Spontaneous Oxygen Radical Generation by Sickle Erythrocytes

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ABSTRACT Since the various membrane abnormalities of sickle erythrocytes might result from excessive accumulation of oxidant damage, we have measured the generation of superoxide, peroxide, and hydroxyl radical by normal and sickle erythrocytes using assays involving reduction of cytochrome *c*, aminotriazole inhibition of catalase, and methane evolution from dimethyl sulfoxide, respectively. Compared with normal erythrocytes, sickle erythrocytes spontaneously generate approximately twice as much superoxide, peroxide, and hydroxyl radical. One possible source of hydroxyl radical generation was identified as hemichrome, excessive amounts of which are bound to sickle erythrocyte membranes. Hemichrome did not generate hydroxyl radical when exposed to superoxide alone or peroxide alone. However, in the presence of both superoxide and peroxide, hemichrome greatly facilitated hydroxyl radical generation. Supporting this, normal erythrocyte membranes induced to acquire sickle hemichrome concomitantly acquired an enhanced ability to mediate hydroxyl radical generation. Finally, sickle erythrocyte membranes greatly enhanced superoxide/peroxide-driven hydroxyl radical generation as compared with normal erythrocyte membranes. These data suggest that an excessive accumulation of oxidant damage in sickle erythrocyte membranes might contribute to the accelerated membrane senescence of these cells. They further indicate that accumulation of oxidant damage could be a determinant of normal erythrocyte membrane senescence.

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INTRODUCTION

Although deoxygenation-induced sickling is the most easily demonstrated property of sickle erythrocytes (HbS RBC),[1] numerous membrane abnormalities have also been described. These include: the "frozen" spectrin/actin shell of irreversibly sickled RBC (1); an abnormal orientation of lipid bilayer phospholipids (2); a deficient calcium-ATPase (3); and a propensity for HbS RBC to adhere to vascular endothelium (4). Although it seems likely that some of these membrane abnormalities contribute to the overall pathophysiology of sickle-cell disease, their etiology remains obscure.

We have been intrigued by the concept that sickle cell anemia may be a disease of accelerated membrane senescence, perhaps involving the accumulation of oxidant damage to crucial membrane components. For example, HbS RBC membranes are deficient in vitamin E (5), which might be the result of, or a predisposing factor towards, abnormal peroxidation of membrane lipids. In addition, HbS RBC contain increased amounts of malondialdehyde, a by-product of lipid peroxidation, and evidence of abnormal amino group cross-linking by malondialdehyde has been demonstrated in lipid extracts of HbS RBC membranes (6).

Although these observations could be explained by the abnormal susceptibility of HbS RBC membranes to lipid peroxidation (6, 7), they might also indicate that HbS RBC membranes are exposed to increased

[1] *Abbreviations used in this paper*: AT, aminotriazole (3-amino-1,2,4-triazole); CH_4, methane; DMSO, dimethyl sulfoxide; G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; HbS RBC, sickle erythrocytes; HC, hemichrome; Hct, hematocrit; H_2O_2, peroxide; O_2^-, superoxide; $\cdot OH$, hydroxyl radical; PBSG, phosphate-buffered saline containing 1 g/liter glucose; SOD, superoxide dismutase; WBC, leukocytes.

amounts of endogenous oxidant. That the latter may be a contributing factor in sickle disease pathophysiology is supported by these experiments that document excessive spontaneous oxygen radical generation by HbS RBC. Of particular interest regarding the development of RBC membrane abnormalities, evidence is presented that suggests that superoxide/peroxide-driven hydroxyl radical (·OH) generation is facilitated by membrane-bound hemichrome (HC), a denatured ferric hemoglobin (Hb) found in excessive amounts bound to HbS RBC membranes (8).

METHODS

Patients. Blood was obtained from adult volunteers shown to have sickle-cell anemia by standard criteria and specialized studies as required (9). On the basis of enzyme electrophoresis, all individuals with the GdA isoenzyme of glucose-6-phosphate dehydrogenase (G6PD) were assayed for activity of G6PD and hexokinase (as an age-dependent control enzyme) (10); none of the patients reported was G6PD deficient.

RBC preparation. Within 36 h of venipuncture, RBC from citrated blood were washed three times with isotonic NaCl, with removal of buffy coat. After suspension in phosphate-buffered saline (PBS), RBC were filtered through Whatman CF11 powdered cellulose (Whatman Inc., Clifton, NJ), after which microscopic inspection and monitoring by hemocytometer revealed an absence of leukocytes (WBC) and platelets. To confirm that RBC preparations were not contaminated with other blood cells, RBC suspensions were assayed for ·OH generation (see below) in the presence and absence of opsonized zymosan, phorbol myristate acetate, or zymosan-activated plasma. Control experiments revealed that contamination could thus be detected by an increment in ·OH generation in the presence of these granulocyte stimulators (11). All RBC incubations were performed under ambient oxygen tension and in PBS containing 1 g/liter glucose (PBSG); to those of >8 h duration penicillin/streptomycin was added.

Detection of superoxide (O_2^-). The O_2^--dependent reduction of horse ferricytochrome *c* was measured using established methods (12). RBC at hematocrit (Hct) 2.5% were incubated in nine replicates (for up to 4 h at 37°C) in PBSG containing 50 μM cytochrome *c*, with and without 200 μg/ml superoxide dismutase (SOD). SOD-inhibitable cytochrome *c* reduction was measured spectrophotometrically in the supernatant. Appropriate blanks were used to correct results for RBC-independent cytochrome *c* reduction and the presence of Hb due to any RBC lysis. In control experiments, the latter were found to overcorrect slightly for Hb contamination. Since HbS RBC tended to lyse somewhat more than normal RBC during these incubations (but always <0.1% lysis), the results tend to minimize the differences between normal and HbS RBC. Control experiments indicated that this technique reliably detects >11.4 nmol cytochrome *c* reduced/ml RBC; this corresponds to >3 OD units above blank values.

Detection of peroxide (H_2O_2). H_2O_2 generation within RBC was detected by the H_2O_2-dependent inactivation of catalase by aminotriazole (AT) (13, 14). Triplicate RBC samples were incubated at Hct 1.0% (for up to 20 h at 37°C) in PBSG containing 50 mM AT. At selected times, triplicate aliquots were removed and assayed for residual RBC catalase activity. Results are simply expressed as percentage of catalase activity lost over the period of incubation, which reflects the H_2O_2-specific inactivation of catalase by AT. There was no catalase inactivation in the absence of AT.

Detection of ·OH. Measurement of ·OH generation within RBC utilized the fact that ·OH releases methane (CH_4) from dimethyl sulfoxide (DMSO) (11). Duplicate RBC samples (Hct 60% in PBSG with 100 μl DMSO/2 ml) were incubated at 37°C for 20 h in sealed glass tubes having 1.4 ml headspace air. After incubation, headspace gas was sampled anaerobically by aqueous displacement and analyzed with a Beckman GC72-5 gas chromatograph (Beckman Instruments, Inc., Fullerton, CA) (6 ft × 2 mm-glass column packed with Carbosphere 80/100 mesh [Altec Associates, Arlington Heights, IL]; flame ionization detector; 2 min CH_4 retention time). Readout was mathematically converted to picomoles ·OH per milliliter RBC based on standard gas readings and the 1:1 molar ratio of CH_4 to ·OH. Results were corrected for CH_4 in the starting headspace air and for any RBC-independent CH_4 evolution. Control experiments revealed that the small amount of lysis (1%) occuring during these incubations had no effect on results. An incubation time of 20 h was selected for these experiments since it allowed adequate separation of normal and HbS RBC results, yet it was accompanied by a maximal drop of 0.2 pH unit in the incubation medium. Under these conditions, the incubated HbS RBC suspensions showed no increase in sickled cells at 7 h and the appearance of only ~5% newly sickled cells at 20 h.

HC. The amount of HC bound to washed RBC membranes was determined as previously described (8).

For "chemical system" experiments below, preformed HC was prepared from membrane-free HbS RBC lysates by vortexing (8) in the presence of 10 mM AT (to inhibit the small amounts of catalase that tend to coprecipitate with the HC), followed by extensive washing of the precipitate with 5 mM sodium phosphate buffer (pH 7.0). Precipitated Hb prepared in this manner had no detectable catalase or SOD activity, and evaluation of its absorbance spectrum confirmed that it was HC (8).

For experiments utilizing endogenous membrane-bound HC, 1.0 ml of normal or HbS RBC were washed five times with 40 vol of 5 mM sodium phosphate buffer (pH 7.4), followed by suspension of the ghosts to 4.0 ml in similar buffer at pH 7.0. Thus, RBC membranes used in chemical system experiments were prepared quantitatively, and each assay used 1.0 ml ghost suspension (i.e., the membranes prepared from 250 μl RBC). This method of quantitation was selected after preliminary experiments proved it to yield more reproducible results than attempted quantitation by measurements of lipid P_i or milligrams membrane protein. The selected method would still tend to minimize differences between normal and HbS RBC due to the slightly less efficient packing of HbS RBC.

Finally, for experiments utilizing induced membrane-bound HC, equal volumes of normal RBC white ghosts (ghosts from 250 μl RBC/ml) and HbS RBC membrane-free lysate (3 mg Hb/ml) containing 10 mM AT were mixed and incubated for 30 min at 37°C in 5 mM sodium phosphate buffer (pH 7.0) containing 20 μM $CaCl_2$. After washing with large volumes of the pH 7.0 buffer until the supernate was Hb-free, the pink ghosts were vortexed. Subsequent washing resulted in no appreciable release of Hb, and spectral analysis confirmed the induced presence of HC. In two experiments half the above amounts of lysate and calcium were used to achieve a lesser degree of Hb binding. Control experiments comparing Hb binding to inside-out and right-

side out RBC ghosts (15) under conditions simulating the initial admixture step described above indicated that >95% of the Hb binds to the inner aspect of the RBC membrane. This is consistent with our assumption that the initial step in our preparation of induced membrane-bound HC is the electrostatic binding of Hb to the inner aspect of the membrane (16).

Chemical system. To evaluate the role of HC in ·OH generation, the ·OH assay system was used with chemical generators of activated oxygen in the presence and absence of HC (preformed, endogenous, or induced membrane-bound, as described above). Each ml of the 2.0 ml assay mixture contained 50 μl DMSO and various combinations of the following reagents in 5 mM sodium phosphate buffer: 0.1 or 0.2 U glucose oxidase, 0.1 or 0.2 U xanthine oxidase, 1 or 2 mM glucose, 1 or 2 mM xanthine, 200 μg SOD, 100 μg catalase, 1 or 2 mg preformed HC, and the open ghosts from 125 μl RBC. Except for xanthine at pH 8.0, all reagents were in pH 7.0 buffer. Mixtures were incubated in triplicate for 20 h at 37°C.

Statistical analysis. Data were examined for significance using Student's *t* test.

RESULTS

Compared with normal RBC, RBC from individuals with sickle-cell anemia spontaneously generated significantly increased amounts of O_2^-, H_2O_2, and ·OH (Table I). Additional experiments supported the specificity of these measurements. O_2^- generation was increased 105±76% (n = 4) by 50 mM diethyldithiocarbamate, an SOD inhibitor (17). Pretreatment of RBC with the anion channel blocker 4,4'-diisothiocyano-2,2'-stilbene disulfonate (DIDS) (18) decreased detection of O_2^- by 70±28% (n = 2). ·OH detection was inhibited by 82±17% (n = 5) by 15 mM thiourea, an ·OH scavenger (11), but not by 15 mM urea (0±3%, n = 5). The less efficient ·OH scavengers ethanol and mannitol (11) inhibited detection by 65±20% (n = 3) and 62±35% (n = 3), respectively. Addition of both SOD and catalase to the incubation medium had no effect on CH_4 evolution, indicating that all detected ·OH generation occurred within the RBC.

·OH generation values were within the normal range for four individuals with sickle trait and for six nonhemoglobinopathic patients with reticulocyte counts ranging from 5 to 20%. Among the individuals with sickle-cell anemia, there was no significant correlation between ·OH generation and either reticulocyte count (r = −0.107) or irreversibly sickled cells count (r = −0.141). For HbS RBC separated by density (4), least- and most-dense layer RBC did not differ significantly from unseparated RBC in terms of ·OH generation or rate of catalase inactivation. Thus, excessive oxygen radical generation by HbS RBC is not explained simply by reticulocytosis or by the presence of sickled RBC.

Amounts of RBC membrane-bound HC were found to correspond with those reported by others (8, 19): 0.023±0.030 (n = 20) and 0.162±0.149% (n = 32) of total cellular heme for normals and sickle-cell anemia patients, respectively (mean±SD). For the 18 individuals on whom both measurements were made, a significant correlation between ·OH generation and membrane-bound HC is apparent (Fig. 1).

Consequently, three types of experiments were devised to determine whether HC might be involved in ·OH generation. Experiments using preformed HC

TABLE I
*Spontaneous Oxygen Radical Generation by Erythrocytes**

	Units	Normal RBC	Sickle RBC	*P*
Superoxide	Cytochrome *c* reduced, *nmol/ml RBC*			
	In 2 h	17±20 (8)	42±10 (4)	<.02
	In 4 h	41±11 (7)	95±35 (6)	<.01
Peroxide‡	Catalase inhibition, %			
	In 8 h	4.9±9.5 (2)	23.0±9.8 (5)	<.1
	In 20 h	8.9±9.7 (9)	33.4±12.4 (11)	<.001
·OH	CH_4 liberated, *pmol/ml RBC*			
	In 7 h	21.8±5.9 (3)	44.5±16.4 (4)	<.1
	In 20 h	71.0±32.8 (9)	128.9±36.2 (22)	<.001

* O_2^- escaping from RBC was detected by the O_2^--dependent, SOD-inhibitable reduction of ferricytochrome *c*. H_2O_2 accumulation within RBC was measured by the H_2O_2-dependent inhibition of catalase by AT. ·OH generation within RBC was detected by the ·OH-dependent evolution of CH_4 from DMSO. Results are shown as mean±SD (number of individuals evaluated).

‡ The mean zero-time catalase activity of HbS RBC was 79.1% that of normal RBC; absolute amounts of catalase inactivated were threefold greater for HbS RBC as compared with normal RBC.

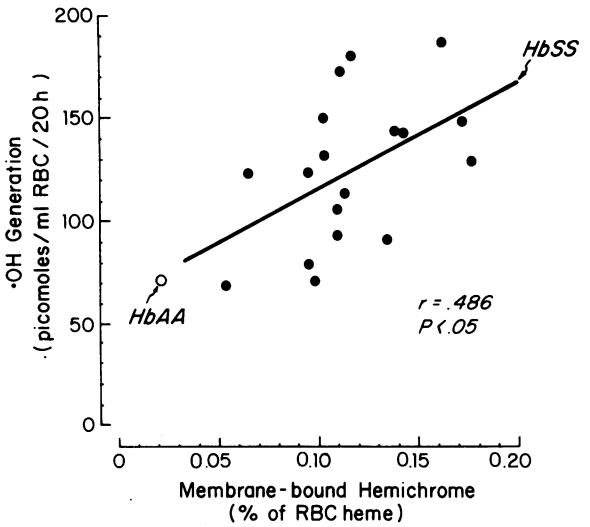

FIGURE 1 Relationship between spontaneous RBC ·OH generation and amount of membrane-bound HC. A modest but significant correlation between ·OH and HC for 18 individuals with sickle-cell anemia (●, HbSS) is apparent ($r = 0.486$, $P < 0.05$). Note that the mean value for nine normals (○, HbAA) falls very close to the regression line calculated for the HbSS data.

(Table II) demonstrated that no detectable ·OH generation results from incubation of HC with chemical generators of O_2^- alone or H_2O_2 alone. However, in the presence of both O_2^- and H_2O_2 (itself sufficient to generate a background of 86 pmol CH_4), HC clearly facilitated ·OH generation.

Suggesting a possible pathophysiologic role for HC, membranes prepared from HbS RBC (and therefore having much greater amounts of endogenous membrane-bound HC) facilitated O_2^-/H_2O_2-driven ·OH generation more efficiently than membranes prepared from normal RBC (Table III).

Finally, normal RBC membranes induced to acquire increased amounts of HC, concomitantly acquired a greatly enhanced ability to facilitate ·OH generation in the presence of O_2^- and H_2O_2 (Table IV).

For all three types of experiment (Tables II, III, IV), HC facilitation of O_2^-/H_2O_2-driven ·OH generation was completely inhibited by 0.6 mM NaCN, which in liganding with Fe^{+++} may prevent HC iron from cycling between ferrous and ferric states (data not shown). In three experiments for each of the three experimental systems, addition of either SOD or catalase significantly inhibited HC-facilitated ·OH generation by >90%.

TABLE II
*Participation of HC in ·OH Generation**

Components in chemical system‡	CH_4	
	pmol	
1 mg/ml HC + O_2^- + catalase	0±0	(3)
1 mg/ml HC + H_2O_2 + SOD	0±0	(3)
O_2^- + H_2O_2	86±22	(4)
1 mg/ml HC + O_2^- + H_2O_2	529±248	(4)
2 mg/ml HC + O_2^- + H_2O_2	959±565	(4)

* ·OH was detected by the ·OH-dependent evolution of CH_4 from DMSO, using a 2.0-ml assay system containing 100 μl DMSO and incubated at 37°C for 20 h. Results are shown as mean±SD (number of experiments).

‡ These experiments used "preformed HC", the precipitated HC derived from vortexing membrane-free lysates of HbS RBC. O_2^- was generated with xanthine/xanthine oxidase, and H_2O_2 was generated with glucose/glucose oxidase. Enzyme and substrate concentrations were 0.1 U/ml and 1.0 mM, respectively.

DISCUSSION

Compared with normal RBC, HbS RBC spontaneously generate significantly increased amounts of O_2^-, H_2O_2, and ·OH (Table I). The enhanced generation of activated oxygen extends to all three species studied, lending internal support to the validity of these observations. It should be noted, however, that the exact magnitude of activated oxygen generation cannot be determined from these studies, since the efficiency of detection is far from perfect. All three forms of activated oxygen are quite reactive and short lived in the presence of biological material. Thus, only that O_2^- that actually escapes from the RBC may be detected by external cytochrome *c*. Only that H_2O_2 that does not first react with Hb, glutathione, or the RBC membrane will be detected by AT-induced inhibition of catalase complex I. Similarly, only ·OH that fails to react with a variety of intracellular organic molecules will be available to react with DMSO, a reaction that itself may be quite inefficient (11).

At least two sources of O_2^- generation within RBC are easily identified. First, the constant turnover of methemoglobin is a source of relentless O_2^- formation from oxyhemoglobin (20) within both normal and HbS RBC. Second, the oxidation state of HC iron (Fe^{+++}) indicates that an electron has been lost during its formation and, therefore, that O_2^- has probably been generated (21–23). Thus, the greatly increased amount of HC in HbS RBC (8, 19) may explain, at least in part, the excessive O_2^- generation by HbS RBC. Dismutation of O_2^- thus formed will readily generate excessive amounts of H_2O_2.

The mechanism of RBC ·OH generation is not as straightforward. Given the presence of both O_2^- and H_2O_2, ·OH may be formed through the Haber-Weiss reaction ($O_2^- + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$). Despite

TABLE III

*Endogenous Membrane-bound HC Facilitates ·OH Generation**

Components in chemical system	Amount of bound HC‡	CH_4§	
		pmol	
Normal RBC membranes	0.039±0.010	10±12	(4)
Sickle RBC membranes	0.139±0.096	44±54	(4)
O_2^- + H_2O_2		89±68	(4)
Normal RBC membranes + O_2^- + H_2O_2	0.039±0.010	761±661	(4)
Sickle RBC membranes + O_2^- + H_2O_2	0.139±0.096	1,924±2195	(4)

* ·OH was detected, and O_2^- and H_2O_2 were generated, as described for Table II. This experiment examines the effect of "endogenous membrane-bound HC" (that HC found naturally adhering to multiple-washed RBC membranes). Each assay used the membranes prepared quantitatively from 250 μl RBC. Results are shown as mean±SD (number of experiments).

‡ HC is expressed as milligrams HC bound to the membranes prepared from 250 μl RBC (mean±SD).

§ Since the presence of any contaminating ferrous Hb in such membrane preparations might contribute to ·OH generation through the reaction of H_2O_2 with Fe^{++}, results are presented after correction of the complete system result (i.e., membranes + O_2^- + H_2O_2) by subtraction of the results from a parallel incubation with H_2O_2 alone (i.e., membranes + H_2O_2).

uncertainty as to whether this reaction really occurs as written, it probably does accurately reflect the stoichiometry of ·OH formation in biological systems, with transition metals acting as catalytic intermediates (24–25).

Thus, in considering possible mechanisms for ·OH generation, we have focused upon iron compounds because of their great abundance within RBC. Specifically, we have studied the role of HC, since it is clearly found in excessive amounts bound to HbS RBC membranes (8, 19) and because its presence correlates modestly with ·OH generation (Fig. 1). That HC iron may be involved in ·OH generation through the Haber-Weiss reaction is suggested by these studies, in that

TABLE IV

*Induced Membrane-bound HC Facilitates ·OH Generation**

Components in chemical system	Amount of bound HC‡	CH_4	
		pmol	
Normal RBC membranes	0.026±0.006	0±0	(3)
Normal RBC membranes with ↑HC	0.187±0.045	0±0	(3)
O_2^- + H_2O_2		1,064±463	(3)
Normal RBC membranes + O_2^- + H_2O_2	0.026±0.006	4,073±1,108	(3)
Normal RBC membranes with ↑HC + O_2^- + H_2O_2	0.099±0.013	9,284±700	(2)
Normal RBC membranes with ↑HC + O_2^- + H_2O_2	0.187±0.045	13,831±1,729	(3)

* These experiments used "induced membrane-bound HC," which is the product of admixing normal RBC membranes with sickle Hb in the presence of calcium, followed by removal of unbound Hb and vortexing of the pink ghosts. Each assay used the membranes prepared quantitatively from 250 μl RBC. Conditions were as described for Table III, except that enzyme and substrate concentrations for these experiments were higher (0.2 U/ml and 2.0 mM, respectively). Results are shown as mean±SD (number of experiments).

‡ HC is expressed as milligrams HC bound to the membranes prepared from 250 μl RBC (mean±SD).

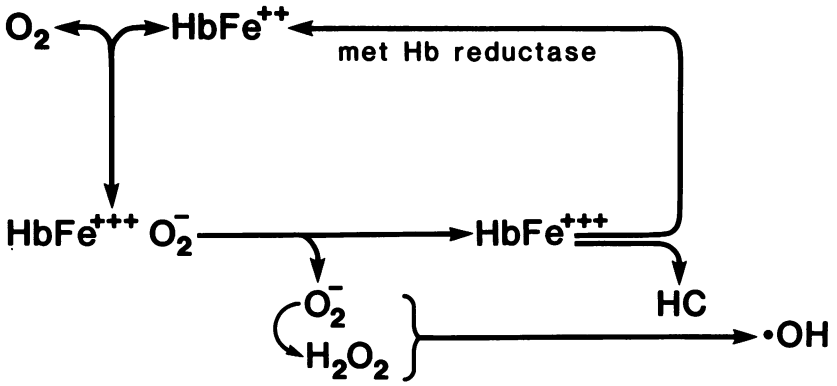

FIGURE 2 Possible mechanism of •OH generation within RBC. The reversible combination of oxygen (O_2) with deoxy Hb ($HbFe^{++}$) results in formation of cytoplasmic (or membrane bound) oxyHb, shown here in its ferric-superoxy state ($HbFe^{+++}O_2^-$), with an electron shared between Hb iron and oxygen (29). The daily turnover of metHb ($HbFe^{+++}O_2^- \rightarrow HbFe^{+++} \rightarrow HbFe^{++}$) is one source of O_2^- and H_2O_2 generation. An additional source of O_2^- and H_2O_2 is the formation of HC ($HbFe^{+++}$ $O_2^- \rightarrow HbFe^{+++} \rightarrow$ HC), which for unknown reasons is excessive in sickle RBC. Given the presence of O_2^-, H_2O_2 and HC iron (all three being present in abnormal amounts in HbS RBC), conditions are met for excessive •OH generation through the Haber-Weiss reaction. To the extent that •OH generation involves membrane-bound HC, membrane components might be the predominant targets for attack by activated oxygen.

HC facilitates •OH generation only in the presence of both O_2^- and H_2O_2. Furthermore, CN^- completely inhibits HC-mediated •OH generation, presumably because it ligands with Fe^{+++} and thereby prevents HC iron from cycling between ferric and ferrous states. Although HC is commonly described as a permanently denatured form of low-spin ferric Hb, there are in fact several species of HC that vary in degree of reversibility/irreversibility and in degree of heme pocket abnormality (26). Our HC measurements do not distinguish between these various species.

Based on these observations, a scheme to account for excessive •OH generation by HbS RBC is presented in Fig. 2. However, it must be emphasized that this scheme is both hypothetical and simplified. Besides HC, there are numerous iron sources within HbS RBC, including various other species of cytoplasmic and membrane-bound Hb and abnormal cytoplasmic deposits of ferritinlike (or hemosiderinlike) iron (27). In addition, there are other potentially important metals such as copper. Finally, the amounts of activated oxygen detected would also depend upon the integrity of cellular antioxidant systems. Thus, increased •OH generation by HbS RBC may also reflect their possible deficiency of glutathione (28). Similarly, while neither membrane-bound HC (19) nor •OH generation appear to vary significantly with HbS/RBC density, the biologic effect might still be age-related if RBC were to progressively deteriorate in terms of antioxidant mechanisms. It would appear that identification of exact mechanisms of excessive activated oxygen formation within HbS RBC will be an exceedingly complex problem.

In terms of the biologic effects of activated oxygen formation within RBC, it seems likely that the location of generation would be at least as important as the quantity of activated oxygen formed. For example, if membrane-bound HC is involved in •OH generation as these data suggest, membrane components might be particularly susceptible to oxidative damage, since the radical generator would be relatively sequestered from the cytoplasmic antioxidant mechanisms. We are currently investigating the effect of oxygen radical exposure upon various membrane components, including structural proteins, phospholipids, and enzymes. For example, our preliminary data indicate that HC-mediated •OH generation may inhibit RBC membrane calcium-ATPase. Insofar as premature RBC death caused by the presence of abnormal hemoglobins may represent an acceleration of normal aging processes, these results may ultimately be applicable to the general phenomenon of cell aging.

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