Oxidant Damage of the Lipids and Proteins of the Erythrocyte Membranes in Unstable Hemoglobin Disease

EVIDENCE FOR THE ROLE OF LIPID PEROXIDATION

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ABSTRACT Since unstable hemoglobins have been considered a source of reactive oxygen radicals, and oxidative membrane damage a prehemolytic event, we examined the erythrocyte membranes of six patients (three splenectomized) with hemoglobin Köln disease. In the hydrogen peroxide stress test, the patients' erythrocytes generated more than twice the malonyldialdehyde (a lipid peroxidative product) than control erythrocytes. Fluorescence spectra of lipid extracts of the patients' erythrocytes showed an excitation maximum at 400 nm and an emission maximum of 460 nm, characteristic of malonyldialdehyde lipid adducts. Two types of membrane polypeptide aggregates were found in the erythrocytes of the splenectomized patients. The first, which were dissociable by treatment with mercaptoethanol, contained disulfide-linked spectrin, band 3 and globin. The second, not dissociable by mercaptoethanol, had an amino acid composition similar to that of erythrocyte membranes and spectrin (unlike globin) and like that of aggregates produced by the action of malonyldialdehyde on normal erythrocyte membranes. Atomic absorption spectroscopy of hemoglobin Köln erythrocytes showed no increase in calcium content implying that these cross-links were not due to calcium-stimulated transglutaminase. Using a micropipette technique, we demonstrated that erythrocytes containing membrane aggregates from splenectomized patients were less deformable while aggregate-free erythrocytes from nonsplenectomized patients had normal deformability. We conclude that the erythrocyte membranes in hemoglobin Köln disease show evidence of lipid peroxidation with production of malonyldialdehyde, and that the nondissociable membrane aggregates formed in this disease are likely cross-linked by malonyldialdehyde. Because the erythrocytes containing membrane aggregates from splenectomized patients with unstable hemoglobin disease show decreased membrane deformability, we hypothesize that this abnormality results in premature erythrocyte destruction in vivo.

INTRODUCTION

Approximately 60 human hemoglobin (Hb)[1] variants have been called unstable by virtue of their increased propensity to denature, precipitate, and form Heinz bodies (1). This type of Hb mutation produces unstable Hb disease, manifested by variable degrees of chronic hemolysis, Heinz body-containing erythrocytes and dipyrroluria (1). Many of the unstable Hb are characterized by an amino acid substitution in the globin chain segment that forms the heme pocket (2). Alterations in the physicochemical properties of the heme pocket secondary to these amino acid substitutions may

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[1] *Abbreviations used in this paper:* G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; MDA, malonyldialdehyde; ME, mercaptoethanol; non-SPX, nonsplenectomized; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; SPX, splenectomized.

TABLE I

High Molecular Weight Membrane Polypeptide Aggregates, Erythrocyte Calcium, and Glutathione Content in Hb Köln Patients

	Glutathione*	Calcium	Aggregates*	Nondissociable aggregates*
	μmol/g Hb	*μmol/liter RBC‡*	*% membrane protein*	
Controls	6.44±0.34	15–50§	0	0
SPX	3.87±0.30‖	33.3	1.22±0.18	0.38±0.13
Non-SPX	6.05±1.78	34.3	0	0

* Results expressed as mean±SD, $n \geq 3$.

‡ Erythrocytes.

§ Range observed in multiple control specimens.

‖ Significantly less than controls and non-SPX patients ($P < 0.005$).

lead to excessive generation of superoxide and other toxic oxygen radicals that could oxidize erythrocyte components (3). Theoretically the formation of toxic oxygen radicals within the erythrocyte could result in oxidative damage to the membrane as well as cytoplasmic constituents, but apart from the observation that erythrocyte membrane sulfhydryls may form mixed disulfide linkages with denatured Hb (4), no direct evidence of oxidant-induced membrane damage has been presented.

We have found disulfide-bonded membrane polypeptide aggregates in the erythrocytes of patients with chronic hemolytic glucose-6-phosphate dehydrogenase (G6PD) mutants (5, 6), and have shown that such oxidant-induced erythrocyte membrane injury is associated with decreased erythrocyte survival in a in vivo model system (7). Jain and Hochstein (8) demonstrated that the malonyldialdehyde (MDA) generated by the peroxidation of membrane lipids, in addition to forming phospholipid adducts, can also cross-link erythrocyte membrane proteins into nondissociable aggregates, in contrast to disulfide-linked aggregates. In an attempt to discover evidence of oxidant-induced membrane damage in unstable Hb disease, we studied the erythrocytes from several members of a family with Hb Köln (9). These studies indicate the presence of oxidative erythrocyte damage involving both membrane lipids and proteins. The nature of the lesions demonstrated suggest that they play an etiologic role in the hemolysis that occurs in unstable Hb disease.

METHODS

Subjects. Six affected members of a family with an unstable Hb were studied. Three were splenectomized (SPX) (ages 37, 37, 40), three nonsplenectomized (non-SPX) (ages 11, 14, 15). Blood samples were drawn into tubes containing sodium heparin or disodium EDTA as were samples from simultaneous normal volunteers. Other control samples from patients with various hemolytic disorders were studied as indicated, when appropriate. One patient's unstable Hb was identified as Hb Köln by determination of its primary structure (9, 10).

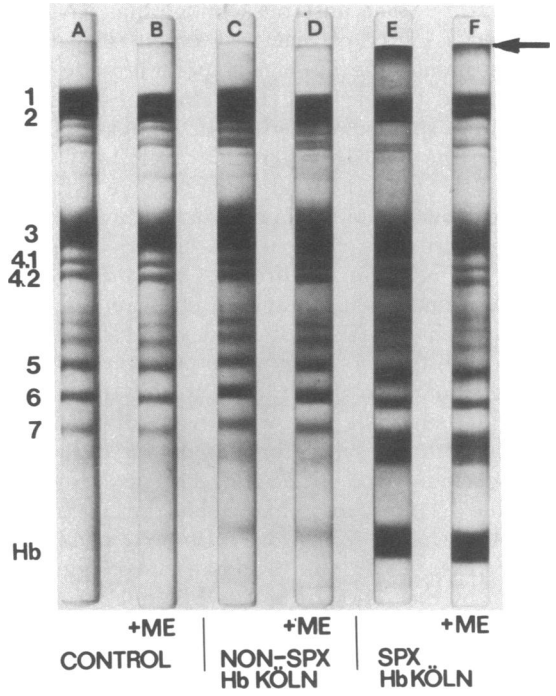

FIGURE 1 SDS-PAGE of erythrocyte membranes of control (A and B), non-SPX Hb Köln patient (C and D) and SPX Hb Köln patient (E and F). Gels A, C, and E were run in the absence of ME, and gels B, D, and F in the presence of ME. High molecular weight polypeptide aggregates are indicated by the arrow and are shown to be only partially dissociable by ME. Bands are numbered according to Steck (28). The heavy bands just below band 7 and at Hb on gels E and F were shown to be mono- and dimeric globin by simultaneous electrophoresis with known globin fractions.

Studies on intact erythrocytes. Complete blood counts, reticulocyte counts, and Heinz body determinations were performed by standard methods. Erythrocyte reduced glutathione levels were determined by the method of Beutler (11). MDA generation from membrane lipids by H_2O_2 stress was analyzed according to Stocks et al. (12). Erythrocyte calcium was measured on three times washed erythrocytes by Dr. John Eaton using atomic absorption spectroscopy (13, 14). Erythrocyte micropipette deformability was measured on cells washed three times in Hanks' buffered saline as before (6).

Studies on erythrocyte membrane proteins. Erythrocyte membranes were prepared in a cold room at 0°–4°C with methods described by Dodge et al. (15) and Fairbanks et al. (16). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) was performed by the method of Fairbanks et al. (16). Protein was measured (17) before electrophoresis or gel filtration chromatography; membrane samples were heated in 1% SDS at 100°C for 2 min with or without 1% mercaptoethanol (ME). Polypeptide aggregates at the origin of the gel were estimated by planimetry in terms of the total membrane proteins present on the gels. A correction for the meniscus of the gels was used. Membrane polypeptide aggregates were also analyzed and isolated by a 2 × 80-cm gel filtration column of Biogel A50m (Bio-Rad Laboratories, Richmond, CA) developed with 0.01 M phosphate buffer pH 7.0 with 0.1% SDS and 0.2% sodium azide. The same column was used to prepare spectrin.

SDS-PAGE using gels containing 4% acrylamide was performed on patient membranes without the addition of ME. One of each set of duplicate gels was stored at −70°C while the other was stained with Coomassie Blue to assess the presence of high molecular weight aggregates at the origin. A 2–3-mm slice was then taken from the top of the unstained gels known to contain aggregates at the origin, immersed in electrophoresis buffer containing 1% ME, heated to 100°C for 2 min and allowed to stand at room temperature for 2 h. The slice was then placed atop a gel containing 5% acrylamide and electrophoresis carried out.

Nondissociable high molecular weight polypeptide aggregates were isolated from erythrocyte membranes by gel filtration chromatography (as above) in the presence of ME. These fractions were then dialyzed against H_2O, the protein hydrolyzed 24 h in 6 N HCl and dried in a rotary still. After addition of β-alanine as an internal standard, the dried hydrolysate was dissolved in H_2O, saturated with sodium bicarbonate and reacted with {3,5-^{3}H}1-fluoro-2,4 dinitrobenzene (24 Ci/mM, New England Nuclear, Boston, MA) diluted to 0.15 mCi/mM with carrier. The resulting dinitrophenyl amino acids were separated by thin-layer chromatography using silica gel plates according to Brenner et al. (18) and quantitated by scraping the appropriate dinitrophenyl amino acids located by UV photography and determining radioactivity on a scintillation counter. Amino acid composition was calculated using dinitrophenyl β-alanine as an internal standard and, for ease of comparison with literature results, was expressed as moles percent of the total amino acids known to be present, not just those amino acids measured by this technique.

Studies on erythrocyte membrane lipids. Erythrocyte membrane lipids were extracted in duplicate with isopropanol/chloroform (3:2) by the method of Rose and Oklander (19). The final extract was filtered through glass wool and the fluorescence excitation and emission spectra obtained at room temperature on an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, MD), standardized with quinine sulfate (1 μg/ml 0.1 N H_2SO_4). The slit arrangement for recording fluorescence spectra was slits 3, 4, and 6, set at 3, 1, and 3 mm, respectively. Fluorescence of simultaneously prepared isopropanol/chloroform blanks was subtracted from erythrocyte extract readings, and corrections were made for variations in

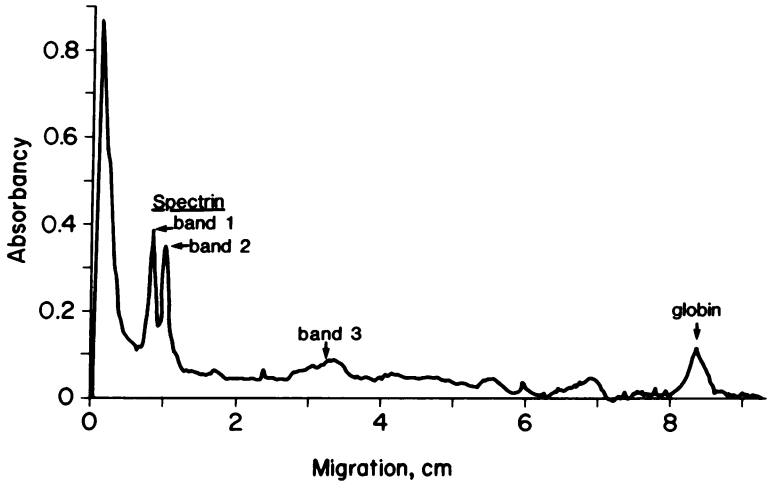

FIGURE 2 Densitometric scan of polypeptide aggregates treated with ME. Gel top slice containing high molecular weight aggregates run without ME (such as from gel E, Fig. 1), was soaked and heated in ME, placed atop another gel and electrophoresis performed. A densitometric scan of one such Coomassie blue-stained gel is shown. The spectrin, band 3, and globin dissociated from the aggregates by sulfhydryl reduction are indicated. Also note the significant amount of polypeptide remaining at the origin despite sulfhydryl reduction.

quinine sulfate fluorescence due to fluctuations in xenon lamp intensity. An aliquot of each erythrocyte extract was submitted to washing and lipid phosphorus determinations by the method of Chen et al. (20). Fluorescence was recorded in arbitrary units based on the above instrument settings as the peak at an excitation wavelength of 400 nm and emission wavelength of 460 nm. Results were then expressed as fluorescence units per microgram of lipid phosphorus in the extract.

Standard statistical methods were used throughout, with results expressed as mean±standard deviation, and the *t* test was used to determine the significance of the difference between means (21).

RESULTS

All six family members studied with Hb Köln had compensated hemolysis (Hb 13.4±0.4 g/dl) with persistent reticulocytosis, SPX: 6.0±1.1%; non-SPX: 10.4±0.4%. Heinz bodies were seen in a significant percentage of erythrocytes only in the SPX patients (58±3%) and were associated with decreased erythrocyte reduced glutathione (Table I). No increase in erythrocyte calcium was present in either the SPX or non-SPX patients (Table I).

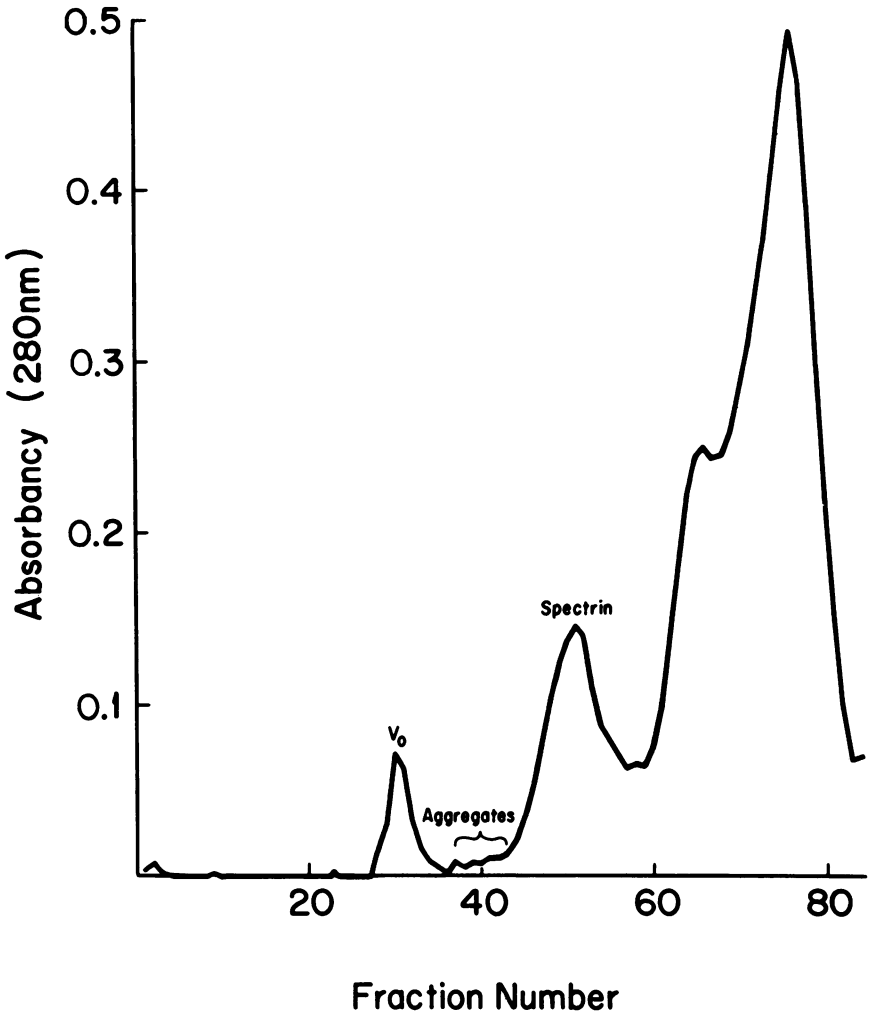

FIGURE 3 Gel filtration chromatography on a 2 × 80-cm column of Biogel A50m of 20 mg of erythrocyte membranes from an SPX patient with Hb Köln disease. The membranes were reduced before analysis (1% SDS, 1% ME, 100°C, 2 min). The column profile plots absorbancy at 280 nm vs. fraction number. Note the presence in the exclusion volume (Vo) of 280 nm absorbing material that was found not to react with either Coomassie blue stain for protein (Fig. 4) or with the Lowry et al. (17) protein method and may be related to material earlier observed by Lux and John (29).

A typical analysis by SDS-PAGE of unreduced and reduced erythrocyte membrane protein samples from control, non-SPX, and SPX Hb Köln patients is shown in Fig. 1. High molecular weight polypeptide aggregates are present at the origin of the gels in SPX patients (Fig. 1). These aggregates were not seen in the membranes of normal control (A, B) or in non-SPX patients (C, D), whether treated with ME or not. In the SPX patients (E, F) a portion of the aggregates was seen to dissociate when the membranes were heated with ME but a significant amount of high molecular weight protein material (hereafter referred to as "nondissociable aggregates") remained despite such sulfhydryl reduction (F).

The components of the high molecular weight aggregates solubilized by disulfide bond reduction were found to be mainly spectrin, band 3, and globin, as shown in the densitometric scan (Fig. 2). Characteristic peaks for spectrin (bands 1 and 2), band 3, and globin were identified by simultaneous electrophoresis with known proteins. These scans also confirmed that a significant amount of the high molecular weight aggregates was not dissociable by sulfhydryl reduction and remained at the origin of the gel (Fig. 2).

The aggregates were also isolated and quantitated by gel filtration chromatography using Biogel A50m (Fig. 3). The aggregates made up 1.2% of the total membrane protein and one-third of these were nondissociable (Table I). Fig. 4 demonstrates the correspondence in relative migration of the high molecular weight aggregates on SDS-PAGE to elution of the column and demonstrates that the apparent high molecular weight of these peptides is not an artefact of overloading either analytic technique, since the amounts analyzed are well within the capacity of the methods. Neither dissociable or nondissociable aggregates were seen in SPX patients with other chronic hemolytic disorders, namely hereditary spherocytosis and pyruvate kinase deficiency.

The composition of the nondissociable aggregates was studied further by amino acid analysis after isolation on gel filtration columns (for example, fractions 37–43, Fig. 3). Amino acid analysis was performed on the aggregate fractions of erythrocyte membranes from patients with Hb Köln disease, on normal spectrin, and erythrocyte membranes (Table II). Note that the aggregates showed no similarity to Hb (Table II).

Membrane lipid peroxidation was detected in both SPX and non-SPX patients. Fluorescence spectra of chloroform/isopropanol lipid extracts of fresh patient erythrocytes showed peaks at an excitation wavelength of 400 nm, and an emission wavelength of 460 nm. Typical examples of such spectra are shown in Fig. 5, which indicate the presence of Schiff base-type condensation products that are seen when lipid peroxidation occurs (8). Comparing these peaks of fluorescence in units per microgram of lipid phosphorus, significantly more of these fluorescent products were found in both SPX and non-SPX patients compared with controls ($P < 0.005$) (Fig. 6). Two additional observations suggest that these spectra were characteristic of lipid extracts of fresh patient erythrocytes and not due to in vitro changes, or precipitated unstable Hb. Storage of the isolated patient membranes, or the extracts did not increase the intensity of the fluorescence spectra of lipid extracts. The fluorescence spec-

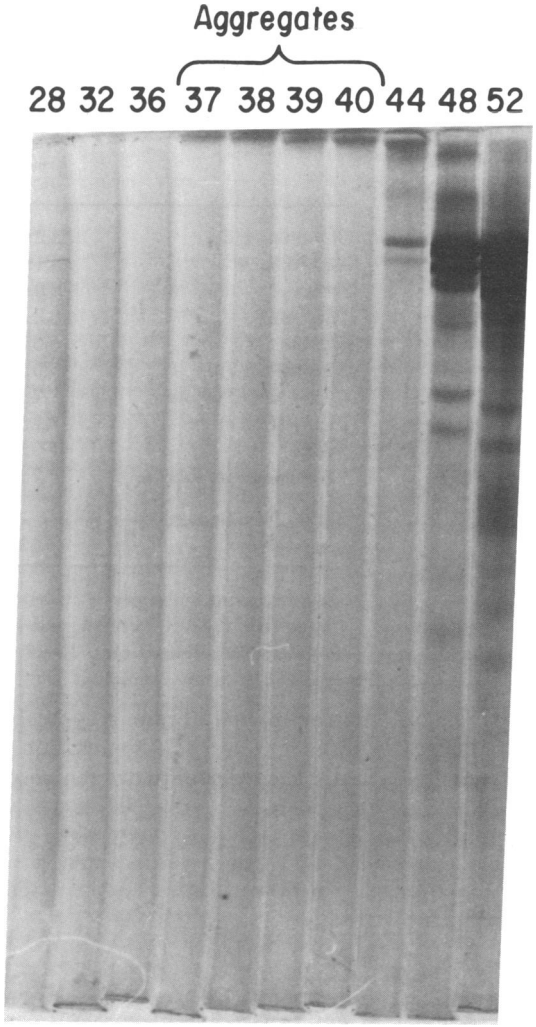

FIGURE 4 SDS-PAGE of effluent fractions of the gel filtration chromatogram shown in Fig. 3. Note the presence of high molecular weight aggregates, partially separated from spectrin in fractions 37–44. No Coomassie blue-staining material was found in the exclusion volume (fractions 28–32).

TABLE II

Comparison of the Amino Acid Composition of Nondissociable Aggregates from Erythrocyte Membranes of Hb Köln Disease with Normal Spectrin and Normal Erythrocyte Membranes

	Erythrocyte membranes (n = 2)	Hb Köln aggregates (n = 4)	Spectrin (n = 3)	Hb*
Glutamic acid	12.2±0.03	14.9±1.0	19.8±0.8	5.6
Aspartic acid	8.1±0.6	9.4±0.2	10.7±1.4	8.7
Serine	6.0±0.3	8.3±0.9	3.7±0.1	5.6
Threonine	6.2±0.6	5.8±0.4	4.5±0.9	5.6
Glycine	6.6±0.1	8.0±0.8	5.6±0.3	7.0
Alanine	8.0±0.7	7.9±0.3	11.0±2.1	12.5
Lysine and tyrosine	7.7±0.3	7.0±0.3	7.9±0.5	9.7
Phenylalanine	4.2±0.3	3.5±0.04	3.2±0.2	5.2
Valine	6.9±0.5	6.0±0.3	5.9±0.2	10.8
Leucine and isoleucine	17.7±0.8	16.6±1.2	17.7±1.2	12.5
Total of amino acids analyzed	83.2	87.3	87.3	83.2

Data represent moles percent±standard deviation.

* Bunn, H. F., B. G. Forget, and H. M. Ranney. 1977. Human Hemoglobins. W. B. Saunders, Co., Philadelphia. 15–17.

trum of the lipid extract of membrane-free heat-precipitated Hb Köln was negative. MDA generation from membrane lipids under the stress of hydrogen peroxide in vitro was also assessed. These results paralleled the fluorescence results, with significantly increased MDA generated in both SPX and non-SPX patient cells compared with controls ($P < 0.005$) (Fig. 7).

The results of a typical experiment comparing the micropipette deformability of control erythrocytes, and erythrocytes from two non-SPX siblings and their SPX mother, all with Hb Köln disease, are shown in Fig. 8. In this experiment 15 erythrocytes from each subject were subjected to 10-cm H_2O negative pressure using a pipette of 0.9-μm i.d., and the micrometers of tongue extension measured. As can be seen, the erythrocyte membranes from the SPX Hb Köln patient are

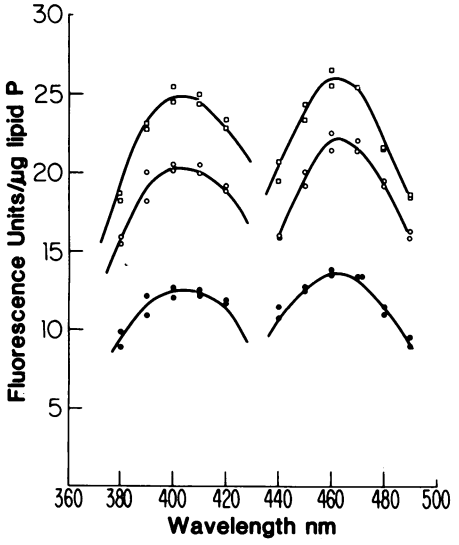

FIGURE 5 Fluorescence spectra of chloroform/isopropanol extracts of fresh erythrocytes. Results are expressed as arbitrary fluorescence units per microgram lipid phosphorus in the extract. One fluorescence unit is equivalent to that produced by 0.1 ng quinine sulfate/ml 0.1 N H_2SO_4. Spectra of representative duplicate extracts from normal control (●), SPX (○), and non-SPX (□) unstable Hb patients are shown.

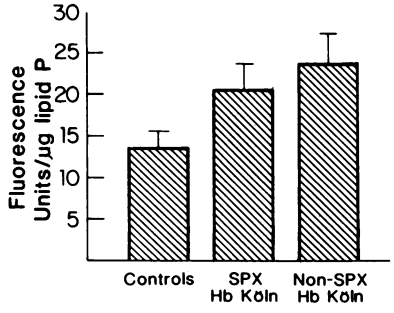

FIGURE 6 Fluorescence in lipid extracts of fresh erythrocytes. Units are expressed as in Fig. 5. Results are shown as mean and standard deviation of peak fluorescence (excitation 400 nm, emission 460 nm). Fluorescence in patient's samples is significantly greater than in controls ($P < 0.005$).

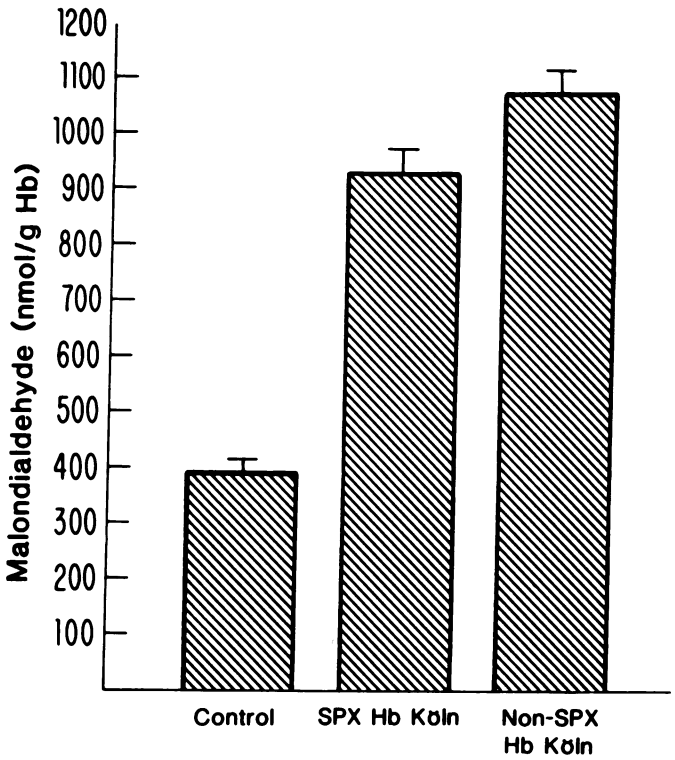

FIGURE 7 MDA generation by H_2O_2 stress. Erythrocytes were stressed in vitro with exogenous H_2O_2 and the resulting MDA assayed. Patient erythrocytes generated significantly more MDA than controls ($P < 0.005$). Abbreviations and symbols are as in Fig. 5.

significantly less deformable than the control erythrocytes, but those of the non-SPX patients are not significantly different from controls.

DISCUSSION

The present studies provide evidence that oxidative damage to both lipids and proteins of the erythrocyte membrane occurs in unstable Hb disease. Depletion of membrane thiols has been previously reported and ascribed to reaction with Heinz bodies (4). In the studies reported here, the membrane damage is detectable in freshly obtained erythrocytes not otherwise oxidatively stressed, and thus likely results from the oxidant-generating capacity of the unstable Hb.

Membrane lipid peroxidation was detected by the presence of increased fluorescence in lipid extracts. Such lipid extractable fluorescence has been shown by Dillard and Tappel (22) to be 10–100 times more sensitive than direct MDA assay as a measure of lipid peroxidation. The major species responsible for this fluorescence appears to be conjugated Schiff base fluorophores formed by cross-linking two primary amines with MDA. In lipid extracts the amino groups would obviously be those of aminophospholipids, but cross-links could also form between amino groups of peptides. Jain and Hochstein (8) have shown that exposing erythrocytes to MDA in vitro produces both fluorescent chromolipids and protein cross-links not dissociable by sulfhydryl reduction. Our patients' membranes had significantly increased amounts of these fluorescent species compared with normals, whether the spleen was intact or had been removed.

In addition to the lipid peroxidation, two types of high molecular weight polypeptide aggregates were seen in the erythrocyte membranes, but only in SPX family members. Two-thirds of these high molecular weight aggregates were dissociable by sulfhydryl reduction analogous to those in chronic hemolytic G6PD mutants (5, 6). Also, like these G6PD mutants, the presence of the disulfide-bonded aggregates was associated with decreased levels of erythrocyte reduced glutathione. However, in unstable Hb disease the components of the disulfide-bonded peptide material were different from that found in G6PD mutants in that globin was a principal constituent. These observations

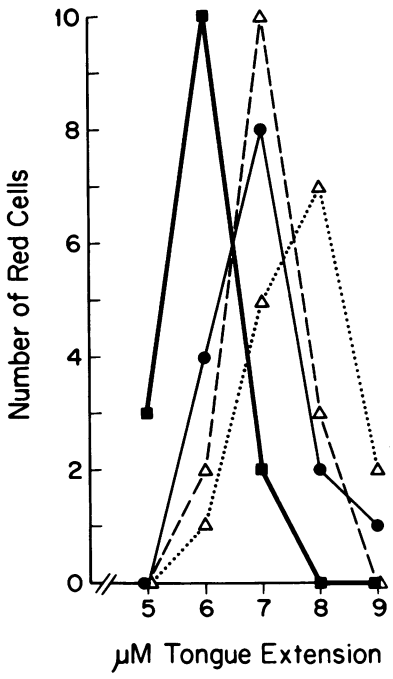

FIGURE 8 Distribution of micropipette deformability in erythrocytes from a control (-●-), two non-SPX Hb Köln patients (-△-) (... △ ...), and one SPX patient (-■-). The number of erythrocytes with a given membrane deformability is plotted against that deformability (micrometers tongue extension±0.5 μm at −10 cm H_2O). The erythrocytes from the SPX patient are significantly less deformable than control erythrocytes ($P < 0.005$), but those of the non-SPX patients are not. Other experiments with slightly different pipette size and negative pressures gave the same results.

are consistent with the earlier concept of disulfide binding of Heinz bodies to the membrane (4), and more recent evidence that Hb is reversibly bound to band 3 (23), to which it can become disulfide-linked by oxidation (24).

These disulfied-bonded aggregates were accompanied by additional high molecular weight polypeptides that remained after sulfhydryl reduction and are likely cross-linked by MDA. At neutral pH, MDA rapidly polymerizes to form longer bifunctional aldehydes, with actually increased capacity for cross-linking and denaturing protein (25). When normal erythrocyte membranes are briefly treated with fresh MDA (26) only spectrin appears to be polymerized, but with incubated MDA more and more membrane polypeptides become included.[2] The amino acid composition demonstrates that these aggregates cannot be aggregated Hb, and the composition resembles aggregated spectrin or erythrocyte membranes.

It is likely significant that both membrane polypeptide aggregates and decreased erythrocyte membrane deformability were detected only in cells from SPX patients. We have shown in an in vivo model system in dogs that erythrocytes with diamide-induced membrane polypeptide aggregates and decreased deformability are preferentially removed from the circulation and sequestered in the spleen (7). Further, our patients' erythrocyte membranes had sustained damage in the form of nondissociable aggregates. The action of MDA on erythrocytes, which produces similar aggregates, has recently been shown to produce significant alterations in erythrocyte deformability with all evidence pointing to effects on the cell membrane as the mechanism (26). Since each of the lesions we describe taken separately can alter erythrocyte deformability, their combination is likely of even greater significance as evidenced by the decreased deformability of our SPX patients' erythrocytes.

Removing the spleen in unstable Hb disease makes the erythrocyte membrane defect and decreased deformability apparent. The modest decrease in reticulocytosis that occurs with splenectomy (6 vs. 10% for non-SPX patients) illustrates the limited value of removal of a prime locus of Heinz body pitting (27) and, we suspect, a likely site also for removal of erythrocytes with cross-linked membrane skeletons (7). Determination of the relative importance of splenic removal of Heinz bodies or stiff membrane skeletons in the hemolytic process will require further investigation.

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