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Favism
$Gd^{Mediterranean}$ heterozygous females
G6PDd activity
hemolytic crises

Favism in $Gd^{Mediterranean}$ Heterozygous Females

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Summary

Fifty-one girls, aged 2-10 yr, who had had hemolytic crises requiring hospitalization after the ingestion of fava beans, were randomly selected and examined for G6PD activity and percent of G6PD deficient red cells. The parents as well as 35 $Gd^{Mediterranean}$ heterozygous females who had never had significant hemolytic crises were also examined. $Gd^{Mediterranean}$ heterozygous females showed a G6PD activity of 2.58 ± 0.69 IU/gHb. The percent of deficient red cells was 60.09 ± 10.1 ranging from 45-85%. A clear correlation between the G6PD activity and percent of enzyme-devoid red cell was found. The distribution of the subjects examined according to the percent of G6PD deficient red cells is significantly shifted in favor of those whose percent of G6PD-red cells is higher than 50%. This is more conspicuous in subjects already hospitalized for a hemolytic crisis as compared to subjects who had never had hemolytic crises. In the group of girls hospitalized for a severe hemolytic crisis after ingestion of fava beans, we observed 39% homozygotes and 61% heterozygotes. Among the heterozygotes, only three subjects developed severe crises as to require transfusion. On the basis of the number of heterozygotes hospitalized for a hemolytic crisis and on the number of crises-free heterozygotes out of the population as a whole, we drew a probability curve showing the risk for G6PD deficient heterozygous females to develop hemolytic crises during childhood. This risk does not exceed an average of 1.3%.

Speculation

The findings that G6PD deficient heterozygous females have a low probability of developing a significant hemolytic crisis during childhood and that such crisis is always moderately severe has a practical utility in genetic counselling and in the prescription of drugs that may be potentially harmful to G6PD deficient female heterozygotes.

G6PD deficient heterozygous females are known to be characterized by the presence of two different red cell populations, *i.e.*, a "normal" and a "deficient" one.

Because the relative proportion of normal and deficient cells is randomly determined, the level of enzymatic activity in these patients varies according to a normal type of distribution. On the average, 50% of the cells are normal and 50% G6PD deficient, but some individuals follow a normal or totally deficient phenotype.

At the end of the distribution curve, there will be a few individuals with either type of cells in excess which will make them appear, respectively, normal or deficient (1, 3, 4, 10).

Actually, recent studies by Rinaldi *et al.* (8) showed that the distribution is shifted in favor of the normal phenotype in the case of heterozygous females selected on genetic criteria.

Female heterozygotes for G6PD deficiency of the $Gd^{Mediterranean}$ type have been found to develop self-limited hemolytic crises after the ingestion of fava beans (9). In these subjects, a correlation has been detected between the intensity of hemolytic crises on one hand and the degree of enzyme deficiency and/or the percent of deficient red cells on the other (4, 9). This correlation depends on selective hemolysis of deficient red cells sparing the normal cells.

The present study was directed to establish the levels of G6PD activity and/or the percent of deficient red cells in female heterozygotes for G6PD deficiency of the $Gd^{Mediterranean}$ type associated with a substantial risk of developing significant hemolytic crises.

MATERIALS AND METHODS

Fifty-one girls, aged 2–10 yr, who had had hemolytic crises requiring hospitalization after the ingestion of fava beans, were randomly selected and examined for G6PD activity and percent of deficient red cells 10–24 months after their crisis. Hospitalization criteria for a hemolytic crisis in our department are: presence of hemoglobinuria and/or serious anemia (Hb < 7 g/dl). The enzymatic assays were done in autumn and winter to avoid the effect of minor hemolytic crises which may occur in spring and in early summer. G6PD activity and deficient red cell percent were also determined in the parents as well as in 35 $Gd^{Mediterranean}$ heterozygous girls, of the same age as the crisis group, who had never had hemolytic crises according to clinical criteria.

Of these, some had ingested fava beans during their life. Others probably did so because this legume is a fundamental part of the diet in many Sardinian villages.

The informed consent of all subjects was obtained.

G6PD activity of the hemolysate was determined according to the method of Zinkham *et al.* (11). In our laboratory this method gave values of 6.0 ± 1.5 IU/gHb in normal males and 0.12 ± 0.2 in hemizygous males in crises-free periods.

The presence of G6PD activity in individual erythrocytes was tested using the methemoglobin elution test (1). For an objective estimate of the proportion of individual red cell phenotypes, two slides per person were scanned with a zig-zag pattern and a total of 1000 cells were classified.

All the determinations mentioned above were completed within 24 hr from the time of collection.

According to Rinaldi *et al.* (8), the thresholds for the normal and the mutant homozygous phenotypes were found with this test to be 5 and 95% of G6PD deficient red cells, respectively. The percent of reticolocytes, assessed in all the subjects examined was within the normal range.

Electrophoretic migration was carried out on each subject, following the technique of Rattazzi *et al.* (7). In fact, in Sardinia, the only variants frequently to be found are the $Gd^{Mediterranean}$ and Seattle-like (8).

The latter is clearly to be distinguished from the $Gd^{Mediterranean}$ on account of the faster electrophoretic migration.

A female with reduced enzymatic activity was considered to be a true heterozygote if: 1) she was the daughter of a deficient father and a normal mother; 2) she was born of a homozygote mother and a normal father; and/or 3) she was born of a heterozygote mother and a normal father.

On the other hand, a female with a fully deficient phenotype was considered to be homozygote if she was the daughter of a deficient father and of a heterozygous or homozygous mother. Actually, such a classification inappropriately includes among homozygotes also the very rare heterozygotes with a totally deficient phenotype, which are daughters of a deficient father and a heterozygote mother.

DATA PROCESSING

The following data were used to define a mathematical model for the description of the risk of severe hemolytic crises developing in heterozygotes: 1) child population, from birth to 14 years, covered by the district: 270,000 in 3 yr; 2) total number of children hospitalized in the above district in the last 3 years: 11,348: 3) number of children hospitalized in the same period for a hemolytic crisis related to G6PD deficiency—326 males, 141 females; 4) incidence of G6PD heterozygotes in the same population. The incidence figure of 38.7% of G6PD heterozygotes was estimated on the basis of the incidence of male hemizygotes according to the Hardy-Weinberg equilibrium.

The risk was defined by calculating the ratio of the number of heterozygotes hospitalized for a hemolytic crisis to the number of crises-free heterozygotes.

In heterozygous females taken as a whole, this "average global risk" is approximately 1.3%.

The distribution of the heterozygotes population according to an 0–6 range of enzymatic activity was also studied. The range 0–6 suggested the use of a type β distribution: $f(x) = cx^{\alpha-1}(6-x)^{\beta-1}$ $0 < x < 6$ were α,β are real positives and c is the factor of normalization.

The figures for risk percent as a function of enzymatic activity were obtained by working out pointwise the ratio of function crisis-free subjects/crisis-prone subjects and multiplying it by the average global risk.

RESULTS

Figure 1 shows the red blood cells of normal subjects and of heterozygous and homozygous females for G6PD deficiency investigated by the methemoglobin elution test for the distribution of G6PD red blood cell phenotype.

Gd^{Mediterranean} heterozygous females showed a percent of deficient red cells of 60.09 ± 10.1 ranging from 45–85%. As can be shown in Figure 2, a clear correlation ($r = 0.544$, $P < 0.001$) between the G6PD activity and percent of enzyme-devoid red cell was found.

This figure shows that distribution of the subjects examined according to the percent of G6PD deficient red cell is significantly shifted in favor of those whose percent of G6PD red cells is higher than 50% (χ^2_{real} 4.81 $\chi^2_{theor.}$ 3.84, $P < 0.05$).

This is more conspicuous in subjects already hospitalized for a hemolytic crisis as compared to subjects who had never had hemolytic crises.

Figure 3 shows that crises-free heterozygotes have an average enzymatic activity of 2.85 ± 0.76 ranging from 1.21–4.3 while crises-prone subjects have an average enzymatic activity of 2.58 ± 0.69 ranging from 1–3.72 (IU/gHb).

The results of G6PD activity and the percent of G6PD deficient red cells in females who had had significant hemolytic crises have been summarized in Figure 4.

It can be seen that out of the females tested, 39% were homozygous and 61% were heterozygous for G6PD deficiency.

Figure 4 also shows that all the homozygotes as well as the possible heterozygotes with totally deficient phenotype required packed red cell transfusion whereas three heterozygotes only were transfused. No correlation between extent of G6PD deficiency and transfusion requirements emerged among heterozygotes.

In our department, an acute hemolytic crisis in G6PD subjects is treated with packed red cell transfusion in the following conditions: 1) Hb <6g/dl; and/or 2) Hb <7g/dl in case of hemoglobinuria.

The lower Hb levels obtained during the crises in each subject examined in relation to the degree of enzyme defect is shown in Figure 5. Among heterozygote females, no significant correlation ($r = 0.34$) between Hb levels and enzymatic activity was exhibited.

Figure 6 shows the probability for a G6PD deficient heterozygote to have a severe hemolytic crisis during childhood, according to the different levels of enzymatic activity. We also compared the distribution of the crises-free heterozygote population with respect to that of the crises-prone heterozygote population.

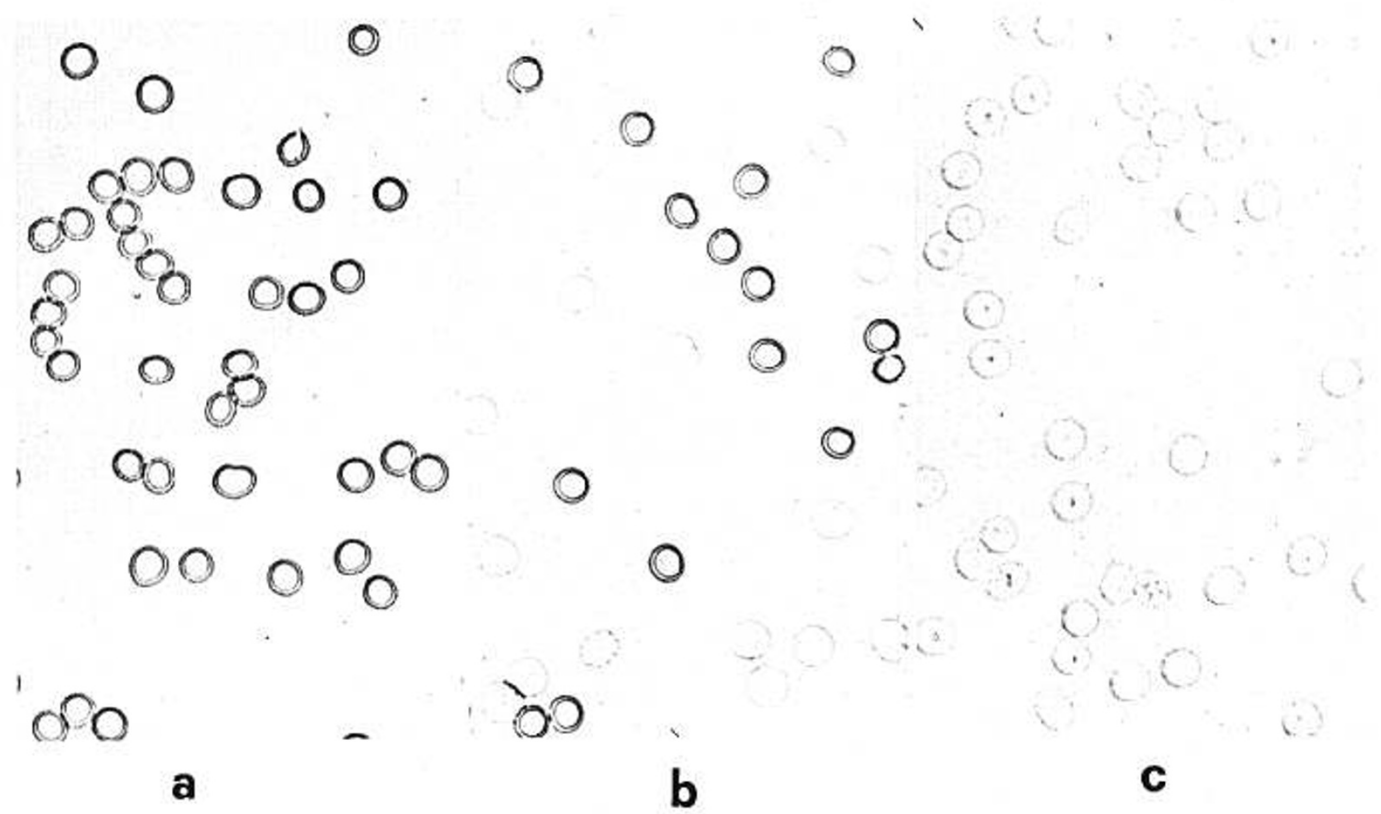

Fig. 1. Methemoglobin elution test for the distribution of G6PD red blood cell phenotype: A) normal subject, B) heterozygous for G6PD defect, C) homozygous for G6PD defect.

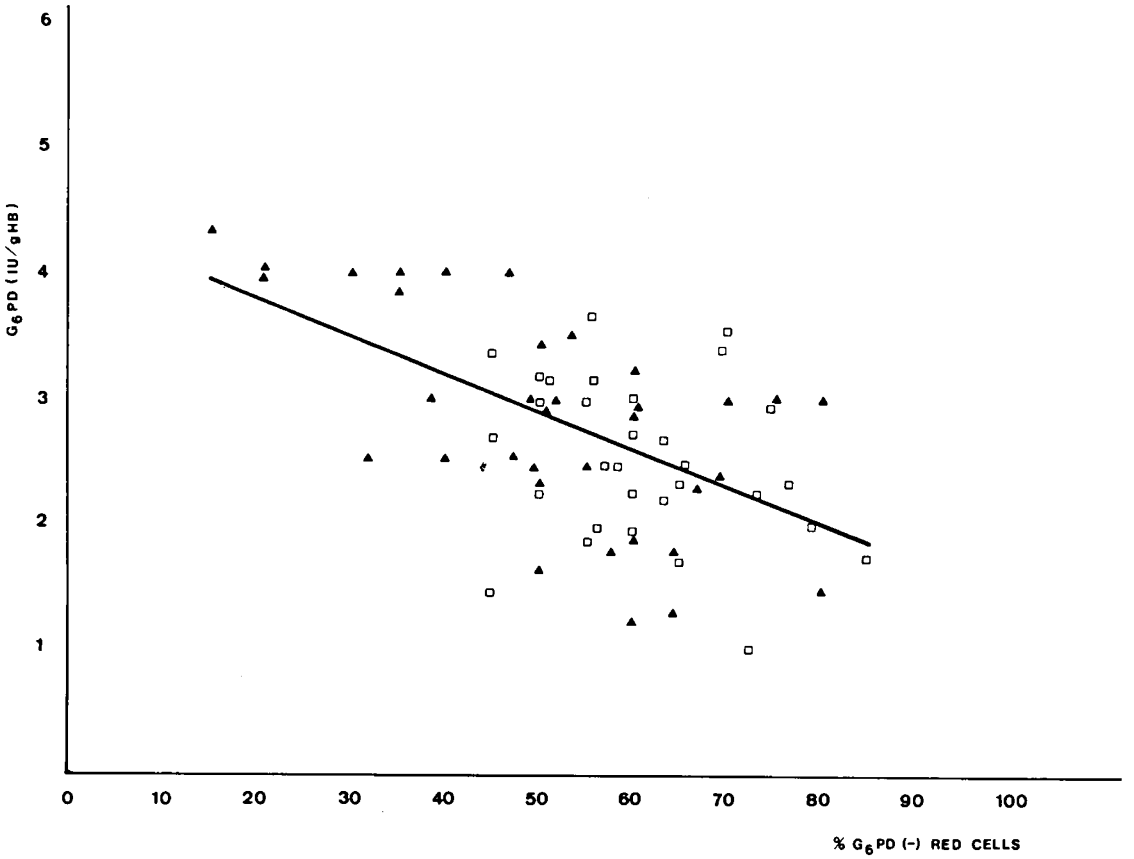

Fig. 2. Correlation between G6PD activity and percent of enzyme devoid red cell ($r = 0.544$; $P < 0.001$). □ heterozygous female who had significant hemolytic crisis, ▲ heterozygous female who never had significant hemolytic crisis.

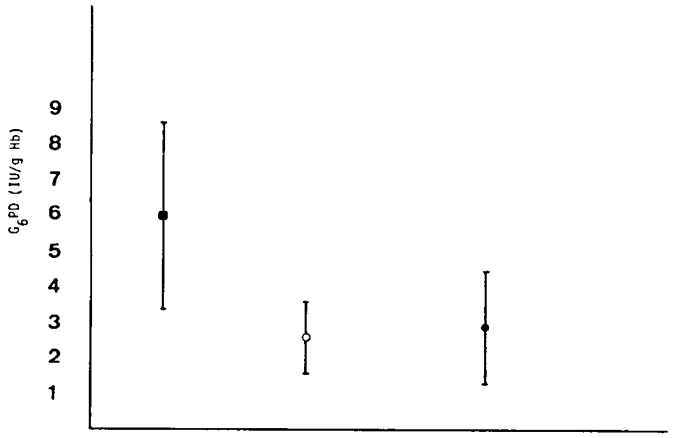

Fig. 3. Distribution of enzymatic activity, ■ normal subject, ○ heterozygous females who had significant hemolytic crisis, ● heterozygous females who never had significant hemolytic crisis. Each point represents the average of enzymatic activity of each group of subjects, and the vertical bars represent the 2 SD.

The comparison between the to normalized curves shown a shift to lower figures of enzymatic activity for crises-prone heterozygotes.

DISCUSSION

As was to be expected, the results of our work show, first of all, the existence of a highly significant correlation between the enzymatic activity, expressed in IU/gHb, and the percent of G6PD deficient red cells.

A similar correlation between enzymatic activity and G6PD cell percent was observed by Kattamis *et al.* (3) with the cyanmethemoglobin elution test and by Rinaldi *et al.* (8) with the histochemical method we ourselves used.

In the work of Rinaldi *et al.* (8) the correlation is less significant than in ours.

In this respect, it is worth noting that Rinaldi *et al.* (8) determined the enzymatic activity with the semi-quantitative method of Motulsky and Campbell-Kraut (5). In the group of heterozygotes we examined, which comprised subjects who had had severe hemolytic crises as well as crises-free subjects, or the percent of G6PD deficient red cells, showed a shift in favor of the deficient phenotype. That was more evident in the first group.

A distribution in favor of the normal phenotype has been observed by Rinaldi *et al.* (8).

The difference between our results as for the first group, and those of Rinaldi *et al.* (8) depends on our having selected the female heterozygotes we examined among those who had been hospitalized for a severe hemolytic crisis, while the subjects examined by Rinaldi *et al.* (8) had been chosen at random and identified on genetic criteria. It is worth noting that we never found subjects with a fully deficient phenotype among the heterozygotes we observed.

After screening for G6PD deficiency the hospitalized women belonging to the same population we examined, Gandini *et al.* (2) selected the subjects with a normal father and a normal son and observed that 1.3% of the heterozygotes had a completely deficient phenotype. The difference between ours and the data of Gandini et al. (2) is probably to be ascribed to the small number of subjects we examined. Heterozygote distribution with respect to enzymatic activity shows that crises-prone subjects very slightly overlap with the values of normal subjects whereas crises-free subjects overlap much more extensively. The subjects we observed belonged to the population of South Sardinia where the incidence of hemizygote males has been demonstrated to be 26.3% (8).

According to the Hardy-Weinberg equilibrium, the frequency of homo- and heterozygotes females is 6.9 and 38.7%, respectively.

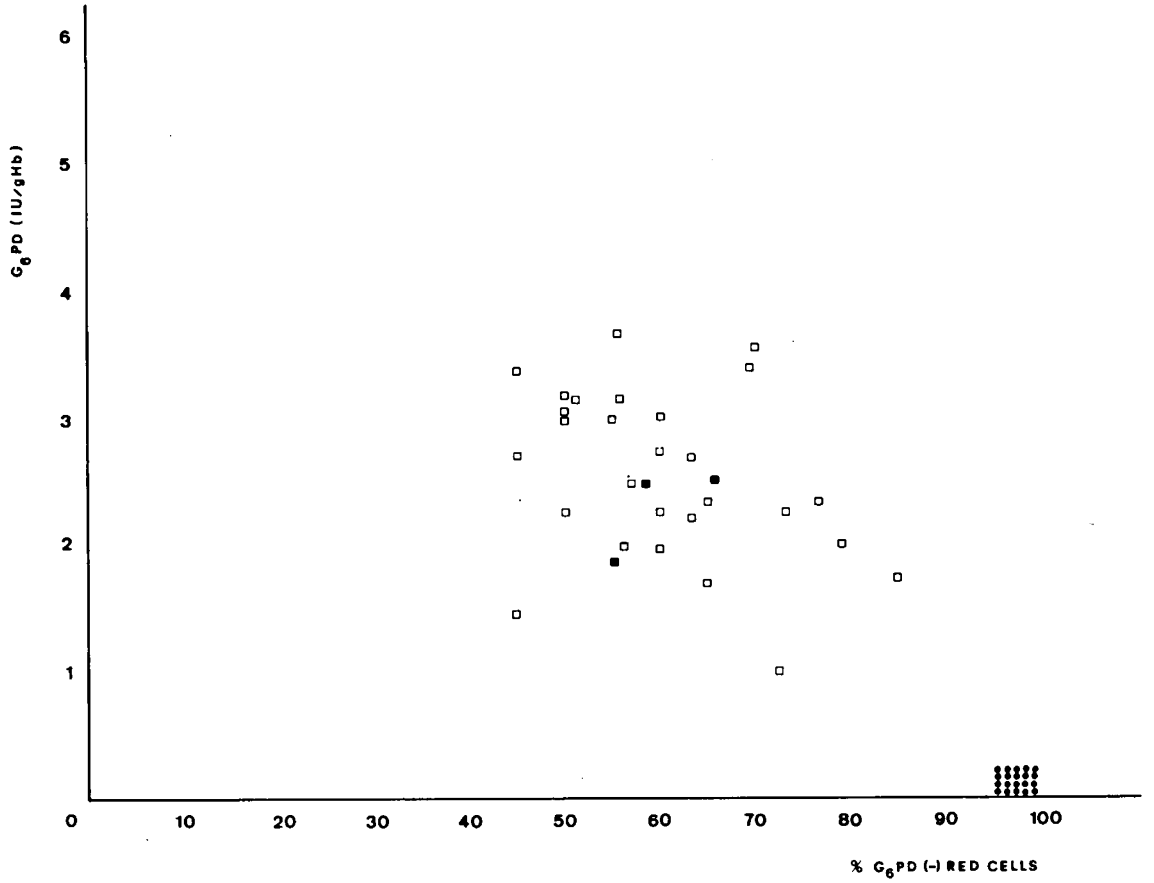

Fig. 4. G6PD activity and percent of G6PD deficient red cells in girls who had significant hemolytic crisis. □ heterozygous not requiring transfusion, ■ heterozygous requiring transfusion, ● homozygous requiring transfusion.

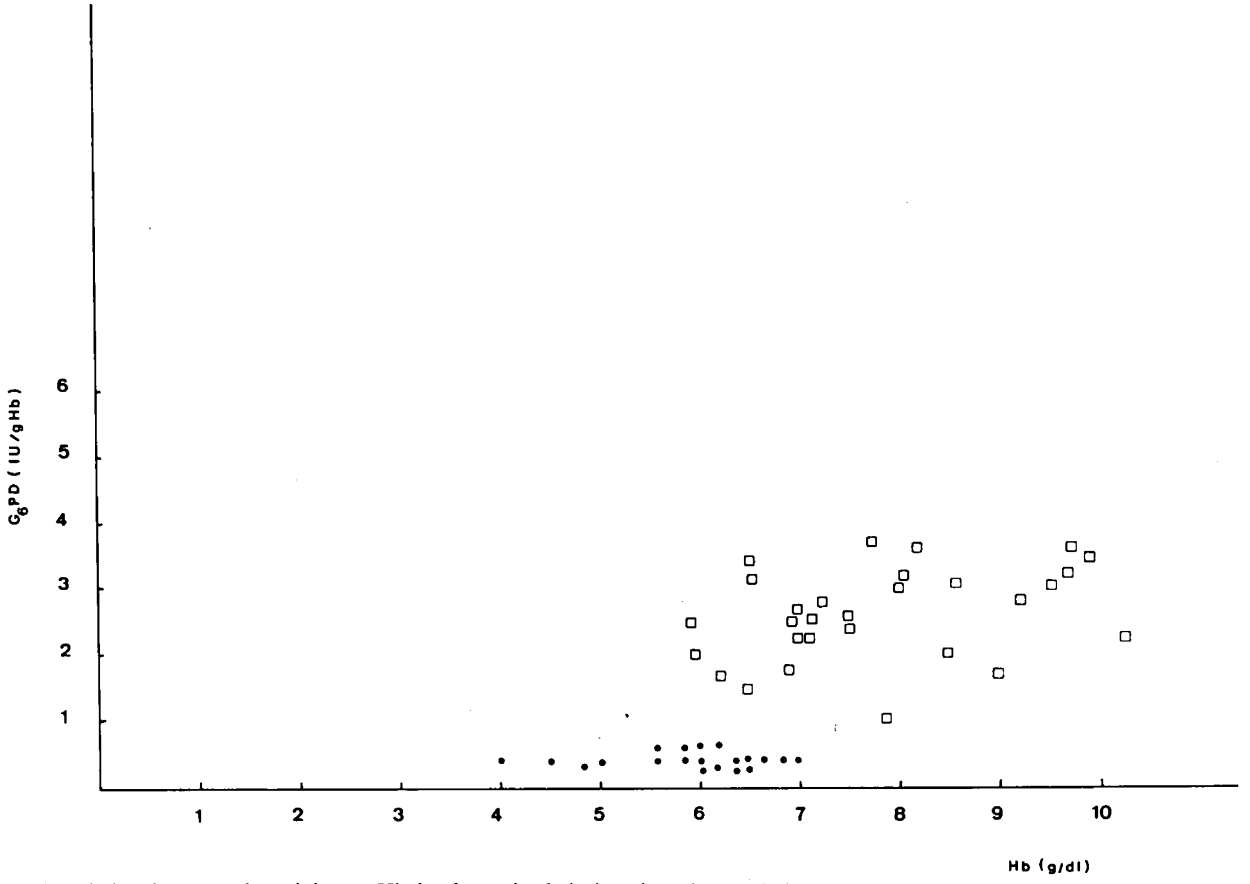

Fig. 5. Correlation between the minimum Hb levels attained during the crises and the degree of enzyme defect. ● homozygous female, □ heterozygous female.

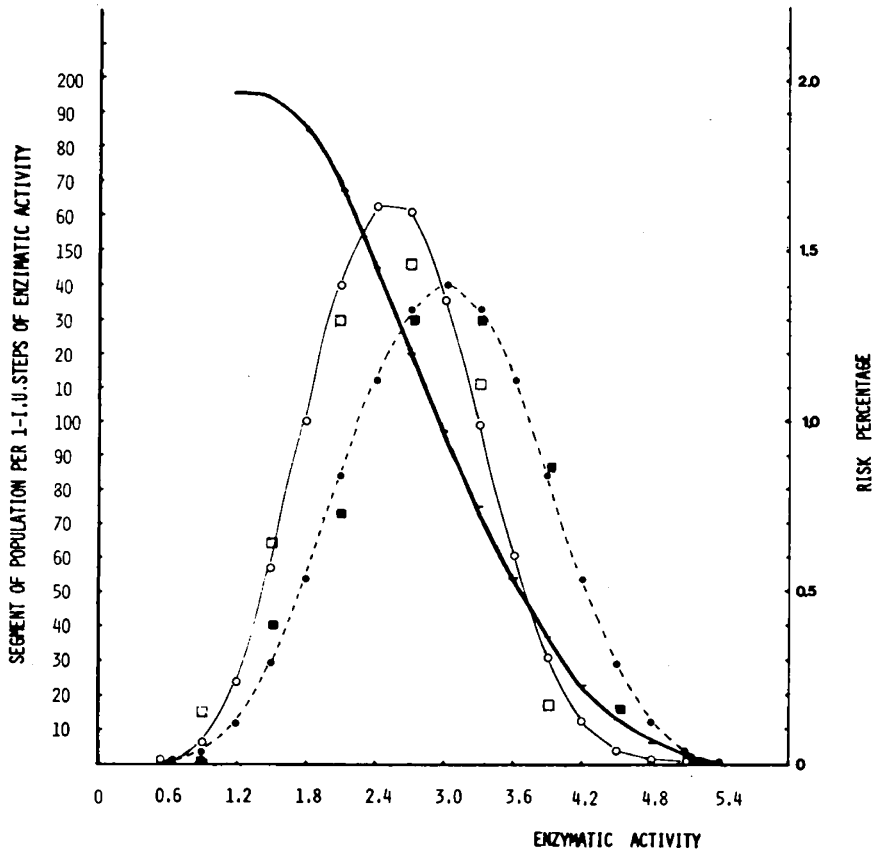

Fig. 6. Probability for a G6PD deficient heterozygote to have a severe hemolytic crisis, according to the different levels of enzymatic activity. --●-- crises-free heterozygote population (■ real values); —○— crises-prone heterozygote population (□ real values); ——— probability curve.

In the group of G6PD deficient girls, hospitalized for a severe hemolytic crisis after ingestion of fava beans, we observed 39% homozygotes and 61% heterozygotes. It is worth noting that only three out of all the heterozygotes required transfusion. On the basis of these two data, we may retain that G6PD deficient heterozygotes are subject to moderately severe crises.

Unlike the observations of Russo *et al.* (9), the minimum Hb levels found during the crisis did not seem to correlate significantly with enzymatic activity. This leads us to retain that the severity of the crisis may be ascribed to other factors too, apart from the enzymatic activity, such as individual differences in the metabolism of oxydizing drugs or of the agent contained in fava beans.

As we can see from the analysis of the probability curve described, the risk for a female heterozygote to develop a significant hemolytic crisis during childhood, is obviously related to the enzymatic activity. However, this risk does not globally exceed an average of 1.3%. The possibility for G6PD subjects who develop a hemolytic crisis after the ingestion of oxidizing drugs or fava beans to have an additional genetic or acquired deficiency is a limiting factor in this context (6).

Recently, a phenotypic variability of individual somatic cells of heterozygotes at the G6PD locus in the same individual was found (8).

However, a significant variation with a shift from 0–11 and 31%, respectively of G6PD deficient red cells was found in only two heterozygotes with a normal phenotype among 77 heterozygotes examined. This variation is probably best explained by a somatic selection that is a shorter survival rate of G6PD deficient red cells in the environment.

This data, therefore, does not limit the validity of the approach presented in this study.

In conclusion, the information produced by this study seems to have a practical utility in genetic counselling and in the prescription of drugs that may be potentially harmful to G6PD deficient female heterozygotes.

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