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Effect of retinol on the hemolysis and lipid peroxidation in vitamin E deficiency

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Abstract. A study on the effect of retinol *in vitro* on the hemolysis of vitamin E deficient rat red blood cells showed that retinol enhanced the lysis of the E deficient cells as compared to the lysis of normal cells. The lipid peroxidation present during hydrogen peroxide induced lysis of E deficient cells was however markedly inhibited in the presence of retinol without affecting the rate of lysis. In an actively peroxidising system of non-enzymatic lipid peroxidation of rat liver or brain homogenates and of brain lysosomes incubated with human erythrocytes, no lysis was obtained; incorporation of retinol in such systems resulted in lysis but no peroxidation. Hydrogen peroxide generating substances almost completely inhibited the lysis of normal human erythrocytes by retinol, but linoleic acid hydroperoxide and auto-oxidised liver or brain homogenates and ox-brain liposomes increased the lysis. It is concluded that vitamin E deficient erythrocyte hemolysis may be augmented by retinol, an anti-oxidant, having a lytic function without the peroxidation of stromal lipids.

Keywords. Normal human cells; vitamin E-deficient red blood cell; retinol lysis; H_2O_2 generating agents; ox-brain liposomes; lipid peroxidation.

Introduction

Among the well documented lytic actions of retinol, red cell hemolysis has been investigated extensively (Krishnamurthy and Kartha 1973; Lucy 1970). Mega dosing of retinol to rats has been shown to produce oxidative lysis of erythrocytes (Kartha and Krishnamurthy, 1978; Thomas George *et al*., 1981). In an earlier communication we have shown that peroxidation of erythrocyte stromal lipids does not. occur during retinol lysis of normal human erythrocytes (Krishnamurthy and Kartha, 1973). On the other hand we have adduced evidences for the involvement of reactive hydroxyl (OH^-) and H_2O_2 radicals in retinol induced lysis (Krishnamurthy *et al*., 1984). Along with normal human and rat cells, another type, *viz.* the α-tocopherol deficient red blood cells which undergo oxidative damage and lysis during exposure to small amounts of H_2O_2, are also investigated in the present study. Both glucose-6-phosphate dehydrogenase deficient (Cohen and Hochstein, 1965) and vitamin E deficient erythrocytes (Cohen, 1975) respond to menadione during *in vivo* hemolysis. Certain other drugs induce hemolysis, *i.e.* Phenylhydrazine and 8-hydroxy quinoline derivatives have been shown to be due to the toxicity of H_2O_2 generated during the interaction of the drug with oxyhemoglobin (Cohen, 1975). Dialuric acid and glutathione have been shown to generate H_2O_2 on *in vivo* incubation with vitamin E deficient red blood cells resulting in

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hemolysis and lipid peroxidation of cell membranes (Brownlee *et al*., 1977; Goldberg and Stern, 1976). In the present study we are reporting the effect of retinol on two types of cells, normal and vitamin E deficient, in the light of the known antioxidant property, of retinol both *in vitro* and *in vivo* (Kartha and Krishnamurthy, 1978). Furthermore, we have studied the effect of H_2O_2 generating agents and actively peroxidising system of non-enzymatic lipid peroxidation on the course of retinol lysis with both normal and vitamin E deficient erythrocytes. Our results demonstrate that the presence of retinol, markedly augmented vitamin E-deficient erythrocyte-lysis even in the absence of any oxidative challenge and the lysis was unaccompanied by stromal lipid peroxidation.

Materials and methods

Synthetic retinol was obtained from Hoffman-la-Roche, Basle, Switzerland; other chemicals were obtained from SISCO Research laboratories, India.

Human blood, freshly drawn from healthy volunteers, was collected in ACD (acid-citrate-dextrose, National Institute of Health formula A of citric acid, sodium citrate and dextrose) and the separated cells were washed three times with saline phosphate (10 mM sodium phosphate buffer, pH 7·4, containing 0·15 M NaCl) and stored as 20% stock suspension in the same buffer at 4–6°C. Saline phosphate, pH 7·4 (of above composition) was used throughout the studies.

Hemolysis studies were done essentially following the procedure described earlier (Krishnamurthy and Kartha, 1973). Retinol (35 μg) in 0·03 ml of aldehyde free ethanol was emulsified by adding 5 ml of saline phosphate buffer to which was added 5 ml of stock cell suspension. Additives were added either in ethanol or the buffer. Aliquots were withdrawn at specified time intervals for measuring hemolysis and thiobarbituric acid reactive products. Hemolysis with distilled water was taken as total hemolysis and the values were expressed as percentage of the total. Thiobarbituric acid reactive products are expressed in terms of absorbance at 535 nm. For oxidative hemolysis the system was the same as above but contained 220 mM H_2O_2 (final concentration).

Preparation of methemoglobin and carboxyhemoglobin cells

Methemoglobin cells were prepared from normal human erythrocytes by treating them with sodium nitrite at a final concentration of 1% as described by Taylor and Hochestein (1977).

Cells containing carboxyhemoglobin were prepared by exposure to approximately 10 % CO by injecting the gas into the air space of the flask through air-tight rubber caps as described by Kellogg and Fridovich (1975).

Tissue homogenates

Ten per cent homogenates of normal rat liver or brain were prepared in saline phosphate, pH 7·4, centrifuged at 10,000 *g* for 10 min and the supernatants used.

Preparation of ox-brain liposomes

Ox-brain phospholipids were extracted and the liposomes were prepared in saline phosphate following the method of Guttridge (1977). Ox-brain (50 g) obtained from

local slaughter house was used. For preparation of liposomes, 10 mg of the phospholipid was dissolved in 0·2 ml of chloroform, the solvent was completely evaporated under vacuum and 10 ml of saline phosphate was added to the lipid film which was later brought into liposomal form by mechanical agitation and was then immediately used.

α-*Tocopherol deficient rate*

Weanling male albino rats (30) were fed a synthetic diet, consisting of vitamin free casein-20 %, rice starch-50 %, safflower oil-20 %, cellulose 5 % and salt mixture 4 % and vitamin mixture except vitamin E. At the end of six weeks (100-120 g), blood was drawn from the tail vein and tested for oxidative (hydrogen peroxide) hemolysis. About 80% hemolysis and lipid peroxidation of the cells (TBA -index 0·13) were obtained within 60 min showing α-tocopherol deficiency. Rats reared on such E deficient diet were sacrificed at the end of 6 weeks, bled by heart puncture while under light ether anaesthesia, into heparinised syringes. Liver α-tocopherol content was determined following the procedure of Bieri *et al.* (1961) at the end of the feeding experiments and it was less than 1 μg/5 g, confirming that the diet was able to produce adequate α-tocopherol deficiency.

Results and discussion

The results presented in table 1 shows that both normal and vitamin E deficient erythrocytes were readily lysed by retinol but unaccompanied by membrane lipid peroxidation. Lysis of vitamin E deficient erythrocytes in the presence and absence of H_2O_2 was augmented by retinol. Oxidative hemolysis of α-tocopherol deficient erythrocytes was associated with membrane lipid peroxidation confirming previous reports (Brownlee *et al*., 1977;Krishnamurthy and Bieri, 1961). The presence of 35 μg of retinol in the oxidative lysis system of vitamin E deficient erythrocytes enhanced the lysis from 65 % to 100 % but inhibited the thiobarbituric acid reactive material by 38 %.

Table 1. Retinol induced lysis of normal and α-tocopherol deficient rat erythrocytes.

	Percentage hemolysis				
	Time in min				
	0	15	30	45	60
Normal rat cells	0	0	2	5	5(0·00)**
Normal rat cells + retinol (35 μg)	4	30	52	68	86(0·00)**
α-Tocopherol deficient cells	2	5	5	7	8(0·00)
α-Tocopherol deficient cells + retinol (35 μg)	2	40	68	95	100(0·00)
α-Tocopherol deficient cells + H_2O_2*	4	18	31	44	65(0·13)
α-Tocopherol deficient cells + H_2O_2 + retinol (35 μg)	5	53	80	100	100(0·08)

* H_2O_2—final concentration 220 mM.
** Values in parenthesis indicate the increase in absorbance at 535 nm over 60 min.
Retinol was emulsified using 0·03 ml ethanol and this had no effect on the hemolysis.

Retinol at concentrations of 70 μg and 100 μg produced 100% lysis at the end of 15 and 0 min but inhibited peroxidation by 69 and 85% respectively (data not given).

In the case of normal cells also 70 μg and 100 μg of retinol produced complete lysis at the end of 15 and 0 min. Thus retinol, hemolytic agent by itself could augment both the autohemolysis and oxidative lysis of α-tocopherol deficient cells to a marked degree more than observed from normal cells. Further, retinol inhibited the stromal lipid peroxidation of vitamin E deficient cells.

Cohen and Hochstein (1965) have shown that hemolytic drugs like Phenylhydrazine, menadione, 1,2-naphthaquinone and hydroquinone could liberate a flux of H_2O_2 intracellularly with simultaneous oxidation of oxyhemoglobin to methemoglobin. Results in table 2 show the effect of such drugs on retinol lysis of normal human erythrocytes. The hemolysis was almost completely inhibited by the hemolytic drugs. In separate experiments, human erythrocytes containing methemoglobin and carboxy-hemoglobin prepared as described under 'materials and methods' were subjected to retinol induced lysis. Only 10 and 16% hemolysis were observed in methemoglobin and carboxyhemoglobin cells as against 91% for normal human red cells (data not given). These results could mean that the presence of oxyhemoglobin is obligatory for the retinol induced lysis.

Table 2. Effect of H_2O_2 generating agents on retinol induced hemolysis of normal human erythrocytes

		Percentage hemolysis			
	Concentration	Time in min			
Additives	(mM)	0	15	30	60
None	—	4	37	60	95
Phenylhydrazine	1·5	0	0	0	3
Menadione	1·5	0	0	2	6
1,2-naphtha quinone-4-sulphonic acid	1·5	0	2	5	5
Hydroquinone	2·0	0	12	16	18

Values are mean of six separate sets of experiments.

McCay *et al.* (1972) have shown that normal erythrocytes were hemolysed when incubated in an actively peroxidising system of rat liver microsomes, ADP, Fe^{2+} and NADPH and have concluded that the hemolysis was a result of red cell membrane damage due to the enzymatic lipid peroxidation of the microsomes. The effect of non-enzymatic peroxidative system on retinol induced lysis of normal human erythrocytes was studied and results are presented in table 3. Three non-enzymatic peroxidising systems were studied—rat liver, brain homogenates and ox-brain liposomes in the presence of Fe^{2+}. These systems by themselves produced no hemolysis of normal human erythrocytes. The presence of retinol along with the peroxidising system resulted in lysis of human erythrocytes. The degree of lysis was the same irrespective of the presence or absence of the peroxidising system. However, retinol markedly inhibited the lipid peroxidation in all the three systems over a period of 60 min.

Preincubation of rat liver or brain homogenates and liposomes with Fe^{2+} produced

Table 3. Lipid peroxidation and retinol induced hemolysis of normal erythrocytes

	Percentage hemolysis			TBA-index at 535 nm	
	Time in min				
	0	30	60	0	60
Cells[a] + retinol[b]	5	60	96	0·02	0·02
5 ml brain homogenate + cells	0	0	0	0·06	0·80
5 ml brain homogenate + cells + retinol	2	60	96	0·06	0·07
5 ml preincubated brain homogenate + cells + retinol	5	86	100	0·08	0·81
5 ml liver homogenate + cells	0	0	0	0·03	0·54
5 ml liver homogenate + cells + retinol	2	65	100	0·05	0·05
5 ml preincubated liver homogenate + cells + retinol	4	82	100	0·55	0·55
Ox-brain liposomes[c] + cells	0	0	0	0·00	0·02
Ox-brain liposomes[d] + Fe^{2+} + cells	0	0	0	0·04	0·29
Ox-brain liposomes + Fe^{2+} + cells + retinol	2	60	98	0·00	0·00
Ox-brain liposomes preincubated with Fe^{2+} + cells + retinol	10	98	98	0·30	0·29
LAHPO[e] + cells + retinol	8	100	100	0·32	0·30

[a] One ml of 20 % cell suspension of human erythrocytes in saline phosphate.
[b] 35 μg of retinol in 0·03 ml ethanol.
[c] Liposomes were prepared with 10 mg of ox-brain phospholipid in 10 ml saline phosphate. One ml of the suspension was used.
[d] Ox-brain liposomes were incubated with Fe^{2+} (0·4 mM) for 30 min. An aliquot was withdrawn and the absorbance at 535 nm was determined.
[e] LAHPO prepared from pure linoleic acid and equivalent to 100 nmol of MDA were incubated in the system.

appreciable amount of thiobarbituric acid reactive material. Addition of these to the hemolytic system enhanced the retinol induced lysis pointing out that preformed lipoperoxides can increase the lysis by retinol. Further, retinol cannot act as an inhibitor of already formed peroxides. This is further supported by the results of additions of linoleic acid hydroperoxides to the hemolysing system which resulted in a complete lysis of cells over a period of 30 min as against only 60% lysis of normal red-cells by retinol.

The lysis of α-tocopherol deficient cells is shown to be peroxidation dependent (Krishnamurthy and Bieri, 1961). According to Brownlee *et al.* (1977) in the E deficient cells undergoing oxidative lysis the stromal lipid peroxidation is initiated by singlet oxygen radical while the lysis is attributed to hydroxyl radical. Retinol has been shown to inhibit mixed function oxidase by its ability to quench singlet oxygen (Hill and Shih, 1974) and the results of the present study may be taken as confirming the ability of retinol to quench singlet oxygen and thus preventing lipid peroxidation but not hydroxyl radicals, which is probably the cause of lysis, as reported by us (Krishnamurthy *et al.*, 1984) in an earlier study.

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