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ROLE OF RED CELL MEMBRANE LIPID PEROXIDATION IN HEMOLYSIS DUE TO PHENYLHYDRAZINE

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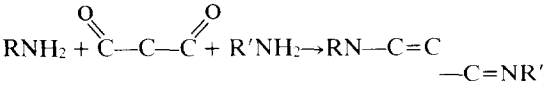
Abstract—Although red cell membrane lipid peroxidation has been identified as a consequence of certain oxidizing hemolytic drugs, the relative contribution of lipid peroxidation to red cell damage leading to hemolysis is unclear. This has been evaluated by studying the response to phenylhydrazine of vitamin E-deficient rats as compared to vitamin E-supplemented rats. Following repetitive phenylhydrazine injections, a lower hematocrit was observed in the vitamin E-deficient group which was associated with higher levels of lipid peroxidation, as indicated by the fluorescence of lipid-containing red cell extracts. However, no significant difference in the initial extent of hemolysis following phenylhydrazine injection was observed. Evidence was also obtained suggesting that malonaldehyde, a decomposition product of polyunsaturated fatty acids, is capable of cross-linking hemoglobin to the red cell membrane. These findings suggest that red cell membrane lipid peroxidation is of relatively minor consequence in the acute response to phenylhydrazine but may be of importance in chronic hemolysis due to this oxidizing drug.

The peroxidation of red cell membrane unsaturated fatty acids has been suggested as contributing to shortened red cell survival in a number of hemolytic states. These include oxidant drug-induced hemolysis, thalassemia major, paroxysmal nocturnal hemoglobinuria, and the anemia of vitamin E-deficient premature infants [1–5]. With the exception of vitamin E-deficient states, the evidence that lipid peroxidation plays a role in hemolysis has been indirect, being based mainly on *in vitro* studies. Recently, we demonstrated the presence of fluorescent products consistent with *in vivo* lipid peroxidation in extracts of red cells freshly obtained from patients receiving the oxidant hemolytic drug diaminodiphenylsulfone, from premature infants deficient in vitamin E, and from rats and rabbits treated with phenylhydrazine or acetylphenylhydrazine [6, 7]. The observed fluorescence is due to the cross-linking of red cell membrane lipid amino groups by malonaldehyde (MDA), a decomposition product of peroxidized polyunsaturated fatty acids [7, 8]. The reaction proceeds through the formation of Schiff bases resulting in an aminoiminopropene derivative:



While the demonstration of such fluorescence indicates that lipid peroxidation does occur *in vivo*, and can occur despite normal serum vitamin E levels, these findings do not conclusively demonstrate that lipid peroxidation contributes to the shortened red cell survival of Heinz body hemolytic processes. In order to test the role of cell membrane lipid peroxidation in oxidant hemolysis, we have studied the effect of vitamin E deficiency on the response of rats to phenylhydrazine. Inasmuch as the major function of vitamin E is to prevent cell membrane lipid peroxidation, vitamin E deficiency should produce an enhanced phenylhydrazine effect if lipid peroxidation does contribute to phenylhydrazine-induced Heinz body hemolysis. In addition, we have assessed the possibility that malonaldehyde might cross-link hemoglobin to the red cell membrane, another potentially deleterious effect of membrane peroxidation.

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METHODS

Animal studies. Sprague–Dawley rats, 200–250 g, were used throughout. Ten days after arrival in the animal quarters, the rats were all begun on a vitamin-fortified tocopherol-deficient test diet (Nutritional Biochemicals, Cleveland, OH). The vitamin E replete groups received thrice weekly intraperitoneal injections of an aqueous emulsion of alpha-tocopherol, 2 mg/kg body wt, using Emulphor 620 (a gift of L. Machlin and M. Brin, Hoffmann-La Roche, Nutley, NJ) as the emulsifying agent. The vitamin E-deficient group was similarly injected with equivalent amounts of Emulphor 620, a polyoxyethylated fatty acid derivative. Phenylhydrazine was prepared in isotonic 0.01 M phosphate-buffered saline, pH 7.4 (PBS), on the day of intramuscular injection.

Tail blood samples were collected in heparinized hematocrit tubes, which were centrifuged for determination of hematocrit. Blood samples were obtained prior to injection of phenylhydrazine on that day. Reticulocyte and Heinz body counts were performed by standard techniques. The dialuric acid hemolysis test was performed as an indirect assay of vitamin E status [9]. Fluorescence was measured as described previously with some modifications due to the small blood sample [6, 7]. After centrifugation, packed cells were expressed from the capillary

hematocrit tubes and washed twice in PBS (0.01 M), pH 7.4 In order to avoid possible fluorescing contaminants in different heparin preparations, the same lot of capillary hematocrit tubes was used throughout. The lipid from 50 μl of washed packed cells was extracted using a scaled-down version of the procedure of Rose and Oklander [10]. After filtration through glass wool, the sample was placed in a quartz microcuvette (0.3 ml) and fluorescence determinations were made in a Hitachi–Perkin–Elmer MPF-3 spectrofluorometer using a microcell holder with instrumental settings as described previously [6,7].

Hemoglobin binding studies. A study of the effects of malonaldehyde on hemoglobin binding to the red cell membrane was performed utilizing human blood. These experiments were carried out in conformance with the Declaration of Helsinki and with approval from the New York University Medical Center Human Experimentation Committee. Blood was washed three times in 310 milliosmolar (mOsM) phosphate buffer, pH 7.0, and resuspended in this buffer to a hematocrit of 10%. Malonaldehyde was prepared as described previously [11] in 310 mOsM phosphate buffer, pH 7.0. Following overnight incubation of red cells in various concentrations of malonaldehyde, hemoglobin that was bound to the red cell membrane was measured by the pyridine hemochromogen method as modified by Dodge *et al.* [12].

Studies of the binding of hemoglobin to the red cell membrane were also performed in a reconstituted system consisting of ghosts and hemoglobin, with or without added malonaldehyde. In these experiments, an aliquot of washed red cells was hemolyzed by freezing and thawing. Following centrifugation at 16,000 g for 30 min, the ghost pellet was discarded and the hemoglobin solution was diluted in 310 mOsM phosphate buffer, pH 7.0, to a concentration of 5 g/100 ml. Red cell membranes were prepared from the remaining red cells by a modification of the procedure of Dodge *et al.* [12] in which the last wash is performed in 5 mOsM Tris buffer, pH 7.4. The ghosts were then resuspended in 310 mOsM phosphate buffer, pH 7.0, to a final concentration of 1 mg/ml. Aliquots of these ghosts were pretreated with 3 mg/ml fluorescamine or with 5 mM *N*-ethylmaleimide for 30 min at 25°. All of the ghost samples were then washed twice in 310 mOsM phosphate buffer, pH 7.0, and resuspended in the hemoglobin solution at a ghost concentration 1 mg/ml. Two aliquots of each treatment condition were prepared, to one of which was added malonaldehyde in 310 mOsM phosphate buffer, pH 7.0, to a final concentration of 4 mM, while the other received an equivalent amount of buffer. After incubation for 16 hr the ghosts were washed three times in buffer and then analyzed for ghost hemoglobin concentration.

RESULTS

Animal studies. Two studies of the effects of a vitamin E-deficient diet on the response of rats to phenylhydrazine were done. In the first, the animals were divided into four groups of six rats, all receiving the vitamin E-deficient diet. Groups I and III were

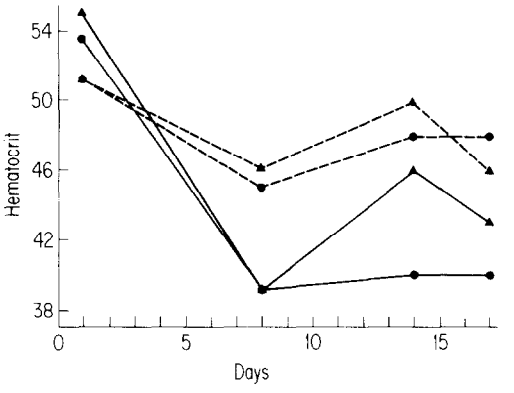

Fig. 1. Hematocrit of vitamin E-deficient diet (●) and control (▲) rats injected with 20 mg/kg phenylhydrazine (———) or with placebo (– – –) daily.

treated with tocopherol (2 mg/kg body wt, three times weekly) and groups II and IV received intraperitoneal injections of placebo. After 10 days of this dietary regimen, groups I and II were begun on daily intramuscular injections of phenylhydrazine (20 mg/kg body wt) in PBS, while groups III and IV received equivalent amounts of PBS alone. No significant difference in the hematocrit or fluorescence of red cell extracts was observed between the vitamin E-deficient and the vitamin E-supplemented groups not injected with phenylhydrazine (Figs. 1 and 2). An identical hematocrit was noted in the two phenylhydrazine-treated groups on day 8 of the study. However, the hematocrit in the vitamin E-deficient group was significantly lower than in the vitamin E-treated group after phenylhydrazine treatment for 14 days (39.7 ± 2.1 vs 45.7 ± 2.2; $P < 0.05$) and 17 days (40.3 ± 1.3 vs 43.3 ± 1.0; $P = 0.05$) (Fig. 1). A significantly higher level of fluorescence in red cell extracts was present in the vitamin E-deficient diet group on day 17 (136.5 ± 8.4 vs 115.7 ± 5.4; $P < 0.05$) but not earlier (Fig. 2). No increase in dialuric acid hemolysis was observed in the vitamin E-deficient diet group injected with PBS until day 14 when mean hemolysis was 17 per cent, and on day 17 when it was 26 per cent (control < 3 per cent). The dialuric acid hemolysis test was found

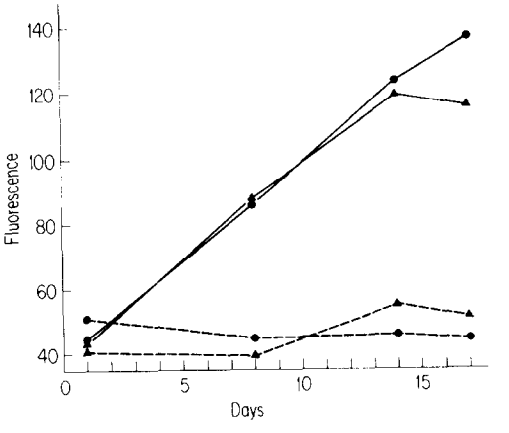

Fig. 2. Fluorescence in red cell extracts of vitamin E-deficient diet (●) and control (▲) rats injected with 20 mg/kg phenylhydrazine (———) or with placebo (– – –) daily.

to be inappropriate for the assay of vitamin E status in phenylhydrazine-treated animals due to the relative resistance of reticulocytes and young red cells to dialuric acid.

These findings were interpreted as suggesting that, as vitamin E deficiency developed, phenylhydrazine injection resulted in an increased level of fluorescence and lower hematocrit. In order to evaluate this further, a subsequent experiment was performed in which the study rats were made more profoundly vitamin E-deficient before being injected with phenylhydrazine. Specifically, the second experiment consisted of two groups of eight rats both placed on a vitamin E-deficient diet without supplementation for 5 weeks at which time there was more than 90 per cent dialuric acid hemolysis. Group I then began receiving vitamin E (2 mg/kg body wt three times weekly) while group II received placebo. One week later, at which time the dialuric acid hemolysis test had returned to normal in Group I, both groups were started on intramuscular injections of phenylhydrazine (15 mg/kg body wt, 5 days/week) while continuing to receive vitamin E or placebo.

No difference in the hematocrit was observed prior to injection of phenylhydrazine, or on day 3 of phenylhydrazine. At subsequent time periods, the hematocrit was significantly lower ($P < 0.05$) in the vitamin E-deficient as compared to the vitamin E-treated group (Fig. 3). The levels of fluorescence in red cell extracts were similar prior to phenylhydrazine injection. Subsequently, higher fluorescence was present in the vitamin E-deficient group (Fig. 4). The differences were statistically significant ($P < 0.05$) except for day 12 ($P = 0.10$). Impaired erythropoiesis, rather than increased hemolysis, is an alternative explanation for the lower hematocrit in the vitamin E-deficient group. This is unlikely inasmuch as at the end of the study the reticulocyte counts in the vitamin E-deficient rats (41.9 ± 2.8) were slightly higher, although not significantly so, than in the control animals (39.8 ± 2.4). In both groups of animals, Heinz bodies were present in more than 90 per cent of the red cells that were not reticulocytes.

Hemoglobin binding studies. Overnight incubation of intact human red cells with malonaldehyde produced an increase in the amount of hemoglobin that remained associated with the red cell membrane following preparation of ghosts (Fig. 5). The extent of hemoglobin binding was linearly related to the concentration range of malonaldehyde used in these studies. Additional washing of the ghosts in 5 mOsM Tris buffer, pH 7.4, or 20 mOsM phosphate buffer, pH 7.4, resulted in less than 10 per cent further decrease in ghost hemoglobin concentration.

The addition of malonaldehyde to a suspension of ghosts incubated overnight in a 2 g/100 ml hemoglobin solution also produced an increase in the binding of hemoglobin to the ghosts. This effect was substantially diminished by pretreating the ghosts with fluorescamine, an amine-blocking agent (Table 1). The latter finding is consistent with the effect of malonaldehyde being manifested through a Schiff base crosslink between the carbonyl groups of malonaldehyde and red cell membrane and hemoglobin amino groups.

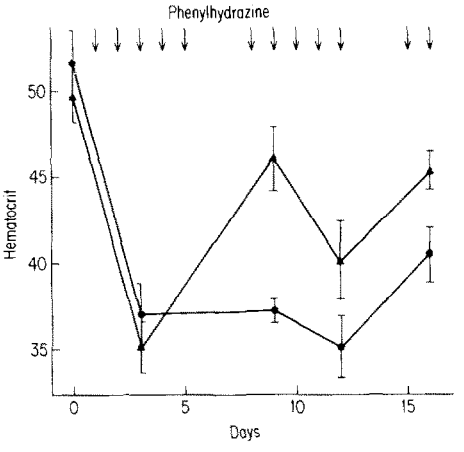

Fig. 3. Hematocrit of vitamin E-deficient diet (●) and control (▲) rats injected with 15 mg/kg phenylhydrazine 5 days/week (indicated by arrows).

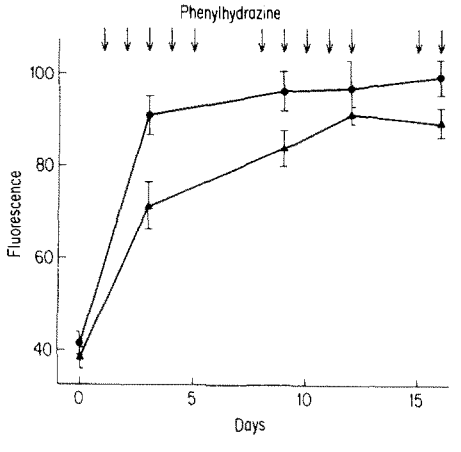

Fig. 4. Fluorescence in red cell extracts of vitamin E-deficient diet (●) and control (▲) rats injected with 15 mg/kg phenylhydrazine 5 days/week (indicated by arrows).

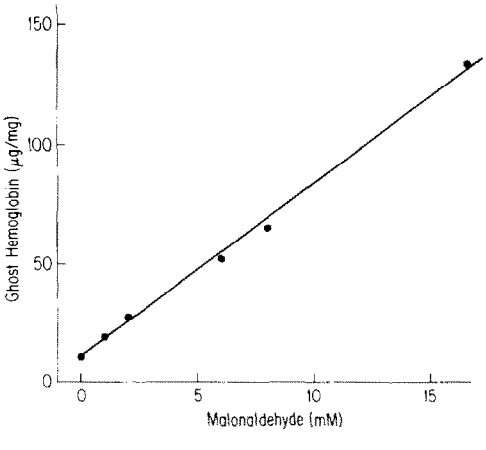

Fig. 5. Binding of hemoglobin to ghosts prepared from 8% red cell suspensions incubated for 16 hr at 25° in various concentrations of malonaldehyde.

Table 1. Ghost hemoglobin levels following incubation of ghosts (1 mg/ml) in hemoglobin for 16 hr with or without malonaldehyde (MDA)*

Pretreatment	MDA (4 mM)	Ghost hemoglobin† (μg/mg protein)
None	+	40.6
		4.2
Fluorescamine (3 mg/ml)	+	6.4
		5.2
N-Ethylmaleimide (5 mM)	+	37.6
		4.6

* Ghost samples were pretreated with fluorescamine, an amine blocking agent, or with *N*-ethylmaleimide, a sulfhydryl blocking agent.

† Mean of duplicate determinations in two studies.

DISCUSSION

Evaluation of patients with Heinz body hemolytic anemias due to the oxidant drug diaminodiphenylsulfone suggested that fluorescent aminoiminopropene membrane cross-links developed relatively slowly and were present primarily in older red cells [6]. That such cross-links accumulate over time was also suggested by the findings in animals receiving phenylhydrazine and *in vitro* studies in which red cells were incubated with preformed MDA [6, 7]. In the present studies, fluorescent evidence of lipid peroxidation also increased over time despite the expected gradual return in the hematocrit toward normal with continued phenylhydrazine administration. Of note is the fact that significantly lower hematocrits were observed in the vitamin E-deficient groups only after continued phenylhydrazine treatment and not as part of the initial acute response. This apparent lack of enhancement of the immediate phenylhydrazine effect by vitamin E deficiency does not rule out a role for lipid peroxidation in acute hemolysis. It does suggest that the many other potentially hemolytic processes resulting from phenylhydrazine, including production of the superoxide anion and other radicals, as well as Heinz body formation and sulfhydryl oxidation, may be of greater immediate significance [13,14]. However, lipid peroxidation does appear to contribute to hemolysis following continued phenylhydrazine administration.

The mechanism by which cell membrane lipid peroxidation produces hemolysis is unknown. It is difficult to distinguish the effects of this process from that of other oxidative events occurring as a result of phenylhydrazine or other inducers of free radicals and active oxygen species. In particular, there is excellent evidence that Heinz bodies potentiate splenic destruction of red cells [15] and that sulfhydryl oxidation leads to shortened red cell survival [16]. Peroxidation of unsaturated fatty acids produces further free radical formation, and it is conceivable that such radicals might result in further oxidative denaturation of hemoglobin as well as oxidation of red cell membrane sulfhydryl groups. However, this would be expected to be a relatively minor source of oxidative stress in comparison to the direct effect of active species derived from the interaction of phenylhydrazine with hemoglobin.

A possible mechanism by which lipid peroxidation may potentiate hemolysis *in vivo* is through alteration of cell membrane fluidity. This could be due directly to the loss of polyunsaturated fatty acids inasmuch as diene bonds contribute to membrane fluidity. In addition, the formation of membrane aminoiminopropene cross-links would have deleterious consequences to normal cell membrane function. We have shown previously that incubation with malonaldehyde produces an increase in red cell membrane protein viscosity [11]. Current concepts of red cell membrane viscosity stress the importance of movement of molecules within a relatively fluid matrix [17,18]. Cross-linking of membrane molecules by MDA presumably interferes with such motion, thereby increasing viscosity. This could, theoretically, result in destruction of red cells by the reticuloendothelial system. The present observation that an increase in such cross-links, as indicated by fluorescence, is associated with a lower hematocrit is at least consistent with such a mechanism.

In this study, we have also explored the possibility that lipid peroxidation might contribute to the binding of hemoglobin to the cell membrane by way of a malonaldehyde cross-link. There have been two suggested mechanisms for the attachment of hemoglobin to the red cell membrane in oxidative hemolytic states: a covalent linkage in which oxidation of both a cell membrane sulfhydryl group and a hemoglobin sulfhydryl group results in a mixed disulfide [19], and a non-covalent hydrophobic bonding interaction [20]. The present findings suggest a third possible mechanism, that of an aminoiminopropene cross-link between hemoglobin and the cell membrane. In view of the relatively slow reaction rates, cross-linking by this process could occur only in a chronic oxidative hemolytic state. Furthermore, it should be emphasized that the evidence supporting such a Schiff base cross-link between hemoglobin and the red cell membrane is indirect, being based on an *in vitro* model using high levels of malonaldehyde. Methodological problems related to potential quenching by heme and its derivatives interfere with direct determination of fluorescence due to aminoiminopropene derivatives of hemoglobin. Additional chemical techniques will be necessary to confirm that such cross-links can occur *in vivo*.

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REFERENCES

1. T. L. Dormandy, *Br. J. Haemat.* **20**, 457 (1971).
2. E. A. Rachmilewitz, B. H. Lubin and S. B. Shohet, *Blood* **47**, 495 (1976).
3. C. E. Mengel, H. E. Kann, Jr. and W. D. Meriwether, *J. clin Invest.* **46**, 1715 (1967).
4. F. A. Oski and L. A. Barness, *J. Pediat.* **70**, 211 (1967).
5. G. E. Mengel, *Am. J. med. Sci.* **255**, 341 (1968).
6. B. D. Goldstein and E. M. McDonagh, *J. clin. Invest.* **57**, 1302 (1976).

7. B. D. Goldstein, M. G. Rozen and M. A. Amoruso, *J. Lab. clin. Med.* **93**, 687 (1979).
8. K. S. Chio and A. L. Tappel, *Biochemistry* **8**, 2821 (1969).
9. C. S. Rose and P. Gyorgy, *Am. J. Physiol.* **168**, 414 (1952).
10. H. G. Rose and M. Oklander, *J. Lipid Res.* **6**, 428 (1965).
11. B. D. Goldstein, G. W. Falk, L. J. Benjamin and E. M. McDonagh, *Blood Cells* **2**, 535 (1976).
12. J. T. Dodge, C. Mitchell and D. J. Hanahan, *Archs Biochem. Biophys.* **100**, 119 (1963).
13. D. W. Allen and J. H. Jandl, *J. clin. Invest.* **40**, 454 (1961).
14. B. Goldberg, A. Stern and J. Peisach, *J. biol. Chem.* **251**, 3045 (1976).
15. R. A. Rifkind, *Blood* **26**, 433 (1965).
16. H. S. Jacob and J. H. Jandl, *J. clin. Invest.* **41**, 1514 (1962).
17. P. L. LaCelle, *Semin. Hemat.* **7**, 355 (1970).
18. R. A. Cooper and S. J. Shattil, *New Engl. J. Med.* **285**, 1514 (1971).
19. H. S. Jacob, *Semin. Hemat.* **7**, 341 (1970).
20. C. C. Winterbourn and R. W. Carrell, *Br. J. Haemat.* **25**, 585 (1973).