, only those data bearing most directly on the sickle membrane have been noted herein; the larger body of relevant but less direct evidence related to this has been reviewed elsewhere. For present purposes, all HbS/membrane interactions are included in the category of Hb instability because data reviewed elsewhere suggest the existence of an intimate relationship between initial electrostatic association of HbS with the membrane and subsequent hydrophobic interaction and manifestation of HbS instability through its surface denaturation behavior. However, this is clearly an area that requires additional investigation to clarify mechanisms underlying the influence of Hb type on stimulation of K/Cl cotransport and the reversible effects of HbS concentration on microrheology.

A MODIFIED HYPOTHESIS

Some years ago, it was recognized that sickle disease clinical diversity is influenced by a series of cellular and genetic modulators of disease severity. In view of the wealth of data developed in the ensuing years, perhaps this view can be modified to recognize that the extreme complexity of sickle disease pathophysiology reflects the presence of two quite different levels of influence (Table 3).

One level comprises those influences that are readily explainable by genotype analysis and thus includes, most obviously, the determinants of cytosolic Hb composition and the regulators of HbF levels; it probably also includes the influence of α globin gene number and β cluster haplotype. The impact of these factors on pathophysiology is to broadly define the basic propensity for clinical disease through their influence on Hb polymerization and cellular sickling. Hence, their role in determining phenotypic diversity is predictable and mathematically solvable, at least in theory. While some optimism in this regard is perhaps justified by the great strides already made in both clinical correlation and in understanding the physical chemistry of HbS, sufficient data already exist to raise concern that even more detailed understanding of these factors will still leave unexplained the two great paradoxes of sickle disease. Why is degree of clinical involvement temporally variable despite constancy of these genotypically predictable cellular characteristics? What accounts for the great heterogeneity among sickle patients in clinical phenotype, so that variability within a specific genotypic profile can be even greater than differences between genotypes?

The present hypothesis finds the answer to these central questions in those factors that are not readily explainable by

---

**Table 3. Determinants of Phenotypic Diversity**

<table>
<thead>
<tr>
<th>Factors Exerting Predictable Effects</th>
<th>Factors Exerting &quot;Random&quot; Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>β globin concentration</td>
<td>Environmental influences</td>
</tr>
<tr>
<td>Presence of other β globins (β^*, β^+, β^0, β^0,-αδε)</td>
<td>(plasma osmolality, pH, proteins, zinc; regional pO2, antioxidant levels)</td>
</tr>
<tr>
<td>β Cluster haplotype</td>
<td>Vascular properties</td>
</tr>
<tr>
<td>α Globin gene number</td>
<td>(vascular dynamics, endothelial injury, endothelial-dependent vasoactive factors)</td>
</tr>
<tr>
<td>Presence of other α globins</td>
<td>Cellular factors</td>
</tr>
<tr>
<td>HbF level</td>
<td>(Psv, enzyme activities, redox status, ATP/DPG levels)</td>
</tr>
<tr>
<td>RBC membrane abnormalities</td>
<td>(abnormal cation homeostasis, PL destabilization, membrane protein defects, abnormal microrheology, band 3/glycerocholate body, red blood cell coagulation, macrophage interactions, endothelial adhesivity)</td>
</tr>
</tbody>
</table>

---

Fig 9. A hypothetical scheme that integrates the abnormal molecular behaviors of HbS and the probable roles of Hb polymerization and RBC membrane defects in sickle disease pathophysiology.
simple genotype definition. This includes not only the non-genetic factors described above as being “vascular” and “environmental” but also most of the sickle membrane defects. As noted already, these are remarkable for their variety, for the extraordinary heterogeneity in their expression, for their potential clinical relevance, and for their apparently intricate interrelationships (in both cause and effect). These facets readily provide opportunity for a degree of randomness in modulation of disease expression that can affect phenotypic diversity in a way that is inexplicable on the basis of traditional genotypic analysis. Insofar as this is explained by HbS instability, these effects are not expected to be truly random, of course, because their occurrence and nature must be governed by certain biochemical principles. However, oxidative pathobiology is so extraordinarily complex that the process of membrane damage may appear to be random; whether it is solvable remains to be seen.

As a consequence, the historic view of sickle disease pathobiology based on HbS polymerization and the “vicious cycle” model of Ham and Castle perhaps should be revised to include a component of “stochastic stasis.” Full integration of HbS polymerization and HbS instability into a comprehensive pathophysiologic scheme clearly must await further study, but Fig 9 hypothesizes a model that is justifiable based on current data. Meanwhile, recognition of a pathophysiologic role for the RBC membrane creates opportunities for possible therapeutic approaches including, but not limited to, interventions designed to improve cellular hydration status, to diminish endothelial adhesivity, to improve membrane micro rheology, or to optimize critical aspects of oxidative pathobiology. On the other hand, it is likely that successful development of creative therapeutic approaches based on the contribution of membrane pathobiology will require a more detailed understanding of its intricacies. Indeed, the appropriateness of the term “stochastic stasis” reflects the level of our ignorance more than it predicts a lack of solvability. It is clear that the sickle membrane continues to present great challenges for investigators of biochemistry, physiology, and cellular and molecular biology.

ACKNOWLEDGMENT

I gratefully thank the several investigators who discussed their cited data with me to help resolve points of contention and/or to educate me, and I thank Carol Taubert for her expert assistance in manuscript preparation.

REFERENCES

20. Ortiz OE, Lew VL, Bookchin RM: Calcium accumulated by sickle cell anemia red cells does not affect their potassium ("Rb") flux components. Blood 67:710, 1986
28. Gopinath RM, Vincenzi FF: (Ca²⁺ + Mg²⁺)-ATPase activity of sickle cell membranes: Decreased activation by red blood cell cytoplasmic activator. Am J Hematol 7:303, 1979
33. Luthra MG, Sears DA: Increased Ca⁺+, Mg⁺⁺, and Na⁺ + K⁺ ATPase activities in erythrocytes of sickle cell anemia. Blood 60:1332, 1982
47. Clark MR, Rossi ME: Cation selective leak in acutely deoxygenated sickle cells. Blood 74:308a, 1989 (abstr)
52. Fabry ME, Nagel RL: The effect of deoxygenation on red cell density: Significance for the pathophysiology of sickle cell anemia. Blood 60:1370, 1982
60. Brugnara C, Bunn HF, Tosteson DC: Regulation of erythrocyte cation and water content in sickle cell anemia. Science 232:388, 1986
67. Bunn HF, Noguchi CT, Hofrichter J, Schechter GP,
86. McCurdy PR, Sherman AS: Irreversibly sickled cells and red cell survival in sickle cell anemia: A study with both DF50 and 51Cr. Am J Med 64:253, 1978
96. DOVER GJ, CHANG VT, BOYER SH, SERJEANT GR, ANTONARAKIS S, HIGGS DR: The cellular basis for different fetal hemoglobin levels among sickle cell individuals with two, three, and four alpha-globin genes. Blood 69:341, 1987
100. Ohnishi ST, Katagi I, Katagi C: Inhibition of the in vitro formation of dense cells and of irreversibly sickled cells by charybdotoxin, a specific inhibitor of calcium-activated potassium efflux. Biochim Biophys Acta 1010:199, 1989
107. Shiffer KA, Goerke J, Dürgunes N, Fedor J, Shohet SB:


131. Ohnishi S-I, Ito T: Clustering of lecithin molecules in phosphatidylserine membranes induced by calcium ion binding to phosphatidylserine. Biochem Biophys Res Commun 51:132, 1973


143. Barsukov LI, Victorov AV, Vasilenko IA, Estvigneeva RP, Bergelson LD: Investigation of the inside-outside distribution, intermembrane exchange and transbilayer movement of phospho-
lipids in sonicated vesicles by shift reagent NMR. Biochim Biophys Acta 598:153, 1980

144. Jain SK: The accumulation of malonyldialdehyde, a product of fatty acid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes. J Biol Chem 259:3391, 1984


154. Jain S, Williams DM: Reduced levels of plasma ascorbic acid (vitamin C) in sickle cell disease patients: Its possible role in the oxidant damage to sickle cells in vivo. Clin Chim Acta 149:267, 1985


166. Francis RB Jr: Protein S deficiency in sickle cell anemia. J Lab Clin Med 111:571, 1988


172. Westerman MP, Diloy-Puray M, Streczyn M: Membrane components in the red cells of patients with sickle cell anemia: Relationship to cell aging and to irreversibility of sickling. Biochim Biophys Acta 557:149, 1979


188. Low PS, Waugh SM, Zinke K, Drenckhahn D: The role of hemoglobin denaturation and band 3 clustering in red blood cell aging. Science 227:531, 1985


189. Sorette MP, Galili U, Clark MR: Comparative binding of antibodies of known specificity to high density and phenylhydrazine treated human RBC. Blood 70:115a, 1987 (abstr)


212. Allen DW, Cadman S: Calcium-induced erythrocyte mem- brane changes: The role of adsorption of cytosol proteins and proteases. Biochim Biophys Acta 551:1, 1979


222. Green MA, Noguchi CT, Keidan AJ, Marwah SS, Stuart J:
243. Sydnerstrcker VP: Further observations on sickle cell anemia. JAMA 83:12, 1924
vascular endothelium: Morphologic correlates and the require-


269. Wick TM, Moske JI, Udden MM, McIntire LV: Unusually large (UL) vWF multimers bind to GPIb-like and integrin receptors on sickle and young non-sickle RBC and on endothelial cells (EC): A mechanism for sickle and other young RBC adhesion to EC. Blood 72:76a, 1988 (abstr)


308. Milner PF, Leibfarth JD, Ford J, Barton BP, Grenett HE, Garver FA: Increased Hbf in sickle cell anemia is determined by a factor linked to the βs gene from one parent. Blood 63:64, 1984
313. Ham TH, Castle WB: Relation of increased hypotonic fragility and of erythrostasis to the mechanism of hemolysis in certain anemias. Trans Assoc Am Physicians 55:127, 1940
Beyond hemoglobin polymerization: the red blood cell membrane and sickle disease pathophysiology

RP Hebbel