INCREASED LEVELS OF SIALIC ACID ASSOCIATED WITH A SIALIDASE DEFICIENCY IN I-CELL DISEASE (MUCOLIPIDOSIS II) FIBROBLASTS

G.H. Thomas, G.E. Tiller, Jr., L.W. Reynolds, C.S. Miller and J.W. Bace

The John F. Kennedy Institute, 707 North Broadway, Baltimore, Maryland, 21205, U.S.A.

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Cultured fibroblasts from three unrelated patients with I-cell disease (mucolipidosis II) have a 3 to 4 fold increase in total sialic acid when compared to control fibroblasts. Sialic acid levels in a number of other lysosomal disorders, i.e., mucopolysaccharidosis I, II, III, VI, metachromatic leukodystrophy, G_{M1} gangliosidosis, mannosidosis, Gaucher's and Sandhoff's disease are within the normal range suggesting that this is a finding specific for I-cells. Additionally, sonicates of cultured fibroblasts from controls were shown to have an acid sialidase capable of removing sialic acid from added fetuin at pH 4.2 in 0.05M acetate buffer. In contrast, I-cell fibroblasts, within the limits of the assay, lack this enzyme activity.

I-cell disease (mucolipidosis II) is an autosomal recessive disorder having many of the clinical features of the more severe forms of the mucopolysaccharidoses but lacking the marked mucopolysaccharidiuria. Prominent features include early onset, severe growth failure, psychomotor retardation, hypertrophy of the gingiva, joint stiffness, dysostosis multiplex and early death (1,2,3). Cultured fibroblasts from these patients contain numerous cytoplasmic granules or inclusions, hence the name I-cell disease (4).

While little information is available concerning the nature of the storage material in I-cell disease, there are some data suggesting alterations in sialic acid storage and/or metabolism. These include reports of increased levels of, and a decreased ability to metabolize, the sialic acid containing glycosphingolipids, GD_3 and GM_3, in I-cell fibroblasts (5); detection of sialic acid rich fractions in the urine of an I-cell patient (6) and a report of increased levels of serum sialic acid (2).

We now present direct evidence that there is also an increase in

total sialic acid (3 to 4 times normal) associated with a marked deficiency of an acid sialidase in I-cell fibroblasts.

METHODS AND MATERIALS

I-cell patients #1 and #2 have been followed at The Johns Hopkins Hospital and The John F. Kennedy Institute. Patient #3 is being followed by Dr. T. E. Kelly at the University of Virginia, Charlottesville, Virginia. Details regarding patient #2 have been reported previously (7,8). Patients #1 and #3, not previously described, also have both the clinical and biochemical features of this disorder. Fibroblast cultures were established from skin biopsies and maintained according to the method previously published (8). Cultures were harvested with trypsin and analyzed seven days after subculture and, when possible, matched for total "culture age". Mycoplasma screening was carried out periodically and no evidence of infection was detected.

For sialic acid analyses, cells from two confluent cultures (75 cm^2 Falcon Flask #3024) containing approximately 2 mg of protein were harvested with trypsin, pooled and washed three times with phosphate buffered saline (0.122 grams KH_2PO_4, 0.147 grams Ca $Cl_2 \cdot H_2O$, 0.228 grams $Na_2HPO_4 \cdot 7H_2O$ and 8.5 grams of NaCl in a final volume of 1000 ml of distilled water) with centrifugation following each step. The washed cells were suspended in one ml of 0.1N H_2SO_4 and ruptured by ultrasonication (three 10-second treatments with 30-second intervals in between for cooling in ice water). 0.2 ml of the sonicate was utilized directly for the determination of free sialic acid (9). Total (bound and free) sialic acid was determined by heating a second 0.2 ml aliquot for one hour at 80°C in the 0.1N sulfuric acid in which the cells were ruptured, prior to the determination of the sialic acid (9). For all determinations, correction was made for interfering substances by determining the optical density readings at both 532 and 549 mμ and inserting these values into equation #2 given by Warren (9). This equation was recalculated to reflect the molecular extinction coefficients obtained for N-acetyl-neuraminic acid and the deoxyribose under our conditions. Total hexose content was determined by the macrosulfuric acid - phenol method (10). 0.1 ml of the cell sonicate (approximately 0.2 mg protein) in 0.1N H_2SO_4 was diluted with distilled water to a final volume of 1 ml. To this was added 1 ml of 5% phenol in water and 5 ml of concentrated sulfuric acid, followed by analysis according to the procedure of Dubois et al. (10).

Sialidase activity was determined by measuring the release of sialic acid from fetal calf serum fetuin Type II (Sigma Chemical Company) according to a modification of the method of Aronson and deDuve (11). Fibroblasts from confluent flasks were harvested 7 days after subculture with trypsin, washed three times with phosphate buffered saline and ruptured by sonication in 0.05M acetate buffer, pH 4.2, in the cold. Enzyme activity was determined by adding 0.1 ml of the cell sonicate (approximately 0.4 to 0.5 mg of protein) in the acetate buffer to 0.1 ml of a solution of fetuin, (10 mg fetuin per ml of 0.05M acetate buffer, pH 4.2). No bacteriostatic agents were utilized. The mixture was incubated for the appropriate period of time at 37°C with suitable controls, i.e., cell sonicate lacking fetuin and fetuin lacking cell sonicate. Following the incubation, the samples were analyzed for free sialic acid (9).

RESULTS

Sialic acid quantitation.

Cultured fibroblasts were ruptured by sonication and analyzed for total sialic acid according to the procedure of Warren (9). Spectral analysis of the extracted solution indicated that not only do the I-cell fibroblasts yield more colored product but also that the product has a different absorption curve (Fig. 1). While the control fibroblasts yield a maximum absorption between 530 and 540 mμ, the I-cell sonicates have a maximum at 545-550 mμ. As shown in Fig. 1, this latter value is identical

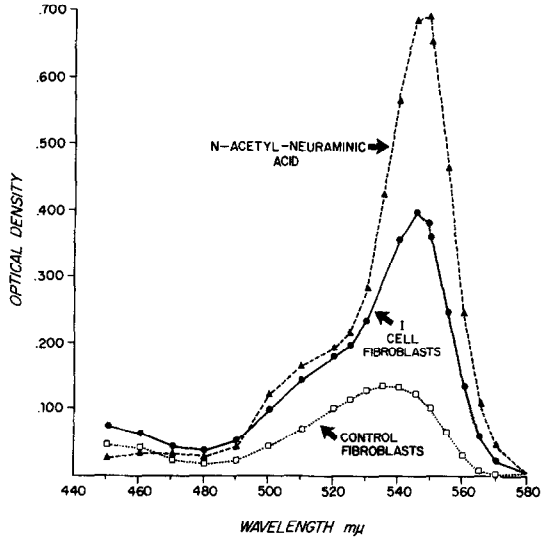

Fig. 1. The absorption spectra of a sonicate of I-cell fibroblasts (0.41 mg protein per assay) and control fibroblasts (0.44 mg protein per assay) treated according to thiobarbituric acid assay after mild acid treatment (80°C for 1 hour in 0.1N H_2SO_4). The concentration of the N-acetyl-neuraminic acid was 0.05 μmoles per assay.

to sialic acid(s), as for example, N-acetyl-neuraminic acid. I-cell fibroblasts treated with neuraminidase in place of acid yield similar results (Table I). Based on these findings, the "thiobarbituric reactive material" is believed to be sialic acid. Correction for interfering substances was made by utilization of equation #2 given by Warren (9).

Table I. Release of "thiobarbituric acid positive" material by either neuraminidase or mild acid treatment.

Cells	Treatment	Sialic acid released nmol/mg protein
I-cell	0.1N H_2SO_4	46
I-cell	Neuraminidase *	46
I-cell	None	4
Control	0.1N H_2SO_4	14
Control	Neuraminidase *	18
Control	None	1
Neuraminidase	0.1N H_2SO_4	0.3

* Clostridium Perfringens - Worthington Biochemical Corporation

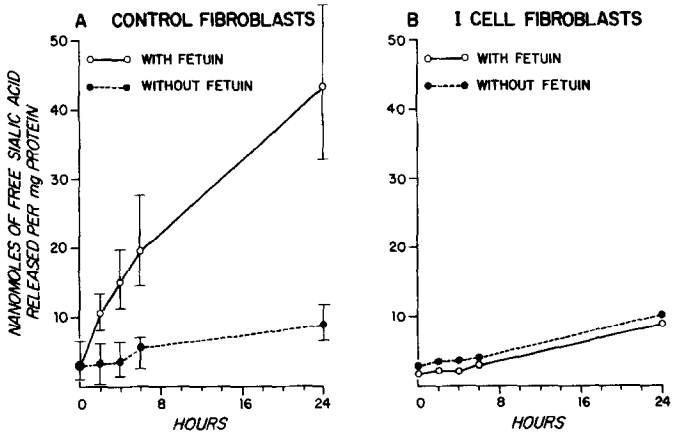

Fig. 2. Release of sialic acid by fibroblast sonicates with and without added fetuin in 0.05M acetate buffer at pH 4.2 at 37°C. Section A: mean and range of the values obtained from 8 determinations carried out on 6 different cell lines from normal individuals. Section B: mean of the values obtained from 5 determinations on fibroblasts obtained from 3 unrelated patients with I-cell disease. The values are corrected by subtraction of fetuin blank values where appropriate.

As shown in Table II, the I-cell fibroblasts have a marked increase in the total sialic acid. Measurement of the free sialic acid present prior to mild acid treatment indicated that the increase is due almost entirely to bound sialic acid. Studies on fibroblasts from patients with a variety of other lysosomal storage disorders yielded normal sialic acid concentrations (Table II).

Table II. Sialic acid and hexose content of cultured fibroblasts.

	Sialic acid[1]		Hexose[2]
	Total	Free	Total
Patients[3]			
I-cell patient #1	49	2	210
I-cell patient #2	69	4	340
I-cell patient #3	63	4	310
Parents			
Mother of patient #1	19	0	435
Mother of patient #2	19	3	240
Father of patient #2	12	3	-
Patients with other lysosomal disorders			
MPS I patient (N=1)	18	0	236
MPS II patients (N=2)	15	0	231
MPS III patient (N=1)	12	2	207
MPS VI patients (N=3)	15	1	205
Metachromatic leukodystrophy (N=2)	19	7	139
Sandhoff's (N=1)	17	1	139
G_{M1} gangliosidosis (N=1)	13	4	163
Mannosidosis (N=3)	18	2	223
Gaucher's (N=1)	10	1	242
Control (N=11)			
mean	16	2	257
range	11 - 23	0 - 6	158 - 354

[1] nanomoles of sialic acid per mg of protein using N-acetyl-neuraminic acid as standard

[2] µg of hexose per mg protein using glucose as standard

[3] Note: The values given for the I-cell patients represent the average of 5 or more determinations carried out on different days on separately grown flasks

Sialidase activity.

Associated with the increased sialic acid levels in the I-cell fibroblasts is an absence of an acid sialidase. As shown in Fig. 2, normal fibroblasts release free sialic acid from fetuin at pH 4.2. When fetuin is added to the I-cell fibroblasts prepared in an identical fashion, no release of sialic acid above the non-fetuin control blank was detectable. In fact,

Table III. Sialidase activity in sonicates of cultured fibroblasts with and without added fetuin.[1]

Patients	a with fetuin[2]	b without fetuin	c a-b
I-cell patient #1	10	12	negative
I-cell patient #2	9	10	negative
I-cell patient #3	8	9	negative
Parents			
Mother of patient #1	63	9	54
Mother of patient #2	42	11	31
Father of patient #2	33	6	27
Normal Controls			
Control #1	36	8	29
Control #2	56	6	49
Control #3	73	17	56
Control #4	25	9	16
Control #5	17	4	13
Control #6	43	8	34
Control #7	44	11	33
Control #8	33	10	23
Control #9	55	7	48
Normal Control Mean	42	9	34
I-Cell + Control	36	12	25

[1] Enzyme activity is expressed as nanomoles of sialic acid released per mg protein per 24 hours at 37°C in 0.05M acetate buffer, pH 4.2, using N-acetyl-neuraminic acid as standard

[2] Corrected for fetuin blank values

the final, calculated results indicated an actual reduction in sialidase activity in the presence of fetuin as compared to the non-fetuin control mixture. (Table III). A mixture of equal amounts of cell sonicates of control and I-cell fibroblasts yielded 55% of control sialidase activity thus indicating that the deficiency was not due to the presence of inhibitors. (Table III).

Attempts to detect sialidase activity in conditioned media from both I-cell and normal fibroblast cultures yielded negative results. Additionally no sialidase activity could be detected in serum from either I-cell patients or normal individuals.

DISCUSSION

The data presented here indicate that sonicates of normal fibroblasts have an acidic sialidase capable of removing sialic acid from exogenous fetuin. In contrast, I-cell fibroblasts from three unrelated patients lack this activity. At the present time it is unclear if the release of "thiobarbituric acid reactive" material from endogenous sources in the absence of added fetuin by both normal and I-cell fibroblasts is due to the same sialidase or another enzyme.

The etiology of the sialidase deficiency in the I-cell fibroblasts is unknown. The defect could represent the primary lesion or alternatively could be part of the non-specific, generalized reduction in lysosomal enzymes previously reported (12).

Regardless of the basic mechanism the deficiency of intracellular sialidase in I-cell disease may account for the 3 to 4 fold increase in total sialic acid rich material shown in Table II.

Furthermore, we speculate that this deficiency could result in the presence of glycoproteins rich in sialic acid including the lysosomal enzymes. This would be in agreement with the suggestion presented by

Vladutiu and Rattazzi (13) that the electrophoretic changes in hexosaminidase in I-cell disease (8), as well as the impaired uptake of lysosomal enzymes excreted from I-cell fibroblasts (14,15), may be due to the presence of additional sialic acid residues on these enzymes. This, in turn, could explain the observations which led to the Hickman and Neufeld hypothesis of a defective enzymatic recognition site in I-cell disease (14). Thus, the sialidase deficiency reported here might be of central importance in the final understanding of this complex disorder.

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REFERENCES

1. Wiesman, U.N., Vassella, F., and Herschkowitz, N.N. (1974) Acta Paediat. Scand., 63, 9-16.
2. Terashima, Y., Tsuda, K., Isomura, S., Sugiura, Y., and Nogami, H. (1975) Am. J. Dis. Child., 129, 1083-1090.
3. Blank, E., and Linder, D. (1974) Pediatrics, 54, 797-805.
4. Leroy, J.G., and DeMars, R.I. (1967) Science, 157, 804-805.
5. Dawson, G., Matalon, R., and Dorfman, A. (1972) J. Biol. Chem., 247, 5951-5958.
6. Orii, T., Chiba, T., Minami, R., Sukegawa, K., and Nakao, T. (1974) Tohoku J. Exp. Med., 112, 373-380.
7. Sensenbrenner, J.A. (1974) Birth Defects: Original Article Series, X, No. 12, 470-477.
8. Lie, K.K., Thomas, G.H., Taylor, H.A., and Sensenbrenner, J.A. (1973) Clin. Chim. Acta, 45, 243-248.
9. Warren, L. (1959) J. Biol. Chem., 234, 1971-1975.
10. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. (1956) Anal. Chem., 28, 350-356.
11. Aronson, N.N., Jr., and deDuve, C. (1968) J. Biol. Chem., 243, 4564-4573.
12. Leroy, J.G., Ho, M.W., MacBrinn, M.C., Zielke, K., Jacob, J., and O'Brien, J.S. (1972) Pediat. Res., 6, 752-757.
13. Vladutiu, G.D., and Rattazzi, M.C., (1975) Biochem. and Biophys. Res. Commun., 67, 956-964.
14. Hickman, S., and Neufeld, E.F. (1972) Biochem. and Biophys. Res. Commun., 49, 992-999.
15. Hickman, S., Shapiro, L.J., and Neufeld, E.F. (1974) Biochem. and Biophys. Res. Commun., 57, 55-61.