Generalized Hexokinase Deficiency in the Blood Cells of a Patient With Nonspherocytic Hemolytic Anemia

By G. Rijken, J. W. N. Akkerman, A. W. L. van den Wall Bake, D. Pott Hofstede, and G. E. J. Staal

In a patient with nonspherocytic hemolytic anemia, a hexokinase deficiency was detected in the red cells (residual activity about 25% of normal) and in blood platelets (20%-35% of normal activity). Although the total hexokinase activity in lymphocytes was normal, the amount of hexokinase type I was decreased to about 50% of normal. However, the deficiency was compensated for by the appearance of type III hexokinase. Compartments studies with controlled digitonin-induced cell lysis showed that this type III enzyme was localized in the cytosol, while almost all hexokinase activity in normal lymphocytes is particulate. No abnormal lymphocyte functions could be detected. The patient was homozygous for the defect. The parents and three of five sibs of the patient were apparently heterozygous with residual activities of 50%-67% of normal in their red cells, but did not show any clinical signs of hexokinase deficiency. The variant enzyme had a slightly decreased affinity for MgATP and a strongly increased inhibition constant for glucose-1,6-P$_2$. Affinity for glucose, heat stability, and pH optimum were normal. In the electrophoretic pattern of red cell hexokinase, only one subtype of hexokinase I could be detected, while in normal red cells, at least three subtypes are present. In the heterozygous individuals, no enzymatic abnormalities could be detected, except for an aberration in the electropherogram of one sib.

H_2EXOKINASE (ADP: d-hexose-6-phosphotransferase, EC 2.7.1.1, HK) is one of the rate-limiting enzymes in erythrocyte glycolysis. The key role of HK in red cell metabolism is illustrated by the manifestation of severe nonspherocytic hemolytic anemia (NSHA) in subjects with a partial deficiency of this enzyme. About ten families with HK-deficient members suffering from NSHA have been reported, as recently reviewed by Paglia et al. Mutations affecting the substrate affinities of the enzyme, regulatory properties, or heat stability have been described. Other variants only show a decreased enzyme activity with no additional enzyme abnormalities. In all these patients the deficiency affected type I HK, the predominant isoenzyme of HK in mammalian tissues. In spite of the presence of mitochondrial energy generation, platelets, lymphocytes, and granulocytes all depend more or less heavily on energy supplied by glycolysis. HK is therefore a likely candidate to play an essential role in the energy metabolism of these cells too. Yet, HK deficiency manifests itself primarily in NSHA, although in some patients a diversity of abnormalities, including multiple malformations, pannymelopahy, and latent diabetes mellitus have been described. The metabolic defects in these patients, however, appear to be acquired rather than truly genetic. One patient with a heterozygous expression of HK deficiency showed an undefined abnormality in the immune response.

Some years ago we described a Dutch patient with NSHA associated with HK deficiency. The mutant enzyme was characterized by a decreased affinity for the inhibitor glucose-1,6-diphosphate (Glc-1,6-P$_2$) and an absent regulation by inorganic phosphate. Some minor abnormalities were found in the type I region of the electrophoretic pattern. In this report we describe a new apparently unrelated patient with NSHA and HK deficiency. We studied the intracellular localization, the kinetic and electrophoretic properties of the mutant enzyme in erythrocytes, as well as in lymphocytes and platelets.

CASE REPORT

The proband was a 19-yr-old woman, anemic since birth, with marked reticulocytosis and variable hyperbilirubinemia. In early infancy, several blood transfusions were necessary. At the age of 2 yr, splenectomy was performed, after which the hemoglobin stabilized at 6 mmole/liter. At the age of 15, cholecystectomy was performed; a liver biopsy taken peroperatively showed massive iron storage. The etiology of the hemolytic anemia remained uncertain until the time of the present investigations: a hexokinase deficiency was detected as the possible cause of the hemolytic anemia. The most recent hematologic data of the patient are shown in Table 1. The patient was slightly anemic with a pronounced reticulocytosis and an increased mean corpuscular volume and mean corpuscular hemoglobin. In the blood smear, 2-25 normoblasts/100 white cells and a moderate polychromasia were observed. A few spherocytes and Howell-Jolly bodies were present. A marked leukocytosis and thrombocytosis was found. Haptoglobin was low; the amount of HbF was increased mean corpuscular volume and mean corpuscular hemoglobin. In the blood smear, 2-25 normoblasts/100 white cells and a moderate polychromasia were observed. A few spherocytes and Howell-Jolly bodies were present. A marked leukocytosis and thrombocytosis was found. Haptoglobin was low; the amount of HbF was slightly increased. The bone marrow showed a very active macrocytic erythropoiesis and an increased megakaryopoiesis. Immunologic response in the patient appeared normal: serum IgM, IgG, and IgA levels were slightly elevated. The numbers of B and T lymphocytes were normal. The in vitro proliferative response of the blood lymphocytes on mitogens (PHA, Con-A, and PWM)

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Submitted March 15, 1982; accepted June 10, 1982.

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0006-4971/83/61/01-002$01.00/0

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and an antigenic stimulation (tetanus toxoid) was normal. In the mixed lymphocyte culture, the lymphocytes behaved normally.

The father and mother of the patient were first cousins. The blood pictures of the parents and of five sibs of the patient were normal, except for a slight and unexplained leukopenia in the mother on one occasion.

**MATERIALS AND METHODS**

Venous blood from the patient, relatives, and controls was collected in either heparin (30 U/ml) or acid-citrate-dextrose (ACD, final citrate concentration 13 mM). Within 1 h after collection of the blood a part of it was deproteinized for the determination of glycolytic intermediates. Deproteinization and determination of glycolytic intermediates were performed according to the methods of Minakami et al. except for 2,3-diphosphoglycerate and adenosine triphosphate (ATP), which were determined with Sigma (St. Louis, Mo.) test kits (Sigma Technical Bulletin 35-UV and 366-UV, respectively).

Red cells were isolated by filtration of whole blood through an α-cellulose–crystalline cellulose mixture. For kinetic and electrophoretic experiments, red cell HK was partially purified by batchwise treatment with DEAE-cellulose A 50 (Pharmacia, Uppsala, Sweden) and concentrated by ammonium sulphate precipitation as described previously. The precipitate was dissolved in 0.2 M Tris-HCl, pH 7.8, containing 3 mM β-mercaptoethanol, and extensively dialyzed against the same buffer.

Platelets were isolated from freshly collected ACD-blood by centrifugation (10 min, 200 g, 22°C) and subsequent gel filtration of the platelet-rich plasma. Platelets were isolated from fresh human blood collected in either ACD or heparin. After density centrifugation (20 min, 1000 g, 22°C) of the blood on Ficoll-Isoaque (Pharmacia, Uppsala, Sweden, density 1.077), the lymphocytes were carefully collected from the interface and treated with ammonium chloride (0.83%). Monocytes were removed by adherence to plastic Petri dishes. The resulting cell preparation consisted of >95% lymphocytes.

Cytosolic and bound HK were separated by discontinuous gradient centrifugation after controlled digitonin-induced cell lysis as described for human platelets by Akkerman et al. This procedure enables the isolation of cytosol and particulate fractions within 1 min after cell lysis. In an Eppendorf Microfuge centrifugation tube the following gradient was established: lower layer, 100 μl Ca-free Tyrode solution containing 10% v/v glycerol; middle layer, 250 μl (platelets) or 500 μl (lymphocytes) of a 3:2 (v/v) mixture of dibutyrylphosphate and dinonylphosphate (Merck, Darmstadt, G.F.R.); and upper layer, 500 μl cell suspension. To the upper layer, 50 μl of a digitonin suspension (Merck, Darmstadt, G.F.R.) was then added in various concentrations and mixed. This procedure was adapted for the fractionation of lymphocytes. The digitonin concentration enabling maximal liberation of cytosolic lactate dehydrogenase (EC 1.1.1.27, LDH) (>90%) and minimal leakage of glutamate dehydrogenase (EC 1.4.1.2, Glu-DH) was dependent on cell type and cell concentration. This optimal digitonin concentration was determined for each cell type by titrating with increasing amounts of digitonin at standard cell concentrations.

After incubation of 10 sec (platelets) or 20 sec (lymphocytes) with the digitonin, the suspension was centrifuged (45 sec, 10,000 g) in an Eppendorf Microfuge equipped with a swingout rotor. Upper layer and lower layer were collected and analyzed. To the lower layer, 400 μl of Ca-free Tyrode was added and the pellet was suspended by mixing with a Vortex whirlmixer. The fractions were analyzed for the activities of HK, LDH as a marker for the cytosol fractions, and Glu-DH as a marker for the mitochondrial matrix. Percentages of HK activity in cytosol and particulate fraction were corrected for the percentage of non-cell lysis as based on the LDH activity in the pellet fraction. Recoveries of total activity in pellet plus supernatant fractions ranged from 90% to 110%.

The erythrocyte glycolytic enzymes were determined according to Beutler and Glu-DH according to Schmidt. Hexokinase was measured at 37°C in a glucose-6-phosphate coupled assay as described previously. HK activities are expressed either in units per gram hemoglobin or in milliunits per 10⁶ cells. Activities of HK and Glu-DH in total cell lysates or in pellet fractions were determined after incubation with 0.5% Triton X-100. The enzyme activities were unaffected by either digitonin or Triton X-100.

HK electrophoresis on cellulose acetate gels and subsequent staining for enzyme activity was performed as described before. Prior to electrophoresis, HK from particulate fractions was solubilized by incubation with 0.5 M KCl (20 min, 20°C) and isolated by subsequent centrifugation (15 min, 48,000 g, 4°C).

**RESULTS**

**Erythrocyte Metabolism**

In the erythrocytes of the patient, the absolute activity of HK was normal or near the lower limit of normal, when compared to normal controls. However, the activities of the other cell age-dependent enzymes—pyruvate kinase and glucose-6-phosphate dehydrogenase—were strongly increased (Table 2), which argues for a relative HK deficiency in the patient. All other glycolytic enzymes were normal or slightly increased (aldolase).

The activities of the erythrocyte glycolytic enzymes in the immediate family were determined also. The parents and three of the sibs (A.Z., H.G.Z., and C.Z.) were found to be heterozygous for the defect. They showed decreased HK activities ranging from 50% to 67% of normal. The other glycolytic enzymes in these subjects were in the normal range.

Some abnormalities could be detected in the con-

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*Investigations by Dr. B. J. M. Zegers, Dept. of Immunology, University Children's Hospital, "Het Wilhelmina Kinderziekenhuis" Utrecht, The Netherlands.
A considerable shift to the cytosolic fraction: only 42% of changed. In normal lymphocytes, over 78% of total activity was found in the pellet fraction. Whereas in the patient’s lymphocytes there was a activity bound to the particulate fraction, as is found in blood platelets. The residual activity was 20%-30% of normal. Essentially all this activity was also found in blood platelets. The residual activity was even above the upper limit of normal. Fructose-6-P were decreased, 2,3-diphosphoglycerate was 40-50% of normal. However, its distribution between soluble and particulate fractions had been normal (Table 3). How- ever, when the high degree of reticulocytosis in the patient is taken into account, the levels of all these metabolites are probably markedly decreased as a result of the hexokinase deficiency.6

Hexokinase Activity and Compartmentation in Lymphocytes and Blood Platelets

The activities of hexokinase were also studied in the lymphocytes and blood platelets of the patient. The results are summarized in Table 4. A deficiency of HK was also found in blood platelets. The residual activity was 20%-30% of normal. Essentially all this activity was bound to the particulate fraction, as is found in normal platelets.

The total HK activity in the patient’s lymphocytes was completely normal. However, its distribution between soluble and particulate fractions had been changed. In normal lymphocytes, over 78% of total activity is bound to the mitochondrial membrane, whereas in the patient’s lymphocytes there was a considerable shift to the cytosolic fraction; only 42% of total activity was found in the pellet fraction.

| Table 2. Erythrocyte Enzyme Activities in Patient, Relatives, and Controls |
|-----------------------------|-----------------------------|-----------------------------|
| Subject                    | Hexokinase                  | Pyruvate Kinase             | Glucose-6-P Dehydrogenase |
| Patient                    | 0.99 ± 0.19 (4)             | 27.4 ± 3.8 (4)              | 22.1 ± 3.5 (3)            |
| Father                     | 0.69                        | 11.1                       | 13.7                      |
| Mother                     | 0.89                        | 10.9                       | 12.4                      |
| Sister A. Z.               | 0.64                        | 10.8                       | 12.8                      |
| Brother E. W. Z.           | 1.38                        | 10.3                       | 13.8                      |
| Brother H. G. Z.           | 0.66                        | 9.9                        | 12.4                      |
| Sister M. Z.               | 1.22                        | 12.0                       | 12.9                      |
| Sister C. Z.               | 0.68                        | 13.5                       | 13.0                      |
| Normals                    | 1.34 ± 0.42 (40)            | 13.5 ± 3.0 (40)            | 10.2 ± 3.3 (40)           |

Enzyme activities are expressed in units per gram hemoglobin. The numbers in parenthesis indicate the number of determinations on different samples.

Enzyme Properties

Kinetics. The kinetic properties of partially purified erythrocyte HK are summarized in Table 5. The $K_m$ glucose of the patient’s enzyme was completely normal; the $K_m$ MgATP was somewhat increased. The inhibition constant for the inhibitor Glc-1,6-P$_2$ varied from sample to sample, but was always between 3 and 10 times normal values. No kinetic abnormalities could be detected in the partially purified preparations from heterozygous subjects. The abnormal behavior of the erythrocyte enzyme of the patient towards the inhibitor Glc-1,6-P$_2$ was also detected for the platelet enzyme (Fig. 1). A marked decrease in inhibition was observed.

Heat stability. The heat stabilities of partially purified erythrocyte hexokinase from patient, heterozygotes, and controls at 44°C were not significantly different. Half of activity was lost between 3 and 4 min of incubation at 44°C.

pH Optimum. A broad pH optimum was found, ranging from pH 6.7 to 8.4 for patient, as well as for heterozygotes and controls.

Electrophoresis. Cellulose acetate electrophoresis of partially purified HK from normal erythrocytes shows a multibanded pattern in the “type I region.” Two subtypes are clearly visible, but more subtypes can be discerned after separation by phosphocellulose chromatography.24 Electrophoresis of the patient’s

<table>
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<th>Table 3. Erythrocyte Glycolytic Intermediates</th>
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<tr>
<td>Glucose-6-P</td>
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<tr>
<td>Fructose-6-P</td>
</tr>
<tr>
<td>Fructose-1,6-di-P</td>
</tr>
<tr>
<td>Glyceroldehyde-P</td>
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<tr>
<td>2,3-Diphosphoglycerate</td>
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<tr>
<td>3-P-glycerate</td>
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<tr>
<td>2-P-glycerate</td>
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<tr>
<td>2-P-enolpyruvate</td>
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<td>ATP</td>
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Concentrations are expressed in nanomoles per milliliter erythrocytes.

<table>
<thead>
<tr>
<th>Table 4. Hexokinase Activity and Compartmentation in Lymphocytes and Platelets</th>
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<tbody>
<tr>
<td>Lymphocytes Activity (mU/10^6 cells)</td>
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<tr>
<td>Percent bound (3)</td>
</tr>
<tr>
<td>Platelets Activity (mU/10^6 cells)</td>
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<td>Percent bound (3)</td>
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The numbers in parenthesis indicate the number of determinations.
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Table 5. Properties of Partially Purified Red Cell Hexokinase

<table>
<thead>
<tr>
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<th>Patient</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{\text{glucose}} (\text{mM}) ) ((\text{MgATP}^2 - 5 \text{ mM}))</td>
<td>0.062 ± 0.007 (3)</td>
<td>0.062 ± 0.014 (5)</td>
</tr>
<tr>
<td>( K_{\text{MgATP}^2} (\text{mM}) ) ((\text{glucose} = 10 \text{ mM}))</td>
<td>1.28 ± 0.15 (5)</td>
<td>0.71 ± 0.09 (7)</td>
</tr>
<tr>
<td>( K_{\text{Glc-1,6-P}_2} (\text{mM}) ) *</td>
<td>0.14 – 0.22 – 0.40†</td>
<td>0.045 ± 0.015 (7)</td>
</tr>
<tr>
<td>Heat stability</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>pH Optimum</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>Altered</td>
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</tbody>
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*The \( K_{\text{Glc-1,6-P}_2} \) was determined at 10 mM glucose and varying \( \text{MgATP}^2 \) concentrations (0.3–5.0 mM) at pH 7.15 and 37°C. Excess of \( \text{Mg}^{2+} \) over ATP was kept at 5 mM in all conditions.
†Each value represents the result of a separate experiment on different blood samples.

The enzyme showed only the most anodal subtype of HK I (Fig. 2). Remarkably, the same pattern was seen in one of the heterozygous sibs (C.Z.), while the other heterozygotes were not discernable from normal.

In normal human lymphocytes, type I HK was predominant, the major part of it being localized in the particulate fraction. No subtypes of HK I were seen. Mitochondrial HK consisted exclusively of the type I isozyme. In the cytosol, circa 20% of total activity was present, distributed between type I (50%–75%) and type III HK (25%–50%). Thus, the contribution of type III HK to the total activity did not exceed 5%–10%. As type III HK is known to be inhibited by high concentrations of glucose, its position in the electropherogram was identified by the insertion of 100 mM glucose in the staining mixture, resulting in a marked reduction in staining intensity (results not shown). In the lymphocytes of the HK-deficient patient, the activity was about equally distributed between type I and type III HK. Type I was found exclusively in the particulate fraction, while type III was localized in the cytosol. The results are shown in Fig. 3.

DISCUSSION

The age dependency of HK in human erythrocytes is well documented now even in quantitative terms. Although the absolute HK activity in the present patient with nonspherocytic hemolytic anemia is about normal, it can be concluded that the activity is only
about 25% of normal, when the degree of reticulocytosis and the activities of the age-dependent enzymes pyruvate kinase and glucose-6-phosphate dehydrogenase are taken into account. The HK activity in the patient is even higher than in the apparently heterozygous parents and sibs. The HK activities in these heterozygous individuals vary between 50% and 70% of normal, while no clinical symptoms of this partial deficiency can be detected. Three of six children are apparently heterozygous; one is homozygous for the deficient trait. As HK deficiency is very rare and as the parents are consanguineous, it is very probable that the patient is a true homozygote, rather than compound heterozygous for two different mutations. It can be concluded that the inheritance of HK deficiency is autosomal recessive, in accordance with most data in the literature. In some studies a dominant mode of inheritance was suggested. However, it seems probable that in these studies patients were investigated with a moderate clinical expression of a heterozygous state, as in the study of Siimes et al. The residual activities of red cell HK in those patients was about 40%-50% of normal. The impact of HK deficiency on red cell metabolism in our patient is rather difficult to evaluate because of the young mean cell age. Glucose-6-P and fructose-6-P are slightly lowered; 2,3-diphosphoglycerate is low normal; while ATP concentration is above the upper limit of normal. However, it is probably reasonable to conclude that due to the HK deficiency, the concentration of all these compounds is strongly decreased with respect to the high reticulocytosis.

Some years ago we described the first Dutch patient with nonspherocytic hemolytic anemia associated with HK deficiency. In this case the mutant enzyme was characterized by a decreased affinity for the inhibitor G1c-1,6-P, an absence of regulation by inorganic phosphate, and a slightly abnormal electrophoretic pattern in the “type I region.” The $K_m$ for MgATP$^2$ was slightly increased too. In the present patient, some of the enzymic abnormalities were similar: an increased $K_m$ MgATP$^2$ and an increased $K_i$ Glc-1,6-P$_2$ were found. However, the regulation of the inhibition by hexose-phosphates by inorganic phosphate was normal (results not shown) and the electrophoretic pattern differed from the former patient. In the present case only the most anodal subtype of HK I was present, while in the former patient, two of three subtypes of normal HK I were visible in the electrophoretic pattern. In the heterozygous subjects no enzyme abnormalities, except an aberrant electropherogram in one, could be detected.

Although no family relationship between both patients could be demonstrated up to now, ancestors of both could be traced to the same rural village. Therefore, and because of the rarity of the defect, it is very likely that the same variant is expressed in both patients, although differences in enzyme properties exist. Direct evidence, however, is still lacking. It was argued that the mutant enzyme in the first patient was subject to increased posttranslational modification of the primary enzyme. The differences in the enzyme properties between the two deficient subjects probably reflect a phenotypic or genotypic variation of these processes. In this respect, the aberrant electrophoretic pattern in one of the heterozygous sibs (C.Z.) is of particular interest.

The study of HK deficiency has been mostly restricted to the red cell. However, as the deficiency concerns the most abundant isoenzyme type I HK, it probably affects other cell types too. As platelets and lymphocytes are strongly dependent on glycolysis for a proper performance of cell functions, we investi-
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The impact of HK deficiency on these cell types. HK activity in the lymphocytes of the patient was normal. Yet, the amount of type I HK was only about 50% of normal. However, the deficiency was compensated for by the appearance of type III HK, which is present in only minor quantities (5%-10%) in normal lymphocytes. Type III HK has a higher affinity for glucose than the type I isoenzyme and is inhibited by high concentrations of glucose. In normal lymphocytes, most of the HK activity is found in the particulate fractions, presumably bound to the mitochondria. The localization of HK on the mitochondrial membrane was suggested to play a key role in the maintenance of a high glycolytic rate. In the HK-deficient patient, the residual HK activity is bound also. How-ever, the compensatory type III HK is found only in the cytosol. It is not clear how these alterations affect lymphocyte metabolism, but no functional abnormalities could be found in the patient's lymphocytes.

In the patient's platelets HK was also deficient. No compensation by type III HK was found, and most of the activity was localized in the particulate fraction as in normal platelets. The mutant properties of the enzyme, in particular the decreased affinity for the inhibitor glucose-1,6-diphosphate, were also detected in the platelet enzyme. Platelet aggregation induced by 0.1-5 U/ml thrombin was normal. In contrast, Ca^{2+} secretion, as a reflection of dense granule release, was slightly reduced. In the presence of the inhibitor of mitochondrial ATP synthesis, antimycin-A (8.25 μg/ml) Ca^{2+} secretion was more inhibited in the patient's platelets than in concurrently run normal subjects. The discrepancy was even greater in the presence of both antimycin-A and the glycolytic inhibitor, glucono-δ-lactone. HK activity in the patient's granulocytes appeared to be normal. As the major isoenzyme in normal human granulocytes is type III HK and the activity and the electrophoretic pattern showed no abnormalities (results not shown), we did not extend our studies of HK to this blood cell type.

ACKNOWLEDGMENT

We gratefully acknowledge the cooperation of the present patient and her family during the course of these investigations, the technical assistance of G. Gorter, M. Streefkerk, and P. J. Bek, and the secretarial efforts of E. L. Huisman-Backer Dirks.

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