

Properties of Vitamin E-Deficient Erythrocytes following Peroxidant Injury

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Summary

Several membrane properties of vitamin E-deficient and normal erythrocytes were studied after incubation of these cells with hydrogen peroxide. Measurements of mean corpuscular volume, cation permeability, membrane Na⁺, K⁺ ATPase activity, red cell filterability through 5 μ millipore filters, and membrane protein pattern on sodium dodecyl sulfate-gel electrophoresis revealed marked alterations before lysis. Vitamin E sufficient cells were unaffected by a similar incubation with hydrogen peroxide. We speculate that the changes in membrane function, which follow peroxidant injury, contribute to the shortened red cell survival in the vitamin E deficient state.

An anemia secondary to vitamin E deficiency has long been recognized in premature infants (20). Dietary supplementation with vitamin E prevents the development of this anemia. Red cell chromium survival studies have demonstrated a shortened life span for red cells from vitamin E-deficient premature infants (22); however, the exact cause of this shortened red cell survival is poorly understood.

In light of the antioxidant role of vitamin E in biologic tissues (28), it is generally believed that the shortened erythrocyte survival in vitamin E-deficient premature infants is the result of membrane damage secondary to enhanced lipid peroxidation. Indeed, many changes in the membrane of vitamin E-deficient red cells after peroxidant injury have been reported. Among these changes is the prelytic loss of phosphatidylethanolamine (9, 10). This observation is consistent with the fact that phosphatidylethanolamine contains a high concentration of polyunsaturated fatty acids and these fatty acids are liable to lipid peroxidation in the absence of appropriate amount of antioxidant. Furthermore, Lubin *et al.* (16) have demonstrated that hydrogen peroxide stimulated the incorporation of fatty acid into vitamin E-deficient red cells but had no effect on the incorporation of fatty acid into normal red cells. They suggested that peroxidant injury to vitamin E-deficient red cells stimulates a membrane repair process. Because products of lipid peroxidation can react with sulfhydryl and/or amino groups of proteins (2, 14, 25), peroxidative process initiated in the membrane lipid moiety will inevitably lead to damage in membrane proteins.

Although Stocks and Dormandy (27) clearly demonstrated that peroxidant injury to red cells can lead to eventual hemolysis, changes in cellular properties before hemolysis after oxidative damage have not been carefully studied. This paper reports changes in the cellular properties of vitamin E-deficient human erythrocytes as a consequence of H₂O₂-induced peroxidant injury *in vitro*. Among the cellular properties reported in this paper are membrane protein alterations, red cell size, cation permeability, membrane Na⁺, K⁺ ATPase activity, and red cell filterability through millipore filters.

MATERIALS AND METHODS

Cell preparation. Heparinized blood was obtained with informed consent of the parents from premature infants whose birth weight

was less than 1500 g. The red cells were washed three times with Krebs Henseleit Buffer (KHB) pH 7.4, and residual leukocytes and platelets were removed. The peroxide hemolysis test, performed by previously reported methods (24), was positive in all patients studied, and % hemolysis ranged from 60-100%. Plasma vitamin E level of all vitamin E-deficient premature infants was below 0.5 mg/dl. Control red cells were obtained from premature infants who were receiving supplemental vitamin E and had a peroxide hemolysis value of less than 5%. All experiments were performed in duplicate. The results indicate the mean values of two individual patients used for each experiment.

Red cell volume distribution. A 4% suspension of red cells was incubated with an equal volume of 1% hydrogen peroxide (final concentration, 147 mM) in a 30-ml beaker. A Coulter electrode connected to a digital plotter recorded the erythrocyte volume distribution before peroxide addition and at 1-min intervals during the subsequent incubation with hydrogen peroxide. The controls consisted of vitamin E-deficient cells incubated in the presence of equal volumes of KHB and normal cells incubated with and without hydrogen peroxide.

Potassium efflux. Red cells previously washed in KHB were incubated for 2 h at 37°C in isotonic saline containing 50 μ Ci of [⁴²K] at a hematocrit of 30%. Unincorporated [⁴²K] was removed by three washes in ten volumes of KHB at 37°C. The cells were then resuspended to a hematocrit of 4% and were incubated in separate tubes for each time-sample with an equal volume of 1% hydrogen peroxide in KHB. An initial sample was reserved to determine the total [⁴²K] incorporated. At 5-min intervals, 0.1 ml of a saturated solution of thymol was added to the successive incubation mixtures to inhibit further peroxidation (31). The suspensions were centrifuged at 5000 rpm for 5 min and the supernatant removed. The radioactivity in aliquots of the supernatant was determined and expressed as a percentage of the total radioactivity incorporated. Percent hemolysis was determined on similar aliquots taken at the same time. The initial cell concentration of [⁴²K] in the reserved cells was determined by counting aliquots of the washed cells that had been dissolved in Beuhler's reagent (1).

Filterability. Cells were incubated with hydrogen peroxide as described above. After 15 min of incubation, concentrated thymol was added to the incubation mixture. After the separation of cells from the supernatant, cells were washed twice with KHB and resuspended to a 2% hematocrit in KHB. The mean corpuscular volume was determined for each sample. Cells were then filtered through a 5 μ millipore filter at 37°C at a pressure of 20 mmHg as reported by Schmid-Schonbein *et al.* (26). The time required for 2 ml of red cell suspension to pass the filter was recorded and was used to calculate the rate of filtration.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Ghosts were prepared by the method of Dodge *et al.* (4) from cells which had been incubated with or without hydrogen peroxide as described above. To carry out SDS-polyacrylamide gel electrophoresis of membrane proteins, aliquots of ghost preparations were treated with equal volumes of 20 mM Tris buffer containing 2% SDS, 20% sucrose, 2 mM EDTA and 1 ml of 1% phenol red, pH 7.9, for 30 min at 37°C. Upon completion of incubation, an

aliquot of approximately 30 λ of ghost incubation mixture containing exactly 40 μg of membrane proteins were loaded onto a 4% Biophore-gels (BioRad, Richmond, CA). After completion of electrophoresis, gels were fixed with isopropanol and stained with Coomassie blue dye by the method of Fishbein (5). Membrane protein concentration of different ghost preparations was determined by the Method of Lowry *et al.* (15).

Membrane Na^+ , K^+ ATPase activity. Because the activity of Na^+ , K^+ ATPase is completely abolished by 0.2 mM ouabain, the activity of this enzyme was determined as the difference between assays in the presence and the absence of ouabain. In our assay procedure, 0.5 mg/ml of membrane protein was suspended in 3 ml of 50 mM Tris buffer (pH 7.4) containing 50 mM NaCl, 120 mM KCl, 5 mM MgCl_2 , with and without 0.2 mM Ouabain. At timed intervals during the 37°C incubation, aliquots of the reaction mixture were taken, mixed with 6% trichloroacetic acid and subsequently assayed for phosphorus in the supernatant.

RESULTS

Corpuscular volume distribution. Figure 1 shows the sequential changes in corpuscular volume distribution in vitamin E-deficient red cells after incubation with hydrogen peroxide. The size distribution in these cells rapidly decreased within the first 5 min after the addition of hydrogen peroxide, and then progressively increased during further incubation until lysis occurred. The same cells incubated in buffer without H_2O_2 showed no change in volume distribution. Normal cells, not shown in the graph, were not altered in volume distribution during the incubation with or without hydrogen peroxide in KHB.

Potassium efflux. The loss of [^{42}K] from vitamin E-deficient red cells to the incubation medium is graphically shown in Figure 2. Potassium permeability was markedly increased immediately upon the addition of hydrogen peroxide. Hemoglobin release was not noted until 30 min after the addition of hydrogen peroxide, at which time 20% of the incorporated [^{42}K] had been lost to the medium. In the absence of hydrogen peroxide, no loss of [^{42}K] was observed in these cells. Furthermore, hydrogen peroxide did not increase the potassium permeability of normal cells.

Filterability. Red cell filterability was markedly decreased in the vitamin E-deficient cells after incubation with hydrogen peroxide for 15 min. (Table 1). In contrast, when normal red cells were similarly incubated, they showed no change in filterability. The 15 min-incubation period was chosen because at this time the mean corpuscular volume of the vitamin E-deficient cells incubated with hydrogen peroxide was not significantly different from the mean corpuscular volume of the vitamin E-deficient cells incubated in KHB (101–102 μ^3 , respectively; also see Fig. 1). Filtration rates for vitamin E-deficient cells in KHB without

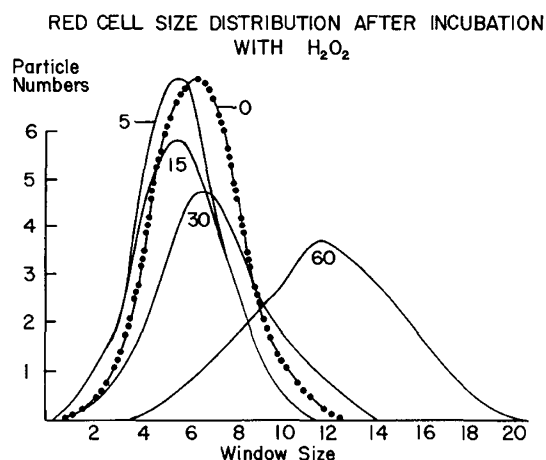


Fig. 1. Red cell size distribution after incubation with H_2O_2 . The size distribution of vitamin E-deficient red cell before incubation with H_2O_2 (0 time) and at 5, 15, 30, and 60 min intervals after incubation with H_2O_2 was recorded by a Coulter electrode connected to a digital plotter.

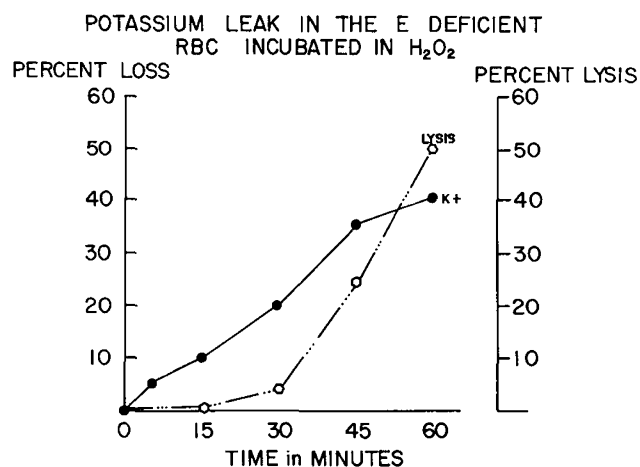


Fig. 2. Effect of H_2O_2 on potassium permeability and hemolysis of vitamin E-deficient red cells. Potassium leakage of H_2O_2 -treated vitamin E-deficient red cells was monitored by measuring the release of [^{42}K] into the incubation medium at different time intervals after the addition of H_2O_2 . The [^{42}K] was first incorporated into red cells before incubation with H_2O_2 . Hemolysis was monitored by the release of hemoglobin from red cells into the incubation medium.

Table 1. Effect of H_2O_2 on red cell filterability

Red blood cell samples	Rate of filtration (mm^3/sec)
Normal (4) ¹	122 \pm 6.5 ²
H_2O_2 -treated normal (4)	149 \pm 8.2
Vitamin E-deficient (4)	142 \pm 7.9
H_2O_2 -treated vitamin E-deficient (4)	49 \pm 6.3

¹ Number in parenthesis indicates number of experiments.

² Mean \pm S.E.

hydrogen peroxide were not significantly different from those of normal cells in KHB with or without hydrogen peroxide.

Membrane protein pattern on SDS-polyacrylamide gel electrophoresis. Figure 3A shows the electrophoresis patterns of the membrane proteins from vitamin E-deficient red cells on 4% polyacrylamide gels in SDS. The membrane protein pattern of vitamin E-deficient red cells (gel E and F) was similar to that of normal red cells (not shown in this Fig.); however, after exposure to hydrogen peroxide, vitamin E-deficient red cells exhibited abnormal membrane protein patterns, particularly expressed by the formation of high molecular weight aggregates (gels A–D). Furthermore, densitometry scanning of these gels (Fig. 3B) clearly demonstrated diminished quantities of spectrin (bands 1 and 2) in vitamin E-deficient red cells exposed to hydrogen peroxide as compared to the same cells that were not exposed to hydrogen peroxide. Incubation of normal red cells with hydrogen peroxide did not alter the membrane protein pattern of these cells.

Membrane Na^+ , K^+ ATPase activity. Figure 4 shows the influence of hydrogen peroxide on membrane Na^+ , K^+ activity in vitamin E-deficient and normal red cells. The enzyme activity was decreased by 60% after vitamin E-deficient red cells were incubated with hydrogen peroxide. In contrast, the enzyme activity was unaffected in normal red cells by a similar incubation with hydrogen peroxide. Furthermore, the initial enzyme activity in vitamin E-deficient red cells was similar to that in normal red cells.

DISCUSSION

The results of these studies demonstrate the diffuse membrane injury in vitamin E-deficient red cells induced by hydrogen peroxide. Peroxidative reactions can lead to membrane damage followed by alteration in cellular properties and eventual cell lysis (3). Although our *in vitro* incubation mixture contained a non-physiologic concentration of hydrogen peroxide, the continuous production of hydrogen peroxide *in vivo* as a consequence of

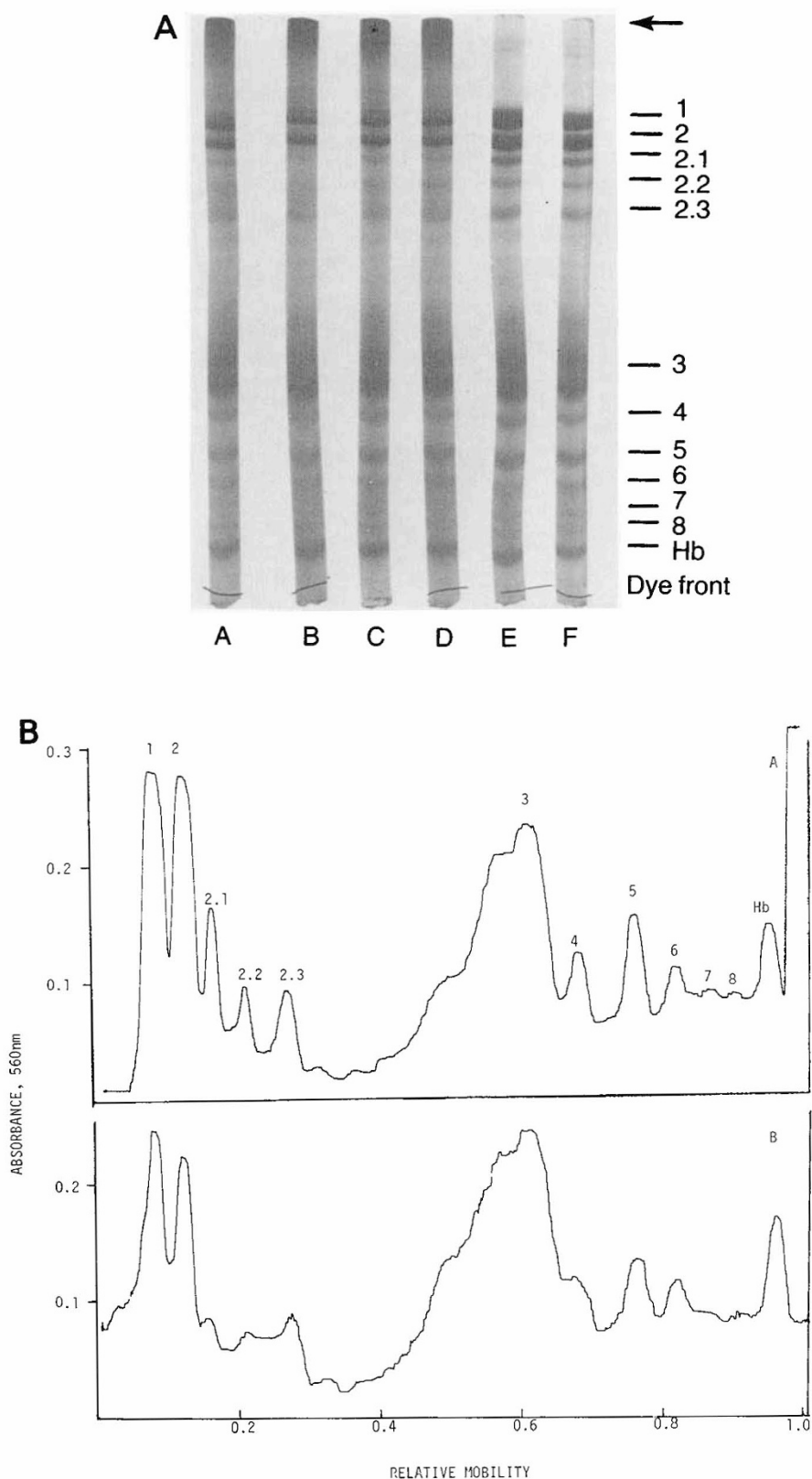


Fig. 3. (*Left hand panel*) alteration of membrane proteins of vitamin E-deficient red cell following H_2O_2 incubation. Membrane proteins were separated by electrophoresis in 4% polyacrylamide gel containing 1% sodium dodecyl sulfate and stained by Coomassie blue. All gels contained exactly $40 \mu\text{g}$ of membrane proteins. Gels A through D shows the membrane protein pattern of vitamin E-deficient red cell following incubation with H_2O_2 . Arrow indicates the formation of large molecular weight membrane protein aggregates on H_2O_2 treated vitamin E-deficient red cells. The aggregates were not present in H_2O_2 -treated vitamin E-sufficient red cells. Gels E and F shows the membrane protein pattern of vitamin E-deficient red cells without H_2O_2 treatment. Bands 1 and 2 are spectrin. (*Right hand panel*) densitometry scanning of membrane protein patterns on sodium dodecyl sulfate-polyacrylamide gels. Panel A shows the membrane protein pattern of H_2O_2 -treated vitamin E-sufficient red cells which correspond to gel E of the figure's left hand panel. Panel B shows diminished spectrin content (bands 1 and 2) of H_2O_2 -treated vitamin E-deficient red cells which corresponds to gel A of the figure's left hand panel.

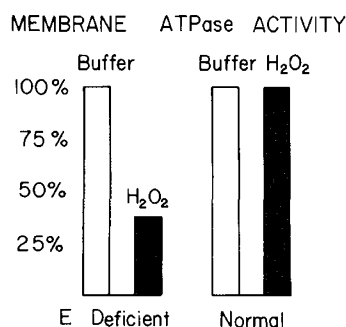


Fig. 4. Decreased membrane Na⁺, K⁺ ATPase activity of vitamin E-deficient red cells after incubation with H₂O₂. In this assay procedure, membrane protein (0.5 mg/ml) was suspended in 3 ml of 50 mM tris buffer (pH 7.4) containing 50 mM NaCl, 120 mM KCl, 5 mM MgCl₂, with and without 0.2 mM quabain. At approximate time intervals, the release of phosphate was assayed. H₂O₂ treatment decreased the ATPase activity in vitamin E-deficient red cells but the enzyme activity was not affected in normal red cells.

normal red cell metabolism such as the conversion of oxyhemoglobin to methemoglobin (18) may produce subtle membrane damage of physiologic significance. Furthermore, our *in vitro* design may mimic an *in vivo* condition in which red cells are exposed to a sudden burst of oxidative stress such as that which occurs after treatment with oxidant drugs (6, 7). It has also been shown that stimulated human neutrophils generate superoxide radicals which can lyse normal human erythrocyte (29).

The exact mechanism underlying the hydrogen peroxide-induced alterations in cellular properties of vitamin E-deficient cells is not clear; however, our results together with those available in the literature strongly suggest that membrane alterations are initiated via a lipid peroxidation scheme. Kesner *et al.* (12) have demonstrated that peroxidized phospholipids are potent inhibitors of human erythrocyte membrane Na⁺, K⁺ ATPase. Because phosphatidyl serine is rich in polyunsaturated acyl side chains, the finding of Roelofsen and van Deenen (23) indicating that phosphatidyl serine plays an important role in monitoring ATPase activity may explain why Na⁺, K⁺ ATPase is very susceptible to oxidative insult (Fig. 4).

Alternatively, the changes in membrane permeability may represent a direct toxic effect of peroxidation on membrane integrity. It has long been recognized that changes in membrane fatty acid composition contribute to alteration of membrane permeability in both artificial membranes (19) and intact red cells (13). Changes in membrane permeability were closely associated with a change in cell volume and were subsequently followed by gradual colloidal lysis. Similar changes have been observed in mitochondrial and lysosomal membranes as a consequence of peroxidant injury (30).

It must be pointed out that multiple factors determine the degree of hemolysis seen in the vitamin E-deficient premature infants. Infants fed formulas containing an elevated concentration of polyunsaturated fatty acid and decreased concentration of vitamin E have a greater incidence of hemolysis than those fed breast milk containing a more appropriate ratio of vitamin E per mole of polyunsaturated fatty acids (PUFA) (22). Moreover, exposure to oxygen at high pressure (11) or the addition of iron have also been demonstrated to accentuate the hemolytic process in the vitamin E deficient state (17). Because both oxygen and iron (3) play an important role in lipid peroxidation, these findings are consistent with the hypothesis that hemolytic anemia in vitamin E-deficient premature infants is the result of erythrocyte membrane damage secondary to enhanced lipid peroxidation.

Peroxidant injury initiated in the lipid components of the membrane can easily be transmitted to neighboring proteins. Sulfhydryl groups in the proteins are extremely susceptible to free radical attack (14, 25). Among the membrane proteins, spectrin has been shown to be particularly susceptible to oxidant injury (8,

21), perhaps due to the proximity of exposed spectrin sulfhydryls within the membrane. Our results (Fig. 3a and b) also demonstrate that spectrin (bands 1 and 2) is more susceptible to oxidant injury than other membrane proteins. Formation of spectrin aggregates as a result of oxidative insult may explain, in part, the reduced filterability seen in hydrogen peroxide-treated vitamin E-deficient cells (Table I).

Taken together, our data suggest that several changes observed in membrane structure and function after oxidant injury *in vitro* could explain the shortened red cell survival observed in the vitamin E-deficient state. Evidence is accumulating to indicate that peroxidative reactions continually take place in human erythrocytes and these peroxidative reactions are potentially deleterious to the red cells. Fortunately, the potential adverse effects of these reactions can be minimized by the normal amount of antioxidant reserve in red cells under most circumstances; however, decreased antioxidant reserve as in the case of vitamin E deficiency, or excess oxidant stress, will lead to irreversible membrane damage and shortened red cell survival.

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