Neurovisceral and Skeletal G_{M1}-Gangliosidosis in Dogs with β-Galactosidase Deficiency

Abstract. *β-galactosidase–deficient siblings in two litters of English springer spaniel puppies showed a progressive neurological impairment, dwarfism, orbital hypertelorism, and dysostosis multiplex. An excess of G_{M1}-ganglioside was found in the brain. Three abnormal oligosaccharides were present in samples of urine, brain, liver, and cartilage. Light microscopy of selected tissue specimens revealed cytoplasmic vacuoles in neurons, circulating blood cells, macrophages, and chondrocytes. Ultrastructural studies demonstrated that these membrane-bound vacuoles were of two types—one containing lamellated membranes and the other, finely granular material. These clinical and pathological findings are similar to those observed in human patients affected by the infantile form of G_{M1}-gangliosidosis.*

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The gangliosidoses are a group of heterogenous metabolic disorders that are transmitted as autosomal recessive traits. They are characterized by genetic deficiency of those lysosomal hydrolases that participate in the catabolism of sialic acid–containing sphingolipids, resulting in the accumulation of one or more undegraded substrates in affected cells (*1*). These disorders are divided into two groups: the G_{M1}- and G_{M2}-gangliosidoses.

G_{M1}-gangliosidosis is caused by a deficiency in the activity of lysosomal acid β-galactosidase and results in the lysosomal accumulation of glycolipids and oligosaccharides with a terminal β-galactosidic linkage. In humans this disease is classified into infantile (type 1), juvenile (type 2), and adult (type 3) forms on the basis of age at onset of symptoms, temporal evolution, clinical and pathological manifestations (*1, 2*), and the excretion pattern of urinary oligosaccharides (*3*). Deficiency of β-galactosidase associated with skeletal lesions is characteristic of the infantile form and of Morquio's syndrome type B (*1, 4*). G_{M1}-gangliosidosis has been identified and studied in cats, cattle, and dogs (*5*). In these species the affected animals do not have skeletal involvement, and their disease has features similar to those of the juvenile form in humans. We describe the occurrence of G_{M1}-gangliosidosis in English springer spaniel dogs with severe neurovisceral and skeletal involvement.

An 8-month-old dog (dog 19), with a 4-month history of progressive neurological impairment, was proportionately dwarfed, with frontal bossing and hypertelorism. He was mentally alert but ataxic and had decreased cranial nerve responses and nystagmus. A blood smear revealed the presence of large vacuoles in neutrophils, eosinophils, lymphocytes (Fig. 1A), and monocytes. Bones were characterized radiographically by irregular intervertebral disk spaces and deformities of both femoral heads. The dog was killed with sodium barbital, and tissues were obtained for morphological and biochemical studies. Two of his siblings (dogs 17 and 18), killed at age 5 months, had a similar neurologic disorder. In addition, dog 18 had radiographic abnormalities similar to those of dog 19. The proband's parents (dogs 2 and 11) were rebred, and an F_1 litter of eight puppies was obtained (Fig. 2). Two of the pups (dogs 23 and 24) developed progressive ataxia at age 4 months, and another (dog 26) developed the ataxia at age 5½ months. Dog 26 and an asymptomatic sibling (dog 28) were killed at age 6 months because of complications arising from infection by the canine parvovirus.

Necropsy of two of the affected dogs (dogs 19 and 26) demonstrated that they were hydrocephalic. Dog 19 had widened intervertebral disk spaces and a variable degree of osteoarthrosis in articular cartilage of both femurs and tibiae. Lesions, seen on microscopy only in dog 19, included hypertrophy and vacuolation of neurons in the brain (Fig. 1B), spinal cord, para-aortic ganglia, and retina. Hypertrophy of macrophages was seen in thymus, lymph nodes, Peyer's patches, and lungs; hepatic Kupffer cells were enlarged and vacuolated. Pancreatic islet and acinar cells, adrenal cortical and medullary cells, pituitary chromophobe cells, renal proximal tubular cells,

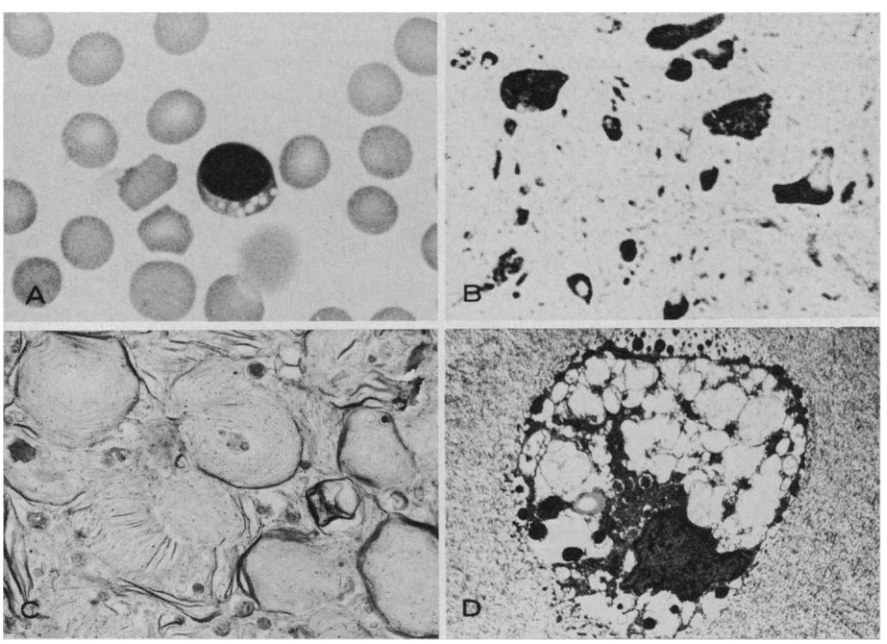

Fig. 1. (A) Vacuolated cytoplasm of a circulating lymphocyte from dog 19 (magnification, ×940). (B) A BS-I–stained paraffin-embedded section of spinal cord from dog 19. The positively stained neurons contain saccharides with terminal α-galactose residues (×198). (C) An electron micrograph of a cerebral neuron reveals numerous densely packed lamellated structures (×15,400). (D) An electron micrograph of a chondrocyte from articular cartilage of the distal femur. The cytoplasm is highly vacuolated and contains fine amorphous granular material (×4900).

and chondrocytes all contained cytoplasmic vacuoles.

Electron microscopy of tissues from dog 19 revealed large neurons containing cytoplasmic, membrane-bound vacuoles, some of which contained only densely packed lamellated membranous structures (Fig. 1C), while others contained either lamellated membranous or fine amorphous granular material. Lamellated membranous structures were also found in endothelial, adrenal medullary, pancreatic islet, pancreatic acinar, and reticuloendothelial cells. In tissues from dog 26, sparse lamellated structures were seen only in neurons. Vacuoles containing amorphous and fine granular material were found only in chondrocytes (Fig. 1D), glomerular epithelium, and mesangial cells of dog 19. Frozen sections of the neurons of dog 19 were positively stained with periodic acid–Schiff, Sudan black, and *Ricinus communis* agglutinin-I (RCA-I) (*6*). Paraffin sections of his neurons stained intensely with *Bandeirea simplicifolia*-I (BS-I), *Dolichos biflorus* agglutinin (DBA), soybean agglutinin (SBA), and *Ulex europaeus* (UEA-I) and stained moderately with *Canavalia ensiformis* agglutinin (Con A), but not with peanut agglutinin (PNA), RCA-I, succinyl–wheat germ agglutinin (S-WGA), and wheat germ agglutinin (WGA). The chondrocytes of this dog were the only cells that stained intensely with RCA-I and WGA (*6*).

Table 1. β-Galactosidase activity in tissues and white blood cells of normal dogs and dogs with G_{M1}-gangliosidosis. The assay mixture contained 20 to 40 μg of enzyme protein (0.05 m*M*) 4-methylumbelliferyl-β-D-galactopyranoside and 0.1*M* sodium acetate buffer (*p*H 3.5) in a total volume of 0.1 ml. Incubation was for 1 hour at 37°C.

Source	Enzyme activity (nmol · mg^{-1} · $hour^{-1}$)
Liver	
Normal dog	611
Dog 19*	35
Kidney	
Normal dog	396
Dog 19*	20
White blood cells	
Normal dog	129
Normal dog	219
Dog 2†	77
Dog 11†	82
Dog 13†	57
Dog 14†	48
Dog 15†	46
Dog 16†	53
Dog 23*	33
Dog 24*	33
Dog 25†	63
Dog 26*	41
Dog 27†	49
Dog 28†	67
Dog 29	121

*Dogs affected clinically. †Heterozygotes.

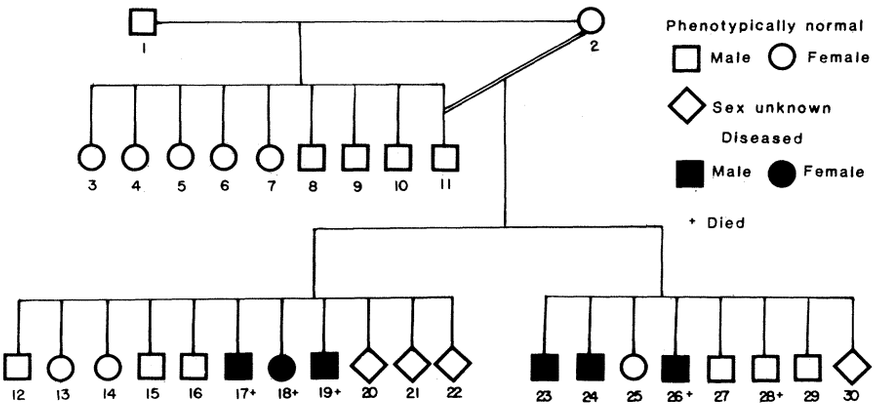

Fig. 2. Family pedigree of dogs with infantile G_{M1}-gangliosidosis.

We examined the activity of lysosomal hydrolases obtained from the liver and kidney of a normal dog and dog 19 and also from the white blood cells of normal dogs and the proband's parents and siblings. Fluorogenic 4-methylumbelliferyl-β-D-galactopyranoside was used as the substrate for β-galactosidase activity (*7*). Decreased activity of β-galactosidase was observed in the clinically affected dogs and in the heterozygote dogs (Table 1).

Brain lipids were extracted, separated, and analyzed from samples of Formalin-fixed cerebral cortex from dog 19 and from an age- and sex-matched normal control (*8*). The brain of dog 19 contained 19.3 mol of lipid-bound sialic acid per gram of dry tissue, whereas the control dog had only 3.44 mol. Thin-layer chromatography (TLC) of a brain lipid extract from dog 19 exhibited an increased amount of ganglioside with a mobility similar to that of G_{M1}. Similarly, TLC of urine oligosaccharides from dog 19 revealed at least three bands of high molecular weight oligosaccharides that were absent from the urine of control dogs and from the urine of the father, sister, and brother of dog 19.

Oligosaccharides were isolated from urine by chromatography on a short column of Bio-Gel P-2, and deionization (*9*). Tissue samples were minced and homogenized, and the constituent oligosaccharides were isolated by ether precipitation (*10*). The urine and tissue oligosaccharides were examined by high-performance liquid chromatography (HPLC) (Fig. 3). In order to make a meaningful comparison of oligosaccharide elution patterns obtained in different chromatographic runs it was necessary to assign "retention indices" (shown in parentheses above the peaks) by reference to an internal standard (see legend to Fig. 3). The pattern of urine oligosaccharides from dog 19 (Fig. 3A) showed a distinctive pattern of three major peaks (retention indices 2.3, 2.55, and 3.5) corresponding to oligosaccharides that contain between six and nine sugar residues.

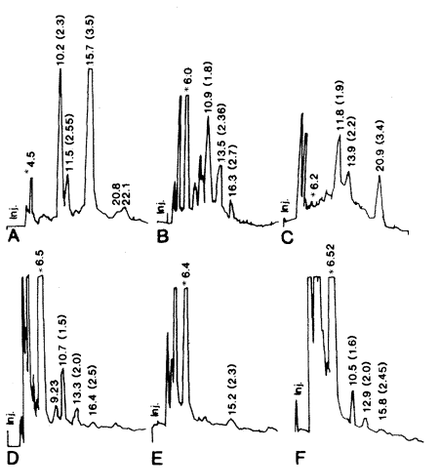

Fig. 3. HPLC separation of oligosaccharide fractions from urine and tissues of dogs homozygous and heterozygous for G_{M1}-gangliosidosis. (A) Dog 19, urine; (B) dog 19, liver; (C) liver from dog with juvenile G_{M1}-gangliosidosis (*12*); (D) spinal cord of dog 26; (E) spinal cord of dog 28; (F) cartilage from dog 26. Isocratic HPLC (B to F) was performed on a Techsphere Ultra 5-μm aminopropyl column (H.P.L.C. Technology, Palos Verdes Estates, California) and a model 5020 chromatograph (Varian Associates, Palo Alto), with a 3:2 mixture of acetonitrile and water as the mobile phase, and detection at 190 nm. Chromatography of sample A was performed on a 5-μm aminopropyl Spherisorb column. When chromatographed on the same column, the major peaks in (C) corresponded to those in (A). The numbers above the peaks are retention times, and "retention indices" in parentheses were derived by dividing these retention times by the retention time of the internal standard of di-*N*-acetylchitobiose (peak marked with a *). The approximate size (that is, numbers of monosaccharide residues) of the oligosaccharides eluting in the major peaks was assigned by comparison with the elution positions of reference oligosaccharides. The significant peaks in this HPLC analysis are those with retention indices of 1.5 or more.

A similar oligosaccharide elution pattern was observed in urine samples from human patients affected with infantile or juvenile G_{M1} (*11*). In contrast the pattern of oligosaccharides extracted from the liver of dog 19 (Fig. 3B) was different from the pattern found in a dog with juvenile G_{M1} (Fig. 3C) (*12*). The latter lacked the major peak retention index 3.5 corresponding to an oligosaccharide with nine sugar residues.

Several different tissues from dog 26 and dog 28 were examined by HPLC for the presence of oligosaccharides. Oligosaccharides from samples of the spinal cord, vertebral disk, and cerebellum were characterized by a chromatographic pattern similar to that seen in the sample from the proband's liver (compare Fig. 3, B, D, and F). The chromatographic pattern obtained from dog 28, which was asymptomatic (Fig. 3E), was different, lacking the characteristic triplet of peaks with retention indices between 1.5 and 3.5 seen in the other extracts and in the urine.

These observations suggest that some English springer spaniel mutants are affected by a genetically determined lysosomal storage disease that closely resembles human infantile G_{M1}-gangliosidosis. These mutant dogs have neurovisceral and skeletal involvement, and an accumulation in affected organs of undegraded oligosaccharides that differ in their HPLC patterns from those reported for the canine model of juvenile G_{M1}-gangliosidosis (*12*). Analysis of the dogs' pedigree and clinical manifestations suggested transmission of the disease through an autosomal recessive pattern of inheritance with variable expressivity. This new canine mutant with β-galactosidase deficiency and multiple organ involvement is of particular interest because it provides a model to study human infantile G_{M1}-gangliosidosis and Morquio's disease type B. Furthermore, this animal model provides a versatile in vivo system for testing various therapeutic modalities such as enzyme replacement (*13*), bone marrow replacement (*14*), and gene insertion.

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Trans-4-Hydroxy-2-Hexenal: A Reactive Metabolite from the Macrocyclic Pyrrolizidine Alkaloid Senecionine

Abstract. *The toxicity of macrocyclic pyrrolizidine alkaloids in the livers of man and animals has been attributed to the formation of reactive pyrroles from dihydropyrrolizines. Now a novel metabolite,* trans*-4-hydroxy-2-hexenal, has been isolated from the macrocyclic pyrrolizidine alkaloid senecionine, in an in vitro hepatic microsomal system. Other alkenals such as* trans*-4-hydroxy-2-nonenal have previously been isolated from microsomal systems when treated with halogenated hydrocarbons or subjected to lipid peroxidation. The in vivo pathology caused by* trans*-4-hydroxy-2-hexenal appears to be identical to that previously attributed to reactive pyrroles. There are similarities between the toxic effects of this alkenal and those of centrilobular hepatotoxins such as CCl_4 and other alkenals formed during lipid peroxidation.*

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Plants containing pyrrolizidine alkaloids (PA's) are found throughout the world (*1*). These naturally occurring compounds are responsible for livestock losses and human poisonings (*2*). Many PA's are hepatotoxins and may cause an irreversible hemorrhagic necrosis, hepatic fibrosis, and megalocytosis (*3*). Some may also contribute to lung injury (*4*), and several have been shown to be teratogenic (*5*), carcinogenic (*6*), and genotoxic (*7*). Grains, bread, milk, honey, and herbal teas contaminated with PA's have either caused human poisonings or represent potential sources of human poisonings (*8*).

The major metabolites from unsaturated PA's, *N*-oxides, and pyrroles are generated by the action of the hepatic microsomal enzyme system (*9*). The pyrroles have been associated with the toxic effects of the PA's while the *N*-oxides are believed to result from a detoxification step in pyrrole metabolism (*10*). The pyrrole may be metabolized further (*11*), but efforts to isolate the final product have failed as the pyrrole derivative (or derivatives) is a reactive substance and rapidly decomposes or polymerizes in an aqueous environment (*12*).

It is possible that numerous metabolites and intermediates are responsible for syndromes associated with PA poisoning. The pyrrolizidine metabolite dehydroretronecine may covalently bind to cysteine, glutathione plus other thiol-containing compounds, as well as purines and pyrimidines such as adenosine monophosphate and deoxyguanosine (*12, 13*). By means of an in vitro mouse hepatic system and the macrocyclic PA senecionine derived from *Senecio vulgaris* (common groundsel), we have demonstrated that senecic acid, senecionine-*N*-oxide, 19-hydroxysenecionine, methoxydehydroretronecine, and hydroxydanaidal are formed from senecionine (*14*) (Fig. 1).

A [^{14}C] senecionine-derived metabolite has been difficult to identify; upon isolation it either decomposes, polymerizes, or forms a dark reddish-brown or purple precipitate after the addition of