Glucose-6-Phosphate Isomerase Deficiency Associated With Nonspherocytic Hemolytic Anemia in the Mouse: An Animal Model for the Human Disease

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The first two mutations causing hereditary glucose-6-phosphate isomerase (GPI) deficiency associated with chronic nonspherocytic hemolytic anemia in nonhuman mammals are described in the mouse. As in humans, the hemolytic syndrome, which is characterized by a diminished erythrocyte number, lower hematocrit, lower hemoglobin, higher number of reticulocytes and plasma bilirubin concentration, as well as increased liver- and spleen-somatic indices, was exclusively manifested in homozygous mutants. In comparison with wild type, heterozygous individuals exhibited neither hematologic differences nor alterations of other physiologic parameters, including plasma concentration of glucose, pyruvate and lactate, body weight, organo-somatic indices of liver, lung, kidney, spleen, and heart, as well as viability. Glycolytic intermediates, adenine nucleotides, and metabolic rate were not significantly altered in erythrocytes from heterozygotes. On the contrary, if allowance is made for the young erythrocyte population, homozygous mutant erythrocytes showed an increased concentration of glucose-6-phosphate and normal or decreased concentrations of glycolytic metabolites following the enzymatic block. The concentration of adenosine triphosphate and the glycolytic rate also appeared to be reduced. Homozygous anemic mice showed hepatosplenomegaly and typical adaptations to hypoxia, such as an elevated heart-somatic index and, for one mutant line, an enhanced lung-somatic index. Further, these animals were characterized by a marked reduction of body weight and an increase of lethality both correlated with the degree of enzyme deficiency in tissues. The latter findings were attributed to a reduced glycolytic capability of the whole organism caused by the enzyme defect in tissues, rather than representing secondary consequences of GPI deficiency in erythrocytes. The similarity in physicochemical and kinetic properties of the mutant murine proteins reported earlier with those of allozymes found in human GPI deficiency, as well as the comparable metabolic and physiologic consequences of this enzyme defect in mice and humans support that these murine mutants are excellent animal models for the human disease.

DEFICIENCY OF glucose-6-phosphate isomerase (GPI; D-glucose-6-phosphate ketol isomerase; EC 5.3.1.9) is a well-known congenital disorder with the typical manifestation of nonspherocytic hemolytic anemia in humans. Since the first description of this recessive enzymopathy in humans by Baugham et al[1] in 1967, more than 40 subjects with hemolysis caused by GPI deficiency have been identified.[2] GPI deficiency is the second most common cause of congenital nonspherocytic hemolytic anemia caused by deficiency of a glycolytic enzyme, the commonest being deficiency of pyruvate kinase.

Despite this relatively frequent occurrence of GPI deficiency associated with hemolytic syndromes in humans, so far no case was detected in other mammalian species that exhibited this enzyme defect together with pathophysiologic signs comparable with those characteristic for the human disorder.[3] Recently, two mouse mutants were found in mutagenicity experiments,[4] which, in the homozygous state, exhibited 80% to 90% GPI deficiency in blood and several other tissues.[3] The extent of the deficiency and the similarity in physicochemical and kinetic properties between the mutant proteins and allozymes found in human GPI deficiency suggested that the GPI-deficient mouse mutants could serve as models for the human enzymopathy. However, the ultimately decisive hematologic data were unavailable at that time.

Therefore, the present investigation presents the physiologic characterization of these GPI-deficient mouse mutants. The results indicated that homozygous mutants suffered from nonspherocytic hemolytic anemia accompanied by other pathophysiologic signs. However, heterozygotes appeared unaffected by the enzyme defect. A clear relationship between in vitro stability of the mutant protein, degree of enzyme deficiency, and severity of pathophysiologic effects was demonstrated in mutant homozygotes. Thus, the physiologic findings substantiate the conclusion drawn from the biochemical studies that these GPI-deficient mutants are excellent animal models for the human disease.

MATERIALS AND METHODS

Animals. The original two GPI-deficient mouse mutants were detected in mutagenicity experiments after spermatogonial treatment of male mice with ethylnitrosourea.[4] Blood GPI activity was decreased to approximately 60% of the wild-type level in heterozygotes and to about 20% in mutant homozygotes. Linkage analysis and biochemical studies confirmed the mutants as resulting from mutations at the *Gpi-1s* structural locus.[3] Therefore, the mutant alleles were designated *Gpi-1s*$^{b\text{-}m1Neu}$ (allele short symbol, b-1N) and *Gpi-1s*$^{b\text{-}m2Neu}$ (allele short symbol, b-2N), respectively. The mutations were backcrossed 10 generations to the inbred strain C3H/El to produce congenic inbred strains. For physiologic characterization, 10-week-old animals of both sexes were used. These were obtained by inter se crossing of heterozygous animals. After weaning, four littermates of the same sex were housed per cage and maintained under constant temperature (22 ± 2°C), with a fixed 12 hour light to 12 hour dark cycle and free access to tap water and standard diet (Altromin 1314; Altromin International, Lage, Germany).

Tissue sampling. Mice were weighed, anesthetized with ether, and blood was collected through heparinized glass capillary tubes

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Submitted May 13, 1992; accepted August 25, 1992.

Supported in part by Contract BI6-E-156-D from the Commission of the European Communities.

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0006-4971/93/8101-0023$3.00/0

Table 1. Hematologic Data for Wild Types, Heterozygous, and Homozygous GPI-Deficient Mutants

	Gpi-1s Genotype				
	b/b	b/b-1N	b-1N/b-1N	b/b-2N	b-2N/b-2N
RBCs ($\times 10^{12}$/L)	8.37 ± 0.13	8.50 ± 0.12	4.82 ± 0.27*	8.46 ± 0.10	4.55 ± 0.20*
Hematocrit (%)	48.2 ± 0.4	48.7 ± 0.6	38.1 ± 1.0*	48.4 ± 0.4	36.6 ± 0.9*
Hb (g/dL)	15.4 ± 0.2	15.7 ± 0.4	9.8 ± 0.5*	15.6 ± 0.4	9.0 ± 0.2*
MCV (fL)	57.6 ± 0.8	57.3 ± 0.5	79.0 ± 3.0*	57.2 ± 1.0	80.4 ± 3.8
MCH (pg)	18.4 ± 0.3	18.5 ± 0.5	20.3 ± 0.6*	18.4 ± 0.4	19.8 ± 0.5*
MCHC (g/dL)	32.0 ± 0.4	32.2 ± 0.5	25.7 ± 0.6*	32.2 ± 0.6	24.6 ± 0.5*
Reticulocytes (%)	2.3 ± 0.4	1.7 ± 0.3	58.0 ± 6.8*	2.5 ± 0.4	68.7 ± 3.8*
MCF (% NaCl)	0.496 ± 0.005	0.497 ± 0.005	0.492 ± 0.005	0.495 ± 0.004	0.486 ± 0.005

Data are given as mean ± SEM of 16 animals.

Abbreviations: b, *Gpi-1s*^{b}: wild-type allele; b-1N, *Gpi-1s*$^{b\text{-}m1Neu}$; b-2N, *Gpi-1s*$^{b\text{-}m2Neu}$: GPI-deficient alleles; MCV, mean cellular volume; MCH, mean cellular hemoglobin; MCHC, mean cellular Hb concentration; MCF, mean cellular fragility (NaCl concentration where 50% of erythrocytes hemolyze).

* Significant differences ($P < .05$) between wild-type and mutants.

from the retroorbital sinus. Fresh blood was used for determination of hematologic parameters, glycolytic intermediates, and metabolic activities of erythrocytes, as well as for measurement of plasma compounds. Immediately thereafter, the mice were killed by cervical dislocation and dissected. Organs were removed and weighed for measuring the organo-somatic indices (organ weight × 100/body weight). A liver specimen was used for determination of liver glycogen. To minimize the effects of circadian rhythm, the procedures were performed between 8 and 10 AM.

Hematologic data and plasma compounds. Hematologic data, including red blood cell (RBC) number, hematocrit, hemoglobin (Hb) concentration, and osmotic fragility, were obtained by standard techniques as previously described.[5] Peripheral blood smears for determination of reticulocyte count were stained by mixing two parts of fresh blood with one part of 1% brilliant cresyl blue in isotonic saline. Measurements of total bilirubin in plasma followed the method of Wahlefeld et al.[6] Plasma glucose was analyzed by the glucose-oxidase-peroxidase method.[7] Determinations of pyruvate and lactate in plasma was performed as described by Czok and Lamprecht[8] and Gutmann and Wahlefeld,[9] respectively.

Glycolytic intermediates and adenine nucleotides in erythrocytes. Measurements of erythrocyte glycolytic intermediates and adenine nucleotides were performed enzymatically according to Minakami et al.[10]

Metabolic activities of erythrocytes. Determinations of glucose consumption and lactate formation of erythrocytes in cell suspension at pH 7.4 and 37°C were performed essentially according to the method of Chapman et al.[11] Erythrocytes were isolated using the sulfoethylcellulose filtration technique described by Nakao et al.[12]

Enzymatic studies in blood. Preparation of blood lysates and determinations of specific activities of GPI and several other erythrocyte enzymes were performed by the methods previously reported.[4] The automatic analyzer ACP 5040 (Eppendorf, Hamburg, Germany) was used for all assays.

Liver glycogen. Liver glycogen was measured by the amyloglucosidase reaction of Keppler and Decker.[13]

Viability of heterozygous and homozygous mutants. Viability of mutant animals was calculated from the breeding data obtained in back- and inter-crosses of heterozygotes. The percent deviation from Mendelian segregation in back- and inter-crosses was the criterion for determining viability of heterozygous and homozygous mutants, respectively. Heterozygous viability was calculated from data of back-crosses as: (number of heterozygotes/number of wild types) × 100. Because heterozygotes were found to be fully viable, viability of homozygous mutants was calculated from data of inter-crosses as: (number of homozygotes/1/3 [number of heterozygotes + number of wild types]) × 100.

Stage of homozygous lethality. Early and late postnatal lethality of mutant homozygotes was estimated by comparing the number of offspring lost during specified periods of postnatal life in back-crosses and inter-crosses (preweaning period: birth to weaning [day 21 postpartum); postweaning period: weaning to classification (day 28 to day 42 postpartum).

Statistical analysis. The χ^2-test was used to compare the segregation ratios obtained in breeding experiments with Mendelian expectations. Data from the homozygous-lethality experiments were compared using a nonparametric method (Wilcoxon rank-sum test). In the physiologic characterization experiments, data from the same number of female and male animals were used to determine the mean and the standard error of the mean (SEM). For statistical comparisons between the different genotypes, Student's *t*-test was used. Differences were stated as significant for P values <.05.

RESULTS

Hematologic data. The results of the hematologic investigations of wild-type, heterozygous, and homozygous mutant littermates are given in Table 1. No significant differences could be observed between wild types and heterozygotes from both mutant lines. In contrast, b-1N and b-2N homozygotes exhibited a marked reduction in the number of RBCs, hematocrit, and in the concentration of Hb compared with wild types. Reticulocyte counts in these anemic mice were approximately 60% to 70% compared with about 2% in wild-type and heterozygous individuals. Consistent with the macrocytosis of young erythrocyte populations, the mean cell volume (MCV) was increased, whereas the mean cellular Hb concentration (MCHC) was decreased in mutant homozygotes. Figure 1 depicts the results of the osmotic fragility test. In comparison with the osmotic fragility curve of wild-type erythrocytes, the curves of erythrocytes from homozygous mutants were flattened and showed a slight shift toward lower NaCl concentrations. This finding indicated that the macrocytic erythrocytes of mutant homozygotes were more resistant to osmotic stress than the normocytes of wild types. Because macrocytic, young cells from normal human erythrocyte populations generally are also more resistant to hypotonic salt solutions than older cells,[14] the alteration of osmotic fragility curve in mutant homozygotes appeared rather

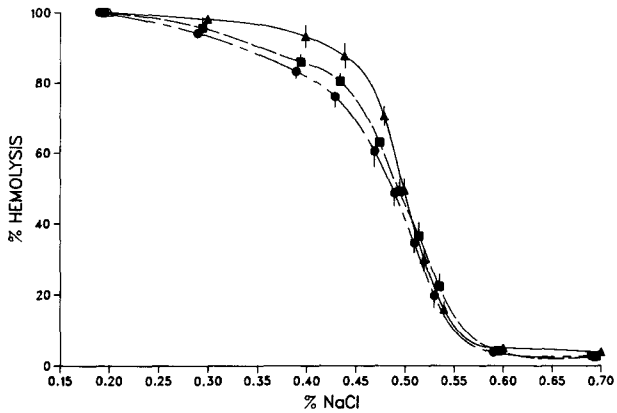

Fig 1. Osmotic fragility of erythrocytes from wild types (triangles) and homozygous b-1N (squares) and b-2N (circles) animals. Data are given as mean ± SEM of 10 animals.

to be a result of reticulocytosis than to be a direct consequence of the enzyme defect in erythrocytes.

Enzyme activities in blood. Table 2 shows the activities of some erythrocyte enzymes determined in blood lysate and expressed in IU/g Hb. Heterozygotes from both mutant lines exhibited approximately 60% of wild-type GPI activity, whereas measurement of erythrocyte enzymes other than GPI yielded normal values. With the exception of GPI, enzyme activities in blood of homozygous mutants were increased to varying extents compared with the wild type. GPI activity was diminished to 21% and 19% of the mean normal activities in b-1N/b-1N and b-2N/b-2N individuals, respectively. In humans it is well established that reticulocytes and young erythrocytes generally have considerably higher activities of most enzymes than older RBCs.[15,16] The elevation of enzyme activities other than GPI in blood from homozygous mouse mutants, which is also a characteristic of GPI deficient anemic humans,[17,18] thus is commensurate with young cell populations in these animals and essentially agrees with the relative elevation of these enzymes in murine erythrocytes produced during phenylhydrazine-induced stress erythropoiesis.[19] Surprisingly, despite a higher reticulocyte count, the mean elevation for all enzymes studied was lower in blood from b-2N/b-2N animals than that in blood from b-1N homozygotes. This less marked elevation of enzyme activity in blood with a higher reticulocyte level might be indicative of reticulocytes leaving the erythropoietic tissues at earlier stages of maturation. A shortening in the maturation time of the erythrocyte was previously shown in a lactate dehydrogenase (LDH)-deficient mouse mutant suffering from severe nonspherocytic hemolytic anemia.[20] In this context, it is noteworthy that the general decrease of activity of enzymes in aging erythrocytes should also be considered for the mutant enzyme. Based on the marked instability of the mutant GPI homodimers[3] and comparable with the situation in humans,[21,22] it is expected that an accelerated loss of GPI activity during aging of RBCs of homozygous mutants occurs. The residual GPI activity in blood hypothesized for a more mature state of the erythrocyte existence, and expected to be incompatible with survival, is estimated to be less than 10% of the wild-type level. Thus, it is in the range of the mean deficiency measured in nonerythrocytic tissues (see Table 7). Because of the absence of marked differences in kinetic properties and pH dependence of the mutant proteins compared to the wild type,[3] the relative enzyme deficiencies determined in vitro probably also reflect the situation in vivo, thus agreeing with the respective GPI deficiency suggested to result in impairment of glycolytic function of human erythrocytes.[22,23]

Metabolic activity, glycolytic intermediates, and adenine nucleotides in erythrocytes. The effects of GPI deficiency on the energy metabolism of erythrocytes may be evaluated based on the concentration of intermediate metabolites and

Table 2. Activity of Several Erythrocyte Enzymes in Blood of Wild Types, Heterozygous, and Homozygous GPI-Deficient Mouse Mutants

	Gpi-1s Genotype				
	b/b	b/b-1N	b-1N/b-1N	b/b-2N	b-2N/b-2N
G6P isomerase	100.0 ± 2.4 (67.8)	62.7 ± 1.0*	22.3 ± 2.8*	60.7 ± 0.8*	19.9 ± 1.4*
Triosephosphate isomerase	100.0 ± 1.4 (724.1)	98.2 ± 3.1	146.0 ± 2.9*	99.4 ± 1.3	142.1 ± 2.0*
3-Phosphoglycerate kinase	100.0 ± 1.7 (71.1)	98.5 ± 1.0	138.7 ± 4.4*	99.3 ± 1.9	131.9 ± 3.0*
Phosphoglycerate mutase	100.0 ± 2.9 (12.4)	100.8 ± 3.5	214.0 ± 5.8*	96.7 ± 4.3	197.5 ± 6.0*
Pyruvate kinase	100.0 ± 1.7 (11.4)	97.1 ± 6.0	151.5 ± 3.2*	103.1 ± 2.1	148.3 ± 4.0*
LDH	100.0 ± 1.5 (222.5)	101.5 ± 2.0	141.1 ± 2.6*	99.4 ± 1.2	136.9 ± 2.0*
G6P dehydrogenase	100.0 ± 1.4 (13.8)	98.1 ± 5.2	177.8 ± 5.4*	101.9 ± 1.6	172.5 ± 2.9*
Glutathione reductase	100.0 ± 1.5 (5.6)	100.4 ± 3.0	161.3 ± 5.2*	97.2 ± 1.5	153.8 ± 3.3*

Data are expressed as percentage of wild-type enzyme activity and given as mean ± SEM of 16 animals. In parentheses the mean specific enzyme activity of wild type is given as IU/g Hb. Same symbols are used as in Table 1.

Table 3. Metabolic Activity, Glycolytic Intermediates, and Adenine Nucleotides in Erythrocytes of Wild Types and b-1N Heterozygous and Homozygous GPI-Deficient Mouse Mutants in Blood

	Gpi-1s Genotype		
	b/b	b/b-1N	b-1N/b-1N
G6P	122 ± 5	115 ± 5	208 ± 15*
F6P	33 ± 3	29 ± 2	42 ± 4*
G6P/F6P	3.70 ± 0.13	3.77 ± 0.20	4.95 ± 0.32*
Dihydroxyacetonephosphate	17 ± 2	16 ± 2	24 ± 2*
3-Phosphoglycerate	78 ± 8	80 ± 8	76 ± 9
Phosphoenolpyruvate	23 ± 2	21 ± 3	24 ± 3
Pyruvate	210 ± 13	205 ± 14	216 ± 25
Lactate	4,450 ± 38	4,360 ± 29	4,320 ± 100
ATP	1,940 ± 150	1,940 ± 110	2,890 ± 190*
ADP	186 ± 23	184 ± 18	275 ± 29*
Glucose consumption	19.4 ± 2.6	19.7 ± 2.4	30.3 ± 2.4*
Lactate formation	35.2 ± 3.0	34.3 ± 2.8	58.3 ± 1.8*

Data are given as mean ± SEM of 10 animals. Glycolytic intermediates and nucleotides are expressed in nmol/mL RBCs. The levels of pyruvate and lactate are given in nmol/mL blood. Glucose consumption and formation of lactate and pyruvate are given in nmol/10^{12} erythrocytes/h. Same symbols are used as in Table 1.

the flux rate of glycolysis. Table 3 shows the measured glycolytic intermediates and adenine nucleotides, as well as glucose consumption and lactate production in erythrocytes of b-1N mutants. No significant differences could be found between heterozygotes and wild types. In comparison with the normal RBC population in wild types and heterozygotes, the assays for glycolytic intermediates in homozygous GPI-deficient erythrocytes showed increased concentrations of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) and unaltered levels for the other glycolytic intermediates. However, the relative increase of G6P, the metabolite preceding the enzyme block, was higher than that of F6P and other glycolytic intermediates, increasing the G6P/F6P ratio, the equilibrium catalyzed by GPI, from approximately 3.7 in wild types to about 5 in mutant homozygotes. The concentrations of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) also were elevated in erythrocytes of homozygous mutants. Moreover, a twofold increase of lactate formation, as well as of glucose consumption, was observed in RBCs from homozygous mutants.

Because glucose metabolism of human erythrocytes is influenced by aging,[16,17,24,25] the interpretation of results obtained from erythrocytes of anemic mice is complicated by the high reticulocyte count. To detect the effect of GPI deficiency on erythrocyte metabolism, a comparison of the results from homozygous mutants with data from RBC populations with similar reticulocyte counts appears more meaningful than the comparison with the values of wild-type erythrocytes. It is well established that the levels of glycolytic intermediates in human erythrocyte populations with increased reticulocyte counts are elevated compared with those in normal RBC populations.[24,25] However, the ratio between metabolites, ie, between G6P and F6P, are not markedly altered. Therefore, it may be concluded that the elevated G6P/F6P ratio in erythrocytes from homozygous mouse mutants was not a result of reticulocytosis but was a direct effect of the reduced GPI activity resulting in an increase of intermediary substrate proximal to the enzymatic block and a decrease distal to it, as well as a reduced glycolytic flux and an impaired energy metabolism. The latter was indicated by an ATP concentration and a glycolytic rate, which both are about 70% of that in a murine RBC population with comparable reticulocyte count.[26,27]

Total bilirubin and several glycolytic metabolites in plasma. The results of the determinations of total bilirubin, glucose, pyruvate, and lactate in plasma are summarized in Table 4. There were no significant differences between heterozygotes and wild types for all parameters studied. In accordance with the expected increased loss and shortened life span of erythrocytes, levels of total bilirubin were increased in homozygotes of both mutant lines. Further, the glucose concentration was significantly elevated in the plasma from

Table 4. Some Plasma Compounds of Wild Types, Heterozygous, and Homozygous GPI-Deficient Mouse Mutants

	Gpi-1s Genotype				
	b/b	b/b-1N	b-1N/b-1N	b/b-2N	b-2N/b-2N
Total bilirubin (mg/dL)	0.30 ± 0.04	0.29 ± 0.04	0.62 ± 0.10*	0.27 ± 0.04	0.84 ± 0.07*
Glucose (mg/dL)	120 ± 3	116 ± 4	154 ± 6*	122 ± 5	134 ± 6*
Pyruvate (mmol/L)	0.21 ± 0.02	0.22 ± 0.02	0.20 ± 0.03	0.23 ± 0.03	0.19 ± 0.02
Lactate (mmol/L)	6.53 ± 0.40	6.20 ± 0.35	6.45 ± 0.38	6.70 ± 0.45	6.26 ± 0.30

Data are given as mean ± SEM of 16 animals. Same symbols are used as in Table 1.

* Significant differences ($P < .05$) between wild types and mutants.

Table 5. Body Weight, Organo-somatic Indices, and Liver Glycogen of Wild Types, Heterozygous, and Homozygous GPI-Deficient Mouse Mutants

	Gpi-1s Genotype				
	b/b	b/b-1N	b-1N/b-1N	b/b-2N	b-2N/b-2N
Body weight (g)	23.0 ± 0.4	22.7 ± 0.5	20.3 ± 0.6*	23.0 ± 0.7	16.8 ± 0.5*
Organo-somatic index (g/100 g body weight)					
Liver	5.33 ± 0.09	5.31 ± 0.10	5.55 ± 0.11*	5.30 ± 0.11	5.59 ± 0.08*
Lung	0.59 ± 0.02	0.60 ± 0.02	0.59 ± 0.03	0.59 ± 0.02	0.65 ± 0.02*
Kidney	1.27 ± 0.07	1.29 ± 0.04	1.21 ± 0.05	1.28 ± 0.04	1.31 ± 0.05
Spleen	0.41 ± 0.03	0.42 ± 0.03	2.10 ± 0.12*	0.41 ± 0.03	2.23 ± 0.14*
Heart	0.39 ± 0.01	0.39 ± 0.01	0.43 ± 0.01*	0.39 ± 0.01	0.44 ± 0.01*
Liver glycogen (g/100 g fresh weight)	9.8 ± 0.4	10.0 ± 0.6	9.2 ± 0.5	9.6 ± 0.4	9.3 ± 0.5

Data are given as mean ± SEM of 16 animals. Same symbols are used as in Table 1.

* Significant differences ($P < .05$) between wild types and mutants.

anemic mice, whereas plasma concentrations of pyruvate and lactate showed normal levels. Because increased glucose values were not observed in the plasma of LDH-deficient anemic mice with similar hematologic findings,[28] it seems unlikely that this effect is a result of anemia. Rather, it might be a consequence of decreased glucose usage caused by reduced total glycolytic capability of mutant homozygotes.

Body weight, organo-somatic indices, and hepatic glycogen. Body weights, somatic indices of several organs, and the liver glycogen concentrations of wild-type and mutant animals are presented in Table 5. Significant differences between heterozygotes and wild types were absent for all parameters studied. Anemic b-1N and b-2N homozygotes, in contrast, exhibited marked body weight reductions, which were about 20% and 30% of the wild type value, respectively, and thus correlated with the mean residual GPI activity in tissues (see Table 7). Investigations of organ weights in mutant homozygotes showed an approximate fivefold increase in spleen-somatic index, slightly yet significantly increased somatic indices of liver and heart and at least in b-2N/b-2N individuals an elevated lung-somatic index. No differences were found in the relative weights of kidney or in the liver glycogen concentrations between wild types and homozygous mutants. The latter finding excluded that the increase of liver-somatic index is a consequence of an elevated glycogen concentration in liver cells.

Viability of heterozygous and homozygous mutants. The results of the breeding experiments are given in Table 6. When progeny of heterozygous and wild-type animals were classified at 4 to 6 weeks of age, homozygous wild-type and heterozygous mutant offspring were found in a ratio of approximately 1:1. This suggests that heterozygotes are fully viable. Inter-crossing amongst heterozygotes yielded wild types, heterozygous and homozygous mutants. However, for both mutant lines there was a significant reduction of approximately 20% to 30% in the number of homozygous mutants compared with Mendelian expectations, suggesting a decreased viability of these animals. Reduction of viability was slightly more pronounced in b-2N homozygotes compared to b-1N/b-1N individuals, and thus correlates with the average of GPI deficiencies in the tissues as well as with the body weight reduction of these animals (Table 7).

Stage of homozygous lethality. The numbers of animals lost between certain periods of postnatal life in back- and inter-crosses are given in Table 6. Whereas among offspring from back-crosses no animal lost during the preweaning period was noted, the losses of offspring in inter-crosses were remarkably higher. Interestingly, the number of animals lost in inter-crosses was within the range of the number of missing b-1N homozygotes. No losses were observed during the postweaning period in back-crosses and inter-crosses. Therefore, these results suggest that lethality of homozygous b-1N mutants essentially occurred during the first 3 weeks after birth.

DISCUSSION

The present study describes the first two mutations causing hereditary deficiency of GPI associated with chronic nonspherocytic hemolytic anemia in the mouse. Padua et al[29] in 1978 detected a GPI-deficient variant in a population of feral mice, but despite 67% enzyme deficiency in blood, increased

Table 6. Breeding Data of Two GPI-Deficient Mouse Mutants

Cross *Gpi-1s* Genotype	*Gpi-1s* Genotype			Postnatal Mortality	
	b/b	b/b-.N	b-.N/b-.N	Preweaning	Postweaning
b/b-1N × b/b	192	200	—	0	0
b/b-1N × b/b-1N	108	193	78	26	0
b/b-2N × b/b	405	358	—		
b/b-2N × b/b-2N	158	280	104		

Postnatal mortality was determined in matings back-crossed more than 15 generations to the inbred strain C3H/El. The number of animals lost between birth and weaning (preweaning) and weaning and classification (postweaning) is given as postnatal mortality. Same symbols are used as in Table 1.

Table 7. Viability, Body Weight, and Mean GPI Activity of Wild Types, Heterozygous, and Homozygous GPI-Deficient Mouse Mutants

	Gpi-1s Genotype				
	b/b	b/b-1N	b-1N/b-1N	b/b-2N	b-2N/b-2N
Viability	100.0	97.2	74.4*	88.4	71.2*
Body weight	100.0 ± 1.7	98.7 ± 2.2	85.2 ± 2.6*	100.0 ± 3.0	71.3 ± 2.2*
Mean GPI activity	100.0	75.4 ± 2.3*	15.7 ± 2.2*	72.5 ± 2.7*	10.2 ± 2.1*

Data are given as percent of wild type. Mean GPI activity was calculated using the percent mean GPI activities in nonerythrocytic tissues reported by Pretsch and Merkle.[3] Viability of heterozygotes and mutant homozygotes was calculated as described in Materials and Methods. Same symbols are used as in Table 1.

* Significant differences ($P < .05$) between wild types and mutants.

autohemolysis was the only indication for hemolytic disease in homozygous individuals. On the contrary, homozygous b-1N and b-2N animals from this study with an approximate 80% GPI deficiency in blood both exhibited severe chronic anemia, pronounced reticulocytosis, and slight hyperbilirubinemia. Heterozygous mice, characterized by an intermediate approximate 40% enzyme deficiency, appeared to be metabolically and physiologically unaffected. Thus, the indicated autosomal recessive mode of inheritance of hemolytic anemia caused by GPI deficiency in the mouse corresponds to that of this enzymopathy in humans, in whom also only homozygotes or double-heterozygotes have been found to manifest the hemolytic syndrome. However, clinical severity varied markedly in the reported cases.[30] At one extreme, affected patients had a mild and well-compensated chronic hemolytic anemia that was diagnosed for the first time in adult life. Increased hemolysis only became clinically important during infections, or possibly as a result of the administration of drugs. On the other extreme there were cases in which affected individuals suffered from severe chronic anemia since birth or early childhood. Comparing the severity of the hematologic signs manifested in the GPI-deficient, anemic mice with that found in GPI-deficient human patients it is indicative that the hematologic feature in the mouse mutants resembles that of the severe, chronic form of the human enzymopathy.

In addition to the phenotypic manifestations in blood and erythrocytes, further prominent pathophysiologic characteristics secondary to GPI deficiency observed in humans such as hepatomegaly and splenomegaly[23] were present in GPI-deficient, anemic mice. In mice with hemolytic anemia caused by LDH deficiency or caused by a defect of the erythrocyte membrane protein spectrin, splenomegaly appears to reflect an immense expansion of splenic erythropoiesis compensating for the hemolytic RBC loss.[20,31,32] The splenomegaly found in GPI-deficient anemic mice is interpreted to be caused by the same cause, but the comparably moderate splenic enlargement in homozygous mutants suggests that erythrocyte loss is lower and life span is longer than in mice with LDH deficiency or spectrin defects.

However, liver and spleen were not the sole organs in GPI-deficient anemic mice demonstrating alterations in relative size. In both mutants the heart, and additionally in b-2N homozygotes the lung, showed increased somatic indices. This may be interpreted as cardiopulmonary adaptations to hypoxic conditions to increase the oxygen-delivering capacity and to counteract the decreased amount of Hb by elevating respiratory function and cardiac index. Whereas cardiac hypertrophy seems to be a common finding in mice suffering from chronic hemolytic anemia,[27,28] this phenomenon has so far seldom been mentioned in anemic humans.[30] Concerning hemolytic anemia secondary to GPI deficiency, enlargement of the heart was noted so far only in two cases.[33] However, one may speculate that the apparent difference in frequency of occurrence of this adaptation between mice and humans may be attributable to a lack of examinations in humans than to a species difference.

It is evident that the above-described pathologic findings, although involving distinct organ systems, are secondary manifestations of the enzyme defect in erythrocytes. The question arises whether the other phenotypic alterations found in GPI-deficient anemic mice, such as body weight reduction or increased early postnatal lethality, are also related to the complications of chronic anemia and thus are indirect results of the erythrocyte enzyme defect or alternatively are systemic effects caused by the enzyme deficiency in other body tissues. However, the absence of similar pathophysiologic manifestations in LDH-deficient mice with a severe anemia similar to that observed in GPI-deficient homozygotes and an even higher degree of reticulocytosis[19,34] contradict the possibility that body weight reduction and increased lethality in GPI-deficient anemic mice is a result of the hemolytic syndrome. On the other hand, mouse mutants with spherocytic anemia exhibiting both retarded growth and impaired viability have been described,[27,35] but anemia in these animals with an approximate reduction of Hb concentration to 40% of the wild-type value was markedly more severe than in anemic LDH- and GPI-deficient mice with a reduced hemoglobin value of 60% of the normal. Thus, the phenotype of these animals appears to be a less suitable argument to address this problem in the reported GPI-deficient mice. However, impaired viability and reduced body growth may well be a systemic effect because the degree of deficiency in nonerythrocytic body tissues of GPI-deficient anemic mice is in the range that would compromise cellular function in erythrocytes. Further, the obvious correlation between reduced GPI activity in tissues and reduction of both body weight and viability argues for a systemic effect. Finally, the observed elevated plasma glucose levels in homozygous mutant mice can be explained by reduced glucose usage of the whole organism because of a reduced glycolytic capacity rather than ascribed to secondary effects of anemia.

The postulated occurrence of systemic effects in the GPI-deficient mouse mutants presented here contrasts with most cases of GPI deficiency in humans, in which the erythrocyte seemed to be the only cell type with a notable functional impairment.[2,23,36,37] Because instability of the variant protein is the main cause of the deficiency in these cases, this phenomenon is generally explained by the different synthetic capabilities of erythrocytes and nonerythrocytic cell types.[31,38] Erythrocytes are incapable of protein synthesis and, therefore, cannot replace lost enzyme activity, whereas in contrast, other tissues may partly compensate for the loss of enzyme. As there is only one structural locus for GPI in humans[39] and mice,[40] the extent of enzyme deficiency may vary between tissues in unstable GPI mutants, as unstable GPI molecules will be present in all cells. In general, the deficiency will be maximal in erythrocytes and may reach the critical level that compromises metabolic function in these but not in other cells.[23] Comparing the heat stability characteristics of several human GPI variants[41] with those of the presented murine mutant proteins,[3] it seems that the lability of the mutant murine GPI proteins is markedly higher than that of the described human GPI variants. As a consequence, the deficiency in nonerythrocytic tissues of the mouse mutants seems to be higher than in comparable human tissues with heat-labile GPI proteins,[17,20,42] and with about 90% might be in the range of the critical deficiency level capable of inducing systemic metabolic dysfunction. This hypothesis is supported by the clinical findings of two exceptional cases of GPI-deficient humans characterized by heat-stable GPI proteins with reduced catalytic efficiency. In accordance with the occurrence of only one isozyme in human tissues, the patients exhibited about 75% to 93% GPI deficiency in blood as well as in nonerythrocytic tissues and suffered from a generalized syndrome including severe hemolytic anemia, unspecific myopathy, and neurologic symptoms.[43,44] Moreover, one of these patients died during early childhood.[43] As in other human GPI-deficient patients, who died around birth or during childhood,[33,36] it is yet not clear whether death is a result of anemia, impaired systemic metabolism, or both.

In this context, it is an interesting problem why a genetic defect that generates a well-defined altered protein despite the same genetic background and similar environmental conditions in one case but not in the other leads to early death of the affected individual. This situation exemplified in homozygous GPI-deficient mouse mutants in the present study appeared to be present also in GPI-deficient human subjects, although in the latter genetic background and environmental conditions are more variable than in inbred mice. Hutton and Chilcote[36] reported a family in which three siblings with clinical histories consistent with GPI deficiency died at the ages of 11 days, 8 months, and 7 years, respectively. A fourth sibling was still alive at the age of 10 years. Whitelaw et al[33] reported hydrops fetalis and neonatal death of a GPI-deficient baby, whereas the sibling with the same genotype and signs of hemolytic syndrome survived. Theoretical and experimental evidence confirm that the presence of GPI activity is essential for life in mice[45,46] and humans.[38] Further, nonregulatory enzymes, such as GPI, are present in excess in tissues from normal individuals, implying also that large fluctuations in enzyme activity have minimal effects on metabolic flux. In contrast, in homozygous GPI-deficient mice of the present study and some GPI-deficient anemic humans the residual GPI activities in tissues seem to represent a critical activity range where small reductions of enzyme activity may markedly affect glucose usage and energy metabolism, and under certain circumstances may even have lethal consequences. These small alterations might represent stochastic effects of biologic variation. On the other hand, in heat labile proteins such as the mutant GPI protein investigated here, these small reductions of enzyme activity may be induced by slight elevations in body temperature. Thus, temperature would be a deciding factor, determining viability or lethality. It is noteworthy that in many GPI-deficient human patients with heat-labile GPI proteins, bacterial or viral infections generally preceded the hemolytic crisis.[30] Although other causes, such as cessation of erythropoietic activity usually occurring during infections,[38] could be responsible for this phenomenon, it might be that a stronger enzyme deficiency induced by the increase of body temperature usually accompanying infections contributes to the episode of exaggerated anemia.

In conclusion, the mouse mutants described here are suitable animal models to clarify these and other open questions concerning hereditary GPI deficiency in humans. Because the underlying genetic defects are probably caused by base-pair changes in a structural gene with largely known base sequence, they, moreover, might provide valuable experimental models for gene therapy experiments.

ACKNOWLEDGMENT

We express our appreciation to Prof U.H. Ehling, Dr J. Favor, and Dr J. Peters for discussions and helpful criticism of the manuscript. The expert technical assistance of M. Ellendorff and S. Wolf is gratefully acknowledged. We also thank M. Steglich for preparing the figure.

REFERENCES

1. Baugham MA, Valentine WN, Paglia DE, Ways PO, Simon ER, DeMarsh QB: Hereditary hemolytic anemia associated with glucosephosphate isomerase (GPI) deficiency—A new enzyme defect of human erythrocytes. Blood 30:850, 1967

2. Jaffé ER, Valentine, WN: Human erythroenzymopathies of the anaerobic Embden-Meyerhof glycolytic and associated pathways, in Nagel RL (ed): Genetically Abnormal Red Cells, vol I. Boca Raton, FL CRC, 1988, p 105

3. Pretsch W, Merkle S: Glucose phosphate isomerase enzyme-activity mutants in *Mus musculus:* Genetical and biochemical characterization. Biochem Genet 28:97, 1990

4. Charles DJ, Pretsch W: Linear dose-response relationship of erythrocyte enzyme-activity mutations in offspring of ethylnitrosourea-treated mice. Mutat Res 176:81, 1987

5. Merkle S, Pretsch W: Characterization of triosephosphate isomerase mutants with reduced enzyme activity in *Mus musculus.* Genetics 123:837, 1989

6. Wahlefeld AW, Herz G, Bernt E: Modification of the Malloy-Evelyn-method for a simple, reliable determination of total bilirubin in serum. Scand J Clin Lab Invest 126:11.12, 1972 (suppl 29)

7. Werner W, Rey H-G, Wielinger H: Über die Eigenschaften eines neuen Chromogens für die Blutzuckerbestimmung nach der GOD/POD-Methode. Z Anal Chem 252:224, 1970

8. Czok R, Lamprecht W: Pyruvate, phosphoenolpyruvate and D-glycerate-2-phosphate, in Bergmeyer, HU (ed): Methods of Enzymatic Analysis, vol 3. New York, NY, Academic, 1974, p 1446

9. Gutmann I, Wahlefeld AW: L-(+)-lactate determination with lactate dehydrogenase and NAD, in Bergmeyer HU (ed): Methods of Enzymatic Analysis, vol 3. New York, NY, Academic, 1974, p 1465

10. Minakami S, Suzuki C, Saito T, Yoshikawa H: Studies on erythrocyte glycolysis. I. Determination of the glycolytic intermediates in human erythrocytes. J Biochem 58:543, 1965

11. Chapman RG, Hennessey MA, Waltersdorph AM, Huennekens FM, Gabrio BW: Erythrocyte metabolism. V. Levels of glycolytic enzymes and regulation of glycolysis. J Clin Invest 41:1249, 1962

12. Nakao M, Nakayama T, Kankura T: A new method for separation of human blood components. Nature New Biol 246:94, 1973

13. Keppler D, Decker K: Glycogen, determination with amyloglucosidase, in Bergmeyer HU (ed): Methods of Enzymatic Analysis, vol 3. New York, NY, Academic, 1974, p 1127

14. Marks PA, Johnson AB: Relationship between the age of human erytrocytes and their osmotic resistance: A basis for separating young and old erythrocytes. J Clin Invest 37:1542, 1958

15. Rubinstein D, Ottolenghi P, Denstedt OF: The metabolism of the erythrocyte XIII. Enzyme activity in the reticulocyte. Can J Biochem Physiol 34:222, 1956

16. Seaman C, Wyss S, Piomelli S: The decline in energetic metabolism with aging of the erythrocyte and its relationship to cell death. Am J Hematol 8:31, 1980

17. Arnold H, Blume KG, Busch D, Lenkeit U, Löhr GW, Lübs E: Klinische und biochemische Untersuchungen zur Glucosephosphatisomerase normaler menschlicher Erythrocyten und bei Glucosephosphatisomerase-Mangel. Klin Wschr 48:1299, 1970

18. Helleman PW, Van Biervliet JPGM: Haematological studies in a new variant of glucosephosphate isomerase deficiency (GPI Utrecht). Helv Paediatr Acta 30:525, 1975

19. Hutton JJ: Glucose-metabolizing enzymes of the mouse erythrocyte: Activity changes during stress erythropoiesis. Blood 39: 542, 1972

20. Kremer J-P, Datta T, Pretsch W, Charles DJ, Dörmer P: Mechanisms of compensation of hemolytic anemia in a lactate dehydrogenase mouse mutant. Exp Hematol 15:664, 1987

21. Arnold H, Blume K-G, Engelhardt R, Löhr GW: Glucosephosphate isomerase deficiency: Evidence for in vivo instability of an enzyme variant with hemolysis. Blood 41:691, 1973

22. Eber SW, Gahr M, Lakomek M, Prindull G, Schröter W: Clinical symptoms and biochemical properties of three new glucosephosphate isomerase variants. Blut 53:21, 1986

23. Paglia DE, Valentine WN: Hereditary glucosephosphate isomerase deficiency. Am J Clin Pathol 62:740, 1974

24. Chapman RG, Schaumburg L: Glycolysis and glycolytic enzyme activity of aging red cells in man. Changes in hexokinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and glutamic-oxalacetic transaminase. Br J Haematol 13:665, 1967

25. Beutler E, Sigalove WH, Muir WA, Matsumoto F, West C: Glucosephosphate-isomerase (GPI) deficiency: GPI Elyria. Ann Intern Med 80:730, 1974

26. Hutton JJ, Bernstein SE: Metabolic properties of erythrocytes of normal and genetically anemic mice. Biochem Genet 10:297, 1973

27. Bernstein SE: Inherited hemolytic disease in mice: A review and update. Lab Animal Sci 30:197, 1980

28. Pretsch W, Merkle S, Favor J: A mutation affecting the lactate dehydrogenase locus *Ldh-1* in the mouse.—II. Mechanism of the LDH-A deficiency associated with hemolytic anemia. (in preparation)

29. Padua RA, Bulfield G, Peters J: Biochemical genetics of a new glucosephosphate isomerase allele (*Gpi-1^c*) from wild mice. Biochem Genet 16:127, 1978

30. Dacie J: The Haemolytic Anaemias, vol. 1: The Hereditary Haemolytic Anaemias (ed 3). Edinburgh, UK, Churchill Livingstone, 1985

31. Brookoff D, Maggio-Price L, Bernstein S, Weiss L: Erythropoiesis in *ha/ha* and *sph/sph* mice, mutants which produce spectrin-deficient erythrocytes. Blood 59:646, 1982

32. Kremer J-P, Datta T, Dörmer P: A model of the hemopoietic stress in a lactate dehydrogenase mouse mutant with hemolytic anemia. Blut 52:179, 1986

33. Whitelaw AGL, Rogers PA, Hopkinson DA, Gordon H, Emerson PM, Darley JH, Reid C, Crawfurd Md'A: Congenital haemolytic anaemia resulting from glucose phosphate isomerase deficiency: Genetics, clinical picture, and prenatal diagnosis. J Med Genet 16:189, 1979

34. Charles DJ, Pretsch W: A mutation affecting the lactate dehydrogenase locus *Ldh-1* in the mouse.—I. Genetical and electrophoretical characterization. Biochem Genet 19:301, 1981

35. Unger AE, Harris MJ, Bernstein SE, Falcone JC, Lux SE: Hemolytic anemia in the mouse. Report of a new mutation and clarification of its genetics. J Hered 74:88, 1983

36. Hutton JJ, Chilcote RR: Glucose phosphate isomerase deficiency with hereditary nonspherocytic hemolytic anemia. J Pediatr 85:494, 1974

37. Glader BE, Nathan, DG: Haemolysis due to pyruvate kinase deficiency and other glycolytic enzymopathies. Clin Haematol 4:123, 1975

38. Kahn A, Kaplan J-C, Dreyfus J-C: Advances in hereditary red cell enzyme anomalies. Hum Genet 50:1, 1979

39. Payne DM, Porter DW, Gracy RW: Evidence against the occurrence of tissue-specific variants and isoenzymes of phosphoglucose isomerase. Arch Biochem Biophys 151:122, 1972

40. Carter ND, Parr CW: Isoenzymes of phosphoglucose isomerase in mice. Nature 216:511, 1967

41. Schröter W, Tillmann W: Congenital nonspherocytic hemolytic anemia associated with glucosephosphate isomerase deficiency: Variant Paderborn. Klin Wschr 55:393, 1977

42. Van Biervliet JPGM, Van Milligen-Boersma L, Staal GEJ: A new variant of glucosephosphate isomerase deficiency (GPI-Utrecht). Clin Chim Acta 65:157, 1975

43. Zanella A, Izzo C, Rebulla P, Perroni L, Mariani M, Canestri G, Sansone G, Sirchia G: The first stable variant of erythrocyte glucose-phosphate isomerase associated with severe hemolytic anemia. Am J Hematol 9:1, 1980

44. Schröter W, Eber SW, Bardosi A, Gahr M, Gabriel M, Sitzmann FC: Generalised glucosephosphate isomerase (GPI) deficiency causing haemolytic anaemia, neuromuscular symptoms and impairment of granulocytic function: A new syndrome due to a new stable GPI variant with diminished specific activity (GPI Homburg). Eur J Pediatr 144:301, 1985

45. Merkle S, Pretsch W: A glucosephosphate isomerase (GPI) null mutation in *Mus musculus:* Evidence that anaerobic glycolysis is the predominant energy delivering pathway in early post-implantation embryos. Comp Biochem Physiol 101B:309, 1992

46. West JD, Flockhart JH, Peters J, Ball ST: Death of mouse embryos that lack a functional gene for glucose phosphate isomerase. Genet Res Camb 56:223, 1990

1993 81: 206-213

Glucose-6-phosphate isomerase deficiency associated with nonspherocytic hemolytic anemia in the mouse: an animal model for the human disease

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.