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β-hexosaminidase
feline GM_2 gangliosidosis
hepatic glycoprotein receptors
lysosomal enzyme replacement
plasma clearance
Sandhoff disease
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Toward Enzyme Therapy in Gm_2 Gangliosidosis: β-Hexosaminidase Infusion in Normal Cats

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Summary

As a first step toward the use of cats with Gm_2 gangliosidosis as models for enzyme replacement therapy, we studied plasma clearance, organ disposition, and subcellular localization of human β-hexosaminidase in normal cats. Plasma half-life of placental β-hexosaminidase at low doses was 3–4 min; both Hex A and Hex B were cleared at approximately the same rate. The half-life of human plasma β-hexosaminidase in contrast, was >60 min. Clearance curves at higher doses approached zero order kinetics, suggesting the existence of a saturable clearance mechanism. Injection of periodate-treated placental β-hexosaminidase resulted in a plasma half-life of ~50 min, strongly suggesting that rapid clearance of both Hex A and Hex B was mediated by carbohydrate-specific mechanisms. Circulatory bypass of liver resulted in plasma half-life of the enzyme of ~60 min, indicating that the liver was the main clearing organ. As both main feline β-hexosaminidase isozymes did not crossreact with antihuman β-hexosaminidase immune sera, independent evidence of preferential hepatic uptake was obtained by immunofixation electrophoresis; immunoelectrophoresis, and immunotitration. The human enzyme detected in liver accounted for ~80% of the injected dose; small amounts of exogenous enzyme were detected in spleen and kidney. Subcellular fractionation of liver showed that human Hex A and Hex B had entered the lysosomal-vacuolar apparatus of hepatic cells.

Speculation

Therapeutic applications of lysosomal enzyme replacement in patients with storage diseases are fraught with difficulties. Normal cats infused with human β-hexosaminidase can be used to develop enzyme replacement methodologies to be tested in cats with genetic Gm_2 gangliosidosis. This unique animal model makes it possible to explore *in vivo* rational approaches to therapeutic intervention in human patients.

Since the discovery that the human genetic storage diseases, Gm_2 gangliosidosis is due to deficiency of lysosomal β-D-N-Acetyl-glucosaminidase (E.C. 3.2.1.30; β-hexosaminidase) (32, 38), a substantial body of data has been gathered on enzymatic aspects of this group of fatal neurodegenerative disorders. This knowledge has found practical applications in screening programs for the detection of heterozygotes for Tay–Sachs disease (TSD: Gm_2 gangliosidosis type I) in the Ashkenazi Jewish population, in which the disease is particularly frequent, and in prenatal diagnosis (24). No treatment for Gm_2 gangliosidosis is available at present; active intervention is thus limited to selective abortion of affected fetuses detected by amniocentesis in pregnancies from couples at risk, identified either through screening programs or after the birth of an affected infant (24).

Treatment of lysosomal storage diseases by administration of normal enzyme seems possible, at least in principle (14), and therapeutic measures could be useful in treating infants with Gm_2 gangliosidosis born to heterozygous couples not detected through screening programs. The difficulties of this therapeutic approach have recently been reviewed (18).

A genetically determined Gm_2 gangliosidosis has recently been described in the domestic cat, *Felis catus* (13). The feline neurogenerative disorder is associated with profound deficiency of β-hexosaminidase, is inherited as an autosomal recessive condition, and appears homologous to human Gm_2 gangliosidosis type II (Sandhoff disease) (38, 44). Cats with Gm_2 gangliosidosis may thus provide a unique opportunity to explore *in vivo* therapeutic strategies based on enzyme replacement, of possible application to human patients.

In order to assess the feasibility of using affected animals as models for replacement therapy in Gm_2 gangliosidosis, we have studied plasma clearance, organ disposition, and subcellular localization of human β-hexosaminidase injected into normal cats.

MATERIALS AND METHODS

PREPARATION OF HUMAN β-HEXOSAMINIDASE

Human β-hexosaminidase was partially purified from term placentae by concanavalin A (Con A) affinity chromatography. About 1200 g of minced tissue, rinsed free of blood with 0.9% NaCl was homogenized (Waring blender) at 4°C in 4 liters of 0.05 M Na-succinate buffer, pH 6.7, containing NaCl (0.5 M); $MgCl_2$, $CaCl_2$, and $MnCl_2$ (all 1 mM); Triton X-100 (0.1%; v:v); and NaN_3 (0.01%; w:v). After centrifugation at 25,000 *g* for 30 min at 4°C and reaction of supernatants with 50 ml of settled Con A-Sepharose (48) overnight at room temperature, the material was washed with ~1500 ml of the above buffer (1.0 M NaCl). The gel was poured into a column and the enzyme was eluted with 500 ml of 0.05 M Na-succinate buffer, pH 6.7, containing 0.5 M NaCl and 0.01% NaN_3. The procedure gave a 67.5-fold purification; β-hexosaminidase specific activity was 596,000 units/mg protein. Eluates containing both Hex A and Hex B were concentrated by ultrafiltration and dialyzed against 0.9% NaCl.

Outdated human plasma was used to prepare β-hexosaminidase-enriched fractions by addition of solid $(NH_4)_2SO_4$ to a 60% concentration or Con A-Sepharose treatment as above. The specific activity of these preparations was 3.5 and 66.0 units/mg protein, respectively. The concentrated preparations were dialyzed against 0.9% NaCl.

Sodium meta-periodate oxidation of human placental β-hexosaminidase was performed essentially as described by Stahl *et al.* (42). The enzyme retained 50% of its activity and had normal electrophoretic mobility; less than 10% of the treated enzyme, however, could be bound to Con A-Sepharose, indicating that the carbohydrate moiety of the enzyme had been altered by the treatment (42).

ANALYTICAL PROCEDURES

β-hexosaminidase was routinely assayed according to Leaback and Walker (26, 35) with the fluorogenic substrate 4-methyl umbelliferyl β-D-N-acetyl glucopyranoside (system 1) (49). As saturation kinetics are not attained in this system, for more precise determinations, system 2 was used, as follows: 10 μl aliquots of the material to be assayed were added to 100 μl of 5.0 mM fluorogenic substrate in 0.1 M citrate-phosphate buffer pH 4.5, containing 0.05% bovine serum albumin and 0.01% NaN_3; after 15 or 30 min at 37° (water bath), the reaction was terminated by addition of 3 ml of 0.25 M sodium carbonate-glycine buffer, pH 10. Enzyme activity in system 2 is higher by a factor 3.1 than that in system 1 over a wide range of dilutions. The concentration of liberated 4-methyl umbelliferone was determined by fluorometry (50) with appropriate calibration lines. One unit of enzyme activity was defined as 1 nmole of 4-methyl umbelliferone liberated per hour at 37°C.

Electrophoresis of β-hexosaminidase was carried out on cellulose acetate gel (Cellogel) (51) at pH 6.0 in Barbital-citrate buffer (35). Immunofixation electrophoresis (3) was carried out on Cellogel. After electrophoresis at pH 6.0, a 1:1 dilution of bovine antihuman β-hexosaminidase immune serum (0.5 ml) was spread on the gel with an applicator (52). After incubation for 1 hr at room temperature in a moist chamber (35), the gels were washed overnight with running 0.9% NaCl containing 0.05% Triton X-100. Immunoelectrophoresis was carried out as described (6, 35). In either case, enzymatically active immune precipitates were detected by incubation with fluorogenic substrate (35). For immunotitration of human β-hexosaminidase in feline organ extracts (29), increasing amounts of antiserum and decreasing amounts of 0.9% NaCl were added to fixed amounts of organ extracts. The mixtures were incubated for 3 hr at 37°C and 18 hr at 4°C. High speed centrifugation supernatants were assayed for residual β-hexosaminidase activity. The values were corrected for enzyme activity in the antiserum, and for the activity of soluble immune complexes by electrophoretic quantitation (35). Bovine and rabbit immune sera used in this work has been described (6). Protein concentrations were determined by the method of Waddell (47).

ANIMAL PREPARATIONS

Healthy adult mongrel cats of both sexes (1.8–4.5 kg) were anesthetized by iv injection of Sodium Pentothal (0.25% solution; ~2 ml/kg body wt) and artificially ventilated with O_2-air mixture. The animals were heparinized at the onset of the experiment. Injection of enzyme and withdrawal of blood samples (1 ml) was done through polyethylene catheters introduced into the right femoral vein and artery, respectively. Enzyme activity injected was adjusted as required, assuming a plasma volume of ~40 ml/kg body wt (19). Plasma from pre- and postenzyme injection blood samples was recovered by centrifugation. To determine the organ distribution of exogenous β-hexosaminidase, 2 hr after enzyme injection, the animals were subjected to open-end cardiac perfusion with ~700 ml of 0.9% NaCl. Organ extracts were prepared by sonication on ice of minced tissue in 10 vol of 0.05 M citrate-phosphate buffer, pH 5.5 containing 0.01% NaN_3, followed by centrifugation at 40,000 *g* for 20 min at 4°C and recovery of supernatants.

Circulatory exclusion of the liver was performed by a modification of the technique described by Glenn *et al.* (22). In anesthetized, heparinized, and ventilated animals, the inferior vena cava was resected above the right renal vein, and two arms of an unequally-armed, T-shaped PVC tube (53) were introduced cephalad and caudad, respectively, into the vena cava, and ligated in place. One arm of the T-tube spanned the liver, reaching the thoracic cavity; a ligature was placed on the vena cava in the bare area of the liver under the diaphragm, thus preventing blood flow from the hepatic veins into the vena cava. The portal vein was resected between the liver and the gastric vein junction and the end proximal to the liver was ligated; the third arm of the T-tube was then introduced into the distal end of the vein and ligated, thus creating an end-to-side porta-caval anastomosis. Finally, the common hepatic artery was ligated, thus completely excluding the organ from the circulation. The abdominal wall was then closed with sutures. After completion of the procedure, the animals received a drip infusion of 5% glucose solution. The shunt remained pervious for the duration of the subsequent enzyme infusion experiment.

PREPARATION OF SUBCELLULAR FRACTIONS OF LIVER

Subcellular fractionation of hepatic tissue was carried out in one animal by a combination of differential and isopycnic centrifugation (12). Two hr after injection of human enzyme, and after open-end cardiac perfusion, the blanched liver was sliced and placed in ice-cold 0.25 M sucrose at pH 7.0 (Tris-EDTA buffer). A tissue aliquot (20 g) was minced, suspended in 180 ml of cold sucrose solution, and gently homogenized in a Potter-Elvejehm glass homogenizer on ice, followed by two strokes in a Dounce homogenizer (loose-fitting pestle). The homogenate was centrifuged at 1000 *g* for 10 min at 4°C; the precipitate was resuspended in 200 ml of sucrose solution and centrifuged at 2000 *g* for 10 min. The supernates were combined (postnuclear supernatant fraction) and centrifuged at 15,000 *g* for 20 min at 4°C. Sediments were resuspended in 0.25 M sucrose (50 ml) and both first supernatants and resuspended sediments were centrifuged once more as above. The supernatants and sediments from the second centrifugation represented the microsomal-soluble fraction, and the lysosomal-mitochondrial fraction, respectively; aliquots were withdrawn for further analysis, and the remainder was subjected to isopycnic centrifugation (Beckman-Spinco L2-65B ultracentrifuge, SW 25-1 rotor) in a discontinuous sucrose gradient, consisting of 6 ml each of 50, 40, 30, and 20% sucrose solutions at pH 7, on top of which the samples (6 ml) were layered. After centrifugation at 4°C for 9 hr at 65,681 *g*/average, fractions (~1 ml) were collected from the top of the gradient. "Latency" of β-hexosaminidase in the subcellular fractions thus obtained was determined by assay of 50 μl aliquots in 1 ml 0.25 M sucrose at pH 4.5 (0.1 M citrate buffer) containing 0.264 mM fluorogenic substrate as in system 1; and by assay of 10 μl aliquots in pH 4.5 buffer without sucrose (system 1), but containing 0.1% Triton X-100 (w:v), after 3 freezing and thawing cycles (solid CO_2-acetone). The density of the sucrose gradient fractions was determined by refractometry (54) using standard sucrose density tables. Liver from a control cat was subjected to the same fractionation procedure.

RESULTS

PLASMA CLEARANCE OF HUMAN PLACENTAL AND PLASMA β-HEXOSAMINIDASE

IV injection of partially purified human placental β-hexosaminidase ($4 \times 10^4 - 1.2 \times 10^5$ units/kg body wt, system 1) as a 0.1–0.5 ml bolus was followed by an abrupt increase in plasma activity in the recipient animal, reaching a maximum at 2 min from injection. The activity was consistent with dilution of the injected dose in the animal plasma compartment (19). The enzyme activity returned to approximately preinjection levels in about 30–40 min (Fig. 1). The low endogenous activity in the animal plasma (30–60 U/ml, system 1) allowed a direct assessment of the nature of the enzyme activity after injection by electrophoresis. Both human Hex A and Hex B were clearly identifiable, and were apparently cleared at approximately the same rate (Fig. 2).

The plasma half-life of the exogenous enzyme (activity at 2 min: 1000–3000 units/ml) was of the order of 3–4 min. Injection of increasing doses of placental β-hexosaminidase, however, resulted in clearance curves approached zero-order kinetics at the highest doses (Fig. 3) suggesting that clearance was mediated by a saturable mechanism.

Injection of the β-hexosaminidase-enriched human plasma frac-

tions in two cats (7.8×10^3 and 4.1×10^3 units, in 16 and 5 ml, respectively) was followed by moderate rise in activity consistent with dilution in the animal plasma of the relatively low dose injected; the increase in activity persisted for a relatively long time

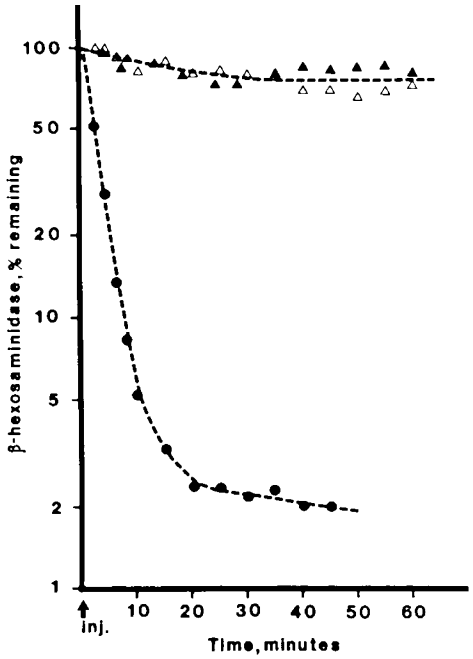

Fig. 1. β-hexosaminidase activity in cat plasma after injection of partially purified human β-hexosaminidase from placenta (*circles*) or from plasma (*triangles*). At 2 min from placental enzyme injection, the activity range was $1\text{–}3 \times 10^3$ U/ml (4 experiments). Corresponding values after plasma enzyme injection were 1 and 3×10^2 U/ml (two separate animals). Activity was expressed as % of theoretical instantaneous distribution, obtained by extrapolation to 0 time of the measured activity. The half-life of human plasma enzyme, estimated by immunotitration, was ~70 min.; that of placental enzyme, estimated directly, was ~3 min.

(Fig. 1), and electrophoresis of plasma samples from the injected animals showed that human plasma β-hexosaminidase was recognizable in the presence of feline plasma enzyme at 2 min, and was still visible at 74 min from injection (Fig. 4). Immunotitration of feline plasma samples with noncrossreacting antihuman β-hexosaminidase serum (discussed later in this paper) indicated that the half-life of the human enzyme was approximately 70 min.

In vitro incubation at 37°C of human placental and plasma β-hexosaminidase diluted in feline plasma to give the same activity measured *in vivo* at 2 min from injection; or of 2 min plasma samples from injected animals showed that inactivation of the human enzyme was minimal, with an estimated half-life of the order of several hours, well above the maximum half-lives (~70 min) observed *in vivo*.

EFFECT OF PERIODATE TREATMENT

To assess whether carbohydrate-specific mechanisms (2, 4, 28, 41–43) may be responsible for clearance of human β-hexosaminidase in the cat, we injected partially purified placental Hex A and Hex B which had been treated with sodium meta-periodate (42).

Sequential injection of the same dose of untreated and periodate-treated enzyme preparations in the same animals showed that the plasma clearance of the treated enzyme was drastically decreased, resulting in a half-life of about 60 min (Fig. 5). Electrophoresis of plasma samples after injection of periodate-treated enzyme (not shown) demonstrated that 1 hr after injection, the ratio Hex A/Hex B was approximately the same as at 2 min postinjection. This strongly suggested that the clearance of both enzyme forms had been affected by periodate treatment and that clearance of both untreated Hex A and Hex B, therefore, was mediated by carbohydrate-specific mechanisms.

ASSESSMENT OF PREFERENTIAL HEPATIC UPTAKE

The role of liver in the rapid clearance of human enzyme from feline plasma was assessed by exclusion of the organ from the circulation and porta-caval anastomosis in four animals. In three of these, a standard dose of placental β-hexosaminidase was first

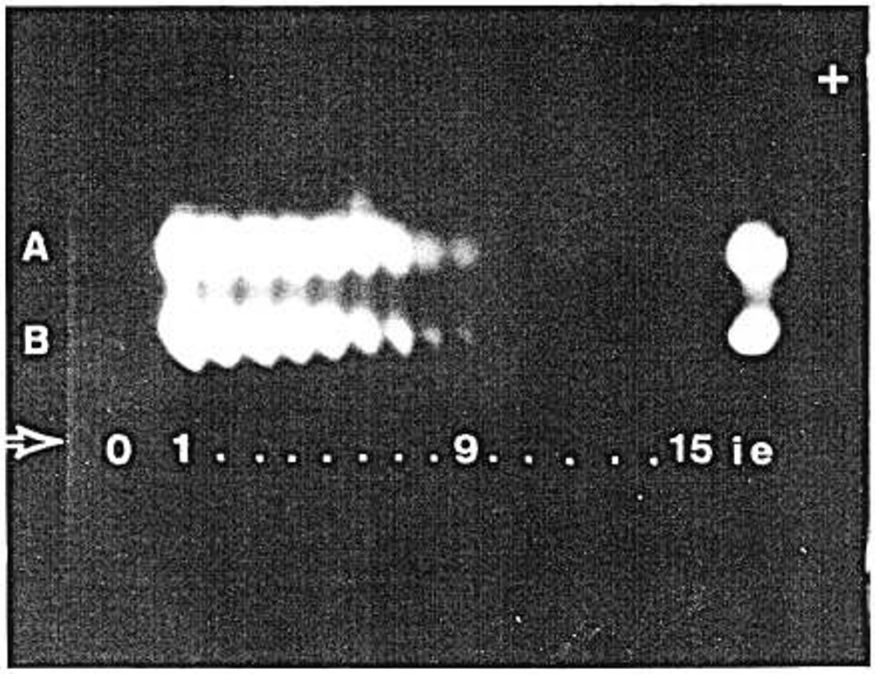

Fig. 2. Fluorescent zones of β-hexosaminidase activity after electrophoresis (Cellogel at pH 6.0) of plasma samples from a cat injected with 4.9×10^5 units of human placental β-hexosaminidase. Lane 0: cat plasma before injection. Lane 1: cat plasma sample 4 min after injection. Lanes 2–9: samples collected at 5- to 6-min intervals up to 50 min. Lanes 10–15: samples collected at 10- to 15-min intervals up to 120 min from injection. Enzyme activity was still visible in lane 13 (90 min) in the original gel. Lane i.e.: injected enzyme. Notice parallel disappearance of human Hex A (*A*) and Hex B (*B*). *Arrow* = point of application.

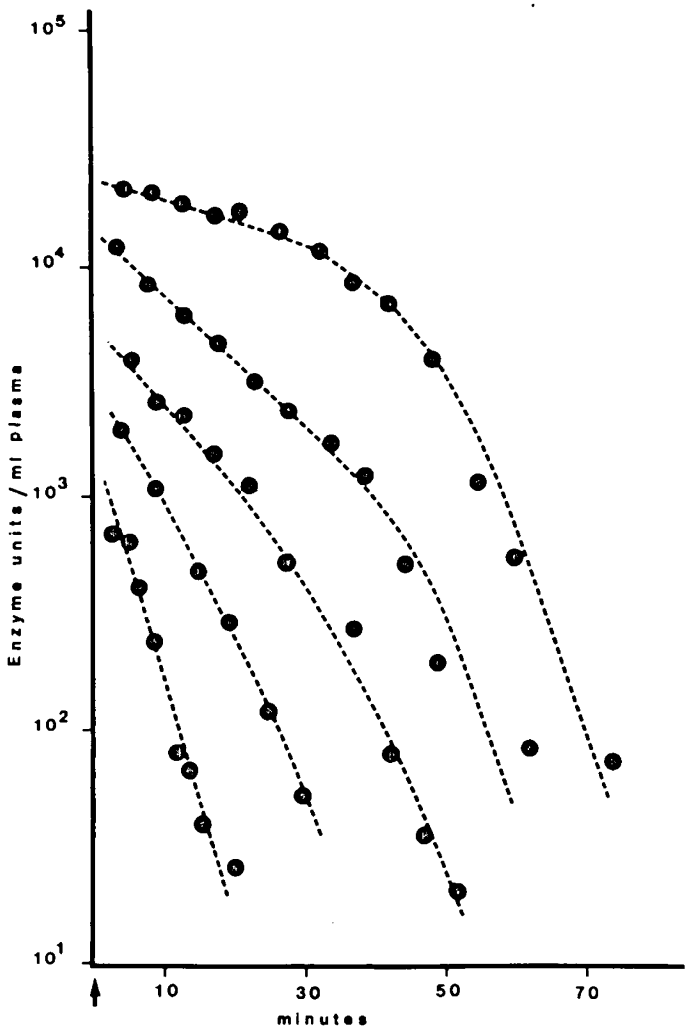

Fig. 3. Dose-dependent plasma clearance of human placental β-hexosaminidase (Hex A + Hex B) after iv infusion into normal cats. The injected doses varied between 1.6×10^5 and 6.8×10^5 U/kg body wt; the endogenous plasma activity in the animals was of the order of 20–60 U/ml. Injection of saline solution, followed by blood sampling for comparable periods in control animals did not produce appreciable increases in endogenous activity. Units: nmoles of 4-methyl umbelliferyl β-D-N acetyl glucopyranoside cleaved/hr at 37°C (system I; *see text*).

injected iv as a control. After 45 min, liver bypass was performed, and a second, identical enzyme dose was injected. Transient, repeated disturbances in the cardiac rhythm and a fall in arterial blood pressure were noted in these animals (22). In the fourth animal, a similar dose of human enzyme was given only once, after liver bypass: no untoward circulatory effects were noted for up to 90 min from the injection. In all animals, β-hexosaminidase clearance was drastically impaired after liver exclusion (Fig. 5), indicating that the liver was the main clearing organ.

A preliminary immunologic characterization of feline β-hexosaminidase was then carried out, and it was found that both main feline β-hexosaminidase isozymes, Hex A and Hex B (13) did not crossreact in immunoelectrophoresis with bovine and rabbit antihuman β-hexosaminidase immune sera. This was confirmed by double immunodiffusion; moreover, immunoprecipitation experiments indicated that this was not due to the formation of enzymatically inactive immune complexes (33).

Thus, independent evidence of preferential liver uptake was obtained by immunofixation electrophoresis of homogenates of brain, lung, liver, spleen, and kidney from enzyme-injected, intact animals. Enzymatically active bands of immune precipitate of human Hex A and Hex B were evident in extracts of liver, but not in those of the other organs, indicating that both isozymes had been preferentially retained by the liver. Using the more sensitive immunoelectrophoresis, however, faint arcs of enzymatically active immune precipitate of human enzyme were visible in kidney and spleen extracts. By immunotitration of human β-hexosaminidase in the extract of liver from 2 animals, it was estimated that the enzyme accounted for about 80% of the injected dose. This subtractive method, however, did not allow quantitation of the much smaller amounts of human enzyme present in kidney and spleen.

INTRACELLULAR LOCATION OF HUMAN β-HEXOSAMINIDASE IN FELINE LIVER

The liver from a cat injected with a large dose (9.76×10^5 units) of human placental Hex A and Hex B was subjected to subcellular fractionation. After differential centrifugation, immunofixation electrophoresis of the soluble-microsomal and lysosomal-mitochondrial fractions showed that a substantial portion of the human enzymes was associated with the lysosomal-mitochondrial fraction; Hex A and Hex B were present in this fraction in approximately the same ratio as in the material used for injection, while the activity associated with the supernatant-microsomal fraction appeared to be due mainly to Hex A (Fig. 6). After isopycnic centrifugation the majority of β-hexosaminidase activity from the lysosomal-mitochondrial fraction sedimented at a median density d=1.20, and exhibited latency, (Fig. 7) both characteristics of membrane-bounded lysosomal enzymes (15). Arylsulfatase and β-hexosaminidase from the control cat liver showed an identical sedimentation profile, and exhibited latency. The activity in the soluble-microsomal fraction sedimented at a median density d=1.07 and also exhibited some latency. Control runs with added human β-hexosaminidase showed that the free enzyme also sedimented in correspondence with fractions 2–13 of the gradient. Finally, electrophoresis and immunofixation electrophoresis of the sucrose gradient fractions showed that, in addition to feline β-hexosaminidase, both human Hex A and Hex B were present in the lysosomal-mitochondrial peak, confirming the results obtained by immunofixation electrophoresis of the subcellular fractions from the differential centrifugation.

DISCUSSION

THE CAT AS AN ANIMAL MODEL FOR ENZYME REPLACEMENT

The principle of enzyme replacement in lysosomal storage diseases (14) has been repeatedly verified in cultured cell systems, but several early attempts at enzyme replacement in patients with lysosomal storage diseases have not given very encouraging results (18). One of the factors that has hindered research on enzyme replacement has been the relative scarcity of animal models of human lysosomal storage diseases (5). The recently described feline Gm_2 gangliosidosis (13), already reasonably well characterized from the anatomopathologic, genetic, biochemical, and enzymatic points of view (36), offers for the first time the opportunity to explore therapeutic strategies of enzyme replacement in a convenient laboratory animal. Thus, these infusion experiments in normal cats should not be viewed simply as a study of clearance and disposition of a lysosomal enzyme in yet another animal species (7, 18, 20, 22, 42), but as a necessary preliminary to enzyme replacement experiments in cats with Gm_2 gangliosidosis.

The choice of human β-hexosaminidase was dictated by several considerations. Structure-dependent properties of human β-hexosaminidase important in enzyme replacement can be assessed *in vivo*, yielding data more directly relevant to therapeutic attempts in man than those obtained by infusion of a feline enzyme very similar to, but not identical with human β-hexosaminidase (13, 36). The absence of immunologic crossreactivity allows detection of human β-hexosaminidase in the presence of feline β-hexosaminidase, and thus, normal cats, rather than enzyme-deficient cats, could be used in these experiments. Although the injection of heterologous enzyme could lead to immunologic complications, this is unlikely to occur in short term experiments (8); on the other

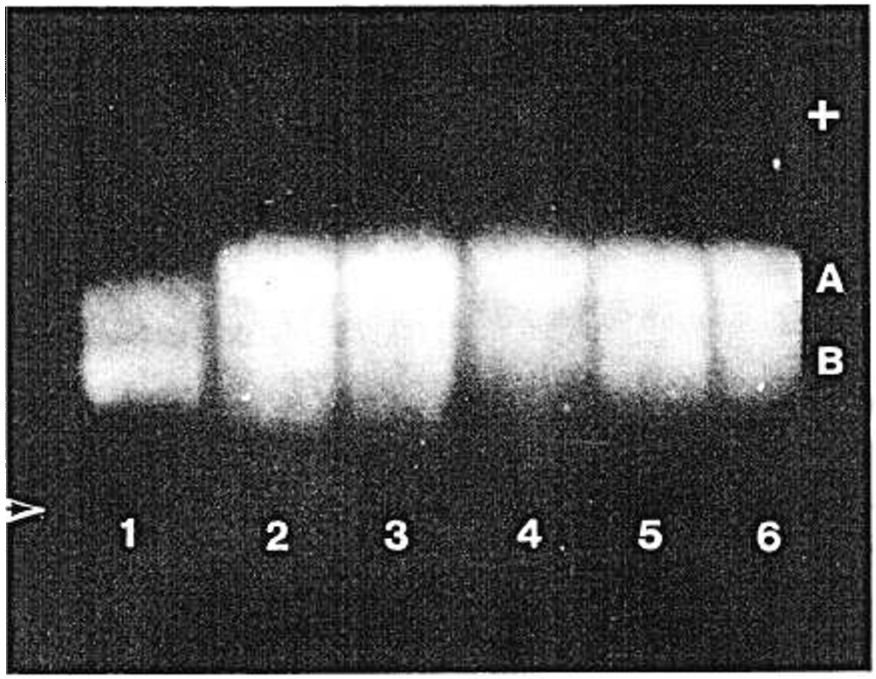

Fig. 4. Fluorescent bands of β-hexosaminidase activity after electrophoresis on Cellogel of plasma from a cat injected with 7.8×10^3 units of human plasma β-hexosaminidase Lane 1: cat plasma before injection. Lane 2: cat plasma with added human plasma β-hexosaminidase, at about the concentration expected after injection. Lanes 3, 4, 5, 6: cat plasma at 4, 24, 54, 74 min from injection, respectively. Notice persistence of recognizable human β-hexosaminidase. Notation as in Figure 2.

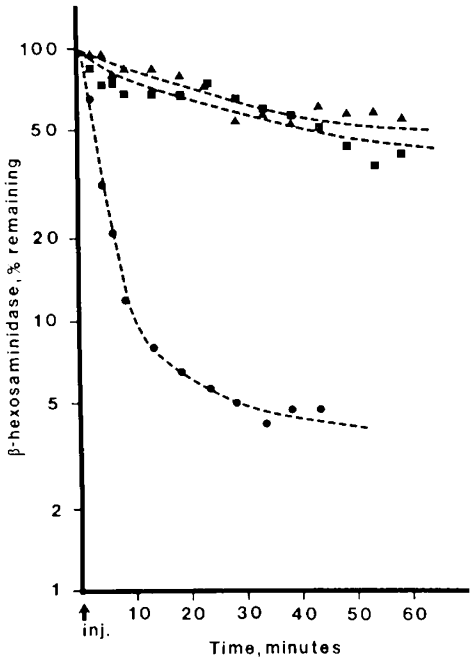

Fig. 5. Inhibition of plasma clearance of human β-hexosaminidase (Hex A + Hex B) after periodate treatment (*triangles*) or liver exclusion (*squares*). The half-life of the enzyme, of the order of 3–4 min in control animals (*circles*), increased to 50–60 min after injection of periodate-treated enzyme or after the surgical procedure. The doses injected were calculated to give similar activity (about 1×10^3 U/ml plasma) in all animals. Residual % activity was calculated as in Figure 1. Each series of points is the average of 3 experiments. As shown by electrophoresis, both human Hex A and Hex B were present in the animals' plasma during the slow clearance phase in the control animals, and at the end of the collection period in the experimental animals.

hand, immunologic complications in chronically infused cats may serve to evaluate the risks of therapeutic infusions in CRM-negative human patients (9). Finally, some controversial aspects of human β-hexosaminidase pathophysiology, such as the role of the different isozymes in the catabolism of Gm_2 ganglioside *in vivo* (30) may be studied in affected animals.

PLASMA CLEARANCE OF HUMAN β-HEXOSAMINIDASE

The low endogenous β-hexosaminidase activity in normal cat plasma allowed a direct assessment of human enzyme clearance kinetics even with relatively low doses of injected enzyme. The rapid clearance of human placental β-hexosaminidase results in a plasma half-life of the order of 3–4 min. This is similar to what has been observed in man (23) and in rat (1, 7) after injection of purified, organ-derived human enzyme; rapid plasma clearance, in fact, appears to be a feature common to many lysosomal enzymes injected iv in man and other species (18, 20, 22, 42). The plasma clearance curve of placental β-hexosaminidase (Fig. 1) apparently consists of a rapid and a slow phase. The estimated half-life of the enzyme in the slow phase is about 45–60 min. Stahl *et al.* (40) have also observed a 2-phase clearance of β-glucuronidase and β-hexosaminidase in the rat, and have proposed that this may reflect heterogeneity of the injected enzyme.

The tendency towards zero-order kinetics at higher doses observed in our experiments (Fig. 3) indicates existence of a receptor mechanism(s), although saturation cannot be ascribed solely to β-hexosaminidase, because the enzyme preparations injected were not homogeneous. This, however, does not invalidate the conclusion that clearance is mediated by a saturable receptor, as contaminants and β-hexosaminidase would have to compete for the same receptor in order for saturation kinetics to be observed. Competitive inhibition of β-glucuronidase clearance by excess β-hexosaminidase and by lysosomal extracts has been observed in the rat (40). Our use of relatively impure enzyme preparations was deliberate, because it offered a convenient way to compare the clearance of Hex A and Hex B in the same animal by simultaneous injection

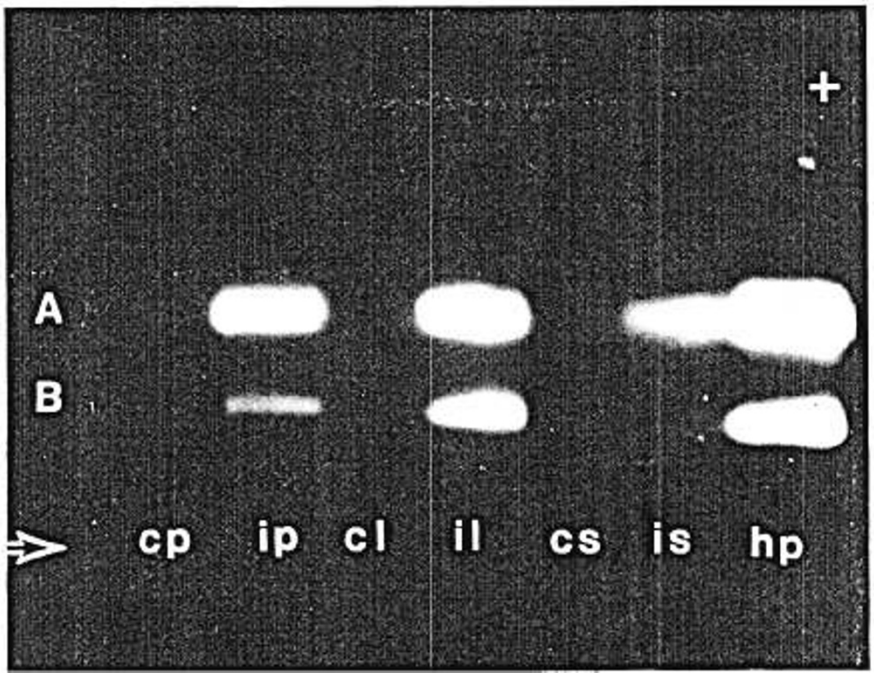

Fig. 6. Immunofixation electrophoresis of subcellular fractions of liver from a cat injected with human β-hexosaminidase (4.8×10^5 U/kg) and from a control animal. Electrophoresis on cellulose acetate gel of extracts of fractions from differential centrifugation was followed by incubation of gels with antihuman β-hexosaminidase immune serum, washing to remove unreacted enzyme, and staining with fluorogenic substrate. Bands of enzymatically active immunoprecipitates corresponding to human Hex A and Hex B (A, B) are visible in postnuclear supernatant (*ip*), lysosomal-mitochondrial (*il*) and soluble-microsomal (*is*) fractions from the injected animal, and in extracts of human placenta (*hp*). No activity is visible in the corresponding fractions (*cp*, *cl*, *cs*) from the control animal. *Arrow* = point of application of samples.

of both isozymes, followed by electrophoresis of plasma. The results (Fig. 2) indicate that, as in the rat (7) Hex B is cleared at approximately the same rate as Hex A, despite the difference in net charge between the two isozymes, from which one might have predicted a more rapid clearance of Hex B (37). Structural differences between the two isozymes may exist, however, which may modulate cellular recognition and uptake in other systems: Hex A, but not Hex B, from human urine is pinocytosed by cultured skin fibroblasts (10).

The much lower clearance rate observed when human plasma-derived β-hexosaminidase was infused in cat (Figs. 1 and 3) agrees well with what has been observed in patients with Gm_2 gangliosidosis after iv infusion of normal plasma (17, 23, 31 and Rattazzi and Davidson, unpublished data) or β-hexosaminidase-rich $(NH_4)_2SO_4$ plasma fractions (17). Although competitive inhibition of clearance by contaminating proteins in the enzyme preparation cannot be excluded, it should be noted that no inhibition of plasma clearance of organ-type β-glucuronidase was observed in the rat by simultaneous injection of plasma proteins in excess (40). Furthermore, the same slow clearance has been observed recently in rats injected with highly purified human plasma enzyme (7). A more likely explanation for the difference in clearance rates between placenta and plasma β-hexosaminidase is the existence of differences in carbohydrate chain composition and/or structure between plasma and organ β-hexosaminidase (11, 21, 27, 35, 46). This may provide a basis for organ-targeting of lysosomal enzymes by manipulation of their carbohydrate composition (25).

HEPATIC UPTAKE

The longer plasma half-life of placental β-hexosaminidase after circulatory bypass of the liver (Fig. 5) indicates that this organ plays a major role in the rapid phase of clearance of the enzyme (Fig. 1). This is confirmed by recovery of a large portion of the injected enzyme in extracts of liver from intact animals. These findings are in good agreement with data obtained in human patients and in experimental animals infused with human β-hexosaminidase and other lysosomal enzymes (1, 17, 18, 20, 39). Other visceral organs may participate in the clearance of the exogenous enzyme, because clearance was not abolished by liver bypass, and small amounts of human enzyme were detected in spleen and kidney. In a preliminary infusion experiment in a β-hexosaminidase deficient kitten (36), the human enzyme detected in the animal liver by direct assay amounted to about 70% of the injected dose, confirming the data obtained by immunotitration in normal animals. The activity in spleen and both kidneys amounted to approximately 0.35 and 1.6% of that in liver, respectively.

There is now convincing evidence that, in the rat, preferential hepatic uptake of lysosomal enzymes is mediated by carbohydrate-specific receptor mechanisms (41). The effect of periodate treatment on the clearance of human β-hexosaminidase in the cat (Fig. 5) strongly suggests that similar mechanisms are also operative in the cat. Studies now in progress in our laboratory (34, 36), indicate that carbohydrate-specific receptors for human β-hexosaminidase do exist in feline liver, and that their specificity (terminal mannosyl and/or N-acetyl-glucosaminyl residues of glycoproteins) is similar to that of the hepatic receptor mechanism described in the rat (2, 41). Knowledge of the specificity of the receptor system may allow to devise means to inhibit hepatic uptake, prolonging the circulatory life of exogenous β-hexosaminidase and increasing the exposure of other target organs. The hemodynamic effects of injection of β-hexosaminidase after liver bypass observed in cats, similar to those observed in dogs (22), indicate that further experiments with purer enzyme preparations, and selective inhibition of β-hexosaminidase uptake in intact animals are needed to evaluate the feasibility of this approach.

SUBCELLULAR LOCATION

The recovery of human enzyme in the mitochondrial-lysosomal subcellular fractions of the recipient animal's liver and its latency

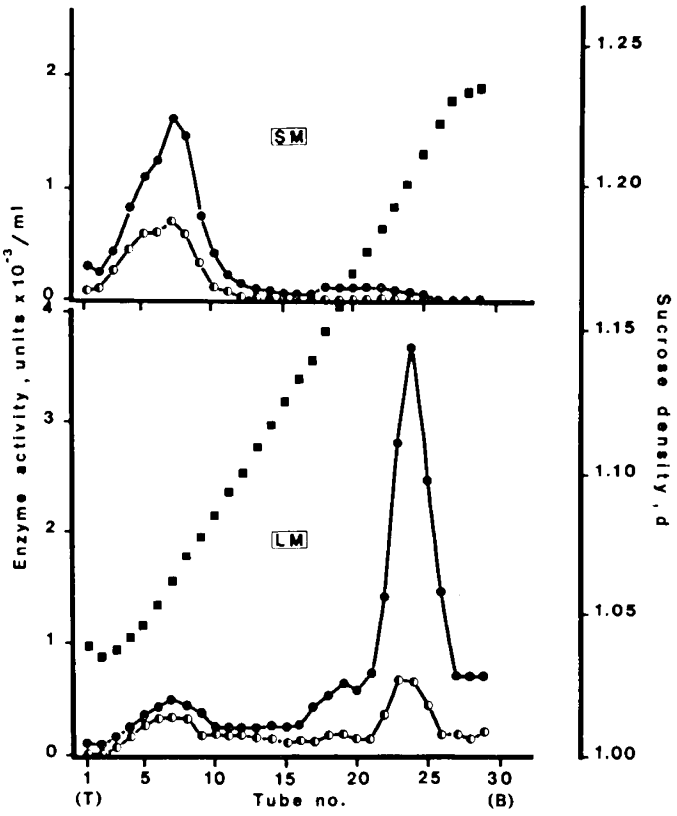

Fig. 7. Isopycnic centrifugation in sucrose gradient (20–50%) of soluble-microsomal fraction (upper half, *SM*) and lysosomal-mitochondrial fraction (lower half, *LM*) from differential centrifugation of liver from a cat injected with human placental β-hexosaminidase. Fractions (1 ml) were collected from the top of the tubes (*T*), and assayed for β-hexosaminidase activity in buffered sucrose (*half-filled circles*), and in buffer containing Triton X-100 (*solid circles*) to determine "latency." The sucrose concentration in each fraction (*solid squares*) was determined by refractometry. One sucrose gradient profile is superimposed to both enzyme sedimentation profiles for ease of comparison.

(Figs. 6–7) indicate that the enzyme was endocytosed and had entered the lysosomal-vacuolar apparatus of the hepatic cells, analogous to what has been observed in the rat (30). Both Hex A and Hex B were recovered in the lysosomal fraction in approximately the same proportions as in the injected material (Fig. 6); it seems possible, therefore, to study *in vivo* the degradation by both human isozymes of stored glycolipids in liver of affected animals. The preliminary data from the infusion experiment in the affected cat (previously discussed) suggest that storage does not significantly affect uptake.

Finally, the presence of human enzyme exhibiting latency in the microsomal-soluble fractions of the isopycnic fractionation may be due, in part, to β-hexosaminidase enclosed in endocytic vacuoles that had not yet undergone fusion with denser primary or secondary lysosomes at the time of removal of the liver (39, 45). The reason for the preponderance of Hex A in this fraction is not clear.

CONCLUSIONS

The data presented above indicate the usefulness of the domestic cat for the study of therapeutic aspects of Gm_2 gangliosidosis. The low endogenous β-hexosaminidase activity in feline plasma and the absence of immunologic crossreactivity between human and feline β-hexosaminidase provide convenient means to study plasma clearance, and organ and subcellular disposition, respectively, of exogenous enzyme in non-β-hexosaminidase-deficient cats. The results so far have shown numerous analogies with those obtained in enzyme replacement experiments in other experimental animals and in human patients. Characterization of hepatic uptake, most likely due to carbohydrate-specific receptors, may contribute to the development of organ targeting methodologies. Most important, the data obtained in normal animals can be applied to critical enzyme replacement experiments in cats with Gm_2 gangliosidosis.

Desnick and Goldberg (16) have recently discussed the numerous obstacles to enzyme replacement in human Gm_2 gangliosidosis, and have pointed out that the prospects for treatment are, at present, extremely discouraging. Although some of these obstacles may well prove unsurmountable, the use of an animal model may allow the formulation and the systematic experimental verification of rational enzyme replacement strategies. These should be thoroughly assessed before the concept of a therapeutic alternative to prenatal diagnosis and selective abortion is abandoned.

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