INVESTIGATION OF DYSMYELINOGENESIS IN CAPRINE β-MANNOSIDOSIS: BIOCHEMICAL, CELL CULTURE, MORPHOLOGICAL, AND ENDOCRINE STUDIES

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ABSTRACT

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Central nervous system (CNS) myelin deficiency is a consistent pathological feature of caprine ß-mannosidosis, an autosomal recessive neurovisceral lysosomal storage disease. The four projects presented in this dissertation were designed to examine some of the factors which could contribute to the myelin deficiency found in affected animals.

Quantification of regional central nervous system oligosaccharide accumulation addressed the possibility of an association between the storage of oligosaccharides and myelin deficits. Results indicate that the extent of regional CNS accumulation of oligosaccharide is not associated with regional differences in severity of myelin deficiency in caprine ß-mannosidosis.

Cell culture studies examined oligodendrocytes from affected and control animals to compare their number, morphology, and immunostaining characteristics and assess the possibility of intrinsic oligodendrocyte defects. Results indicate that differentiated oligodendrocytes from affected animals do not show morphological abnormalities in culture. However, increased numbers of galactocerebroside-negative bipolar cells, which may be glial progenitor cells, were present in affected animal cultures, suggesting the possibility of a defect in

differentiation to mature oligodendrocytes, with persistence of the undifferentiated glia during late stages of development.

Astrocyte changes at various stages of myelination of the optic nerve were examined by in vivo immunocytochemical studies to assess developmental features of astrocytic abnormalities. Results suggest that astrocyte changes are present in the affected animal optic nerve even during early stages of myelination and that changes are not progressive. The increased density of astrocyte processes appears to be due to a greater number of processes extending from astrocytes, although the possibility of an increased number of astrocytes with redistribution of glial fibrillary acidic protein from cell bodies into processes has not been ruled out.

Examination of thyroid morphology and function revealed that extensive and developmentally progressive morphological abnormalities as well as statistically significant thyroid function deficits are present in affected goats. Thus, a role for reduced thyroid hormone levels in hypomyelination in caprine β-mannosidosis is possible.

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INTRODUCTION

In caprine ß-mannosidosis, an autosomal recessive disease of glycoprotein metabolism (28), deficient activity of the lysosomal enzyme ß-mannosidase (EC 3.2.1.25) is associated with tissue and body fluid accumulation of the enzyme's putative substrates, including a large amount of a trisaccharide (TS, mannosylß1-4N-acetylglucosaminylß1-4N-acetylglucosamine), a smaller amount of a disaccharide (DS, mannosylß1-4N-acetylglucosamine), and much smaller quantities of more complex oligosaccharides (19,29,30,38,39,47,48). Clinical and pathological abnormalities in affected goats include facial dysmorphism; neurological signs include deafness, ataxia, and tremor; neurovisceral cytoplasmic vacuolation; and central nervous system hypomyelination (37,41).

ß-Mannosidase is one of many enzymes involved with catabolism of the oligosaccharide portion of glycoproteins. One or both of two enzymes can cleave N-linked oligosaccharides from glycoproteins, a step which precedes oligosaccharide degradation. An endo-ß-N-acetylglucosaminidase can cleave the chitobiosyl GlcNAcß1-4GlcNAc bond while an endo-ß-aspartylglucosaminidase can cleave the GlcNAcß1-1asparagine bond. The predominant storage of TS and secondary storage of DS in goats affected with ß-mannosidosis suggests that endo-ß-aspartylglucosaminidase activity predominates but that significant endo-ß-N-acetylglucosaminidase activity is present too (30). Once the glycoprotein is cleaved, the oligosaccharide unit is sequentially degraded in a series of enzyme-mediated steps (21). ß-Mannosidase activity act on the last step of the catabolic scheme and cleaves a ß1-4 linkage between mannose and N-acetyl-glucosamine. Two forms of ß-mannosidase exist (56), a cytoplasmic form that appears to be

localized exclusively in caprine liver, and a lysosomal form that has been found in all tissues assayed for it. The cytoplasmic form of the enzyme is deficient in animals affected with ß-mannosidosis (58), and will be referred to here as ß-mannosidase. Deficient ß-mannosidase activity leaves the enzyme's substrates uncatabolized, and lysosomal storage of the substrates is thought to explain the cytoplasmic vacuolation seen in most cell types of affected animals, although non-lysosomal cytoplasmic storage and extracellular storage may also be present. The presence of oligosaccharides in urine from affected animals and in affected animal fibroblast cell culture media indicates that oligosaccharides exist outside of lysosomes as well as inside them. In fact, the TS:DS ratio is nearly equal in urine and cell culture media, compared to the TS predominance in tissue, indicating a greater diffusion of DS out of lysosomes, probably consistent with the smaller molecular size of DS.

A caudal-to-rostral pattern of increasingly severe central nervous system (CNS) myelin deficiency is a major pathological feature of caprine ß-mannosidosis. In affected goats, the spinal cord, which is myelinated relatively early in development, has a moderate degree of myelin deficiency while the cerebral hemispheres (CH), which are myelinated later in development, have a severe deficiency of myelin. Both quantitative (43) and qualitative observations (44,45) have suggested that oligodendrocyte number is reduced in regions where myelin deficiency is present. Other in vivo observations include: (a) a greater proportion of oligodendrocytes with vacuoles and dark cytoplasm in affected goats, beginning prior to development in corpus callosum (Lovell, in

preparation); (b) no ultrastructural difference in CNS myelin sheaths between affected and control animals; (c) no evidence for defective axon-oligodendrocyte interaction; and (d) absence of either oligodendrocyte or myelin degeneration (37,44,45). The possibility that affected animal oligodendrocytes produce proportionally fewer myelin sheath segments than control animal oligodendrocytes has been suggested but not definitively addressed. Taken together, these findings suggest that, in affected animals, (a) myelination is disrupted during development and (b) some alteration in oligodendrocyte proliferation, differentiation, and/or function is present.

Oligodendrocytes are glial cells which produce and maintain CNS myelin. Recent evidence from mixed glial cell cultures from rat optic nerve suggests that oligodendrocytes and type II astrocytes arise from a common progenitor cell (55). As compared to Schwann cells in the peripheral nervous system, where a single cell contributes myelin to a single axon, CNS white matter oligodendrocytes contribute myelin internodes to as many as 50 axons. Oligodendrocytes are also found in the gray matter, where they are called "satellite oligodendrocytes." Oligodendrocytes develop through a maturation scheme which progresses, as seen ultrastructurally, from light in appearance with abundant cytoplasm and a large nucleus to dark in appearance with scanty cytoplasm and a compact nucleus. At all stages of maturation, prominent cytoplasmic Golgi and endoplasmic reticulum content is present in oligodendrocytes.

No good explanation is yet formulated to explain the pathogenesis of the unique pattern of hypomyelination in caprine ß-mannosidosis. Figure 1

summarizes what is known and not known about the connection between the genetic defect in caprine ß-mannosidosis and hypomyelination. Solid arrows indicate connections that have been established while dashed arrows indicate uncertain connections. The fundamental genetic defect which leads to deficient enzyme activity in goats is likely to be restricted to the ß-mannosidase locus. If so, the pathogenesis of abnormalities present in ß-mannosidosis, including deficient myelination, would be directly or indirectly linked to deficient activity of ß-mannosidase. At the cellular level, possible explanations of direct effects of deficient ß-mannosidase activity on oligodendrocytes include (1) a toxic, inhibitory, or space-occupying effect of oligosaccharide accumulation, and (2) effects due to altered glycoprotein metabolism, possibly through a toxic intermediate from an alternate metabolic pathway. Alternatively, effects on oligodendrocytes may be secondary to effects of deficient ß-mannosidase activity on another organ, for instance the thyroid, which would have implications for oligodendrocyte development and function. Less likely, but still possible, are effects of the genetic defect other than on the ß-mannosidase locus that could have a direct or indirect effect on oligodendrocytes.

Clinical manifestations of ß-mannosidosis are less severe in humans than in goats. Of the 5 cases of human ß-mannosidosis described in the literature, all had mental insufficiency and 3 had some degree of hearing and speech impairment, however, none showed neurological signs consistent with gross myelin deficiency (18,24,71). Computerized tomography scan of one patient found no lesions indicative of myelin deficiency (18). The difference in oligosaccharide

storage in human and caprine ß-mannosidosis provides a possible explanation of the differences in phenotypic expression. Of potential importance, DS is the only oligosaccharide detected in the urine of human ß-mannosidosis patients (17,24,71), sharply contrasting with the TS storage predominance in caprine ß-mannosidosis. However, the results of the oligosaccharide study in this dissertation suggest that extent of oligosaccharide accumulation is not associated with the degree of myelin deficiency. Thus, the differences in oligosaccharide storage between caprine and human ß-mannosidosis probably do not help to explain the species differences in myelination.

With respect to other neurovisceral lysosomal storage diseases, α-mannosidosis, associated with deficient activity of lysosomal α-mannosidase, another enzyme in the N-linked oligosaccharide catabolic pathway, is the most closely related to ß-mannosidosis. Many similarities in CNS pathology, especially with respect to the presence of axonal spheroids and the distribution of cytoplasmic vacuolation exist between α-mannosidosis and ß-mannosidosis (44). However, central nervous system myelin deficiency is not an early feature of bovine (35,36) and human α-mannosidosis (50,61), although it has been reported in feline α-mannosidosis at 33 days of age and beyond (67). Thus, the factor or factors that cause myelin deficiency in ß-mannosidosis are probably distinct from general CNS storage of oligosaccharides or storage of the specific complex oligosaccharides in α-mannosidosis.

Oligodendrocyte deficiency is a major feature of several dysmyelinating disorders including the jimpy mouse (49,60), the myelin-deficient rat (23), and the

twitcher mouse (63). The etiology of oligodendrocyte deficiency in each of these diseases appears to be quite different from that in ß-mannosidosis. For instance, caprine ß-mannosidosis differs in vivo from both twitcher and jimpy mutants in several ways but, most importantly, no evidence of oligodendrocyte degeneration has been seen in ß-mannosidosis (44,45), although degeneration is a major feature in the twitcher mouse (62) and jimpy mouse (40). Cell culture studies in the jimpy mouse (10) and the twitcher mouse (51) have found relatively normal numbers and morphological characteristics of oligodendrocytes in their early stages of development but abnormalities become apparent within 7 to 10 days of culture. Abnormalities included degeneration of oligodendrocytes in twitcher mice and decreased numbers and altered morphologic features and immunofluorescent labeling in jimpy mice, features consistent with what is found in vivo in the respective disorders. As presented in this dissertation, in vitro characteristics of oligodendrocytes isolated from animals affected with ß-mannosidosis that contrast with the in vitro findings in twitcher and jimpy include: (1) no evidence of cell degeneration, (2) no evidence of morphological and immunolabelling abnormalities, and (3) the presence of increased numbers of bipolar cells, possibly glial progenitor cells. While it is important to note that the caprine oligodendrocyte cultures were initiated with relatively mature cells, compared to the immature cells studied in the mouse myelination mutant cultures, the mechanism of dysmyelination is different in caprine ß-mannosidosis than in twitcher or jimpy mice.

Astrocytes respond to central nervous system (CNS) pathological changes with "gliosis." Gliosis consists of (1) astrocyte proliferation and/or (2) astrocyte hypertrophy, with an increased number of extended processes and an increased expression of the astrocyte-specific intermediate filament glial fibrillary acidic protein (GFAP) (15,42). Gliosis has been described in a variety of CNS pathological conditions including trauma, inflammatory diseases, hepatic encephalopathy, degenerative diseases, and aging (15,27,31,68). Additionally, gliosis has been identified in the CNS of various myelination mutant mice, including jimpy, quaking, shiverer, and mld mutants (11). Of considerable interest, findings similar to those seen in the optic nerve in ß-mannosidosis have been identified in various CNS regions in the jimpy mouse. Gliosis in various CNS regions preceding myelination or in early stages of myelination was recently reported in the jimpy mouse and was attributed to glial cell hypertrophy and not to an increased number of astrocytes (12).

Thyroid hormones play an important role during the development of the central nervous system (1,32,33) and considerable evidence indicates that thyroid hormones have a direct impact on myelination (33,69,70). For example, fetal sheep from experimentally hypothyroid ewes have a decreased number of cells, presumably including oligodendrocytes, and deficient myelination, and both situations are reversible by injection of ewes with iodized oil (52). In the rat, thyroxine deficiency leads to myelin deficiency (6, 59). Additionally, cell culture studies have shown that thyroid hormones are important for the differentiation and function of oligodendrocytes (2). In general, hyperthyroidism has been

associated with early initiation and premature termination of myelination while hypothyroidism has been associated with deficient myelination (69). In summary, both in vivo and in vitro studies have shown quite clearly that thyroid hormones have significant effects on oligodendrocyte differentiation and function (2,6,13,14,16,20,46,53,59).

Recent evidence in sheep, an animal similar to goats with respect to central nervous system development, indicates that decreased thyroid hormone levels may play a causative role in the hypomyelination of border disease (3). Severe central nervous system hypomyelination, involving spinal cord, brainstem, and cerebral hemispheres, is a major pathological feature of border disease, a disease of sheep caused by in utero infection of fetuses with the border disease virus (BDV) (5,7-9,54). A reduced thyroid hormone level, possibly secondary to follicular cell invasion by BDV, has been postulated to be the causative factor in the severe CNS myelin deficiency (3). Considerable similarity exists between border disease and ß-mannosidosis. Average T_3 and T_4 levels in border disease sheep were 60% and 74% of control values, respectively, similar to the extent of reduction in ß-mannosidosis. Clinical features of border disease which have similarities to features of caprine ß-mannosidosis (37,41) include skeletal abnormalities such as doming of the skull, small and abnormally placed orbits, and abnormalities of limb bones (4,7,66), and neurological signs including spasm, tremor, and locomotor defects (8). In fetal sheep from ewes with experimentally induced iodine deficiency, doming of the skull, retrognathia, subluxation of the front joints, and hypomyelination are prominent morphological abnormalities and

each of these features can be fully or partially reversed after administration of iodine to the ewe at 100/150 days gestation (53). Thus, even though the etiology of ß-mannosidosis, border disease, and prenatal iodine deficiency are distinctly different, it is possible that a common pathogenic mechanism of hypomyelination and other phenotypic features is reduced thyroid hormone levels secondary to thyroid dysfunction.

Several lines of evidence suggest that ß-mannosidase plays an important role in thyroid metabolism. First, thyroglobulin is the predominant glycoprotein in the thyroid and its degradation includes lysosomal catabolism of N-linked oligosaccharides (34,64,72), requiring ß-mannosidase activity. Second, control goat thyroid ß-mannosidase activity is the highest of any organ tested (unpublished data, and (52)). Third, large quantities of oligosaccharides have been isolated and characterized from thyroids of affected goats (29).

Thyroglobulin is a glycoprotein containing a Manß1-4GlcNAc residue in its N-linked oligosaccharides (64,72). The direct impact of ß-mannosidase deficiency on thyroglobulin metabolism would occur during lysosomal catabolism of thyroglobulin. It follows that a thyroglobulin degradation impediment or defect is the most likely alteration that would lead to decreased thyroid hormone secretion in ß-mannosidosis. Absence of affected goat lysosomal ß-mannosidase activity and accumulation of oligosaccharides could have an adverse effect on thyroglobulin degradation, and then on thyroid hormone release, through (a) decreased availability of primary lysosomes due to impaired recycling of secondary lysosomes (22,57) , and (b) interference in cell function by storage

vacuole accumulation. Relevant to both possibilities, a recent study identified a possible post-endocytosis regulation step in thyroglobulin catabolism, where there is a lag period between fusion of endosomes with primary lysosomes to form secondary lysosomes (57). Alterations in the availability of primary lysosomes, due to alterations in synthesis or in accessibility, might adversely affect post-endocytosis regulation. Thyroid metabolic alterations other than disruption of catabolism--such as a thyroglobulin synthesis defect, an organification defect, a hormone receptor abnormality, or a thyrotropin deficiency--are much less likely to be involved in the thyroid hormone deficits in ß-mannosidosis. It is possible, however, that metabolic perturbations secondary to abnormal glycoprotein metabolism could adversely affect thyroglobulin metabolism.

The four separate research projects presented here were designed to explore some of those factors which could have an impact on abnormal oligodendrocyte development and hypomyelination in caprine ß-mannosidosis. In the first project, quantitation of regional CNS oligosaccharide accumulation addressed the possibility of an association between the stored oligosaccharides and myelin deficits. Results indicate that the extent of regional CNS accumulation of TS and DS is not associated with regional differences in severity of myelin deficiency in caprine ß-mannosidosis. Thus, it is unlikely that oligosaccharide accumulation plays a pathogenetic role in hypomyelination.

In the second project, oligodendrocytes from affected and control animals were examined in vitro to compare their numbers, morphology, and immunostaining characteristics and assess the possibility of intrinsic

oligodendrocyte defects. Results from the cell culture study are consistent with in vivo morphological observations and suggest that differentiated oligodendrocytes from affected animals do not show morphological abnormalities in culture. However, increased numbers of galactocerebroside-negative bipolar cells, which may be glial progenitor cells, were present in affected animal cultures. Morphologically similar cells have been identified as glial progenitor cells in mixed glial cultures from neonatal rats (55,65) and fetal sheep (26), using anti-galactocerebroside and anti-A_2B_5, a monoclonal antibody directed against a complex ganglioside (25). Presence of the bipolar cells in increased numbers in affected animals, if they indeed are glial progenitor cells, suggests the possibility of a defect in differentiation to mature oligodendrocytes, with persistence of the undifferentiated glia during late stages of development. Additional studies are needed to further investigate bipolar cells and to determine the role of impairment of oligodendrocyte development in hypomyelination in caprine ß-mannosidosis. Studies need to include, initially, full documentation of the bipolar cells with anti-A_2B_5 and possibly with fluorescent microbeads. A_2B_5-positive labeling would identify the cells as glial progenitor cells while microbead uptake would identify the cells as macrophages. Additionally, assuming that the cells are glial progenitor cells, isolation of bipolar cells from affected animal CNS and experimentation with addition of various factors -- e.g. thyroid hormone and nerve growth factors -- to see if an actual block in differentiation is present would be of interest.

In the third project, astrocyte changes at various stages of myelination of the optic nerve were addressed by in vivo immunocytochemical studies. This study suggests that astrocyte changes are present in the optic nerves of affected animals even during early stages of myelination and that changes are not progressive. Increased astrocyte process density at a time early in myelination suggests that gliosis may not be simply a reaction to a decreased number of myelin sheaths but may take place in response to other abnormalities, possibly in response to a decreased number or abnormal development of oligodendrocytes or perturbations in oligosaccharide metabolism. Additionally, the increased density of GFAP+ astrocyte processes appears to be due to a greater number of processes extending from astrocytes, although the possibility of an increased number of astrocytes with redistribution of GFAP from cell bodies into processes has not been ruled out. Further examination of the gliosis, in spinal cord, cerebellum, and corpus callosum, will help to define the extent of the change and allow assessment of the association between gliosis and regional variations in myelin deficiency. Additionally, examination of the corpus callosum will allow for examination of white matter before myelination has begun and then will allow for examination of the issue of whether gliosis is present before myelination begins.

In the fourth project, examination of morphology and function of the affected animal thyroid was designed to assess whether decreased hormone secretion could be a factor leading to impaired myelination. The thyroid study documents the presence of extensive and developmentally progressive

morphological abnormalities in the thyroid of goats affected with ß-mannosidosis. Additionally, evidence suggesting deficient thyroid function in affected goats is presented. This functional abnormality may be due, at least in part, to the extensive thyroid follicular cell morphological abnormalities or to metabolic perturbations secondary to deficient ß-mannosidase activity. A role for reduced thyroid hormone levels in the hypomyelination and other clinical and pathological abnormalities seen in goats affected with ß-mannosidosis is possible and needs further investigation.

Since thyroid hormone deficiency appears to be a strong candidate for explaining hypomyelination in ß-mannosidosis, further examination of the relationship between thyroid hormone deficiency and hypomyelination are in order. The following hypothesis may help explain the role deficient thyroid hormone levels may play in the disruption of myelination in ß-mannosidosis. At the onset of thyroid function, at between 50 and 60 days gestation, thyroid hormone levels may be normal, or at least sufficient to support relatively normal myelination. However, as development proceeds, with continual degradation of thyroglobulin and progressive accumulation of uncatabolized oligosaccharides within lysosomes, progressively greater disruption of thyroid function may result. Progressively decreased amounts of thyroid hormone over time could lead to increasingly severe myelin deficiency in later myelinating regions. Both (a) substantial morphological alterations present as early as 96/150 days gestation and (b) documentation of the progressive nature of accumulation during development offer circumstantial support for the hypothesis. Evaluation of

thyroid hormone levels at various stages of in utero development of affected and control fetal animals will help assess the possibility of a role for thyroid hormone deficiency in the hypomyelination of ß-mannosidosis. Clearly, further investigation of thyroid function in ß-mannosidosis in warranted. Further work could include (a) verification of the current thyroid function findings in both fetal and neonatal affected animals, (b) assessment of affected and control animal thyroid colloid immunocytochemically, (c) assessment of anterior pituitary morphology at both the light and electron microscopic levels, and (d) characterization of degree of vacuolation and secretory granule content in anterior pituitary thyrotrophs ultrastructurally and possibly immunocytochemically. The absence of a thyroid stimulating hormone (TSH) assay that works in goats limits our ability to measure this important parameter. However, it may be worthwhile to attempt to stimulate thyroid hormone secretion by injection of affected and control goats with TSH releasing hormone (TRH) to challenge their thyroid secretory capacity.

Four major projects were carried out as part of this dissertation in an attempt to help define the pathogenesis of myelin deficiency in ß-mannosidosis (Fig. 1). The two most promising results were (a) the finding of an increased number of bipolar cells, possibly glial progenitor cells, in affected animal white matter cultures and (b) the documentation of deficient thyroid function in affected animals. The increased number of bipolar cells may indicate that a block in differentiation of progenitor cells to mature oligodendrocytes is present in affected animals. Deficient thyroid hormone levels in affected animals may help explain both blocked differentiation and abnormal function. This is the first

report suggesting that the pathogenesis of CNS lesions in a storage disease could involve endocrine perturbations. Thus, the current findings may underscore the importance of integrated models of cell to cell interactions. In this case, abnormal function of thyroid follicular cells in affected goats may have important implications for the development and function of oligodendrocytes.

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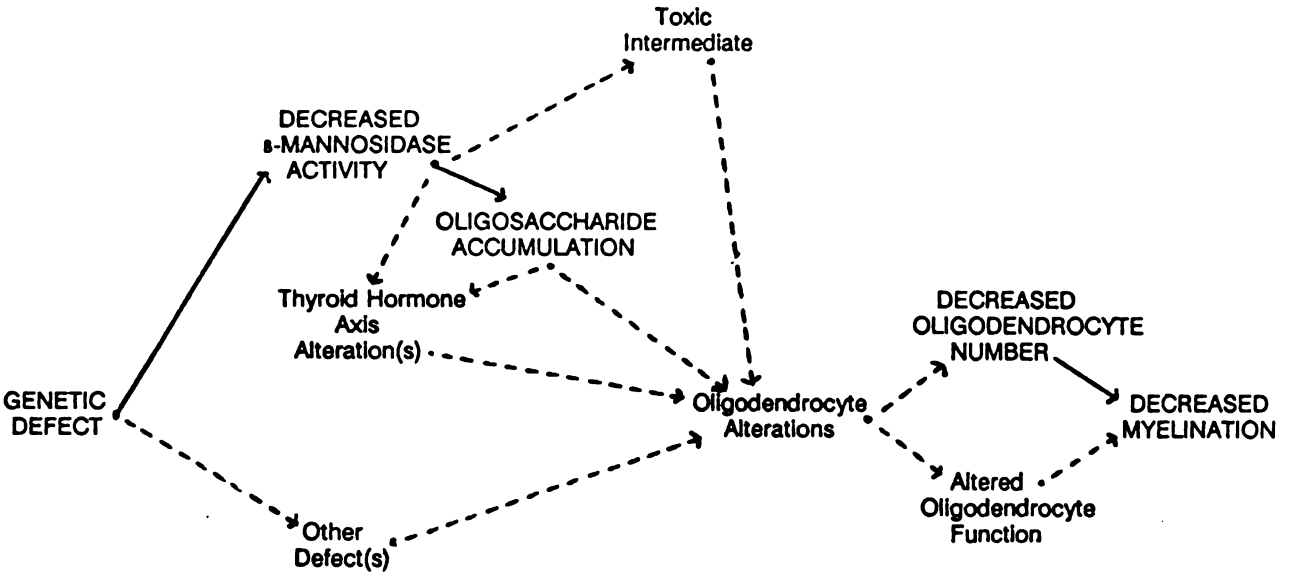
Fig. 1 Summary of known (complete lines) and possible (dotted lines) connections between the genetic defect in ß-mannosidosis and decreased myelination. Capital letters indicate features of ß-mannosidosis that have been confirmed experimentally.


Toxic
Intermediate
DECREASED
β-MANNOSIDASE
ACTIVITY
OLIGOSACCHARIDE
ACCUMULATION
Thyroid Hormone
Axis
Alteration(s)
GENETIC
DEFECT
Oligodendrocyte
Alterations
DECREASED
OLIGODENDROCYTE
NUMBER
DECREASED
MYELINATION
Altered
Oligodendrocyte
Function
Other
Defect(s)

Regional Central Nervous System Oligosaccharide Storage in Caprine β-Mannosidosis

ABSTRACT

Goats affected with ß-mannosidosis, an autosomal recessive disease of glycoprotein metabolism, have deficient activity of the lysosomal enzyme ß-mannosidase along with tissue storage of oligosaccharides, including a trisaccharide (mannosylß1-4N-acetylglucosaminylß1-4N-acetylglucosamine) and a disaccharide (mannosylß1-4N-acetylglucosamine). CNS myelin deficiency, with regional variation in severity, is a major pathological characteristic of affected goats. This study was designed to investigate regional CNS differences in oligosaccharide accumulation to assess the extent of correlation between oligosaccharide accumulation and severity of myelin deficits. The concentrations of accumulated disaccharide and trisaccharide and the activity of ß-mannosidase were determined in cerebral hemisphere gray and white matter and in spinal cord from three affected and two control neonatal goats. In affected goats, content of trisaccharide and disaccharide in spinal cord (moderate myelin deficiency) was similar to or greater than content in cerebral hemispheres (severe myelin deficiency). Thus, greater oligosaccharide accumulation was not associated with more severe myelin deficiency. Regional ß-mannosidase activity levels in control goats were consistent with the affected goat oligosaccharide accumulation pattern. The similarity of trisaccharide and disaccharide content in cerebral hemisphere gray and white matter suggested that lysosomal storage vacuoles, more numerous in gray matter, may not be the only location of stored CNS oligosaccharides.

Key words: lysosome, myelination, storage disease, goat

Running title: OLIGOSACCHARIDE STORAGE IN ß-MANNOSIDOSIS

In caprine ß-mannosidosis, an autosomal recessive disease of glycoprotein metabolism (Fisher et al., 1986), deficient activity of the lysosomal enzyme ß-mannosidase (EC 3.2.1.25) is associated with tissue and body fluid accumulation of a large amount of the enzyme's putative substrates, including a trisaccharide (TS, mannosylß1-4N-acetylglucosaminylß1-4N-acetylglucosamine), a smaller amount of a disaccharide (DS, mannosylß1-4N-acetylglucosamine), and much smaller quantities of more complex oligosaccharides (Jones and Laine, 1981; Matsuura et al., 1981; Jones et al., 1984; Matsuura and Jones, 1985; Dahl et al., 1986; Frei et al., 1986; Hancock et al., 1986). Clinical and pathological abnormalities in affected goats include facial dysmorphism; neurological signs including deafness, ataxia, and tremor; cytoplasmic vacuolation; and CNS hypomyelination (Jones et al., 1983; Kumar et al., 1986). The genetic abnormality which leads to deficient enzyme activity in goats is currently under investigation. It is likely that the genetic defect is restricted to the ß-mannosidase locus. If so, the pathogenesis of abnormalities present in ß-mannosidosis would be directly or indirectly linked to deficient activity of ß-mannosidase. At the cellular level, toxic or inhibitory effects of stored, uncatabolized substrate or perturbed glycoprotein metabolism are possible contributing factors.

A caudal-to-rostral pattern of increasingly severe CNS myelin deficiency is a major pathological feature of caprine ß-mannosidosis. In affected goats, the spinal cord, which is myelinated relatively early in development, has a moderate degree of myelin deficiency while the cerebral hemispheres (CH), which are myelinated later in development, have a severe deficiency of myelin. This pattern

and other evidence (Lovell and Jones, 1983; Lovell and Boyer, 1987) suggests disruption of the myelination process during development. Regional variations in dysmyelination could be due to accumulation of different amounts of a substance that impairs oligodendrocyte proliferation, differentiation, or function.

Clinical manifestations of ß-mannosidosis are less severe in humans than in goats and myelin deficiency has not been detected in human ß-mannosidosis (Cooper et al., 1986; Wenger et al., 1986; Dorland et al., 1988). Of potential importance, DS is the only oligosaccharide detected in the urine of human ß-mannosidosis patients (Wenger et al., 1986; Cooper et al., 1988; Dorland et al., 1988), sharply contrasting with the TS storage predominance in caprine ß-mannosidosis. Differences between human and goat oligosaccharide storage suggest that an accumulation of TS or more complex oligosaccharides could play a role in the pathogenesis of dysmyelination in caprine ß-mannosidosis.

The major objectives of the study were to (1) determine the content of both TS and DS and the TS:DS ratio in CH gray and white matter and in spinal cord; (2) assess a possible association of oligosaccharide content with the severity of myelin deficiency in these regions; and (3) measure control animal ß-mannosidase activity in these regions to correlate with the extent of affected animal TS and DS accumulation. Definition of the regional distribution of ß-mannosidase substrate accumulation in the CNS and determination of the role of oligosaccharide accumulation in dysmyelination in caprine ß-mannosidosis were expected outcomes of this investigation.

MATERIALS AND METHODS

Materials

T-61 was purchased from American Hoechst (Somerville, NJ). Bio-Gel P-2, 400 mesh, was purchased from Bio-Rad (Richmond, CA). Redi-Plate silica gel G thin layer chromatography (TLC) plates and 1-butanol, ACS certified grade, were purchased from Fisher Scientific (Pittsburgh, PA). Leupetin and pepstatin A were purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals were of reagent grade and purchased from Sigma (St. Louis, MO).

Tissue acquisition

Following euthanasia with T-61, brain and spinal cord from two control and three affected 2- to 3-day-old animals were removed, sliced, and frozen. For each animal, 2.2 g samples were excised from frozen spinal cord, CH white matter, and CH gray matter. CH white matter excision was limited to centrum semiovale.

Oligosaccharide isolation and analysis

Tissue extract preparation. Using modifications of methods previously described (Matsuura and Jones, 1985), tissue samples were minced, brought to a total volume of 5 ml with triple deionized water, and sonicated for four minutes using a Heat Systems Ultrasonics model W185 sonifier at a power setting of 3. Two 0.23 ml samples, containing 0.1 g of tissue each, were withdrawn and frozen for later β-mannosidase and protein assays. The remaining sonicate was centrifuged and the supernatant was removed. The pellet was twice dispersed in water, sonicated, and centrifuged. Supernatants were combined, made 15% in

acetic acid by the addition of glacial acetic acid, and after 30 minutes centrifuged at 12,000g for 20 min. The pellet was twice resuspended in 0.5 ml water and centrifuged. Supernatants were combined and lyophilized.

Bio-Gel P-2 chromatography. Lyophilized material was solubilized in 1.0 ml water, applied to a 1.0 X 100 cm, 400 mesh Bio-Gel P-2 column, and eluted with water. Fractions of 1.0 ml were collected.

Oligosaccharide analysis. The hexose content in a 200 ul sample from each fraction was determined by the phenol-sulfuric acid colorimetric method (DuBois et al., 1956). Thin layer chromatography (TLC) was used to determine the identity of oligosaccharides contained within the fractions. Samples (40 ul) from each fraction, along with oligosaccharide standards ((a) glucose oligomer from acid-digested dextran and (b) a mixture of authentic oligosaccharide samples purified from affected goat thyroid, see Fig. 1), were applied to silica gel G TLC plates, and developed using a butanol, acetic acid, water mixture (3:3:2, by volume). Hexose was visualized with orcinol-sulfuric acid reagent and heating at 100°C, as previously described (Matsuura and Jones, 1985).

ß-Mannosidase assay

Enzymatic activity of ß-mannosidase was determined, as previously described (Jones et al., 1984), except enzyme extraction was in water containing 0.01 M sodium citrate, 0.05 M sodium chloride, 10 mM calcium chloride, 10 mM manganese chloride, 0.02% (wt/vol) sodium azide, 5% glycerol (vol/vol), 0.2 mg/l leupetin, and 0.7 mg/l pepstatin A instead of plain water. Assays were run in duplicate, at pH 5.5, and 37°C from 25 ul samples of sonicates of each tissue

sample, using 4-methylumbelliferyl-ß-D-mannopyranoside as substrate. Fluorescence levels were assessed using a Gilford FluoroIV fluorimeter. Values were expressed as nmol 4-methylumbelliferyl released per h per mg protein.

Protein assay

Protein content of both the crude tissue preparations from the oligosaccharide isolation samples and the supernatants of ß-mannosidase assay samples were determined, in triplicate, by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. Optical absorbance of samples and dilutions of the standard were analyzed using a Beckman DU-64 spectrophotometer.

RESULTS

Oligosaccharide isolation, quantitation, and characterization

Characterization of crude tissue extracts by thin layer chromatography (Fig. 1) identified major accumulations of monosaccharide, DS, and TS in affected animal samples (lane 4) while control animal samples contained monosaccharide but neither TS nor DS (lane 3). The major oligosaccharides separated by Bio-Gel P-2 chromatography from affected goat samples included DS (lane 5) and TS (lane 6). As determined by phenol-sulfuric acid assay, accumulation of TS and DS varied somewhat among animals (Fig. 2). However, in each animal, TS content was consistently higher in spinal cord than in either CH white or gray matter. DS levels were higher in spinal cord than in either CH region in two of three animals. Mean spinal cord TS and DS levels were each

approximately 1.4 times higher than mean levels in either CH region. TS and DS levels in the affected animals were not substantially different between CH white matter and gray matter. The TS:DS ratios were not consistently different among regions. In control animals, TS and DS were not detected in either cerebral hemisphere or spinal cord tissue.

Regional ß-mannosidase activity levels

For both control animals, ß-mannosidase activity levels in spinal cord were higher than in either CH region. Within the cerebral hemispheres, gray and white matter activity levels were not appreciably different (Fig. 3). ß-Mannosidase activity was not detectable in affected animal samples.

DISCUSSION

These results are the first report of regional CNS oligosaccharide accumulation and ß-mannosidase activity levels in caprine ß-mannosidosis. Given the approximately 2 fold higher level of ß-mannosidase activity in control goat spinal cord compared to CH white or gray matter, the finding of generally higher levels of oligosaccharide storage in affected goat spinal cord than in CH white or gray matter is not surprising. Previous studies of thyroid, kidney, and CH gray matter also have suggested that the level of ß-mannosidase activity in control animal tissues is correlated with the accumulation of oligosaccharides in affected animal tissues (Jones and Dawson, 1981; Jones and Laine, 1981; Matsuura et al., 1981; Jones et al., 1984; Pearce et al., 1987). However, the functional significance of higher ß-mannosidase activity in the spinal cord than in CH white

or gray matter is not clear.

No determination was made of the molecular structures of the DS and TS isolated in this study. However, given the conserved nature of the core sugar unit in N-linked glycoproteins (Schachter, 1984), the molecular structure of CNS TS and DS is likely to be the same as, and can be inferred from, previous structural determinations in CH gray matter (Jones and Laine, 1981), peripheral nerve (Frei et al., 1984), kidney (Matsuura and Jones, 1985), and thyroid (Frei et al., 1986).

A major goal of this research was to assess in specific CNS regions whether an association existed between oligosaccharide content and severity of myelin deficiency. In affected goats, the average content of both TS and DS was 40% higher in spinal cord, an area of moderate myelin deficiency, than in CH, an area of severe myelin deficiency. Although the number of samples was too low for meaningful statistical analysis, the consistently higher spinal cord levels of TS suggest that the etiology of dysmyelination in caprine β-mannosidosis is not related to accumulation of oligosaccharides. The accumulation of oligosaccharides in the spinal cord white matter was not separately measured; however, results for CH gray and white matter were similar, and thus appreciable spinal cord gray and white matter differences are unlikely. The current research does not rule out the possibility of inhibitory or toxic effects of complex oligosaccharides, which have been difficult to study because of their very low tissue concentrations (Matsuura and Jones, 1985). However, it is probable that the complex oligosaccharides accumulate somewhat proportionally to TS and DS. Since

affected animal CNS has a relatively low concentration of TS and DS, compared to visceral tissues, the concentration of complex oligosaccharides in CNS is expected to be low also, and to be distributed similarly to TS and DS distribution. The existence, in affected goat CNS, of a toxic compound generated by an alternate metabolic pathway, analogous to galactosylsphingosine in the twitcher mouse (Igisu and Suzuki, 1984), remains a possible etiologic mechanism.

A potential limitation in the present study was its focus on postnatal animals. Myelination in goats is nearly complete at birth, and so tissues at the "end-stage" of disease were studied. A prenatal developmental study would be needed to assess oligosaccharide accumulation during active myelin formation to completely rule out developmentally significant regional differences. However, although regional differences may exist temporally, it seems likely that, in each region, the accumulation of oligosaccharides would increase according to that region's developmental schedule, and the maximum accumulation would be reflected in the values measured after birth.

The similarity in storage levels of TS and DS in affected goat CH gray and white matter and the higher content in spinal cord were unanticipated results. Morphologically, a greater degree of lysosomal storage vacuolation is present in CH gray matter cells (predominantly in neurons) than in CH white matter or spinal cord cells (Jones et al., 1983; Lovell and Jones, 1983; Lovell and Jones, 1985). It was expected, given the distribution of vacuolation, that CH gray matter oligosaccharide storage would be greater than CH white matter and spinal cord storage. The current findings, however, suggest that considerable extra-

lysosomal oligosaccharide storage, i.e., cytoplasmic and/or extracellular, is present in affected goat CNS tissues. This distribution may be unique to the CNS, since the level of oligosaccharide storage in thyroid, kidney, and liver is roughly proportional to the extent of cytoplasmic vacuolation in these organs (Jones et al., 1983; Jones et al., 1984; Frei et al., 1986).

In summary, the data indicate that the extent of regional CNS accumulation of TS and DS is not associated with regional differences in severity of myelin deficiency in caprine ß-mannosidosis. Although these results do not directly assess complex oligosaccharide accumulation, it is nonetheless unlikely that the etiology of dysmyelination in caprine ß-mannosidosis is related to accumulation of oligosaccharides. Thus the differences in oligosaccharide storage between caprine and human ß-mannosidosis do not explain the species differences with respect to myelination.

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FIG. 1. Thin layer chromatography of oligosaccharide standards, CH white matter crude tissue extracts, and Bio-Gel P-2 isolated oligosaccharides. Samples were applied to silica gel G TLC plates; plates were developed in butanol, acetic acid, and water (3:3:2, by volume); and hexose was visualized with orcinol-sulfuric acid reagent. 1, glucose oligomer standards; 2, authentic standards: from top, monosaccharide, DS, TS, pentasaccharide; 3, crude extract of control animal white matter; 4, crude extract of affected animal white matter; 5, purified DS from affected animal white matter; 6, purified TS from affected animal white matter.

FIG. 1

FIG. 2. Regional CNS DS and TS content in affected animals. Samples (2 g) from CH white matter and gray matter and from spinal cord (combined white and gray matter) of 3 affected (A1, A2, and A3) and 2 control animals were sonicated with water, and oligosaccharides in water extracts were separated by Bio-Gel P-2 column chromatography. Oligosaccharides within fractions were identified by thin layer chromatography mobility with respect to standards (see Fig. 1) and the hexose content of each fraction was measured by phenol-sulfuric acid assay. Additionally, protein content of sonicates was determined (see methods). In control animals, DS and TS were undetectable. Bars represent, for affected animal regions, DS or TS content expressed per mg protein.

FIG. 3. Regional CNS ß-mannosidase activity in control animals. Samples (0.1 g) from CH white matter and gray matter and from spinal cord (combined white and gray matter) of 3 affected and 2 control (C1 and C2) animals were sonicated with an extraction buffer (see methods) and centrifuged. ß-mannosidase activity in each region was determined in supernatant samples by fluorometric quantitation of the amount of 4-methylumbelliferyl (4-MU) released from 4-MU-ß-D-mannopyranoside added to the samples. Additionally, protein content of supernatants was determined (see methods). In affected animals, enzyme activity was undetectable in all regions. Bars represent, for control animal regions, means of duplicate ß-mannosidase activity measurements expressed per mg protein.

FIG. 2

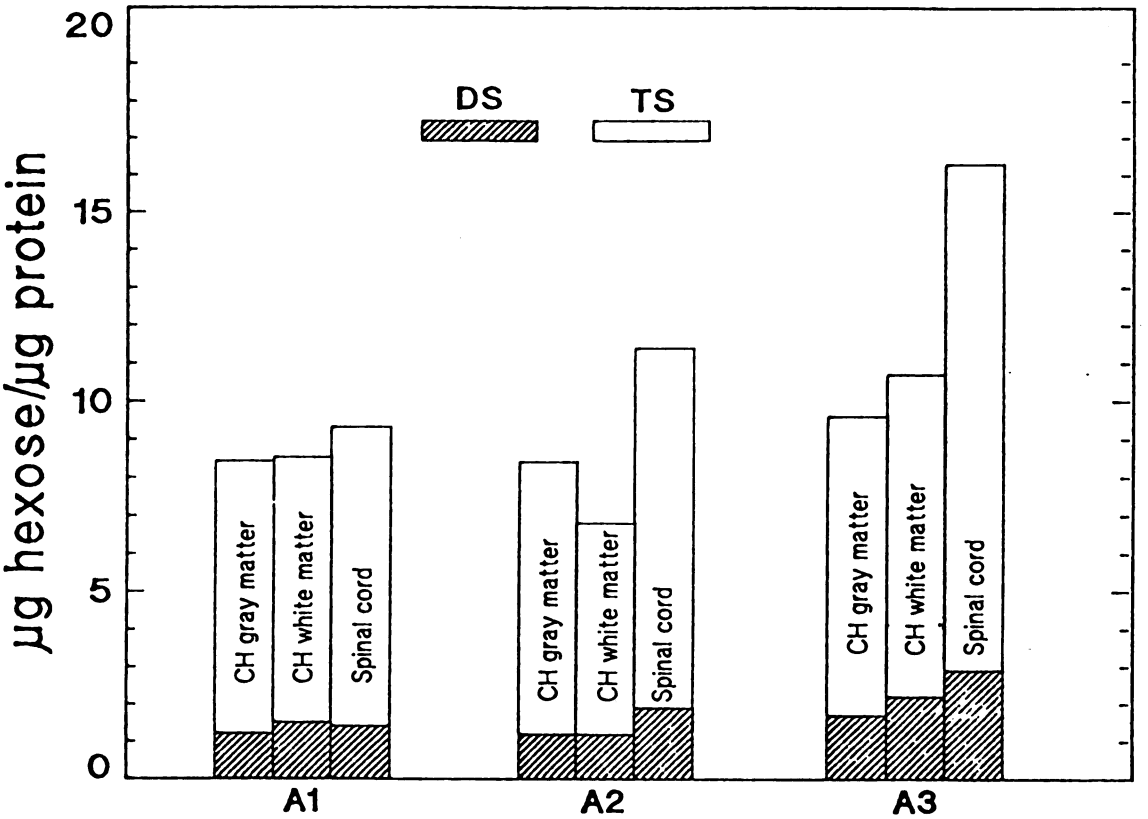
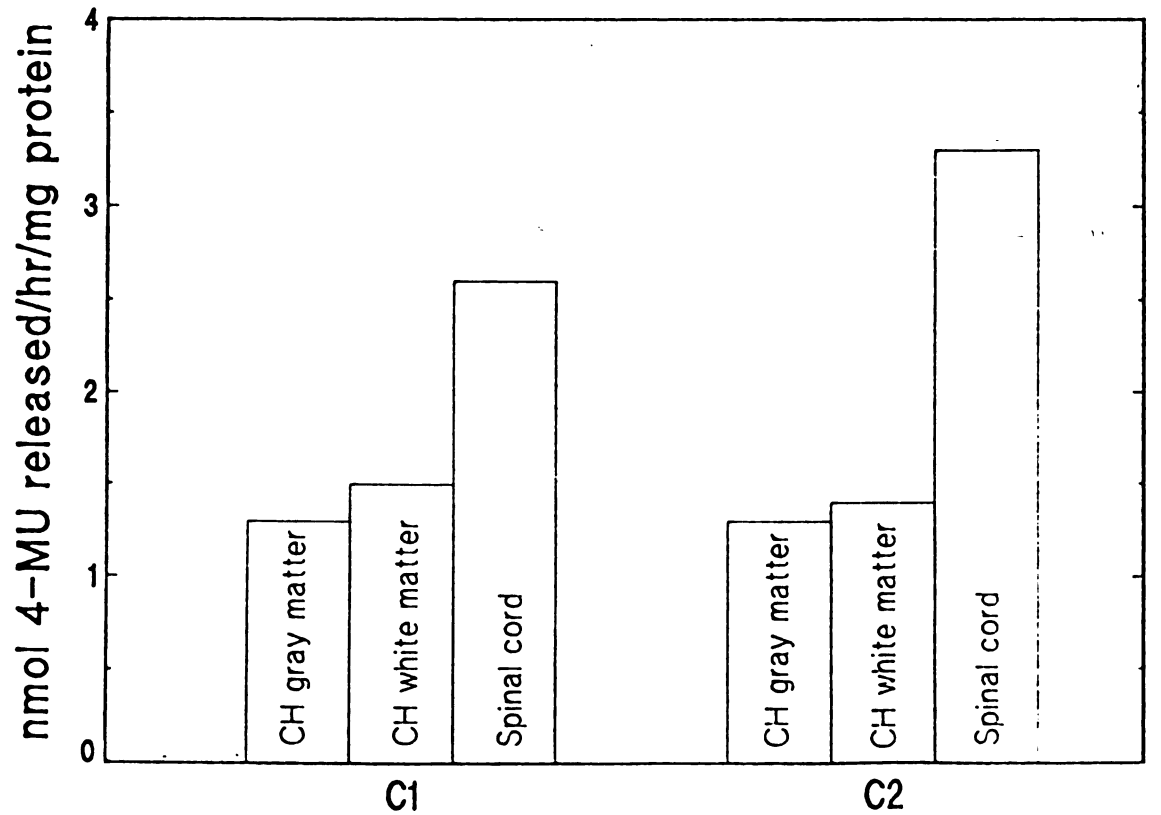

20
15
10
5
0
μg hexose/μg protein
DS
TS
CH gray matter
CH white matter
Spinal cord
A1
A2
A3

FIG. 3


4
3
2
1
0
nmol 4-MU released/hr/mg protein
CH gray matter
CH white matter
Spinal cord
C1
C2

Investigation of Dysmyelinogenesis in Caprine β-Mannosidosis: In Vitro Characterization of Oligodendrocytes

ABSTRACT

Central nervous system myelin deficiency is a consistent feature of caprine β-mannosidosis, an autosomal recessive neurovisceral lysosomal storage disease. To investigate the possibility of an intrinsic oligodendrocyte defect in β-mannosidosis, oligodendrocyte-enriched glial cultures from the cerebral hemisphere white matter of 2 affected and 6 control goats were compared with respect to culture yield and morphology. Fewer oligodendrocytes were cultured per gram of white matter from affected animals than from control animals. Galactocerebroside-positive oligodendrocytes from all animals were similar morphologically at all stages of culture by phase contrast and fluorescence microscopy. These findings are consistent with in vivo morphological observations and suggest that differentiated oligodendrocytes from affected animals do not show morphological abnormalities in culture. However, increased numbers of galactocerebroside-negative bipolar cells, which may be glial progenitor cells, were present in affected animal cultures. Their presence in increased numbers in affected animals suggests the possibility of a defect in differentiation to mature oligodendrocytes, with persistence of the undifferentiated glia during late stages of development.

INTRODUCTION

Myelin deficiency, with regional variation in severity, is a major pathological feature of the lysosomal storage disease caprine ß-mannosidosis. In a caudal to rostral pattern of increasingly pronounced myelin deficiency, affected goat spinal cord white matter tracts have moderate myelin deficiency, optic nerve and brainstem regions have an intermediate degree of myelin deficiency, and cerebral hemisphere white matter has severe myelin deficiency (Lovell and Jones, 1983). In optic nerve from an affected-control pair of 124/150 days gestation fetuses, a 40% reduction in the number of oligodendrocytes was present in the affected animal (Lovell and Boyer, 1987). This finding, in combination with qualitative observations in other studies (Lovell and Jones, 1983; 1985), suggested that oligodendrocyte number is reduced in regions where myelin deficiency is present. The possibility that affected animal oligodendrocytes produce fewer myelin sheath segments than control animal oligodendrocytes has been suggested but not definitively addressed. Other in vivo observations include: a greater proportion of oligodendrocytes with vacuoles and dark cytoplasm in affected goats, beginning prior to development in corpus callosum (Lovell, in preparation); no ultrastructural difference between affected and control animal CNS myelin sheaths; and absence of either oligodendrocyte or myelin degeneration (Jones et al., 1983; Lovell and Jones, 1983; 1985). Taken together, these findings suggest that some alteration in oligodendrocyte proliferation, differentiation, and/or function is present in animals affected with ß-mannosidosis.

Culturing of oligodendrocytes from the CNS of goats affected with β-mannosidosis is important to (1) determine if in vivo and in vitro findings are consistent, and (2) define any abnormalities that might indicate that an intrinsic oligodendrocyte defect is present in β-mannosidosis. Thus, the present study was designed to characterize affected and control goat oligodendrocytes in vitro with respect to yield per gram white matter and morphological characteristics.

METHODS AND MATERIALS

Materials

Animals studied included six newborn control goats and two newborn affected goats. Following euthanasia with T-61 (American Hoechst, Somerville, NJ), the brain was removed and either one or both cerebral hemispheres were placed in sterile Hanks' balanced salt solution without calcium and magnesium containing 10 ug/ml gentamicin and 0.24 ug/ml Fungizone (HBSS-GF), and transported to tissue culture facilities. Tissue culture procedures were begun within 30 to 60 minutes of animal death. Unless otherwise noted, reagents were obtained from GIBCO.

Cell Culture Methods

CNS mixed cell cultures were prepared with modifications of the Percoll gradient methods described by Hirayama et al. (1983) and Kim et al. (1983). Cerebral hemispheres were rinsed 5-7 times with HBSS-GF and cut coronally into six sections. White matter was carved from each section with a scalpel, minced with two single-edged razor blades held in tandem, weighed, and incubated in HBSS-GF containing 2.5% trypsin and 10 ug/ml DNAse (Sigma)

for 30 min at 37°C in an Orbit shaker bath (Lab-Line). After cooling in an ice bath for 3-5 min, the solution was spun at 750 g for 4 min in a swinging bucket centrifuge (International Equipment Company) and the supernatant was poured off. Minimal essential medium (MEM) was added to the pellet and the tissue was agitated by trituration, passed through 210 um and 50 um mesh Nitex screens (Tetco), and diluted to a maximum volume of 40 ml. In each of two polycarbonate centrifuge tubes, 20 ml of the screened solution was combined with 9 ml Percoll (Sigma) and 1 ml 10X HBSS-GF, mixed thoroughly, then spun at 31,000 g for 30 min at 10°C in a Beckman J2-21 centrifuge in a J17 25° fixed angle rotor. From each centrifuge tube, the maximal quantity of the clear "oligodendrocyte-rich" area of the resulting gradient, between the myelin layer on top and the "cloudy" and red blood cell layers near the bottom (Hirayama et al., 1983), was withdrawn, diluted to a total volume of 50 ml in MEM, mixed thoroughly, and spun at 1000 g for 5 min in a swinging bucket centrifuge. The supernatant was poured off and the pellet was resuspended in 5 ml MEM, triturated, diluted to 50 ml with MEM, and spun at 750 g for 5 min. The supernatant was poured off and the pellet was suspended in media. A sample of the cell suspension was taken for counting by hemocytometer in all 6 control animal cultures and in 1 of 2 affected animal cultures. The remaining suspension was placed into Petri plates, and diluted further with medium. Medium consisted of MEM with D-valine (GIBCO # 320-2570), 10% heat-treated fetal calf serum (Hyclone Laboratories), an additional 6 mg% glucose, 0.12 ug/ml gentamicin, and 0.12 ug/ml Fungizone. Cells were incubated in an humidified chamber with

5% CO_2 at 37°C. On day 3, medium, containing floating clumps of cells, was removed from Petri plates, triturated, and spun at 1,000 g for 10 min. The cell pellets were resuspended in media, plated on 15-mm-diameter glass coverslips (Bellco) precoated with 1 mg/ml poly-L-lysine (Sigma), and incubated in Petri plates. Medium was added to Petri plates after 12-16 hours. Subsequently, cells were fed every 3-4 days by replacement of half of the total medium volume with fresh medium.

Immunolabelling

For immunofluorescence labeling of oligodendrocytes, coverslips were rinsed in fresh MEM, then incubated for 15 min with monoclonal anti-galactocerebroside (anti-GC, provided by Dr. Joyce Benjamins) diluted 1:50 in MEM. Coverslips were rinsed in MEM, then incubated for 30 min with fluorescein-conjugated goat anti-mouse IgG (ICN Biochemicals) diluted 1:20 in MEM. Coverslips were rinsed in MEM, cells were fixed in 4% paraformaldehyde for 10-15 min., and coverslips were rinsed with 0.05 M Tris and mounted as below. For immunofluorescence labeling of astrocytes, cells were rinsed in MEM, fixed first in 4% paraformaldehyde for 10 min and then in -20°C methanol for 20 min. Cells were labeled for 60 min with a prediluted monoclonal anti-GFAP cocktail (Biomedical Technologies), then, after rinsing in Tris, incubated for 30 min with FITC-conjugated goat anti-mouse antibody (ICN Biochemicals) diluted 1:20 in Tris and then rinsed with Tris. For staining controls, normal mouse serum (Dako), diluted 1:50, and prediluted myeloma protein control solution (Biomedical Technologies) replaced primary antibody. Coverslips were mounted

onto glass slides with Aquamount (Lerner Laboratories). All procedures, with the exception of methanol fixation, were carried out at room temperature.

Microscopy

Cells were examined and photographed at various stages of culture using a Nikon TMS inverted microscope. Immunofluorescence labelled cells were examined and photographed using a Leitz Ortholux II fluorescence microscope equipped with phase contrast optics. Morphological assessment of cultured cells included examination by phase contrast and fluorescence microscopy over a period of 0 (at plating) through 30 days on coverslips (DOC). Phase contrast morphological assessment included examination of cell body and processes and assessment of changes during culture. Additionally, immunolabelled cells were examined by (1) fluorescence then phase contrast, and (2) phase contrast then fluorescence to (a) assess the approximate proportion of GC+ cells among plated cells; (b) assess GC-immunofluorescence pattern of oligodendrocytes; and (c) check for GC+ staining in cells with phase contrast oligodendrocyte-like morphology.

RESULTS

Cells from Control Animals

Control animal cell yield from the "oligodendrocyte-rich" layer of the Percoll gradient ranged from 0.86 X 10^6 to 3.25 X 10^6 cells per gram white matter. Cell viability, assessed by trypan blue exclusion, was consistently greater than 93%. In all cultures, after 3 days in Petri plates, numerous clumps of cells

were seen floating in the media while other cells had attached and typically had a flat appearance. The vast majority of clumped cells were uniform in appearance (Fig. 1 A). Most were round, had a phase bright halo, and had a yellow-orange hue. Even after vigorous trituration, many floating cells spun down from medium removed from the Petri plates remained in clumps at plating on poly-lysine coated coverslips, although some individual cells were present also. Within 2-5 days on coverslips (DOC) most control animal cells had established processes. Processes were initially short, extending about 2-5 um and often curving back toward the cell body. Cell body diameters were typically 8 to 15 um. When examined between 3-5 DOC, over 70% of plated cells were GC+ (Fig. 2 A,B). Between 7 and 30 DOC, oligodendrocyte morphology was similar to that reported in oligodendrocyte cultures isolated from the brains of mature sheep (Yim et al., 1986), cows (Lisak et al., 1981), rats (Hirayama et al., 1983), and humans (Kim, 1983). The number, extent of branching, length, and diameter of processes varied among oligodendrocytes. Most oligodendrocytes established relatively simple, GC+ processes (Fig. 3 A), although some developed elaborate, fine branches off of main processes. Some cells elaborated GC+ membranous sheets from their processes or from their cell bodies (arrow, Fig. 3 D). Occasional oligodendrocytes developed long, thick, GC+ processes which extended as far as 50 um with node-like areas periodically along their shafts. Most oligodendrocytes remained in direct contact with coverslips. Using both phase contrast and fluorescence microscopy, all cells with phase contrast oligodendrocyte-like appearance were found to be GC+, and few GC- cells were

seen early in control animal cultures. Occasional small, round, bipolar, GC- cells with 4-8 um cell body diameter were present (arrows, Fig. 4 A). These cells plated out exclusively on top of flat cells which had the appearance of astrocytes. By 10-14 DOC, a virtual monolayer of flat, predominantly GFAP+ cells was present on coverslips which made phase contrast observations of oligodendrocytes difficult.

Cells from Affected Animals

Affected animal cell yield from the "oligodendrocyte-rich" layer of the Percoll gradient from one animal was 0.29 X 10^6 cells per gram white matter. Cell viability, assessed by trypan blue exclusion in one animal, was 94%. As in control animal cells, floating clumps of cells were noted in the medium during the Petri plate step. However, while some clumped cells from affected animals were similar in size and phase contrast appearance to control cells, many cells were small and phase dark (arrowhead, Fig. 1 B). After plating of cells spun down from Petri plate media, only a sparse population of oligodendrocytes was identified by immunofluorescence. Only 10% to 20% of plated affected goat clumped cells were GC+ at 3 DOC (Fig. 2 C,D). Affected animal oligodendrocyte appearance, with respect to process and sheet formation, was similar to that of control animal oligodendrocytes by both phase contrast and immunofluorescence parameters at all stages of culture (Fig. 3 B,C,E). GC-labelling characteristics of affected animal oligodendrocytes, including pattern of fluorescence and qualitative intensity of fluorescence, were similar to control animal oligodendrocyte characteristics. Using both phase contrast and

fluorescence microscopy, all cells that had phase contrast oligodendrocyte-like appearance were GC+. Additionally, bipolar, GC- cells (arrows, Fig. 4 B), were present in much greater numbers in affected animal cultures. Cell body diameter of bipolar cells was between 4 and 8 um. The eventual development of a monolayer of flat, GFAP+ cells was similar to the control culture pattern. Neither cytoplasmic vacuolation nor evidence of oligodendrocyte degeneration or death was noted in affected or control cultures.

DISCUSSION

This is the first report of the culture of oligodendrocytes from animals affected with ß-mannosidosis. An important result of this study is the observation that affected and control animal oligodendrocytes are morphologically very similar in vitro. Similarities are present with respect to (1) appearance of processes and cell bodies, (2) appearance of sheet-like membranous proliferation from a similar proportion of cells, and (3) pattern of immunofluorescence on cells identified by phase contrast as oligodendrocytes. These results are consistent with in vivo morphological observations, i.e., affected animal oligodendrocytes were reduced in number but produced normal-appearing myelin sheaths (Lovell and Boyer, 1987; Lovell and Jones, 1983; 1985). In vitro evaluation of cells by electron microscopy will be needed to assess how cultured oligodendrocytes compare with respect to the in vivo finding of an increased proportion of oligodendrocytes with dark cytoplasm and vacuoles in affected animals (Lovell,

in preparation).

Two lines of evidence from this research indicate that significantly fewer oligodendrocytes were present in affected animal cultures than in control animal cultures. First, counts of cells isolated from the Percoll gradient from one affected animal found between 10% and 40% of the number of cells isolated in the six control animal cultures. While these counts include some proportion of "contaminating" cells like astrocytes, they nonetheless reflect the number of cells present in the "oligodendrocyte-rich region" of the Percoll gradient. Second, a much lower percentage of affected animal clumped cells were GC+ after plating on coverslips and there was a greater percentage of GC- bipolar cells. It is possible that differences in oligodendrocyte buoyancy, due to cytoplasmic vacuolation, might lead to failure in isolation of a higher percentage of affected animal oligodendrocytes. However, a large portion of the Percoll gradient was harvested, to include a relatively broad band of densities, and loss of some oligodendrocytes due to buoyancy differences would not explain the lower percentage of GC+ cells on coverslips. Thus, it is most likely that the decreased number of oligodendrocytes observed in vitro reflects the decreased number in vivo in areas of affected goat CNS with severe myelin deficiency (Jones et al., 1983; Lovell and Boyer, 1987; Lovell and Jones, 1983; 1985).

The genetic defect in ß-mannosidosis most likely affects the ß-mannosidase enzyme gene locus. Decreased levels of tissue and plasma activity of the lysosomal form of this enzyme are found in all affected animals (Jones and Dawson, 1981; Jones et al., 1984) and produce tissue accumulation of the

putative oligosaccharide substrates for the enzyme (Jones and Laine, 1981; Jones et al., 1984; Matsuura and Jones, 1985; Matsuura et al., 1981). Oligosaccharide storage is thought to explain the cytoplasmic vacuolation seen in vivo in most cell types of affected animals, although cytoplasmic storage may also be present outside of lysosomal storage vacuoles. While mild to moderate vacuolation is seen in vivo in oligodendrocytes (Jones et al., 1983; Lovell and Boyer, 1987; Lovell and Jones, 1983; 1985), no cytoplasmic vacuolation is seen by phase contrast microscopy in cultured oligodendrocytes up to 30 DOC or in astrocytes up to 90 DOC. Phase contrast microscopy should provide excellent visualization of vacuoles if they are present. This apparent lack of vacuolation of cultured affected animal glial cells is consistent with similar observations in affected animal fibroblast cultures. Fibroblasts contain vacuoles in vivo (Jones et al., 1983). However, in vitro, both the presence of oligosaccharides in medium and in cytoplasm have been demonstrated in fibroblast cultures, in the absence of cytoplasmic vacuolation (Hancock et al., 1986; and unpublished observations). The absence of vacuoles in cultured cells could be related to cell isolation procedures, which cause some disruption of the normal architecture of cells. Thus, loss of some portion of membrane and cytoplasm, and possibly lysosomal storage vacuoles, could occur during the isolation. Lack of formation of vacuoles during the culture period may be related to different behavior of the cells in culture than in vivo, possibly with (1) different storage locations for uncatabolized oligosaccharides (e.g. cytoplasmic versus lysosomal), (2) altered metabolism with less accumulation of oligosaccharides and thus fewer or smaller storage vacuoles,

and (3) exocytosis of uncatabolized oligosaccharides into the medium as opposed to storage.

Although not specifically quantitated, a substantial increase in the percentage of GC- bipolar cells was present in affected animal cultures compared to control animal cultures. Bipolar cells had Percoll gradient density characteristics within the range of oligodendrocytes and floated and clumped together, along with oligodendrocytes, during the Petri plate step. Morphologically similar cells have been identified as glial progenitor cells in mixed glial cultures from neonatal rat (Raff et al., 1983; Temple and Raff, 1986) and fetal sheep (Elder et al., 1988), using anti-GC and anti-A_2B_5, a monoclonal antibody directed against a complex ganglioside (Eisenbarth et al., 1979). A small number of bipolar cells was also seen in all control goat cultures, but the ratio of bipolar cells to oligodendrocytes was clearly low in control cultures. If the bipolar cells are glial progenitor cells, these results may suggest that an increased number of glial progenitor cells remain undifferentiated in affected animals. Since the affected animal cells were isolated from cerebral hemisphere white matter, where a decreased number of mature oligodendrocytes are found, an abnormality or block in differentiation of progenitor cells to oligodendrocytes may be indicated. The possibility remains, however, that affected and control animal white matter contain comparable numbers of bipolar cells per gram tissue and that the appearance of an increased proportion of bipolar cells in affected animal cultures is due to the substantial reduction in number of mature oligodendrocytes. Additional studies are needed to further investigate bipolar

cells and to determine the role of impairment of oligodendrocyte development in caprine ß-mannosidosis.

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Fig. 1. Control (A) and affected (B) animal cells after 3 days in culture in Petri plates. Floating cells isolated from both affected and control animal CNS white matter were clumped together. Control animal cells were relatively uniform in size and most had a phase bright appearance (arrow). Most affected animal cells were small and phase dark (arrowhead) although some phase bright cells were present (arrow). 250X.

Fig. 2. Phase contrast (left) and GC immunofluorescence-labelled (right) micrographs from identical fields from control (A,B) and affected (C,D) animal cells 3 days after plating of floating cells on poly-L-lysine coated glass coverslips. Most clumped control animal cells were GC+ (B) while many clumped affected animal cells (e.g. C, arrows) were GC- (D). 840X.

Fig. 1

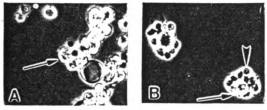


Fig. 2

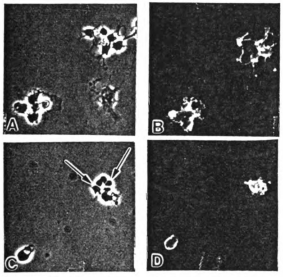


Fig. 3. Control (A,E) and affected (B-D,F) animal cells between 12 and 21 days on coverslips labelled by GC immunofluorescence. Although there were fewer oligodendrocytes present in affected animal cultures, the number and length of oligodendrocyte processes in control (A,E) and affected (B-D,F) animal cultures was quite similar. Also, formation of GC+ membranous sheets was similar in control (E, arrow) and affected (F, arrow) cultures. 630X.

Fig. 4. Control (A) and affected (B) animal cells at 8 days on coverslips. Many bipolar cells (arrows) were present in affected animal cultures while few were present in control animal cultures. No cytoplasmic vacuoles were noted by phase contrast microscopy in control or affected animal cells. 160X.

Fig. 3

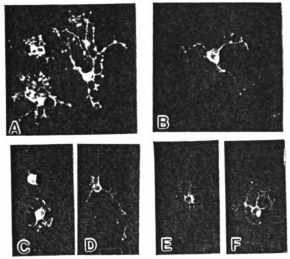
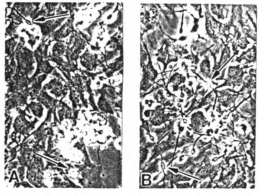


Fig. 4



Astrocyte Abnormalities in Caprine β-Mannosidosis: An Immunocytochemical Analysis of Optic Nerve

ABSTRACT

Central nervous system abnormalities in caprine β-mannosidosis, an autosomal recessive disease of glycoprotein catabolism, include myelin deficiency with regional variation in severity and reduced numbers of oligodendrocytes in areas of severe myelin deficiency. A previous ultrastructural and immunocytochemical study demonstrated an increased density of astrocyte processes in CNS regions with moderate to severe myelin deficiency. The current study sought to assess developmental aspects of gliosis through an immunocytochemical investigation of glial fibrillary acidic protein (GFAP) reactivity in optic nerves of animals ranging in age from 115/150 days gestation to 31 days postnatal.

The density of GFAP+ astrocyte processes was quantitatively greater in affected animal optic nerves than in control animal optic nerves as early as 115/150 days gestation. However, astrocyte process density did not increase substantially with increasing age. Additionally, no increase in the number of GFAP+ astrocyte cell bodies was present in affected goat optic nerves compared to control goat optic nerves. These results suggest that astrocyte changes are present in the affected animal optic nerve early in the process of myelination and that the extent of change, compared to controls, does not increase substantially with age. Increased astrocyte process density at a time early in myelination suggests that gliosis is not secondary to deficient myelination alone and may instead take place in response to other abnormalities, possibly in response to a decreased number or abnormal development of oligodendrocytes. Additionally,

the increased density of GFAP+ astrocyte processes appears to be due to a greater number of processes extending from each astrocyte, although the possibility of an increased number of astrocytes with redistribution of GFAP from cell bodies into processes has not been ruled out.

INTRODUCTION

A caudal-to-rostral pattern of increasingly severe central nervous system (CNS) myelin deficiency is a major pathological feature of caprine ß-mannosidosis, an autosomal recessive disease of glycoprotein metabolism (20). In affected goats, the spinal cord, which is myelinated relatively early in development, has a moderate degree of myelin deficiency while the cerebral hemispheres (CH), which are myelinated later in development, have a severe deficiency of myelin. This pattern and other evidence suggest that disruption of the myelination process takes place during development. Both quantitative (19) and qualitative observations (20,21) have suggested that oligodendrocyte number is reduced in regions where myelin deficiency is present.

Astrocytes respond to central nervous system (CNS) pathological changes with "gliosis." Gliosis consists of (1) astrocyte proliferation and/or (2) astrocyte hypertrophy, with an increased number of extended processes and an increased expression of the astrocyte-specific intermediate filament glial fibrillary acidic protein (GFAP) (3,17). Gliosis has been described in a variety of CNS pathological conditions including trauma, inflammatory diseases, hepatic encephalopathy, degenerative diseases, and aging (3,7,10,29). Additionally, gliosis has been identified in the CNS of various myelination mutant mice, including jimpy, quaking, shiverer, and mld mutants (1).

A previous immunohistochemical and electron microscopic study in goats affected with ß-mannosidosis demonstrated an increased density of astrocyte processes without an increased number of cell bodies in CNS regions with

moderate to severe myelin deficiency (18). Possible explanations of this gliosis include reactive response to (1) deficient myelin formation, (2) oligodendrocyte abnormalities, or (3) metabolic perturbations inherent to affected animals. Additionally, it is possible that a primary abnormality in astrocyte multiplication in β-mannosidosis could lead to inhibition of myelination. To help clarify some of these issues, the current study was designed to assess developmental aspects of gliosis through an immunocytochemical investigation of GFAP reactivity in optic nerves of animals ranging in age from 115/150 days gestation to 31 days postnatal. Objectives were to (1) assess whether gliosis was present during early myelination, (2) determine whether gliosis increased during development, and (3) describe features of gliosis in the optic nerve for later comparison with features in other CNS regions.

METHODS AND MATERIALS

Tissue Acquisition and Handling

Age-matched pairs of affected and control animals at 115/150 and 124/150 days gestation (1 pair at each age), 3-5 days postnatal (2 pairs), and 4 weeks postnatal (1 pair) were used in this study. Animals were classified as affected, carrier, or normal by phenotypic characteristics (12,14,16) and plasma ß-mannosidase assay (4,6,8). Both carrier and normal animals were used as controls. T-61 euthanasia was used for neonatal and older goats. Fetuses, obtained by cesarean section, were perfused with a 2% paraformaldehyde and 2% glutaraldehyde buffered solution before necropsy. Optic nerve samples from within 0.5 cm of the orbit were excised and (1) fixed in 4% glutaraldehyde for 2-4 hrs, post-fixed in 1% osmium tetroxide, and embedded in an Epon-Araldite mixture and (2) fixed in 4% paraformaldehyde for 24 hours and stored in 0.05 M Tris buffer containing 0.02% sodium azide until Vibratome sectioning. Thick sections (1 um) were cut from Epon blocks, placed on glass slides, and either left unstained or stained with toluidine blue. Sections of 50 or 60 um were cut with a series 1000 Vibratome (Technical Products International) and placed in Tris containing 0.02% sodium azide.

Immunocytochemistry

Epon immunocytochemistry was carried out using modifications of the methods of Trapp et al. (28). Glass slides containing 1 um semithin sections were heated overnight in a 68°C oven; etched by (a) incubation of slides for 15 min in sodium ethoxide (saturated sodium hydroxide in absolute ethanol aged for

more than 2 weeks, diluted 1:2 with absolute ethanol), (b) a 1 min rinse in each of three absolute ethanol baths, and (c) incubation in 0.2% sodium hydroxide in water for 5 min; and rinsed in 0.05 M Tris buffer bath (pH=7.4). All subsequent rinses were for 15 min in Tris. All incubations were carried out at room temperature. All antibody dilutions were in Tris containing 1% swine serum. Blocking serum (Tris containing 3% swine serum (Dako)) was applied to sections for 60 min, then blotted off. Sections were incubated for 60 min with primary antibody (1:100); rinsed; incubated for 60 min with secondary antibody (1:40); rinsed; incubated in peroxidase-anti-peroxidase complex (Dako) (1:80); and rinsed. For staining control slides, Tris and normal rabbit serum (Dako) (1:100) were substituted for primary antibody. Peroxidase labeling was revealed by incubation for 10 min with diaminobenzadine (Sigma), 0.5 mg/ml, in water containing 0.01% hydrogen peroxide. DAB staining was intensified by 5 min exposure of sections to 2% osmium tetroxide. Finally, sections were dehydrated, cleared, and coverslipped using Permount (Fisher Scientific) as a mounting media.

Vibratome immunochemistry, using methods modified from Koeppen et al. (15), was carried out in tissue culture wells. Sections were incubated overnight in Tris containing 1.5% sodium chloride, rinsed, and incubated for 1 h in a "suppressor serum" consisting of 10% swine serum, 4% bovine serum albumen (Sigma), and 0.1% Triton X-100 (Sigma) in Tris. Suppressor serum was pipeted off and sections were incubated in a 1:100 dilution of anti-GFAP, or 1:100 rabbit serum or Tris for staining controls, each containing 1% swine serum and 0.1%

Triton X-100. Remaining solutions and procedures were as described for paraffin sections, except DAB incubation was for 1-2 min. After mounting on glass slides precoated with a aqueous mixture of 0.01% chrome alum and 0.1% gelatin, sections were dehydrated, cleared, and coverslipped.

Light Microscopy

GFAP-labelled Epon, paraffin and Vibratome sections were examined and photographed with a Nikon Microphot-FX photomicroscope. A qualitative assessment of distribution of GFAP+ reactivity and number of astrocyte cell bodies was made.

Image Analysis

The proportion of areas occupied by GFAP reaction product was quantified using a Nikon-Joyce Loebl Magiscan IIa image analyzer system. Three GFAP-labelled sections from either 2 or 3 blocks from fetal pairs at 115/150 and 124/150 days gestation, two pairs of 3-day-old animals, and one pair of 3-week-old animals were examined. Fifteen consecutive oil immersion fields, occupying a total of 1.2 X 10^4 um^2, were analyzed on each section. Fields were adjusted to exclude cell bodies, perifascicular connective tissue, and vascular regions.

RESULTS

Assessed qualitatively, a substantially greater density of GFAP+ astrocyte profiles was present in the optic nerve of affected animals compared to control optic nerves as early as 115/150 days gestation and as late as four weeks of age (Fig. 1). At higher magnification (not shown) it was found that affected animal

astrocyte process profiles were both more numerous and of larger cross-sectional area than control animal profiles. As quantitated by image analysis, the proportion of area covered by GFAP reaction product was consistently greater in affected animals than in their age-matched controls (Fig. 2). However, comparing affected animal results, only a modest difference in GFAP reaction product density was present between the 115/150 and the 124/150 days gestation fetal optic nerves, and no difference was present among optic nerves of the 124/150 days gestation fetus and older animals. Although a greater density of GFAP+ astrocyte profiles is present in optic nerve of affected animals, no corresponding increase in the number of GFAP+ cell bodies was present (Fig. 3).

DISCUSSION

The optic nerve was chosen for analysis to (1) represent an area of intermediate myelin deficiency in affected animals and to (2) provide a morphologically uniform area for comparisons between affected and control animals and among animals of different ages. Because of the well documented glial cell and blood vessel variations along the length of the optic nerve (26,27), this study was restricted to sections of optic nerve nearest the orbit.

A pronounced increase in the proportion of area occupied by GFAP-reactive astrocyte processes was present in all affected goat optic nerves examined, and qualitative light microscopic evidence from GFAP-reactivity in the

affected animal optic nerve Vibratome sections found that cell body density is not increased above normal levels. These findings suggest that the astrocyte process density increase in affected animals is probably secondary to astrocyte hypertrophy, with an increase in number of processes extended from each astrocyte, without increased astrocyte number. Although astrocyte proliferation is an element of gliosis in some CNS pathological processes (10,17), astroglial hypertrophy without concomitant proliferation leads to marked gliosis as well (24,25). Additionally, a study in the myelination mutant jimpy mouse attributed gliosis to glial cell hypertrophy after ruling out astrocyte proliferation (2). Nonetheless, identification of astrocyte cell bodies in the current study rests on the labeling of GFAP. Redistribution of GFAP away from the cell bodies of astrocytes and into the newly extended processes could leave some proportion of cell bodies undetected by the immunocytochemical staining procedure employed here. An earlier electron microscopic and immunocytochemical study suggested that such a pattern may occur in the cerebral hemisphere white matter of affected goats (18). This possibility needs further investigation.

A decreased volume of tissue in which the same number of astrocytic processes are more densely packed could lead to the appearance of increased density of astrocyte processes. An increased amount of interfascicular connective tissue and a decrease in fascicle area in the affected animal optic nerve (Lovell, unpublished observations), so condensation of fascicle contents is a possibility. However, cross-sectional area in optic nerves from a pair of affected and control fetuses at 124/150 days gestation, during early myelination, was nearly identical

(19), and there is no corresponding increase in packing density of GFAP-reactive cell bodies. Nonetheless, gliosis is present early in myelination, and may well compensate for decreased tissue density by playing a space-occupying role.

A substantial difference in astrocyte profile density between control and affected animals was present as early as 115/150 days gestation, an early point in myelination. Similar findings were recently reported in the jimpy mouse, with gliosis present in various CNS regions, at a point preceding or early in myelination in the region (2). Increased astrocyte density in the optic nerve of animals affected with ß-mannosidosis at an early stage in myelination suggests that gliosis is not entirely a reaction to deficient myelination. Other elements possibly responsible for triggering the gliotic reaction may include abnormalities in oligodendrocyte differentiation and maturation or the accumulation of oligosaccharides.

Animals affected with caprine ß-mannosidosis have deficient activity of the lysosomal enzyme ß-mannosidase and an associated tissue and body fluid accumulation of a large amount of the enzyme's putative substrates, including a trisaccharide (mannosylß1-4N-acetylglucosaminylß1-4N-acetylglucosamine), a smaller amount of a disaccharide (mannosylß1-4N-acetylglucosamine), and much smaller quantities of more complex oligosaccharides (5,9,13,14,22,23). Astrocytes, like oligodendrocytes, have a variable but generally modest degree of cytoplasmic vacuolation, with presumed lysosomal accumulation of oligosaccharides (12). A recent study has suggested that levels of oligosaccharide storage are not

associated with myelin deficiency (Boyer et al., in preparation). However, it is nonetheless possible that astrocytes, which appear to play a key metabolic and homeostatic role in maintaining the microenvironment of the CNS (3,7,11), could respond by gliosis to the abnormal presence of oligosaccharides or to an undefined CNS injury secondary to oligosaccharide presence.

These results suggest that astrocyte changes are present in the affected animal optic nerve early in myelination and that the extent of change, compared to controls, does not increase substantially with age. Increased astrocyte process density early in myelination suggests that astrocytosis may not entirely be a reaction to deficient myelination and may take place in response to other abnormalities, possibly defects in oligodendrocyte development. Additionally, the increased density of GFAP+ astrocyte processes appears to be due to a greater number of processes extending from astrocytes, although the possibility of an increased number of astrocytes with redistribution of GFAP from cell bodies into processes has not been ruled out. Further work is needed to characterize astrocyte changes in regions of both severe myelin deficiency, such as the corpus callosum, and in regions of mild myelin deficiency, such as the spinal cord.

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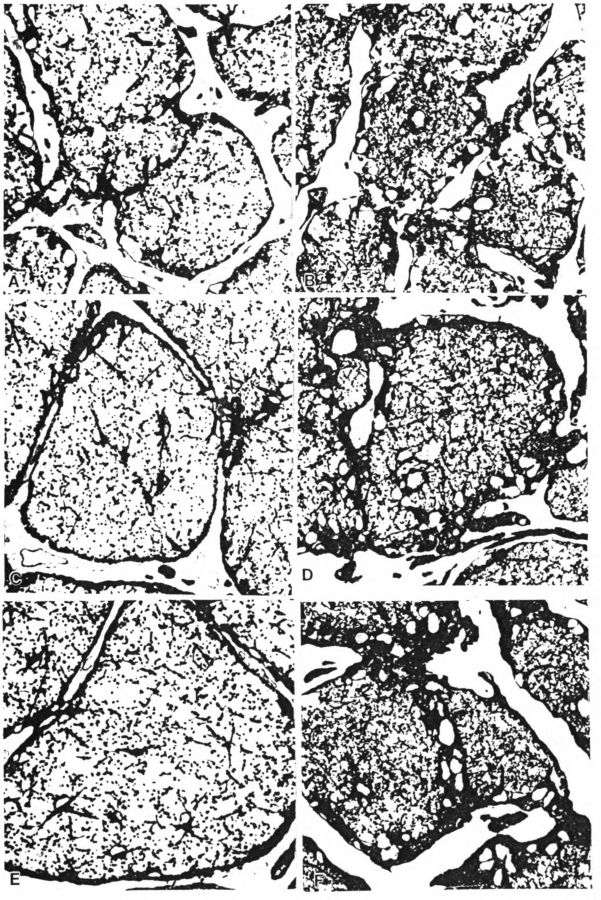
Fig. 1. GFAP-labelled, Epon-embedded optic nerve sections from age-matched control (left) and affected (right) goats at 115/150 days gestation (A,B), 3 days postnatal (C,D), and 4 weeks postnatal (E,F). A substantial increase in the number of GFAP+ astrocyte profiles is present in the affected animal sections. The difference between affected and control animals is present as early as 115/150 days gestation. 790 X.


A
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E
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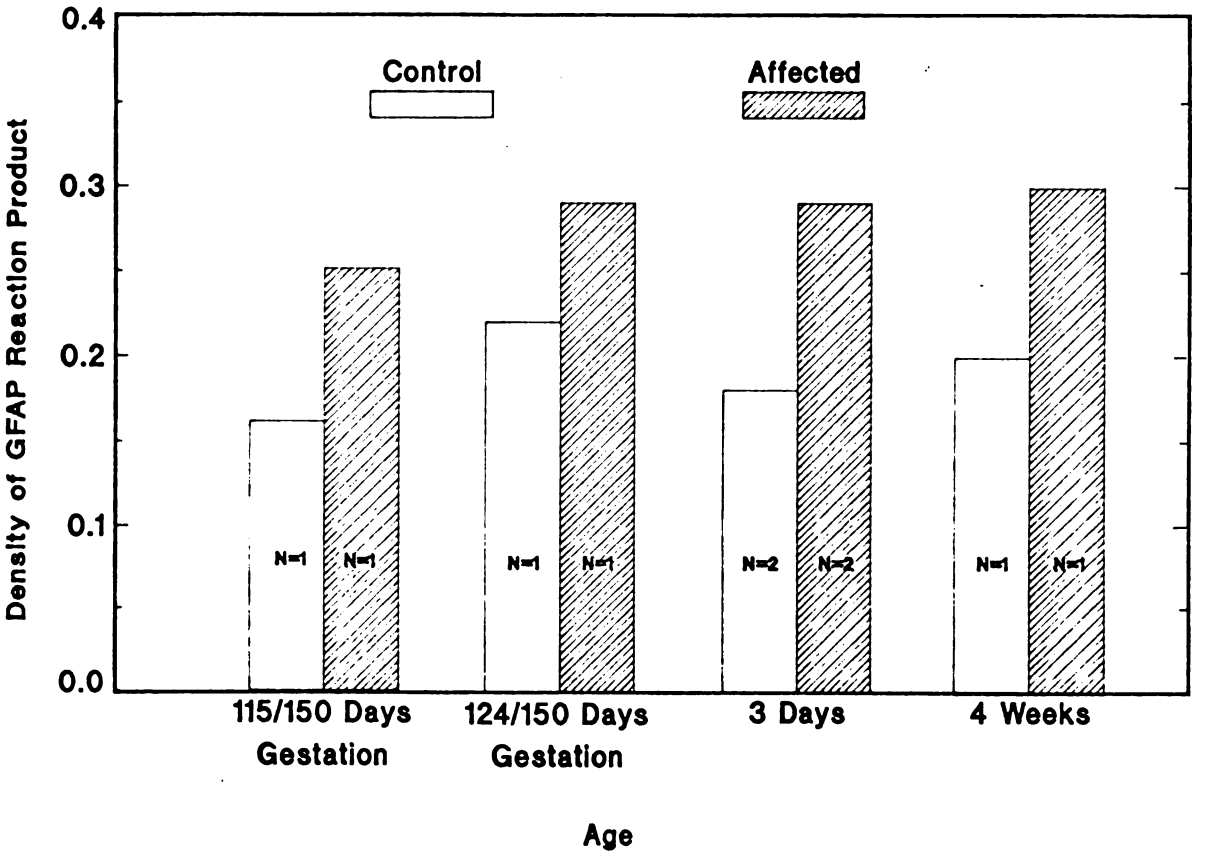
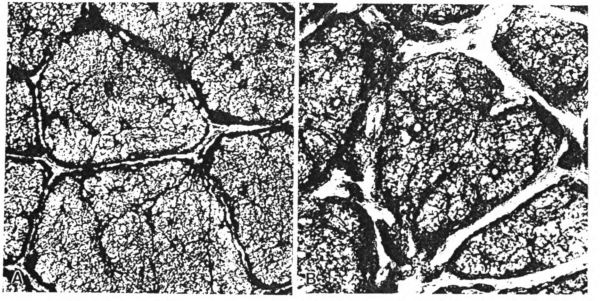


Fig. 2. GFAP reaction product density determined by image analysis in age-matched control and affected animal optic nerves. Density is defined as the ratio of area occupied by GFAP reaction product to total area. Bars represent staining density in $1.2x10^4$ um^2 of optic nerve at each age. Density of the 3-5 day old animals was averaged (labelled "3 Days"). Affected animals have a greater density of GFAP reaction product at all ages examined, however, affected animal GFAP reaction product density does not rise appreciably with age.

Fig. 3. GFAP-labelled 60 um Vibratome sections of optic nerve from 3-day-old control (A) and affected (B) goats. Although a greater density of GFAP+ astrocyte processes is present in the affected animal, no corresponding increase in the number of GFAP+ cell bodies is present. 315 X.


A
B

Caprine β-Mannosidosis:

Abnormal Thyroid Structure and Function in a Lysosomal Storage Disease

ABSTRACT

Deficient activity of the lysosomal enzyme ß-mannosidase leads to widespread tissue accumulation of oligosaccharides in caprine ß-mannosidosis, an autosomal recessive neurovisceral storage disease. Severe thyroid morphological abnormalities found in a previous light microscopic survey of tissues from neonatal affected goats suggested the possibility of impairment of function. Since considerable evidence indicates that thyroid hormones play an important role in regulation of myelination, deficient thyroid hormone concentrations, if present, could be a factor in the hypomyelination seen in affected animals. Thus, this study was designed to characterize thyroid structure and function in ß-mannosidosis.

To investigate developmental aspects of structural abnormalities, thyroids from six pairs of affected and control animals ranging in age from 96/150 days gestation to 3 days postnatal were analyzed by light and electron microscopy. Major findings in affected animal thyroids, as early as 96/150 days gestation, included follicle irregularities and pronounced presence of lysosomal storage vacuoles in all cell types, particularly in follicular cells. The degree of cytoplasmic vacuolation increased with advancing age. To assess thyroid function, thyroid hormone concentrations were determined in six age-matched, neonatal pairs of affected and control goats. Significantly decreased thyroid hormone concentrations were present in affected animals. It is hypothesized that reduced thyroid hormone function plays a role in the pathogenesis of hypomyelination in affected animals. This study comprises, to our knowledge, both the most

complete description of developmental abnormalities and the first report of abnormal function in an endocrine organ in a lysosomal storage disease. Further, this report suggests that the pathogenesis of central nervous system lesions could involve systemic perturbations induced by a genetically determined deficiency of a lysosomal hydrolase.

INTRODUCTION

The fundamental genetic defect in caprine ß-mannosidosis, an autosomal recessive disease of glycoprotein metabolism, is thought to affect the ß-mannosidase gene. Deficient activity of the lysosomal enzyme ß-mannosidase in affected goats leads to tissue accumulation of related oligosaccharide substrates (26,34,35). Previous light microscopic tissue surveys in four affected and two control neonatal animals showed that some degree of cytoplasmic vacuolation was present in most parenchymal, vascular, and connective tissue cells in affected goat tissues, but that thyroid follicular epithelial cells were particularly heavily vacuolated (25). The extensive degree of vacuolation raised the issue of possible functional alteration due to morphological abnormalities or to metabolic perturbations resulting from deficient ß-mannosidase activity. Although endocrine organs are the site of considerable substrate deposition in some lysosomal storage diseases (22,47), the possibility of a coexisting functional abnormality has not previously been addressed. This study was designed to investigate structure-function correlations in a severely affected endocrine organ in a lysosomal storage disease and to consider the possibility that the pathogenesis of hypomyelination in caprine ß-mannosidosis is secondary to abnormal thyroid function.

EXPERIMENTAL DESIGN

Fetal and newborn goat kids were obtained from breedings within two goat herds: herd A, consisting of normal goats (homozygous unaffected for the

genetic defect), and herd B, consisting of carrier goats (heterozygous for the genetic defect). Experimental animals were classified as affected, carrier, or normal by phenotypic characteristics (25,27,29) and plasma ß-mannosidase assay (10,14,17). Both carrier and normal animals were used as controls.

To characterize developmental aspects of thyroid structural abnormalities in ß-mannosidosis, thyroid samples from age-matched pairs of affected and control fetuses at 96, 115, and 124/150 days gestation and from three affected and control animal pairs at 3-5 days of age were studied by light and electron microscopy. Morphological features evaluated included: (a) follicular structure; (b) extent of vacuolation in specific cell types; and (c) ultrastructure of vacuoles and follicular cells. Additionally, thyroid weights from 10 affected and 29 control animals compiled from the ß-mannosidosis research program necropsy records were organized by age into 4 groups: 96-124/150 days gestation, 0-8 days, 1 month, and 4 months. Because of the small sample size in three of the four groups, only the 0-8 day group was tested for statistical significance, using Student's unpaired t-test.

Two separate standardization studies were carried out. A thyroid hormone release study sought to determine whether release was random or pulsatile and sought to establish the validity of single, morning blood samples for thyroid hormone assay in neonatal goats. Also, samples were collected during 14-day neonatal periods to determine normal goat T_3 and T_4 concentrations during the first two weeks of life and examine the possibility of abnormal thyroid hormone concentrations in carriers of ß-mannosidosis. In the thyroid hormone release

study, serum samples were collected every 15 minutes from four animals from each herd at 7 days of age. The thyroid hormone release data for each animal were analyzed by comparison of coefficients of variation to assess evidence of a pulsatile release pattern. In the 14-day study, blood was drawn within 3 hours after birth and then daily up to 8 days postnatally for five herd A and ten herd B animals; and between 9 and 14 days postnatally for five animals in each herd. Herd and day of draw differences and interactions were assessed by a split plot analysis of variance (19) and daily means of the two herds were compared by Bonferroni's t-test. To determine whether T_3 and T_4 concentrations were influenced by sex, assay results from male and female animals between days 1 and 14 from both herds were compared by the Mann-Whitney rank-sum test.

To assess thyroid function in β-mannosidosis, results of serum T_4, free T_4, and free T_3 assays from six newborn affected-control pairs of animals were compared by the Wilcoxon signed-rank test. Pairs consisted of animals 1 or 2 days old, and four of the six pairs were composed of littermates. Data were compiled between 1983 and 1987. The upper limit of sensitivity of the T_3 assay did not allow for statistical analysis of T_3 data.

RESULTS AND DISCUSSION

Thyroid weights

Mean thyroid weights were greater in affected animals than in control animals in all four age groups (Table 1). A significant difference ($p<0.001$) between affected and control thyroid weights was present in the 0-8 day postnatal

group, the only group in which differences were compared statistically.

Morphological abnormalities in affected animal thyroids

By light microscopy, extensive cytoplasmic vacuolation was seen in most cells of affected goat thyroid as early as 96/150 days gestation (Fig. 1 B). Subjective evaluation indicated that the severity of cytoplasmic vacuolation increased with increasing age (Fig. 1 B,C,D). Some variation in the extent of cytoplasmic vacuolation was present both (a) among different thyroid sections from any one animal and (b) among the three neonatal affected animals, but was markedly greater in each of them than in the thyroid from the oldest (124/150 days gestation) affected fetus. In contrast, only occasional, isolated vacuoles were present in control animal thyroid cells (Fig. 1, A). Affected animal thyroid follicles were less regularly defined than control animal follicles, especially in the oldest animals (Fig. 1 C,D). This irregularity appeared to be primarily due to the distention of follicular cells into follicle lumina by cytoplasmic vacuolation.

By electron microscopy, cytoplasmic vacuolation was present in all cell types in affected goat thyroid, including follicular, perifollicular, endothelial, and perithelial cells (Fig. 2 B), and fibroblasts (not shown), compared to the normal appearance of control animal thyroid cells (Fig. 2 A). Follicular cells contained many more vacuoles than other cell types. Vacuole size was relatively constant within a cell type, but varied among cell types. Endothelial cell and fibroblast vacuoles were smaller than follicular cell vacuoles. The size of follicular cell vacuoles in the 96/150 days gestation fetus was more variable than in other affected animal cells, a variability similar to what was seen in the proximal renal

tubular cells of that animal (27). Vacuoles in affected animal thyroid cells were identified ultrastructurally as lysosomal storage vacuoles (Fig. 2 C). They were bordered by a defined limiting membrane which enclosed a floccular, amorphous material containing occasional membrane-like structures. The occasional control animal thyroid cytoplasmic vacuoles seen by light microscopy were typically found to be swollen endoplasmic reticulum cisternae by electron microscopy, and no lysosomal storage vacuoles were encountered in normal animal thyroid cells. In follicular cells, the appearance of both follicular cell rough endoplasmic reticulum and luminal aspects of plasma membrane surfaces were morphologically similar in affected and control thyroids at all ages. Colloid was ultrastructurally similar in affected and control animals.

Perifollicular cells, identified ultrastructurally, were only infrequently found in affected animal thyroids. The lysosomal storage vacuoles in those perifollicular cells clearly identified by secretory granules differed from follicular cell vacuoles in that they were both generally smaller in size and less numerous. Occasional affected goat thyroid cells with a lysosomal storage vacuole appearance similar to perifollicular cells but depleted of secretory granules were noted (large arrow, Fig. 2 B). No comparable secretory granule-depleted perifollicular cells were seen in control thyroid.

Thyroid hormone release in neonatal goats

The 4-hour pattern of thyroid hormone release in 7-day-old goats (Fig. 3) was relatively constant for all four animals from both herds. The average coefficient of variation among the eight animals was 0.07 (range 0.08 to 0.05) for

T_4 and 0.08 (range 0.15 to 0.06) for T_3. Thus, there was no evidence of a pulsatile release pattern. In the 14-day evaluation of newborn animal thyroid hormone concentrations, serum T_4 concentration decreased significantly ($p<0.05$) during the first week of life and then leveled off in both herds (Fig. 4 A). Also, T_4 concentration in herds A and B differed significantly ($p<0.05$) between 2 and 6 days of age. T_3 concentration in both herds were relatively stable during the first two weeks of life, and did not vary significantly between herds (Fig. 4 B). Neither T_4 nor T_3 varied significantly with sex.

Affected and control animal thyroid hormone concentrations

Comparison of T_4, FT_3, and FT_4 concentrations in serum drawn from six age-matched affected-control animal pairs (Fig. 4) found affected animal values to be significantly lower ($p<0.05$) than control values. For the six pairs, there was an average reduction in affected animal T_4 concentration to 66% of control concentration. T_3 concentration exceeded the highest standard in four pairs and could not be analyzed statistically.

Discussion

This study represents, to our knowledge, the most complete description of developmental changes and the first report of abnormal function in an endocrine organ in a lysosomal storage disease. Further, this is the first report suggesting that the pathogenesis of central nervous system (CNS) lesions in a storage disease could involve endocrine perturbations. Many storage diseases have associated clinical and morphological CNS abnormalities, but the impact of the storage disease on the CNS is poorly understood and has often been

attributed to direct effects of accumulated substrate (42,45).

Some of the increased thyroid weight in affected animals may be explainable by the extreme degree of lysosomal storage in the organ. In a previous study, thyroid from an affected goat was found to contain 9.9 mg of the predominant storage oligosaccharides per gram wet weight of thyroid (18), constituting only about 1% of the weight of tissue. Although this value does not take into consideration the weight of the water likely to be found in lysosomal storage vacuoles along with the oligosaccharides, storage of oligosaccharides probably does not by itself account for all of the increased mass of affected animal thyroids. The possibility of increased thyrotropin concentration, which may lead to thyroid hyperplasia, has not yet been assessed in affected animals because of lack of a TSH assay that detects the goat hormone.

Little information is available in the literature concerning thyroid function in neonatal goats, although a number of studies have reported thyroid hormone concentrations in goats 3 weeks old or older (28,39,44). The timing of blood draws with respect to hormone secretion patterns can be important for accurate assessment of thyroid hormone concentrations. This is of particular importance in neonatal animals, since thyroid hormone concentrations are generally high at birth and drop substantially during the first week of life (16). The present study demonstrated that T_3 and T_4 concentrations in neonatal animals are relatively constant within a 4-hour period and no evidence of pulsatile release was present in the thyroid hormone release study (Fig. 3). Thus, single morning samples, during at least the first week of life, should be representative of baseline thyroid

hormone concentrations and not an artifact of pulsatile release. The significant difference in T_4 concentration between the herds during the first week of life in the 14-day study was an unexpected finding (Fig. 4A). Factors that may help to explain the differences include (a) induction of parturition in herd B but not in herd A, with herd B animals 2 to 3 days younger in gestational age at delivery than herd A animals; (b) environmental differences, such as differences in feed eaten by the lactating does; and (c) ß-mannosidosis carrier status or other herd genetic factors. Nonetheless, the results indicate that no evidence of carrier goat thyroid hypofunction is present.

Results from thyroid hormone assays of affected-control pairs compiled between 1983 and 1987 indicate that thyroid hormone concentrations are significantly reduced in affected animals. Since lysosomal storage vacuoles occupy a major proportion of neonatal thyroid follicular cells, it is somewhat surprising that a T_4 concentration of even 65% of normal is achieved. The absence of validity testing of the T_4, FT_3 and FT_4 assays with normal goat serum is a weakness in this study. However, extensive comparisons of canine T_4 concentration using both the Becton Dickinson T_4 kit, used in the affected-control pair study (no longer available as originally formulated), and the Ciba Corning T_4 kit, currently in use, found substantially similar readings (unpublished data). Additionally, a recent 1-day-old affected animal had a T_4 level of 89.5 nmol/l, similar to the values for the two 1-day-old affected animals in the pair study. Thus, the data suggest that affected animals have deficient thyroid hormone concentrations, and indicate that further investigation of thyroid function is

warranted.

Central nervous system (CNS) hypomyelination, a major pathological feature of caprine ß-mannosidosis, is characterized by regional variation in extent of hypomyelination (31). Results of several studies have suggested that developmental defects may lead to the reduced number of oligodendrocytes present in myelin deficient CNS regions of affected animals (25,30-32). Thyroid hormones play an important role during development of the central nervous system (1,20,21) and considerable evidence indicates that thyroid hormones have a direct impact on myelination (21,46,48). Hypothyroidism has been associated with deficient myelination (46) and impaired oligodendrocyte differentiation and function (2,8,9,11,12,33,36,41). In addition, in border disease, a disease of sheep caused by in utero infection of fetuses by the border disease virus (BDV) (4-7,37), a reduced thyroid hormone level, possibly secondary to follicular cell invasion by BDV, has been postulated to be the causative factor in the disease's severe CNS myelin deficiency (3). Average T_3 and T_4 concentrations in border disease sheep were 60% and 74% of control values, respectively, similar to the extent of reduction in ß-mannosidosis. Thus, thyroid hormone deficiency in goats affected with ß-mannosidosis may be a major factor in the pathogenesis of hypomyelination. Whether the reduction in thyroid hormone concentrations seen in newborn affected goats is indicative of a comparable reduction in utero, and whether a reduction in T_4 concentration to 65% of control is enough to impair myelination to the extent seen in the cerebral hemispheres of affected goats remains to be determined. If vacuolation is either

by itself the major factor in decreased thyroid hormone concentrations or is symptomatic of metabolic perturbations that might disrupt thyroid function, the increased vacuolation during development may suggest that a progressive decrease in hormone release takes place as development proceeds. Decreasing concentrations of thyroid hormones during development could explain the generally increased severity of myelin deficits in regions that myelinate late in CNS development (31).

Thyroglobulin is a glycoprotein and contains Manß1-4GlcNAc bonds in its N-linked oligosaccharides (43,49). The direct impact of ß-mannosidase deficiency on thyroglobulin metabolism would occur during lysosomal catabolism of thyroglobulin, and a degradation impediment or defect is the most likely alteration that would lead to decreased thyroid hormone secretion in ß-mannosidosis. Absence of affected goat lysosomal ß-mannosidase activity and accumulation of oligosaccharides could have an adverse effect on thyroglobulin degradation, and then on thyroid hormone release, in several ways, including through (a) decreased availability of primary lysosomes due to impaired recycling of secondary lysosomes (13,40), and (b) interference in cell function by storage vacuole accumulation. Relevant to both possibilities, a recent study identified a possible post-endocytosis regulation step in thyroglobulin catabolism, involving a lag period between fusion of endosomes with primary lysosomes to form secondary lysosomes (40). Alterations in the availability of primary lysosomes, due to alterations in synthesis or in accessibility, might adversely affect post-endocytosis regulation. Thyroid metabolic alterations other than disruption of

catabolism--such as a thyroglobulin synthesis defect, an organification defect, a hormone receptor abnormality, or a thyrotropin deficiency--are much less likely to be involved in the thyroid hormone deficits in ß-mannosidosis. It is possible, however, that metabolic perturbations secondary to abnormal glycoprotein metabolism could adversely affect thyroglobulin metabolism. The thyroid in ß-mannosidosis may provide a good system in which to study the role of lysosomes in thyroglobulin metabolism.

Little information is available about endocrine morphology and function in storage diseases. Some thyroid pathological changes have been reported in α-mannosidosis, the lysosomal storage disease most closely related to ß-mannosidosis. Thyroid epithelial cell vacuolation was not found neonatally in neonatal bovine α-mannosidosis (24), but vacuolation has been reported in the thyroid of cattle which ingested the α-mannosidase inhibitor swainsonine (23). Additionally, in feline α-mannosidosis, the thyroid was found to have the greatest storage of oligosaccharides, per unit wet weight, of any tissue analyzed (47). Thyroid hormone serum concentrations have not been reported in α-mannosidosis or in swainsonine poisoning.

In this study, the extensive and developmentally progressive morphological abnormalities present in the thyroid of goats affected with ß-mannosidosis are documented. Additionally, evidence suggesting deficient thyroid function in affected goats is presented. This functional abnormality may be due, at least in part, to the extensive thyroid follicular cell morphological abnormalities or to metabolic perturbations secondary to deficient ß-mannosidase activity. Thyroid

hormone deficiency in sheep has been proposed to cause clinical features such as doming of the skull, limb abnormalities, and hypomyelination, each prominent abnormalities in caprine ß-mannosidosis. Thus, a role for reduced thyroid hormone concentrations in the myelin deficits and other clinical and pathological abnormalities seen in goats affected with ß-mannosidosis is possible and needs further investigation.

METHODS

Animal acquisition

Animals were obtained from two goat herds: herd A, consisting of normal goats, and herd B, consisting of carrier goats. In herd A, pregnancies were established by natural breeding of cycling does, parturition was natural, and gestation time varied from 146 to 150 days. Pregnancies in herd B were established in a planned parturition program with natural breeding. Doe estrus cycles were synchronized using sponges impregnated with 45 mg fluorogestone acetate (Intervet, Boxmeer, Netherlands) and ovulation was induced by injection of 600 IU PMSG (Intervet) (15). Fetal animals, all from herd B, were delivered by cesarean section. Parturition in herd B was induced by injection of does with 0.5 cc Estrumate (Butler, Brighton, MI), a synthetic prostaglandin $F_{2\alpha}$ analog, at day 143, and delivery was at 144-145 days gestation.

Tissue preparation

Thyroid samples, collected after T-61 (American Hoechst, Somerville, NJ) euthanasia, were fixed in 4% glutaraldehyde, post-fixed in 1% osmium tetroxide, processed routinely, and embedded in an Epon-Araldite mixture. For light microscopy, semithin sections (1 um) were cut from between four and eight blocks per animal and stained with toluidine blue. For electron microscopy, ultrathin sections were cut from at least two blocks per animal and stained with uranyl acetate and lead citrate.

Thyroid hormone assays

For 4-hour and 14-day studies, serum T_4 and T_3 concentrations were estimated by radioimmunoassay (RIA). For T_4 assay, a Ciba Corning Magic kit was used. Twenty five ul of standard or unknown sample and 100 ul of kit tracer/buffer solution were pipetted into polystyrene tubes and 500 ul of antibody-buffer solution was added to all tubes except the total count tubes. The contents of the tubes were mixed by vortexing and the tubes were incubated at room temperature for 1 h. The tubes were placed in magnetized racks for 5 minutes to separate the antibody-bound fraction from the free fraction. The liquid content of all tubes except the total count tubes were poured off and the tubes were allowed to drain on absorbent paper for 2 minutes. Antibody-bound ^{125}I was quantified with a gamma emission spectrometer. Assay sensitivity was estimated at 13 nmol/L, the point of 90% of total binding on the standard curve. Assay specificity was determined with a serial dilution method. Dilution of caprine sera with 0 standard paralleled standard curves, but dilution with distilled water or gelatin-phosphate buffer underestimated expected T_4 indicating a matrix effect. When 10, 25, 50 or 100 ng/ml of T_4 were added to caprine serum to determine assay accuracy, 100, 101, 100, and 97% of exogenous T_4 was measured, respectively. Intraassay coefficients of variation (CV) for two caprine control sera were 1.1% (mean = 185 nmol/L, number of assays = 3) and 3.3% (mean = 167 nmol/L, number of assays = 3). Interassay CV's for the same sera were 1.7% and 3.8% respectively (number of assays = 3). Procedures for the TT_3 radioimmunoassay have been described previously for bovine sera (38). The

antiserum was used at a final dilution of 1:6000 in assay buffer. The assay sensitivity was estimated at 0.02 nmol/L, the concentration calculated at 90% of total binding. Dilution of caprine sera paralleled standard curves. When 0.25, 0.5 or 1.0 ng of T_3 was added to caprine serum to determine assay accuracy, 102%, 101%, and 101% of exogenous hormone was measured in the assay, respectively. Dilution of caprine serum with 0 standard paralleled standard curves, but dilution with distilled water or gelatin-phosphate buffer indicated that parallelism was lost at the 1:8 dilution. Intraassay CV's for two caprine sera were 21.5% (mean = 4.15 nmol/L, number of assays = 3) and 4.0% (mean = 4.3 nmol/L, number of assays = 3). Interassay CV's for the same serum pools over 3 assays were 6.2% and 7.9%, respectively.

Affected-control pair assays of T_4, free T_4, and free T_3 concentrations, all run between 1983 and 1987, were made using Becton Dickinson assay kits. Validity testing with goat serum was not performed with these assays, however, extensive assessment of canine T_4 concentration using both the Becton Dickinson T_4 kit (no longer available as originally formulated) and the Ciba Corning T_4 kit found substantially similar readings (unpublished data). The T_3 assay was the same as described above.

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TABLE 1. MEAN THYROID WEIGHTS AT VARIOUS STAGES OF DEVELOPMENT

	Control		Affected	
Age	n	weight (g)	n	weight (g)
96-120/150 Days Gest.[a]	5	0.36 (0.24-0.42)[b]	2	0.82 (0.73-0.90)
0-8 Days	15	0.68 (0.51-1.00)	5	1.17[c] (0.93-1.45)
1 Month	4	0.82 (0.59-0.95)	2	2.12 (2.03-2.20)
4 Months	5	2.29 (1.42-3.00)	1	3.00 --

[a] days gestation
[b] range
[c] $p < 0.001$

TABLE 2. THYROID HORMONE ASSAY SUMMARIES FROM SIX AFFECTED AND CONTROL ANIMAL AGE-MATCHED PAIRS

Age (days)	ß-Man. Status[a]	T_4 (nmol/l)	T_3 (nmol/l)	FT_4 (pg/ml)	FT_3 (pg/ml)
1	C	133.3	>6[b]	22.8	-[c]
	A	92.7	>6	12.7	-
1	C	117.5	>6	14.5	7.9
	A	100.6	>6	10.3	5.9
2	C	91.9	-	-	6.9
	A	51.9	-	4.5	5.3
2	C	96.1	6.86	18.7	16.1
	A	63.5	5.94	12.4	10.6
2	C	95.6	6.48	13.1	11.1
	A	55.3	2.8	3.9	3.0
2	C	89.3	5.2	10.4	8.6
	A	49.8	3.3	4.1	3.5

[a] ß-mannosidosis status: C, control; A, affected
[b] value exceeded highest standard value
[c] indicates test not done or insufficient sample size to run test

FIG. 1. Light micrographs of toluidine blue stained 1um Epon sections from control and affected animals at 96/150 days gestation (A,B), and affected animals at 124/150 days gestation (C), and 3 days postnatal (D). Extensive vacuolation was present in affected goat thyroid cells as early as 96/150 days gestation (B), and became more pronounced with advancing age (C,D) 275 X.

FIG. 1

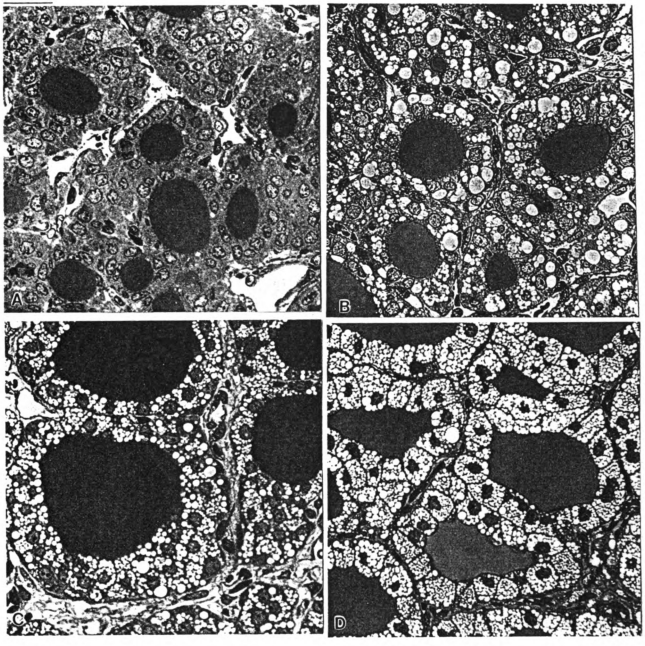


FIG. 2. Electron micrographs of thyroid follicular cells from 3-day-old control (A) and affected (B,C) goats. Control animal thyroid follicular and perifollicular cells (A, small and large arrows, respectively) contained only occasional cytoplasmic vacuoles. A large number of cytoplasmic vacuoles were present in all affected animal thyroid cell types (B) including follicular cells (small arrow), endothelial cells (arrowhead), perithelial cells (double arrow), and perifollicular cells (large arrow), shown degranulated here. Vacuole size and number varied among cell types. Higher magnification demonstrated that cytoplasmic vacuoles in affected animal thyroid cells were consistent with lysosomal storage vacuole structure (C). Their limiting membrane (arrows) was distinct from adjacent rough endoplasmic reticulum. A,B 4,940 X; C 27,000 X.

FIG. 2

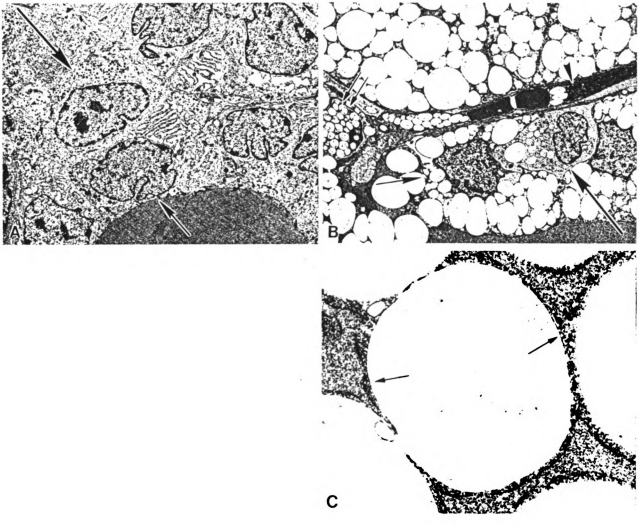


FIG. 3. T_3 and T_4 release pattern assessed in serum samples drawn at 15 minute intervals over 4-hours, between 8:00 am and 12:00 noon, from a representative herd A (normal animals) and herd B (carriers of ß-mannosidosis) animal.

FIG. 4. T_4 (A) and T_3 (B) mean values from blood draws at birth (day 0) and between days 1 and 14 postnatal from herds A (n = 5) and B (n = 10, days 0-8; n = 5, days 9-14). Error bars show standard deviation. * indicates statistically significant difference ($p<0.05$) between herds A and B at indicated age

FIG. 3

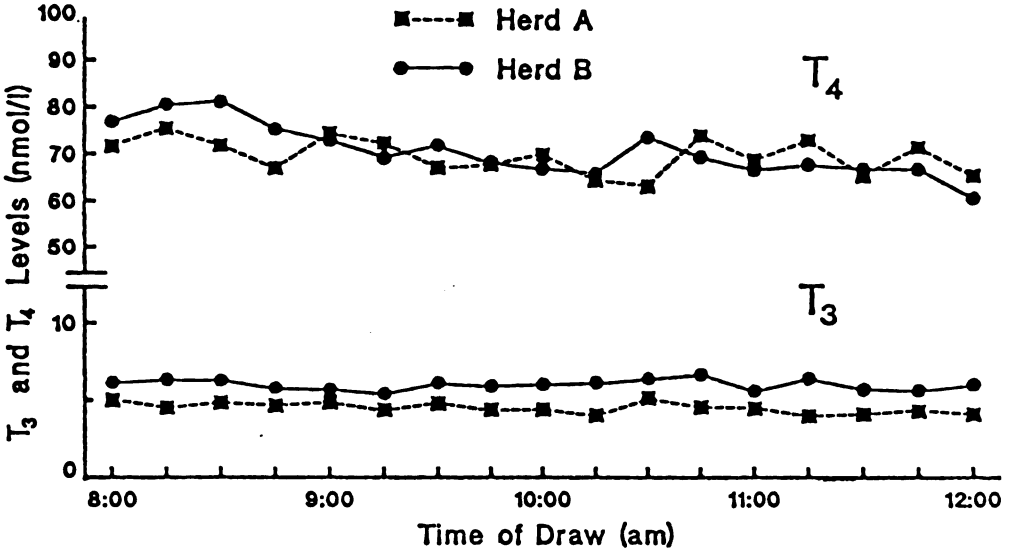
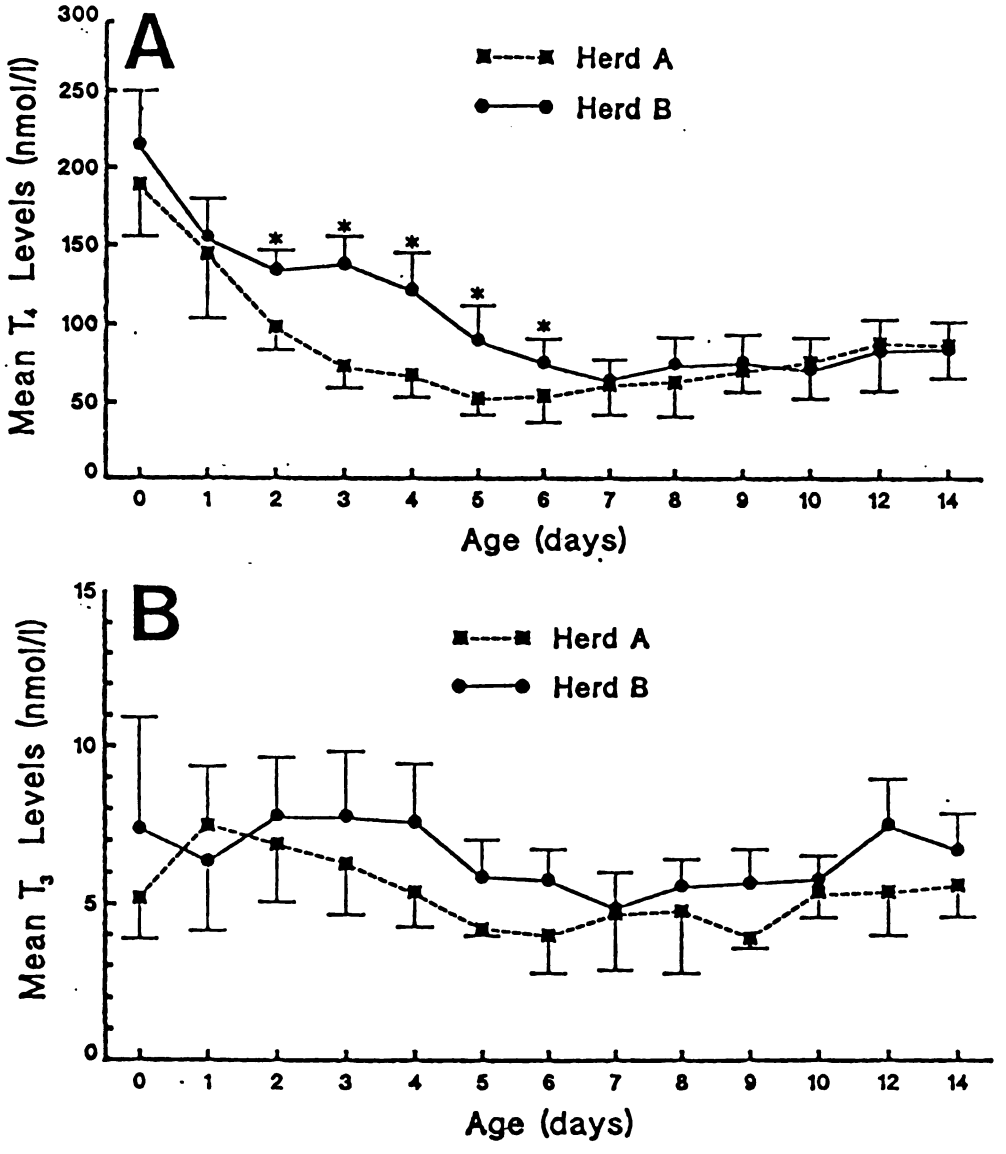

Herd A
Herd B
T_4
T_3
T_3 and T_4 Levels (nmol/l)
100
90
80
70
60
50
10
0
8:00
9:00
10:00
11:00
12:00
Time of Draw (am)

FIG. 4


A
Herd A
Herd B
Mean T_4 Levels (nmol/l)
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 12 14
Age (days)
B
Herd A
Herd B
Mean T_3 Levels (nmol/l)
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 12 14
Age (days)