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Bone marrow transplantation corrects the enzyme defect in neurons of the central nervous system in a lysosomal storage disease

(α-mannosidosis/genetic disease/lysosomal enzyme)

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ABSTRACT **Neuronal storage disorders are fatal neurodegenerative diseases of humans and animals that are caused by inherited deficiencies of lysosomal hydrolase activity. Affected individuals often appear normal at birth but eventually develop progressive neurologic symptoms including sensory and motor deficits, mental retardation, and seizures. We have examined efficacy of bone marrow transplantation as a means of enzyme replacement, using cats with the lysosomal storage disease α-mannosidosis. Treated animals showed little or no progression of neurologic signs 1–2 years after transplant, whereas untreated cats became severely impaired and reached end-stage disease by 6 months of age. Increased lysosomal α-mannosidase activity was found in brain tissue of the treated animals, and electron microscopy revealed no evidence of lysosomal storage within most neurons. Histochemical localization of acidic α-D-mannoside mannohydrolase (EC 3.2.1.24), using 5-bromo-4-chloro-3-indolyl α-D-mannopyranoside, showed that functional enzyme was present in neurons, glial cells, and cells associated with blood vessels. This study provides direct evidence that bone marrow transplantation as treatment for a neuronal storage disease can lead to significant levels of a missing lysosomal hydrolase within neurons of the central nervous system and to compensation for the genetic metabolic defect.**

Lysosomal storage disorders are autosomal recessive diseases caused by deficient activity of specific acid hydrolases. Defective hydrolytic activity within lysosomes results in accumulation of unmetabolized substrates and an expanding cascade of metabolic derangements within cells. A variety of such conditions are known and many, like Tay–Sachs disease, primarily exhibit central nervous system (CNS) dysfunction. Affected individuals often appear normal at birth but subsequently demonstrate a range of progressive symptoms including sensory deficits, movement disorders, mental retardation or dementia, and seizures.

Bone marrow transplantation (BMT) as a means of treatment for neuronal storage diseases has been examined in children (1) and in animal models (2), but its effectiveness remains controversial. The rationale for its use is based on delivery of enzyme from normal, donor-derived hematopoietic cells to diseased host cells after transplantation (3). Although evidence for enzymatic correction in liver and other visceral tissues in storage diseases after BMT is substantial, similar findings on enzyme entry and substrate reduction in neurons and other cells of the CNS has been more difficult to achieve, especially in children.

Animal models of lysosomal storage diseases offer a unique opportunity to examine mechanisms of pathogenesis and to evaluate therapeutic strategies. In this paper we report the results of studies evaluating efficacy of BMT for treatment of the neuronal storage disorder α-mannosidosis, using a disease model available in cats. α-Mannosidosis in children is characterized by mental retardation, skeletal dysplasia, facial coarsening, motor incoordination and ataxia, and hearing loss (4). Activity of acidic α-D-mannoside mannohydrolase (α-mannosidase) is markedly reduced or absent and intracellular storage of mannose-rich oligosaccharides is widespread in all tissues. Inherited deficiency of lysosomal α-mannosidase in cats is well-documented and shows clinical, morphological, and biochemical features closely resembling those of the human disease (5–7). Our studies using BMT to treat feline α-mannosidosis indicate that it is remarkably effective in ameliorating the disease process. A preliminary report of these findings has appeared (8).

MATERIALS AND METHODS

BMT was performed on three kittens with α-mannosidosis at ages 8, 10, and 12 weeks (animals 192, 171, and 191, respectively) by using phenotypically normal siblings as donors. (Since α-mannosidosis is an autosomal recessive condition, this group included homozygous and/or heterozygous normal animals.) Newborn kittens from matings of obligate heterozygotes for α-mannosidosis were identified as homozygous recessive—i.e., diseased—on the basis of levels of acidic α-D-mannosidase activity in homogenates of tail tissue biopsies. Animals with α-mannosidosis displayed virtually no enzyme activity, whereas phenotypically normal animals demonstrated activity ranging from 150 to 750 nmol of substrate (4-methylumbelliferyl α-D-mannopyranoside) cleaved per hr per mg of protein.

Kittens lacking α-mannosidase activity exhibit vacuolation of neurons and other cells during the early postnatal period, with these changes becoming progressively more severe with time. Clinical signs are first observed at about 6 weeks of age and consist of mild dysmetria and intention tremor. Cerebellovestibular signs progress steadily and advance to opisthotonos and ataxia. Affected kittens are smaller than phenotypically normal littermates and by 2 months of age do not respond to sound. End-stage disease is reached by 6–8 months of age.

Abbreviations: BMT, bone marrow transplantation; CNS, central nervous system; CSF, cerebrospinal fluid; X-Man, 5-bromo-4-chloro-3-indolyl α-D-mannopyranoside.

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BMT Procedure. The affected animals and their donors were shipped to the Feline Marrow Transplant Laboratory at Colorado State University a week before the planned transplant. A standard BMT protocol adapted for cats was used (9). Total body irradiation was delivered to the kittens with α-mannosidosis in 10.0-Gy doses given in six fractions over 3 days with a 6-MV linear accelerator. Immediately after the sixth fraction of irradiation, kittens were given $1.2–1.8 \times 10^9$ donor-origin nucleated marrow cells per kg of body weight by intravenous injection. A second injection of donor marrow cells ranging from 4.7×10^5 to 1.2×10^9 cells per kg was given 2 days later. Cyclosporin (15 mg/kg, orally once daily) as prophylaxis against graft-versus-host disease was administered on days -1 and 0 and daily on days 8–100 post-BMT. To prevent infection following immunosuppression, selective decontamination of the intestinal tract was achieved by administering oral ciprofloxacin (Baytril, 2.5 mg/kg, twice per day) from day -6 to day $+14$. Mefoxin (5 mg/kg, three times per day) was injected subcutaneously from day 5 to day 21. By days 10–14, the treated cats showed evidence of engraftment consisting of increased leukocyte counts and concomitant elevated levels of acidic α-mannosidase in leukocyte preparations. The animals receiving transplants at 10 and 12 weeks of age were allowed to survive for 21 and 11 months post-BMT, respectively, whereas the animal transplanted at 8 weeks is currently 26 months old.

Tissue Collection. Tissues from two cats receiving BMT, their respective normal controls (nos. 172 and 193), and two untreated kittens with α-mannosidosis, aged 2 weeks (no. 199) and 9 weeks (no. 174), were evaluated in this study. Animals were preanesthetized with acepromazine (0.5 mg/kg) and atropine (0.03 mg/kg) and anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally). In some cases a cerebrospinal fluid (CSF) tap was performed and 0.5 ml of CSF was removed from the cisterna magna. A craniotomy, allowing exposure of the lateral suprasylvian gyrus area was followed by a thoracotomy for intracardiac perfusion. For the first group of animals (nos. 171, 172, and 199) cold saline perfusion to clear the vasculature was followed by removal and freezing of biopsy samples of cerebral cortex, liver, and other tissues. The animals then were perfused with 1 liter of cold fixative (4% paraformaldehyde/0.1% glutaraldehyde) and the brain and selected tissues were removed and immersed in additional fixative for a total of 3 hr (cold), followed by storage in phosphate buffer (4°C). Some of the collected tissues were placed in 20% sucrose/0.1 M phosphate buffer (pH 7.2) and subsequently frozen for cryostat sectioning. For the second group of animals (nos. 191 and 193), cold saline perfusion was followed by immediate removal of the brain. Tissues were dissected by region and frozen, along with samples of visceral tissues, for later enzyme analysis. Several small blocks of cerebral cortex, cerebellum, and visceral tissues were immersion-fixed for 5–6 hr in the same fixative described above, or in 4% paraformaldehyde/2% glutaraldehyde, and were stored in cold phosphate buffer. For all of the above animals small samples of brain and viscera were taken and placed in a 4% paraformaldehyde/2.5% glutaraldehyde fixative overnight and then processed for routine EM. The second, untreated mutant animal (no. 174) was anesthetized and perfused according to the protocol given above, but without biopsy removal, followed by EM processing of brain and visceral tissue. Procedures in this study were performed in accordance with recommendations and approval of the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

Enzyme Analysis. The assay for α-mannosidase activity was carried out on tissue samples homogenized with 20 volumes of 10 mM sodium phosphate buffer (pH 7.2). Homogenates were centrifuged at $10,000 \times g$ for 10 min, and supernatants were diluted 10-fold in sodium phosphate buffer. Samples of 10 and 20 μl were assayed in a final volume of 200 μl of 0.05–0.1 M citrate–phosphate buffer (pH 3.8) with 1.5 mM $ZnCl_2$ and 4-methylumbelliferyl α-D-mannopyranoside (Sigma) for 1 hr at 37°C. CSF samples were processed without centrifugation and at dilutions no greater than 1:2. For all samples, the reaction was stopped with 2 ml of 0.2 M glycine/NaOH (final pH 10.6) and fluorescence was read (365-nm excitation, 448-nm emission). Protein determination was done by the Lowry method (10). All assays were run in duplicate.

Enzyme Histochemistry. Histochemical demonstration of acidic α-mannosidase activity was performed as follows. The reaction mixture contained the substrate 5-bromo-4-chloro-3-indolyl α-D-mannopyranoside (X-Man, 15 mg/ml; Sigma) solubilized with dimethyl sulfoxide, 1.5 mM $ZnCl_2$, 5–20 mM $K_4Fe(CN)_6$, 5–20 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$ in 0.05 M citrate buffer (pH 3.8). This mixture was placed as droplets ($\approx$0.2 ml) over 20-μm cryostat sections mounted on gelatinized slides. Alternatively, 35-μm vibratome sections were reacted free floating in vials containing reaction mixture. After overnight incubation at 37°C, the sections were washed three times in citrate buffer and processed for light microscopy. Some sections were counterstained with safranin. For EM, vibratome sections were reacted as described above, dehydrated, and embedded in Epon. Ultrathin sections were examined without additional staining.

Other Morphologic Studies. Immunocytochemical staining procedures for glutamate decarboxylase, as a marker of axonal spheroid formation, and for GM2 ganglioside, a known secondary storage compound, were performed according to published procedures (11, 12). To identify microglial cells, vibratome sections were processed by using *Griffonia simplicifolia* I-B_4 isolectin conjugated to peroxidase (Sigma) (13).

RESULTS

During the posttransplant period, all of the treated animals were alert and active, were responsive to sound, gained weight, and appeared healthy. The cat receiving a transplant at 10 weeks of age (no. 171) was neurologically normal when killed 21 months later but exhibited bilateral grade 1 medial patellar luxations and a left craniodorsal coxofemoral luxation. The cat receiving a transplant at 12 weeks (no. 191) and killed 11 months later exhibited pelvic limb joint deformities as well as mild cerebellar ataxia and positional vertical nystagmus. The cat transplanted at 8 weeks (no. 192), which is still alive, lacks skeletal system deformities but exhibits mild, nonprogressive gait and postural abnormalities attributable to cerebellar dysfunction.

Light and electron microscopic studies of CNS tissue available from two cats receiving BMT revealed an absence of storage vacuoles in most cells, whereas the untreated 9-week-old kitten with α-mannosidosis exhibited ubiquitous intracellular storage in brain (Fig. 1 *A* and *B*). In treated cats, neurons and most other cell types in the cerebral cortex, thalamus, caudate, brainstem, and spinal cord appeared entirely normal. Conspicuous numbers of clear cytoplasmic vacuoles characteristic of oligosaccharide storage were present only in occasional non-neuronal-like cells scattered throughout the brain (Fig. 1*C*). These cells subsequently were identified as microglia by B_4 isolectin binding (Fig. 1*D*). Neurons with identifiable cytoplasmic vacuoles were found only in the cerebellum, but the degree of storage was considerably less than that observed in the 9-week-old untreated affected animal. A mild loss of Purkinje cells also was evident in the two treated animals. Immunocytochemical staining of the cerebellum with antibodies to glutamate decarboxylase, the enzyme synthesizing γ-aminobutyric acid, revealed occasional spheroids within axons of Purkinje cells and other

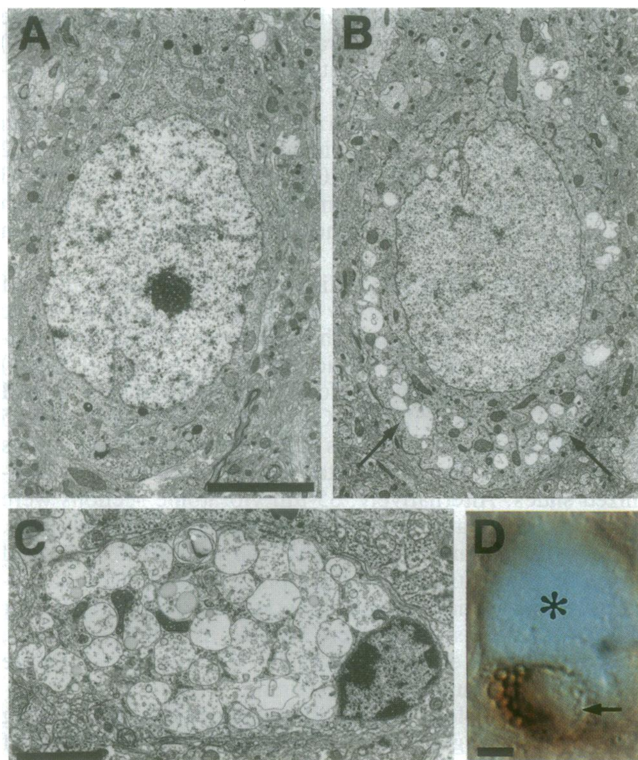

FIG. 1. Cells in the cerebral cortex of cats with α-mannosidosis after BMT (*A*, *C*, and *D*) and without treatment (*B*). (*A*) EM of a representative pyramidal neuron from a treated cat (no. 171) showing the complete absence of storage vacuoles. (*B*) EM of a pyramidal neuron from a 9-week-old untreated affected cat (no. 174) showing characteristic cytoplasmic vacuoles (arrows). (*C*) EM of a microglial-like cell in the treated cat (no. 171) exhibiting cytoplasmic vacuoles characteristic of α-mannosidosis. (*D*) B_4 isolectin staining indicating microglial status of a cell like that in *C*. Asterisk, adjacent unstained neuron. (Bar in *A* = 5 μm and also applies to *B*; bar in *C* = 2 μm; bar in *D* = 5 μm.)

cerebellar neurons, but the extent of this neuroaxonal dystrophy was minimal compared with that observed in untreated cats with α-mannosidosis (11). Secondary abnormalities known to accompany the primary lysosomal defect in cerebral cortex of cats with α-mannosidosis—namely, the accumulation of GM2 ganglioside within neuronal somata and the growth of ectopic dendrites on pyramidal neurons (12)—also were seen infrequently compared with untreated affected animals.

Activity of acidic α-mannosidase was determined in tissues from the BMT-treated animals, normal animals, and an untreated 2-week-old affected kitten. As shown in Table 1, enzyme activity attained in various tissues from the treated animals differed but in all cases was greater than that measured in an untreated animal with α-mannosidosis. The liver, for example, demonstrated nearly half the α-mannosidase activity of liver of a normal cat, whereas activity in kidney was only 3–6% of normal. For the CNS, α-mannosidase activity ranged from 9% to 40% of normal. Greater increases in activity (>100% of normal) were found in CSF and in bone marrow of the treated animals.

Histochemical staining using X-Man allowed for determination of the exact location of acidic α-mannosidase activity in tissue sections. When carried out on brain tissue from normal cats, this procedure revealed α-mannosidase activity within the cytoplasm of individual neurons and other cells, with the histochemical reaction product appearing as small, blue, granular or crystalloid puncta distributed in a lysosomal-like pattern (Fig. 2*A*). In contrast, the untreated cat with α-mannosidosis showed little or no detectable reaction product within the cytoplasm of cells (Fig. 2*B*). Affected cats receiving BMT exhibited positively labeled neurons, glial cells, and cells associated with blood vessels (Fig. 2 *C* and *D*). Occasional neurons exhibited blue reaction product in the cytoplasm that was nearly equivalent to that observed in cortical neurons in normal animals, whereas other cells contained only one or two positive crystals or granules, or no clearly distinguishable reaction product. Cells exhibiting the greatest amount of X-Man staining were found in association with blood vessels, contained cytoplasmic vacuoles with lipid-like material, and at the ultrastructural level resembled perivascular microglia (Fig. 3).

Table 1. Activity of α-mannosidase in tissues collected in this study

Tissue type	Activity, nmol per hr per mg (% normal)				
	No. 171 BMT	No. 172 normal	No. 191 BMT	No. 193 normal	No. 199 diseased
Cerebral cortex	22 (12)	182	27 (14)	186	0
Cerebellum	ND	ND	27 (14)	195	ND
Thalamus	ND	ND	11 (9)	119	ND
Medulla	ND	ND	31 (15)	201	ND
Spinal cord	ND	ND	48 (40)	120	ND
CSF	238 (182)	131	109 (—)	ND	ND
Liver	176 (40)	443	153 (50)	305	1.1
Kidney	31 (6)	551	19 (3)	557	0.4
Pancreas	60 (18)	336	62 (27)	231	0.4
Bone marrow	548 (112)	489	588 (255)	231	ND
Plasma	34 (94)	36	14 (54)	26	0.1

Cat 171 underwent BMT at 10 weeks of age and was killed 21 months later. Enzyme activity is compared with that of an age-matched, phenotypically normal sibling (no. 172) and percentage of normal activity is given in parentheses. Cat 191 underwent BMT at 12 weeks of age and was killed 11 months later. Enzyme values are compared with that of its donor (no. 193) and percentage of normal is given in parentheses. Enzyme data for an untreated 2-week-old kitten with α-mannosidosis (no. 199) also are shown. In some cases no tissue was available for enzyme study (not done, ND).

DISCUSSION

This study demonstrates that BMT in feline α-mannosidosis results in the appearance of acidic α-mannosidase activity in neurons and other cells of the CNS concomitant with an absence of intracellular storage vacuoles. The most likely explanation for this finding is that α-mannosidase present in cells derived from donor bone marrow is available to and can enter cells of the CNS after transplantation. The α-mannosidase present in diseased cells appeared to be metabolically active, since no storage was present in most neurons even 2 years after treatment. Our study also indicates that storage was not only prevented but also reversed, since most of the vacuolation known to be present in neurons at the time of transplantation (Fig. 1*B*) disappeared after treatment.

The mechanism allowing for this putative transfer of acidic α-mannosidase from donor-derived cells to neurons of the CNS following BMT is unknown. Previous experience with therapeutic strategies utilizing purified enzyme has underscored the problems of the blood–brain barrier and the low endocytic rate of neurons. In view of the low concentration of lysosomal enzyme in plasma, it is unlikely that neurons acquired α-mannosidase through uptake of extracellular enzyme derived from the circulation, unless a highly efficient adsorptive endocytic mechanism was involved (14). It may be more likely that neuronal enzyme replacement was achieved following establishment of locally high concentrations of enzyme by cells of hematogenous origin that invaded the

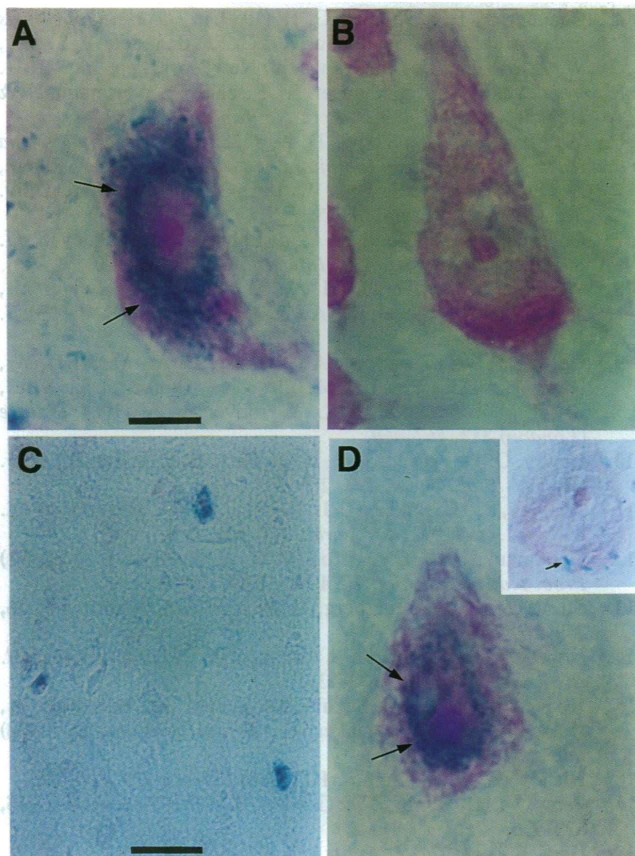

FIG. 2. Histochemical (X-Man) staining of tissue sections of cerebral cortex in a normal cat, in an untreated cat with α-mannosidosis, and in a cat with α-mannosidosis that received BMT. (*A*) Cortical neuron from a normal cat (no. 172) demonstrating X-Man staining as blue puncta distributed in a lysosomal-like pattern in the perikaryal cytoplasm (arrows). (*B*) A pyramidal neuron from a cat with α-mannosidosis that did not receive BMT (no. 199) reveals no X-Man staining. (*C*) Low-power photomicrograph of cerebral cortex from a cat with α-mannosidosis that received BMT (no. 191) demonstrates prominent X-Man staining in three neurons. (*D*) Higher-power photomicrograph of a pyramidal neuron in the same tissue as *C*, showing X-Man staining with a perikaryal distribution (arrows) similar to that seen in normal neurons. Other neurons (*D Inset*) exhibited only occasional blue cytoplasmic puncta (arrow). (All sections were counterstained with safranin; bar in A = 10 μm and also applies to *B* and *D*; bar in *C* = 50 μm.)

CNS—i.e., brain macrophages or microglia (1)—and our finding of strongly X-Man-positive microglial-like cells near blood vessels is consistent with this possibility. The presence of these cells could be a consequence of pathologic events occurring in brain secondary to the disease itself, as has been suggested by BMT experiments on mice with Krabbe disease (15) or, alternatively, a physiologic process associated with the proposed *normal* invasion of monocytes that is believed to at least partly underlie the establishment and turnover of the resident macrophage or microglial population of brain (16, 17). It is conceivable that these cells represent the source of lysosomal enzyme, since some microglia contain abundant lysosomal enzyme activity (18), and cells of hematogenous origin have the capacity to pass lysosomal hydrolases, including α-mannosidase, to enzyme-deficient fibroblasts in cocultures (19, 20). Indeed, some experimental studies have suggested that transfer of lysosomal enzymes occurs normally between cells in a variety of tissues (21, 22).

Not all brain regions in cats receiving BMT showed the same degree of effective enzyme replacement. The cerebellum, in particular, revealed the presence of occasional neu-

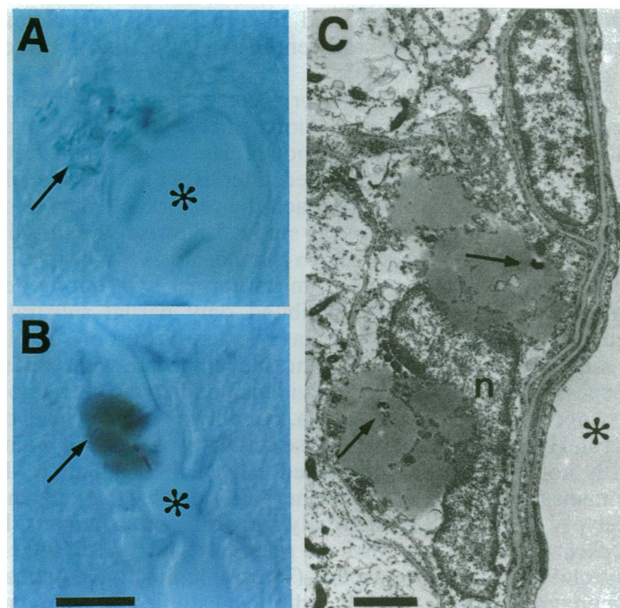

FIG. 3. Histochemical (X-Man) staining of perivascular cells of cerebral cortex in a cat with α-mannosidosis that received BMT (no. 171). (*A*) A microglial-like cell (arrow) adjacent to a blood vessel (asterisk) and exhibiting robust X-Man staining (vibratome section). (*B*) Similar cell as seen in a 2-μm plastic section. Note the presence of lipid inclusions in addition to X-Man reaction product (arrow). (*C*) EM of a cell like those in *A* and *B*, shows perivascular location, microglial-like nucleus (n), lipid inclusions, and X-Man reaction product (arrows). These cells were easily distinguished from typical endothelial cells (see adjacent cell with unlabeled nucleus), which did not contain lipid or detectable amounts of X-Man reaction product. Asterisks, blood vessel lumen. (Bar in *B* = 10 μm and also applies to *A*; bar in *C* = 2 μm.)

rons with storage vacuoles and axonal spheroids, as well as a loss of Purkinje cells. Clinical evidence of cerebellar disease was observed in two of the three cats with α-mannosidosis given BMT. Yet enzyme analysis showed α-mannosidase activity in cerebellum to be equivalent to that of cerebral cortex, and X-Man histochemistry revealed enzyme within surviving Purkinje cells. The severe cerebellar pathology and dysfunction predominating in untreated cats with α-mannosidosis has suggested that this brain region is particularly vulnerable to a deficiency of lysosomal α-mannosidase (6). Such vulnerability may indicate that pathologic changes occur very early and that clinical disease would be prevented only if treatment were initiated earlier than in the present study. Alternatively, Purkinje cells may have a greater requirement for α-mannosidase and be effectively treated only if a higher level of enzyme delivery can be achieved.

The critical threshold of lysosomal hydrolase activity required to compensate for the metabolic defect and prevent storage in different kinds of cells in neuronal storage disorders is not precisely known. Recent studies attempting to understand the late-onset chronic forms of lysosomal β-hexosaminidase A and arylsulfatase A deficiencies, using substrate-fed fibroblasts in culture, have indicated that this threshold can be as low as 10–15% of normal (23), a level comparable to the α-mannosidase activity achieved in many tissues in the present study. Earlier BMT studies using canine models of fucosidosis (24) and mucopolysaccharidosis type I (25) and murine mucopolysaccharidosis type VII (26) demonstrated enzyme activity (α-L-fucosidase, α-L-iduronidase, and β-glucuronidase, respectively) in brain ranging from 1% to 20% of normal, concomitant with reduced amounts of intraneuronal storage. These findings provide additional support for the view that BMT can result in transfer of lysosomal

hydrolases into the CNS and that the levels of enzyme activity achieved can be sufficient to at least partially correct a genetic metabolic defect.

Whether there are disease- or species-related factors that make feline α-mannosidosis more amenable to BMT than other storage disorders is unknown. The reduced substrate levels in CNS neurons reported after BMT in the models described above did not approach the remarkable degree of correction seen in the present study. Likewise, a naturally occurring chimeric calf with α-mannosidosis which had a transplant of lymphocytes from its normal twin during development *in utero* also exhibited only partial substrate reduction in brain (27). There has been one report of BMT in a child with α-mannosidosis who succumbed 18 weeks after successful engraftment (28). Evidence of enzyme correction was detected in liver and spleen, but only a very low level of normal lysosomal α-mannosidase activity was found in brain, with no evidence of decreased neuronal vacuolation. It remains to be determined whether this indicates that enzyme delivery to the CNS is inherently more difficult in humans. It is perhaps more likely that the relatively later age at which this child received a transplant (7 years) limited the potential for enzyme transfer. Clearly, additional studies using animal models are warranted as a means to determine the exact mechanism of enzyme delivery and the relative efficacy of transfer of different lysosomal enzymes involved in different storage diseases. The impact of other factors, such as substrate mobility and accessibility, the chronology and rate of substrate accumulation, and the specific processes contributing to pathogenesis of neuronal dysfunction, also must be determined, since all of these factors may differentially influence the outcome of BMT. Nevertheless, the present study provides direct evidence that a lysosomal hydrolase present in cells derived from donor bone marrow can gain access to CNS neurons in one of these diseases and significantly compensate for the genetic metabolic defect.

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