LOW LEUKOCYTE PHOSPHORYLASE IN HEPATIC PHOSPHORYLASE-DEFICIENT GLYCOGEN STORAGE DISEASE

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Glycogen storage disease was first recognized as a clinical entity by Von Gierke in 1929 (1). Subsequently numerous clinical and biochemical studies have indicated that this syndrome is composed of several distinct entities (2, 3). The present-day classification of glycogen storage disease into five subgroups is based on the clinical picture, and in some, on the demonstration of specific enzymatic defects (4). The most common type of the disease (Von Gierke's disease) results from the lack of glucose-6-phosphatase in the liver (5). The term Pompe's disease is used to denote a group of children with generalized glycogen deposition, especially in the heart, who usually die before 8 months of age of cardiac complications (6). The third type of the disease represents cases in which debranching enzyme has been low in the livers of the patients studied (7). In the fourth type, branching enzyme is thought to be deficient on the basis of abnormal glycogen structure (8), but no definite assay has been performed to confirm this. Under the fifth subgroup, two separate syndromes have been described—patients with a progressive myopathy and absent muscle phosphorylase (9, 10), and patients with hepatomegaly and low liver phosphorylase (11).

In 1959 Hers (11) presented data on three patients with glycogen storage disease who had low levels of liver phosphorylase, but normal levels of glucose-6-phosphatase and amylo-1,6-glucosidase; muscle phosphorylase was normal. Recently we have studied two brothers with glycogen storage disease who were also found to have low liver phosphorylase but normal glycogen structure and glucose-6-phosphatase activity. The clinical data on these two patients will be presented in detail elsewhere (12).

In view of the observation that leukocyte glycogen is elevated in glycogen storage disease (13) and the desirability of establishing the nature of the enzymatic defect without resorting to liver biopsy, methods were devised to measure the activities of leukocyte phosphorylase and glucose-6-phosphatase. These were then applied to the above two patients, their relatives and a group of control subjects. The results of this study form the basis of this report.

MATERIALS AND METHODS

I. Biochemical studies. The two patients studied were brothers, aged 7 and 9, who had enlarged livers, with increased glycogen content but normal glycogen structure and glucose-6-phosphatase activity. Fasting blood sugars were somewhat low but the hyperglycemic response to epinephrine and glucagon was adequate in both. Muscle glycogen was normal. Liver phosphorylase was found to be low in both patients when assayed on specimens obtained by open liver biopsy.[1] The remaining family members included the father, aged 38, mother, aged 37, brother, aged 12, and sister, aged 11. In none of them was there a history of liver disease nor was hepatomegaly found on examination.

II. Leukocyte studies. Twenty ml of blood was drawn by venipuncture from patients in the fasting state. White blood cells were obtained by the fibrinogen sedimentation technique (14) using 3 per cent bovine fibrinogen in Krebs-Ringer bicarbonate buffer (pH 7.4). The total number of leukocytes was estimated in the supernatant after a 20-minute sedimentation. After centrifugation of the supernatant at 755 G for 20 minutes, the button of cells, which contained approximately a 1:1 ratio of leukocytes to erythrocytes, was suspended in 1 ml of NaF (0.1 M). After homogenization by ultrasonication (10 kc) for 2 minutes, phosphorylase activity was determined by the method of Sutherland (15). Three-tenths ml of the sonicate was added to tubes containing 0.1 ml of 0.02 M adenosine-5'-phosphate and 0.1 ml water. At zero time, 1 ml of a glucose-1-phosphate solution (0.05 M, pH 6.1) containing 5.7 mg glycogen per ml and NaF (0.05 M) was added. Immediately, a 0.5 ml aliquot was added to 2 ml of 10 per cent trichloroacetic acid, and inorganic phosphorus was determined by the method of Fiske and Subbarow (16). The tubes were then incubated for 30 minutes at 37° C in a Dubnoff

[1] We are indebted to Dr. Barbara Illingworth Brown, Washington University of St. Louis, for performing the studies on the liver biopsy specimens.

TABLE I

Leukocyte phosphorylase and glycogen levels in patients with glycogen storage disease and their family

Patient	Age	Relation	Condition	Glycogen	Phosphorylase
				μg/10⁷ leukocytes	*μg P liberated/10⁷ WBC*
Jo.F.A.	38	Father	Normal		33.2
A.A.	37	Mother	Normal		10.2*
Ja.A.	12	Son	Normal		33.6
R.A.	10	Son	Affected	35.4	7.1*
Jo.S.A.	7	Son	Affected	29.2	5.5*
Normal (20)†				34.3 ± 0.9‡	29.6 ± 1.2‡

* Average of four separate determinations.
† Number of control subjects.
‡ Standard error.

metabolic shaker. At the end of incubation another 0.5 ml aliquot was removed for inorganic phosphorus determination. Appropriate blanks without glucose-1-phosphate and without sonicate were included in each experiment. Results were expressed as micrograms of inorganic phosphorus liberated per 10^7 leukocytes per 30-minute incubation.

Glycogen was determined by the method of Carroll, Longley and Roe (17) on the leukocyte button suspended in 3 ml of 5 per cent trichloroacetic acid. After sonication for 2 minutes the solution was filtered through acid-washed filter paper. To 1 ml of this filtrate was added 5 ml of 99 per cent ethanol, and glycogen precipitation was allowed to occur overnight at room temperature. The precipitate was centrifuged at 1,475 G for 10 minutes and the supernatant carefully decanted. The pellet was dissolved in 2 ml of water and glycogen was determined with anthrone. Results were expressed as micrograms of glycogen per 10^7 leukocytes. Glucose-6-phosphatase was determined in sonicated leukocytes, using the method of Swanson (18).

The activation of phosphorylase by glucagon was studied by the method of Sutherland and Cori (19). The leukocyte-rich supernatant solution obtained from sedimentation with 3 per cent fibrinogen (usually about 20 ml) was preincubated in 50-ml siliconized flasks in an atmsophere of 95 per cent O_2 and 5 per cent CO_2 for 30 minutes in a Dubnoff metabolic shaker at 37° C. At the end of this incubation 0.1 mmole of glucagon was added to the appropriate flasks and incubation continued for another 30 minutes. Equal amounts of buffer without glucagon were added to appropriate flasks which served as controls. Phosphorylase activity of the leukocytes was then determined as described above.

Bovine fibrinogen was obtained from Armour Pharmaceutical Company. Glycogen was purchased from

TABLE II

Leukocyte phosphorylase activity in control subjects

Patient	Age	Sex	Condition	Leukocyte phosphorylase activity
				μg P liberated/10⁷ WBC
P.D.	17	F	Normal	30.8
E.M.	18	F	Normal	20.6
C.G.	35	M	Normal	21.4
K.S.	20	F	Normal	32.5
P.M.	18	F	Normal	28.8
H.W.	28	M	Normal	31.0
S.H.	10	F	Normal	22.4
J.P.	18	F	Normal	37.4
H.K.	35	M	Normal	37.4
C.C.	26	M	Malignant insulinoma	27.2
C.M.	7	F	Leukoencephalopathy	39.4
A.F.	26	F	Thyrotoxicosis	27.6
P.T.	9	F	Thyrotoxicosis	41.6
S.M.	3	M	Hurler's syndrome	28.4
C.M.	8	M	Hurler's syndrome	29.9
B.M.	6	F	Hurler's syndrome	23.8
L.B.	11	M	Galactosemia	29.8
J.C.	19	F	Mongolism and hypothyroidism	21.8
D.D.	1	F	Von Gierke's disease	31.1
H.S.	14	M	Adenocarcinoma thyroid	30.6
Mean				29.6 ± 1.2*

* Standard error.

Nutritional Biochemicals Corporation and was reprecipitated twice from water with 99 per cent ethanol before use. Glucose-1-phosphate as the dipotassium salt was obtained from Schwarz Bioresearch, Inc. and adenosine-5'-phosphate as the sodium salt from Sigma Chemical Company. Glucose-6-phosphate was purchased from Nutritional Biochemicals Corporation as the barium salt. Glucagon was obtained from Eli Lilly and Company.

RESULTS

I. Glycogen storage disease. The levels of leukocyte phosphorylase activity in the family studied are summarized in Table I. Both Jo.S.A. and R.A. have levels below 10 μg per 10^7 leukocytes. While the mother's level is midway between the lower limit of normal and that of the two patients, it was also lower than any of the control patients studied. The father and unaffected brother have normal levels of leukocyte phosphorylase. Despite these low levels of leukocyte phosphorylase in the affected members of the family, the glycogen content of the leukocytes was not elevated, as was liver glycogen. Sidbury, Cornblath, Fisher and House (20) have recently reported elevated erythrocyte glycogen in patients with debranching enzyme deficiency but normal levels in other types of glycogen storage disease. However, their series did not include patients with low hepatic phosphorylase.

II. Normal values. Leukocyte phosphorylase levels were determined in 20 patients and normal subjects, a total of 27 determinations being made (Table II). The mean level was 29.6 μg inorganic phosphorus liberated per 10^7 leukocytes with a standard error of $\pm$ 1.2 μg and a range of 20.6 to 41.6 μg. The youngest patient tested, a 1 year old female child (D.D.) with clinical Von Gierke's disease,[2] had a level of activity near the mean for the group; the values in the other children tested were indistinguishable from those found in adults. One of the control subjects, P.M., was studied on 4 different days and the level of leukocyte phosphorylase varied between 25.4 and 31.7 μg.

Wagner has reported that most of the glycogen in leukocytes resides in the polymorphonuclear types (13). The clear-cut difference between the patients with glycogen storage disease and the controls remained when the phosphorylase levels were corrected for percentage of polymorphonuclear leukocytes present in the fibrinogen supernatant. Since there was a 1 : 1 contamination of leukocytes with erythrocytes in the supernatant, phosphorylase activity of the erythrocyte was measured. No phosphorylase activity was detected in the erythrocytes, using approximately 1,000 times as many erythrocytes as the usual number of leukocytes in any one determination. Glucose-6-phosphatase activity was not demonstrable in leukocytes.

[2] Enzyme studies on the liver from this patient were not available at the time of writing.

TABLE III

In vitro effect of glucagon on leukocyte phosphorylase

Patient	Leukocyte phosphorylase	
	Control level	After glucagon
	μg P/10^7 WBC	
A.A.	3.7	11.5
Jo.S.A.	2.3	7.2
R.A.	0	1.4
Controls*	29.1 $\pm$ 3.9†	40.5 $\pm$ 6.5†

* Average of 5 separate determinations.
† Standard error; the mean stimulatory effect of glucagon is significant ($p < 0.05$).

In vitro addition of glucagon caused a 20 per cent ($p < 0.05$) stimulation of active phosphorylase in normal leukocytes. When glucagon was added *in vitro* to leukocytes from R.A., Jo.S.A. and A.A. there was a slight stimulation of phosphorylase activity in each, but in no case did the stimulated value approach the normal range (Table III). Adenosine-5'-monophosphate (0.02 M) had no significant effect on leukocyte phosphorylase activity.

When an aliquot of the sonicated leukocytes from R.A. was added to an equal volume of normal sonicated leukocytes, no inhibition of phosphorylase activity in the normal leukocytes was noted.

DISCUSSION

The demonstration of abnormally low levels of phosphorylase in the leukocytes of two patients with glycogen storage disease of the hepatic phosphorylase-deficient type indicates the usefulness of this type of study in establishing the diagnosis. The accessibility of these cells as contrasted with liver emphasized their advantage from a diagnostic point of view. Unfortunately it was not possible to study leukocyte phosphorylase in all the types of glycogen storage disease, although the level in a patient with the glucose-6-phosphatase-

deficient type was normal. It will be important to determine leukocyte phosphorylase levels in all the types before this test can be said to be entirely diagnostic of the hepatic phosphorylase-deficient type. Since there is no significant phosphorylase activity in erythrocytes, contamination of leukocytes with small numbers of erythrocytes does not alter the results. In these two patients there was good correlation between the levels of liver phosphorylase determined in biopsy specimens and the values found in leukocytes; i.e., liver phosphorylase in Jo.S.A. was estimated to be about one-third normal and in R.A., about one-sixth normal. Neither case had complete absence of phosphorylase and in this respect they are similar to the cases previously reported by Hers (11).

A relationship between liver and leukocyte phosphorylase is also apparent from the observation that in leukocytes from control subjects it was possible to activate phosphorylase by the *in vitro* addition of glucagon. This compound increases active phosphorylase in liver but has no effect on the muscle enzyme (21). The slight stimulation of leukocyte phosphorylase in the two patients and their mother does not permit a differentiation between a deficiency of the phosphorylase activating system and a decrease in the total amount of inactive phosphorylase present. Conversion of inactive liver phosphorylase to the active form is stimulated by epinephrine and glucagon through an adenyl cyclase system which increases the amount of adenosine-3′,5′-(cyclic) monophosphate (22). This in turn stimulates a specific kinase system requiring ATP and Mg^{++}, thereby converting inactive phosphorylase to the active form (23). Since leukocyte phosphorylase seems to respond to glucagon, this same system presumably should be operative in leukocytes, and low levels of the enzymes involved in this activating system might give decreased leukocyte phosphorylase activity, as would a low level of inactive phosphorylase. Nirenberg (24) has described a lack of both total and inactive phosphorylase in ascites tumor cells, where a normal activation system exists.

A defect in liver phosphorylase has only recently been described by Hers (11) in three patients with glycogen storage disease. In these patients (one male, two females) liver phosphorylase was found to be about one-seventh of normal, while muscle phosphorylase was normal, as was glucose-6-phosphatase and amylo-1,6-glucosidase. Hers was unable to stimulate active phosphorylase in the liver of these patients by addition of ATP and Mg^{++}, or adenosine-3′,5′-(cyclic) monophosphate. Another condition involving phosphorylase has been described by Mommaerts and co-workers (9) and Schmid, Robbins and Traut (10) in a syndrome represented by a "progressive myopathy" in which muscle phosphorylase was absent. Leukocyte phosphorylase has not been studied in patients with myopathy characterized by low muscle phosphorylase, but since leukocyte phosphorylase appears to mimic the liver enzyme, normal leukocyte phosphorylase levels would be expected in this disease.

The low level of leukocyte phosphorylase in these patients supports the theory that a specific phosphorylase-deficient type of glycogen storage disease exists as a distinct entity. The presence of a low leukocyte phosphorylase level in the mother suggests that the heterozygous state may be demonstrable in relatives of patients with this disease. However, the normal leukocyte phosphorylase in the father is against an autosomal recessive type of inheritance, such as has been suggested for other forms of glycogen storage disease (4). A sex-linked type of inheritance is unlikely in that the three patients presented by Hers (11) were both male and female. Further study of families with this disease may help settle this problem.

The recent studies of Leloir, Olavarria, Goldemberg and Carminatti (25) have shown a uridine diphosphoglucose-linked system for glycogen synthesis which is now thought to be the major synthetic pathway for glycogen in the body. The presence of normal leukocyte glycogen with low leukocyte phosphorylase in these two patients is thought to be further proof that the pathways of glycogen synthesis and breakdown are separate.

SUMMARY

Phosphorylase activity in leukocytes has been measured and found to be low in two cases of glycogen storage disease of the hepatic phosphorylase-deficient type. There was good correlation between the relative amounts of the enzyme in the liver and the leukocytes of these patients.

The mother had levels intermediate between the patients and control subjects but the remainder of the family members had normal levels. Since leukocyte phosphorylase seemed to vary with hepatic phosphorylase and since leukocytes are easily obtainable, it is suggested that this may be used as a diagnostic test for this condition. The low level of leukocyte phosphorylase in the mother of the two patients suggests that this test may be useful in determining the heterozygote state in this condition.

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