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Clinical and Biochemical Studies in an American Child with Sialuria

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Sialuria is a rare inborn error of sialic acid (NeuAc) metabolism resulting from failure of CMP-NeuAc to adequately feedback inhibit the rate-limiting enzyme in sialic acid synthesis, UDP *N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase. We describe the fourth reported sialuria patient, T.W., whose clinical features include developmental delay, coarse facies, and massive urinary excretion of sialic acid. Biochemical studies of T.W. fibroblasts revealed a 200-fold increase in free NeuAc content compared with normal. Bound NeuAc was only slightly elevated. The free NeuAc was predominantly in the cytosol fraction of fibroblasts after differential centrifugation, with only 4% of the free NeuAc content in other (nuclear, granular, and microsomal) cellular compartments. CMP-NeuAc inhibited UDP-GlcNAc 2-epimerase by 80% in normal fibroblasts but inhibited the epimerase of T.W. (sialuria) cells by only 13%. Cytidine feeding of sialuria fibroblasts decreased the intracellular free NeuAc content by 47%; this was accompanied by a fourfold increase in CMP-NeuAc, which may be sufficient to feedback inhibit the mutant epimerase and reduce free NeuAc production. Cytoplasmic pH was determined by the pH sensitive fluorescent indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein, pentaacetoxymethylester (BCECF/AM) using the H+ equilibration method. The intracellular pH of sialuria fibroblasts, 7.18 ± 0.04, was not found to be significantly different from that of normal cells (7.19 ± 0.08).

N-Acetylneuraminic acid (NeuAc),[1] or sialic acid, is a negatively charged sugar present within cells in both bound and free forms (1). Bound NeuAc refers largely to NeuAc covalently attached to glycoconjugates and is considered to play a

[1] Abbreviations used: NeuAc, *N*-acetylneuraminic acid; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; CT, computed tomographic scan; DMEM, Dulbecco's modified Eagle's medium; PAD, basic pulse amperometric detector; NaOAc, sodium acetate; BCECF/AM,2′7′-bis(carboxyethyl)-5(6)-carboxyfluorescein, pentaacetoxymethylester

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regulatory role in many cellular functions. The function of free NeuAc remains unknown.

Several different human diseases can result from perturbations in NeuAc metabolism. Sialidosis is a lysosomal storage disorder of bound sialic acid caused by a deficiency in lysosomal neuraminidase activity (2). Salla disease (3,4) and infantile free sialic acid storage disease (5,6) are lysosomal storage disorders of free sialic acid resulting from impaired NeuAc egress from lysosomes (7). Finally, sialuria is a metabolic disorder resulting from relatively unregulated synthesis of sialic acid (8). Specifically, CMP-NeuAc fails to adequately feedback inhibit the rate-limiting enzyme in NeuAc synthesis, UDP *N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase (9,10). This results in excess free NeuAc concentrations in the cytoplasm of affected cells.

Three patients with sialuria have been reported to date (10–13). Their clinical courses have been characterized by variable degrees of developmental delay, the presence of coarse facial features, and massive urinary excretion of unconjugated sialic acid. We now describe the clinical and biochemical characteristics of a fourth patient with sialuria, with special reference to studies performed in cultured fibroblasts. In particular, we investigated the effect on cytoplasmic pH of the exceptionally high intracellular NeuAc content of sialuria cells.

Case Report

T.W. was the 1700-g Caucasian male product of a 30-week gestation born to a 15-year-old woman. His neonatal course was complicated by hospitalization for 2 weeks for suspected hydrocephaly as well as low birth weight associated with his prematurity. Initially, he required nasogastric feeding, but quickly gained weight and became an active feeder. The brain was evaluated by cranial ultrasound, which showed minimal ventricular enlargement verified on two subsequent CT scans over the next year. There was mild hepatomegaly and rib flaring apparent on chest radiograph at 6 months of age. During infancy and early childhood, the patient experienced obstructive sleep apnea confirmed by oxygen desaturation measurements and requiring bilateral tonsillectomy and adenoidectomy at 4 years of age. These procedures improved the apnea-induced hypoxia. The patient also experienced seizures beginning at 1 month of age and currently controlled with phenobarbital. Family history was unremarkable with a normal younger sibling.

On physical examination at 4 ½ years of age, the patient's height (106 cm), weight (17.7 kg), and head circumference (50.5 cm) were at approximately the 50th percentile for age. His facies (Fig. 1) was coarse with an apparent fullness, a widened nasal bridge, epicanthal folds, synophrys, low set ears, low posterior hairline, and generalized hirsutism. He had a high ridged palate and normal tongue. His chest was small with hypoplastic nipples. His abdomen protruded with moderate hepatomegaly. Abnormal skeletal features included pes planus and mild thoracic scoliosis. Hearing was normal. Developmental assessment at 6 years of age revealed a full scale IQ of 68 with difficulties in short-term memory and quantitative reasoning. In contrast, he demonstrated average to low average abilities in solving visual/spatial tasks, expressing verbal concepts and using long-term memory.

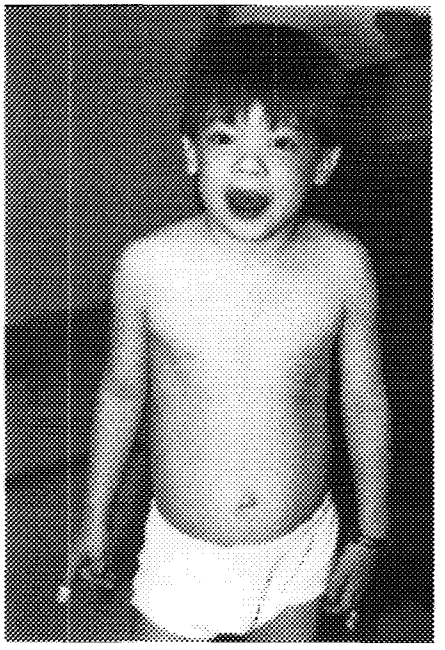

FIG. 1. T.W. at 4 years of age showing coarse features, widened nasal bridge, and epicanthal folds.

Laboratory studies performed by one of us (D.W.), revealed a urine sialic acid content of 117,288 nmol/mg creatinine which is 500- to 1000-fold elevated above normal.

MATERIALS AND METHODS

Cell culture and fractionation. Control fibroblasts (GM 5757) were obtained from the Human Mutant Cell Repository (Camden, NJ). T.W. and normal fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (GIBCO), 2 m*M* glutamine, and supplemented with antibiotics. Cells were washed three times with phosphate-buffered saline (PBS) and trypsinized for harvesting as previously described (10). For fractionation studies, the cells were washed once with cold PBS and once with cold 0.25 *M* sucrose. Fibroblast pellets were resuspended in 2–3 ml ice-cold 0.25 M sucrose, ruptured in a nitrogen cavitation bomb (Kontes, Vineland, NJ) at 30 psi for 10 min, and gently homogenized. The homogenate was further fractionated by centrifugation at 1000*g* for 7 min to prepare a nuclear pellet. The supernatant was then centrifuged at 17,000*g* for 10 min to obtain a granular fraction pellet. The post 17,000*g* supernatant was centrifuged for 1 h at 100,000*g*, yielding the microsomal and cytosol fractions.

Sialic acid measurement. This determination was performed under alkaline conditions by anion exchange chromatography. Monosaccharides were separated using

a CarboPac TM PA Guard column and Dionex PA1 Ion Pack column with pulse amperometric detection using a basic pulse amperometric detector (PAD) with gold electrode (Dionex Bio LC, Sunnyvale, CA). The following settings were used: E1, 0.05; E2, 0.06; E3, 0.06; T1, 5 s; T2, 3 s; T3, 1 s; range, 2. The flow rate was 1 ml/min. Twenty microliter of cell homogenate, subfraction or NeuAc standard (50–1000 pmol) in H_2O, was run isocratically using 0.06 N sodium acetate (NaOAc) + 0.01 N NaOH for NeuAc and 0.22 N NaOAc + 0.1 N NaOH for CMP-NeuAc determinations.

Total sialic acid was determined as above, except that the samples were hydrolyzed in 0.1 M sulfuric acid by incubation for 1 h at 80°C prior to analysis. Bound sialic acid was calculated as the difference between total and free sialic acid. Protein was measured using a bicinchoninic acid kit from Pierce Chemical Co.

UDP-GlcNAc 2-epimerase. UDP-GlcNAc 2-epimerase was assayed in fibroblasts from patients and controls as previously described (9,10). The assay measures the conversion of ^{3}H-UDP-glucosamine to *N*-^{3}H-acetylmannosamine.

Intracellular pH determinations. Intracellular pH was determined by an adaptation of the method of Moolenaar *et al.* (14). Briefly, human fibroblasts were trypsinized after washing three times with PBS. Cell pellets were then washed in ice-cold Buffer A (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, pH 7.4) and were resuspended in ice-cold Buffer A without calcium; 5 μM 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein, pentaacetoxymethylester (BCECF/AM) (Calbiochem Corp.) was then added. After 15 min of incubation at 37°C, 1 mM $CaCl_2$ was added and incubation continued for 30 min. Cells were then washed twice in ice-cold Buffer A and resuspended in either Buffer A (control) or in K-Buffer (150 mM KCl, 10 mM Hepes, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) containing 14 μM nigericin (Sigma Chemical Co., St. Louis, MO). Fluorescence intensity in the control tube was recorded using a Perkin-Elmer 650 10S fluorescence spectrophotometer (excitation wavelength 500 nm, emission wavelength 530 nm). Calibration of the linear relationship between pH and fluorescence intensity was determined on cells exposed to nigericin, which equilibrates the intracellular and extracellular hydrogen ion concentration. After exposure to nigericin, the pH of the cell suspension was varied by addition of HCl or NaOH and the fluorescence change was recorded. The fluorescence intensity varied in a linear fashion over a pH range of 6.8–7.8.

RESULTS

The cultured fibroblasts of T.W. displayed a free NeuAc content of 95.9 ± 54.3 nmol/mg cell protein (mean ± SD; n = 6; range, 54.3 to 203). This represented a 120- to 450-fold increase over normal values concurrently determined (n = 7, 0.44 ± 0.47 nmol/mg protein) or previously reported (n = 11, 1.0 ± 0.6 nmol/mg protein) (10). The free NeuAc concentration of T.W.'s fibroblasts was consistent with the 70- to 200-fold increased levels previously reported for other sialuria patients (10). Bound NeuAc measured in T.W.'s fibroblasts was 41.1 ± 11.2 nmol/mg cell protein (n = 6); this was slightly elevated compared with normal values of 24.4 ± 5.1 nmol/mg cell protein (n = 7). These bound

TABLE 1
Free Sialic Acid in Subcellular Fractions of Normal and T.W. Fibroblasts

	Percentage of Recovery	
	Normal[a]	T.W.[b]
Nuclear	18.3	3.8 ± 2.4
Microsomal	6.4	1.3 ± 1.7
Granular	21.3	3.9 ± 0.9
Soluble	53.9	91.0 ± 4.2

Note. Fibroblasts were harvested and fractionated to yield subcellular compartments, and their sialic acid content was determined as described under Materials and Methods.

[a] Mean free NeuAc content 0.3 nmol/mg protein; $n = 2$.

[b] Mean free NeuAc content 116.7 nmol/mg protein; $n = 3$.

NeuAc values for both T.W. and normal cells are slightly higher than previously published values (10).

Subcellular fractionation of T.W. fibroblasts revealed that 91% of the free NeuAc was located in the soluble subfraction, representing the cytoplasmic compartment (Table 1). The granular subfraction, containing lysosomes, mitochondria, and other membrane vesicles, contained less than 4% of the free NeuAc content. This subcellular distribution closely resembles that for each of the other three known sialuria cases (10,15).

The definitive diagnosis of sialuria rests on demonstrating the known metabolic defect, i.e., failure of CMP-NeuAc to inhibit UDP-GlcNAc 2-epimerase in a normal fashion (9,10). In a lysate of T.W.'s fibroblasts, 50 μM CMP-NeuAc inhibited the epimerase 13%, compared to an 80% inhibition of the epimerase by the same concentration of CMP-NeuAc in a normal fibroblast lysate. These data confirm the diagnosis of sialuria (9,10) in T.W.

An additional finding in sialuria fibroblasts has been the ability of exogenous cytidine to decrease the intracellular free NeuAc content (10), presumably by increasing intracellular CTP concentrations. This could increase CMP-NeuAc to high enough levels to feedback inhibit even the mutant epimerase, thus reducing free NeuAc production (10). The results of cytidine feeding studies in T.W. fibroblasts support this hypothesis (Table 2). After inclusion of 5 mM cytidine in typical tissue culture media for 68 h, the free NeuAc content of the cells decreased 47% while the cellular content of bound NeuAc remained the same. The CMP-NeuAc content increased fourfold with cytidine feeding.

Cytoplasmic pH was determined in both normal (GM 5757) and T.W. cells using the pH sensitive fluorescent indicator BCECF/AM, a membrane permeable ester (16). The cytoplasmic dye fluorescence was calibrated by the H^+ equilibration

TABLE 2
Effect of Cytidine Feeding on NeuAc and CMP NeuAc Content of T.W. Fibroblasts

	nmol/mg protein		
	Free NeuAc	Bound NeuAc	CMP-NeuAc
Control	67.3	32.3	3.0
Cytidine	35.9	39.9	12.0

Note. Cells were incubated in medium without (control) or with 5 mM cytidine for 68 h and harvested, and their sialic acid content was determined.

method, using nigericin (16–18). After recording the spontaneous intracellular fluorescence of BCECF, fluorescence was calibrated under conditions of varying pH following the equilibration of the extracellular and intracellular hydrogen ion concentrations using nigericin, a K^+/H^+ ionophor. The intracellular pH for T.W. cells, 7.18 ± 0.04 (n = 4), did not differ from that of normal cells, 7.19 ± 0.08 (n = 4), and was the same as previously reported for human fibroblasts in culture, i.e., 7.18 ± 0.02 (17).

DISCUSSION

Sialuria, a disease caused by an inborn error of metabolism involving impaired feedback inhibition of NeuAc synthesis, has been reported previously in three patients (9,10). T.W., the fourth reported case, has a clinical history remarkable for prematurity, coarse facies, dysmorphic features, hepatomegaly, and developmental delay similar to the other sialuria patients. The biochemical features, including increased urinary excretion of sialic acid, approximately 200-fold excess free sialic acid localized to the cytosol fraction of cultured fibroblasts (Table 1), and impaired feedback inhibition of UDP-GlcNAc 2-epimerase by CMP-NeuAc, confirmed the diagnosis. Cytidine feeding of the mutant fibroblasts led to increased CMP-NeuAc concentrations capable of feedback inhibiting the mutant epimerase and reducing NeuAc production (Table 2). This supports the previous suggestion that cytidine may be a useful therapeutic intervention for this disease (10).

Studies of the intracellular pH of T.W. and normal cells showed that the excess free sialic acid present did not affect the intracellular pH. Intracellular pH, which influences several cellular functions, appears to be closely regulated by a Na^+/H^+ exchange mechanism in the plasma membrane (14). Moolenaar *et al.* (17) have postulated that under normal conditions the Na^+/H^+ exchanger is relatively inactive, but its activity may be altered if the intracellular pH changes. Sialuria cells may employ this mechanism to compensate for the persistently increased metabolic acid present in the form of NeuAc. In any event, the documentation of additional cases of sialuria will help to define the limits of this disorder's clinical heterogeneity. Longitudinal follow-up can give an indication of prognosis, and further biochemical investigations may assist in correlating metabolic aberrations with clinical manifestations.

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