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Glycosaminoglycan Accumulation with Partial Deficiency of β-Glucuronidase in the C3H Strain of Mice

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Young (60–80 days) mice of the low β-glucuronidase strain, C3H/HeJ, showed no differences in hepatic levels of glycosaminoglycans (GAGs) when compared to the randombred, "normal" Swiss-Webster mice of the same age. However, by 12 months of age hepatic GAG is nearly twice as high in C3H/HeJ mice as in Swiss-Webster mice. Studies of β-glucuronidase, β-galactosidase, and N-acetyl-β-glucosaminidase in four tissues of the two types of mice at the two ages revealed that glucuronidase was the only enzyme with lower activity in the C3H/HeJ strain.

KEY WORDS: β-glucuronidase; lysosomal enzymes; mucopolysaccharidosis; glycosaminoglycans; mice.

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[6] *Abbreviations:* GAGs, glycosaminoglycans; C3H, C3H/HeJ; SW, Swiss-Webster.

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INTRODUCTION[6]

Mucopolysaccharidosis VII, a severe clinical disease, is thought to be due to a deficiency of lysosomal β-glucuronidase (Sly *et al.*, 1973). Fibroblasts from patients with this disorder have 1–2% residual β-glucuronidase (Gehler *et al.*, 1974; Beaudet *et al.*, 1975). It has been known for some time that mice of the C3H/HeJ strain have 5–10% the normal (i.e., the level in most strains of mice) amount of acid β-glucuronidase in several organs (Morrow *et al.*, 1949). These low levels of β-glucuronidase have allowed the use of this strain of mice as a model of enzyme therapy (Thorpe *et al.*, 1975), but there has been no evidence for accumulation of glycosaminoglycans (GAG) in this strain of mice. We herein delineate GAG accumulation in the liver of C3H mice.

MATERIALS AND METHODS

Mice

Inbred C3H/HeJ (C3H) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Randombred Swiss-Webster (SW) albino mice were obtained from Carworth Farms, Gilroy, California. Shavings were used as bedding and the mice were kept in a room with controlled lighting (14 hr light, 10 hr dark). "Young" mice were 60–80 days old, while "old" mice were 12 months old. All mice used were females.

Determination of Glycosaminoglycans

Mice were killed by cervical dislocation and livers were quickly removed and frozen on dry ice. They were later thawed and minced, and the minced livers were defatted by two 24-hr extractions with 20–50 vol of acetone–ether (1:1) and dried at 100 C for 4 hr. The dried livers were ground in a mortar and 200–500 mg portions of the livers were rehydrated in 10 vol of 0.1 M phosphate buffer, *p*H 7.5, and heated at 100 C for 20 min. After cooling, 2 vol of 0.25% Pronase B (Calbiochem) was added and the tissues were incubated at 57 C. After 12–16 hr another 2 vol of 0.25% Pronase B was added and the incubation continued for 24 hr. Sufficient 100% w/v trichloroacetic acid was added to make the final concentration 5%. Following centrifugation the supernatant extract was decanted and the precipitate was reextracted with 2 ml of 5% trichloroacetic acid. The GAGs were precipitated from the combined extract by addition of 3 vol of ethanol containing 5% potassium acetate. After standing overnight at 4 C, the precipitates were collected by centrifugation and washed with alcohol–ether (1:1). The precipitates were dissolved in 2.5 ml of 0.15 M NaCl and clarified by centrifugation. One milliliter of 5% cetylpyridinium chloride was added to 2 ml of the solution of GAGs. After standing at 37 C for 4 hr, the tubes were centrifuged and the supernatant solution was

decanted. One milliliter of 1.25 M $MgCl_2$ was placed in each tube and the sides of the tube were carefully wetted with this solution. Three volumes of ethanol were then added and the tubes were held overnight at 4 C. After centrifugation, the tubes were drained and the GAGs were taken up in a small volume of 0.15 M NaCl. Aliquots were used for assay of uronic acid according to Bitter and Muir (1962).

Determinations of Glycosidases

Two grams percent homogenates of fresh or fresh-frozen liver, kidney, and spleen were prepared in distilled water using a Potter-Elvehjem homogenizer. They were then frozen and thawed three times and cleared by centrifugation at 12,000 *g* for 30 min in a Sorval R2 refrigerated centrifuge. The supernatants, or sera, were assayed for the lysosomal acid hydrolases (1) β-galactosidase, (2) *N*-acetyl-β-glucosaminidase, and (3) β-glucuronidase with the fluorometric 4-methylumbelliferyl substrates at 0.5 mM (Sandman *et al.*, 1973). The buffers were slightly different with each substrate: (1) 0.1 M, *p*H 4.5 sodium acetate with 4-methylumbelliferyl-β-D-galactopyranoside; (2) 0.1 M, *p*H 4.75 sodium acetate with 4-methylumbelliferyl-β-D-glucuronide; and (3) 0.21 ionic strength, *p*H 4.6 citrate phosphate with 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside. Incubations were carried out for 1 hr at 37 C in the presence of 1 mg/ml bovine serum albumin for enzyme stabilization. The assays were linear during this time. Proteins were determined by the method of Lowry.

RESULTS

Young C3H mice had lower (but not statistically significant) levels of liver GAGs than did control, SW, mice (Table I). However, liver GAGs increased slightly with increasing age in SW mice while they increased markedly in C3H mice. The nearly twofold greater levels of GAGs in old C3H as compared to old SW mice were compared by Student's *t* test and found to be significant at $p < 0.01$.

We had chosen to study the GAGs in the two strains of mice because of

Table I. Determination of GAGs in Liver of SW and C3H Mice (Old and Young)

	SW young	SW old	C3H young	C3H old
μg GAG-uronic acid/g of dry fat-free liver	(15) 230 ± 63[a]	(4) 277 ± 56	(12) 178 ± 47	(11) 488 ± 84

[a] (Number of mice examined) mean ± SD.

the known deficiency of β-glucuronidase in the C3H strain. We measured several GAG-degrading enzymes in the experimental and control mice to show that β-glucuronidase levels of Swiss mice were comparable to those in *Gus*[a] and *Gus*[b] homozygous mice and that other acid hydrolases were also not deficient in C3H mice. Studies on β-glucuronidase, β-galactosidase, and β-glucosaminidase in liver, kidney, spleen, and serum in young and old C3H and SW mice revealed marked deficiencies of β-glucuronidase in C3H liver (Table II). The enzyme values showed much greater variability in the SW

Table II. Lysosomal Hydrolase Activities in Tissues and Serum: SW and C3H (Young and Old)

	SW young[a]	SW old[a]	C3H young[a]	C3H old[a]
	nmol/hr/mg protein			
Liver				
β-Galactosidase	85 ± 32[b]	92 ± 40	30 ± 1.3	79 ± 20
	(46–125)[c]	(53–139)	(29–32)	(67–109)
N-Acetyl-β-glucosaminidase	447 ± 83	1200 ± 408	178 ± 24	465 ± 28
	(382–568)	(907–1803)	(143–197)	(434–488)
β-Glucuronidase	221 ± 13.4	191 ± 41	16.2 ± 1.5	30.5 ± 24.5
	(204–235)	(145–245)	(15–18)	(16–67)
Kidney				
β-Galactosidase	394 ± 162	162 ± 88	151 ± 7.5	177 ± 12
	(191–585)	(92–276)	(146–162)	(162–187)
N-Acetyl-β-glucosaminidase	1176 ± 112	1525 ± 574	1135 ± 44	1758 ± 78
	(1055–1317)	(1104–2353)	(1075–1180)	(1646–1827)
β-Glucuronidase	117 ± 18	54 ± 6.8	39 ± 14	23 ± 3
	(98–142)	(47–63)	(28–58)	(20–27)
Spleen				
β-Galactosidase	236 ± 107	265 ± 199	199 ± 13	205 ± 38
	(114–374)	(159–565)	(188–218)	(165–246)
N-Acetyl-β-glucosaminidase	1697 ± 112	3195 ± 626	1772 ± 125	1949 ± 116
	(1615–1856)	(2653–4088)	(1629–1902)	(1786–2044)
β-Glucuronidase	333 ± 38	290 ± 67	122 ± 7.1	86 ± 13
	(297–384)	(240–390)	(117–133)	(73–103)
	nmol/hr/ml serum			
Serum				
β-Galactosidase	29 ± 12	32 ± 11	14 ± 3.9	19 ± 2.4
	(13.5–44)	(22–48.5)	(10.5–19.5)	(15.5–21)
N-Acetyl-β-glucosaminidase	1427 ± 332	2827 ± 401	728 ± 51	891 ± 212
	(1024–1722)	(2450–3334)	(686–803)	(712–1198)
β-Glucuronidase	36 ± 4.0	47 ± 24	4.1 ± 1.4	5.6 ± 1.4
	(30.5 ± 40)	(28–81)	(3.0–6.0)	(4.0–7.5)

[a] Six female mice.
[b] Mean ± SD.
[c] Range.

mice, as is expected for randombred mice. The livers of young C3H mice contained 7.3% as much β-glucuronidase as did young SW livers; at 12 months the level was 16% of normal. The deficiency was less severe in kidney (33% of SW young, 42% of SW old) and increased with age in spleen (37% of SW young, 30% of SW old). Although β-galactosidase was relatively deficient in young C3H liver, kidney, and serum, old C3H mice were comparable to old SW mice in liver and kidney. Similarly, although *N*-acetylglucosaminidase in C3H liver was 40% of SW levels in both young and old mice, comparable and higher levels were found in young and old kidneys, respectively, while comparable levels were found in young spleens. The levels of lysosomal enzymes generally increased with age in both strains of mice except for β-glucuronidase, which tended to decrease with age in C3H mice. Thus, among the enzymes studied, only β-glucuronidase was markedly decreased in C3H mice, liver and serum showing the largest decreases.

DISCUSSION

The structural gene for β-glucuronidase in mice, *Gus*, is located near the end of chromosome 5, and is known to have three common alleles (Paigen *et al.*, 1976). Gus^a and Gus^b determinine electrophoretically distinguishable forms, while Gus^h, the allele characteristic of the C3H strain, determines a heat-labile enzyme which has not been shown to be altered in its other physical and chemical properties. Radiopulse-immunoprecipitation studies have shown that the rate of synthesis of β-glucuronidase in the C3H strain is proportional to the enzymatic activity, and that the ratio of synthesis to activity is the same in C3H as in other strains (Paigen *et al.*, 1976). Thus the lowered activity of β-glucuronidase in C3H mice does not seem to be due to increased *in vivo* lability or to the presence of inhibitors.

It is a moot point whether or not one could expect external evidence of β-glucuronidase deficiency in C3H mice. Human patients with nearly complete deficiencies have been diagnosed in the first year of life (Sly *et al.*, 1973; Beaudet *et al.*, 1975), although the features of another patient (Beaudet *et al.*, 1975) did not suggest a storage disease until the second decade of life. Smaller animals, mice included, have higher fluxes of compounds involved in energy metabolism, but few data are available on the rates of synthesis or turnover of structural macromolecules such as GAGs. The twofold increase in hepatic GAGs in 12-month-old C3H mice is small compared to the hundredfold increase found in affected human liver (Brown, 1957). One might expect some evidence of storage in the nervous system, but neurological examinations are difficult to perform in mice—it took years for users of C3H mice to realize that they were blind from about 3 weeks of age because of homozygosity for a second mutation, retinal degeneration (Noell, 1958).

It is highly likely that the hepatic storage of GAGs in aged C3H mice is due to their relative deficiency of β-glucuronidase. Studies of two other lysosomal enzymes did not reveal major deficiencies and did not suggest that a general decrease had occurred in lysosomal enzymes, due to complexing with undegraded substrate, as might have occurred with accumulation of GAGs due to some other cause (Kint, 1974). However, it is possible that some other of the about ten enzymes already identified as involved in GAG degradation is deficient in C3H mice. A congenic line of mice with the Gus^h allele homozygous on a non-C3H background has been developed (LaVail and Sidman, 1974), and would clearly resolve the question, but was not available to us at this time.

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