ABNORMAL LYSOSOMAL HYDROLASES EXCRETED BY CULTURED FIBROBLASTS IN I-CELL DISEASE (MUCOLIPIDOSIS II)

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The isozymes of β-D-N-acetylhexosaminidase excreted by cultured skin fibroblasts derived from patients with I-cell disease (ICD) differ electrophoretically from the isozymes excreted by fibroblasts from normal individuals and from the intracellular isozymes of both cell types. The higher electronegative charge at pH 6 presumably results from sialic acid residues not present on normal and ICD intracellular enzyme or on normal fibroblast-excreted enzyme, since neuraminidase treatment only affects the mobility of the ICD fibroblast-excreted enzyme. A higher electronegative charge than that of normal fibroblast-excreted or intracellular enzymes has also been detected in ICD fibroblast-excreted arylsulfatase, β-glucuronidase and α-L-fucosidase. Additional sialic acid residues on the ICD-excreted hydrolases may result from abnormal exocytosis, and may be responsible for the inability of these enzymes to enter normal or ICD fibroblasts by adsorptive pinocytosis.

I-cell disease (ICD; Mucolipidosis II) is a fatal neurodegenerative disease of childhood, transmitted with an autosomal recessive mode of inheritance, clinically similar to the mucopolysaccharidoses, but usually with normal urinary excretion of mucopolysaccharides (1,2). ICD is characterized by numerous cytoplasmic inclusions visible by phase-contrast microscopy in cultured skin fibroblasts (2,3), and by increased activity of several lysosomal hydrolases in the plasma of these patients (4,5). The activities of at least five lysosomal enzymes are greatly reduced in extracts of cultured ICD fibroblasts, and are correspondingly elevated in the culture fluid (6). The ICD fibroblast-excreted lysosomal enzymes are taken up by non-ICD fibroblasts much less readily than normal fibroblast-excreted enzymes, while non-ICD fibroblast-excreted enzymes are taken up at a normal rate by ICD fibroblasts (7,8). It has thus been proposed that a "recognition marker" on lysosomal enzymes, necessary for normal reentry of the excreted enzymes into cells, is altered in ICD fibroblast-excreted enzymes (7). We present data suggesting that the impaired uptake of ICD fibroblast-excreted lysosomal enzymes may be due to the presence of

additional sialic acid residues on these enzymes, not present on enzymes excreted by normal fibroblasts.

MATERIALS AND METHODS

Skin fibroblasts from one ICD patient were a gift from Dr. George Thomas (Johns Hopkins University). Monolayers of both ICD and normal fibroblasts were maintained in 75 cm^2 Falcon flasks with Ham's F-10 medium containing 10% fetal calf serum (Grand Island Biologicals Inc.). Two cell lines from obligate heterozygotes for the ICD gene, not related to the ICD patient, were obtained from the Mammalian Genetic Mutant Cell Repository (Camden, N. J.) and were maintained in either modified McCoy's 5a medium with 20% fetal calf serum or the Ham's F-10 medium as above. Preparations of excreted enzymes were obtained by incubation of approx. 70%-confluent monolayers in half the normal volume (5 ml) of serum-free medium (SFM) after extensive washing of the monolayers in approx. 50 ml Hanks' balanced salt solution. After 72 hr incubation, the SFM from 6-8 Falcon flasks was pooled and concentrated 20-30 fold by ultrafiltration at 4°C (XM 100 membrane; Amicon Corp.). Cells were harvested using Viokase (0.25%; Grand Island Biol.) washed in Hanks' solution, resuspended in saline, extracted by sonication, and centrifuged at 48,000 x g for 20 min at 4°C. β-hexosaminidase activity in the extracts and the SFM was assayed fluorometrically using a 4-methyl-umbelliferyl (4-MU) derivative of β-D-N-acetylglucosaminide (Pierce Chemical Co.) as a substrate (9). Electrophoretic and immunodiffusion procedures were carried out on cellulose acetate gel (Cellogel; Kalex, Manhasset, N. Y.) in either 25 mM sodium citrate-citric acid buffer, pH 5.5, or 36 mM sodium barbital-citric acid buffer, pH 6.0, or 36 mM sodium barbital acetic acid-acetate buffer, pH 7.0, as described (10). Fluorogenic substrates used included 4-MU-N-acetylglucosaminide, 4-MU sulfate (11), 4-MU glucuronide (Pierce Chemical Co.), and 4-MU α-L-fucopyranoside (Research Product International Corp.). An antiserum against human β-hexosaminidase B (Hex B) was prepared in the rabbit (12), and a specific antiserum against the immunologically distinct α-site of β-hexosaminidase A (Hex A) was prepared by absorption (12,13) of an anti-human β-hexosaminidase bovine serum (14). Susceptibility to neuraminidase was assessed by incubation (3 hr at 37°C) of 100 µl aliquots of cell extracts, normal human serum, and SFM (adjusted to pH 5 with 0.1 volumes of 1.0 M sodium citrate-sodium phosphate buffer) with 5 µl of a 12.6 u/ml solution of Cl. perfringens neuraminidase (15) (Type VI, Sigma Chemical Co.), followed by electrophoresis.

RESULTS AND DISCUSSION

Electrophoresis at pH 5.5 and pH 6.0 of extracts and culture medium of fibroblasts derived from normal individuals, showed that the excreted β-hexosaminidase isozymes were identical to Hex A and Hex B from cell extracts (Fig. 1A). Examination of culture medium of fibroblasts derived from the patient with ICD showed a pattern consisting of two enzyme bands, with higher mobilities than Hex A and Hex B of normal fibroblasts (Fig. 1B). The intracellular β-hexosaminidase from ICD fibroblasts consisted of two bands with lower electrophoretic mobilities than the corresponding bands of intracellular β-hexosaminidase from normal fibroblasts (Fig. 1B), as previously described by

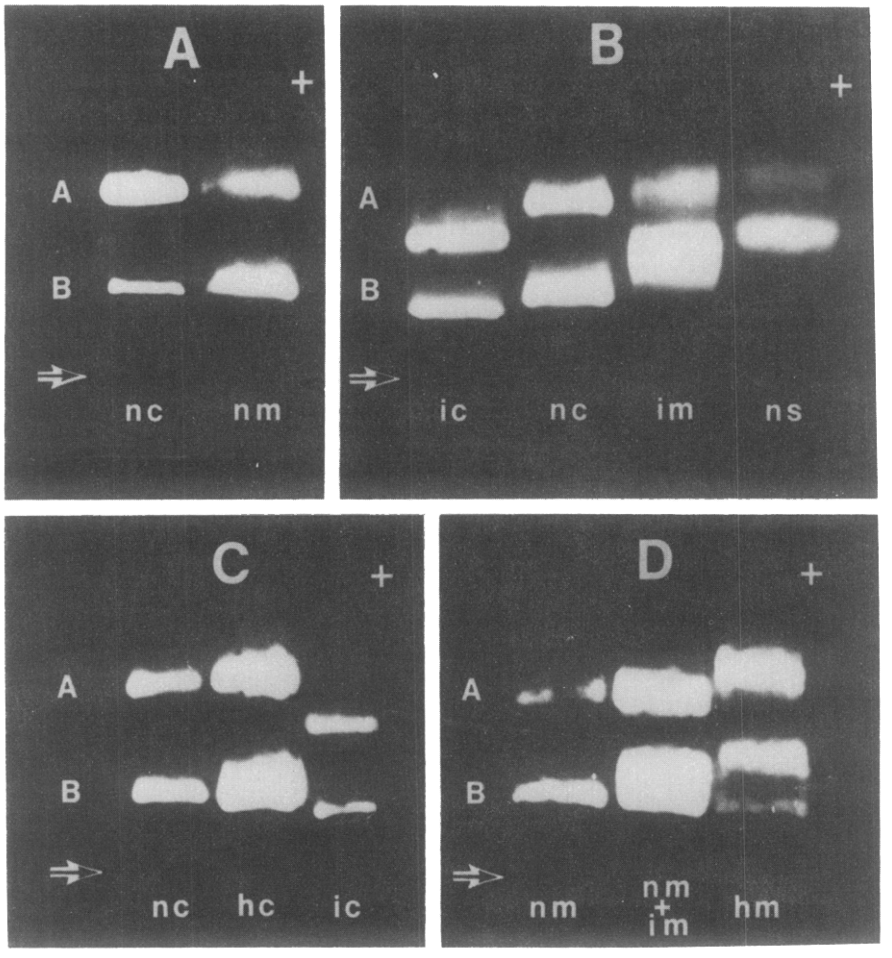

Fig. 1. Fluorescent bands of β-hexosaminidase activity after electrophoresis at pH 6.0 on cellulose acetate gel and development with fluorogenic substrate. A: Comparison of normal cell extract (nc) and normal culture medium (nm); B: Comparison of ICD fibroblast extract (ic); normal cell extract (nc); ICD culture medium (im); and normal human serum (ns); C: Comparison of normal cell extract (nc), ICD-heterozygote cell extract (hc), and ICD cell extract (ic). D: Comparison of normal culture medium (nm), mixture of normal and ICD culture media (nm + im) and ICD heterozygote culture medium (hm). A,B: Hex A and Hex B from normal fibroblast extracts. Arrow: point of application.

Lie et al. (16). In both intracellular and excreted β-hexosaminidase from ICD fibroblasts, the two bands corresponded to Hex A and Hex B, respectively, from normal fibroblasts, as verified by immunoelectrophoresis: in both ICD patterns, both bands reacted with the anti-Hex B serum, while only the more anodal bands reacted with the specific anti-Hex A serum. Thus the alteration affecting the

electrophoretic mobilities of the β-hexosaminidase forms in ICD does not affect either the common antigenic determinant, β, of Hex A or Hex B, or the unique antigenic determinant, α, of Hex A (12). This was confirmed by a reaction of identity in immunodiffusion.

The electrophoretic pattern of β-hexosaminidase excreted by ICD fibroblasts resembled that of the enzyme from normal plasma or serum (Fig. 1B). It has been shown (10,17) that, unlike Hex A and Hex B from cells and organs, plasma Hex A and Hex B are susceptible to the enzymatic action of neuraminidase, which results in a lower electrophoretic mobility of both enzyme forms[1]. Treatment of normal and ICD fibroblast extracts and normal fibroblast culture fluid with Cl. perfringens neuraminidase did not affect the electrophoretic patterns of β-hexosaminidase. In contrast, similar treatment of ICD-fibroblast culture fluid caused a marked decrease in the electrophoretic mobility of the excreted β-hexosaminidase bands (Fig. 2). Direct comparison by co-electrophoresis showed that the patterns of both neuraminidase-treated, ICD-excreted enzyme and neuraminidase-treated normal serum were very similar to if not identical with the ICD intracellular β-hexosaminidase. These results, obtained in several experiments with two different preparations of Cl. perfringens neuraminidase, strongly suggest that the characteristic electrophoretic mobility of ICD-excreted β-hexosaminidase results from the addition of sialic acid residues to the intracellular enzyme. The apparent molecular weight by gel filtration (18) of ICD-excreted β-hexosaminidase (178,000) was similar to that of normal plasma β-hexosaminidase (185,000), and higher than that of the intracellular enzymes (150,000); however, the molecular weight by sucrose gradient centrifugation of ICD intracellular enzyme, normal cell enzyme and normal plasma enzyme was

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1: The apparent conversion of human organ Hex A to Hex "B-like" upon treatment with Vibrio cholerae neuraminidase observed by Robinson and Stirling (Biochem. J. 107, 321-327 (1968)) has been shown to be due to merthiolate present in the commercial neuraminidase preparations as a preservative (Carmody, P. J. and Rattazzi, M. C., Biochim. Biophys. Acta, 371, 117-125 (1974)). The preparations of Cl. perfringens neuraminidase used in the present work did not contain merthiolate.

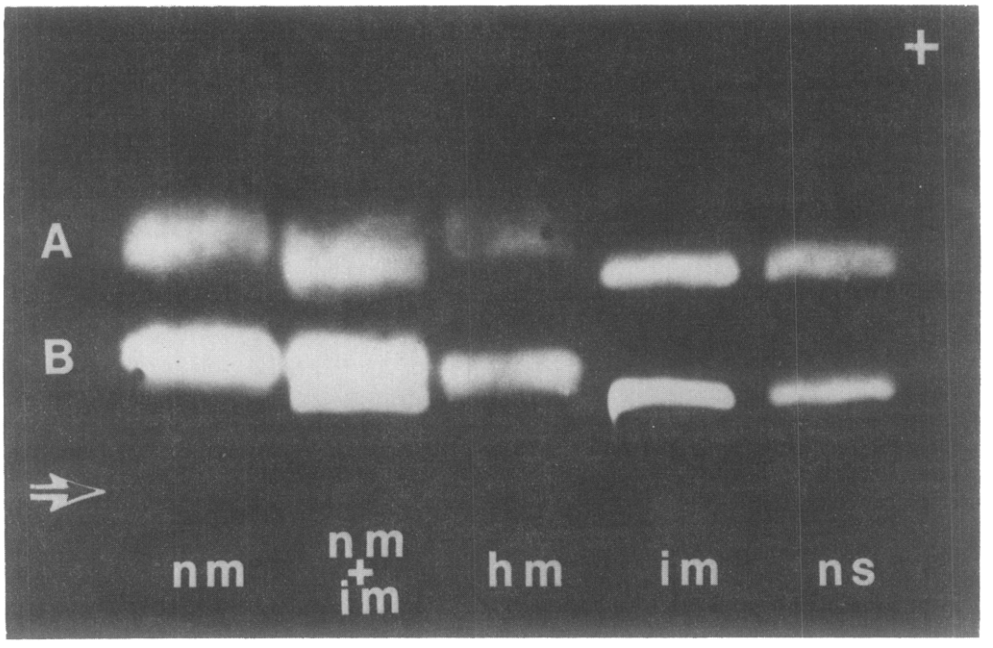

Fig. 2. Effect of neuraminidase treatment on the electrophoretic patterns of β-hexosaminidase from culture media. Medium from normal fibroblasts (nm); mixture of media from normal and ICD fibroblasts (nm + im); medium from ICD-heterozygote fibroblasts (hm); medium from ICD fibroblasts (im); normal human serum (ns). Conditions and notation as in Fig. 1, except that all samples were incubated with neuraminidase as described in Methods.

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the same (approx. 100,000). Thus the observed electrophoretic differences may result from small negatively charged residues, presumably carbohydrates, which may account for the higher apparent molecular weight observed by gel filtration (20).

Three additional lysosomal enzymes, arylsulfatase, α-L-fucosidase, and β-glucuronidase were examined by electrophoresis on cellulose acetate gel. In initial experiments, the patterns of the normal fibroblast-excreted enzymes were identical with those of the corresponding intracellular enzymes. In contrast, the overall patterns of all three ICD fibroblast-excreted enzymes were more electronegative than the normal and the ICD intracellular enzymes, as exemplified by the arylsulfatase patterns in Fig. 3. The mobilities of α-L-fucosidase and β-glucuronidase were also similar to those of the correspond ing enzymes from normal plasma, which are more electronegative than the organ-

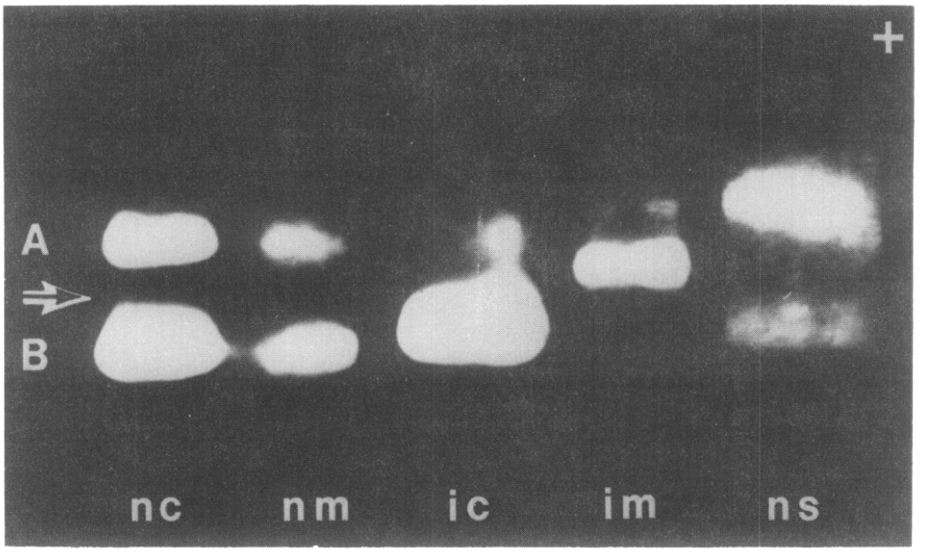

Fig. 3. Fluorescent bands of arylsulfatase activity after electrophoresis on cellulose acetate gel at pH 7.0 and development with fluorogenic substrate. Normal cell extract (nc); normal culture medium (nm); ICD fibroblast extract (ic); ICD-culture medium (im); normal human serum (ns). A,B: Arylsulfatase A and B from normal fibroblast extracts. Arrow: point of application.

derived enzymes (21,22). These data suggest that a higher electrophoretic mobility may be a feature common to several lysosomal hydrolases excreted by ICD fibroblasts. However, further experiments are necessary to establish whether the additional charge on ICD-excreted enzymes is due exclusively to sialic acid.

It has been shown that the uptake by rat liver cell plasma membranes of glycoproteins with exposed galactose residues is favored over the uptake of glycoproteins with sialylated galactose residues (23). We propose analogously that the presence of additional sialic acid on ICD-excreted lysosomal hydrolases may be the cause of the observed impaired uptake of ICD-excreted enzymes by normal or non-ICD fibroblasts (7,24) by masking or sterically hindering the hypothesized carbohydrate "recognition marker" on these enzymes (24). It is worth noting that the more electronegative β-hexosaminidase and β-glucuronidase from normal plasma, when injected intravenously, have half-lives considerably

longer than those of similarly administered preparations of organ-type enzymes (25-27).

ICD fibroblasts can retain endocytosed normal enzymes (7,8); the elevated level of lysosomal hydrolases in ICD cell culture medium thus is not due to "leaky" secondary lysosomes. We propose instead that in these cells, abnormal exocytosis may occur by preferential fusion of the Golgi-derived primary lysosomes with the plasma membrane, bypassing fusion with the pinocytic vacuoles, as observed in osteoclasts during bone resorption (28). The coincidence in electrophoretic mobility between ICD intracellular β-hexosaminidase and neuraminidase treated β-hexosaminidase from normal serum, suggests that the ICD intracellular enzyme is not abnormal. The less electronegative forms of β-hexosaminidase in ICD fibroblast extracts may represent incompletely glycosylated normal enzyme forms derived from the endoplasmic reticulum (29). These would be fully sialylated in the Golgi complex (29) and excreted immediately, instead of being desialylated in the digestive vacuoles (29). A single gene mutation affecting the control of exocytosis could thus account for all the abnormal biochemical findings in ICD fibroblasts.

Our observations on fibroblasts from two obligate heterozygotes for the ICD gene are consistent with this hypothesis. β-hexosaminidase activity (nmoles 4-MU liberated/cell/hr) in culture medium from these cells was increased 1.5-3.5 times over normal levels: a similar increase has been found in the serum of parents of children with ICD (30). While the electrophoretic patterns of intracellular β-hexosaminidase were indistinguishable from normal (Fig. 1C), the patterns of excreted β-hexosaminidase were an apparent composite of normal and ICD-excreted enzymes (Fig. 1D). After neuraminidase treatment, the intermediate band was no longer visible, while the Hex B region band increased in intensity, as expected from the observed effect of this treatment on ICD-excreted β-hexosaminidase; however the pattern did not exactly coincide with that of a neuraminidase-treated mixture of ICD-excreted and normal fibroblast-excreted enzymes (Fig. 2).

Thus the ICD gene defect is expressed in fibroblasts from ICD heterozygotes, and this may form the basis for diagnostic procedures.

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