CONCISE REPORT

The Interference of Leukocytes and Platelets With Measurement of Glucose-6-Phosphate Dehydrogenase Activity of Erythrocytes With Low Activity Variants of the Enzyme

By A. Morelli, U. Benatti, L. Lenzerini, B. Sparatore, F. Salamino, E. Melloni, M. Michetti, S. Pontremoli, and A. De Flora

Complete removal of leukocytes and platelets from whole blood showed that the glucose-6-phosphate dehydrogenase (G6PD) activity in "pure" erythrocytes from G6PD-deficient hemizygous Sardinian subjects is consistently lower than reported in the literature. Thus, although none of 27 hemizygous subjects showed undetectable erythrocyte G6PD activity, their levels ranged between 0.0015 and 0.0008 IU/g Hb, as compared with a mean value of 4.5 IU/g Hb in normal subjects. Most of the biochemical properties that were formerly ascribed to erythrocyte G6PD appear to be those of the enzyme from contaminating leukocytes and/or platelets.

THE MEDITERRANEAN variety of glucose-6-phosphate dehydrogenase (G6PD) deficiency is characterized by severely decreased catalytic activity in the affected erythrocytes and is therefore classified as belonging to class II in the usual tabulations of G6PD variants. However, evidence has been provided that in Mediterranean countries there are several polymorphic G6PD variants sharing activity levels below 10% of those found in normal subjects.

We have now investigated in greater detail the actual levels of G6PD in erythrocytes from G6PD-deficient hemizygous Sardinian subjects. We show here that after complete removal of leukocytes and platelets, the G6PD activity of pure erythrocytes from these subjects is far lower than reported previously. Accordingly, most of the biochemical properties of the mutant enzyme protein that were formerly ascribed to erythrocyte G6PD should be regarded as those of the enzyme from contaminating leukocytes and/or platelets.

MATERIALS AND METHODS

Blood samples from 30 male subjects were drawn and investigated according to the principles of the Helsinki declaration. Out of these, 3 with normal G6PD activity had the wild-type enzyme (B). The other 27, of Sardinian ancestry, were born in the town of Nuoro or in its neighbors (22), in the town of Sassari (3) and in villages of southern Sardinia (2). All samples were processed immediately or 0.0015 described by Beutler et al., as reported previously. If some residual leukocytes and platelets were eluted from the column, a further step of filtration was followed. Hemolysates from pure erythrocytes were obtained by hypotonic lysis with 9 volumes of deionized water containing 0.2% β-mercaptoethanol (v/v) and 1 mM EDTA and immediately after G6PD was partially purified according to two alternative procedures: (1) DEAE-cellulose chromatography and fractionation with ammonium sulfate and (2) a single step method of affinity chromatography on adenosine 2',5'-bisphosphate-agarose.

Lymphocytes were prepared by centrifugation of whole blood on Ficoll-Hypaque density gradients. Assays of G6PD activity were carried out at 25°C according to the following procedures.

Spectrophotometric Assay

This was performed in the mixture recommended by WHO, using a Gilford model 2400 recording spectrophotometer operated at a full scale setting of 0.25 absorbance units. The threshold of sensitivity of this assay method proved to be 0.020 IU/g Hb, i.e., at 95% confidence limits, 0.020 IU/g Hb was significantly different from zero activity.

Radiometric Assay

This was performed in two steps and was based on the time-dependent release of ¹⁴CO₂ from ¹⁻¹⁴C-glucose-6-P (New England Nuclear, 0.15 mCi/mmol).

Other components of the assay mixture were as for the spectrophotometric assay, with the addition of 0.12 IU of yeast 6-phosphogluconate dehydrogenase (Boehringer Mannheim, F.R.G.), preliminarily freed of contaminating G6PD activity by means of affinity chromatography on adenosine 5'-phosphate-agarose. The mixtures containing varying amounts of the hemolysates or of purified G6PD samples were incubated at 25°C for 20 min using 20-mI vials fitted with a rubber cap and a plastic center well containing 0.2 ml of 2 N NaOH. The reactions were stopped by addition of 0.5 ml of 0.2 N HCl, followed after 10 min by 0.05 ml of 1.5 N NaOH. The second step of incubation was started by addition of 0.2 mM NADP (final concentration) and 0.12 IU of purified 6-phosphogluconate dehydrogenase and stopped after 60 min at 37°C with 0.7 ml of 4 M perchloric acid. After further stirring for 20 min at 37°C, the wells were removed and counted for radioactivity.

The threshold of sensitivity of the radiometric assay procedure was found to be 0.001 IU/g Hb.

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Regeneration of GSH

This procedure, followed with the hemolysates only, is equivalent to that described for estimating the glucose-dependent capacity of intact erythrocytes to reduce glutathione after an extensive oxidation of it by means of t-buty1 hydroperoxide, i.e., a substrate for glutathione peroxidase.1 Briefly, 0.44 mM (final concentration) of t-buty1 hydroperoxide (Fluka, Buchs, Switzerland) was added to 4 ml of CO-saturated hemolysates. After 2 min, the first sample was withdrawn for determination of GSH and both 0.2 mM glucose 6-P and 0.5 mM NADP (final concentrations) were added to the mixtures that were incubated for 60 min at 25°C. At different time intervals, 0.5-ml aliquots were removed and submitted to determination of GSH according to Beutler et al.12 The threshold of sensitivity of this assay procedure was found to be 0.0015 IU/g Hb.

RESULTS

Table 1 shows the levels of G6PD activity in pure erythrocytes from a G6PD-deficient subject. The high extent of interference afforded by leukocytes and platelets on the estimate of erythrocyte G6PD activity is indicated by comparison of the values observed in the presence and in the absence of these cells, respectively. Since the actual G6PD activity measured in "pure" erythrocytes is close to and, in the case of the spectrophotometric assay, lower than the threshold of sensitivity of the assay procedures, the most reliable estimates were those performed on the purified G6PD samples because of their marked concentration over the starting hemolysates.

Similar levels of erythrocyte G6PD activity were observed in all G6PD-deficient subjects screened in this respect. Thus, we never observed undetectable enzyme activity, while the highest recorded activity among G6PD-deficient subjects was 0.008 IU/g Hb. Reference experiments performed in normal subjects indicated a mean value of 4.5 IU/g Hb.

A mixture of four monoclonal antibodies directed to human erythrocyte G6PD-B13 results in virtually complete disappearance of detectable enzyme activity in G6PD-deficient hemolysates (Table 2). This finding indicates that the low levels of catalytic activity estimated in the G6PD-deficient erythrocytes can be safely referred to mutant G6PD and not for instance, to the autosomal hexose phosphate dehydrogenase.14

The amount of erythrocyte enzyme that could be purified was insufficient in quantity to permit biochemical characterization. Figure 1 shows the pH curve of G6PD purified both from "pure" lymphocytes and from whole blood cells from a G6PD-deficient subject without any prior removal of leukocytes and platelets, respectively. The striking identity between the two pH profiles, which exhibit the typical biphasic pattern referred to the "Mediterranean" G6PD variant,15 is further proof that this as well as other biochemical properties that were formerly ascribed to the erythrocyte G6PD are indeed inherent to the leukocyte and/or platelet enzyme.

DISCUSSION

The present findings represent a further example of the requirement of removing leukocytes and platelets completely before investigating biochemical properties of erythrocytes.3 Thus, allowing for the consistently higher G6PD activity of these nucleated cells over that

| Table 2. Effect of Four Monoclonal Antibodies to Human G6PD (Type B)13 on the Catalytic Activity of a G6PD-Deficient Hemolysate |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Addition        | Radiometric GSH | G20.1 + G 14.5  |
| Unrelated antibody (anti-δ) | 0.0024 | Undetectable |
| G 1.6 + G 32.4 + * | 0.0027 | Undetectable |
| G20.1 + G 14.5 | Undetectable | |

*The incubation mixture contained 1 ml of hemolysate, 0.02 ml of pure monoclonal G 1.6 anti-G6PD antibody,13 0.1 ml of G 32.4 (ascites),13 0.1 ml of G 20.1 (ascites),13 and 0.02 ml of G 14.5 (ascites).13 This mixture proved to be the most effective among several combinations of monoclonal anti-G6PD antibodies tested. After 2 hr at 37°C the mixtures were centrifuged and the supernatants assayed for G6PD activity.

Table 1. Levels of Erythrocyte G6PD Activity (IU/g Hb) in Hemolysate and Purified G6PD Samples

<table>
<thead>
<tr>
<th>Assay</th>
<th>Hemolysate</th>
<th>WHO7</th>
<th>Ado 2' 5'-P2-Agarose*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectrophotometric</td>
<td>0.120</td>
<td>0.106</td>
<td>0.092</td>
</tr>
<tr>
<td>Radioactive</td>
<td>0.095</td>
<td>0.114</td>
<td>0.098</td>
</tr>
<tr>
<td>GSH Regeneration</td>
<td>0.112</td>
<td>0.112</td>
<td>0.0025</td>
</tr>
<tr>
<td>+ WBC*</td>
<td>Undetectable</td>
<td>0.0024</td>
<td>0.0019</td>
</tr>
<tr>
<td>- WBC‡</td>
<td></td>
<td>0.0018</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

*Blood samples collected in EDTA (1 mg/ml) as anticoagulant, followed by removal of the buffy coat at each washing of cells.
‡No white cells detectable by manual or automatic counting.
§Reference to 1 g Hb in the starting hemolysate was maintained for standardization purposes, even after removal of Hb by means of either procedure of purification.

§0.71 leukocytes per 1000 erythrocytes.
Fig. 1. pH optimum curves of G6PD purified from blood cells still containing appreciable amounts of leukocytes and platelets (i.e., treated as in the legend to Table 1, III) and from isolated lymphocytes (C) of a G6PD-deficient male. The pH profile of G6PD-B purified from erythrocytes of a normal subject is also shown for comparison (O). Activities were determined spectrophotometrically using the assay mixtures described by Kirkman et al. of pure erythrocytes, a contamination of as little as 1% by the initial content of leukocytes and platelets would contribute 0.003–0.005 IU/g Hb, i.e., a value of the same order of magnitude as that of G6PD-deficient erythrocytes from Sardinian subjects. Accordingly, the catalytic activity of the G6PD variants included in class II and partially in class III should be regarded as being in most cases overestimated. Moreover, the intrinsically low complement of mutant G6PD in the affected erythrocytes represents a serious limitation to both purification and biochemical characterization of the erythrocyte enzyme.

These results tend to decrease considerably the lowest threshold value of G6PD activity believed to be compatible with erythrocyte survival. Nevertheless, the minute levels of G6PD activity assayed in the hemolysates from G6PD-deficient subjects are still higher by 2–4-fold than the maximum hexose monophosphate shunt (HMS) activity of the corresponding intact erythrocytes (data not shown). Accordingly, removal of leukocytes and platelets does not eliminate, although decreasing its extent, the still unexplained “restraint” of intracellular G6PD activity within the G6PD-deficient erythrocytes.

REFERENCES

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