

# Globoid Cell Leukodystrophy in Cairn and West Highland White Terriers

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**Krabbe disease or globoid cell leukodystrophy (GLD) is an autosomal recessive disorder resulting from the defective lysosomal hydrolysis of specific galactolipids found primarily in myelin. This leads to severe neurological symptoms including seizures, hypotonia, blindness, and death, usually before 2 years of age in human patients. In addition to human patients, several animals, including dog, mouse, and monkey, have the same disease caused by a deficiency of galactocerebrosidase (GALC) activity. In this article we describe studies in cairn and West Highland white terriers (WHWT) affected with GLD. Through a screening test based on the molecular defect found in these breeds, over 50 cairn terrier carriers have been identified and a colony of five carrier dogs has been established. Affected dogs from this colony plus an affected WHWT were available for study. An affected WHWT was evaluated by magnetic resonance imaging at 6 and 11 months of age and pronounced changes in the T-2 weighted fast spin-echo images were found. Biochemical and pathological evaluation of the same dog after euthanasia at 12 months of age showed a large accumulation of psychosine in the brain and white matter filled with globoid cells. Some comparisons were made to younger affected and carrier dogs. Studies have shown successful transduction of cultured skin fibroblasts from an affected dog and normal canine bone marrow using a retroviral vector containing the human GALC cDNA. Successful treatment of this canine model will lead to studies in some humans with GLD.**

Globoid cell leukodystrophy (GLD), or Krabbe disease, is a neurological disease caused by mutations in the gene for galactocerebrosidase (GALC). This enzyme is responsible for the lysosomal degradation of specific galactolipids including several important in the production of compact, stable myelin. A failure to adequately degrade galactosylceramide and psychosine (galactosylsphingosine) results in the characteristic pathological findings observed in tissue from humans and animals affected with GLD. Most human patients present with symptoms before six months of age and die before 18 months of age [see Wenger (1997) for a review]. Older children and adults are also diagnosed with GLD. Since the cloning of human GALC cDNA (Chen et al. 1993; Sakai et al. 1994) and gene (Luzi et al. 1995), molecular studies on these patients could be undertaken. The phenotypic differences in the human patients result from the wide range of mutations identified in human patients [see Wenger (1997) for a review]. In addition to humans with GLD, naturally occurring animal models have been de-

scribed (Baskin et al. 1989; Fankhauser et al. 1963; Fletcher et al. 1966; Johnson 1970; Kobayashi et al. 1980; Pritchard et al. 1980; Suzuki et al. 1970; Suzuki and Suzuki 1985). The cloning of mouse (Sakai et al. 1996), dog (Victoria et al. 1996), and rhesus monkey (Luzi et al. 1997) GALC genes and the identification of the disease-causing mutations will facilitate the use of these models in studies to evaluate pathogenetic mechanisms and treatment options. This is feasible because of the ability to accurately identify carriers and affected fetuses by DNA analysis of small samples.

Through a testing program for cairn terriers and West Highland white terriers (WHWT) we have identified more than 50 carriers of GLD, and 5 carrier cairn terriers have been donated to the School of Veterinary Medicine at the University of Pennsylvania. We also identified an affected WHWT and were able to perform magnetic resonance imaging (MRI) on this dog at 6 and 11 months of age. When this dog was sacrificed, biochemical and pathological studies were performed to establish

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the long-term consequences in an untreated dog.

Previous studies (Rafi et al. 1996) have demonstrated that human GALC cDNA in a retroviral vector can readily transduce GALC-deficient cells, and these overexpressing cells can transfer GALC activity to other deficient cells. Similar studies were done with affected canine fibroblasts and normal canine bone marrow cells. The availability of this well characterized canine model will permit treatment trials in the near future.

## Materials and Methods

### Establishment of a Colony of Cairn Terriers and Genotyping of Individual Dogs

Using the method of mutation analysis previously described (Victoria et al. 1996), more than 350 cairn terriers have been screened using small blood samples. DNA was isolated from 0.1–0.5 ml heparinized blood samples using the materials and methods provided by Qiagen. The DNA was dissolved in 0.1 ml distilled water and 5–10  $\mu$ l aliquots were subjected to polymerase chain reaction (PCR) amplification. Following amplification, the fragment was digested with *Mnl*I and the products were visualized by ethidium bromide staining in 3.5 % metaphore (FMC) gel (Victoria et al. 1996). More than 50 carrier dogs have been identified, and five were donated to start our breeding colony. At the end of April 1997 the first litter was born. Three puppies—one carrier, one affected, and one noncarrier—were born dead, and two puppies—one affected and one carrier—survived. In addition, an affected WHWT was diagnosed by low GALC activity in leukocytes and by mutation analysis.

### Magnetic Resonance Imaging of an Affected WHWT at 6 and 11 Months of Age

Anesthesia was induced with intravenous oxymorphone, diazepam, and thiopental, an endotracheal tube was passed, and anesthesia was maintained with the inhalant gas isoflurane. Imaging was performed at 1.5 Tesla (T) using a dedicated research MR scanner (Signa, General Electric Corp., Milwaukee, WI). An extremity coil was used in transmit-receive mode to acquire the images. Imaging sessions consisted of sagittal conventional spin-echo imaging with short TR/TE localizer, axial conventional spin-echo imaging with short TR/TE, and axial fast-spin echo imaging with long

TR/TE sequences. T1-weighted images were generated using TR = 550 ms, TE = 10 ms, 128 phase encode steps, and 256 data points. T2-weighted images were generated using TR = 4000 ms, TE = 113 ms, 192 phase encode steps, and 256 data points. Gadolinium dimeglumine (Gd-DTPA; Magnevist; Berlex Laboratories, Wayne, NJ) was administered intravenously at a dose of 0.3 ml/kg in order to generate contrast-enhanced, T1-weighted images.

### Pathology of Brain and Sciatic Nerve Samples from the Affected Newborn Cairn Terrier and the 12-Month-Old Affected WHWT

Euthanasia of the 12-month-old WHWT was performed using concentrated sodium pentobarbital in accordance with the American Veterinary Medical Association guidelines. Tissues from the affected cairn terrier who died near birth were also examined. Tissues samples for light microscopy were fixed in buffered 10% formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin.

### Lipid Analysis of Brain Samples from Affected Cairn Terriers and the 12-Month-Old Affected WHWT

Small samples of brain from an affected cairn terrier who died near birth, 4-month-old affected and carrier cairn terriers, and an affected 12-month-old WHWT were extracted according to the method of Fujita et al. (1996). Because of the condition of the brain it was not possible to separate white and gray matter in the brain of the youngest puppy. The psychosine concentration was determined on 20–40 mg tissue samples using high-pressure liquid chromatography as described by Matsu-moto et al. (1998).

### Transduction of Canine Cells with Retrovirus Containing Human GALC cDNA

Human GALC cDNA containing the modified initiation codon region was placed in the MFG vector as described previously (Rafi et al. 1996). Virus-containing supernate from the  $\psi$ -crip amphotropic packaging cell line was used to infect cultured skin fibroblasts from an affected cairn terrier and bone marrow cells from a normal dog growing on a stromal layer. Virus in Dulbecco's Modified Eagle medium supplemented with 10% fetal calf serum and 2 mM glutamine was placed on the cells for 24 h. After three or two rounds of infection (fibroblasts or bone marrow cells, respec-

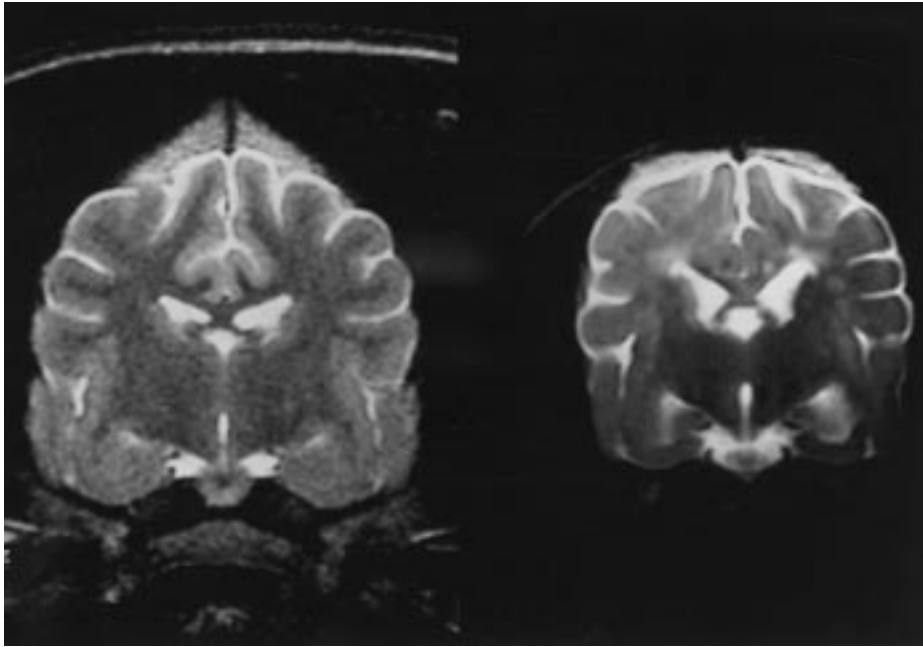
tively), the cells were harvested and assayed for GALC activity using galactose-labeled galactosylceramide as described (Rafi et al. 1996).

## Results

Since the identification of the mutation (A to C transversion at cDNA position 473, changing tyr 158 to ser) causing GLD in WHWT and cairn terriers, we have screened more than 350 cairn terriers and have identified 54 carriers. This high carrier rate does not reflect the true carrier frequency in these breeds as it reflects bias of ascertainment because many of the dogs tested came from breeders and owners with dogs from a line that has GLD. This method provides rapid and accurate genotyping for all WHWT and cairn terriers using any tissue sample available. Mutation analysis for carrier identification is superior to measurement of GALC activity because of the wide range of GALC values in peripheral blood leukocytes. It also will permit prenatal testing using small fetal samples such as chorionic villus biopsy and amniotic fluid cells.

Through the efforts of the carrier screening program for cairn terriers, five carriers (three females, two males) have been donated for breeding purposes. Five puppies were born to one of these young female carriers; three died at birth and two survived. Genotyping revealed that the dead puppies consisted of one affected, one carrier, and one noncarrier, and the living puppies consisted of one affected and one carrier. The affected puppy was not treated and it was euthanized at 4 months of age. Samples from the dead puppies and the affected 12-month-old WHWT, who was euthanized at 1 year of age when symptoms (blindness, tremors, incontinence, severe hind leg weakness) became overwhelming, were obtained for lipid analysis and pathological examination.

The affected WHWT was diagnosed at 6 months of age and MRI was performed. Imaging at 1.5 T revealed mild hydrocephalus and increased signal intensity in the corpus callosum on T1-weighted images and bilaterally symmetrical increases in signal intensity of the corpus callosum, centrum semiovale, internal capsule, corona radiata, and cerebellar white matter on T2-weighted images (Figure 1) (Cozzi et al., in press). A decrease in signal intensity of the thalamus and caudate nucleus was found on T2-weighted images. Gadolinium-enhanced T1-weighted images

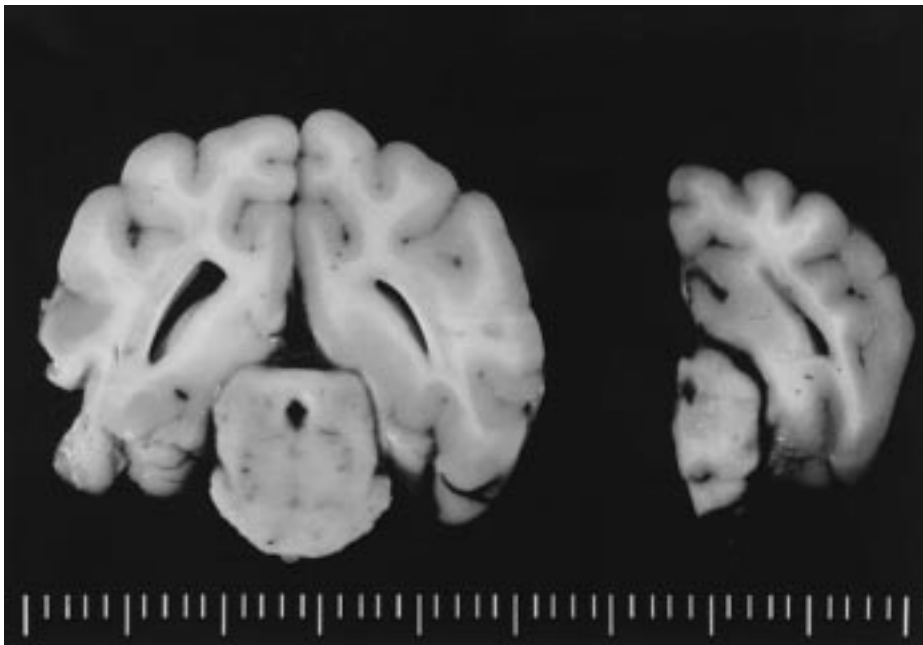


**Figure 1** T-2 weighed fast spin-echo images of two dogs at the level of the pituitary gland. The image on the right is from the 6-month-old affected WHWT; the image on the left is from an age-matched healthy dog. Used with permission, J Small Anim Pract.

showed symmetrical enhancement of the corpus callosum, internal capsule, and corona radiata. T2-weighted images generated at 11 months of age showed progression of the signal abnormalities throughout the white matter and increased gadolinium-enhancement of the white matter (not shown).

Grossly the lateral ventricles of the brain of the 12-month-old affected WHWT

appeared slightly dilated, and the white matter was prominent and bright (Figure 2). Histologically there were massive accumulations of globoid cells in the white matter throughout the brain (Figure 3), extending to the limits of the corona radiata, which correlated with the findings made by MRI. There were areas of extensive gliosis and lymphocytic perivascular cuffs in the white matter. The gray matter ap-



**Figure 2** Gross anatomy of the brain at the level of the midbrain from the 12-month-old affected WHWT (right side) and age-matched control (left side).

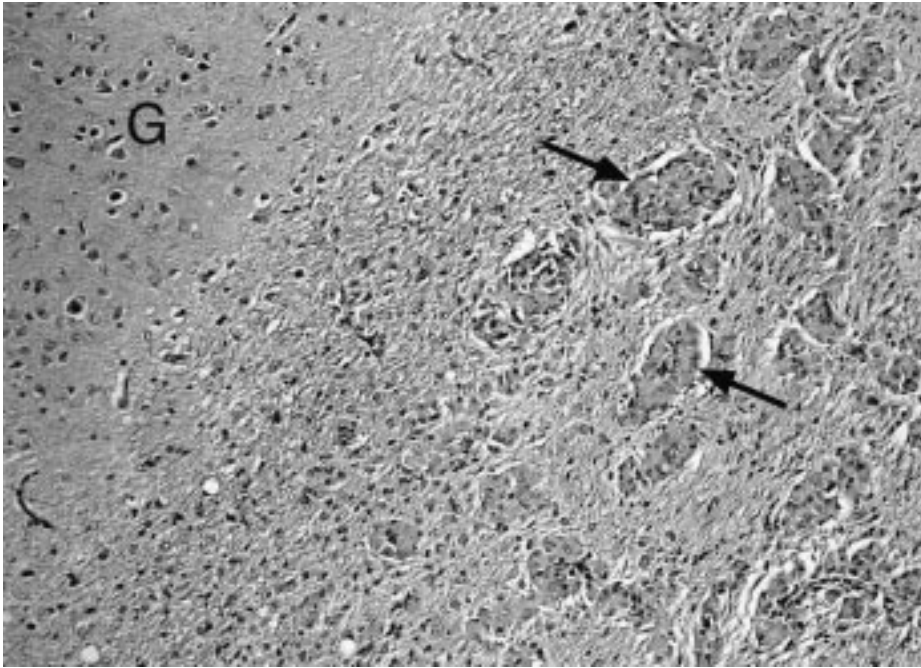
peared normal. No globoid cells were seen on hematoxylin and eosin-stained sections of the sciatic nerve of the newborn affected dog, but they were present in the 12-month-old WHWT. Histologically there were no abnormalities observed in the brain of the newborn affected dog (with hematoxylin and eosin staining). This probably is due to the lack of myelination at this time. However, preservation of the tissue samples from the puppy that was dead and delivered by C-section was not optimal.

Lipid analysis of the brain of the affected dog that died near birth showed no increase in the level of psychosine (Table 1). Due to the condition of the brain samples, white and gray matter could not be separated. However, lipid analysis of the 4-month-old affected cairn terrier and 12-month-old affected WHWT showed great increases in the amount of psychosine in the white matter of the brain (Table 1). A small amount was detected in the white matter of the 4-month-old carrier cairn terrier. There was a generalized decrease in the concentration of other lipids normally found in white matter (data not shown).

Initial studies demonstrating the expression of human GALC activity were performed on cultured skin fibroblasts from an affected cairn terrier and bone marrow from a normal dog. The MFG-human GALC vector was capable of infecting fibroblasts from an affected dog, resulting in the production of GALC activity about four times normal (7.5 versus 1.5–2.0 nmol/h/mg protein) (Figure 4). The activity after transduction is more than 200 times the activity of untransduced cells from an affected dog. The slowly dividing marrow cells from a normal dog showed about a four-fold increase in GALC activity after two rounds of infection.

## Discussion

The canine model of GLD was the first animal model of a lysosomal disease described (Fankhauser et al. 1963). This diagnosis was confirmed by enzyme analysis showing low GALC activity (Suzuki et al. 1970). Recent molecular studies, showing the nature of the mutation in the GALC gene in WHWT and cairn terriers (Victoria et al. 1996), provided the impetus for establishment of a colony of carrier dogs for research purposes. There is some variability in the onset of clinical signs in affected dogs, ranging from 6 to 20 weeks of age. This may result from the presence of a very small but significant amount of



**Figure 3** Light micrograph of hematoxylin and eosin stained section of brain from the 12-month-old affected WHWT. Note the numerous globoid cells (arrows) in the white matter next to normal cortical gray matter (G). (Magnification 31.2 $\times$ .)

GALC activity produced from the two copies of the mutated gene containing a missense mutation, and/or other genetic or environmental factors. This mutation compares to the twitcher mouse, which has a nonsense mutation (Sakai et al. 1996), and the rhesus monkey, which has a 2 bp deletion and frame shift resulting in a stop codon after 15 abnormal amino acids (Luzi et al. 1997).

MRI findings in the affected dogs are identical to those found in human patients with Krabbe disease (Percy et al. 1994), providing an excellent way to monitor treatment. The replacement of white matter by globoid cells in the older dog is typical of all species with GLD. Lipid analysis revealed that psychosine was not present in the brain of an affected dog at birth, but was greatly elevated in the brains of the 4- and 12-month-old affected dogs. As ex-

**Table 1. Psychosine concentration<sup>a</sup> in the brains of dogs with globoid cell leukodystrophy**

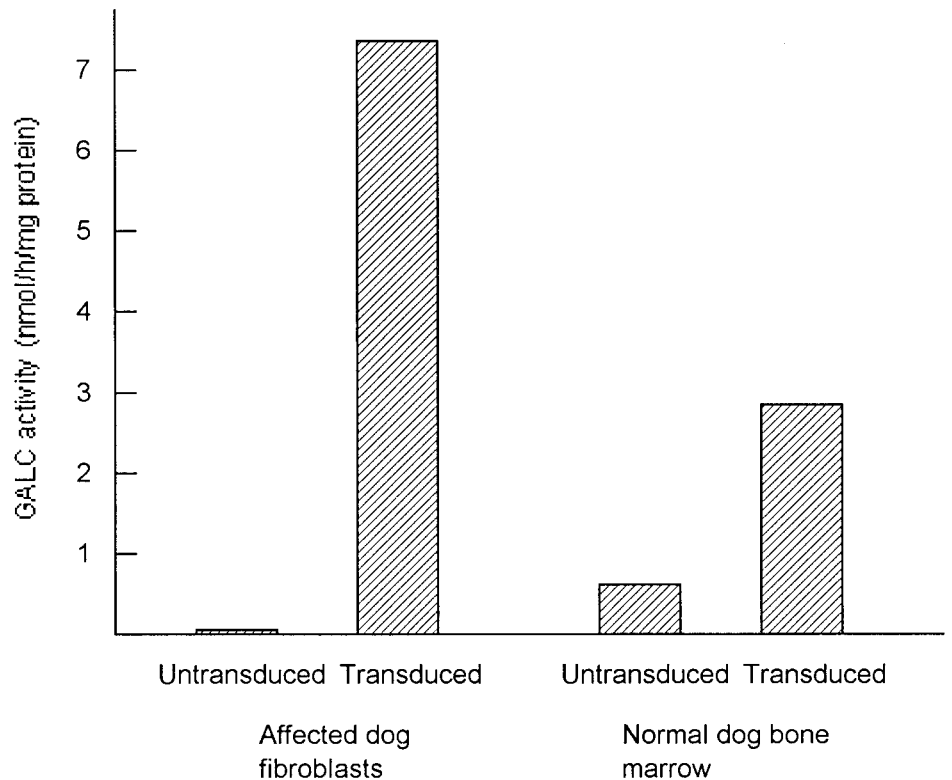
Sample (age)	Genotype	Gray matter	White matter	Total brain
K105 (newborn)	Affected			<1
K106 (4 months)	Carrier	3	45	
K104 (4 months)	Affected	50	690	
Dinah (13 months)	Affected	50	2100	
# (1.8 months) <sup>b</sup>	Affected			90
#2 (7 months) <sup>b</sup>	Affected	22	930	

<sup>a</sup> pmol/mg protein.

<sup>b</sup> Recalculated from data published by Igisu and Suzuki (1984).

pected, the increase was most prominent in the white matter. This confirms the earlier studies performed by Igisu and Suzuki (1984) demonstrating that psychosine accumulates with time in the dog and mouse models of GLD. Psychosine is probably synthesized during the period of most active myelination due to the high expression of uridine diphosphate galactose:ceramide galactosyltransferase. As this may be a dead end, and with no GALC activity to degrade it, psychosine accumulates and produces its cytotoxic effects on the myelin-forming cells in the central and peripheral nervous systems (Yajima et al. 1977). The lipid and pathological changes, which are primarily limited to macrophages in white matter in the GLD dogs, differ significantly from the findings in dogs with other lysosomal disorders such as mucopolysaccharidoses I (Shull et al. 1982) and VII (Haskins et al. 1984) and fucosidosis (Kelly et al. 1983). In these diseases, hydrophilic substrates are stored in lysosomes in many cell types.

Numerous studies involving bone marrow transplantation have been performed



**Figure 4** Expression of GALC activity in cells infected with MFG retroviral vector containing human GALC cDNA. Cultured skin fibroblasts from an affected dog were infected three times and bone marrow cells from a normal dog were infected two times before harvesting and assaying them for GALC activity.

on the twitcher mouse (Hoogerbrugge et al. 1988a,b; Ichioka et al. 1987; Yeager et al. 1984). These studies have shown that early transplantation prolongs the lives of affected mice from 40 days to about 100 days, that donor-derived macrophages can be found in the brains of transplanted mice, and that areas of the brain show evidence of remyelination. A very recent study showed that twitcher mice with a GALC transgene expressing subdetectable GALC levels live longer and accumulate less psychosine than untreated affected mice (Matsumoto et al. 1998). A number of human patients with GLD have received bone marrow transplants (Shapiro et al. 1991). In those patients who had successful engraftment before symptoms became too severe, there was a slowing of the neurological regression and stabilization of the disease process. One fetus predicted to be affected with GLD was given a bone marrow transplant in utero using the father as a donor (Bambach et al. 1997). Unfortunately the fetus died about 20 weeks gestation; however, studies in fetal liver showed engraftment of donor cells. While these results are exciting, it would be helpful to evaluate treatment protocols in a larger animal model such as the dog. Advantages include the similarities in the timing of myelination in dogs and humans, the protracted course in dogs providing time for intervention after birth, the similarities in selection of transplantation donors and ablation techniques, the ability to perform in utero and postnatal bone marrow transplantation on multiple dogs, the ability to secure repeat pathological samples for analysis, and the ability to use noninvasive MRI to evaluate the effects of treatment. These dogs will provide an excellent bridge between studies of treatment under way in the twitcher mouse and human patients with GLD.

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