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A STUDY OF SUBJECTS WITH ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY. II.
INVESTIGATION OF LEUKOCYTE
ENZYMES *

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The susceptibility to hemolysis following administration of primaquine, naphthalene and other drugs, as well as after the ingestion of fava beans was described in individuals with a defect in their erythrocyte metabolism (1, 2). These erythrocytes manifest a number of abnormalities, namely, a low reduced glutathione (GSH) level, a glutathione instability when exposed to acetylphenyl hydrazine, a low glucose-6-phosphate dehydrogenase activity (G6PD), high aldolase and a decreased incorporation of glycine into GSH *in vitro* (3-7). The primary defect of these cells is probably related to the low G6PD.

This erythrocyte abnormality is transmitted by an incompletely dominant sex-linked gene (8, 9). Recently we demonstrated that the platelets of these individuals exhibit a decreased G6PD activity (10). On the other hand, Marks, Gross and Hurwitz have found a normal G6PD activity in the leukocytes of such individuals; the activity of this enzyme in their livers was found to be slightly decreased (11).

The investigation of G6PD in leukocytes in our population is described in the present communication. Contrary to the findings of Marks and associates, we found significantly lowered activity of this enzyme in leukocytes of subjects with the erythrocyte defect.

MATERIAL AND METHODS

Blood was obtained from: a) individuals, mostly males, whose erythrocytes demonstrated a marked glutathione instability by the method of Beutler, Robson and Buttenweiser (5) and very low G6PD activity (these subjects are referred to as "sensitive"); b) females with intermediate levels of this enzyme; c) control group.

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Leukocytes were isolated from 20 ml. blood (12). The anticoagulant solution used was 1 per cent disodium sequestrene, 1 per cent tritone (W.R. 1339) in 0.7 per cent NaCl. All operations were performed at +4° C. The blood was centrifuged for eight minutes at 200 G. The supernatant platelet-rich plasma was discarded. The buffy coat was removed, mixed with one-fifth of its volume polyvinyl pyrrolidone (Teva) and allowed to sediment for half an hour at +4° C. in a tube at a 45° angle. The supernatant solution was removed and centrifuged at 250 G for 10 minutes.

In order to obtain a pure leukocyte suspension the remaining red cells were lysed by the addition of 3 ml. distilled water to the leukocyte sediment. After 20 seconds 1 ml. of 3.4 per cent NaCl solution was added in order to readjust the isotonicity of the medium. The mixture was centrifuged at 1,000 G for 10 minutes and the pure leukocyte sediment was resuspended in 0.5 ml. isotonic KCl, pH 7.4. The leukocytes were disrupted by freezing and thawing three times, using a mixture of dry ice and acetone. Leukocyte counts were performed before and after this procedure. No more than 5 per cent of cells remained intact. The leukocyte extract was spun down at 20,000 G for 10 minutes and the supernatant was used for enzyme determinations. Glucose-6-phosphate and 6-phosphogluconic dehydrogenases (6PGD) were determined by a slight modification of the methods of Kornberg and Horecker (13) and Horecker and Smyrnotis (14), respectively, as described in a previous communication (10). The volumes of the leukocyte extract used were such that the increase in the optical density at 340 m μ following reduction of triphosphopyridine nucleotide (TPN) was between 0.07 and 0.015 per 30 seconds. The results were expressed in units. A unit of enzyme activity is expressed as the change in optical density per minute per 10⁹ cells (O.D. per minute per 10⁹ cells). We used this type of calculation in order to compare our results with those of Marks and associates (11).

RESULTS

The results are summarized in Table I. The G6PD activity in leukocytes of the control group was found to be 34.7 ± 11.2 units, while in the "sensitive" group the respective mean was $7.59 \pm$

2.72 units ($p < 0.001$), and in the intermediate group 13.29 ± 4.46 units. No significant difference in the 6PGD activity was detected between "sensitive" and nonsensitive subjects.

The G6PD and 6PGD activities are much higher in leukocytes than in platelets or erythrocytes. Table II illustrates the G6PD activity in leukocytes, platelets and red blood cells of normal and "sensitive" subjects.

Table III depicts the residual G6PD activity in leukocyte extracts heated for five minutes at 37°C . As can be seen, leukocyte extracts of "sensitive" subjects are more heat labile. However, since the initial enzyme levels in abnormal cells are much lower than those of the control group the validity of these results could be questioned.

TABLE I

Glucose-6-phosphate and 6-phosphogluconic dehydrogenase activities in leukocytes of normal and "sensitive" subjects

Subjects	Number	G6PD activity*	Number	6PGD activity*
Control	26	34.70 ± 11.20	18	8.66 ± 3.06
Severe deficiency				
red cell G6PD	25	7.59 ± 2.72	11	8.64 ± 2.63
Intermediates	7	13.29 ± 4.46	2	9.00

* O.D. per minute per 10^9 leukocytes \pm standard deviation.

DISCUSSION

Our increasing knowledge of the heterogeneity of enzymes raises two questions: a) Are the enzymes performing the same function in different tissues identical or different? b) Are they controlled by the same gene or do different genes regulate their functions?

In congenital galactosemia, the galactose 1-P uridyl transferase is absent in red cells and liver (15). In hypophosphatasia, the enzymatic defect was demonstrated in serum, liver, bone, kidney, intestine and white blood cells (16). We have demonstrated a decreased G6PD activity in platelets of subjects exhibiting this abnormality in their erythrocytes (10). Marks and colleagues, however, demonstrated a normal enzyme level in leukocytes of affected subjects and suggested that this enzymatic defect of the erythrocytes is not a primary but a more remote consequence of another genetic defect (11). On the other hand, we

TABLE II

Comparison of glucose-6-phosphate dehydrogenase activities in leukocytes, platelets and erythrocytes of normal and "sensitive" subjects

Subjects	Leukocytes*	Platelets*	Erythrocytes*†
Control	34.7 ± 11.2	0.127 ± 0.038	0.52 ± 0.10
"Sensitives"	7.59 ± 2.72	0.024 ± 0.017	0.08 ± 0.02

* O.D. per minute per 10^9 cells \pm standard deviation.

† Data obtained with a new batch of TPN on 16 controls and 5 "sensitive" males.

demonstrated a low enzymatic activity in leukocytes of our affected subjects. It is of great interest that the enzyme level in normal leukocytes is much higher than in erythrocytes or platelets. The enzyme activity of leukocytes and platelets of affected individuals is about 20 per cent of the normal activity. This could be related to the relatively similar survival time of platelets and leukocytes, which is of the order of 5 to 20 days, while the red cell survival is 120 days.

Since the 6PGD activity is the same in normal and affected cells, the differences in G6PD activity could not be related to an incomplete release of the enzyme into the extract. This conclusion is strengthened by the fact that in several experiments we calculated the enzyme activity on the basis of protein concentration in the extract and obtained the same results (17). To exclude the possibility that the difference was caused by an increased TPNH¹ oxidation in the extracts of affected cells, we measured TPNH oxidation rate of extracts obtained from platelets and leukocytes of normal and affected subjects and found practically no TPNH oxidation during a 20 minute

TABLE III

Residual glucose-6-phosphate dehydrogenase activity of leukocyte extracts heated at 37°C . for five minutes

Subjects	Number	Residual enzymatic activity in leukocytes
Control	23	% 60-90
"Sensitives"	14	0-25

¹ We are indebted to Dr. Mager, Department of Biochemistry, Hebrew University Hadassah Medical School, Jerusalem, for his many valuable suggestions and for supplying us with TPNH.

incubation period. The experiment was performed under conditions identical to those for the G6PD assay (17).

We have no explanation at present for the discrepancy between our results and those of Marks and associates (11). It should be pointed out that we found previously a lower erythrocyte G6PD level in normal subjects and practically no enzyme activity in the erythrocytes of affected males. We are at present investigating this discrepancy. It appears that it was related to the TPN batch. We have lately investigated several batches of TPN and with one of the batches found erythrocyte enzyme values similar to those of Marks. These data are presented in Table II.

Our results show that the enzymatic defect in our population is not limited to the erythrocytes and can be demonstrated in platelets, leukocytes and probably in liver.

We investigated the G6PD and 6PGD activities on liver obtained during operation from two "sensitive" females and 10 controls and found values of 0.100 to 0.150 O.D. per minute per 100 mg. wet liver tissue in normals and 0.030 and 0.025 O.D. per minute per 100 mg. wet liver tissue in the "sensitive" subjects. The 6PGD activities were similar in both groups. It will be of interest to measure the enzyme level in other tissues. The obtained results would strengthen the hypothesis, previously suggested by us and others, that we are dealing with an enzyme that is qualitatively abnormal and becomes degraded more rapidly (10, 11, 18). This would explain our finding in "sensitive" subjects that a cell population with a low mean age, characteristic of platelets and leukocytes, has a higher enzyme activity than cells with a high mean age, for instance, erythrocytes. In order to demonstrate a qualitative difference of the enzyme we tried to investigate the heat stability of the leukocyte extracts. On incubating these extracts for five minutes at 37° C., we found a proportionally greater decrease of the enzyme activity in the extracts of abnormal cells than in normal cells. However, since the enzymatic activity in the affected cells is much lower than in normals, the interpretation of these results is difficult. Only isolation of the enzyme from normal and affected cells and its investigation will further our knowledge in this field.

SUMMARY

Glucose-6-phosphate (G6PD) and 6-phosphogluconic dehydrogenase (6PGD) activities were determined in leukocytes of individuals whose erythrocytes showed a deficiency of the former enzyme. Also a significantly lower level of G6PD was found in their leukocytes, whereas the 6PGD activity was found to be normal.

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