Unusual Thermolability Properties of Leukocyte $\beta$-Hexosaminidase: Implications in Screening for Carriers of Tay–Sachs Disease

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Tay–Sachs disease (TSD), an autosomal recessive neurodegenerative condition, is the result of a deficiency of $\beta$-hexosaminidase A (hex A). Heterozygotic individuals are screened by analysis for hex A and hex B activities; the percent of hex A is the critical determinant of carrier vs noncarrier status. Most laboratories use a heat-inactivation assay that exploits the differential thermolability of the isoenzymes. However, we have found a reciprocal relation between the apparent leukocyte hex A activity and the amount of the sample used in the assay; i.e., a significant increase in the percent of hex A activity with decreasing amounts of sample. Three sets of data indicate that this phenomenon was caused by an effect on the hex B isoenzyme and not on hex A. This variation in hex A activity with sample amount was not observed when a hex A-specific substrate was used. This phenomenon was also not seen in assays of leukocytes from carriers for Sandhoff disease, a condition associated with a reduction in the amount of hex B. Finally, when leukocytes from a TSD homozygote, containing almost no hex A, were analyzed, marked increases in the percent of hex A were observed with decreasing sample concentrations. These data indicate that misdiagnoses could result from variations in sample concentrations used for TSD carrier testing and support the view that the leukocyte concentrations used for these assays should be standardized.

Indexing terms: genetic disorders · Sandhoff disease · heterozygosity · analytical error

Tay–Sachs disease (TSD) is an autosomal recessive neurodegenerative disorder caused by a deficiency of the lysosomal enzyme $\beta$-hexosaminidase A (hex A, EC 3.2.1.52).6 Heterozygosity for this disorder occurs with increased frequency among persons of Ashkenazi (Eastern European) Jewish ethnicity and persons of French-Canadian background (1). Over the past two decades, TSD prevention programs have been established around the world to provide carrier testing for these groups, and to provide prenatal diagnosis and genetic counseling for couples who are at risk of having a child with TSD. The effectiveness of these programs represents a major public health genetics success story (1, 2).

Testing for TSD heterozygosity consists of analysis of the $\beta$-hexosaminidases A and B (hex A and B) in blood. For most individuals, testing of serum hex A and B is sufficient to determine TSD carrier status. However, because of the effects of hormonal changes on plasma concentrations of these isoenzymes during pregnancy, analysis of leukocyte hex A and hex B is necessary for the determination of TSD carrier status in pregnant women. Also, any individual whose serum test is inconclusive requires analysis of leukocyte hex A and B. In view of the clinical importance of the leukocyte assay, it is important to understand its potential limitations.

The standard method of analysis for serum and leukocyte $\beta$-hexosaminidases is based on the differential thermal stabilities of hex A and B, particularly that of hex A (3). The artificial fluorescent substrate, 4-methylumbelliferyl-2-deoxy-2-acetamido-$\beta$-D-glucopyranoside, can be hydrolyzed by both hex A and hex B. Sample aliquots are assayed before and after heating at 52°C. The activity observed in the unheated sample is due to both hex A and B; the activity in the heat-treated sample is due to hex B. By comparing the two results, the percent of the total that is due to each isoenzyme can be determined.

The differential thermolability of hex A and hex B is a function of their subunit structures. Hex A is a dimer of one $\alpha$- and one $\beta$-subunit, whereas hex B is a dimer of $\beta$-subunits (1). TSD is caused by mutations in the $\alpha$-subunit gene; therefore, heterozygosity is diagnosed on the basis of the percent of hex A. TSD heterozygotes have a lower percent of hex A than do noncarriers. Tay–Sachs heterozygotes typically have <55% hex A, whereas noncarriers have >60% hex A, although the specific values vary between different clinical laboratories (3).

The leukocyte assay is performed with a fixed volume of a leukocyte sonicate (3). The concentration of the sample in the sonicate can vary widely, depending on the size of the initial leukocyte pellet and the amount of water added to the pellet before sonication. Thus, as specified by the commonly used protocol, the sample concentration in the assay tubes is different for each patient tested. Total leukocyte hexosaminidase activity is normalized relative to the amount of sample protein in the assay and is reported as nanomoles of substrate hydrolyzed per milligram of protein per hour. The percentage of each isoenzyme is assumed to be independent of the amount of sample used.

During the course of performing thousands of leukocyte $\beta$-hexosaminidase assays for the determination of TSD carrier status, we noticed that significantly different values for the percent of hex A could be obtained for a single patient by varying the sample concentration. This report details the study of this heretofore undescribed phenom-
We further suggest methods to minimize the likelihood of inaccurate diagnoses that could result from the failure to recognize this unusual enzyme behavior.

Materials and Methods

Materials

We purchased 4-methylumbelliferyl-2-deoxy-2-acetamido-β-D-glucopyranoside and 4-methylumbelliferone from Sigma Chemical Co. (St. Louis, MO). We purchased 4-methylumbelliferyl-N-acetylglucosamine-6-sulfate (MUGS) from HSC Research and Development Ltd. Partnership (Toronto, Canada).

Samples

We obtained leukocyte samples from apparently healthy normal control subjects and from patients referred to our Tay–Sachs Disease Prevention Program. Included in the analysis were three obligate heterozygotes for Sandhoff disease, i.e., persons who had a child with Sandhoff disease. The remaining samples were from individuals who were diagnosed as carriers of TSD or Sandhoff disease on the basis of our laboratory studies. One individual who was homozygous for TSD was also included in the study. These experiments were done in accord with the ethical standards of the E. K. Shriver Center.

Heat-Inactivation Assay

We determined heat-labile (hex A) and total hexosaminidase activities in leukocytes by the heat denaturation (52 °C) method by using 4-methylumbelliferyl-2-deoxy-2-acetamido-β-D-glucopyranoside as the substrate, as previously described (3). Leukocyte pellets were sonicated in cold distilled water and then diluted with bovine serum albumin (BSA), 6 g/L in 6 mmol/L citrate-10 mol/L phosphate buffer, pH 4.4. Dilutions were made with 400 µL of buffered BSA, an appropriate amount of sonicate to yield the desired sample concentration, and enough distilled water to yield a final volume of 500 µL. The amount of buffered BSA and water was the same in each assay tube. Aliquots (50 µL) of the BSA-diluted samples were subjected to heat-inactivation at 52 °C for 1 and 2 h. We assayed hexosaminidase activity, and the results for the 1- and 2-h inactivations were averaged.

We assayed hex A directly with MUGS. Dilutions into BSA and heat-inactivation were carried out as described above, except that samples were heat-treated for 0, 0.25, 0.5, 1.0, and 2.0 h. To the 50-µL sample aliquots, 100 µL of 3 mmol/L MUGS in 0.1 mol/L citrate-0.2 mol/L sodium phosphate buffer, pH 4.0, was added. The incubation was carried out at 37 °C for 30 min, and the reaction was stopped with 1.9 mL of 0.17 mol/L glycine-carbonate buffer, pH 10. Fluorescence was determined as described elsewhere (3). Protein concentration was determined by the method of Lowry et al. (4).

Results

Effects of Sample Concentration on the Heat-Inactivation Analysis of Hex A and Hex B

To determine the effect of varying the concentration of the sonicate on the apparent percent of leukocyte hex A activity, we performed the standard heat-inactivation assay with 5, 10, 15, or 20 µg of sample protein per assay tube. Figure 1 shows the results obtained with samples from persons diagnosed as noncarriers for TSD (A) and from persons diagnosed as TSD heterozygotes (B).

For all of the noncarrier controls tested, a significant decrease in the percent of hex A activity was observed with increasing sample protein concentration. The average decrease with 5 to 20 µg of sample protein was 7.0% (SD 2.3), with a range of 3.5%-10.3%. A similar decrease in the percent of hex A was noted when samples from TSD heterozygotes were analyzed. The leukocyte samples from TSD carriers had an average decrease of 5.6% (SD 1.6), with a range of 3.4%-7.5%.

The heat-inactivation assay for β-hexosaminidase is also used to identify heterozygotes for Sandhoff disease, which is caused by mutations in the β-subunit of β-hexosaminidase. Sandhoff disease carriers typically have a low amount of total β-hexosaminidase, with a high percent of hex A (>70%) (5). This means that they have much less hex B than noncarriers (5). Figure 2 shows the effect of varying the concentration of the leukocyte sonicate in the heat-inactivation assay of samples from Sandhoff disease heterozygotes. Although a slight de-

Fig. 1. Effect of sample concentration on apparent hex A activity

Leukocyte sonicates from TSD noncarriers (4, n = 7) and from TSD carriers (8, n = 7) were assayed as described in Materials and Methods. Assays were performed with 5, 10, 15, and 20 µg of sample protein for each sonicate; each symbol represents a different test sample.
crease in the percent of hex A was observed, it was much less than that seen in the noncarriers and Tay–Sachs carriers. The average decrease with 5 to 15 μg of sample protein was 1.6%, with a range of 1.1%–4.7%. This result is significantly different from those of the noncarrier and the TSD heterozygote samples (P ≤ 0.01), as determined by the Student’s t-test.

The effect of a 10-fold variation in sample concentration (2 and 20 μg of protein) was analyzed in two normal controls and one TSD patient (Table 1). Persons with TSD have little or no hex A because of mutations in the α-subunit gene of hexosaminidase. As seen in Table 1, the apparent percent of hex A changes dramatically with different amounts of sample analyzed. When only 2 μg of sample protein was used, this patient had 28.6% hex A, a value consistent with heterozygosity, not homozygosity, for TSD. However, at 20 μg of sample protein, there was 12.0% hex A, consistent with the patient’s true diagnosis of TSD. Similar variations were observed in the noncarrier control subjects.

Direct Analysis of Hex A

The heat-inactivation assay described above indirectly yields the percent of hex A activity, because it is assumed that the heat treatment inactivates only hex A, and the remaining activity is due to hex B. To study directly the effects of varying sample concentration on hex A activity in the leukocyte assay, we used the hex A-specific substrate MUGS. This sulfated substrate cannot be hydrolyzed by hex B (6–9), and therefore provides a direct determination of hex A activity. We noted that the MUGS assay was linear with respect to sample concentration, from 5 to 20 μg of sample protein, the range over which the percent hex A changed in the heat-inactivation assay, and that the specific activity of hex A in the sample was unaffected by the change in sample concentration (data not shown).

We also determined whether the sample concentration could affect the rate of heat inactivation of hex A. Figure 3 shows the time course for the heat inactivation of hex A in one sample from a noncarrier, at four different sample concentrations, assayed with the MUGS substrate. At each sample concentration tested, the hex A rate of heat inactivation was the same. Similar results were obtained with a second noncarrier control (data not shown).

Discussion

This report describes an unusual and previously undocumented effect of sample concentration on the apparent activity of hex A when measured by the commonly used heat-inactivation assay. With increasing sample concentration, the percent of total hexosaminidase attributed to hex A decreased significantly when assayed in leukocytes from noncarriers of TSD and individuals heterozygous and homozygous for TSD.

Our results indicate that hex B, but not hex A, is affected by the change in sample concentration. Because the heat-inactivation assay indirectly measures hex A (total minus hex B), any effect on hex B would appear as an opposite effect on hex A. Three sets of data support this hypothesis.

First, when the effects of sample concentration in the heat-inactivation assay were studied in leukocytes from Sandhoff disease heterozygotes, the apparent decrease in the amount of hex A was much less than that seen in noncarriers and TSD carriers. Sandhoff disease carriers typically have a high percent of hex A and reduced total hexosaminidase activity because the mutation they

Table 1. Effect of Sample Concentration on Hexosaminidase Activity in Tay–Sachs Disease Leukocytes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein, μg</th>
<th>% Hex A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control 1</td>
<td>2</td>
<td>80.7</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>63.9</td>
</tr>
<tr>
<td>Normal control 2</td>
<td>2</td>
<td>74.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>67.0</td>
</tr>
<tr>
<td>Tay–Sachs Disease</td>
<td>2</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of sample concentration on apparent hex A activity in Sandhoff disease carriers
Leukocyte sonicates from Sandhoff disease carriers were assayed as described for Fig. 1; n = 5

Fig. 3. Time course for heat inactivation of hex A at various sample concentrations
A noncarrier control leukocyte sonicate was assayed by the MUGS method as described in Materials and Methods. Tubes contained 5 (●), 10 (■), 15 (▲), or 20 (▲) μg of sample protein
carry is in the β-subunit gene. The amount of hex B, which is made up of two β subunits, is greatly reduced in Sandhoff disease heterozygotes. Therefore, if the response to sample concentration is the result of an effect on hex B, one would expect to see less of a decrease in the amount of hex A in Sandhoff disease carriers, as was demonstrated in this study (Figure 2).

Second, when hex A was assayed directly by using the hex A-specific substrate MUGS, we found no effect of sample concentration on hex A activity. The MUGS assay was linear with respect to sample concentration, and the same specific activity was observed at all sample concentrations tested. Also, when the rate of heat inactivation of hex A was examined, no differences were found when different sample concentrations were used. These data eliminate changes in hex A activity as the cause of the apparent decrease in the amount of hex A observed with the standard heat-inactivation assay.

Finally, in a leukocyte sample from a TSD homozygote, in which little hex A exists, a similar concentration-dependent discrepancy in the apparent percentage of hex A was observed. Since hex B is nearly all of the hexosaminidase present in such a sample, this result is further evidence that the observations were due to effects on hex B and not hex A.

The apparent increase in hex A activity noted at low sample concentrations is the result of a decrease in hex B activity. This could be caused by a number of factors. At lower sample concentrations, hex B could be more heat-labile than at higher sample concentrations. Since the heat-inactivation assay assumes that all activity lost during heat treatment is that of hex A, the inactivation of a portion of the hex B would appear as an increase in hex A. This effect would not be the result of a nonspecific stabilization by protein, because the inactivation assay is performed in the presence of a high concentration of BSA (4.8 g/L), so that the total protein concentration of the assay system is essentially the same for each concentration of sonicate. Another explanation is that hex B activity is protected or enhanced by a cellular component whose function as an effector is reduced at low sample concentrations and increased at high sample concentrations. Such an effector could be a protein, a lipid, or other molecule. This explanation is also consistent with our data.

Variations in the percent of hex A pose a problem for the accurate diagnosis of TSD carrier status. The diagnosis of carrier or noncarrier status is determined by the percent of hex A in a patient’s sample. In the case of persons who are truly noncarriers, a high sample concentration in the assay could yield an inconclusive or a false-positive result. Likewise, for persons who are truly TSD heterozygotes, a low sample concentration could yield an inconclusive or false-negative result. The former case can result in unnecessary anxiety in a person who is not really at risk to produce a child with TSD. In the latter case, the true risk is not known for that person and other family members. In addition, a very low concentration of leukocyte sample could cause a diseased patient to appear as a carrier.

The importance of the leukocyte β-hexosaminidase assay in diagnosing carriers for a serious neurodegenerative disease and the marked effects of seemingly minor variations in sample concentration on the apparent concentration of the key analyte, hex A, underscore the need for stringent guidelines for the amount of sample used in the leukocyte TSD carrier test. Although there is no "right" amount of sample, we recommend using either a standard amount of sample protein (e.g., 10 μg per tube) or a range of 8 to 12 μg of sample protein per tube. Each testing laboratory should establish its own protocol and reference ranges to maximize test reliability.

Additionally, the use of DNA diagnostics for the molecular genetic analysis of common α subunit gene mutations may be a valuable adjunct in cases of patients whose test results are inconclusive after careful leukocyte hexosaminidase analysis (10-12).

We thank David Newburg for critical reading of the manuscript. This work was supported in part by the Commonwealth of Massachusetts Department of Mental Retardation (contract #1002000293C).

References