COMPARATIVE PATTERNS OF 'IN VITRO' OXIDATIVE HEMOLYSIS OF NORMAL AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE (G6PD)-DEFICIENT ERYTHROCYTES

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1. Introduction

The Mediterranean variety of genetically inherited G6PD deficiency is a convenient model system for investigations on the mechanisms of oxidative hemolysis. In fact:

(i) This enzyme disorder is characterized by a particularly severe deficiency of catalytic activity, being $\lesssim$0.1% of the G6PD activity found in erythrocytes from normal subjects [1];

(ii) It is associated, although in a characteristically non-constant way, with episodes of acute intravascular hemolysis which are triggered by exogenous agents such as fava beans and (or) drugs resulting in a severe oxidative challenge to the affected erythrocytes [2,3].

Peroxidation of membrane lipids has long been considered as an obligatory step of oxidative hemolysis [4]. A preliminary study on the structure of membrane lipid components failed to show significant differences among erythrocytes from: (a) normal subjects; (b) healthy G6PD-deficient subjects; (c) favic patients after hemolytic crisis, excepting for a distinctive content of palmitic acid [5]. This is an investigation exploring, on a comparative basis between control and G6PD-deficient erythrocytes, the dynamic changes in these lipid components which underlie the oxidative hemolysis.

Abbreviations: G6PD, glucose 6-phosphate dehydrogenase; SOD, superoxide dismutase; HMS activity, hexose monophosphate shunt activity; PBS, phosphate-buffered saline (pH 7.2) [20]; MDA, malonyldialdehyde; GSH, reduced glutathione; DDC, diethyldithiocarbamate

2. Materials and methods

2.1. *Blood samples and enzyme assays*

Blood samples from normal male subjects and G6PD-deficient males of Sardinian ancestry were drawn and processed as in [1], in order to remove leukocytes and platelets completely from erythrocytes [6]. Assays of G6PD activity in hemolysates were done as in [1]. Homogeneous superoxide dismutase (SOD) purified from human erythrocytes was a kind gift by Professor G. Rotilio. Entrapment of SOD within normal and G6PD-deficient erythrocytes was performed as in [7]. Hexose monophosphate shunt (HMS) activity of intact erythrocytes was estimated as in [7].

2.2. *Incubations of erythrocytes with the H_2O_2–NaN_3 system*

These were performed essentially as in [8], with some modifications. Briefly, mixtures (3.0 ml) containing isosmotic PBS, erythrocytes at a final hematocrit of 2.5%, 5 mM glucose, 10 mM NaN_3 and H_2O_2 at varying concentrations as indicated, were incubated for 2 h at 37°C under H_2O-saturated oxygen with constant stirring. In few experiments the supply of H_2O_2 was made according to the vapour-diffusion technique as in [8], which allowed to reach final H_2O_2 concentrations of 4–6 mM. Aliquots were removed and submitted to estimation of malonyldialdehyde (MDA) as in [8], of the extent of hemolysis by recording the absorbance at 577 nm and to lipid analyses.

2.3. *Analysis of lipid components*

This was done as in [5] using the standards reported. Besides to these, 3β-hydroxy-5-α-hydroperoxy-Δ^6-

cholestene (kindly provided by Professor R. Strom) was also used as a standard for gas–liquid chromatographic analysis of cholesterol. In this procedure [9], the two compounds showed sharply different retention times.

3. Results

3.1. *Effects of the H_2O_2-NaN_3 system on the HMS activity*

Table 1 shows that the HMS activity of normal erythrocytes is enhanced significantly over the situation of resting state by the oxidative stress used in this study. The extent of such stimulation depends primarily on the actual concentrations of H_2O_2. In the G6PD-deficient erythrocytes, whose HMS activity in resting conditions is lower than in the normal cells, the H_2O_2–NaN_3 system fails to stimulate the oxidative pathway. This differential response of the HMS activity of the normal *vs* the G6PD-deficient erythrocytes to an oxidative stress can be explained on the basis of extensive cellular reoxidation of both GSH and NADPH [10,11].

3.2. *Dynamic changes in membrane lipids*

Repeated experiments indicated that most membrane lipid components are unaffected by the H_2O_2–NaN_3 system, both in the normal and in the G6PD-deficient erythrocytes. Specifically, cholesterol, total phospholipids, phosphatidylcholines, sphingomyelins, phosphatidylserines and the major fatty acids (with the exception of arachidonic acid) remain at their starting levels [5] over the range of H_2O_2 concentrations used. On the contrary, the oxidative system used in this study produces a remarkable decrease of phosphatidylethanolamines (fig.1) as well as of arachidonic acid (fig.2). Both variations are similar in extent in the normal and in the G6PD-deficient erythrocytes.

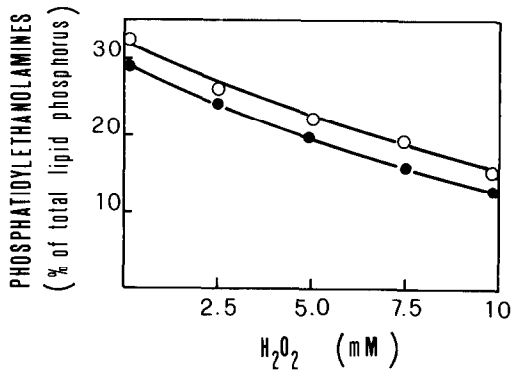

Fig.1. Effect of the H_2O_2–NaN_3 system on the levels of phosphatidylethanolamines: (•) normal erythrocytes; (○) erythrocytes from a G6PD-deficient subject. Experimental details in section 2.

3.3. *Lipid peroxidation and hemolysis*

Fig.3, referring to the same experiment illustrated by fig.1,2, shows the effect of the H_2O_2–NaN_3 system in terms of malonyldialdehyde (MDA) formation and of hemolysis. Several such experiments indicated that lipid peroxidation occurs already at those H_2O_2 concentrations which still elicit only negligible hemolysis. Since MDA formation is related, also from a quantitative standpoint, to the fall in arachidonic acid and it precedes the hemolysis temporally [8], the decrease in lipid components shown in fig.1,2 can be regarded as being a pre-hemolytic event. On the other hand, a close correlation had been established between depletion of membrane phosphatidylethanolamines and H_2O_2-induced hemolysis of rat erythrocytes [4].

Table 1
Effect of various oxidative stresses on the HMS activity of normal and G6PD-deficient erythrocytes[a]

Subjects	G6PD activity (IU/g Hb)[b]	HMS activity (nmol $^{14}CO_2$. ml erythrocytes^{-1} . h^{-1} at 37°C)[c]				
		–	MB[d] (100 μM)	NaN_3 (10 mM)	NaN_3 (10 mM) + H_2O_2 (1 mM)	NaN_3 (10 mM) + H_2O_2 (5 mM)
Normal	4.1	32	1611	102	302	560
G6PD-deficient	3.6×10^{-3}	14	22	17	12	11

[a] Incubation mixtures were as in section 2, excepting for the components outlined and for presence of [^{14}C]glucose [7]; [b] assayed according to [1]; [c] assayed according to [7]; [d] methylene blue

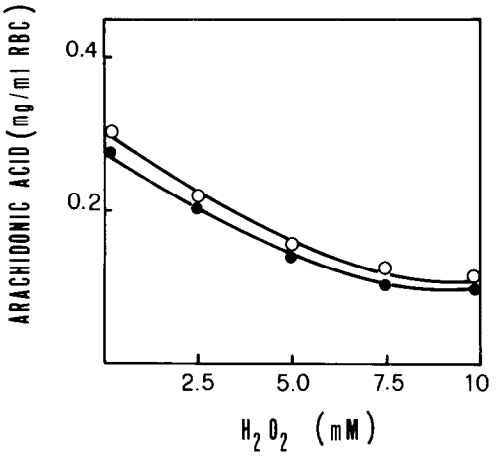

Fig.2. Effect of the H_2O_2–NaN_3 system on the levels of arachidonic acid: (•) normal erythrocytes; (○) erythrocytes from a G6PD-deficient subject. Experimental details in section 2. Arachidonic acid was quantitated by using as internal standard heptadecanoic acid which was added to the lipid extracts.

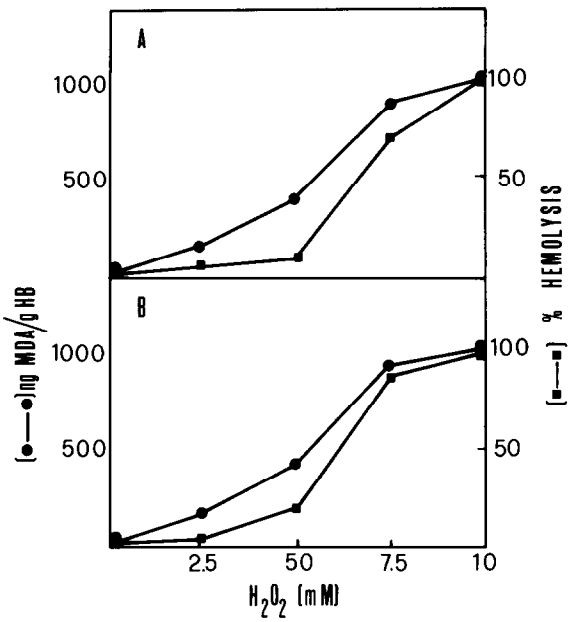

Fig.3. Effect of the H_2O_2–NaN_3 system on MDA formation and hemolysis: (A) normal erythrocytes; (B) erythrocytes from a G6PD-deficient subjects; (•) MDA formation; (▪) hemolysis. Experimental details in section 2.

Inspection of fig.3 shows the absence of significant differences between normal and G6PD-deficient erythrocytes in respect to the extent and the dependence on H_2O_2 concentrations of both MDA formation and hemolysis.

The patterns of oxidative hemolysis were also investigated in normal *vs* G6PD-deficient erythrocytes after:

(i) Entrapment in both types of cells of homogenous human superoxide dismutase (SOD);
(ii) Inhibition of endogenous SOD with diethyldithiocarbamate (DDC).

Procedure (i) did not modify at all the various events under study, i.e., dynamic changes in membrane lipids, MDA formation and hemolysis, even at SOD levels as high as nearly 20-fold those assayed in the native erythrocytes. However, DDC (ii) was found to modify considerably the patterns of oxidative hemolysis afforded by the H_2O_2–NaN_3 system (fig.4): thus, all polyunsaturated fatty acids, besides to arachidonic

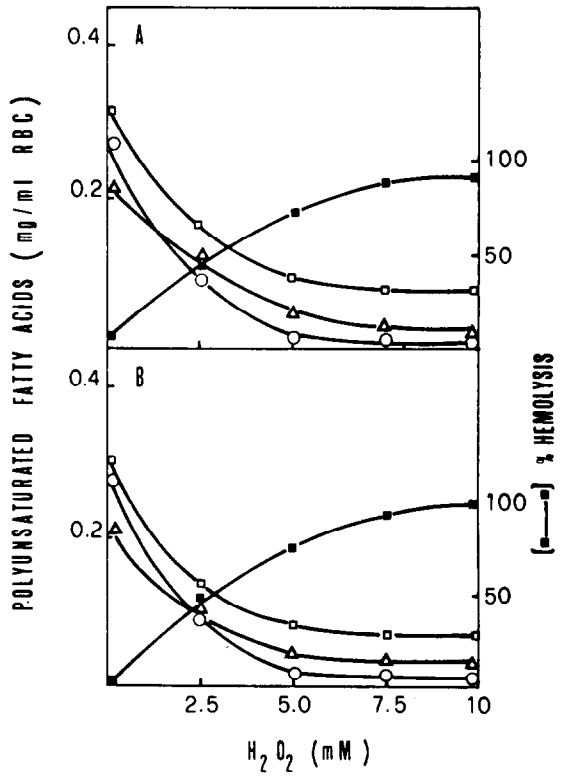

Fig.4. Combined effects of DDC and of the H_2O_2–NaN_3 system on the levels of the major polyunsaturated fatty acids and on hemolysis: (A) normal erythrocytes; (B) erythrocytes from a G6PD-deficient subject; (□) oleic acid; (△) linoleic acid; (○) arachidonic acid; (▪) hemolysis. Fatty acids were quantitated by using heptadecanoic acid as internal standard. Experimental details in section 2. DDC was used at a final concentration of 10 mM.

acid, were destroyed by the oxidative stress, MDA production was negligible, in agreement with [12] (not shown) and the extent of hemolysis was largely increased over that observed in the absence of DDC. In spite of these marked modifications in the biochemical features of oxidative hemolysis, DDC failed to reveal significant differences between normal and G6PD-deficient erythrocytes.

4. Discussion

The involvement of both H_2O_2 and O_2^- in the mechanism of oxidative hemolysis [13,14] prompted us to use an experimental system whereby these 2 oxygen derivatives interact with each other leading to erythrocyte destruction. The role of NaN_3 in this system is 2-fold:

(i) It acts as a powerful inhibitor of endogenous catalase [8], thus reinforcing the effects of added H_2O_2;

(ii) It determines the nucleophilic displacement of O_2^- from HbO_2 [15], thereby accounting for the concomitant formation of methemoglobin that occurs in these conditions.

The latter can be reduced back to Hb in the presence of glucose and HbO_2 is reconstituted as a source of O_2^-. Use of varying concentrations of H_2O_2 confers flexibility to the system the effects of which can therefore be explored on a dose-dependent basis.

An obvious disadvantage of this oxidative system is its remarkable strength which might be expected to produce inactivation of specific enzyme proteins. That however this is not the case of G6PD and of the overall HMS activity is shown by the considerable stimulation of the oxidative pathway produced by the H_2O_2–NaN_3 system in the normal erythrocytes (table 1). Such effect rules out also specific inactivations of glutathione reductase and glutathione peroxidase since both enzymes are sequentially involved together with the HMS in detoxifying the erythrocyte from H_2O_2.

A sharply distinctive effect on the HMS activity of normal *vs* G6PD-deficient erythrocytes has been observed also with the toxic compounds of fava beans, divicine and isouramil [16], as well as with a number of oxidative drugs entailing a hemolytic risk in the G6PD-deficient subjects [17]. The reason for this different behaviour is the rate-limiting role that G6PD itself plays in the overall pathway of glucose oxidation within the deficient but not in the normal cells, when these are challenged with an oxidative stress resulting in re-oxidation of NADPH [7,18]. It is the acute failure to counteract the cellular effects of an oxidant injury, i.e., oxidative draining of both GSH and NADPH, that is commonly thought to cause irreversible damage of G6PD-deficient erythrocytes and to precipitate the hemolytic crisis. However, this view can hardly be reconciled with the closely related features of 'in vitro' oxidative hemolysis shown in a wide range of experimental conditions by 2 types of erythrocytes whose G6PD activities differ by 1000-fold from each other. Therefore, these findings seem to indicate that an oxidative stress alone such as that used here could not be sufficient to determine the acute hemolysis of the G6PD-deficient subjects and that additional, probably extra-erythrocytic factors, may be involved in the mechanism of erythrocyte disruption. This view is consistent with the well-known variability of the hemolytic episodes among G6PD-deficient subjects still within homogeneous genetic groups or even throughout the life of single affected individuals [2,3,19].

Acknowledgements

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