Ganglioside GM_2 Storage Diseases: Hexosaminidase Deficiencies in Cultured Fibroblasts

SHINTARO OKADA,[1] M. LOIS VEATH,[1] JULES LEROY,[2] AND JOHN S. O'BRIEN[1]

INTRODUCTION

Ganglioside GM_2 is stored in three inborn errors of ganglioside metabolism, Tay-Sachs disease, Sandhoff's disease, and juvenile GM_2 gangliosidosis. Each appears to be transmitted as an autosomal recessive trait. Hexosaminidase A is absent in Tay-Sachs disease [1], both hexosaminidase A and B are absent in Sandhoff's disease [2], and a partial deficiency of hexosaminidase A has been demonstrated in juvenile GM_2 gangliosidosis [3, 4]. Descriptions of each disease are presented in a recent review [5]. We report here deficiencies of hexosaminidase components in cultured fibroblasts from patients with these three diseases, and document intermediate enzymic deficiencies in their parents.

MATERIALS AND METHODS

Skin biopsies were taken aseptically from the forearm or subscapular region after subcutaneous infiltration with 1% xylocaine. Specimens were immediately placed in sterile tubes containing medium F10, to which antibiotics and 15% fetal calf serum were added (Grand Island Biological Company). Biopsies were cut into 1–2-mm pieces, placed in 35-mm petri dishes under a cover slip, and 2 ml of the above medium was added. Petri dishes were then placed in an incubator maintained at 37° C, in an atmosphere of 95% oxygen and 5% carbon dioxide saturated with water vapor. Cultures were fed three times a week. After sufficient cell growth had occurred to cover the dish with a monolayer of outgrowing fibroblasts (two and one-half to three weeks), cells were subcultured by treatment with 0.25% trypsin. Frozen cell lines were obtained by freezing at the rate of 1° per minute in media containing 10% glycerol. Cells were stored in liquid nitrogen in glass ampules.

Enzyme assays were carried out on cell lines which had been subcultured at least once. Cells were harvested for assay by replacing the media with 0.85% saline, scraping the cells from the surface of the petri dish with a rubber policeman, centrifuging the cells at 3,000 RPM, and discarding the supernatant. Harvested cell lines could be stored frozen at −20° C prior to assay without affecting the activity of the enzymes studied here.

Received July 10, 1970; revised August 27, 1970.

Portions of this study were supported by National Multiple Sclerosis grant no. 430, National Cystic Fibrosis Foundation grant, National Institutes of Health grant NB 08682, National Foundation for Neuromuscular Diseases grant-in-aid, Futures for Children-Los Angeles, Quick Children bequest, donation from the Children's Brain Diseases, National Institutes of Health Program project grant GM 17702-01, and a grant from the Belgian government to Jules Leroy.

[1] Department of Neurosciences, School of Medicine, University of California at San Diego, La Jolla, California 92037.

[2] Rijksuniversitair Centrum, Middelheimlaan 1, Antwerp, Belgium.

ENZYME ASSAYS

The β-D-N-acetylglucosaminidase activity was assayed according to the procedure described previously by Okada and O'Brien [1]. The wet weight of the cultured skin fibroblasts was obtained and cells were frozen and thawed 20 times prior to homogenization in a ground glass Tenbroeck homogenizer in 10 volumes of distilled water. (One volume is equivalent to 1 g wet weight of cells per milliliter of homogenate.) Two microliter aliquots of the fibroblast homogenate were taken and diluted with 20 μliter of 0.04 M citrate phosphate buffer (*p*H 4.3). To each sample was added 60 nanomoles of 4-methylumbelliferyl-β-D-N-acetylglucosamine (Pierce Co.) dissolved in 20 μliter of the same citrate-phosphate buffer described above. Samples were then incubated at 37° C for 15 and 30 minutes. The reaction

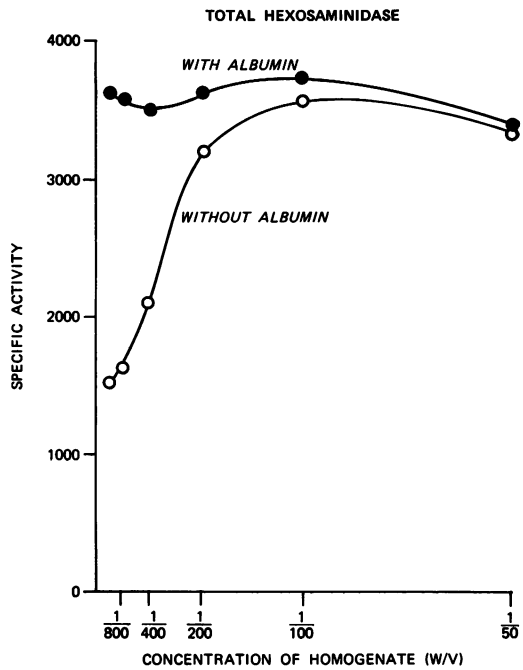

FIG. 1.—Effect of dilution of fibroblast homogenate with buffer on hexosaminidase-specific activity (nanomoles of 4-methylumbelliferyl-β-D-N-acetylglucosamine cleaved per milligram protein per hour). Lower curve—specific activity at varying concentrations of homogenate in buffer in the absence of albumin. Upper curve—0.1% human serum albumin added to each sample prior to assay.

was terminated by adding 2.5 ml of 0.17 M glycine-carbonate buffer (*p*H 10). Fluorescence was read in a Turner fluorometer at an excitation wavelength of 365 mμ and an emission wavelength of 450 mμ. Protein determinations were carried out by the method of Lowry et al. [6].

It was found that if dilute fibroblast homogenates (greater than one in 200) were used for enzyme assay, the hexosaminidase activity was lower than in concentrated homogenates (fig. 1). Addition of human serum albumin (grade III, Sigma Co.) to dilute homogenates resulted in the restoration of activity to levels found in concentrated homogenates (fig. 1). For this reason, 0.075% human serum albumin was routinely added to all homogenates. Optimal enzyme activity occurred at a concentration of 0.05%–0.1% albumin. At concentrations less than 0.05%, activity rapidly fell. The human albumin used had negligible hexosaminidase activity.

Hexosaminidase A and hexosaminidase B were quantified by a method which exploits the different thermal stabilities of each [7]. This method, devised for human serum, was used essentially unchanged for fibroblasts except for the addition of 0.075% serum albumin to each homogenate.

Starch gel electrophoresis studies were carried out on aliquots of skin fibroblast homogenates according to the method of Okada and O'Brien [1]. Hexosaminidase A and hexosaminidase B were detected by the fluorescence produced after incubation of the gels with 4-methylumbelliferyl-β-D-N-acetylglucosamine. The results of the electrophoretic experiments confirmed the quantitative studies (fig. 2). Both hexosaminidase A and B were present in cultured fibroblasts from controls, and approximately half the activity was due to hexosaminidase A. Hexosaminidase A was absent in cultured fibroblasts from patients with Tay-Sachs disease. Both hexosaminidase A and B were undetected in the fibroblasts from the patient with Sandhoff's disease. Fibroblasts from a patient with juvenile GM_2 gangliosidosis had a reduced, but not totally absent, activity of hexosaminidase A.

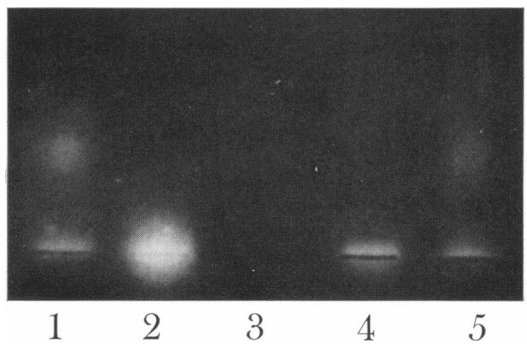

FIG. 2.—Starch gel electrophoresis of hexosaminidases from skin fibroblasts of patients with ganglioside GM_2 storage diseases. Upper spot is hexosaminidase A, lower spot, hexosaminidase B. Twenty μliter of a one-in-10 homogenate was applied to each slot. Control subjects—slots 1 and 5, Tay-Sachs disease patient—slot 2, Sandhoff's disease patient—slot 3, and juvenile GM_2 gangliosidosis patient—slot 4. Hexosaminidase A was clearly visible on the original gel in sample 4, but did not photograph well.

Subjects studied in this report included 20 control subjects, ranging in age from birth to 46 years. Also studied were eight parents of children with Tay-Sachs disease, five children with Tay-Sachs disease, one child with Sandhoff's disease and his mother, and five members of a family (mother, father, patient, brother, and sister) in which the oldest girl had juvenile GM_2 gangliosidosis.

RESULTS

The specific activity of total hexosaminidase (A plus B) varied according to the growth of the culture (fig. 3). Immediately after subculture, a drop in hexosaminidase activity occurred, with the lowest activity four days after subculture. Specific activity increased progressively to 22 days and then leveled off. The cultures were heavily confluent by examination at 10 days after subculture. The total protein content per culture dish (fig. 4) increased linearly over the same time period.

The ratio of hexosaminidase A to hexosaminidase B did not change appreciably during the growth of the culture (fig. 5). The activity of hexosaminidase A in skin fibroblasts from the parent of a child with Tay-Sachs disease averaged approximately 60% of normal at all stages of growth (fig. 5).

To minimize variation of enzyme activity resulting from culture growth, all cultures were harvested for enzyme assay at 21–30 days after subculture. Total hexosaminidase activity in the fibroblasts from patients with Tay-Sachs disease, their parents, a patient with juvenile GM_2 gangliosidosis, and her parents fell within the range of controls (fig. 6). Total hexosaminidase activity in the patient

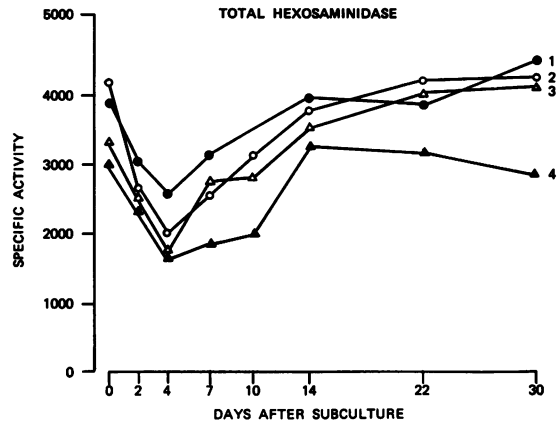

FIG. 3.—Hexosaminidase activity in cultured skin fibroblasts at different days after subculture. Strain 1—normal four-year-old girl, strain 2—parent of child with Tay-Sachs disease, strain 3—40-year-old woman with mental retardation, and strain 4—foreskin from normal newborn boy. Specific activity is expressed as nanomoles cleaved per milligram protein per hour.

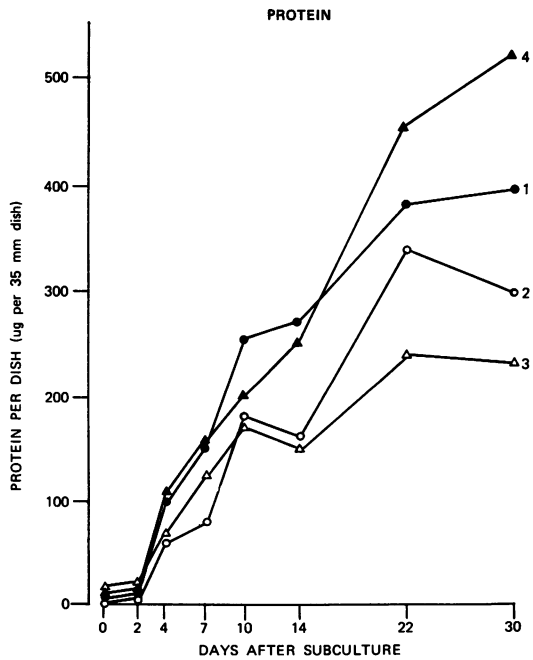

FIG. 4.—Protein content per culture dish at different days after subculture. For identification of strains, see figure 3.

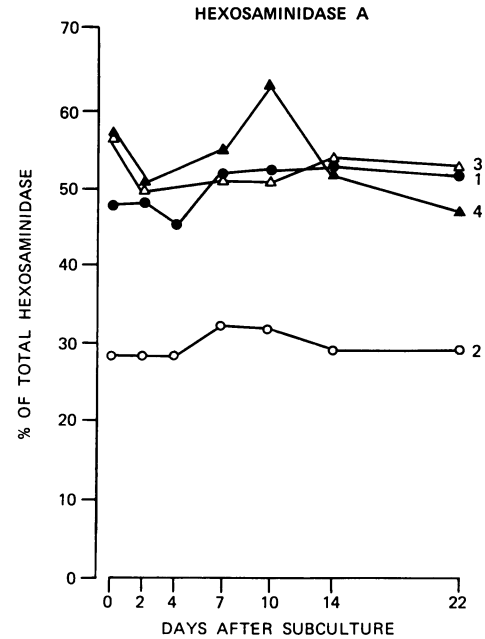

FIG. 5.—Hexosaminidase A activity at different days after subculture expressed as percentage of the total hexosaminidase. For identification of strains, see figure 3.

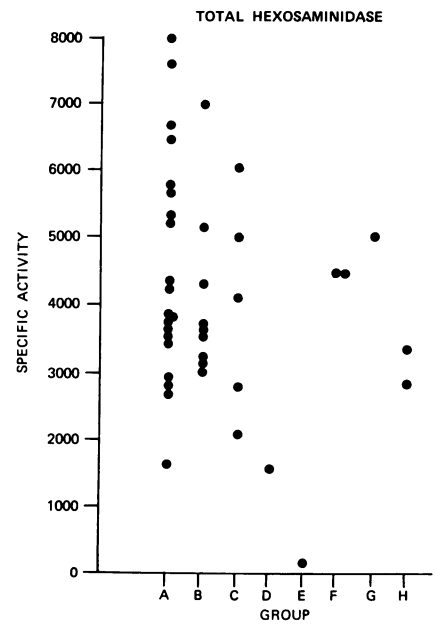

FIG. 6.—Total hexosaminidase activity in skin fibroblasts three weeks or more after subculture. Group A—controls, B—parents of patients with Tay-Sachs disease, C—patients with Tay-Sachs disease, D—mother of patient with Sandhoff's disease, E—patient with Sandhoff's disease, F—parents of patient with juvenile GM_2 gangliosidosis, G—patient with juvenile GM_2 gangliosidosis, and H—siblings of patient with juvenile GM_2 gangliosidosis.

with Sandhoff's disease was less than 5% of the control values. Activities in this patient's mother averaged 35% of the mean of control values.

In control subjects, hexosaminidase A comprised 49% ± 6% of the total hexosaminidase (fig. 7). In eight parents of children with Tay-Sachs disease, hexosaminidase A comprised 23% of the total hexosaminidase. In five children with Tay-Sachs disease, the value was 3.4% of the total hexosaminidase. There was no overlap between the values obtained from control subjects, heterozygotes, and homozygotes. The nearly normal activity of total hexosaminidase in cells from children with Tay-Sachs disease is due to a two-fold elevation of hexosaminidase B. In the cells

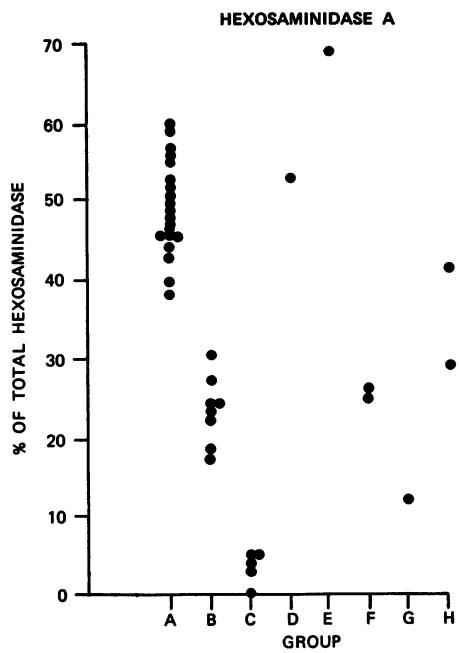

FIG. 7.—Hexosaminidase A activity in skin fibroblasts three weeks or more after subculture. For identification of groups, see figure 6.

from the boy with Sandhoff's disease and his mother, hexosaminidase A was deficient to the same extent as hexosaminidase B, thus the ratio of one to the other was nearly normal.

In the patient with juvenile GM_2 gangliosidosis, hexosaminidase A comprised 12% of the total hexosaminidase, a value 25% of normal. Both parents of the patient with juvenile GM_2 gangliosidosis had deficient levels of hexosaminidase A. Their values fell within the range of heterozygotes for Tay-Sachs disease. Two siblings of the patient were also assayed. One had deficient levels of hexosaminidase, indicating he is heterozygous; the other had normal levels.

DISCUSSION

Stage of culture influences the specific activity of hexosaminidase. A similar effect has been previously published by Leroy and DeMars [8] for another lysosomal

enzyme, β-glucuronidase. The increase in activity as the culture matures may reflect a change in metabolism from one in which cell division predominates to one in which more highly differentiated functions, including lysosomal hydrolase enzyme synthesis, become more important. This effect is important in establishing values for the specific activity of lysosomal enzymes in cell culture. It is especially important where enzyme-specific activity is used in diagnostic studies, such as amniocentesis and prenatal diagnosis. Control values must be established for cells which are at the same stage of culture as the patient's sample. As demonstrated here, the ratio of two closely related enzymes can be used to assess the deficiency of one or the other, as long as it is established that the relationship of the two is constant during different stages of culture growth.

These studies demonstrate the persistence of hexosaminidase deficiencies in cells derived from patients with each of the ganglioside GM_2 storage diseases. The fact that fibroblasts from parents of patients with each disease have intermediate reductions of enzyme activity is a further indication that the enzyme deficiency is a manifestation of the fundamental genetic defect in each. Diagnosis of homozygotes and detection of heterozygotes may be carried out on cultured cells. Fibroblast cultures will be useful in determining the relationship of the genetic defect to the enzymic abnormality in these inborn errors of ganglioside metabolism.

SUMMARY

Hexosaminidase A and hexosaminidase B were assayed in cultured skin fibroblasts derived from patients with three ganglioside GM_2 storage diseases (Tay-Sachs disease, Sandhoff's disease, and juvenile GM_2 gangliosidosis) and compared to controls. Stage of culture and conditions of enzyme assay markedly influenced enzyme activity. Both hexosaminidase A and B were diminished to less than 5% of control values in cells from a patient with Sandhoff's disease; cells from his mother had an intermediate deficiency of these enzymes. Hexosaminidase A was diminished to less than 10% of control values in cells from patients with Tay-Sachs disease; cells from their parents had intermediate deficiencies of this enzyme. Hexosaminidase A was diminished to 25% of control values in cells from a patient with juvenile GM_2 gangliosidosis; cells from her parents had intermediate deficiencies of this enzyme.

REFERENCES

1. Okada S, O'Brien JS: Tay-Sachs disease: generalized absence of a β-D-N-acetylhexosaminidase component. *Science* 165:698–700, 1969
2. Sandhoff K: Variation of β-N-acetylhexosaminidase pattern in Tay-Sachs disease. *Fed Europ Biochem Soc Letters* 4:351–354, 1969
3. O'Brien JS: Five gangliosidoses. *Lancet* 2:805, 1969
4. Okada S, Veath ML, O'Brien JS: Juvenile GM_2 gangliosidosis: partial deficiency of hexosaminidase A. *J Pediat.* In press, 1970
5. O'Brien JS: Ganglioside storage diseases. *Fed Proc.* In press, 1970
6. Lowry O, Rosebrough NJ, Farr AL, et al: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275, 1951
7. O'Brien JS, Okada S, Chen A, et al: Tay-Sachs disease: detection of homozygotes and heterozygotes by serum hexosaminidase assay. *New Eng J Med* 283:15–20, 1970
8. Leroy JG, DeMars RI: Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. *Science* 157:804–806, 1967