Journal of Clinical Investigation
Vol. 46, No. 3, 1967

Peroxidative Hemolysis of Red Blood Cells from Patients with Abetalipoproteinemia (Acanthocytosis) *

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Summary. The effect of peroxidative stress on tissue was studied by exposure of red blood cells (RBC) from patients with abetalipoproteinemia to minute amounts of H_2O_2 *in vitro*. Red blood cells from untreated patients showed a marked sensitivity to H_2O_2, as evidenced by hemolysis and lipid peroxidation (peroxidative hemolysis).

The appearance of lipid peroxidation products in sensitive cells after exposure to H_2O_2 was indicated by *1*) increases in the 2-thiobarbituric acid (TBA) reaction of trichloroacetic acid extracts, *2*) increases in ultraviolet light absorbency of lipid extracts, and *3*) decreases in polyunsaturated fatty acids. These changes were accompanied by a decrease in phosphatidyl ethanolamine and phosphatidyl serine in the RBC lipid extract. Similar lipid changes on exposure to H_2O_2 were observed in the RBC from vitamin E–deficient rats.

Treatment of the patients with *d*-α-tocopherol polyethylene glycol succinate by mouth, or addition of *dl*-α-tocopherol to the incubation medium protected the RBC from peroxidative hemolysis. Tocopherol appears to provide a primary biologic defense against peroxidative hemolysis.

The presence of nitrite or carbon monoxide, which produced methemoglobin and carboxyhemoglobin, respectively, inhibited peroxidative changes, suggesting a catalytic role for oxy- or deoxyhemoglobin.

Substances that prevented lipid peroxidation also prevented hemolysis; in addition, lipid peroxidation appeared to precede hemolysis. These observations suggested that hemolysis was a consequence of lipid peroxidation.

Introduction

Lipids extracted from human red blood cells are highly susceptible to autoxidation (2). The possibility of a comparable lipid alteration occurring in the intact cell has been suggested by the appearance of substances reactive with 2-thiobarbituric acid when RBC from vitamin E–deficient animals were exposed *in vitro* to hydrogen peroxide (3–6) or dialuric acid (3, 7), or *in vivo* to hyperbaric oxygen (8). Indeed, lipid peroxidation has been implicated as a mechanism for some of the manifestations of vitamin E deficiency in

* Submitted for publication July 1, 1966; accepted November 10, 1966.

This investigation was supported by U. S. Public Health Service grants HE-02907, HE-01045, and HE-06481.

A preliminary report was published in abstract form (1).

† This work was done during the tenure of an Advanced Research Fellowship of the American Heart Association.

‡ Recipient of U. S. Public Health Service Career Research Development Award K3 HE-14-828.

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experimental animals (3, 4, 7, 9, 10) and man (5, 11, 12). Recently, Kayden and Silber reported that patients with abetalipoproteinemia (acanthocytosis) were deficient in vitamin E; in fact, the serum tocopherol levels were the lowest reported for man (13). These patients presented a unique opportunity to investigate lipid oxidation in intact human RBC. Rats with experimental vitamin E deficiency were also studied. Red blood cells were exposed to minute amounts of H_2O_2 as a stressing agent; hemolysis and lipid peroxidation (peroxidative hemolysis) resulted. This report is the first description of alterations in the RBC phospholipid fatty acids that characterize peroxidative hemolysis of RBC from patients or experimental animals with vitamin E deficiency. Moreover, evidence suggests that these lipid alterations portend hemolysis.

Methods

Each of the four following patients with abetalipoproteinemia has been included in previous investigations: A.V. (13), a girl born in 1957; and R.B. (13–17), M.S. (13, 16–18), and R.I. (15, 19–21), boys born in 1956, 1954, and 1953, respectively. Control subjects were healthy males and females between the ages of 20 and 40 who were taking no medication. Vitamin E deficiency was produced in male and female Wistar rats by feeding a "tocopherol-deficient diet" [1] from weaning until the age of 6 months, when this study was performed.

We determined plasma tocopherol levels by a micromethod using the Emmerie–Engle reaction (22). The autohemolysis test was performed by sterile incubation of defibrinated blood at 37° C for 48 hours essentially as described by Young, Izzo, Altman, and Swisher (13, 23); samples were run in duplicate and average values reported.

Exposure of RBC suspensions to H_2O_2 vapor. Heparinized [2] venous blood was stored between 0 and 4° C for up to 18 hours before the RBC were isolated by centrifugation, washed three times in an equal volume of isotonic saline at room temperature, and studied. A 10% suspension of the washed RBC in isotonic saline–phosphate buffer, pH 7.4 (24), was prepared; 4.5-ml samples were placed in 25-ml Warburg flasks and exposed to minute amounts of H_2O_2 by the method of Cohen and Hochstein (25, 26). The 0.3 ml of 30% H_2O_2 that was placed in the center well diffused into the RBC suspension at a rate of approximately 2.3 μmoles per hour [3] while the flasks were shaken in a Dubnoff metabolic incubator 90 to 100 times a minute at 37° C. In order to facilitate lipid extraction in later experiments in which phospholipid and fatty acid analyses were performed, we increased the hematocrit of the RBC suspension to between 30 and 40%.[4]

Analysis of the incubation suspension. The per cent hemolysis was calculated from the hemoglobin concentration, which was determined by a cyanomethemoglobin method (26) on the supernatant of the incubation suspension after centrifugation and appropriate dilution. The value for 100% hemolysis was obtained by measurement of hemoglobin concentration in a sample of the RBC suspension lysed in the saline–phosphate buffer diluted to be equivalent to 0.1% saline.

The TBA test was performed on a trichloroacetic acid extract of a sample of the incubation suspension with absorbency measurement at 532 mμ (7). This test provides an index of lipid peroxidation by determination of the malonylaldehyde released on acid hydrolysis (27, 28).

Analysis of RBC lipid. Lipids were extracted at room temperature from 3.0 ml of the 10% suspension of RBC. After centrifugation at $3,000 \times g$ and aspiration of the supernatant, about 0.3 ml of packed RBC (or ghosts) remained, and this amount was taken as 1 vol for calculation of the quantity of reagents used. One vol of methanol that contained the antioxidant 2,6-di-*tert*-butyl 4 methyl phenol (BHT),[5, 6] 5 mg per 100 ml, and 9 vol of methanol alone were added, mixed, and allowed to stand for 30 minutes. Ten vol of chloroform [7] was added, mixed, and allowed to stand for an additional 30 minutes.

[1] Nutritional Biochemicals Corp., Cleveland, Ohio.

[2] Heparin sodium, 1,000 IU USP per ml, containing benzyl alcohol, 9 mg per ml, from the Upjohn Co., Kalamazoo, Mich., was used in preliminary experiments. The benzyl alcohol interfered, however, with ultraviolet light absorbency measurements. Therefore, when spectrophotometry was performed, another preparation was used: crystalline heparin sodium, 120 IU USP per mg, Connaught Medical Research Labs., Toronto, Canada.

[3] Approximately 5.5×10^{-10} μmole of H_2O_2/RBC per hour.

[4] Approximately 2×10^{-10} μmole of H_2O_2/RBC per hour.

[5] "Ionol, CP," a gift from the Shell Chemical Co., New York, N. Y.

[6] Approximately one part BHT per 100 parts of total lipid, by weight. This amount of BHT was approximately 10 times more than was necessary to prevent autoxidation of dried lipid extracts of normal human RBC (2). As a result of our first experiments with RBC from patients with abetalipoproteinemia in which an antioxidant was not employed, we found it necessary to add BHT in order to prevent the artifactual development of large amounts of lipid peroxides in the extract. BHT did not appear to reduce lipid peroxides, specifically hydroperoxides, once they developed. This conclusion was based on studies of cod liver oil during various stages of autoxidation, which showed that the peroxide value, determined by an iodometric method, did not decrease when BHT was added after autoxidation had occurred. Because BHT has a characteristic absorbence in the ultraviolet region of the spectrum (29), absorbency data were corrected for its presence.

[7] Chloroform was purified by distillation in an all glass system and stabilized with methanol 1:50 (vol/vol).

After centrifugation at 3,000 × *g* for 5 minutes, the supernatant was collected and 10 vol of chloroform followed by 6 of 0.05 M KCl was added, mixed, and allowed to stand overnight at −25° C. When warmed to room temperature, the phases separated cleanly. The lower phase was collected and evaporated *in vacuo* at 40° C. Complete exclusion of the upper phase was essential for reproducible ultraviolet light absorbency measurements. The lipid extract was dissolved in 5 vol of chloroform and analyzed the same day. In experiments where the hematocrit of the incubation suspension was between 30 and 40%, lipids were extracted without prior centrifugation from a 2.0-ml sample that was taken as 1 vol for calculation of the quantity of reagents.

The absorbency at 234 and 268 mμ of the lipid extract dissolved in methanol provides a convenient index of peroxidation of the RBC lipids (2), as conjugated dienes and conjugated trienes that absorb at these wavelengths, respectively, are intermediates during autoxidation and peroxidation of polyunsaturated fatty acids (30). The extinctions were expressed in terms of the molar concentration of lipid phosphorus. To determine lipid phosphorus, an adaptation of Marinetti's modification of Bartlett's method was used (31).

We determined the RBC phospholipid distribution by thin-layer chromatography (TLC) on silica gel HR,[8] 0.5 mm thick, with the developing solvent chloroform–methanol–glacial acetic acid–water 25:15:4:2 (vol/vol) (32) to which we added BHT at a concentration of 50 mg per 100 ml to prevent autoxidation during chromatography (33). The spots were made visible in ultraviolet light by spraying with a dichlorofluorescein solution, and the phosphorus content of each spot was analyzed (31).

To prepare RBC total phospholipid fatty acid methyl esters, we employed TLC of the lipid extract on silica gel HR, 0.5 mm thick, in hexane–diethyl ether–glacial acetic acid 70:30:1 (vol/vol) containing BHT at a concentration of 50 mg per 100 ml. The phospholipid, which remained at the origin, was scraped into an ampul, and boron trifluoride–methanol reagent[9] was added; the ampul was flushed with nitrogen, sealed, and heated in boiling water for 90 minutes according to the method of Morrison and Smith (34). Four ml of pentane[10] and 1.0 ml of water were added and mixed, and the fatty acid methyl esters were recovered in the pentane phase. Long chain hydroxy fatty acid methyl esters, if present, were not recovered quantitatively by the single extraction with pentane (34).

Methyl esters of RBC total lipid fatty acids were prepared without prior lipid extraction as follows: A 1.0-ml sample of the incubation suspension was hydrolyzed in 5.0 ml of aqueous 2 N HCl, which contained 25 μg of *dl*-α-tocopherol[1,11] and 12 μg of BHT, in a sealed tube at 110° C for 18 hours. The fatty acids were extracted with diethyl ether that contained BHT, 5 mg per 100 ml, and the ether extract was dehydrated over Na_2SO_4–$NaHCO_3$, 4:1, transferred to an ampul, and evaporated to dryness with a stream of nitrogen. The fatty acids were converted to their methyl ester derivatives by adding 1.0 ml of BF_3-methanol reagent and heating the sealed ampul for 30 minutes at 100° C. The fatty acid methyl esters were extracted from the BF_3-methanol with pentane and purified by TLC on silica gel HR with development in benzene (34) that contained BHT, 50 mg per 100 ml.

Gas–liquid chromatography (GLC) was performed with a Barber–Colman instrument, model 5000, equipped with paired 8-foot columns of EGSS-X 8% on Gaschrom P, 100/120 mesh,[9] and dual flame ionization detectors. The nitrogen flow rate was 50 ml per minute at the inlet. The column temperature was maintained at 165° C for 10 minutes after injection of the sample, then increased at 2° C per minute to 200° C. Thirty-eight peaks were observed and identified on isothermal runs at 185° C by use of a semilogarithmic plot of retention time data and calculation of type I and type II separation factors according to Ackman and Burgher (35). Known fatty acid methyl ester mixtures (NHI-type mixtures KB, KD, and KF, and mixtures K107, L203, L205, and L207)[9] were used as primary reference standards and cod liver oil fatty acid methyl esters as secondary reference standards (36). Identification was confirmed by GLC of the RBC total phospholipid methyl esters on a less polar column, EGSS-Y 12%,[9] at 190° C, and by GLC of the methyl ester fractions separated according to their degree of unsaturation by TLC of the acetoxymercuri-methoxy derivatives (31, 37).

Peak areas were calculated by means of a Disc chart integrator. Quantitative results with National Heart Institute fatty acid methyl ester standard mixtures KB, KD, and KF[9] agreed with the stated composition data with a relative error of less than 2% for major components (> 10% of the mixture) and less than 3.5% for minor components. BHT eluted with a retention time similar to that of methyl myristate (2). When taken through the BF_3-methanol methylation procedure (34), BHT gave rise to an additional peak, which had a retention time similar to that of methyl palmitoleate (2). Therefore, myristic and palmitoleic acids were not quantified. We recalculated the per cent composition data for RBC samples exposed to H_2O_2 by multiplying the value for each fatty acid by the factor that made the per cent of palmitic acid in the exposed sample equal to the per cent in the control sample. Palmitic acid was selected as the reference peak because it is saturated and should be affected relatively less by peroxidation than unsaturated moieties, and because it is a large component and may be accurately quantified. This method of calculation shows the relative changes that occur in the concentration of the individual acids if palmitic acid is assumed to be constant. Should palmitic acid be lost, however, this method for expression of the data would underestimate losses of other fatty acids. The increase in the relative amount of lignoceric acid (24:0) (Tables

[8] Brinkmann Instruments, Inc., Westbury, N. Y.

[9] Applied Science Laboratories, Inc., State College, Pa.

[10] Distilled in an all glass system over $KMnO_4$ and contained BHT, 5 mg per 100 ml.

[11] One mg = 1 IU.

III, IV, V) does indicate a loss in palmitic acid with peroxidation, presumably because some peroxidized phosphatidyl ethanolamine and phosphatidyl serine, which contain palmitic acid but almost no 24:0 (31), were not in the extract.

Results

RBC from the untreated patients with abetalipoproteinemia showed a marked sensitivity to H_2O_2 (Figure 1). Seventy-nine to 95% of the cells from two untreated patients lysed during 4 to 6 hours of exposure to minute amounts of H_2O_2, whereas less than 3% of the RBC from normal subjects lysed during 10 hours, and less than 7% during 21 hours of exposure.

Evidence was found for lipid peroxidation in the RBC from untreated patients with abetalipoproteinemia after exposure to H_2O_2 *in vitro* (Table I). This evidence consisted of striking increases in the TBA reaction (malonylaldehyde) on the trichloroacetic acid extracts of the cell suspensions and increases in the absorbencies at 234 and 268 mμ (conjugated dienes and conjugated trienes, respectively) of the lipid extracts. Since it appears that certain peroxidized lipids may be lost, in part, during the RBC extraction procedure (see below), the ultraviolet absorbency measured may underestimate the degree of peroxidation that has occurred. Addition of *dl*-α-tocopherol,[12] glucose, or sodium nitrite to the incubation solution prevented hemolysis as well as increase in the TBA reaction during the exposure period; partial protection of the cells was provided by carbon monoxide. In a single experiment with the RBC from patient R.B., the exposure to H_2O_2 was continued for 24 hours; at that time, the to-

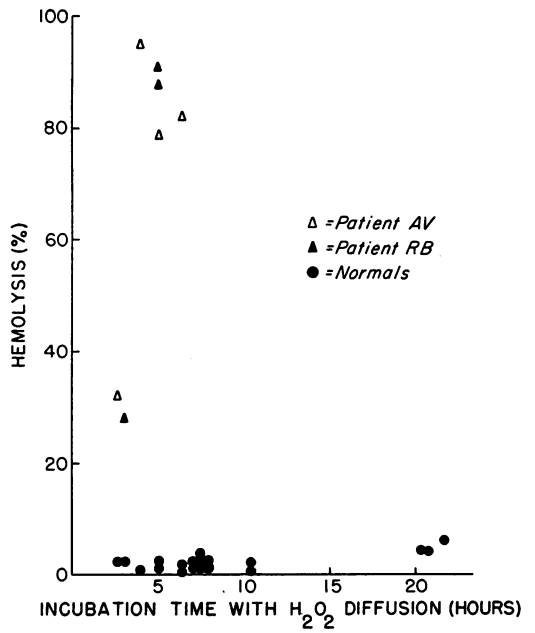

FIG. 1. EFFECT OF EXPOSURE OF RED BLOOD CELLS (RBC) FROM UNTREATED PATIENTS WITH ABETALIPOPROTEINEMIA TO H_2O_2 IN VITRO. Two patients, A. V. and R. B., who had not received vitamin E, were studied on three separate occasions. The control determinations were performed on samples from 10 normal subjects on seven separate occasions.

[12] Calbiochem, Los Angeles, Calif.

TABLE I

Effect of H_2O_2 in vitro on red blood cells (RBC) from untreated patients with abetalipoproteinemia

Conditions	Analyses after incubation of RBC with H_2O_2 diffusion*			
	Analyses of suspension		Absorbency of total lipid†	
	Hemolysis	TBA test	234 mμ	268 mμ
	%	*moles* $\times 10^{-8}$ *malonylaldehyde per ml RBC*	$\epsilon \times 10^{-3}$	
Not incubated		0.8		
Incubated in air	1.5	1.6	1.0	0.4
Incubated with H_2O_2 diffusion‡	87.0	13.9	3.6	2.0
+ *dl*-α-Tocopherol, 0.22 mg per 100 ml	1.5	1.4		
+ Glucose, 200 mg per 100 ml	1.5	1.6		
+ $NaNO_2$, 28 mg per 100 ml	1.5	1.4		
+ CO, 10 ml per 100 ml of atmosphere	5.0	4.8		

* Data are averaged from two experiments with RBC from patient A.V. and one experiment with RBC from patient R.B. The hematocrit of the RBC suspension was 10%. Exposure time was 4 to 6 hours.

† Lipids were extracted without 2,6-di-*tert*-butyl 4 methyl phenol.

‡ In all of the samples incubated with H_2O_2 diffusion alone, hemolysis was >79.0%, 2-thiobarbituric acid (TBA) >12.3, and absorbency at 234 mμ >3.5 and at 268 mμ >1.9. Values for normal controls are shown in Table II.

TABLE II

Effect of vitamin E therapy of patients with abetalipoproteinemia on the sensitivity of their RBC to H_2O_2 in vitro

Patient	Treatment duration	Autohemolysis test	Analyses after incubation of RBC with H_2O_2 diffusion				
			Incubation time	Hemolysis	TBA test	Absorbency of lipid extract 234 mμ	Absorbency of lipid extract 268 mμ
	weeks	%	*hr*	%	*moles × 10^{-8} malonylaldehyde per ml RBC*	$\epsilon \times 10^{-3}$	
A.V.	0	55	4.0	95	14.3		
	1	6.4	4.5	>80*		2.1	0.8
	8	7.2	7.0	2	2.1	0.8	0.3
	12	6.8	4.0	2	1.4	1.2	0.4
R.B.	0	58	4.5	91	14.7		
	1	7.5	4.5	>80*		2.7	1.2
	8	5.8	7.0	2	2.1	0.6	0.2
R.I.	Treated	3.6	7.0	2	1.6		
M.S.	Treated		10.0	2			
	Off 5 weeks	6.0	22.0	16			
	Off 20 weeks	5.6	4.0	94	19.3	1.9	0.8
Normal		†	6.5	1‡			
			7.0	1.4 ± 0.2§	2.1 ± 0.2§	0.9 ± 0.1§	0.3 ± 0.1§
			10.0	2‡			
			20.5	4‡			

* Estimated by visual comparison.
† Normal values in this laboratory are <4.5%.
‡ Averaged from two normal subjects; one was A.V.'s mother.
§ Average ± standard deviation from six normal subjects.

copherol–treated specimen was protected (TBA = 1.3×10^{-8} mole of malonylaldehyde per ml RBC, hemolysis = 10%), but the glucose–treated specimen had exhibited peroxidative hemolysis (TBA = 7.2×10^{-8} mole, hemolysis = 84%).

We provided further evidence for the importance of lipid peroxidation in the hemolysis observed on exposure of RBC from patients with abetalipoproteinemia to H_2O_2 *in vitro* by studying the effect of vitamin E therapy in four patients (Table II). The RBC from two patients, A.V. and R.B., were studied before and during the administration of vitamin E (*d*-α-tocopheryl polyethyleneglycol succinate) [18] equivalent to 750 mg of α-tocopherol daily by mouth. Protection of the RBC against autohemolysis, but not H_2O_2, was noted after 1 week of therapy. After 8 weeks, the RBC were fully protected for at least 7 hours of exposure to H_2O_2. Maintenance of this improvement was documented for patient A.V. after 12 weeks of treatment. Two patients, R.I. and M.S., had received vitamin E in a variety of dosage forms when these tests were first performed. Their RBC gave normal responses to H_2O_2 in this system on incubation for 7 and 10 hours, respectively. Patient M.S. discontinued vitamin E therapy, and his RBC exhibited sensitivity to H_2O_2 when tested several months later; however, at that time his autohemolysis test was not strikingly abnormal.

The fatty acid and phospholipid distribution of the RBC from these patients during vitamin E therapy (38) was similar to that reported previously for untreated patients with abetalipoproteinemia (15, 18, 39), suggesting that the protective effect of vitamin E in these studies was not due to correction of the abnormal RBC lipid distribution. Vitamin E administered orally in a water–soluble form protected the RBC, even though tocopherol could not be determined in the plasma. This discrepancy may be explained either by increased plasma levels that were still below the useful range of the method employed (< 0.08 mg per 100 ml) or by increased tissue levels not reflected in the plasma because of the absence of beta lipoprotein, where most of the plasma tocopherol is found normally; tissue levels were not determined.

Sequential measurements of lysis and lipid peroxidation during exposure to H_2O_2 diffusion were carried out on the RBC of patient M.S., who had not received vitamin E for 6 months. The results from the two experiments done 4 weeks apart (experiments A and B) are shown in Table III. The

[18] Provided by Dr. Stanley Ames, Distillation Products Industries, Rochester, N. Y.

TABLE III

Sequential changes during exposure of RBC from an untreated patient with abetalipoproteinemia to H_2O_2 in vitro*

	Experiment A					Experiment B			
Incubation time with H_2O_2 diffusion (*hours*)	0.0	1.5	2.5	4.5	4.5	0.0	1.8	2.8	4.0
dl-α-Tocopherol added (*μg per ml RBC suspension*)					2.5				
Hemolysis (%)	1	1	7	97	1	1	1	42	69
2-Thiobarbituric acid test (*moles* × 10^{-8} *malonylaldehyde/ml RBC*)	1.4	2.0	6.9	11.9	1.6	1.3	1.9	8.5	12.7
Absorbency of total lipid ($\epsilon \times 10^{-3}$)									
234 mμ						0.88	0.95	1.35	1.75
268 mμ						0.33	0.35	0.53	0.75
Fatty acid distribution in total phospholipid† (*weight* %)									
16:0	20.2	20.2‡	20.2‡	20.2‡	20.6	20.7			20.7‡
18:0 + 18:1 aldehyde§	16.1	16.5	15.1	10.6	17.0	17.3			13.0
18:1	10.7	10.4	10.0	8.8	10.8	10.7			10.0
18:2	2.8	2.8	2.6	2.2	2.7	2.7			2.2
20:3ω6 + 22:0	2.8	2.1	2.2	2.0	2.5	2.4			2.2
20:4ω6 + 22:1‖	14.5	12.7	11.6	5.4	14.4	14.7			6.8
24:0	3.6	3.8	3.6	4.0	3.7	3.6			4.8
22:4ω6 + 24:1¶	13.2	12.6	12.6	10.3	13.2	13.3			13.2
22:5ω6	2.6	2.0	1.9	0.7	2.5	2.3			1.2
24:2	1.4	1.1	1.2	1.1	1.3	1.2			1.6
22:5ω3	2.3	2.0	1.7	0.7	2.4	2.2			1.3
22:6ω3 + 26:0**	4.0	4.5	3.2	1.2	3.9	4.0			1.8
Sum of 10 minor peaks	5.8	4.5	5.1	3.9	5.0	4.9			4.0
Total	100.0	95.2	91.0	71.1	100.0	100.0			82.8
Phospholipid distribution†† (*molar* %)									
Phosphatidyl ethanolamine + polyglycerolphosphatide‡‡						30.4			15.5
Phosphatidyl serine						16.8			10.1
Phosphatidyl inositol‡‡						0.2			2.5
Phosphatidyl choline						18.7			17.4
Sphingomyelin						33.7			33.6
Lysophosphatidyl choline						0.2			0.8
Origin						0.0			0.5
Total						100.0			80.4

* Patient M.S., who had not received vitamin E for 6 months before experiment A and 7 months before experiment B.

† In this abbreviation of the fatty acids, the first two digits state the number of carbon atoms, the third digit states the number of double bonds, and the digit after the omega states the end carbon chain length.

‡ The fatty acid percentages in these incubated samples were recalculated by means of the factor that made the per cent of palmitic acid equal to its value in the control sample.

§ The dimethyl acetal was eluted with this peak and accounted for 12% of the area in control samples; this component remained after exposure to H_2O_2.

‖ 22:1 accounted for 3% of this peak in control samples.

¶ 24:1 accounted for 55% of this peak in control samples.

** 26:0 accounted for 9% of this peak in control samples.

†† Based on the per cent of phosphorus in each spot on thin layer chromatography. Only 80.4% of the lipid phosphorus of the control RBC was obtained in the lipid extract of cells that were exposed to H_2O_2.

‡‡ Not positively identified.

data from experiment A suggest that lipid changes preceded lysis, as *1*) the relative amount of arachidonic acid (20:4ω6) was decreased 12% after 1.5 hours of incubation, although no abnormal hemolysis was evident; and *2*) the TBA reaction was increased 58% of the maximal value after 2.5 hours, when hemolysis was only 7%. The temporal relationship between parameters of lipid peroxidation and hemolysis for experiment A is diagrammed in Figure 2. In experiment B, lysis had already proceeded too far at 2.8 hours, and the temporal relationship could not be determined.

The distribution of the individual phospholipids was analyzed in experiment B (Table III). The lipid phosphorus content of the extract of incubated RBC was only 80.4% of the control value. This finding along with the low values for phosphatidyl ethanolamine (PE) and phosphatidyl

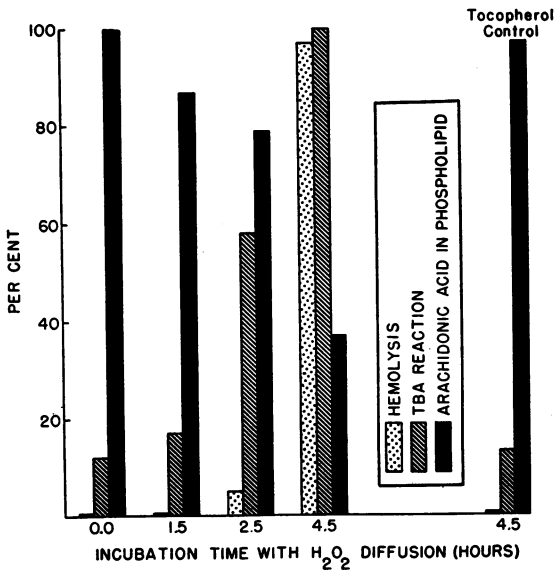

FIG. 2. EFFECTS OF EXPOSURE OF RBC FROM AN UNTREATED PATIENT WITH ABETALIPOPROTEINEMIA (M.S.) TO H_2O_2 IN VITRO, DIAGRAMMED FROM DATA IN TABLE III, EXPERIMENT A. Per cent TBA reaction refers to per cent of 2-thiobarbituric acid reaction at 4.5 hours, and per cent arachidonic acid to per cent of value at 0 time.

serine (PS) suggested that most of the missing PE and PS was not extracted from the RBC or was removed by the washing procedure. This interpretation was supported by the observation of a decreased amount of Ninhydrin staining on the TLC plate. A small amount of Ninhydrin staining in the region of phosphatidyl inositol indicated that the apparent increase shown for that moiety probably represented peroxidized "cephalin" that trailed during chromatography. Large decreases were observed in the relative concentrations of the polyunsaturated fatty acids (Table III). The large amount of arachidonic acid normally present in RBC made it the most useful polyunsaturated fatty acid to quantify as an index of the H_2O_2 effect. The relative concentration of stearic acid (18:0), however, also showed a large decrease. The decrease of this saturated fatty acid probably was related to the decreased phospholipid in the lipid extract after exposure of the RBC to H_2O_2. The stearic acid of human RBC is found mainly in PE and PS and is by far the most abundant fatty acid in the latter phospholipid (31, 40, 41); loss of cephalin from the lipid extract, therefore, may account for a decrease of stearic acid.

Because the decreased extraction of peroxidized phospholipid may influence results of fatty acid analyses of lipid extracts, the fatty acid composition of the total lipid in the incubation suspension was determined without prior extraction. This was accomplished by acid hydrolysis of the RBC suspension, conversion of the fatty acids to methyl esters, and GLC of these derivatives. The results indicated that mainly the polyunsaturated fatty acids were affected by exposure of the RBC to H_2O_2 (Table IV). The decrease of a given fatty acid moiety appeared to be related to the number of double bonds that it contained. The decrease of the peak that contained the polyunsaturated fatty acid 22:4ω6 probably would have been larger if the peak did not also contain a large amount of nervonic acid (24:1). By this method of analysis, stearic acid (18:0) was decreased only 7%.

To assess further the role of vitamin E deficiency in the RBC alterations observed, the RBC from vitamin E–deficient rats were tested in the same manner. The RBC changes that occurred (Table V) were similar to those seen with RBC

TABLE IV

*The effect of H_2O_2 exposure on the RBC total lipid fatty acid distribution determined without prior extraction**

Incubation time exposed to H_2O_2	RBC sample 0	RBC sample 4.0 hours
Hemolysis (%)		>80†
Fatty acid distribution in total lipid‡ (*weight %*)		
16:0	20.0	20.0§
18:0	17.7	16.4
18:1	12.6	10.9
18:2	2.8	2.3
20:3ω6 + 22:0	2.2	1.9
20:4ω6 + 22:1	15.3	5.9
24:0	2.6	3.0
22:4ω6 + 24:1	13.0	10.3
22:5ω6	2.6	1.2
24:2	0.8	1.3
22:5ω3	2.2	0.9
22:6ω3 + 26:0	3.2	1.1
Sum of 10 minor peaks	5.0	5.0
Total	100.0	80.2

* The RBC were from patient M.S., who had not received vitamin E for 7 months before this study.

† Estimated by visual comparison.

‡ Fatty acid methyl esters were prepared by aqueous HCl hydrolysis of the incubation suspension followed by methylation of the extracted fatty acids (see text). Explanation for abbreviations of the fatty acids is given in footnote to Table III.

§ The fatty acid percentages in the incubated sample were recalculated to make the per cent of palmitic acid equal to that of the control sample.

TABLE V

*Effect of exposure of RBC from vitamin E–deficient rats to H_2O_2 in vitro**

	Experiment A						Experiment B	
Incubation time with H_2O_2 diffusion (*hours*)	0.0	0.3	0.7	1.3	2.0	2.0	0.0	1.5
dl-α-Tocopherol added (*μg per ml RBC suspension*)						7.5		
Hemolysis (%)	9	9	12	54	93	11	6	91
2-Thiobarbituric acid test (*moles* × 10^{-8} *malonyl-aldehyde/ml RBC*)	1.3	2.0	3.8	15.1	14.3	1.2	1.2	16.4
Fatty acid distribution in total phospholipid† (*weight* %)								
16:0							25.0	25.0‡
18:0 + 18:1 aldehyde							15.7	12.6
18:1							11.7	10.5
18:2							5.7	5.0
20:4ω6 + 22:1							24.5	9.9
24:0							1.6	1.7
22:4ω6 + 24:1							5.9	4.6
22:5ω6							1.3	0.6
24:2							0.3	0.4
22:5ω3							0.6	0.3
22:6ω3 + 26:0							1.7	0.7
Sum of 11 minor peaks							6.0	4.1
Total							100.0	75.4
Phospholipid distribution§ (*molar* %)								
Phosphatidyl ethanolamine + polyglycerolphosphatide‖							24.0	12.7
Phosphatidyl serine							11.6	6.7
Phosphatidyl inositol‖							5.4	7.3
Phosphatidyl choline							42.1	41.1
Sphingomyelin							13.1	15.3
Lysophosphatidyl choline							3.6	5.4
Origin							0.2	1.5
Total							100.0	90.0

* The hematocrit of the RBC suspension was between 30 and 40%, as in the comparable experiments with human RBC (Table III).

† Explanation for abbreviations of the fatty acids is given in footnote to Table III.

‡ The fatty acid percentages in the incubated sample were recalculated to make the per cent of palmitic acid equal to that of the control sample.

§ Only 90% of the RBC lipid phosphorus in the control sample was obtained in the lipid extract of cells that were exposed to H_2O_2. The individual phospholipids were identified only by mobility on the thin layer plate.

‖ Not positively identified.

from the patient with abetalipoproteinemia. Lipid peroxidation again appeared to precede hemolysis; at 0.7 hour the TBA reaction was elevated, but hemolysis was not increased significantly. Considerable hemolysis was evident, however, before H_2O_2 exposure, and the hemolysis proceeded at a more rapid rate.

Alpha-tocopherol, when added to the incubation medium before exposure to H_2O_2, prevented all of the changes observed in the RBC of patients with abetalipoproteinemia (Tables I and III) and of rats with vitamin E deficiency (Table V).

Discussion

The present study demonstrates the striking vulnerability to H_2O_2 of RBC from untreated patients with abetalipoproteinemia, as evidenced by lipid peroxidation and hemolysis (peroxidative hemolysis). The lipid peroxidation was similar to that seen in spontaneously autoxidized lipid extracts from normal human RBC (*2*). Vitamin E deficiency appeared to be responsible for this vulnerability, as *1*) the patients were shown to be deficient in vitamin E, *2*) vitamin E is a major biologic lipid antioxidant, *3*) administration of vitamin E to the patients or addition to the RBC *in vitro* prevented peroxidative hemolysis, and *4*) the RBC damage in response to H_2O_2 could be duplicated in RBC from vitamin E–deficient rats.

Rose and György were the first to describe the increased hemolysis of RBC from vitamin E–de-

ficient rats upon exposure to H_2O_2 added directly *in vitro;* administration of the vitamin *in vivo* or addition to the RBC suspension *in vitro,* furthermore, prevented hemolysis (42). Slight modifications of their method for H_2O_2 exposure have been used for evaluation of vitamin E levels in premature infants (43, 44), newborn full-term infants (44, 45), and children and adults in a variety of clinical circumstances that includes dietary alterations (5, 11, 44, 46) and malabsorption syndromes (12, 44, 46). In the present study, minute amounts of H_2O_2 were added by continuous diffusion into a suspension of RBC during incubation as described by Cohen and Hochstein (25, 26); this method provides an approximation to the continuous formation of H_2O_2 as it might occur under physiologic conditions. The amount of H_2O_2 required for hemolysis in this system was approximately three orders of magnitude smaller than in the direct H_2O_2 addition test described by Rose and György (42). The low levels of H_2O_2 provided by the diffusion system have helped to elucidate the importance of the enzyme glutathione peroxidase as a primary defense mechanism of RBC against the minute amounts of H_2O_2 that may arise *in vivo* from the action of certain hemolytic drugs (26, 47–49). This H_2O_2 diffusion system was employed in these studies of patients with abetalipoproteinemia to demonstrate the possible consequences of deficiency of vitamin E, another primary biologic defense against peroxidative stress.

Previous studies have shown that hemolysis on exposure to H_2O_2 of RBC from vitamin E–deficient man (5) and animals (4, 6) is accompanied by an increase in the TBA reaction of the lysate. It was also shown that replacement of vitamin E *in vivo* by oral or parenteral routes, or addition of the vitamin to the incubation suspension before exposure to H_2O_2, prevented the increase in the TBA reaction as well as the hemolysis (4–6). The protection afforded by vitamin E appears to be based on its antioxidant properties, because lipid–soluble antioxidants that are not of biologic origin protect vitamin E–deficient RBC during exposure to H_2O_2 *in vitro* (5). These lines of evidence have suggested that peroxidation of lipids may be an important factor in the hemolysis of vitamin E–deficient RBC on exposure to H_2O_2.

Evidence for lipid peroxidation in the present study is based on analyses of the ultraviolet light absorbency and the fatty acid composition of lipid extracts of the incubation suspension, as well as on the results of the TBA test and the protective effect of vitamin E *in vitro* and *in vivo.* Increases in ultraviolet light absorbency paralleled the increase in the TBA reaction. The increased absorbencies at 234 and 268 mμ are consistent with the appearance of conjugated dienes and conjugated trienes, respectively, which are well–established intermediate structures formed during autoxidation of polyunsaturated fatty acids (30). Fatty acid analyses showed a decrease in unsaturated moieties that appeared to be proportional to their number of double bonds. The rates of fatty acid autoxidation have been reported to be many times greater for polyunsaturated than for saturated moieties (50). A similar hierarchy for the susceptibility of tissue lipids based on their degree of unsaturation was shown for heme–catalyzed peroxidation (9, 51).

Evidence was obtained supporting a hypothesis that peroxidation of membrane lipids is the basis for hemolysis on exposure to H_2O_2 *in vitro* of RBC from patients with abetalipoproteinemia and vitamin E deficiency. A time sequence study showed a decrease in the relative amount of phospholipid arachidonic acid before abnormal hemolysis occurred and a large increase in the TBA reaction when hemolysis was starting. Similar TBA changes were observed in the RBC from vitamin E–deficient rats. All procedures tested that prevented peroxidation *in vitro* (i.e., addition to the incubation system of tocopherol, glucose, nitrite, or carbon monoxide) also prevented hemolysis. Finally, after treatment of the patients with vitamin E, their RBC showed increased resistance to H_2O_2 *in vitro.* This effect was presumably due to the antioxidant properties of the vitamin rather than to some possible secondary influence of the vitamin, such as a change in the abnormal distribution of phospholipids and fatty acids in their RBC, because detailed analyses of RBC lipids performed concurrently with this study showed no correction of the altered lipid distribution as a result of treatment with vitamin E (38).

Heme–containing compounds have been recognized as important catalysts of lipid peroxidation in various animal tissues (9). Lipid peroxidation in RBC from patients with abetalipoproteinemia

appeared to be catalyzed primarily by either oxy- or deoxyhemoglobin, because nitrite and carbon monoxide, which produced methemoglobin or carboxyhemoglobin, respectively, inhibited peroxidation. These findings were in agreement with those of Goldstein and Cohen on RBC from vitamin E–deficient rats (6).

The partial protection of RBC from patients with abetalipoproteinemia by addition of glucose to the incubation suspension may be explained by the action of glutathione peroxidase, which catalyzes the destruction of H_2O_2 after it diffuses into the cell (24). In the absence of glucose, the protective glutathione is lost by oxidation; in the presence of glucose, the NADPH required for reduction of oxidized glutathione is derived from hexose monophosphate shunt activity. Tocopherol, however, protected for a longer incubation time than glucose, in agreement with similar observations with RBC from vitamin E–deficient rats (6). These findings indicate that tocopherol provides a primary biologic defense against peroxidative hemolysis.

Anemia is not generally found in patients with abetalipoproteinemia; however, Simon and Ways (16) and Farquhar and Ways (52) have presented evidence and have summarized the work of others that strongly suggests that intermittent hemolysis may occur. Although the causes of this hemolysis have not been defined, H_2O_2 formed *in vivo,* such as by *Mycoplasma pneumoniae* or *M. laidlawii* (53, 54), 8-aminoquinoline antimalarials (47), or menadione (47, 48), may be responsible. Aspirin, phenacetin, and sulfonamides are examples of other drugs that have, in theory, this same capability (47). In addition, exposure of vitamin E–deficient animals to hyperbaric oxygen causes a hemolytic response (4), which appears from recent evidence to be based on peroxidation of membrane lipids (8). Therefore, treatment of patients with abetalipoproteinemia with H_2O_2–producing drugs or hyperbaric oxygen should be approached with caution.

Should peroxidative changes occur in the lipids of RBC *in vivo,* the clinical manifestations may be tempered by that cell's considerable capacity for replacement. In contrast, the nervous system exhibits greater functional differentiation with limited regenerative capacity and has low levels of the protective enzymes glutathione peroxidase and catalase (55). Lipid peroxidation of this tissue may be expected to produce diverse and cumulative changes. Indeed, vitamin E deficiency in laboratory animals is associated with characteristic neurological and neuropathological finding (56–58). A major disability in abetalipoproteinemia is a progressive neurologic disorder (52, 59). The possibility arises, therefore, that the neurologic as well as other clinical manifestations in patients with abetalipoproteinemia may result from lipid peroxidation.

Acknowledgments

The technical assistance of Carole Rockmore, Cathy Berzin, Mildred Medick, Dorothy Dembiec, and Judith Marcus is gratefully acknowledged.

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