Glucose-6-Phosphate Dehydrogenase Red Blood Cell Phenotype in Gd^{Mediterranean} Heterozygous Females and Hemizygous Males at Birth

G. SANNA, F. FRAU, S. DE VIRGILIIS, P. PIU, F. BERTOLINO, AND A. CAO⁽¹⁷⁾

Department of Obstetric and Gynecology University of Cagliari [P. P.], Mathematical Institute for Engineering [F. B.];
and Second Pediatric Clinic University of Cagliari, Sardinia, Italy, [A. C.]

Summary

The distribution of red blood cell G₆PD phenotype was studied by means of the methemoglobin elution test in newborn (46) and adult (50) Gd^{Mediterranean} heterozygous females and newborn (20) and adult (30) hemizygous males. Newborn heterozygotes had a statistically significant ($P < 0.0005$) lower mean red blood cell G₆PD enzymatic activity (3.23 ± 1.04) than did normal newborns (8.78 ± 1.91), whereas there was no significant difference ($P > 0.30$) from the mean of adult heterozygotes (2.93 ± 0.86). Like adults, newborn heterozygous females showed: (1) a clear correlation ($P < 0.001$) between the percentage of enzyme-deficient red blood cells and G₆PD enzymatic activity; and (2) the expected two red blood cell population, i.e., one deficient and the other normal (mosaicism). However, in newborns, the distribution of the subjects according to G₆PD-deficient red blood cell percentage (mean percent, 43.67) was significantly shifted ($P < 0.025$) in favour of the normal phenotype, unlike adult heterozygotes, who showed a symmetrical distribution of G₆PD positive and negative red blood cells (mean percent G₆PD-deficient red blood cells, 53.27; $P > 0.20$). Newborn hemizygous males showed a consistent percentage (average, 8.28 ± 2.2) of stained red blood cells due to the presence of young erythrocytes (pseudomosaicism) unlike the occasional stained cells (≤ 5) seen in adults. The prevalence of hyperbilirubinemia in hemizygous males and heterozygous females was 10.22 and 2.2%, respectively, whereas in G₆PD normal newborns it was 5.1%. The practical implication of this study is that the diagnosis at birth of the heterozygous state for G₆PD deficiency of the Mediterranean type may be more difficult than in adults. Therefore, very sensitive methods, such as the methemoglobin elution test, should be carried out.

Speculation

Inasmuch as the distribution of Gd^{Mediterranean} newborn heterozygous females according to the percent of G₆PD-deficient red blood cells is significantly shifted in favour of the normal phenotype, the diagnosis at birth of the heterozygous state for G₆PD deficiency of the Mediterranean type may be more difficult than in adults. Therefore, very sensitive methods for detecting enzymatic activity in individual red blood cells, such as the methemoglobin elution test should be used.

Glucose-6-phosphate dehydrogenase-deficient heterozygous females have been shown to have two different red blood cell populations, i.e., one "normal" and the other "deficient." On the average, there are 50% normal and 50% G₆PD-deficient red blood cells. However, at the extremes of the distribution curve, there are heterozygous individuals with an excess of G₆PD-deficient or

normal red blood cells who therefore appear deficient or respectively normal at phenotype level (5-7, 13, 16). Actually, Rinaldi *et al.* (11) showed that there was a shifting towards the normal phenotype in Gd^{Mediterranean} adult heterozygous females chosen at random and identified on genetic criteria. However, in heterozygous children of the same population who had already been hospitalized for a severe hemolytic crisis, we found a shift in favour of the deficient phenotype (12).

The practical implication of these studies is that using screening methods such as the methemoglobin reduction test or the fluorescence spot test (1, 2) and even with quantitative assay, there is a consistent percentage of Gd^{Mediterranean} heterozygotes which escapes diagnosis (7). Moreover, increasing difficulties in establishing the diagnosis has been found in a percentage of female heterozygous newborns (16).

In this study, the distribution of red cell G₆PD phenotypes in Gd^{Mediterranean} heterozygous females and hemizygous males was evaluated at birth by means of the methemoglobin elution test.

MATERIALS AND METHODS

From January to March 1979, all newborn females delivered in the Obstetric Department of Cagliari University were examined for red blood cell G₆PD activity and G₆PD red blood cell phenotype. Forty-six subjects with reduced enzymatic activity and percent G₆PD-deficient red blood cells higher than 5% were found. They were considered to be true G₆PD-deficient heterozygotes being daughters of a deficient father and a normal mother, a homozygote mother and a normal father, or a heterozygote mother and a normal father.

In addition, 20 newborn hemizygous G₆PD-deficient males, 50 adult heterozygous females (relatives of the newborn heterozygous females included in this study), 30 adult hemizygous males, and 114 normal newborns were examined.

Meanwhile and for the rest of the year, all newborn males delivered in the same department were screened for G₆PD deficiency by the Beutler method (2).

Hyperbilirubinemia was diagnosed when bilirubin levels were higher than 7, 12, 14, and 16 mg/dl at 24, 48, 72, and 96 hr from birth, respectively.

Informed consent from all subjects or parents was obtained. G₆PD activity of the hemolysate was determined according to the method of Zinkham *et al.* (15) In our laboratory, this method gave values of 6.0 ± 1.5 IU/g Hb in normal males and 0.12 ± 0.2 in hemizygous males in crisis-free periods. Reticulocyte count was performed in each subject.

The presence of G₆PD activity in individual erythrocytes was tested using the methemoglobin elution test (5). This method combines the elution-staining technique for detecting individual methemoglobin containing red blood cells (8) with the methemo-

globin reduction test (1). In the elution staining method, individual red blood cells containing mainly methemoglobin are unstained as the methemoglobin is eluted, whereas cells containing predominantly hemoglobin are stained. Inasmuch as the amount of methemoglobin reduced during *in vitro* incubation with methylene blue or Nile blue is closely related to the G₆PD activity of the erythrocytes, cells which are relatively deficient in G₆PD activity do not reduce methemoglobin efficiently and remain unstained upon elution of methemoglobin.

For an objective estimate of the proportion of individual red blood cell phenotype, two slides per person were scanned with a zigzag pattern, and a total of 1,000 cells was classified. All the determinations mentioned above were completed within 24 hr from the time of collection. The thresholds for the normal and the mutant homozygous phenotypes were found with this test to be 5 and 95% of G₆PD-deficient red blood cells, respectively.

Electrophoretic migration was carried out following the technique of Rattazzi *et al.* (10). In fact, in Sardinia the only variants frequently to be found are the Gd^{Mediterranean} and Seattle-like. The latter is clearly to be distinguished from the Gd^{Mediterranean} because of the faster electrophoretic migration.

The statistical significance of the correlation between the percentage of G₆PD-deficient red blood cells and G₆PD enzymatic activity was evaluated in both newborns and adults with the unpaired Student *t* test. Then a comparison of the correlation coefficient obtained in newborns (*r* N) and in adults (*r* A) was carried out by Student's unpaired *t* test. The shift from mean G₆PD-deficient red blood percentage (50%) was evaluated in both newborns and adults using the Student *t* test. The distribution of percentage G₆PD-deficient red blood cells and G₆PD enzymatic activity was studied by χ^2 goodness of fit test. Mean percentage G₆PD-deficient red blood cells and mean G₆PD enzymatic activity of newborn and adults were compared by the unpaired *t*-test. The cumulative distribution function (Kolmogorov-Smirnov two sample K-S-test) of both parameters, *i.e.* percentage of G₆PD-deficient red blood cells and G₆PD enzymatic activity and the variance for only enzyme activity (χ^2 test), were compared in the two populations (newborns and adults). Moreover, the linear regression (Student's *t* test) and linear correlation (Fisher's *z* test) have been compared in both groups.

RESULTS

Inasmuch as electrophoretic migration constantly showed a normal pattern, all the subjects examined were considered as having the Gd^{Mediterranean} variant.

Newborn heterozygotes have mean red blood cell G₆PD enzymatic activity of 3.23 ± 1.04 ranging from 1.28 to 5.80, whereas normal newborns have an average enzyme activity of 8.78 ± 1.91 (range, 5.16 to 11.8). There is a highly statistically significant difference ($P < 0.0005$) between mean values of these two groups.

On the other hand, there is no significant difference (*t* test; $P > 0.30$) (K-S-test; $P > 0.10$) between mean G₆PD red blood cell enzyme activity of newborn and adult heterozygotes who have an average enzymatic activity of 2.93 ± 0.86 (range, 1.21 to 4.39).

Figure 1 shows red blood cells of a heterozygous G₆PD-deficient newborn female and a hemizygous G₆PD-deficient newborn male investigated by the methemoglobin elution test for the distribution of G₆PD red blood cell phenotypes. As can be seen, newborn heterozygous females showed the expected two red blood cell populations, *i.e.*, one deficient and the other normal (mosaicism). However, newborn hemizygous males had a consistent percentage of stained red blood cells due to the presence of reticulocytes or young erythrocytes (pseudomosaicism), unlike the occasional stained red blood cells seen in hemizygous G₆PD-deficient adult males. Figure 2 shows the regression lines of percentage deficient red blood cells (*x*) and enzymatic activity (*y*) alternatively considered as independent variables. As can be seen, there is a clear correlation between G₆PD enzymatic activity and percentage of enzyme-deficient red blood cells for both newborns and adults (*r* N = 0.5093; *r* A = 0.4804). Moreover, it can be seen that the

Fig. 1. Red blood cells of a hemizygous-deficient newborn male (A) and a heterozygous G₆PD-deficient newborn female (B).

percentage of enzyme-deficient red blood cells (mean, 43.67; $P < 0.025$), is significantly left-shifted in newborns, whereas no significant shift was seen in adults (mean, 53.27%; $P > 0.20$). There is a statistically significant difference ($P < 0.001$) between the two correlation coefficients (*r* N and *r* A). Furthermore, there is a significant difference between both the cumulative distribution function and thus of the histograms (two-sample K-S-test; $P < 0.025$) and mean values ($P < 0.025$) of these parameters (*x* and *y*) between the two populations, *i.e.* newborns and adults. Although shifted one from the other, regression analysis shows that the two populations maintain the same dispersion ($P > 0.30$ for linear regression coefficient of both populations).

Newborn G₆PD-deficient males have a mean enzymatic activity of 0.30 ± 0.3 (range, 0 to 0.85) and a consistent percentage of G₆PD stained red blood cells varying from 5 to 12% (average, 8.28 ± 2.2), unlike adult G₆PD-deficient males who have only occasional (always $< 5\%$) G₆PD-positive red blood cells.

Reticulocyte counts were 34.4 ± 8.6 in heterozygous newborn females, 35.1 ± 10.9 in hemizygous newborn males, 35.8 ± 9.4 in normal newborns, 12.3 ± 5.3 in heterozygous adult females, and 11.4 ± 4.4 in hemizygous adult males, respectively.

In this series, none of the hemizygous males and two of the heterozygous females developed hyperbilirubinemia. In one of the latter, the cause was ABO isoimmunization whereas in the other no common causes of neonatal jaundice could be found. Therefore, the percentage of heterozygotes, developing hyperbilirubinemia, probably due to G₆PD deficiency, is 2.2%.

Meanwhile, this study was carried out, and for the rest of the year, all newborn males delivered in the Obstetric Department were screened for G₆PD deficiency with the Beutler method (2).

Of the 1744 males tested, including 313 hemizygotes (17.94%) and 1433 normals, 74 non-G₆PD-deficient (5.1% of normals) and

Fig. 2. Correlation between G₆PD enzymatic activity (y) and percentage of G₆PD-deficient red blood cells (x) in newborns (A) and adults (B): a_x and b_x , regression lines obtained considering x as independent variable; a_y and b_y , regression lines obtained considering y as independent variable. Newborns: $n = 46$; mean percentage G₆PD-deficient red blood cells, 43.67; $r = -0.5093$. Adults: $n = 50$; mean percentage G₆PD-deficient red blood cells, 53.27; $r = -0.4804$.

32 G₆PD-deficient (10.22% of hemizygotes) subjects developed hyperbilirubinemia which was not attributable to common neonatal causes (Rh and ABO isoimmunization). Newborn G₆PD-deficient subjects had not been exposed to any of the drugs known to cause hemolysis in sensitive subjects. However, the mothers of the babies were usually given oxytocin.

The percentage of enzyme-deficient red blood cells in the heterozygote developing hyperbilirubinemia not due to isoimmunization was 82%.

DISCUSSION

As was to be expected, this study shows a highly significant correlation between the G₆PD enzymatic activity, expressed as IU/g Hb and the percent of G₆PD-deficient red blood cells in newborn G₆PD-deficient heterozygous females, as was already found in G₆PD-deficient adult females (6, 11, 12).

The distribution of G₆PD-positive and G₆PD-negative red blood cells of newborn heterozygotes is skewed towards the G₆PD-positive cell type, unlike the symmetrical distribution found in adult heterozygotes in this study.

Previous studies in adults and children heterozygotes for the G_d^{Mediterranean} mutant have shown a distribution in favour of the normal phenotype (11) or of the deficient one (12) respectively.

In the studies by Rinaldi *et al.* (11) and Sanna *et al.* (12), heterozygotes were ascertained differently from this investigation

because the adult subjects examined by Rinaldi *et al.* (11) had been chosen at random and identified on genetic criteria, while Sanna *et al.* (12) obtained their data by the examination of children who had been hospitalized for a severe hemolytic crisis.

Our results in G₆PD-deficient heterozygous newborns may be explained assuming a faster removal of G₆PD-deficient red blood cells from the blood stream in newborns than in adults. In fact, newborn red blood cells, even in normals, have a reduced life span and are particularly defective in the enzymatic machinery required to maintain hemoglobin the reduced state because NADH-dependent diaphorase as well as glutathione peroxidase is less active in newborn than in adult red blood cells (9). Therefore, the additional deficiency of G₆PD might make the G₆PD-negative red blood cells of newborn heterozygotes even more prone to hemolysis and therefore to faster removal from the blood stream.

Alternatively and perhaps more likely, the presence in newborns of a younger mean red blood cell population may determine a shift towards the normal cell type because the G₆PD-negative reticulocytes or young red blood cells have greatly reduced levels of enzymatic activity but could be still stainable by the methemoglobin elution test. The last interpretation is supported by our finding of a consistent percentage of G₆PD-positive red blood cells in hemizygous newborns for the G_d^{Mediterranean} defect.

In this study, the prevalence of hyperbilirubinemia in hemizygous males and heterozygous females was 10.22 and 2.2, respectively whereas in G₆PD normal newborns it was 5.1%. Highly variable prevalence figures have been found in other series (3, 4, 14), suggesting the role of different genetic constitutions or environments in the phenotypic manifestations of G₆PD deficiency in the neonatal period.

It is worth noting that the only heterozygote developing hyperbilirubinemia in this series had the highest percentage of enzyme-devoid red cells.

Whatever the cause, our results, confirming and expanding previous studies (7, 16) showed that in the neonatal period the diagnosis of the heterozygous state for G₆PD deficiency of the Mediterranean type may be more difficult than in adults. Thus, to identify the major part of heterozygous newborns, very sensitive techniques such as the study of individual red blood cell G₆PD phenotype with the methemoglobin elution test should be carried out.

However, even with this technique, few heterozygotes, expressing almost extreme phenotype at the left end of the distribution, will be missed. Moreover, it is likely that newborn heterozygotes with less extreme shifting towards the normal phenotype but developing neonatal jaundice due to G₆PD deficiency, will escape diagnosis because the G₆PD-deficient red blood cells will probably have been completely cleared from the blood stream at the time of blood withdrawal.

However, it must be pointed out that using less sensitive techniques, such as those used in screening and even with the quantitative enzymatic assay, the percentage of newborn heterozygotes which will escape diagnosis is much higher. Thus mass neonatal screening for G₆PD deficiency, suggested for prevention of neonatal jaundice, seems to be ineffective and unnecessary at least in females.

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17. Requests for reprints should be addressed to: Professor Antonio Cao, 2a Clinical Pediatrica, Università Studi Cagliari, Via Porcell, 1, (Casella Postale 251), 09100 Cagliari, Sardinia, Italy.
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