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The Hydrogen Peroxide Fragility Test and Serum Tocopherol Level in Anemias of Various Etiologies

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Increased in vitro erythrocyte sensitivity to the hemolytic effects of hydrogen peroxide (H_2O_2) in the H_2O_2 fragility test has been widely associated with vitamin E deficiency. In the present study, H_2O_2 red cell fragility was abnormally elevated in many types of acquired and congenital anemias in children who were tocopherol sufficient. In addition to abnormal red cell H_2O_2 sensitivity known to occur in vitro in conditions involving defects in cellular devices for disposal of peroxides, it is likely that a wide variety of erythrocyte dysfunctions result in increased in vitro H_2O_2 hemolysis even in situations where the usually adequate mechanisms for peroxide detoxification are present.

RENEWED INTEREST IN THE BIOLOGIC FUNCTIONS of vitamin E and the effects of vitamin E deficiency in humans has led to a wider use of the erythrocyte hydrogen peroxide (H_2O_2) fragility test.[1-4] Originally devised by György and Rose[5] and modified by Gordon and associates,[6] this procedure measures the in vitro sensitivity of red blood cells (RBC) to the hemolytic effects of low concentrations of H_2O_2. Because of the known antiperoxidant function[7] of vitamin E and its attendant ability to aid in maintaining the integrity of the red cell membrane, an abnormally increased degree of RBC hemolysis on incubation with H_2O_2 has generally been felt to reflect vitamin E deficiency.[1-6] Accordingly, a study was undertaken in a variety of anemic states in order to investigate the H_2O_2 fragility–vitamin E interrelationship.

Materials and Methods

One hundred and twenty-eight infants, children, and young adults with anemias of various etiologies were studied. The unequivocal diagnoses of the different hematologic conditions were made by accepted methods of clinical examination and laboratory evaluation. The majority of these patients were studied during visits to the Pediatric Hematology Clinic of University Hospitals, Cleveland, Ohio. Studies were also conducted on a number of patients during hospitalization at Babies and Childrens Hospital necessitated solely by

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their hematologic problems. The total length of observation ranged from several weeks to years. Thirty hematologically normal children and young adults served to provide normal control values. Tocopherol levels were also determined in 26 premature infants with birth weights of less than 1500 Gm. at 4 weeks of age.

Hemoglobin, microhematocrit, RBC count, and reticulocyte count determinations were performed in standard fashion. A slight modification of the method of Gordon, Nitowsky, and Cornblath[6] was used to study red cell fragility on in vitro exposure to H_2O_2, and several salient features of the method are herein reviewed. Blood was obtained by venipuncture and anticoagulated in a citrate-saline solution. No other anticoagulants were employed. Precleaned, disposable glassware was employed during all stages of the procedure, and H_2O_2 fragility tests were begun within an hour following the drawing of blood. Washing, resuspension, and preincubation of the red cells prior to the addition of H_2O_2 were then carried out according to the original method. However, as well as preparing a 5.0 per cent RBC suspension in saline prior to H_2O_2-buffer addition, a second series of samples was diluted so that the RBC suspension contained approximately 5.0×10^8 RBC per millimeter. Addition of the H_2O_2-phosphate buffer mixture to this red cell suspension in a 1:1 volume ratio resulted in a final incubation suspension of approximately 2.5×10^8 RBC per millimeter. Incubation of both sets of samples was then performed. After completion of the incubation period, the Gordon method calls for a calculation of per cent RBC hemolysis determined by a calculation of the ratio of the mean free hemoglobin present in the saline-buffer supernatants of three H_2O_2-challenged samples compared with that of a fourth peroxide-stressed sample in which RBC lysis was completed by the addition of distilled water. A fifth sample was handled in a manner identical to that of the triplicate H_2O_2-stressed samples throughout the procedure except that H_2O_2 was not added in this control tube, and the triplicate H_2O_2-stressed samples from which the mean value was obtained in calculation of per cent hemolysis varied less than $\pm$ 5.0 per cent. H_2O_2 fragility is herein reported as both per cent hemolysis per volume of red cells and per cent hemolysis per number of RBC.

Serum free tocopherol levels were performed according to the method of Qualife et al.[8] on 0.6 ml. of serum, with proportional readjustment of the reagents. In accordance with previous work, serum tocopherol values below 0.50 mg. per cent were felt to indicate vitamin E deficiency.[1,6,8] Samples run in duplicate yielded values varying less than $\pm$ 0.05 mg. per cent. Bound serum iron (BSI) and total iron capacity (TIBC) were determined by the procedure of Schade et al.[9] All Negro individuals in study and control groups were tested for glucose-6-phosphate dehydrogenase (G6PD) deficiency by the dye-reduction screening technique of Motulsky and Campbell-Kraut,[10] and follow-up assays of G6PD activity were determined by the method of Zinkham et al.[11] Assays of erythrocyte catalase activity were performed in normal control subjects and in patients with sickle cell disease and iron deficiency anemia according to the method of Tarlov and Kellermeyer.[12]

Usually, aliquots of the same blood specimen were used in the performance of each of the above-mentioned determinations on each occasion that a patient was studied. In patients with diseases that were chronic, generally stable in character, and not amenable to specific therapy (such as sickle cell disease and other hemoglobinopathies), repeat H_2O_2 fragility tests and other hematologic studies were also obtained at intervals ranging from 1 week to 1 year after the initial determinations. In patients with aplastic anemia, H_2O_2 fragilities, serum tocopherol levels, and other determinations were performed before blood transfusion therapy was instituted. These patients had essentially stable RBC transfusion requirements at intervals of from 4 to 10 weeks during the study period. Consequently, repeat H_2O_2 fragilities and serum tocopherol levels were carried out at the end of the period between transfusions. Determinations on erythroblastotic infants were always performed before exchange transfusion was carried out.

Results

A compendium of the basic hematologic data is presented in Table 1. In Table 2 are shown the various disorders in which H_2O_2 fragilities were within

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Table 1.—Data on Control and Study Patients

Diagnosis	No. of Patients	Mean Age (yrs.)	Sex M	Sex F	Mean Hgb. (Gm. %)	Mean Hct. (Vol. %)	Mean Retic. (%)
Erythroblastosis fetalis	15	1 (day)	8	7	10.1	28.9	13.8
Infantile pyknocytosis	3	42 (days)	3	0	8.1	26.0	5.9
Iron-deficiency anemia	35	1.5	18	17	7.2	24.7	3.5
Sickle-cell disease	22	9.5	11	11	7.6	22.6	15.9
Hemoglobin C disease	3	11.0	1	2	9.9	28.0	5.6
Sickle-cell hemoglobin C disease	4	4.0	2	2	9.3	28.5	3.4
Sickle cell thalassemia	2	11.5	2	0	10.2	32.0	3.4
Thalassemia intermedia	1	18.0	1	–	8.3	22.0	6.6
Thalassemia minor	3	15.0	0	3	10.2	31.3	4.6
Anemia associated with acute leukemia	13	7.0	6	7	8.5	24.3	0.7
Aplastic anemia	6	11.5	2	4	5.4	16.0	0.1
Hereditary spherocytosis	6	7.0	4	2	11.7	30.2	5.2
Acute blood-loss anemia	5	10.5	4	1	7.3	21.2	7.9
Autoimmune hemolytic anemia	3	10.5	2	1	9.3	27.5	9.5
Hereditary sideroblastic anemia with G6PD deficiency	3	8.0	3	0	10.3	32.0	1.8
Juvenile pernicious anemia	2*	2.5	1	1	8.8	25.5	0.7
Folic-acid-deficiency anemia	2	16.5	0	2	11.2	32.5	0.8
Normal control subjects	30	6.5	10	10	13.5	39.4	0.8
Premature infants	26	4 (wks.)	16	10	–	–	–

* One patient had already been started on vitamin B_{12} therapy 1 month prior to these studies.

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Table 2.—H_2O_2 Fragility and Serum Vitamin E Levels in Anemias Associated with Normal H_2O_2 Hemolysis

Diagnosis	No. of Patients	H_2O_2 Fragility Mean and Range RBC Vol./RBC# (% Hemolysis)*	Vitamin E Mean and Range (Mg. %)*
Aplastic anemia	6	14.3/14.0	0.95
		0–16/0–16	0.70–1.50
Juvenile pernicious anemia	2	11.0/14.0	0.72
		8–14/12–16	0.65–0.80
Folic-acid-deficiency anemia	2	7.0/6.5	0.80
		6–8/5–8	0.75–0.85
Sickle-cell thalassemia	2	10.0/19.0	0.90
		18–20/18–20	0.86–0.94
Thalassemia intermedia	1	0.0/5.0	1.24
		—	
Thalassemia minor	3	6.3/7.2	0.88
		0–11/4–10	0.68–1.05
Anemia associated with acute leukemia	13	8.0/8.0	0.94
		0–16/0–16	0.65–1.43
Acute blood-loss anemia	5	10.2/10.0	0.72
		4–15/5–15	0.60–0.95
Hereditary sideroblastic anemia with G6PD deficiency	3	7.2/6.2	0.88
		0–9/3–9	0.68–1.05
Normal control subjects	30	6.3/6.1	0.94
		0–14/0–12	0.64–1.30
Premature infants	26	80.1/83.6	0.32
		42–100/40–100	0.00–0.46

* p value (comparison with normal controls) N.S. except for premature infants, where $p < 0.001$.

the normal range. Included are aplastic anemia, juvenile pernicious anemia, folic acid deficiency anemia, thalassemia intermedia and thalassemia minor, acute blood loss anemia, anemia of acute lymphoblastic leukemia, and hereditary sideroblastic anemia with associated G6PD deficiency. The mean H_2O_2 fragilities in these groups did not differ significantly from those of the control group, and the range of H_2O_2 fragility was relatively narrow. All individuals had H_2O_2 fragilities below 21 per cent. Mean serum tocopherol levels in these individuals were comparable to that of normal control subjects. None were below 0.50 mg. per cent. The "abnormal control" levels used for comparison were obtained from premature infants and, as anticipated,[13] were uniformly below 0.5 mg. per cent. Repeat determinations of both H_2O_2 fragility and serum tocopherol levels in study patients remained within the normal range and mirrored initial findings.

The groups of patients in whom red cell H_2O_2 fragilities were found to be abnormal are shown in Table 3, where mean H_2O_2 fragilities and serum tocopherol levels are compared with those of control subjects. As shown, the H_2O_2 fragilities were elevated in anemias of widely different etiologies, including hemoglobin SS, CC, and S-C diseases, autoimmune hemolytic anemia, hereditary spherocytosis, infantile pyknocytosis, and iron deficiency anemia.

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Table 3.—H_2O_2 Fragility and Serum Vitamin E Level Associated With Abnormal H_2O_2 Hemolysis

Diagnosis	No. of Patients	H_2O_2 Fragility Mean and Range RBC Vol./RBC# (% Hemolysis)	*p* Value Comparison With Normal Control	Vitamin E Mean and Range (Mg. %)	*p* Value Comparison With Normal Control
Hemoglobin C disease	3	87.5/84.0		0.84	
		86–89/82–86	< 0.01	0.62–1.05	N.S.
Sickle-cell disease	22	52.2/50.0		0.72	
		19–100/18–100	< 0.001	0.58–1.35	N.S.
Sickle-cell hemoglobin C disease	4	38.2/35.7		0.65	
		22–70/24–62	< 0.01	0.60–0.75	N.S.
Erythroblastosis fetalis (E sufficient)	11	80.7/80.0		0.58	
		74–100/73–100	< 0.001	0.50–0.65	0.05
Erythroblastosis fetalis (E deficient)	4	78.3/75.2		0.40	
		70–95/70–90	< 0.001	0.31–0.45	0.01
Autoimmune hemolytic anemia	3	68.0/68.0		0.92	
		56–80/61–80	< 0.01	0.90–0.95	N.S.
Infantile pyknocytosis	3	69.0/70.0		0.64	
		58–80/61–80	< 0.01	0.60–0.72	N.S.
Hereditary spherocytosis	6	63.1/68.0		0.82	
		35–85/41–94	< 0.001	0.62–1.23	N.S.
Iron-deficiency anemia	35	20.6/18.2		0.71	
		8–52/5–64	< 0.001	0.62–1.50	N.S.
Normal control subjects	30	6.3/6.1		0.84	
		0–14/0–12	–	0.64–1.30	–
Premature infants	26	80.1/83.6		0.32	
		42–100/40–100	< 0.001	0.00–0.46	< 0.001

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The elevation of H_2O_2 fragility in each group is markedly greater than that of the normal control group. Where the number of patients in each group was sufficient to allow statistical comparison with the normal controls, differences were significant, with p values ranging from less than 0.001 to 0.01. With the exception of infants with erythroblastosis fetalis, all groups with abnormal H_2O_2 fragilities were found to have normal tocopherol levels (more than 0.50 mg. per cent). Newborn full-term infants with erythroblastosis fetalis had serum tocopherol levels somewhat lower than those of other groups.[13,14] This group was therefore subdivided according to serum tocopherol level. Although the differences in mean tocopherol between both vitamin E-sufficient and -deficient erythroblastotic infants and that of normal control subjects are significant (p values < 0.05 and < 0.01, respectively), no significant difference in mean red cell H_2O_2 fragility was found between infants with serum tocopherol levels above and below 0.50 mg. per cent. In those patients considered in Table 3, whose hematologic conditions remained stable during the study period (hemoglobin SS, CC, and S-C diseases, autoimmune hemolytic anemia, and hereditary spherocytosis), repeat determinations of H_2O_2 fragility and serum tocopherol level were similar to initial values.

No statistically significant correlation between abnormal H_2O_2 fragility and erythrocyte catalase activity in patients with sickle-cell disease or iron-deficiency anemia was noted. The catalase levels in these patients were similar to those of normal control subjects. Although elevated H_2O_2 fragility was often found in anemias associated with increased reticulocytosis, within each group of patients with these anemias, no relationship was noted between the degree of reticulocytosis and the extent of the H_2O_2 fragility elevation. Only in iron-deficiency anemia was there a correlation between H_2O_2 fragility levels and the degree of anemia.[15] No correlation was observed between H_2O_2 fragility and BSI or TIBC, in other than iron-deficient patients.

Discussion

Previous observations on the use of the erythrocyte H_2O_2 fragility determination in humans and in experimental animals have stressed the relationship between the abnormal in vitro RBC H_2O_2 fragility and vitamin E deficiency. In a study of malabsorptive disorders, for example, Binder et al.[1] stated that an H_2O_2 hemolysis value greater than 20 per cent is indicative of vitamin E lack. Several other investigators have described a close correlation between low serum tocopherol and elevated H_2O_2 red cell fragility in full-term and premature infants.[2-6] The relationship between abnormal in vitro H_2O_2 fragility and vitamin E deficiency is therefore well substantiated in such situations. Examination of the present data reemphasizes, however, that in many clinical situations of widely disparate etiologies the degree of RBC hemolysis does not necessarily reflect the level of antiperoxidant protection afforded by vitamin E. It is therefore important to evaluate hematologic status before equating elevated in vitro H_2O_2 fragility with tocopherol deficiency.

Peroxidation of lipid components of cell membranes involves the nonenzymatic interaction of peroxides, polyunsaturated fatty acids, and oxygen,[16,17] resulting in the production of lipid radicals and additional peroxide

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radicals. This series of reactions is self-perpetuating, and may ultimately result in a selective "leeching" of specific lipid components of the RBC membrane.[18] The relevance of the in vitro H_2O_2 fragility test to in vivo sensitivity of RBC membrane lipids to peroxidation has properly been questioned.[19] However, this determination does provide a gross estimation of the adequacy of the mechanisms which protect red cell lipids from in vitro peroxide attack. Both in vitro and in vivo resistance of erythrocytes is in part dependent on the proper function of a number of cellular antiperoxidant mechanisms other than vitamin E. Decreased ability to deal with peroxides has been noted in several distinct deficiencies of RBC enzymes that have roles in detoxification of H_2O_2.[20-22] Although no correlation between RBC catalase levels and H_2O_2 fragility was seen in iron deficiency and sickle-cell anemia in this study, evaluation of this enzyme and others involved in the hexose monophosphate shunt was incomplete in many patients. The three patients with G6PD deficiency coincident with hereditary sideroblastic anemia had normal H_2O_2 fragilities, which might suggest that the G6PD-deficient RBC has the ability to protect against peroxide attack under conditions of the H_2O_2 fragility test, if not previously challenged by unusual oxidative stress. The combination of these two conditions is, however, uncommon,[23] and may not be representative of the usual G6PD deficiency.

Increased in vitro H_2O_2 fragility can be seen in congenital or acquired defects in RBC function that are not directly related to inadequacies in the usual cellular antiperoxidant mechanisms. Increased peroxide-induced hemolysis has been related to exogenous agents which enhance the rate of lipid peroxidation, such as iron[15] and hyperbaric oxygen.[24] In addition, distinctly different RBC defects may render the red cell more susceptible to in vitro peroxidation by creating instability of membrane lipids. Such may be true in paroxysmal nocturnal hemoglobinuria[25] and autoimmune hemolytic disease.[26]

In the present study, anemias associated with elevated H_2O_2 fragility without coincident vitamin E lack are considered "hemolytic"; that is, abnormal factors present in the structure or environment of the RBC lead to early senescence and demise in the peripheral circulation. An objection might be raised in regard to iron-lack anemia, but significantly decreased RBC survival has been noted in moderate to severe degrees of this condition.[27] The anemias associated with normal H_2O_2 fragility are, with the exception of acute blood loss, states of hypoproliferation or dyspoiesis within the marrow erythron. The contrast in H_2O_2 fragility in the various anemias is most striking within the group of so-called hemoglobinopathies. In hemoglobin C disease, sickle-hemoglobin C disease and sickle-cell disease, H_2O_2 fragility was markedly elevated. Fragility was slightly increased in sickle-thalassemia, and normal in thalassemia minor and intermedia. Thus H_2O_2 fragility was abnormal in those hemoglobinopathies where peripheral RBC destruction plays the major role in the production of anemia, and was normal in states where anemia is primarily the result of ineffective erythropoiesis.

In vivo lipid peroxidation has become the focus of increasing interest as a major factor in the senescent changes which occur in many body tissues.[17] Whether the abnormal in vitro erythrocyte H_2O_2 fragility seen in several of the

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anemias studied herein implicates in vivo peroxide stress as a cause of accelebrated red cell destruction in these conditions remains to be further evaluated.

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