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PARTIAL CHARACTERIZATION AND STUDIES OF FIBROBLAST AND LEUCOCYTE NEURAMINIDASE ACTIVITIES TOWARDS SIALYLOLIGOSACCHARIDES IN ADULT SIALIDOSIS AND MUCOLIPIDOSIS II AND III

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We described the partial characterization and some properties of fibroblast and leucocyte neuraminidase towards 2 → 3 and 2 → 6 sialyllactose, and 2 → 3 and 2 → 6 sialylhexasaccharide which were isolated from the urine of a patient with adult sialidosis with partial β-galactosidase deficiency. Neuraminidase activities were assayed using the radioactive-labeled derivatives of these saccharide substrates. These neuraminidases (acylneuraminyl hydrolase, EC 3.2.1.18) were partially inactivated by homogenization, sonication and freeze-thawing treatment. The leucocyte neuraminidase was more labile than that of fibroblasts. Fibroblast neuraminidase had about a 10-fold higher activity than leucocyte neuraminidase towards the respective substrates. The neuraminidase from fibroblasts and leucocytes were each able to hydrolyze 2 → 3 isomers 2–3-times faster than 2 → 6 isomers and the sialyllactoses 1.5–3.0-times faster than sialylhexasaccharides. Neuraminidase activities towards all four substrates were deficient in fibroblasts and leucocytes from the patients with adult sialidosis. Loss of activity was especially prominent in fibroblasts, while considerable residual activities (about 20–30%) remained in leucocytes. In mucolipidosis II and III patients, these neuraminidase activities showed normal levels in leucocytes, although they were decreased in fibroblasts. The discrepancy between neuraminidase activities towards 2 → 3 and 2 → 6 isomers was not found in all the cases.

Neuraminidase deficiency has been found in several clinically distinct disorders [1], but the variability of clinical phenotypes and molecular genetics in these disorders has not been elucidated. To clarify this, the investigation on neuraminidase activities towards the different sialylcompounds is very important and an immediate problem at the present time. We have devised a sensitive method to assay for neuraminidase activity utilizing radioactive-labeled sialyloligosaccharide substrates (unpublished data). In this paper, we describe the partial characterization of fibroblast and leucocyte neuraminidase towards 2 → 3 and 2 → 6 sialyllactose (*N*-acetylneuraminosyl lactose) and 2 → 3 and 2 → 6 sialylhexasaccharide (*N*-acetylneuraminosyl hexasaccharide) by the assay procedure we have devised, and discuss the properties of these neuraminidases towards these substrates.

The deficiency of neuraminidase has been demonstrated in the cultured skin fibroblasts [1]. However, assaying for neuraminidase in leucocytes is clinically

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more preferable, because the cells are easily available. We have developed standard assay conditions for the determination of neuraminidase activities towards four sialyloligosaccharide substrates not only in cultured fibroblasts but also in leucocytes, and present here a precise examination of neuraminidase activities in patients with adult sialidosis with partial deficiency of β-galactosidase, mucolipidosis II and III.

Materials and Methods

Skin fibroblast cultures were maintained with Eagle's minimum essential medium supplemented with 10% fetal calf serum and antibiotics. The cultured fibroblasts were harvested at confluence 7 days after subculture. After treatment with 0.25% trypsin, the cells were washed with cold phosphate-buffered saline (pH 7.2). Leucocytes were separated from the heparinized blood by the method of Tsuboyama et al. [2], and the isolated leucocytes were washed with cold 0.9% saline. These washed cells were suspended in distilled water, and homogenized with a glass homogenizer or sonicated with a Bronson Sonifier Cell Disrupter, Model 185 (Bronson Sonic Power Co., U.S.A.) equipped with a microtip, at 20 KHz. Sonication was carried out for three 5-s periods with the sample immersed in an ice-water bath. The enzyme activities were also measured after rapid freezing of the cells in a solid CO_2/acetone mixture, and then thawing. The diseased samples of cultured fibroblasts and leucocytes were obtained from two patients with adult sialidosis (cases 1 and 2) whose clinical and biochemical details have been already reported [3–5], one with mucolipidosis II and one with mucolipidosis III.

2→3 Sialylhexasaccharide (AcNeu α2→3Gal β1→4GlcNac β1→2Man α1→3Man β1→4GlcNac) and 2→6 sialylhexasaccharide (AcNeu α2→6Gal β1→4GlcNac β1→2Man α1→3Man β1→4GlcNac) were isolated from the urine of case 1 by the method of Strecker et al. [6] with slight modifications [7,8]. These sialylhexasaccharides were reduced with tritiated sodium borohydride ($NaB[^3H]_4$) (New England Nuclear, Boston, U.S.A.) in the presence of 0.01 M NaOH, overnight at room temperature. Subsequently, unlabeled $NaBH_4$ was added and the reduction allowed to proceed for an additional 4 h. Excess $NaBH_4$ was decomposed by adding several drops of glacial acetic acid, and borate was eliminated as volatile methyl borate by adding methanol and evaporating under an N_2 stream. The tritiated reduced sialyloligosaccharides were separated by a Bio-Gel P-2 column (Bio-Rad Laboratories). Further purification was then performed by paper chromatography, DEAE-Sephadex A-25 (acetate form) and Sephadex G-25 column chromatography, *N*-Acetylneuraminosyl lactose (Type II) was purchased from Sigma Chemical Company (St. Louis, U.S.A.) and 2→3 and 2→6 sialyllactoses were isolated by the method of Schneir and Farelson [9]. In addition, 2→6 sialyllactose was also obtained from the human milk by the method of Koseki and Tsurumi [10]. 2→3 and 2→6 sialyllactoses were reduced by the same method described above. Specific radioactivities of reduced 2→3 sialylhexosaccharide (2→3-[^{3}H] sialylhexasaccharidiitol), 2→6 sialylhexasaccharide (2→6[^{3}H] sialylhexasaccharidiitol), 2→3 sialyltose (2→6[^{3}H] sialylactitol) and 2→6 sialyllactose (2→6[^{3}H] sialyllactitol) were 28.8, 43.8, 27.9 and 12.5 Ci/mol, respectively. *N*-Acteyl neuraminic acid was determined by the resorcinol-HCl reagent [12].

Neuraminidase activities were assayed with an incubation system containing 20–35 nmol substrate/50–200 μg enzyme protein/4.5 μmol acetate buffer (pH 4.0 or 4.5) in a total volume of 50 μl. All assays were carried out in duplicate, and the enzyme activity was corrected by subtracting the radioactivity in appropriate blanks containing water in place of enzyme protein. The mixture was incubated at 37°C and the hydrolyzed asialo substrate was determined by the method of Bhavanandan et al. [12] with a slight modification. After incubation, the mixture was diluted with 3 ml distilled water, and applied to a minicolumn of DEAE-Sephadex A-25 (acetate form, 0.5 × 2.0 cm) to separate hydrolyzed substrates from the unhydrolyzed ones. These hydrolysate were eluted from the column with 2 ml distilled water. Radioactivity in 0.5 ml eluate was determined on a scintillation counter. Protein was measured by the method of Lowry et al. [13].

Results

Enzyme preparation. We preliminarily examined the stability of neuraminidase, esepcially the effects of

some techniques of cell rupture on its activity determined by using $2 \rightarrow 3$[^{3}H] sialyllactitol as substrate. Fibroblasts neuraminidase activity was markedly reduced by sonication, i.e., a 25% reduction for 5 s, 40% for 10 s and 59% for 15 s. Homogenization also showed a tendency to reduce the activity, but mild homogenized (10 strokes with a glass homogenizer) fibroblasts had almost same activity as the intact whole cells. Rapid freeze-thawing treatment reduced the enzyme activity by 12% after five times and by

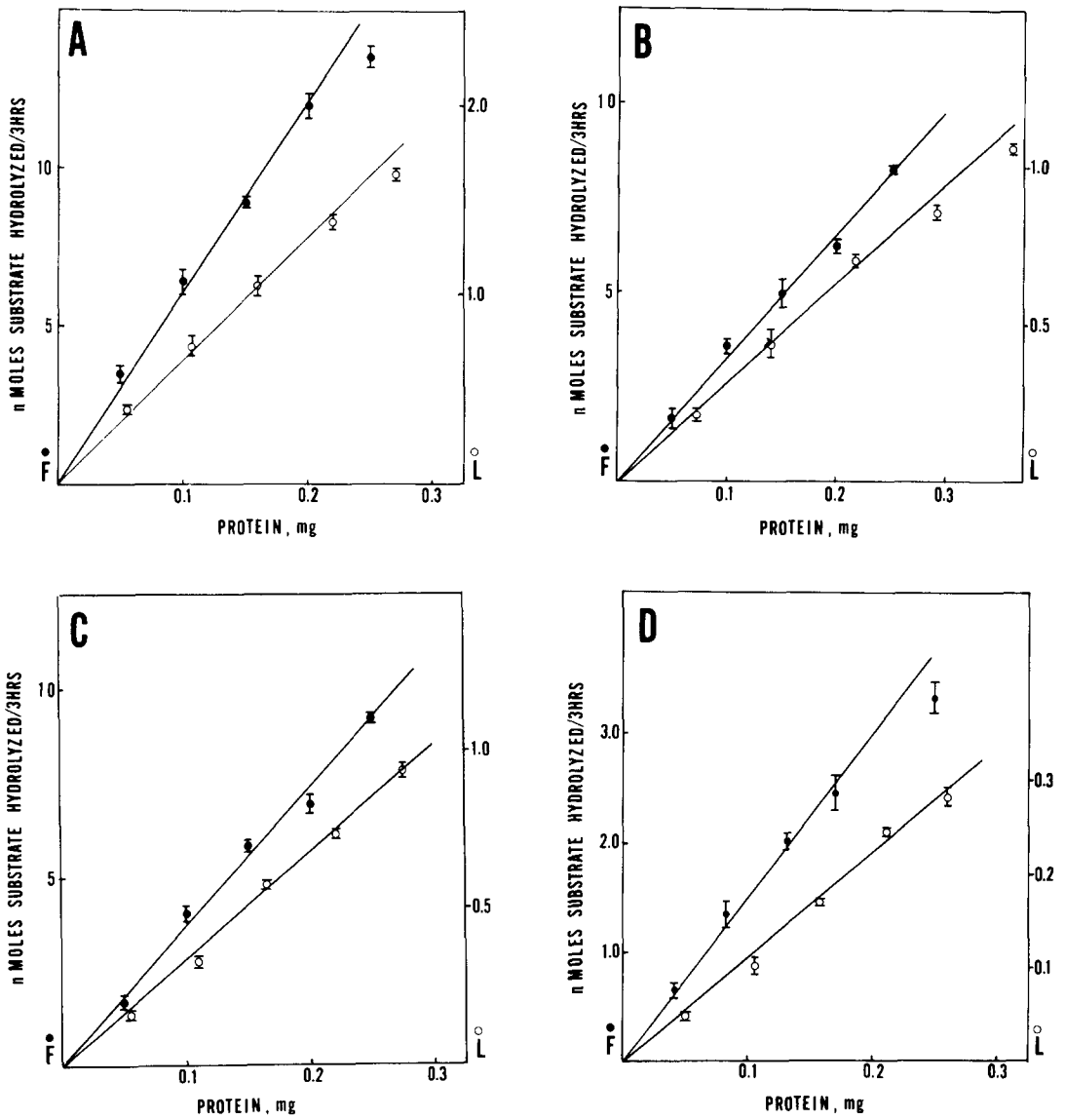

Fig. 1. The effects of enzyme concentration on fibroblast and leucocyte neuraminidase activities. A, $2 \rightarrow 3$[^{3}H] sialyllactitol; B, $2 \rightarrow 6$[^{3}H] sialyllactitol; C, $2 \rightarrow 3$[^{3}H] sialylhexasaccharidítol; D, $2 \rightarrow 6$[^{3}H] sialylhexasaccharidítol. Fibroblast neuraminidase activities (•) were assayed with 33 nmol (A), 33 nmol (B), 28 nmol (C) or 35 nmol (D) substrate and the protein concentrations shown, in the present of pH 4.0 (B, C, D) or 4.2 (A) acetate buffer for 3 h. Leucocyte neuraminidase activities (○) were assayed with 33 nmol (A), 33 nmol (B), 32 nmol (C) or 26 nmol (D) of substrate and the protein concentrations shown, in the presence of pH 4.4 (A, B, D) or 4.4 (C) acetate buffer for 3 h.

45% after ten times. Leucocyte neuraminidase was more labile than that of fibroblasts. Even mild homogenization of leucocytes reduced in a 20–40% decrease in neuraminidase activity as compared to intact leucocytes. Therefore, mildly homogenized fibroblasts and intact leucocytes were used for the enzyme assays. The homogenates were used within 30 min after preparation for the assays without freezing of the homogenates.

pH optimum. The pH optima of fibroblast neuraminidase were 4.2 towards $2 \rightarrow 3[^3H]$ sialyllactitol and 4.0 towards $2 \rightarrow 6[^3H]$ sialyllactitol, $2 \rightarrow 3[^3H]$ sialylhexasaccharidito1, $2 \rightarrow 6[^3H]$ sialylhexasaccharidito1. The effects of pH on fibroblast neuraminidase activities were similar among all cases. On the other hand, the maximum activities of leucocyte neuraminidase were found at pH 3.8–4.0 towards $2 \rightarrow 3[^3H]$ sialyllactitol, $2 \rightarrow 6[^3H]$ sialyllactitol and $2 \rightarrow 6[^3H]$ sialylhexasaccharidito1, and at pH 4.4 towards $2 \rightarrow 3[^3H]$ sialylhexasacchaeidito1. Leucocyte neuraminidase showed considerable activity under acidic conditions, lower pH 3.8, towards all substrates except $2 \rightarrow 3[^3H]$ sialylhexasaccharidito1. In all cases, the activity pH 4.6 was less than 60% of the maximum activity.

Time course. The assays were carried out at the optimal pH condition for each substrate. The enzyme activity increased almost linearly for the first 2 h of incubation in all cases. Increases in substrate hydrolyzed were noticed for 8 h, although the rate of substrate hydrolysis gradually decreased after the first 2 h.

Enzyme concentration. Fibroblast and leucocyte neuraminidase activities towards all four substrates increased almost linearly from 50 to 200 μg protein preparation under optimal pH conditions and during 3 h incubation (Fig. 1).

Substrate concentration. The enzyme activities were assayed under optimal pH conditions using a 3 h incubation period and using 50–200 μg fibroblast homogenate or intact whole leucocyte cells. Table I shows the K_m and V values for fibroblast and leucocyte neuraminidase activity towards the four substrates.

Standard assay condition and neuraminidase activities in normal controls and patients. From the results described above, optimal and standard assay conditions were chosen to be the following: an acetate-buffered solution at pH 4.0–4.4; a 3 h incubation period, and the use of 50–100 μg fibroblast homogenate or 50–200 μg leucocyte intact whole cells per assay. The substrate concentrations used were 20–35 nmol/50 μl ($0.4–0.7 \cdot 10^3$ mol/l), because higher amounts of substrates were not available. Table II shows the fibroblast and leucocyte neuraminidase activities in normal controls and patients. The enzyme activity towards the four substrates was profoundly deficient in both fibroblasts and leucocytes

TABLE I

OPTIMAL pH, K_m AND V OF NEURAMINIDASE ACTIVITIES TOWARDS SIALYLOLIGOSACCHARIDES

	$2 \rightarrow 3[^3H]$-sialyllactitol [a]	$2 \rightarrow 6[^3H]$-sialyllactitol [b]	$2 \rightarrow 3[^3H]$sialyl-hexasaccharidito1 [c]	$2 \rightarrow 6[^3H]$sialyl-hexasaccharidito1 [d]
Fibroblast neuraminidase				
optimal pH	4.2	4.0	4.0	4.0
K_m ($1 \cdot 10^{-3}$ M)	2.17	1.25	2.22	4.17
V [e]	83.3	55.6	76.9	28.6
Leucocyte neuraminidase				
optimal pH	4.0	3.8	4.4	3.8
K_m ($1 \cdot 10^{-3}$ M)	1.36	2.94	1.54	4.55
V [e]	9.1	7.5	7.1	5.0

[a] Tritium-labeled reduced AcNeu α2 → 3Gal β1 → 4Glc.
[b] Tritium-labeled reduced AcNeu α2 → 6Gal β1 → 4Glc.
[c] Tritium-labeled reduced AcNeu α2 → 3Gal β1 → 4GlcNac β1 → 2Man α1 → 3Man β1 → 4GlcNac.
[d] Tritium-labeled reduced AcNeu α2 → 6Gal β1 → 4GlcNac β1 → 2Man α1 → 3Man β1 → 4GlcNac.
[e] nmol substrate hydrolyzed/mg protein per h.

TABLE II

NEURAMINIDASE ACTIVITIES IN CULTURED FIBROBLASTS AND LEUCOCYTE CELLS

	Neuraminidase activities [a]			
	$2 \rightarrow 3[^3H]$-sialyllactitol	$2 \rightarrow 6[^3H]$-sialyllactitol	$2 \rightarrow 3[^3H]$sialyl-hexasacchariditol	$2 \rightarrow 6[^3H]$sialyl-hexasacchariditol
Fibroblasts				
Adult sialidosis				
Case 1	0.42 (3.8%)		0.99 (12.1%)	0.55 (12.5%)
Case 2	0.14 (1.3%)		0.40 (4.9%)	0.03 (0.7%)
M L II	0.76 (6.8%)		1.25 (15.3%)	0.33 (7.5%)
M L III	4.35 (38.9%)		2.14 (26.2%)	1.89 (42.9%)
Normal controls [b]	11.17 ± 0.22 (100%)		8.18 ± 0.45 (100%)	4.41 ± 0.09 (100%)
Leucocytes				
Adult sialidosis				
Case 1	0.63 (28.3%)	0.20 (19.8%)	0.26 (21.8%)	0.08 (21.1%)
Case 2	0.46 (20.7%)	0.21 (20.8%)	0.26 (21.8%)	0.07 (18.4%)
M L II	2.45 (110%)	1.24 (123%)	1.26 (106%)	0.37 (97.4%)
M L III	2.13 (95.9%)	1.07 (106%)	1.28 (108%)	0.30 (78.9%)
Normal controls [c]	2.22 ± 0.24 (100%)	1.01 ± 0.25 (100%)	1.19 ± 0.15 (100%)	0.38 ± 0.06 (100%)

[a] nmol substrate hydrolyzed/mg protein per h.
[b] Mean ± S.D. (n = 3).
[c] Mean ± S.D. (n = 7).

from the two patients with adult sialidosis. In mucolipidosis II and II, fibroblast neuraminidase activities were also found to be decreased, but leucocyte neuraminidase activities were within normal limits.

Discussion

In human fibroblasts and leucocytes, little is known about the specificity of neuraminidase for the different kinds of sialyloligosaccharide substrates, especially for sialyl linkages. The kinetics of neuraminidase activity towards four kinds of sialyloligosaccharides were studied in human cultured fibroblasts and leucocyte cells. We have developed a standard assay using radioactive-labeled substrates established on the basis of these enzyme kinetic data to optimal assay conditions. This method was applied to measure fibroblast and leucocyte neuraminidase activities in patients with adult sialidosis, mucolipidosis II and III.

Fibroblast and leucocyte neuraminidase showed optimal activity towards all four substrates under acidic (pH 3.8–4.4) conditions, which is one of the characteristics of a lysosomal enzyme. However, these neuraminidases were labile enzymes in contrast to other lysosomal enzymes and they were partially inactivated by sonication, homogenization or treatment by rapid freeze-thawing. Thomas et al. [14] have reported similar results for the fibroblast neuraminidase. In addition, our studies indicated that the leucocyte neuraminidase was more labile than the fibroblast neuraminidase. Consequently, mildly homogenized fibroblasts and intact leucocytes were used for the neuraminidase activity assays.

Our comparative studies on enzyme kinetics showed that the fibroblast neuraminidase had about a 10-fold higher activity towards respective sialyloligosaccharide substrates than the leucocyte neuraminidase. The neuraminidase from fibroblasts and leucocytes was able to hydrolyze the $2 \rightarrow 3$ isomers ($2 \rightarrow 3[^3H]$ sialyllactitol and $2 \rightarrow 3[^3H]$ sialylhexasaccharidtol) 2–3-times faster than $2 \rightarrow 6$ isomers ($2 \rightarrow 6$-$[^3H]$ sialyllactitol and $2 \rightarrow 6[^3H]$ sialylhexasaccharidtol) and was able to hydrolyze sialyllactose with three sugars ($2 \rightarrow 3[^3H]$ sialyllactitol and $2 \rightarrow 6[^3H]$ sialyllactitol) 1.5–3.0-times faster than sialylhexasaccharide with six sugars ($2 \rightarrow 3[^3H]$ sialylhexasaccharidtol and $2 \rightarrow 6[^3H]$ sialylhexasaccharidtol). These data suggested that the $2 \rightarrow 3$ isomers seemed

to be degraded faster than the 2→6 isomers, and short chain sialyloligosaccharides faster than long chain ones in fibroblasts and leucocytes.

Strecker and Michalski [15], O'Brien [16] and Cantz and Messer [17] have studied the neuraminidase activities towards sialyloligosaccharides which were accumulated in the urine of the patients, and have reported the deficiencies in the patients with mucolipidosis I, II, III, variant form and cherry red spot-myoclonus syndrome. Strecker and Michalski [15] have reported the deficiencies of neuraminidase activities towards radioactive-labeled 2→3 and 2→6 sialylhexasaccharitol in fibroblasts and leucocytes of the patients with mucolipidosis I, II, III and unclassified mucolipidosis, and proposed that the clinical phenotypes of these disorders might depend on a differential loss of neuraminidase activity towards 2→3 and 2→6 isomers. However, on the basis of our observations in mucolipidosis II and III patients, neuraminidase activities showed normal levels in leucocytes, although they were decreased in fibroblasts. Moreover, the discrepancy between neuraminidase activities towards 2→3 and 2→6 isomers, not only 2→3[^{3}H] sialyllactitol and 2→6[^{3}H] sialyllactitol but also 2→3[^{3}H] sialylhexasaccharitol and 2→6[^{3}H] sialylhexasaccharitol, could not be observed in fibroblasts and leucocytes of adult sialidosis, mucolipidosis II and III. It has been reported that some kinds of virus have a specific neuraminidase for a paticular type of sialyl linkage [18,19]. However, in human fibroblasts and leucocytes, the neuraminidase does not seem to be specialized for 2→3 and 2→6 sialyl linkages in sialyloligosaccharides, though the hydrolysis rate for 2→3 isomers is slightly faster than for 2→6 isomers.

Neuraminidase activities towards four sialyloligosaccharide substrates were deficient in fibroblasts and leucocytes in the two patients with adult sialidosis. Loss of activity was especially prominent in the fibroblasts, while considerable residual activity (about 20–30% of normal activities) remained in the leucocytes. It was suuggested that another neuraminidase (at least one or perhaps more) which was not involved in adult sialidosis might exsist in leucocytes.

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