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The role of membrane protein sulfhydryl groups in hydrogen peroxide-mediated membrane damage in human erythrocytes

L.M. Snyder [a], N.L. Fortier [a], L. Leb [a], J. McKenney [a], J. Trainor [a], H. Sheerin [b] and N. Mohandas [c]

[a] *St. Vincent Hospital, Worcester, MA,* [b] *Worcester Foundation for Experimental Biology, Shrewsbury, MA and* [c] *University of California, San Francisco, CA (U.S.A.)*

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The formation of spectrin-hemoglobin complex following treatment of red cells with hydrogen peroxide (H_2O_2) has previously been shown to be associated with alterations in cell shape, decreased membrane deformability and increased recognition of modified cells by anti-IgM immunoglobulin in a phagocytic assay by monocytes. Prior treatment with carbon monoxide completely inhibited the H_2O_2-associated membrane changes, indicating a role for oxidized hemoglobin in the complex formation. Also, in a cell-free system, blockage of sulfhydryl (SH) groups on purified spectrin by *N*-ethylmaleimide significantly reduced the complex formation, suggesting a role for SH groups of spectrin in crosslinking process. The present study was undertaken to examine the role of SH blockade by *N*-ethylmaleimide on intact red cells undergoing oxidative damage. Pretreatment of erythrocytes with *N*-ethylmaleimide at concentrations ranging from 0.1 to 0.2 mM resulted in decreased lipid peroxidation and spectrin hemoglobin crosslinking. Moreover, pretreatment with *N*-ethylmaleimide resulted in less marked alterations in cell shape and membrane deformability as well as reduced recognition of peroxidized cells by antiglobulin serum. *N*-Ethylmaleimide treatment had no effect on methemoglobin formation. Studies with ^{14}C-labeled *N*-ethylmaleimide showed that over 50% of *N*-ethylmaleimide was incorporated into spectrin. Pretreatment of cells with higher concentrations of *N*-ethylmaleimide (over 0.2 mM) was associated with membrane dysfunction independent of H_2O_2. These results imply that blocking of reactive SH groups leads to reduced interaction of spectrin with oxidized globin. These data, along with our prior observations, indicate that SH groups on spectrin play an important role in hemoglobin oxidation-induced formation of spectrin-hemoglobin complex and the resultant deleterious effects on membrane properties.

Introduction

Heightened sensitivity to oxidant damage has been demonstrated in certain hereditary red cell disorders, such as sickle cell disease and other hemoglobinopathies [1], glucose-6-phosphate dehydrogenase deficiency [2], hereditary xerocytosis [3] and vitamin E deficiency [4]. Functional alterations of the red cell membranes are manifested as result of this increased oxidant damage. A membrane skeleton system consisting of a structural matrix of spectrin-actin, band 4.9 and band 4.1 linked to band 3 in the lipid bilayer through ankyrin (band 2.1) appears to be responsible for

Abbreviations: DI, deformability index; DATD, *N,N*-diallyltartardiamide.

Correspondence: L.M. Snyder, Division of Laboratory Medicine, St. Vincent Hospital, 25 Winthrop St., Worcester, MA 01604, U.S.A.

many of the mechanical properties of the red cell membrane [5]. Various specific skeletal protein interaction have been identified as being important in regulating membrane mechanical properties of deformability and mechanical stability [6].

Recent studies have demonstrated deleterious effects of oxidant drugs and sulfhydryl-reaction agents on membrane function in red cells. *N*-ethylmaleimide has been shown to affect spectrin-ankyrin interaction [7] and to decrease spectrin self-association, resulting in membrane instability [8]. The bifunctional thiol reagent diamide has been demonstrated to alter membrane lipid asymmetry [9] and membrane deformability [10] and to cause a decrease in vivo red cell survival [11]. Recently, Becker et al. [12] studied the effect of diamide on purified spectrin and showed that oxidized spectrin exhibits decreased ability to interact with band 4.1. We have previously shown covalent crosslinking of hemoglobin to spectrin following treatment of human red cells with hydrogen peroxide (H_2O_2) [13,14]. These modified cells underwent echinocytic transformation and exhibited decreased membrane deformability and increased interaction with antiglobulin serum [14]. Prior treatment with carbon monoxide, but not with the antioxidant butylated hydroxytoluene, totally inhibited these membrane changes, demonstrating a role of hemoglobin oxidation in the crosslinking reaction and membrane functional alterations [14]. While the protein complex, once produced, could not be reduced by various reducing agents, its formation could be inhibited in a cell free system by pretreatment of spectrin, but not hemoglobin, with the SH group blocking reagent *N*-ethylmaleimide [13]. The present study was thus undertaken to investigate the ability of varying concentrations of *N*-ethylmaleimide in intact red cells to (1) block the oxidative formation of spectrin/hemoglobin complex and (2) to determine whether *N*-ethylmaleimide can thus protect red cell from undergoing various membrane alterations induced by in vitro peroxidation.

Materials and Methods

Preparation of erythrocyte suspension

Normal human erythrocyte suspensions were prepared from heparinized fresh human blood obtained from laboratory volunteers. After centrifugation to remove the plasma and leukocytes, the erythrocytes were washed three times in a 10 mM phosphate-buffered saline (pH 7.4) enriched with 5 mM glucose. The washed, packed erythrocytes were adjusted to a hematocrit of 10% in glucose-enriched phosphate-buffered saline and incubated with varying concentrations (0.1–0.5 mM) of *N*-ethylmaleimide or ^{14}C-labeled *N*-ethylmaleimide for 15 min at 37°C in a shaking water bath. The red cells were then washed three times in the same buffer and incubated with 5 mM Dithioerythritol for additional 15 min to remove non-reacting *N*-ethylmaleimide. The cells were washed an additional three times in buffer and adjusted to an hematocrit of 20% in glucose-enriched phosphate-buffered saline (pH 7.4) containing 1 mM sodium azide. Hydrogen peroxide was added to the cell suspension to a final concentration from 315 to 810 μM. The cell suspension was incubated at 37°C for 10 min. ^{14}C-labeled *N*-ethylmaleimide solution of known specific activity was made by diluting ^{14}C-labeled *N*-ethylmaleimide (3.7 mCi/mmol) with phosphate-buffered saline buffer to yield a stock solution of 0.1 mM. 0.05 ml of this solution yielded $1.1 \cdot 10^6$ CMP.

Chemical determinations

Methemoglobin and erythrocyte indices and red cell GSH levels were determined by standard procedures [14,15]. The extent of lipid peroxidation was quantified by measuring the fluorescent amino immunopropene derivatives as described by Goldstein et al. [16], using a scaled-down version of the Rose and Oklander [17] chloroform/isopropanol extraction procedure, using 0.05 ml of washed, packed red cells. The fluorescent derivatives were measured in an Amico Bowman spectrofluorometer (American Instrument Co., Silver Springs, MD) at 25°C with an excitation maximum at 360 nm and emission at 440 nm. Fluorescence was recorded in arbitrary units at these settings. The reading obtained with 10^{-8} M quinine sulfate in H_2SO_4 was set as 50 fluorescent units at standard instrument settings.

Electrophoretic analysis of membrane proteins

The membrane ghosts were prepared by hypo-

tonic hemolysis as described previously [14]. Equal amounts of SDS-solubilized membrane proteins approx. 10 μg, determined by the method of Lowry et al. [35] obtained from a defined number of ghosts were run on cylindrical 4% polyacrylamide gels as previously described [14]. In the case of ^{14}C-labeled *N*-ethylmaleimide-treated cells, 0.5% *N,N*-diallyltartardiamide (DATD) was used in place of *N,N'*-methylenebisacrylamide. The percentage of spectrin complex was calculated by integrating the Coomassie blue stain profile on the densitometric scans as previously described [14].

The reaction of ^{14}C-labeled N-ethylmaleimide with normal human red cells

SDS-solubilized ghost membranes prepared from ^{14}C-labeled *N*-ethylmaleimide-treated red cells were run on maximally reduced cylindrical DATD SDS-polyacrylamide gels (approx. 10 μg/gel) as described above. After electrophoresis, the gels were stained with Coomassie blue and destained to remove any non-covalent bound *N*-ethylmaleimide from the gels. The various stained membrane protein polypeptides were also sampled to be used as background controls. Each portion of the gel was placed in a scintillation vial and 1 ml of 0.5% (w/v) periodic acid was added and incubated either at 50 °C for 1 h or at room temperature overnight. To each vial an additional 50 μl of ethanolamine were added and 4 ml of Aquosol-2 (New England Nuclear) counting medium were also added to each vial. Vials which were not completely clear were discarded. Radioactivity of each slice was determined in a scintillation counter with an efficiency of 85%, and the counts per minute per polypeptide band were determined and plotted. Quenching of radioactivity by either coomassie blue stain or protein was ruled out. In some experiments, 0.15 mM *N*-ethylmaleimide was added to red cells prior to oxidation with 315 μM hydrogen peroxide. In other studies, peroxidation occurred prior to the addition of ^{14}C-labeled *N*-ethylmaleimide. Approx. 5–10% of the initial radioactivity was covalently bound to various membrane polypeptides.

Western blot analysis using anti-human hemoglobin antibody

The red cell membrane proteins from red blood cells treated with *N*-ethylmaleimide and H_2O_2 were separated by SDS-polyacrylamide gel electrophoresis on 4% acrylamide gels by the procedure of Fairbanks [18] or on 7% acrylamide gels by the procedure of Laemli [19]. The electrophoretic conditions for the transfer of protein from the SDS gels to nitrocellulose were from Towbin et al. [20]. The transfer buffer consisted of 25 mM Tris base-192 mM glycine/20% (v) methanol at pH 8.3. Transfers were performed in a Bio-Rad Trans-Blot apparatus for 5–18 h at constant amount of 110 mAmps and 60 V at room temperature without cooling.

The protein blots were made using nitrocellulose with a pore size of 0.45 or 0.20 μM (BA-85, BA-83; Schleicher and Schuell). Immediately after protein transfer, the nitrocellulose paper was air dried. One of two halves of the paper with the same protein samples were either stained with 0.1% amido black (naphthol blue black, C.I. 20470 Allied Chemical) or developed utilizing a modified version of the immunostaining procedure described by King et al. [21].

Hemoglobin crosslinked skeletal proteins were detected using affinity purified rabbit anti-human Hb polyclonal IgG antibodies, a gift from Dr. F. Garver, Medical College of Georgia.

Spectrin dimer extraction and oxidation with hemoglobin

Spectrin dimer was extracted from white ghosts and purified by sucrose zonal density gradient as previously described [22]. Purified spectrin at a concentration of 1 mg/ml was incubated with various concentrations of *N*-ethylmaleimide ranging from 0.15 to 0.30 mM for 15 min, in a low-ionic-strength lysing buffer. In order to remove non-reactive *N*-ethylmaleimide, the extracts were dialyzed extensively with frequent changes of low-ionic-strength buffer. The *N*-ethylmaleimide-treated spectrin was then mixed with 3 μM hemoglobin and varying amounts of hydrogen peroxide. The reaction mixtures were solubilized in SDS and run on 4% SDS-polyacrylamide gels and the presence of spectrin hemoglobin crosslinking was determined [22].

Scanning electron microscopy for morphology

Erythrocyte morphology was determined by the

method described previously by Snyder et al. [14]. 500 cells were counted at random and the present of erythrocytes with echinocyte morphology was determined for each *N*-ethylmaleimide concentration and peroxide concentration used.

Deformability measurements

The deformability of intact erythrocytes and resealed erythrocyte ghosts was quantitated using an ektacytometer. This device imposes a well-defined laminar shield stress field on the cells, while simultaneously monitoring the extent of cell deformation by laser diffractometry. A deformability index (DI) is obtained which is equivalent to the ellipticity of the deforming red cells [23,24]. In the standard mode of operation, DI is recorded continuously as a function of shear rate. For measurement of intact erythrocyte suspension were thoroughly mixed with 3 ml of poly(vinyl pyrolindone) (molecular weight, 360000; 4 g/dl weight per volume; 23.6 cP (centipoise) at 20°C; 290 mosmol/kg; pH 7.4). This suspension produced a maximum stress of 170 dyn/cm^2 at 100 rpm. Resealed membranes for deformability measurements were prepared by a procedure [25] adopted from Johnson [26]. Blood from normal volunteers and patients were washed three times in 5 mM Tris and 150 mM NaCl (pH. 7.4). The erythrocytes were then lysed in 40 vol. of 7 mM NaCl and 5 mM Tris (pH 7.4). The membranes were pelleted by centrifugation, resuspended in 10 vol. of 5 mM Tris and 140 mM NaCl (pH 7.4) and incubated for 60 min at 37°C for resealing. Subsequent centrifugation of resealed membranes produced a concentrated ghost suspension for membrane deformability measurements.

To measure membrane deformability 30 μl of packed resealed membranes were suspended in 3 ml of stractan II (290 mosmol, 22 cP, pH 7.40 and examined by ektacytometry, a laser diffraction method previously described [27]. In brief, suspended cells are exposed to an increasing shear stress (0–125 dyn/cm^2) and the change in their laser diffraction pattern from circle to ellipse measured. This photometric measurement produces a signal designated deformability index (DI) which quantitates cell ellipticity. By an automatic image analysis system, the DI is recorded as a continuous function of applied shear stress. For resealed membranes the shear stress required to obtain a defined value of DI is determined by the property membrane deformability, without contributions from either internal viscosity or cell geometry [25]. This is a correlation between changes in deformability measured by this technique and those measured using micropipette [27]. Analysis of the DI curve generated by the ektocytometer thus provides a measure of membrane deformability.

Assay of erythrocyte–monocyte interaction

Mononuclear leukocytes obtained by Ficoll-Hypaque sedimentation [28] were washed and brought to a concentration of 2–20 monocytes/ml complete medium (RPMI-1640, GIBCO, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. A 0.5-ml aliquot was placed on 13-mm diameter glass cover slips in 16-mm diameter wells (Linbro, Titer Tech, VA). After incubation at 37°C for 120 min in a moist incubator with 5% CO_2, the non-adherent cells were removed by extensive washing with phosphate-buffered saline, the adherent monocytes were covered with 0.5 ml of complete medium and used in the phagocytic assay.

Heparinized group O-Rh$^+$ red blood cells were opsonized with 1 : 20 dilution of anti-Rh (D), and used as positive controls for monocyte Fc receptors. The *N*-ethylmaleimide/peroxide-treated erythrocytes adjusted to 5×10^8 cells/ml in phosphate-buffered saline were opsonized with rabbit polyspecific antihuman globulin (Ortho Diagnostics, Piscataway, NJ), for 45 min at 37°C. The cells were washed and suspended at a final concentration of 2×10^8 cells/ml in complete medium and used in the phagocytic assay. Negative controls were untreated, non-opsonized and treated non-opsonized red blood cells.

Monolayers of monocytes incubated with varying red blood cell preparations at a ratio of 50 red blood cells/monocytes for 60 min at 37°C were washed twice with Hanks' balanced saline solution, fixed with 1.25% glutaraldehyde in phosphate-buffered saline and stained with Wright-Giemsa stain. 500 monocytes were counted at random on each slide, and the results are presented at % monocytes ingesting red cells.

Results

The effect of N-ethylmaleimide on methemoglobin formation, lipid peroxidation, hemoglobin spectrin crosslinking and GSH levels

Pretreatment of normal red cells with 0.1–0.25 mM *N*-ethylmaleimide had no significant effect on the formation of methemoglobin, lipid peroxidation or spectrin–hemoglobin crosslinking (Table I). However, when red cells pretreated with *N*-ethylmaleimide were exposed to 540 μM hydrogen peroxide, a *N*-ethylmaleimide dose-dependent decrease in spectrin hemoglobin crosslinking occurred (Table I). Pretreatment with *N*-ethylmaleimide also decreased the amount of lipid peroxidation induced by H_2O_2, but had no significant effect on methemoglobin formation (Table I). Spectrin hemoglobin crosslinking was detected as an additional band migrating at the trailing edge of the alpha chain of spectrin, with a molecular weight of approx. 255 000 (Fig. 1A). As demonstrated in Fig. 1A, a progressive decrease in high-molecular-weight complex is noted with increasing concentrations of *N*-ethylmaleimide in peroxide-treated red cells. In order to define further the inhibitory effect of *N*-ethylmaleimide on spectrin's ability to crosslink with hemoglobin, purified spectrin was then exposed to hemoglobin in an oxidizing state and the resultant sample analyzed. Fig. 1B and Table II demonstrate dose-dependent inhibitory effect of low concentrations of *N*-ethylmaleimide on spectrin's ability to form crosslinks with hemoglobin. Red cell GSH levels were not appreciably affected by short-term incubations

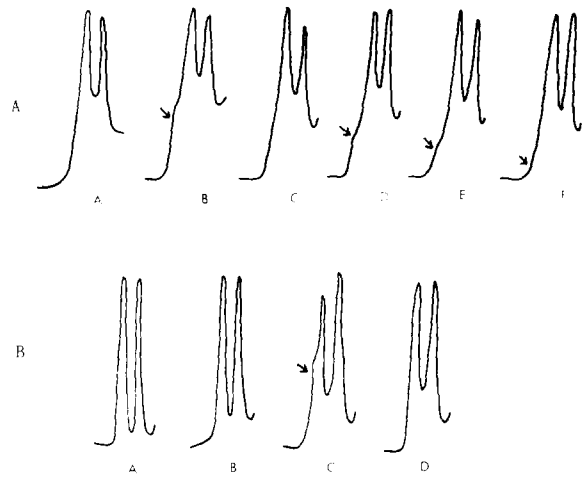

Fig. 1. (A) Densitometric scans of the spectrin region of SDS/4% polyacrylamide gel from *N*-ethylmaleimide and H_2O_2-treated erythrocytes. Arrow points to spectrin hemoglobin crosslinking. A, Control; B, 540 μM H_2O_2-treated cells; C, 0.25 mM *N*-ethylmaleimide control; D, 0.1 mM prior to 540 μM H_2O_2; E, 0.15 mM *N*-ethylmaleimide prior to H_2O_2; F, 0.25 mM *N*-ethylmaleimide prior to H_2O_2-treated cells. (B) Densitometric scans of isolated bands 1 and 2 of SDS/4% polyacrylamide gel from peroxide-treated partially purified spectrin (0.25 mg + 0.5 μM hemoglobin + 315 μM H_2O_2). A, spectrin control; B, 0.25 mM *N*-ethylmaleimide control; C, 315 μM H_2O_2; D, 0.15 mM *N*-ethylmaleimide prior to treatment of spectrin with hemoglobin and peroxide. Arrows point towards hemoglobin spectrin crosslinking.

TABLE I

EFFECT OF *N*-ETHYLMALEIMIDE PRETREATMENT ON METHEMOGLOBIN, LIPID PEROXIDATION AND SPECTRIN–HEMOGLOBIN CROSSLINKING FORMATION FOLLOWING H_2O_2 EXPOSURE IN NORMAL HUMAN ERYTHROCYTES

GSH = μmol of GSH/g hemoglobin, two separate studies. The arbitrary units represent fluorescent changes standardized with quinine sulfate. n.d., not performed.

Pretreatment *N*-ethylmaleimide (mM)	H_2O_2 (μM)	GSH	Methemoglobin (% ± S.D., $N = 4$)	Lipid peroxidation (arbitrary units, ± S.D., $N = 4$)	Spectrin–hemoglobin crosslink (% ± S.D., $N = 3$)
0.0	0	5.9, 5.4	0.4 ± 0.09	3.5 ± 0.4	0
0.15	0	1.9, 2.3	0.1 ± 0.02	4.0 ± 1.0	0
0.5	0	0, 0	0.25 ± 0.08	3.8 ± 0.7	0
0.0	540	5.5, 5.2	23.0 ± 3.2	11.5 ± 1.5	8.0 ± 1.2
0.1	540	n.d.	23 ± 4	5.5 ± 0.8	4.5 ± 0.8
0.15	540	1.8, 2.5	21 ± 6	6.5 ± 1.2	3.7 ± 0.4
0.2	540	1.3, 0.9	21 ± 3	6.0 ± 0.8	n.d.
0.25	540	0, 0	21 ± 3.5	6.0 ± 1.0	1.5 ± 0.08

TABLE II

EFFECT OF *N*-ETHYLMALEIMIDE PRETREATMENT ON ISOLATED SPECTRIN'S ABILITY TO CROSSLINK WITH HEMOGLOBIN FOLLOWING EXPOSURE TO H_2O_2

Pretreatment *N*-ethylmaleimide (mM)	H_2O_2 (μM)	Spectrin–hemoglobin crosslinking (%)
0.0	0	0
0.25	0	0
0.0	315	9.6
0.15	315	4.8
0.25	315	2.1
0.0	540	12.0
0.15	540	5.1
0.25	540	3.5

with H_2O_2; however, treatment with *N*-ethylmaleimide resulted in a dose-dependent decrease in GSH levels (Table 1).

Western blot analysis of spectrin-globin complex

The technique of Western blotting was used to detect covalent irreducible hemoglobin crosslinking to spectrin in hydrogen peroxide and *N*-ethylmaleimide-treated red cells. As shown in Figs. 2A′ C′, the hemoglobin antibody did not react with bands in the spectrin domain of the gel in control membranes, but showed reaction with trailing edge of band 1 on the oxidized sample (Fig. 2E′). This localization of hemoglobin is consistent with our prior protein analysis using SDS-polyacrylamide gel electrophoresis. In contrast, prior treatment with low doses of *N*-ethylmaleimide (0.15 or 0.25 mM) resulted in decrease of hemoglobin crosslinking with spectrin (Fig. 2B′, D′).

^{14}C-labeled N-ethylmaleimide incorporation into membrane proteins

In order to study the distribution of *N*-ethylmaleimide-reactive sites in membrane proteins, red cells were incubated with varying concentrations of ^{14}C-labeled *N*-ethylmaleimide (0.08–0.25 mM). Fig. 3A demonstrates that at low doses of *N*-ethylmaleimide (0.08 mM) a majority of the radioactivity appears in spectrin (band 1 greater than band 2), bands 2.1 and 4.2. Increasing the dose of ^{14}C-labeled *N*-ethylmaleimide not only results in an increase in the total amount of radioactivity associated with each of the above-mentioned proteins but labelling of additional polypeptides such as band 4.1 and band 3 (Fig. 3b and 3c). AT an *N*-ethylmaleimide concentration of 0.25 mM, band 5 is also labelled (Fig. 3C). Fig. 4 demonstrates that pretreatment of red cells with 0.15 mM *N*-ethylmaleimide prior to peroxidation results in a decrease in spectrin hemoglobin crosslinking (compare Fig. 4A and C) as well as ^{14}C-labeled *N*-ethylmaleimide radioactivity similar to that of non-oxidized control (Fig. 4B). Fig. 4C shows the

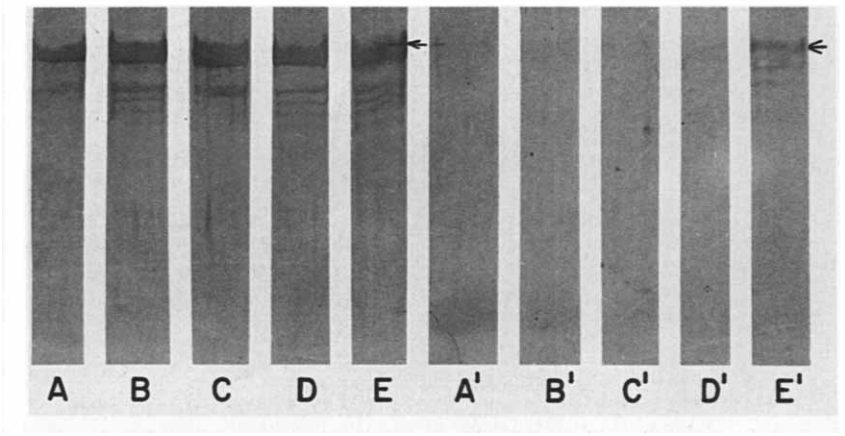

Fig. 2. Western blot of *N*-ethylmaleimide and H_2O_2-treated red blood cell membrane proteins. The blots were treated with alpha spectrin antibody (A–E) and adult hemoglobin antibody (A′–E′). Arrows indicate spectrin hemoglobin crosslinking. A, controls + 0.15 mM *N*-ethylmaleimide; B, controls + 0.15 mM *N*-ethylmaleimide prior to 485 μM H_2O_2; C, controls + 0.25 mM *N*-ethylmaleimide; D, control + 0.25 mM *N*-ethylmaleimide prior to 485 μM H_2O_2; E, controls + 485 μM H_2O_2.

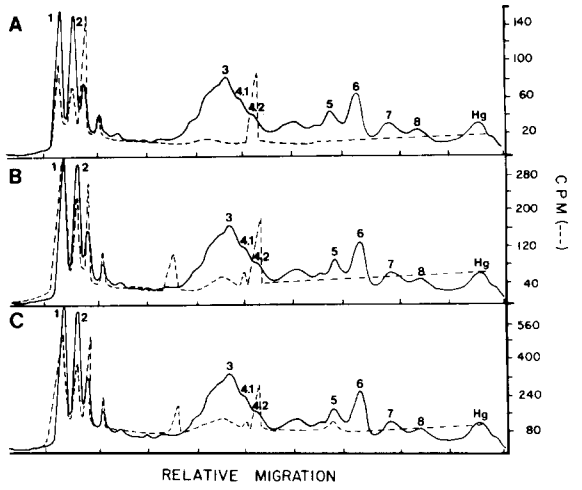

Fig. 3. Densitometric scans of SDS/5% polyacrylamide from ^{14}C-labeled *N*-ethylmaleimide-treated red cells. The ^{14}C-labeled *N*-ethylmaleimide distribution in red cell membrane proteins. ------, ^{14}C-labeled *N*-ethylmaleimide; ———, Coomassie blue. (A) 0.08 mM ^{14}C-labeled *N*-ethylmaleimide. (B) 0.15 mM ^{14}C-labeled *N*-ethylmaleimide. (C) 0.25 mM ^{14}C-labeled *N*-ethylmaleimide.

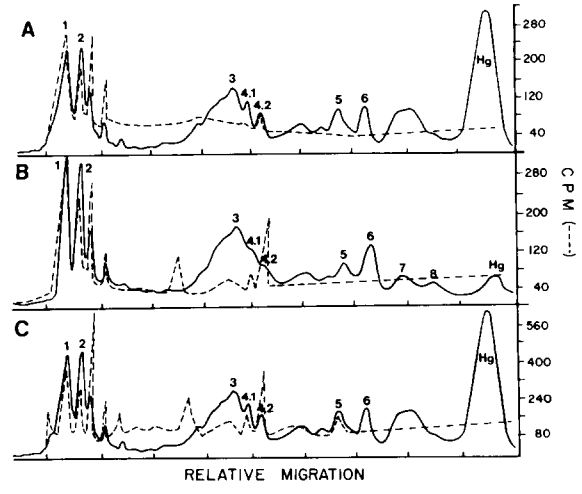

Fig. 4. Densitometric scans of SDS/5% polyacrylamide gel from ^{14}C-labeled *N*-ethylmaleimide-treated red cells. ^{14}C-labeled *N*-ethylmaleimide distribution in red cell membrane proteins treated with *N*-ethylmaleimide and hydrogen peroxide. ------, ^{14}C-labeled *N*-ethylmaleimide; ———, Coomassie blue. (A) 0.15 mM ^{14}C-labeled *N*-ethylmaleimide treated prior to oxidation with 315 μM H_2O_2. (B) 0.15 mM ^{14}C-labeled *N*-ethylmaleimide, control. (C) 315 μM H_2O_2 treatment prior to 0.15 mM ^{14}C-labeled *N*-ethylmaleimide incubation.

results obtained with red cells which were oxidized prior to exposure to 0.15 mM ^{14}C-labeled *N*-ethylmaleimide. In addition to the formation of spectrin hemoglobin complex the amount and distribution of ^{14}C-labeled *N*-ethylmaleimide among the different polypeptides in these oxidized cells was significantly altered. Increased incorporation of radioactivity into various polypeptide species as well as labelling of additional polypeptides, such as those at the trailing edge of band 1, the area between band 2.2 to 2.6 and the area between 4.5 and band 5 were observed.

Shape changes

Fig. 5 illustrates the morphology of erythrocytes after exposure to hydrogen peroxide with and without *N*-ethylmaleimide pretreatment. Approx. 20% of the cell population was echinocytic following treatment with 810 μM hydrogen peroxide. Pretreatment of the erythrocytes with varying doses of *N*-ethylmaleimide in the range of 0.15–0.5 mM caused a dose-dependent inhibition of echinocyte formation.

Erythrocyte membrane deformability

As we have previously documented, deformability measurements on intact erythrocytes showed that treatment with hydrogen peroxide results in a decrease in whole cell deformability, and the observed reduction in cellular deformability was the result of increase membrane rigidity [14]. Direct evidence for increased membrane rigidity of treated erythrocytes was obtained by measuring deformability characteristics of resealed membranes. Fig. 6 shows DI vs. shear rate for resealed membranes prepared from control and *N*-ethylmaleimide-treated cells, before and after exposure to H_2O_2. Membranes from peroxide-treated controle erythrocytes had reduced deformability compared with control membranes at all values of applied shear stress (sheat stress = shear rate × suspending medium viscosity). Pretreatment of erythrocytes with low-dose 0.2 mM *N*-ethylmaleimide inhibited peroxide-induced increases in membrane rigidity, as seen by decrease in the applied shear stress required to reach equivalently deformation (Fig. 6A). These effects are illustrated in detail in Fig. 6B. When the deformability data from resealed membranes were plotted as DI vs.

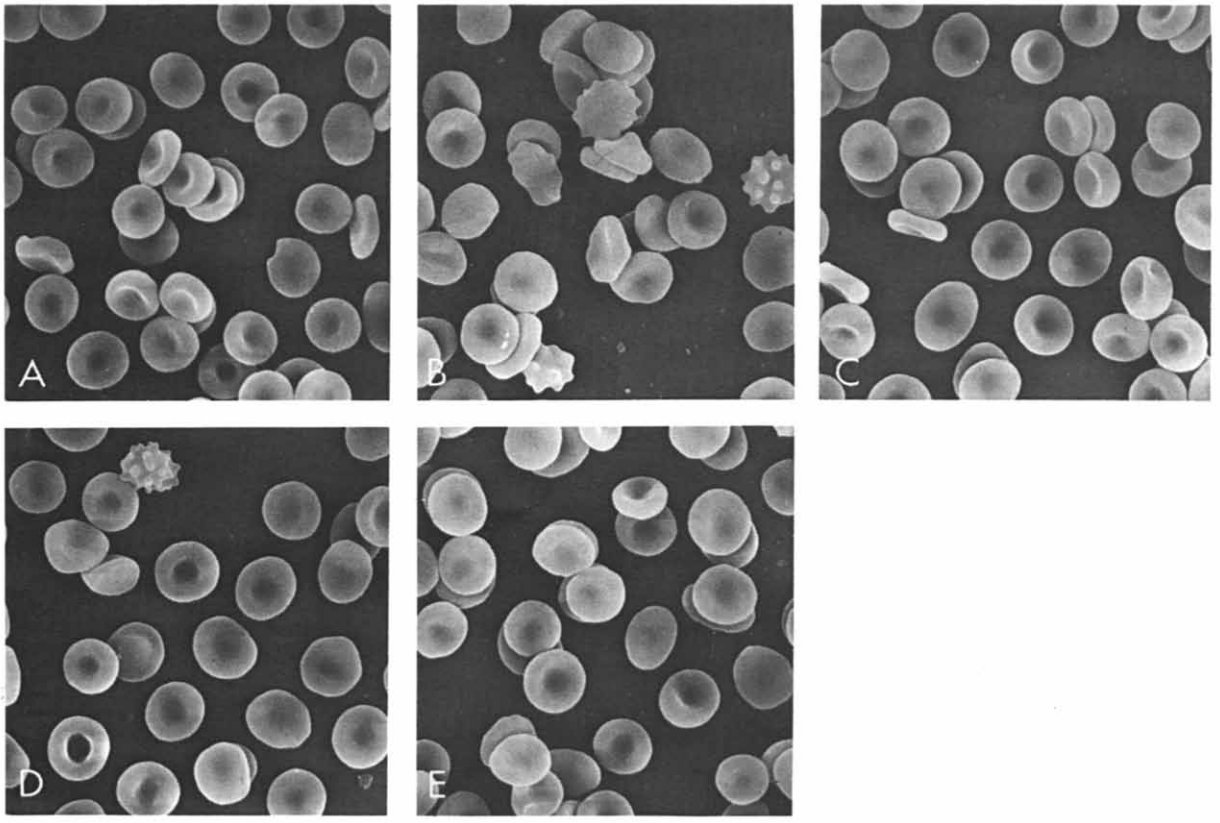

Fig. 5. Cell shape. Scanning electron microscopy of glutaraldehyde-fixed erythrocytes following exposure to both *N*-ethylmaleimide and H_2O_2. 500 cells counted and the percentage of the number of echinocytes determined. (A) Control (< 1% echinocytes), (B) 810 μM H_2O_2 (22% echinocytes), (C) 0.5 mM *N*-ethylmaleimide control (< 1% echinocytes), (D) 0.15 mM *N*-ethylmaleimide prior to H_2O_2 (less than 5% echinocytes), (E) 0.5 mM *N*-ethylmaleimide prior to H_2O_2 (less than 1% echinocytes). A–E, ×1400.

logarithm of shear rate, a linear relationship was seen between the two variables (Fig. 6B). The deformability of control membranes is shown on the top curve. Treatment of cells with increasing concentrations of *N*-ethylmaleimide caused a dose-dependent decrease in the DI obtained at all values of applied shear rate. Since the lines are parallel, one can calculate that membranes treated with 0.1, 0.2 and 0.3 mM *N*-ethylmaleimide required 1.10-, 1.12- and 1.88-fold greater shear rate from control membranes to reach equivalent deformation. Moreover, it can be seen that control membranes treated with H_2O_2 require 2.75-times higher shear rate than untreated membranes to reach equivalent deformation, implying that peroxide treatment increases membrane rigidity 2.75-fold. In contrast, rigidity of membranes from cells pretreated with 0.1, 0.2 and 0.3 mM *N*-ethyl-

TABLE III

PHAGOCYTOSIS OF MODIFIED RED CELLS BY PERIPHERAL BLOOD MONOCYTES, EFFECT OF *N*-ETHYLMALEIMIDE PRETREATMENT ON TARGET ACTIVITY INDUCED BY HYDROGEN PEROXIDE

Pretreatment: 10% suspensions of washed red cells in phosphate-buffered saline were exposed to *N*-ethylmaleimide at the concentration indicated for 15 min at 37°C. Peroxide treatment: 0.315 mM H_2O_2, applied under conditions described in Materials and Methods. Phagocytosis assay: Antiglobin-mediated phagocytosis by fresh monocytes expressed as % of monocytes ingesting red cells ($\pm$S.D., $N = 4$).

Pretreatment (*N*-ethylmaleimide, mM)	Phagocytosis (% $\pm$ S.D.)	
	$-H_2O_2$	$+H_2O_2$
None	4.0 $\pm$ 0.6	9.1 $\pm$ 0.7
0.1	4.7 $\pm$ 0.6	8.2 $\pm$ 1.0
0.15	5.7 $\pm$ 0.7	5.9 $\pm$ 0.9
0.2	6.4 $\pm$ 0.8	5.0 $\pm$ 1.3
0.25	8.2 $\pm$ 0.5	8.8 $\pm$ 0.8

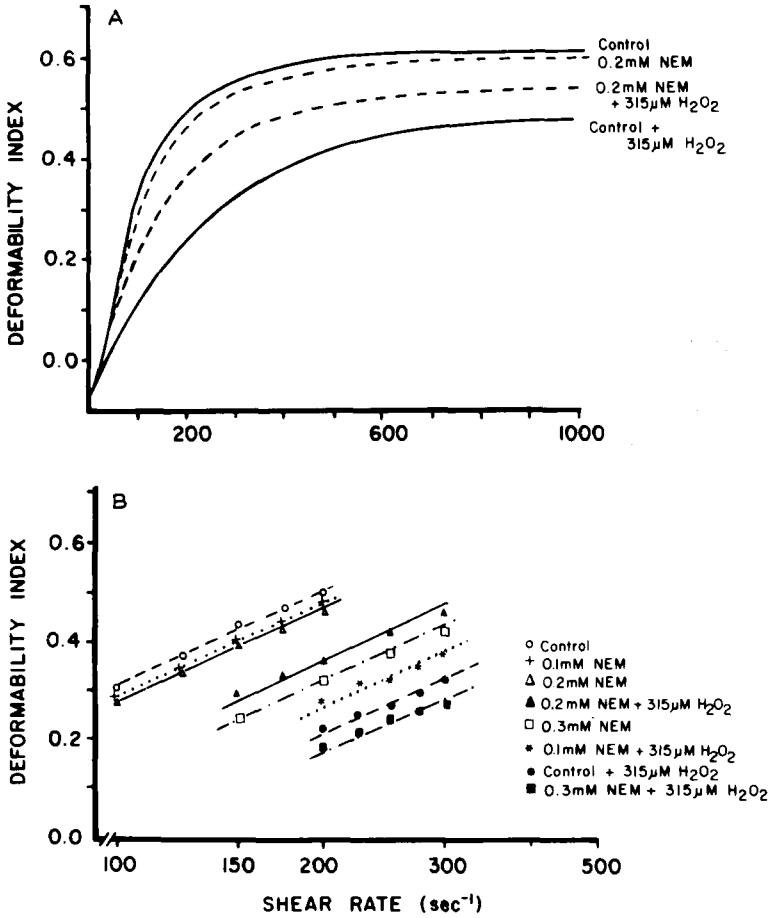

Fig. 6. (A) DI vs. shear stress for resealed erythrocyte membranes from *N*-ethylmaleimide (NEM) + H_2O_2-treated erythrocytes. (B) Deformability data from resealed membranes from control cells; *N*-ethylmaleimide-pretreated control cells; control + H_2O_2-treated cells, *N*-ethylmaleimide-pretreated control + H_2O_2-treated erythrocytes plotted as DI vs. logarithm of shear rate.

maleimide increased only 2.06-, 1.46- and 1.71-fold following treatment with H_2O_2. This data demonstrates the protective effect of low concentrations of *N*-ethylmaleimide on peroxide induced alterations in membrane deformability.

Surface alterations as manifested by the antiglobulin-mediated monocyte phagocytos

Table III shows data on phagocytosis by peripheral blood monocytes of variously modified red cells. Pretreatment with low doses of *N*-ethylmaleimide (0.1–0.2 mM) caused a dose-dependent decrease in recognition of oxidized cells by the antiglobulin serum. However, at concentrations greater than 0.20 mM *N*-ethylmaleimide this protective effect was abolished. Moreover, at higher concentrations, *N*-ethylmaleimide treatment of red blood cells appear to induced increased opsonization by antiglobulin serum with red blood cells which subsequently induced increased monocyte phagocytosis; the magnitude of this opsonization seemed to be similar to that seen following oxidation with H_2O_2 alone.

Discussion

The data obtained in the present study identifies an important role for SH groups of membrane proteins in modulating membrane alterations resulting from oxidative damage. The ability of low concentration of *N*-ethylmaleimide to inhibit not only spectrin hemoglobin complex formation, but

also the myriad of cellular changes lends support to this hypothesis. Our findings of a biphasic effect of *N*-ethylmaleimide on oxidized and non-oxidized human red cells is also interesting. Low doses of *N*-ethylmaleimide (0.1–0.2 mM) decreased H_2O_2 generated spectrin-hemoglobin crosslinking and protected the membrane from secondary effects of peroxidation as evidenced by a decrease in formation of echinocytes, and increase in membrane deformability and a decrease in antiglobulin-mediated phagocytosis by peripheral blood monocytes when compared to an appropriate hydrogen peroxide-treated control. In contrast, higher doses of *N*-ethylmaleimide (0.25 mM) were much less effective in protecting the cell from oxidant-induced alterations in membrane deformability and cell surface in spite of inhibiting the generation of spectrin-hemoglobin complexes. At these concentrations, *N*-ethylmaleimide by itself produced alterations in both deformability and on surface characteristics, suggesting a direct role for sulfhydryl groups in regulating these critical membrane functions. Morever, *N*-ethylmaleimide alone at higher doses has been shown to have a profound effect on erythrocyte membrane cytoskeleton integrity [7,8], which may directly affect deformability and indirectly affect surface characteristics.

These data also point out the importance of small amounts of spectrin–hemoglobin crosslinking in altered membrane function. In addition, the fact that *N*-ethylmaleimide did not inhibit the hydrogen peroxide-induced methemoglobin formation supports the hypothesis that direct oxidation of sulfhydryl groups on spectrin as well as hemoglobin oxidation plays an important role in the complex formation. This is further supported by the ability of *N*-ethylmaleimide to inhibit isolated spectrin from crosslinking with hemoglobin in a cell-free system.

The present data also suggests that the red blood cell GSH levels do not play a major role in peroxidative membrane damage. Following H_2O_2 treatment, a myriad of membrane alterations occurred in spite of maintenance of normal GSH levels (Table I). In contrast, a low dose of *N*-ethylmaleimide, 0.15 mM, which resulted in a significant decrease in GSH levels (Table I), was able to protect the cell from the peroxidative membrane alterations. These experiments reveal that the GSH concentration does not seem to affect certain membrane functions. However, these studies are still consistent with our hypothesis that maintenance of optical membrane function may depend on preservation of specific membrane sulfhydryl groups in the reduced state. Moreover, the implication is that certain membrane thiols may be inaccessible to GSH. This notion is supported by the results with sickle cell erythrocytes which generate an increased amount of activated oxygen [1], and that the membranes have undergone oxidation of protein thiols [29], despite the fact that the GSH levels are essentially unchanged [30].

Previous studies employing much higher levels of ^{14}C-labeled *N*-ethylmaleimide (10–20 mM) than used in the present study failed to demonstrate a selective labelling of a particular membrane protein, but rather mimicked the Coomassie blue stain pattern [31,32,33]. However, in the present study by employing much lower concentrations of *N*-ethylmaleimide we were able to show a majority of early labelling of ^{14}C-labeled *N*-ethylmaleimide occurs in spectrin (alpha chain approx. 30% greater than beta chain), band 2.1 and 4.2. As yet, no known function has been ascribed to band 4.2 while, in contrast, band 2.1 has clearly been shown to play a pivotal role in linking the skeletal systems through spectrin to the intrinsic membrane band 3 [6]. Conceivably, this interaction may be the pathway through which alterations in the membrane skeleton transmits a signal (via band 2.1-band 3 interaction), ultimately resulting in alterations in the cell surface. Only after increasing the concentrations of *N*-ethylmaleimide to 0.15 mM or greater did other SH groups on different membrane proteins such as bands 4.1, 3 and 5 become labeled with ^{14}C-labeled *N*-ethylmaleimide. These findings suggest that certain sulfhydryl groups on spectrin are more accessible than others. Perhaps these sites may be identical to those which are involved in covalently binding to globin.

Our present studies also demonstrate that *N*-ethylmaleimide is capable of decreasing lipid peroxidation following hydrogen peroxide treatment. At first glance, these results would support the idea of a relationship between lipid peroxidation, spectrin–hemoglobin crosslinking and other membrane alterations. However, in previous studies

pretreatment of red cells with carbon monoxide prior to exposure to H_2O_2 completely inhibited hemoglobin-spectrin crosslinking as well as other membrane alterations failed to inhibit lipid peroxidation, thus clearly separating the effect of lipid peroxidation from membrane protein oxidation [14].

We thus postulate that during red cell peroxidation, hemoglobin is oxidized and free radicals are generated which cause particularly susceptible SH groups on the spectrin molecule to be oxidized, possibly by forming mixed disulfides with globin. Such a reaction facilitates covalent and non-reducible complexing of skeletal proteins with neighboring globin molecules. By blocking these reactive sulfhydryl groups with low doses of *N*-ethylmaleimide the subsequent reactions of spectrin with globin is inhibited. Such a hypothesis suggests that the spectrin domain containing the highly reactive sulfhydryl groups is either the same as the domain involving globin binding or is in a position such that a conformational change in the domain caused by SH reactivity alters the ability of a different spectrin domain to interact with globin. Higher doses of *N*-ethylmaleimide probably cause major alterations in the spectrin conformation independent of hemoglobin–spectrin crosslinking, leading to profound alterations in the spectrin self-association [8], spectrin 4.1 binding [34] or spectrin 2.1 binding [7], which in turn may result in altered membrane function, as manifested by a decrease in membrane deformability and changes in surface characteristics, as noted in the present study.

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