DECAY OF A SPECIFIC NADP(H)-BINDING PROTEIN DURING AGING OF NORMAL AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE-DEFICIENT ERYTHROCYTES

A. DE FLORA, A. MORELLI, U. BENATTI and M. FRASCIO
Institute of Biochemistry, University of Genoa, Viale Benedetto XV/1, 16132 Genoa

and

G. F. GAETANI
Institute of Haematology, University of Genoa, Italy

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1. Introduction

Human erythrocytes contain a specific NADP(H)-binding protein, tentatively designated FX [1,2], whose molecular properties have been previously described [3]. A radioimmunoassay, based on double-antibody technique, was developed in order to quantitate this holoprotein in crude as well as in purified systems [4]. Preliminary application of the radioimmunoassay showed that the amount of FX cross reacting material (CRM) is significantly enhanced in hemolysates from individuals affected by the Mediterranean type of glucose 6-phosphate dehydrogenase (G6PD) deficiency when compared with hemolysates from normal subjects. This finding appeared to be of interest because:

(i) Little is known on the pathophysiology of the G6PD-deficient erythrocytes.

(ii) The biological function of FX is still undetermined.

Accordingly, the technique was improved by developing a solid-phase radioimmunoassay whereby the content of FX CRM could be estimated throughout the life-span of the erythrocytes. The results obtained are consistent for distinctive patterns of decline of FX CRM during aging of normal and of G6PD Mediterranean erythrocytes, these patterns accounting for the different levels of the protein in whole cell populations from the two groups of subjects.

2. Materials and methods

2.1. *Blood samples*

These were obtained from male subjects only. G6PD phenotypes were classified according to [5], as described elsewhere [4,6]. Blood samples were defibrinated with glass beads and erythrocytes were subjected to three washings with 5 vol. 0.15 M KCl, then submitted to enzyme assays or to fractionation according to age (see below).

2.2. *Assays of G6PD activity and determination of protein*

Hemolysates were prepared by diluting 1 vol. packed erythrocytes with 6 vol. cold H_2O containing 10 μM $NADP^+$. G6PD activity in normal hemolysates was estimated according to the Glock and McLean method [7] in the assay conditions recommended by [5]. In the deficient subjects (screened by brilliant cresyl blue method [5]), G6PD activity was measured in the presence of saturating amounts of G6PD and $NADP^+$ only [8].

Protein was determined according to Lowry et al. [9] using standard solutions of ovalbumin.

2.3. *Separation of erythrocytes according to age*

Packed erythrocytes were centrifuged on discontinuous gradients of Stractan II as reported by

Corash et al. [10]. Four erythrocyte layers appeared following centrifugation, which were collected by suction with a Perpex LKB peristaltic pump. These were defined as Fractions I, II, III and IV, respectively, from the top to the bottom of each tube and corresponded to erythrocytes of increasing age, as indicated by the patterns of decline of G6PD activity [11] and of the reticulocyte percentage.

2.4. *Antisera and purified FX preparations*

FX was purified from pooled human erythrocytes as previously described [2]. The purity of the final preparations was tested by centrifugation on a Spinco Mod. E ultracentrifuge and by polyacrylamide gel electrophoresis in the presence of SDS [2].

Antiserum to homogeneous FX was raised in rabbits as previously reported [4]. The anti-FX serum was heat inactivated and adsorbed sequentially with human erythrocytes and with a purified IgC fraction from pooled human sera [4]. The specificity of the antiserum was checked by radial immunodiffusion against crude hemolysates. The immunoadsorbent was prepared by reacting 100 mg purified IgC fraction from the anti-FX serum with 20 g BrCN-activated agarose (150 mg BrCN/*1* g Sepharose 4 B). The reaction was allowed to proceed for 24 h at 4°C in 16 ml 0.1 M $NaHCO_3$, pH 9.0. Then the resin was reacted with 40 ml 1 M ethanolamine–HCl, pH 9.0, for 2 h at 4°C, washed with H_2O and treated with 2 M Na acetate, pH 4.0, before being equilibrated with PBS.

2.5. *Solid-phase radioimmunoassay of FX*

Radioiodination of homogeneous FX was carried out using the lactoperoxidase catalyzed method of Marchalonis [12], as previously described [4]. The radioimmunoassay was based upon the capacity of crude hemolysates or of FX preparations of varying degrees of purity to inhibit the reaction between the above immunoadsorbent and ^{125}I-labeled FX. Different volumes (at least three) of each hemolysate tested were mixed in plastic tubes with a constant amount of immunoadsorbent which had been established in previous experiments to bind 50% of radioiodinated FX (1 μg) in the absence of unlabeled FX. Ovalbumin and concentrated PBS were added in order to normalize the assay mixtures for protein concentration (25 mg/ml) and PBS molarity (20 mM Na phosphate, pH 7.0, containing 0.15 M NaCl), since both parameters were found to affect the radioimmunoassay critically. After rotating the tubes for 150 min at 4°C, 1 μg ^{125}I-labeled FX (in PBS containing 3% ovalbumin, w/v) was added and the mixtures were rotated for additional 150 min at 4°C. The tubes were centrifuged at 2500 rev./min and both the supernatants and the pellets (after one washing with 1.0 ml PBS) were counted for radioactivity. Blank assays were run in parallel, in which the usual immunoadsorbent was replaced by a Sepharose-bound IgG fraction from anti-bovine serum albumin serum. The amount of FX CRM present in the sample tested was estimated by interpolation on a curve obtained by allowing known amounts of pure FX to compete with ^{125}I-labeled FX for the immunoadsorbent.

3. Results

3.1. *Levels of immunoreactive FX in normal and G6PD Mediterranean erythrocytes*

Table 1 reports average values of FX CRM determined in hemolysates from nineteen control subjects (carrying the G6PD B phenotype) and from twenty-seven individuals having the G6PD Mediterranean variant, respectively. As shown in the table, FX levels were significantly enhanced in G6PD Mediterranean erythrocytes as compared with normals. However, this increase was not generalized because five out of the twenty-seven G6PD-deficient subjects examined had a normal or slightly decreased erythrocyte content of FX CRM. No correlation was apparent, within either group of subjects, between actual levels of residual G6PD activity and levels of FX.

Table 1
Levels of FX CRM in erythrocytes from normal and G6PD mediterranean subjects

G6PD phenotype	FX CRM (μg/g Hb)
B (19)	45.46 ± 10.57[a]
Mediterranean (27)	77.51 ± 32.48[a]

[a]Values are means ± SD
The number of subjects is given in parentheses
Significance between means, $p<0.0005$

3.2. *Decay of immunoreactive FX during aging of erythrocytes*

The content of FX CRM was investigated in erythrocytes of different age together with values of G6PD activity which is a typical marker of erythrocyte aging [11,13,14]. Figure 1 illustrates the results obtained in six normal individuals. Although a certain variability was observed among the subjects under study, there was a tendency to a progressive decline in the FX levels during the life-span of the control erythrocytes.

The situation was entirely different in the group of subjects carrying the G6PD Mediterranean variant (fig.2). Thus, apart from the consistently enhanced rate of inactivation of G6PD during aging [11], the average content of FX in the young erythrocytes appears to be higher than in the corresponding fraction from the normal subjects. Moreover, no decay in FX levels from the young to the old erythrocyte fractions could be detected in any of the subjects investigated.

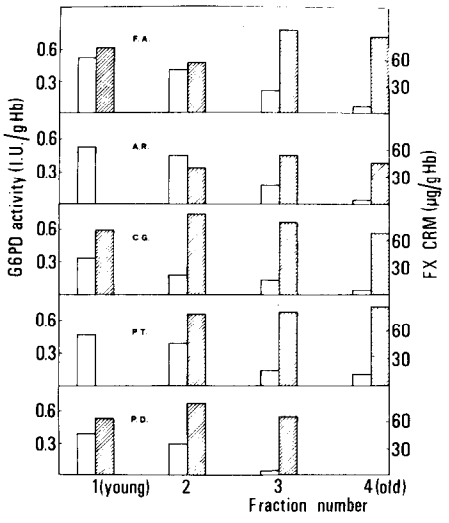

Fig.2. Levels of G6PD activity and FX CRM in differently aged erythrocyte fractions from G6PD-deficient subjects carrying the Mediterranean variant. White columns, G6PD activity. Hatched columns, FX CRM. Fraction IV could not be assayed in subject P.D. because of lack of material. FX CRM in Fraction I from subjects A.R. and P.T. was not determined.

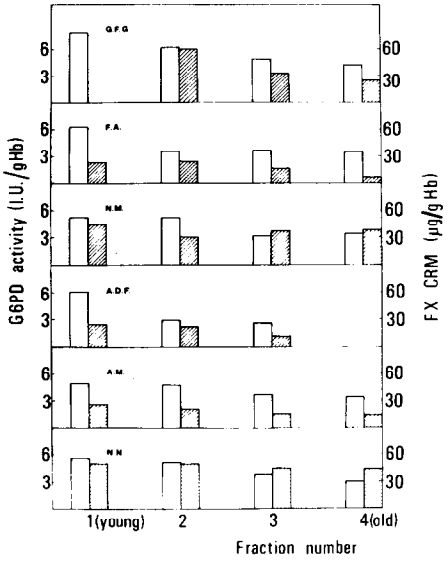

Fig.1. Levels of G6PD activity and FX CRM in differently aged erythrocyte fractions from normal subjects (carrying the G6PD B phenotype). White columns, G6PD activity. Hatched columns, FX CRM. Fraction IV could not be assayed in subject A.D.F. because of lack of material, while assay of FX CRM in Fraction I from subject G.F.G. was not performed.

4. Discussion

The enhanced content of immunoreactive FX in the G6PD Mediterranean erythrocytes cannot be accounted for by direct conversion of the G6PD variant to FX itself, since the two proteins seem to be coded by different structural genes [4]. However, the imbalance between levels of the two NADP(H)-binding proteins appears to be a characteristic feature in the Mediterranean type of G6PD deficiency.

The data reported in this paper provide some information as to the mechanisms resulting in the above-mentioned steady-state levels of both FX and G6PD. Thus, FX shows a decline throughout the life-span of the normal erythrocyte, yet such decay is not as pronounced as for other proteins including fructose 1,6-biphosphate aldolase [15], pyruvate kinase [16], acetylcholinesterase [17], adenylate cyclase [18], protein kinase [18], purine nucleoside phosphorylase [19] and G6PD [11,13]. In the G6PD Mediterranean erythrocytes the amount of FX CRM is higher within the young cells and remains constant during aging,

both facts leading to the increased content of this protein in whole erythrocyte populations (table 1).

The consistently accelerated breakdown of G6PD Mediterranean [6], as compared with the normal B phenotype, can be easily explained on the assumption that the underlying structural mutation renders the protein more susceptible to removal by the still unknown cellular systems involved therein. Conversely, the concurrent resistance of FX to degradation can hardly be explained in terms of a modified protein structure, since:

(i) No evidence is still available for an additional mutation affecting also the FX locus.

(ii) Even if such mutation were involved, it would be expected to determine an enhanced degradation of the protein.

Rather, failure to observe any decrease in the FX content during aging could reflect a 'spare' mechanism secondary to the accelerated removal of mutant G6PD, thus suggesting that common systems may be responsible for the decay of both NADP(H)-binding proteins.

The finding of a small proportion of G6PD Mediterranean subjects showing normal rather than increased levels of immunoreactive FX remains so far unexplained. Further studies are required in order to verify whether such unusual feature is related to any genetic polymorphism within the Mediterranean type of G6PD deficiency.

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