Effect of Reduced Glutathione on Erythrocyte Lipid* (32620)

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Previous studies in this laboratory demonstrated that red cells from patients with paroxysmal nocturnal hemoglobinuria (PNH) lysed excessively and formed abnormally large quantities of lipid peroxides during incubation with H_2O_2(1). Those studies suggested an abnormality of the lipid in PNH RBCs with respect to peroxidation. More recently it was demonstrated that normal red cells incubated with sulfhydryl compounds acquired the usual *in vitro* lytic features characteristic of PNH(2,3). The purpose of the present investigation was to study the effect of incubation of normal red cells in solutions of reduced glutathione with respect to lysis and lipid peroxidation of intact cells by H_2O_2 and peroxidation of extracted lipid by ultraviolet radiation.

Materials and Methods. 1. Blood drawn by venipuncture from normal laboratory personnel and defibrinated in sterile containers with glass beads was centrifuged at 3000 rpm for 10 min and the erythrocytes washed three times with physiologic saline after removal of serum and buffy coat.

2. Fourteen ml of the washed packed erythrocytes were incubated in 56 ml of reduced glutathione[1] (GSH) (210 mg/ml at pH 8.0) (0.67 *M*) at 37°C by shaking in a 50 ml Erlenmeyer flask on a Dubnoff metabolic incubator at 60 oscillations/min for 15 min. Lower concentrations of GSH achieved the same results but less consistently. Therefore, the data included here relates to the higher (0.67 *M*) concentration. Control samples of washed packed red cells in saline at a comparable pH and ionic strength were incubated simultaneously and carried through all procedures. This control eliminated the possibility that changes observed were due to hypertonicity. After incubation the red cells were centrifuged at 3000 rpm for 5 min, the supernatant removed and the cells washed five times with physiologic saline. All washes at this point were titrated for measureable —SH groups None were ever demonstrated in the fourth and fifth wash.

3. Acid-serum lysis, thrombin lysis(4) and sucrose lysis(5) tests were performed on each sample of GSH-treated cells to confirm their PNH-like features.

4. Studies of red cell lysis and lipid peroxidation by H_2O_2 were carried out exactly as described previously(6).

5. Peroxidation of lipid extracted from RBCs was carried out as follows. Four ml of packed cells were hemolyzed to a final volume of 25 ml with H_2O and used for lipid extraction, performed as described by DeGier *et al.* (7) except that 30 ml of 0.05 *M* KCl was used instead of water to wash the final chloroform extracts. The final chloroform layer was evaporated to dryness at 27°C under vacuum and collected in a final total volume of 9 ml of physiologic saline. The lipid extract was used immediately or frozen at —70°C under nitrogen for storage. Lipid extracts thus obtained were white and opalescent and no gross differences were observed among the study groups. The total lipid content of the lipid extracts was quantitated as lipid phosphorus and measured according to the method of Lowry(8). Quantities of lipid (as lipid P) extracted from each batch of cells (control and GSH-treated) did not vary significantly. Thorough mixing of the lipid extract "suspension" before sampling resulted in reproducible concentrations of lipid in each sample. Therefore, the quantities of lipid used in subsequent studies were the same in each experiment for control and GSH-treated cells. The volume and lipid content of control and test samples were the same in each simultaneous study. The diene and triene content of the lipid extracts were determined by absorbance

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[1] Sigma Chemical Co.

TABLE I. *In Vitro* Lytic Features of PNH Erythrocytes.

Sample	Hemolysis (%)[a]			
	Serum			Sucrose
	No acid	Acid	Acid and thrombin	
Control RBCs	0	0	0	0
"Natural" PNH RBCs	0	15–40	25–60	6–30
Normal RBCs + reduced glutathione	0	20–60	40–80	5–25

[a] Values given represent the ranges obtained with 12 separate determinations.

TABLE II. Lysis and Lipid Peroxide Formation in Normal GSH-Treated RBCs during Incubation with H_2O_2.

Sample	Lysis[a] (%)	Lipid peroxides[b] (abs. at 535 mμ)
Control RBCs	17 ± 4.5	.122 ± .030
Normal RBCs + reduced glutathione	54 ± 11	.152 ± .015

[a] Values represent the mean ± 1 SD of 6 separate determinations, each carried out in triplicate.
[b] Values represent the mean ± 1 SD of 11 separate determinations, each carried out in duplicate.

in absolute methanol at 234 and 268 mu respectively(9) as an index of peroxidation of lipid(10).

Portions of lipid extract (2–4 ml) were shaken at 100 oscillations/min in a 100-ml quartz Erlenmeyer flask 11 cm below the ultraviolet source (General Electric G15-T8) for 2 hours. After incubation, 2 ml of the extract were mixed with 2 ml of 10% trichloroacetic acid and filtered through Whatman no. 3 paper. One ml of the filtrate was then mixed with 1.2 ml of 0.67% thiobarbituric acid (TBA), heated in a boiling water bath for 15 min, allowed to cool to room temperature and the absorbance read at 535 mμ. Tetraexythypropane (which hydrolyzes to ethanol and malonylaldehyde) was used in the TBA reaction as a standard and lipid peroxides were expressed as mμmoles malonylaldehyde/μg lipid phosphorus(11).

Results. The development of PNH-like *in vitro* lytic features in normal cells after incubation with GSH is shown in Table I. All values obtained were similar to those of "natural" PNH red cells. No lysis in acidified serum occurred after heating of serum at 56°C for 1 hour. These cells also consistently had lower GSH content than normal cells or their control-incubated counterparts. As shown in Table II cells incubated with GSH also showed an increased lytic sensitivity to H_2O_2 ($p<.001$) and formed larger quantities of lipid peroxides than control cells ($p<.025$). Data of diene and triene content of lipid extracts are presented in Table III. Lipid extracted from GSH-treated cells consistently gave significantly ($p<.01$) higher values than that of the control cells. The effect of UV radiation of red cell lipid extracts is shown in Table IV. Lipid extracts from GSH-treated cells consistently formed significantly greater quantities of lipid peroxides than lipid of control cells ($p<.001$).

Discussion. In previous efforts to define

TABLE III. Diene and Triene Content of Erythrocyte Lipid Extracts.

	Absorbance[a]	
	Dienes (234 mμ)	Trienes (268 mμ)
Control RBCs	.139 ± .050	.065 ± .014
Normal cells + reduced glutathione	.266 ± .070	.152 ± .044

[a] Values given represent the mean ± 1 SD of 6 separate determinations, each performed in duplicate.

TABLE IV. Effect of Ultraviolet Radiation on Red Cell Lipid.

Sample	Lipid peroxides[a] (mμmoles malonylaldehyde/10^{-2} μgP)	
	Before UV	After UV
Control RBCs	0	69 ± 12
Normal RBCs + reduced glutathione	0	143 ± 34

[a] Values given represent the mean ± 1 SD of 6 separate determinations.

the intracorpuscular defect of PNH we showed that PNH erythrocytes lysed excessively and formed abnormally large quantities of lipid peroxides during incubation with H_2O_2. In addition, lipid extracted from PNH erythrocytes formed greater than normal quantities of lipid peroxides during exposure to ultraviolet radiation. Since normal RBCs incubated with reduced glutathione were shown to acquire *in vitro* lytic features considered typical of the PNH red cell, the present study was undertaken to investigate the possibility that abnormal behavior of lipid with respect to peroxidation was also a feature of these "induced" PNH-like cells.

These studies have shown that normal RBCs incubated with GSH lyse excessively during incubation with H_2O_2 and that the lipid of these cells is altered in a manner which renders it more susceptible to lipid peroxidation. The increased diene and triene content immediately after GSH incubation, which can be considered good evidence of lipid peroxidation, suggested that peroxidation of lipid had been initiated during incubation with GSH. That this effect occurred simultaneously as the cells acquired other *in vitro* lytic features of "natural" PNH cells may be taken as additional evidence that peroxidation of lipid is involved in the corpuscular defect of PNH.

Since lysis of PNH cells in acidified serum and sucrose solutions of low ionic strength presumably results from enhanced attachment or activity of complement under these conditions, the present studies may also provide clues to a better understanding of that fundamental reaction.

The mechanism by which GSH-incubation produces these effects has not been established. That no GSH could be found in the final washes of the cells after GSH incubation and cells actually had lower GSH *after* the incubation made it unlikely that excess GSH had been carried over into the lipid extract and accounted for this difference. Although the present studies indicate an ultimate effect on RBC lipid, sulfhydryl compounds are known to oxidize both protein and lipids(13) and may result in accumulation of H_2O_2 under appropriate conditions (14). These alternatives or associated possibilities are currently under investigation.

Summary. The data presented herein demonstrate that as normal cells incubated with GSH assume *in vitro* lytic features usually considered typical of PNH erythrocytes, they also lyse excessively and form greater than normal quantities of lipid peroxides when incubated with H_2O_2. The results suggest that GSH in creating the PNH-like cell ultimately affects erythrocyte lipid. These observations are consistent with our hypothesis that peroxidation of red cell lipid may be involved in the PNH corpuscular lesion.

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