Enzymatic Diagnosis of Aspartylglycosaminuria by Fluorometric Assay of Glycosylasparaginase in Serum, Plasma, and Lymphocytes

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Serum, plasma, and lymphocytes from aspartylglycosaminuria (AGU) patients and carriers and from normal controls were incubated with a fluorescent glycosylasparaginase substrate, l-aspartic acid β-(7-amido-4-methylcoumarin), and the release of 7-amino-4-methylcoumarin was measured fluorometrically after incubation for 1–4 h. The mean glycosylasparaginase (EC 3.5.1.26) activity in normal serum, plasma, and lymphocytes was 20.2 (SD 5.0) mU/L (n = 24), 17.5 (SD 5.0) mU/L (n = 24), and 242 (SD 108) mU/g protein (n = 17), respectively. The corresponding values in the Finnish AGU patients were 0.7 (SD 0.4) mU/L (n = 10), 0.3 (SD 0.3) mU/L (n = 10), and 6.0 (SD 4.6) mU/g protein (n = 7). No overlapping values were obtained between the AGU patients and the carriers in any of the samples, but the values between the carriers and controls were overlapping in 28 of 29 serum, 22 of 29 plasma, and 4 of 21 lymphocyte samples. Thus, the fluorometric glycosylasparaginase assay in various blood samples allows specific detection of the enzyme defect in AGU, but cannot be used for reliable detection of carriers of the disease.

Indexing Terms: lysosomal storage disease/heritable disorders/carrier detection/aspartylglucosamine

Aspartylglycosaminuria (AGU; McKusick 20840) is a recessively inherited lysosomal storage disease that is especially common in the Finnish population, with an estimated frequency of up to 1:4000 and carrier rate of 1:30 in eastern Finland (1). The disease is caused by deficient activity of glycosylasparaginase (glycoasparaginase; aspartylglucosaminidase; EC 3.5.1.26), which cleaves the N-acetylglucosaminyl-asparagine linkage of N-linked glycoproteins (2, 3). The clinical picture is similar to that of mild mucopolysaccharidosis with progressive mental retardation (4). The metabolic defect results in the accumulation of asparaglycosamine (2-acetamido-1-L-β-aspartamido-1,2-dideoxy-β-D-glucose; GlcNAc-Asn) in tissues and body fluids (4). Assay of GlcNAc-Asn in urine can be used in detection of the disease (1, 4, 5). The diagnosis is confirmed by demonstration of the deficient glycosylasparaginase activity in blood samples or cultured fibroblasts (6–8). The gene defect in the Finnish-type AGU consists of two single-base changes in the glycosylasparaginase gene (9, 10), and the two nucleotide changes are always associated (11).

A recent study on the mechanism of action of glycosylasparaginase (3) demonstrated that the enzyme acts as an exohydrolase toward the L-asparagine moiety of its substrates. On the basis of this, we developed a fluorometric assay for glycosylasparaginase activity, using L-aspartic acid β-(7-amido-4-methylcoumarin) (AspAMC) as substrate (12). The method is 40–100 times more sensitive than other published methods for glycosylasparaginase activity, and it enables the diagnosis of AGU by assaying leukocytes and cultured fibroblasts (12). We now report a modification of the assay that allows rapid enzymatic diagnosis of AGU with serum and plasma samples. We found that isolated lymphocytes can be used for glycosylasparaginase assay in diagnosis of the disease, but none of the blood samples is suitable for carrier detection of AGU because of overlap between results for carriers and normal controls.

Materials and Methods

Materials. The substrate AspAMC and the standard 7-amino-4-methylcoumarin (AMC) were purchased from Bachem AG, Bubendorf, Switzerland. Ficoll-Paque was purchased from Pharmacia, Uppsala, Sweden. All other reagents were of analytical grade and were used without further purification.

Samples. Serum and plasma samples were obtained from apparently normal Finnish individuals (n = 24; mean age 41 years, range 16–69), Finnish-type AGU patients (n = 10; mean age 31 years, range 4–49), and carriers of the disease (n = 29; mean age 45 years, range 8–87). The patients had typical clinical signs of AGU (4), excreted large amounts of GlcNAc-Asn in their urine (5), and lacked glycosylasparaginase activity in their lymphocytes (8). The patients were shown to be homozygous with regard to the two point mutations typical of the Finnish-type AGU (9, 10); the carriers had the same mutations in one of their glycosylasparaginase alleles, and the normal controls were lacking those nucleotide changes in both of their alleles. In addition, random serum samples were obtained from newborn babies (n = 20; mean age 7 days, range 0–88) and plasma samples from young infants (n = 14; mean age 18 days, range 1–88). We cultured fibroblast cell lines from two American patients with different mutations in their glycosylasparaginase gene (13, 14) and from one Tunisian patient with an unknown genotype, and measured glycosylasparaginase activity in them as described (12). We isolated lymphocytes by density gradi-

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5 Nonstandard abbreviations: AGU, aspartylglycosaminuria; GlcNAc-Asn, 2-acetamido-1-L-β-aspartamido-1,2-dideoxy-β-D-gluc-

ose; AMC, 7-amino-4-methylcoumarin; AspAMC, l-aspartic acid

β-(7-amido-4-methylcoumarin); and PCR, polymerase chain reac-

tion.

Received June 25, 1993; accepted October 22, 1993.
ent centrifugation with Ficoll-Paque according to the manufacturer's instructions. Samples were stored frozen at -20°C until analysis.

To study the effect of hemoglobin concentration on the assay, we hemolyzed a normal blood sample by freezing 10 mL of it for 24 h and then thawing; serum was separated after centrifugation at 3000g for 10 min and mixed with normal serum in different ratios. The hemoglobin concentration (15) and glycosylasparaginase activity in the serum mixtures were determined. We studied bilirubin interference by adding to a normal serum sample (bilirubin concentration 10 μmol/L) bilirubin concentrations of 100, 200, and 300 μmol/L before the glycosylasparaginase assay. We determined the effect of glucose by analyzing plasma samples containing 6, 10, 14, and 20 mmol/L glucose. Bilirubin and glucose were assayed with a Hitachi 717 automatic analyzer (Hitachi, Tokyo, Japan).

Demonstration of the genotype. We isolated DNA from leukocytes, and amplified the glycosylasparaginase gene region mutated in the Finnish-type AGU by using the polymerase chain reaction (PCR) as described (10). The PCR products were isolated by ethanol precipitation, digested with EcoRI and DdeI, and analyzed by gel electrophoresis on a 2% agarose gel to demonstrate the presence or absence of the two nucleotide changes typical of the Finnish-type AGU (9, 10).

Glycosylasparaginase assay. We assayed the glycosylasparaginase activity in plasma and serum by incubating 100 μL of sample with 10 μL of AspAMC (10 mmol/L in ethylene glycol) and 90 μL of Tris-HCl buffer (50 mmol/L, pH 7.5) containing 10 mL/L ethylene glycol for 60–240 min at 37°C. We measured lymphocyte glycosylasparaginase activity with 0.5 mmol/L substrate in 50 mmol/L Tris-HCl, pH 7.5, containing 10 mL/L ethylene glycol and 100–500 μg of protein, in a final volume of 200 μL (12). Fluorescence was measured with an IL Multistat III Plus fluorescence centrifugal analyzer (Instrumentation Laboratory, Lexington, MA) at 350 nm (excitation) and 450 nm (emission). Glycosylasparaginase activity in plasma and serum was expressed as mU/L and that in lymphocytes as mU/g protein. One unit of glycosylasparaginase liberates 1 μmol of AMC from the substrate per minute at 37°C. Protein was determined with a protein assay kit (Bio-Rad Labs., Richmond, CA) and a Kone Compact clinical analyzer (Kone Instruments, Espoo, Finland).

Results and Discussion

The proportion of hydrolysis of the substrate AspAMC in serum, plasma, and lymphocyte homogenate was linear with time for several hours (tested up to 4 h). The pH for the assay was selected as 7.5, the optimum for both the purified enzyme (16) and the glycosylasparaginase in biological samples (8). The within-day and between-day CVs of the method were <5.3% and <5.7%, respectively (n = 10 each), for a serum sample containing glycosylasparaginase activity of 24.8 mU/L.

Glycosylasparaginase activity in serum, plasma, and lymphocytes of normal controls, carriers of AGU, and AGU patients is illustrated in Fig. 1. The mean enzyme activity was 20.2 mU/L (SD 5.0; range 10.6–28.2, n = 24) in normal serum and 0.7 mU/L (SD 0.4; range 0.0–1.2, n = 10) in AGU serum (Fig. 1A). The corresponding plasma values (Fig. 1B) were 17.8 mU/L (SD 5.0; range 10.2–26.0, n = 24) in the normal individuals and 0.3 mU/L (SD 0.3; range 0.0–0.9, n = 10) in the AGU patients. The mean serum and plasma values in the carriers of AGU were 15.3 mU/L (SD 4.5; range 9.9–26.2, n
The mean glycosylasparaginase activity in the carriers of AGU was 72% of the normal plasma activity, 75% of the normal serum activity, and 25% of the normal lymphocyte activity. The values for the carriers and controls overlapped in 28 of 29 serum samples, 22 of 29 plasma samples, and 4 of 21 lymphocyte samples. The lowest glycosylasparaginase activity detected in serum or plasma of any of the controls or carriers was ninefold the highest value detected in any of the AGU patients, demonstrating the good applicability of the assay to diagnosis of the disease. The lowest value in control lymphocytes was >7 times and that of carriers 1.7 times higher than the highest value in AGU lymphocytes.

Photometric assay of glycosylasparaginase by the Morgan–Elson reaction indicated that normal controls and carriers of AGU could be differentiated on the basis of glycosylasparaginase activity in separated lymphocytes, and that activities for affected heterozygotes and homozygotes overlapped (6). However, the present, more-specific glycosylasparaginase assay demonstrated that values for the carriers of AGU and normal individuals overlapped even in lymphocyte samples. Absolute heterozygote testing is not possible by glycosylasparaginase assay; instead, a DNA test should be used for carrier detection.

The presence of hemoglobin in the incubation mixture had a considerable inhibiting effect on the assay (Fig. 2A). With a hemoglobin concentration of 1400 mg/L, the serum sample had an intense red color, and the detected glycosylasparaginase activity was 16% of that in the original, nonhemolyzed serum. Thus, a very low glycosylasparaginase activity in hemolyzed plasma or serum samples should be confirmed with a nonhemolyzed specimen. The presence of bilirubin (tested up to 300 µmol/L) in the serum sample caused almost a linear decrease in the glycosylasparaginase activity assayed (Fig. 2B); at the highest bilirubin concentration, 42% of the original glycosylasparaginase activity was detected and the value was about fivefold more than the highest value detected in any of the AGU samples. Moreover, the interferences of hemoglobin and bilirubin in the assay are additive. Plasma glucose at concentrations up to 20 mmol/L had no effect on the glycosylasparaginase activity in this assay system (data not shown).

In the random plasma and serum samples from newborn babies and young infants, the mean glycosylasparaginase activity was 20 mU/L (n = 14; range 4.8–53) and 13 mU/L (n = 20; range 4.3–26), respectively. The mean bilirubin concentration in the serum samples was 111 µmol/L (range 5–262 µmol/L). These results suggest that the glycosylasparaginase assay in plasma and serum samples can be used for detection of AGU in newborn babies and young infants also; however, the inhibitory effect of hemoglobin and bilirubin must be considered when low assay values are encountered.

We tested the current glycosylasparaginase assay in three non-Finnish AGU fibroblast cell lines (see Materials and Methods). In all those cases the glycosylasparaginase activity was the same as that in the Finnish AGU patients, 0.00 mU/g protein, whereas the mean activity in the control fibroblasts was 46 mU/g protein (n = 7; range 21–76). Although we may assume that the enzymatic activity in serum and plasma of those patients would also be very low or undetectable, we cannot rule out the potential existence of mutations in the glycosylasparaginase gene that would cause AGU and be associated with residual glycosylasparaginase activity.

In summary, we have described a rapid, sensitive, and specific assay for glycosylasparaginase activity in plasma and serum specimens. Serum and plasma glycosylasparaginase activity can be analyzed by the present method after a short incubation of 1 h instead of the 12–18 h required for earlier methods (6–8). The values of glycosylasparaginase activity in plasma are very similar to those obtained by HPLC analysis of the substrate, GlcNAc-Asn, and product, aspartate (8). No
false-positive or false-negative result has been obtained so far in detection of AGU patients. All the patients who lacked the enzyme activity in their plasma or serum excreted large amounts of GlcNAc-Asn in their urine. No patient with normal glycosylasparaginase activity in plasma or serum has been diagnosed as having AGU. The current assay will be useful for laboratories diagnosing lysosomal storage diseases. It is especially useful for infants, because the assay does not require isolation of leukocytes or culture of fibroblasts. The cost of a glycosylasparaginase activity assay is currently <10% of that of the DNA tests currently available for the Finnish-type AGU.

We thank Ulla Korhonen for technical assistance. This work has been financially supported by The Academy of Finland, Sigrid Juselius Foundation, Technology Development Centre TEKES, and The Sevo Foundation for High Technology (I. M.).

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