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HYDROXYUREA HAS THE CAPACITY TO INDUCE DAMAGE TO HUMAN ERYTHROCYTES WHICH CAN BE MODIFIED BY RADICAL SCAVENGERS

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The treatment of human erythrocytes with hydroxyurea /HU/ results in the azide-dependent changes in osmotic fragility and in increased methemoglobin formation. Similar changes were induced by H_2O_2 treatment. However when H_2O_2 in the presence of azide stimulated malondialdehyde production, in the HU-treated cells no malondialdehyde was detectable. When subjected to an oxidant stress /sodium ascorbate/ HU-treated erythrocytes were more fragile and revealed changes in the absorption spectrum of the TBA-reactive material in comparison with the cells treated with ascorbate alone. Partial protection by radical scavengers against certain HU-induced changes can be achieved. The results indicate that HU can damage erythrocytes and suggest the radical origin of these effects.

It has recently been proposed that HU, a recognized inhibitor of ribonucleotide reductase, may exert its side toxic action through free radical reactions /1,2/. Since the generation of free radicals following irradiation and other pathological conditions is known to be extremely noxious to the cell membranes it seems to be important to investigate the HU-induced toxic effects, particularly in respect to the cell membranes, not only in the proliferating target cells, but also in the non-proliferating non-target cells. It has been found in our laboratory that HU-treatment of human granulocytes in vitro induced a marked suppression of their phagocytic activity which could be ameliorated by various antioxidants /Szczepańska et al., to be published/. Erythrocytes were chosen as the model for the present study.

The purpose of this communication is to report some preliminary results which indicate that HU-treatment can induce several physico-chemical RBC alterations. Some of the observed

Abbreviations: HU, hydroxyurea; RBC, erythrocyte; MDA, malondialdehyde; Hb, hemoglobin; MetHb, methemoglobin; TBA, thiobarbituric acid.

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changes could be partially prevented by the radical scavengers. H_2O_2-treatment of the same RBCs induced several analogous alterations.

MATERIALS AND METHODS

Normal human blood was freshly withdrawn by venepunkture. As an anticoagulant heparin was added.

Hydroxyurea was obtained from Z.F. Polfa, ascorbic acid and sodium benzoate from POCH and human coeruloplasmin from Biomed /Poland/. All the reagents were of the highest purity comercially available. α-tocopherol was prepared as an emulsion, 1 part tween to 4 parts tocopherol. Control systems contained tween in the same concentration. All of the solutions added to erythrocytes were prepared just before use in saline-phosphate buffer and were maintained at pH 7.4 taking care to maintain isotonisity.

Osmotic fragility was determined as described in /3/. The degree of haemolysis in hypotonic buffered NaCl solution / 1 hour, 37°C / was expressed as the percentage of total hemolysis of the same volume of erythrocytes in water.
MDA level and the absorption spectra of the TBA-reactive material were estimated as in /4/.
Erythrocyte ghosts were prepared as in /5/.
MetHb measurement was performed according to /6/.
The oxidant sensitivity /7/ was determined as the effect of sodium ascorbate on the hemolysis of HU-treated and untreated erythrocytes after 3 hours of incubation at 37°C in isotonic NaCl solution. The degree of hemolysis was expressed as the percentage of total hemolysis of the same volume of erythrocytes in water.

RESULTS AND DISCUSSION

Osmotic hemolysis. Erythrocytes in a blood sample /normal/ are hemolyzed over a range of hypotonic salt concentrations. The alterations in the osmotic fragility are thought to be connected with the changes in the physico-chemical properties of the erythrocyte membrane /8/. Thus we compared at first the degree of osmotic hemolysis in HU-treated and untreated RBCs. The effect was determined after incubation of the cells in hypotonic HU-supplemented solutions /Fig. 1/ or in HU-supplemented isotonic solutions followed by hypotonic shock in HU-free solution /Table 1/. It can be seen that HU-treatment induced marked alterations in the osmotic fragility. However the character of these alterations was variable in different conditions. Treatment of the erythrocytes by H_2O_2 induced similar change. Addition of α-tocopherol at suitable concentration /60 µg/ml/ afforded partial protection /$p<0.05$/ against HU-induced damage. The addition of another radical scavenger - sodium benzoate - was ineffective, at least at concentrations

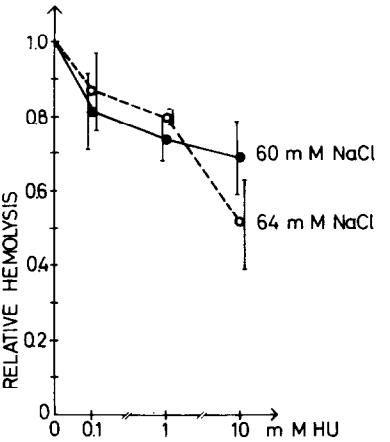


Fig. 1. Lysis of erythrocytes induced by various HU concentrations after 1 hour of incubation at 37°C in hypotonic NaCl solutions. Relative lysis is calculated as $\frac{\text{percent lysis of HU-tr. RBCs}}{\text{percent lysis of untreated RBCs}}$ Each value represents an average from 3 donors /in duplicate/.

Table 1

Osmotic hemolysis of erythrocytes after various conditions of HU treatment

Conc. of HU /mM/	Incubation time with HU /hr/	Temp. of incubation /°C/	Scavenger	Relative hemolysis /control = 1.0 ± S.D./
1	1	37	-	1.36 ± 0.10
1	24	37	-	1.76 ± 0.24
1	24	4	-	0.77 ± 0.04
10	1	37	-	1.49 ± 0.05
10	24	4	-	0.71 ± 0.08
10	1	37	-	1.65 ± 0.04
			tocopherol	
10	1	37	60 µg/ml	1.20 ± 0.10
10	1	37	90 µg/ml	1.80 ± 0.14
			sodium benzoate	
10	1	37	2 mM	1.70 ± 0.08
10	1	37	3 mM	1.74 ± 0.02
Hydrogen peroxide				
10	1	37	-	1.56 ± 0.18

Washed erythrocytes were treated with HU or H_2O_2 alone or with HU in the presence of radical scavenging agent in isotonic NaCl solution followed by hypotonic /60 mM NaCl/ solution for 1 hour at 37°C. Each value represents an average from 3 donors /in duplicate/.

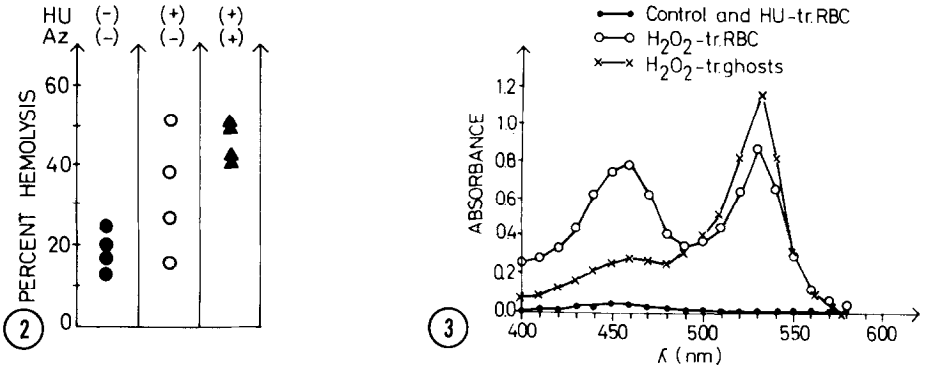


Fig. 2. Lysis of erythrocytes after treatment with 1 mM HU for 1 hour at 37°C in isotonic NaCl solution in the presence or absence of 0.25 mM sodium azide /Az/ followed by incubation for 1 hour in hypotonic /64 mM/ HU-free solution. Cells lysed by water equalled 100 % lysis. Each point represents an average of duplicate measurements of individual donors.

Fig. 3. Absorbance spectrum of TBA-reactive material from erythrocytes or their ghosts incubated for 3 hours at 37°C in 10 mM HU or 10 mM H_2O_2 in the presence of 0.25 mM sodium azide.

effective in granulocyte protection /Szczepańska et al., to be published/. Because the response of RBCs from different individuals to HU-treatment revealed marked variability, the osmotic hemolysis of cells treated with HU in the presence or absence of azide was compared. Fig. 2 indicates much smaller individual variability in azide-pretreated cells. Such an effect of azide was similar to that in H_2O_2-treated cells /4,9/ and suggested that an enzymatic defense mechanism against oxygen radicals is involved in HU-induced effects.

Based on the hypothetized analogy with radiation- or H_2O_2-induced RBCs damage we assumed that in HU-induced damage peroxidation of the membrane lipids is involved. Thus we next investigated lipid peroxide formation measured as TBA-reactive material.

Lipid peroxidation. It is now well established that free radicals can peroxidate unsaturated phospholipids of the membranes /10/. The process used to be estimated by measuring one of the products of the reaction: MDA, which in TBA-complexes gives maximal light absorption at 532 nm /4/. However, as Fig. 3 shows, while in intact erythrocytes or membrane ghosts, treated with H_2O_2 in the presence of sodium azide, a significant quantity of MDA was produced, in TBA-reactive material from HU-treated intact erythrocytes or ghosts no light absorption at 532 nm was detectable. Moreover, in TBA-complexes from H_2O_2-

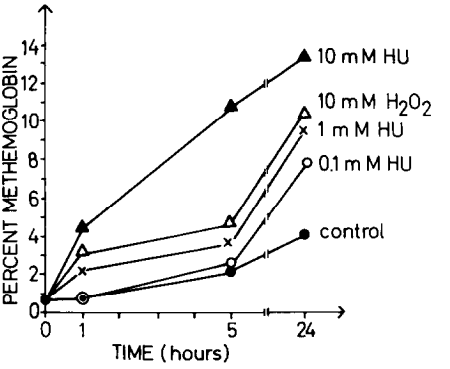


Fig. 4. MetHb level in erythrocytes incubated for indicated periods of time at 37°C in HU- or H_2O_2- supplemented solutions.

treated cells the second peak at 460 nm, considered to be a function of Hb /11/, was visible. From HU-treated cells such peak was also absent.

Another manifestation of the intracellular generation of free radicals can be the production of MetHb /12/. Thus we estimated MetHb level in HU- and H_2O_2-treated RBCs in the presence or absence of radical scavengers.

Methemoglobin production. Fig. 4 shows that similarly to H_2O_2, HU stimulated MetHb production even at such a low concentration as 0.1 mM if the time of exposure was sufficiently long. This toxic effect could be partially prevented by antioxidants /Table 2/.

In view of the reports that the age-related changes in the erythrocyte fragility, supposed to be connected with the increase in lipid peroxidation, correlated with the changes in the oxidant sensitivity /7/, in the next series of experiments we compared the degree of lysis of HU-treated and untreated RBCs, subjected to an oxidant stress /sodium ascorbate/. The experiments were based on the assumption that HU-induced membrane alteration can render the cells more susceptible to lysis and less resistant to peroxidation.

Oxidant stress. In the preliminary experiments RBCs were incubated in an isotonic NaCl solution with sodium ascorbate at concentrations 1, 2, 5, 10, 20 and 50 mg/ml. After 3 hours of incubation increasing, concentration-dependent hemolysis was observed. In the absence of the oxidant no lysis was noted. When HU-treated and untreated RBCs were compared under the same conditions of oxidant stress an increasing lysis of HU-treated cells was observed. The effect was however limited to ascorbate

Table 2

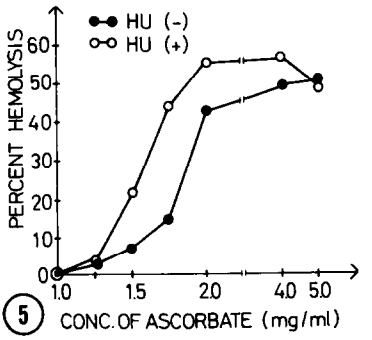
Amelioration of HU-induced methemoglobin formation by scavengers

No of donor	Scavenger	Percent of methemoglobin		
		Control	HU	HU with scavenger
	Tocopherol			
1	30 µg/ml	0.58	2.88 /100/	2.48 /86.1/
2	30 µg/ml	0.33	2.82 /100/	2.62 /92.9/
	45 µg/ml			2.53 /89.7/
3	90 µg/ml	0.35	2.11 /100/	1.56 /74.0/
	180 µg/ml			1.73 /81.9/
4	60 µg/ml	0.81	2.72 /100/	2.21 /81.2/
	90 µg/ml			2.42 /89.3/
	Sodium benzoate			
5	1 mM	0.35	2.23 /100/	1.60 /71.7/
	2 mM			1.58 /70.8/
	3 mM			1.37 /61.4/
	4 mM			1.80 /80.7/
6	3 mM	0.76	2.65 /100/	1.84 /69.4/
	Coeruloplasmin			
7	500 µg/ml	1.36	2.30 /100/	1.35 /58.7/
			H_2O_2	H_2O_2 with scavenger
	Tocopherol			
1	30 µg/ml	0.58	2.08 /100/	1.72 /82.7/
2	45 µg/ml	0.33	3.23 /100/	1.55 /48.0/
3	180 µg/ml	0.35	1.60 /100/	1.24 /77.5/

Washed erythrocytes were treated with 10 mM HU or 10 mM H_2O_2. Where indicated a radical scavenging agent was added. After 1 hour incubation at 37°C the level of MetHb was measured. Each value represents the average from two samples of the same donor. Data in parentheses express the percentage value.

concentration in the range 1 - 5 mg/ml /data not shown/. Fig. 5 indicates that in this narrow range of ascorbate concentration HU-treatment render the RBC more fragile.

One factor which may contribute to the increased tendency of HU-treated erythrocytes to lyse under oxidant stress is an oxidant-induced acceleration of the lipid peroxidation initiated by the HU-derived free radicals. To check this possibility the absorbance spectrum of TBA-reactive material from ascorbate- or ascorbate + HU-treated RBCs was compared. Fig. 6 /A/ shows that



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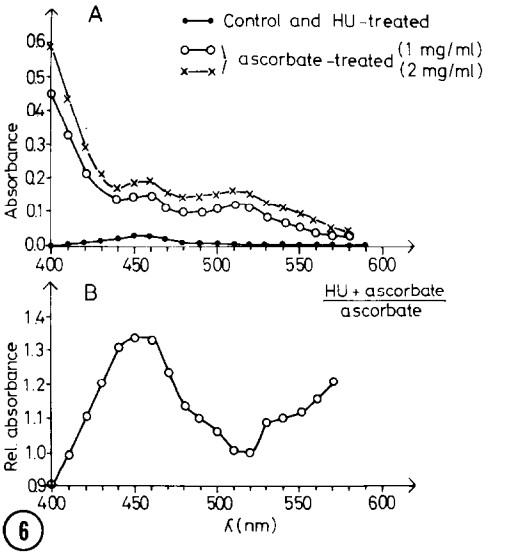

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Fig. 5. Lysis of HU-treated and untreated erythrocytes incubated for 3 hours at 37°C in isotonic NaCl solution supplemented with various concentrations of sodium ascorbate. Cells lysed by water equalled 100 % lysis.

Fig. 6. A. Absorbance spectrum of TBA-reactive material from erythrocytes incubated for 3 hours at 37°C in isotonic solution supplemented with HU /10 mM/ or sodium ascorbate /1 or 2 mg/ml/.
B. Absorbance spectrum of TBA-reactive material from erythrocytes incubated for 3 hours at 37°C in isotonic solution supplemented with sodium ascorbate /2 mg/ml/ in the presence or absence of HU /10 mM/. Data are expressed as relative absorbance A $\frac{\text{ascorbate + HU}}{\text{ascorbate}}$.

while control and HU-treated cells revealed essentially no TBA-reactive material, the ascorbate-treated cells revealed certain absorbance. When the absorbance spectrum of ascorbate + HU-treated and ascorbate alone-treated RBCs was compared /Fig. 6 B/ the data, /expressed as the ratio A $\frac{\text{ascorbate + HU}}{\text{ascorbate}}$ / revealed that HU-treatment generates additional peak of light absorption at 450 - 460 nm and very low, if any, in the region of 530 - 540 nm.

In the last series of experiments with several lectins it was found that serological properties of HU-treated erythrocytes remained unaffected.

In conclusion, the presented data indicate that HU-treatment of RBC in vitro can induce changes in osmotic fragility, MetHb formation and sensitivity to oxidant stress. Such an effect is difficult to explain unless radical side toxicity of this drug is considered. Further confirmation of this suggestion is partial protection afforded by radical scavengers.

More work is obviously necessary for the establishment of the nature of HU-induced RBC damage. However regardless of how far this effect can be understood, the possibility that some RBC damage may occur after HU administration in vivo seems to be of clinical importance. Such conclusion as well as the recent report on the production of hydroxyl radicals by adriamycin in red blood cells /13/ turn the attention to the erythrocyte as one of the target sites for side toxicity of radical generating antineoplastic drugs.

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