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# The Lesions of an Ovine Lysosomal Storage Disease

## *Initial Characterization*

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***An inherited disease associated with deficiencies of β-galactosidase and α-neuraminidase has been identified recently in sheep. The clinical signs, the deficiency of lysosomal enzymes, and the familial nature of the disorder suggested that the condition was a lysosomal storage disease. Four affected sheep were necropsied and their tissues were examined by histopathologic and histochemical methods to determine if the lesions were consistent with a lysosomal storage disease. Central nervous system neurons were enlarged with finely to coarsely granular cytoplasmic material, or less often, neurons were distended with multiple, variably-sized vacuoles. Loss of neurons without gliosis was evident and the Nissl substance was either dispersed and fragmented or condensed around the nuclei of remaining neurons. Neurons of intestinal and other peripheral ganglia, retinal ganglion cells, and heart Purkinje fibers were enlarged similarly. White matter of the cerebrum and spinal cord had numerous spheroid to ellipsoid axonal enlargements. Periportal hepatocytes and renal epithelial cells were enlarged with marked vacuolation. The neuronal storage material stained intensely with periodic acid-Schiff/alcian blue, with Luxol fast blue, for acid phosphastase, and moderately with oil red O stains. Renal and hepatocyte storage material stained intensely with oil red O and moderately with periodic acid-Schiff/alcian blue and Sudan black B stains. The lesions in these sheep were consistent with those of a lysosomal storage disease. Both neuronal and visceral storage occurred, but the neuronal storage was more severe. (Am J Pathol 1989, 134:263–270)***

Lysosomal storage diseases comprise a group of inherited deficiencies of various lysosomal enzymes.[1–4] The resultant deficiencies of these enzymes lead to the storage of uncatabolizable substrates. Storage of material can occur as primarily neuronal, primarily visceral or both (neurovisceral) and the distribution and severity of storage determines clinical signs of each lysosomal storage disease.[1–4]

Animal models of many of these diseases have been identified. Glycogenosis II, III and VIII, mannosidosis, $GM_1$ and $GM_2$ gangliosidosis, Gaucher's disease, globoid cell leukodystrophy, metachromatic leukodystrophy, Niemann-Pick disease types A and C, and mucopolysaccharidosis VI and VII are identified animal models in various species of domestic animals.[5] There is a vast potential use of animal models of lysosomal storage diseases for the development of therapeutics, diagnostics, and for further elucidation of basic mechanisms of lysosome function.

We have recently described, in preliminary reports, an inherited disease of sheep associated with deficiencies of lysosomal β-galactosidase and α-neuraminidase.[6–11] Clinically the sheep become ataxic at 4–6 months of age and the ataxia progresses in severity over 2 weeks to 2 months after becoming apparent.[8] Fibroblasts from affected sheep have less than 5% of the β-galactosidase activity present in fibroblasts from normal sheep and fibroblasts of ewes that produce affected lambs have intermediate enzyme activity.[6–8] Fibroblasts from affected sheep have approximately 20% of the α-neuraminidase

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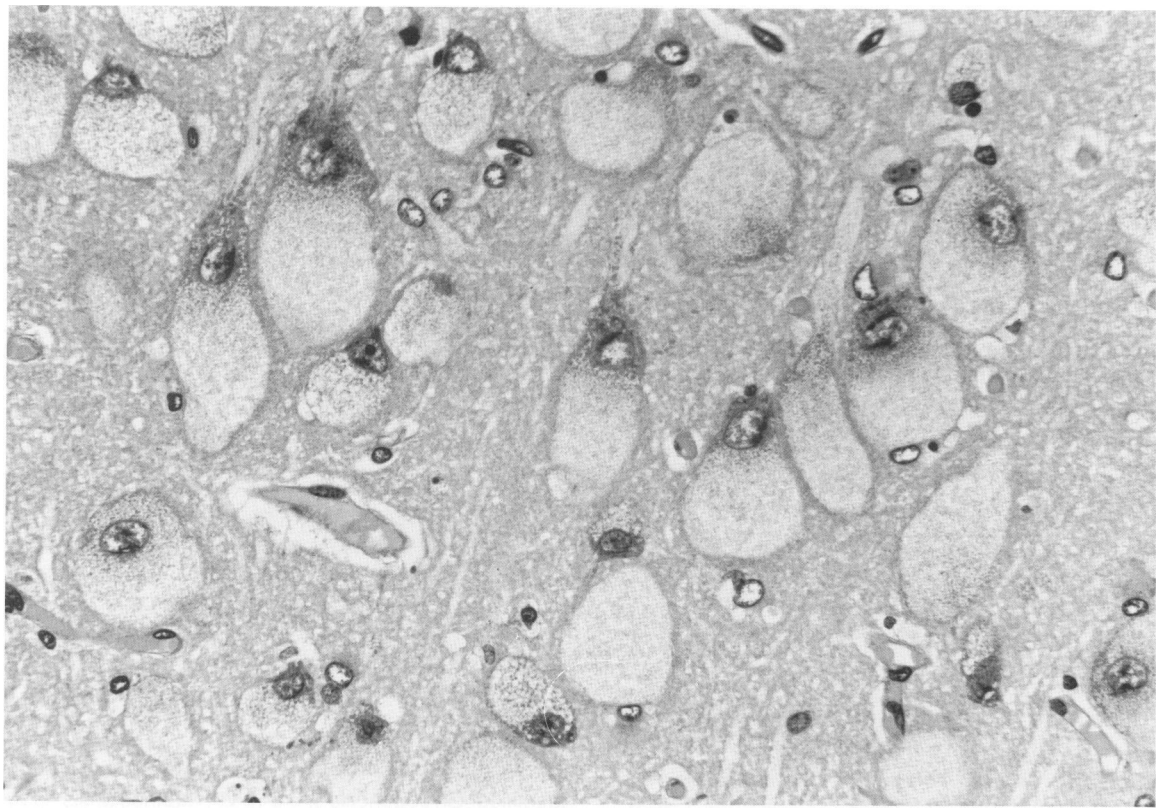

**Figure 1.** *Cerebral cortex of an affected sheep. The cortical neurons have marked cytoplasmic enlargement and condensation of Nissl substance around eccentrically displaced nuclei. Hematoxylin and methylene blue-basic fuchsin, ×385.*

activity present in fibroblasts of normal sheep.[7,8] Furthermore, cerebrum, kidney, lung, spinal cord, and spleen from affected sheep have less than 8% of the β-galactosidase activity present in the respective tissues of normal sheep, and ewes producing affected sheep have intermediate enzyme activity in these tissues.[9] The disease is inherited as an autosomal recessive condition.[10,11] A genetic basis, deficiencies of lysosomal enzymes, and clinical signs consistent with a loss of neuronal function suggested that the disease was a neuronal lysosomal storage disease. The purpose of this study was to evaluate the lesions of this ovine disease to determine if they were consistent with a neuronal lysosomal storage disease.

## Materials and Methods

A group of four (3 female and 1 male), approximately 5-month-old Suffolk crossbred sheep with signs of ataxia and decreased weight gain were presented. The sheep were from an inbred pastured flock. The signs had progressively worsened over the previous month. The sheep were treated with electrolyte solutions, antibiotics, and vitamins B, E, and selenium but no improvement was observed. Complete blood counts and cerebrospinal fluid analyses (total protein, number and types of cells) were performed on samples from three sheep. Blood glucose, urea nitrogen and creatinine, serum enzyme concentrations (gamma glutamyl transferase, sorbitol dehydrogenase, creatine kinase, and alkaline phosphatase), serum total protein and albumin, and serum calcium and phosphorus were quantitated for each of the sheep. At the time the blood and cerebrospinal fluid samples were obtained, all sheep were markedly ataxic with loss of proprioception of the rear legs and difficulty in rising. The sheep were eventually euthanatized with intravenous sodium pentobarbital and necropsied.

At the time the postmortem examinations were conducted, it was not known that the sheep had lysosomal enzyme deficiencies. Tissue samples from each sheep were placed in 10% neutral buffered formalin. Portions of brain, spinal cord, liver and kidney were frozen. Formalin-fixed specimens were routinely processed, paraffin embedded, and sections were stained with hematoxylin and eosin (H&E), Bielschowsky's, cresyl violet, and cresyl violet with Luxol fast blue. Formalin-fixed brain, spinal cord, liver and kidney were also embedded in methacrylate,

sectioned at 0.5 $\mu$ and stained with hematoxylin and methylene blue-basic fuchsin. Frozen sections of brain, spinal cord, liver and kidney were stained with Sudan black B, oil red O, periodic acid-Schiff (PAS)/alcian blue at pH 2.6 and 1.0. Methacrylate-embedded sections were also stained with PAS/alcian blue at pH 2.6 and 1.0. Sections of selected frozen tissues were stained for acid phosphatase with acetate buffer at pH 5.2.[12] Eyes from two sheep were placed in Zenker's solution, routinely processed, and sections were stained with H&E and PAS. Controls of all stains were performed with tissues from normal sheep of similar ages.

## Results

The results of the complete blood counts on the three sheep were normal. No vacuolation in any leukocyte in the Wright's stained blood films was observed. Blood glucose concentrations were within the normal range. The serum enzyme panels disclosed increases in gamma glutamyl transferase (79 and 93 IU/dl; controls, 35–67 IU/dl) in two of the sheep. Three sheep had an increased serum phosphorous (7.1–11.3 mg/dl; controls, 4.1–6.9 mg/dl) and normal serum calcium, whereas the fourth had an increased serum calcium (12.2 mg/dl; controls 8.0–10.4 mg/dl) and a decreased serum phosphorous (3.6 mg/dl). The sheep with the increased serum calcium also had an increased blood urea nitrogen level (43 mg/dl; controls, 12–25 mg/dl), creatinine (2.0 mg/dl; controls, 1.1–1.5 mg/dl) and sorbitol dehydrogenase (95 IU/dl, controls 14–41 IU/dl) levels. The results of these analyses were normal in the other three sheep. The results of the cerebrospinal fluid analyses, serum total proteins, serum albumin, and the remainder of serum enzyme assays were normal.

The only gross abnormalities seen at necropsy were hemorrhages in muscle and connective tissue ventral to the sternum secondary to prolonged recumbency.

Histologic lesions in the central nervous system, retina, ganglia of various organs, heart Purkinje fibers, livers, kidneys, and histiocytes in the ileum and in mesenteric and hilar lymph nodes were consistently observed in sections from all sheep. Neurons of the cerebrum, cerebellum, brain stem, and spinal cord were the most severely affected.

Nearly all neurons of the central nervous systems of the sheep were enlarged (Figures 1 and 2). The only exceptions were in the cerebellum in which the lesions were restricted to Purkinje, Golgi, and stellate basket cells (Figure 3). The enlargement was cytoplasmic, and in the majority of neurons the cytoplasm was stippled pink to purple and was finely to coarsely granular with H&E staining. Vacuolation of the cytoplasm with multiple variably-sized

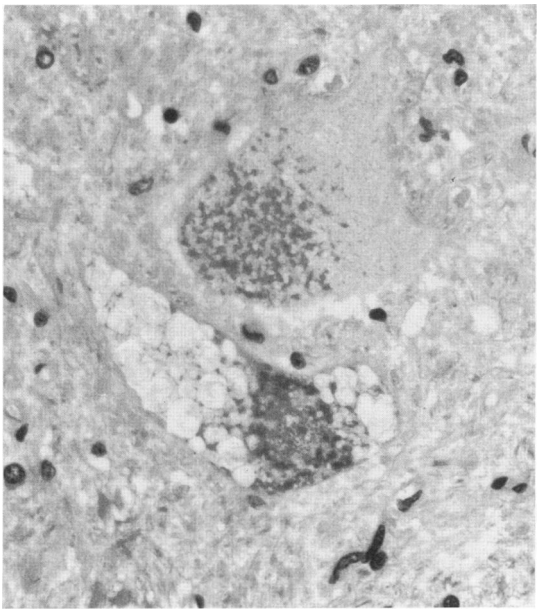

**Figure 2.** *Spinal cord neurons of an affected sheep. There is marked cytoplasmic enlargement and vacuolation with condensation of Nissl substance around the nucleus of one neuron, and a second neuron with granular cytoplasmic enlargement and fragmentation of Nissl substance. Hematoxylin and methylene blue-basic fuchsin, ×390.*

vacuoles was also seen frequently. The more severely affected neurons of the cerebrum and midbrain had eccentrically displaced nuclei. The Nissl substance, as seen with cresyl violet staining, was fragmented and dispersed to completely absent or condensed around the nuclei. Neuronal loss was apparent but gliosis was not evident. Nuclear and cytoplasmic margins of many neurons were indistinct. Bielschowsky's staining of the cerebrum and cerebellum disclosed numerous spheroid to ellipsoid axonal enlargements in the white matter (Figure 4). Similar axonal enlargements were less prevalent in the white matter of the spinal cord.

The cytoplasm of nearly all retinal ganglion cells was markedly enlarged with irregular granular material and some vacuoles (Figure 5A). Many ganglion cell nuclei were swollen, nuclear lysis was occasionally apparent, and nuclear and cytoplasmic margins, in many cells, were indistinct. A few scattered cells of the inner nuclear layer were also enlarged with finely granular cytoplasm (Figure 5B). The neurons of peripheral ganglia including those of the intestines and adrenal glands were similarly but less severely affected. Approximately 25 to 35% of heart Purkinje fibers were enlarged with solitary or a few cytoplasmic vacuoles. The neuronal storage material in frozen and methacrylate embedded sections stained intensely red (PAS+) with PAS/alcian blue at pH 1.0 and 2.6 (Figure 6), and in frozen sections stained moderately with oil red O, but not with Sudan black B. Neuronal cytoplasmic storage

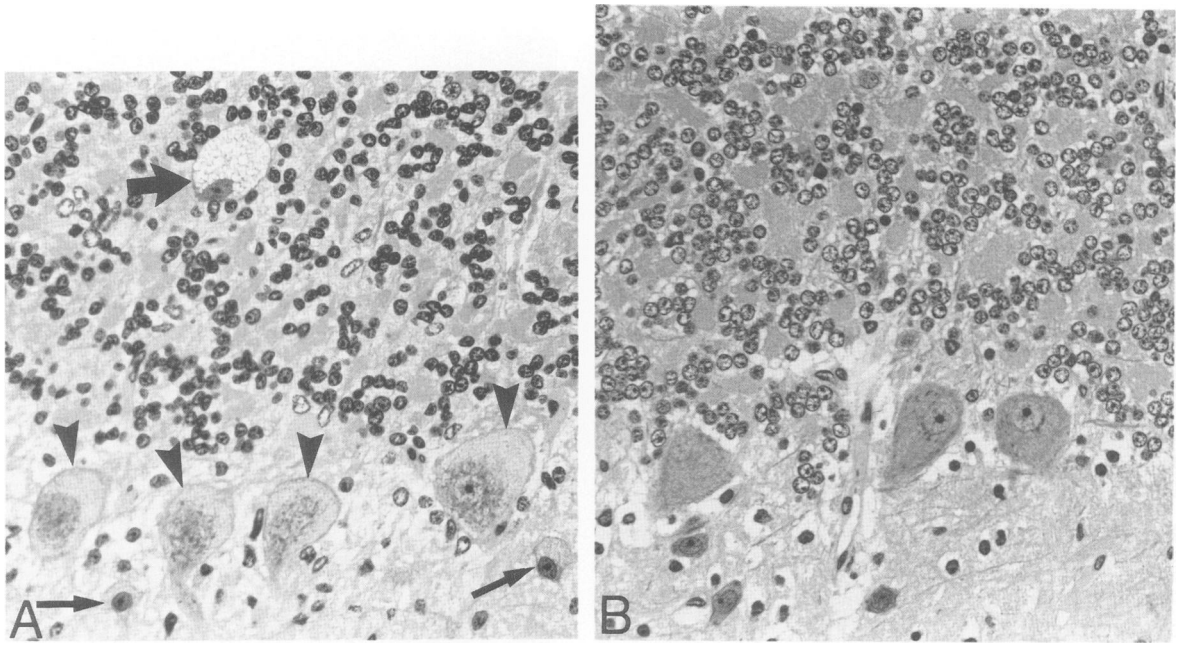


**Figure 3.** *Cerebellum; granular and Purkinje cell layers. Hematoxylin and methylene blue-basic fuchsin.* **A:** *Affected sheep with cytoplasmic enlargement of Purkinje cells (arrowheads), stellate basket cells (small arrows), and vacuolation of a Golgi cell (large arrow) (×280).* **B:** *Cerebellum from control sheep (×280).*

material stained strongly for acid phosphatase in a granular pattern in frozen sections. Neurons, in formalin-fixed central nervous system tissue, also stained intensely with Luxol fast blue.

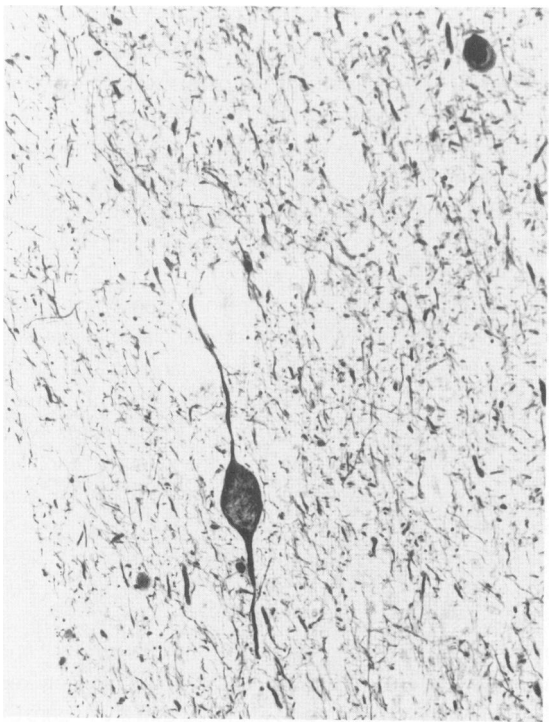

**Figure 4.** *Cerebral white matter of an affected sheep. Cross and longitudinal sections of axonal swellings. Bielschowsky's stain, ×265.*

Hepatic lesions were distributed periportally and decreased in severity in centrilobular areas (Figure 7). The periportal hepatocytes were enlarged with finely vacuolated cytoplasm. These lesions moderated to enlarged cells with foamy, coarsely granular cytoplasm with sporadic vacuolation in centrilobular areas. Normal centrilobular hepatocytes were seen in all sheep, though of varying quantity. The hepatocyte nuclei were normal histologically.

The kidneys had the greatest variation in the pattern of the lesions, but all sheep had some degree of damage in all regions of the kidney (Figure 8). The most severely affected glomeruli had both visceral and parietal epithelial cells with markedly enlarged, foamy, or finely vacuolated cytoplasm. Other glomeruli had milder lesions and all but one sheep had some morphologically normal glomeruli. All four sheep had multifocal lesions of varying degrees in convoluted tubules and collecting ducts. The most marked changes were epithelial cells with enlarged, finely vacuolated and reticulated cytoplasm. These gradated to cells with occasional vacuoles.

The cytoplasm of hepatocytes and renal epithelial cells stained intensely with oil red O and moderately with Sudan black B and PAS/alcian blue at pH 1.0 and 2.6 in frozen sections. Staining for cytoplasmic acid phosphatase was positive in enlarged hepatocytes and renal epithelial cells in frozen sections.

Histiocytes distributed multifocally within Peyer's patches in ileum and in mesenteric and hilar lymph nodes had distended, palely eosinophilic, granular cytoplasm.

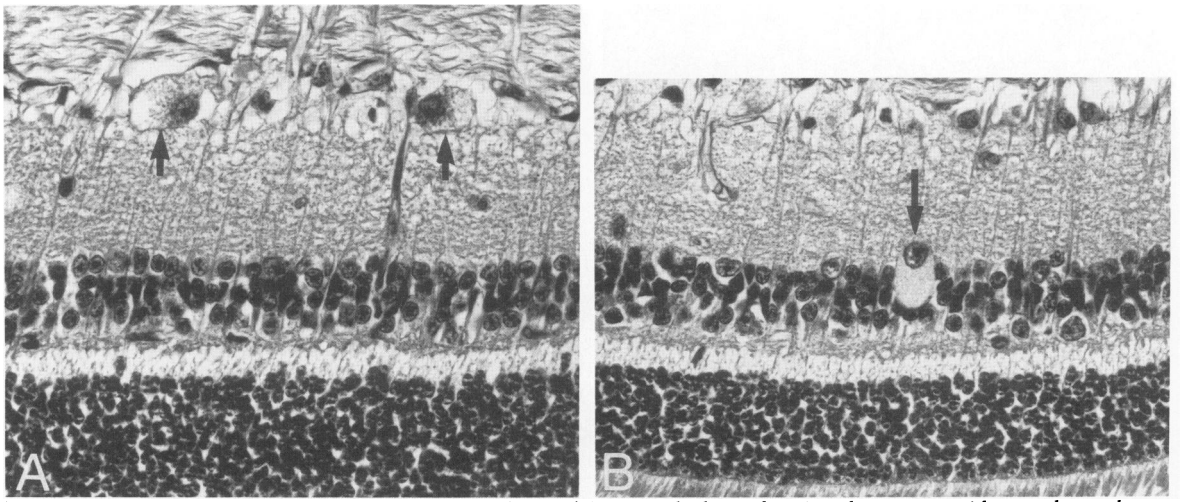


**Figure 5.** *Retina of an affected sheep.* **A:** *Ganglion cells (arrows) have marked cytoplasmic enlargement, with granular and vacuolated cytoplasm.* **B:** *Enlarged cell (possibly an amacrine cell) (arrow) with granular cytoplasm in the inner nuclear layer (H&E, ×330).*

Bone was normal histologically. Moderate numbers of intracytoplasmic vacuoles in chondrocytes from growth plates and articular cartilage were seen in affected sheep but similar, though less frequent, vacuoles were present in normal controls. Incidental lesions of *Sarcocystis* cysts were observed in cardiac muscle fibers.

## *Discussion*

No consistent clinicopathologic abnormalities were observed in the affected sheep. The abnormalities that were present appeared in individual sheep, and probably represented secondary manifestations of advanced disease. It is concluded that no consistent abnormalities that are detectable by standard veterinary clinicopathologic methods, develop in affected sheep. However, measurement of $\alpha$-neuraminidase and $\beta$-galactosidase activity in plasma or serum of at-risk lambs may allow diagnosis of the condition before clinical signs develop.

It can be concluded that lesions in the central nervous system and the visceral organs; the vacuoles in the cytoplasm; are consistent with those in lysosomal storage diseases. The storage of material was both neuronal and visceral (neurovisceral). Considering the severity of the neuronal lesions coupled with the lack of clinicopathologic abnormalities suggestive of impairment of liver or kidney function, however, it can be concluded that the neuronal storage was responsible for clinical signs. Adequate amounts of histologically normal renal and hepatic parenchyma remained for maintenance of respective functions. The skeletal system was normal grossly and histologically, although the degree of vacuolation of chondrocytes appeared slightly increased in affected lambs. Ultrastructural evaluation of chondrocytes would clarify this.

The signs of ataxia in lysosomal storage diseases with neuronal storage were originally believed to result from the displacement of neuronal cytoplasmic organelles by

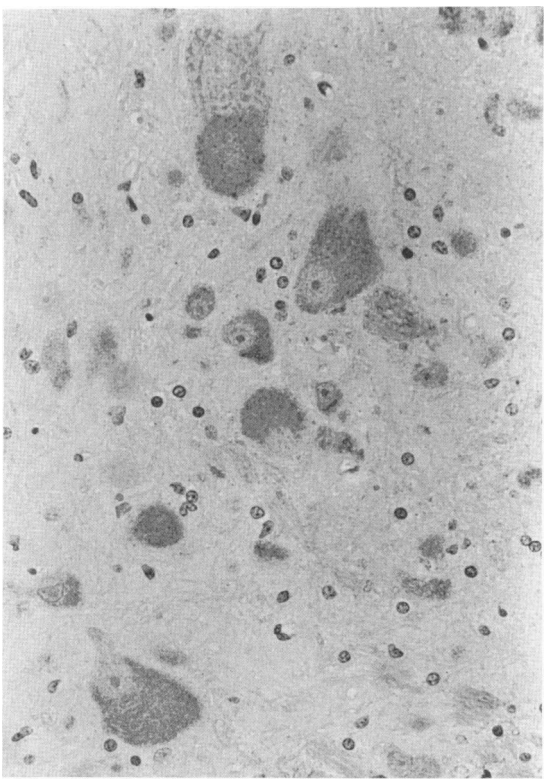

**Figure 6.** *Enlarged cervical spinal cord neurons of an affected sheep with marked cytoplasmic staining (dark areas) by PAS/alcian blue. PAS/alcian blue pH 2.6, ×295.*

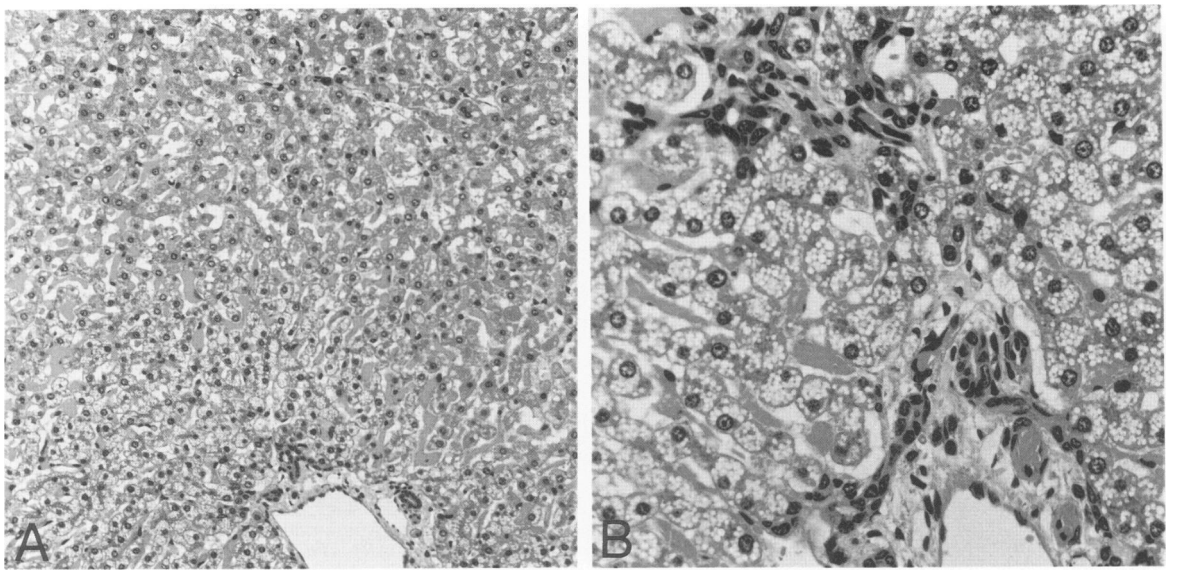


**Figure 7.** *Periportal hepatocytes of an affected sheep. Hematoxylin and methylene blue-basic fuchsin.* **A:** *Cytoplasmic vacuolation of the periportal hepatocytes, (×110).* **B:** *Higher magnification of periportal hepatocytes (×280).*

the massively enlarged lysosomes.[13] The observations made with the application of the Golgi method to these diseases have led to the hypothesis that the neurologic signs are the result of meganeurites and associated secondary neurites that form aberrant synapses of cortical pyramidal neurons that alter normal integrative operations and cause abnormal neuronal activity.[14,15] Recent work indicates that lysosphingolipids accumulate in sphinglipidoses and that these inhibit protein kinase C activity.[16] This observation suggests that it is the inhibition of protein kinase C that leads to neuronal dysfunction.[16] It is possible that all of these factors contribute to the loss of neural function.

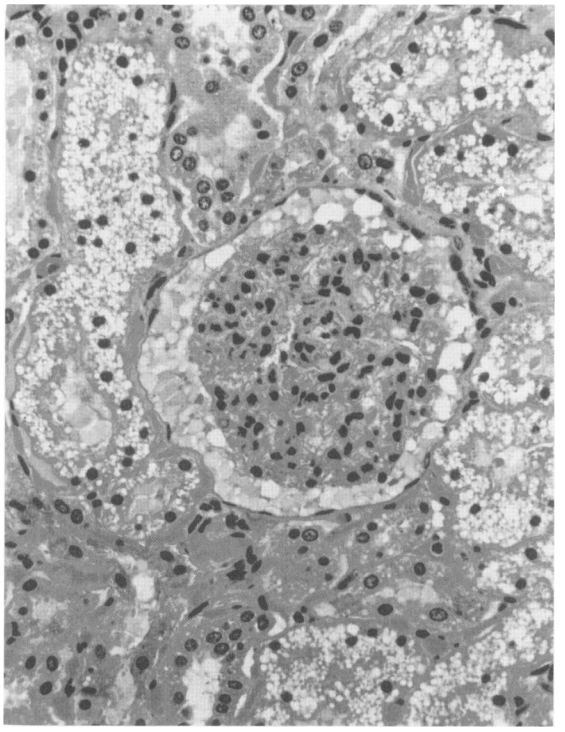

**Figure 8.** *Vacuolation of the renal tubular epithelial and the visceral and parietal epithelial cells of an affected sheep. Hematoxylin and methylene blue-basic fuchsin, ×270.*

In humans, primary defects of $\beta$-galactosidase present as two phenotypically distinct syndromes, Morquio B and $GM_1$ gangliosidosis.[1-4,17-24] Morquio B patients have severe skeletal abnormalities with dwarfism but normal intelligence whereas $GM_1$ gangliosidosis patients have variable intellectual impairment associated with neuronal storage, visceral storage, and highly variable skeletal involvement.[2,17-24] Primary deficiencies of $\alpha$-neuraminidase are known as sialidosis or mucolipidosis I.[25,26] A syndrome consisting of combined deficiences of $\beta$-galactosidase and $\alpha$-neuraminidase is known as galactosialidosis, and is phenotypically very heterogeneous with skeletal, neurologic, ocular, and cutaneous abnormalities.[27-29]

Morquio B and $GM_1$ gangliosidosis have been believed to be due to structural mutations of $\beta$-galactosidase resulting in kinetically altered enzymes.[19,30-33] More recent studies suggest $GM_1$ gangliosidosis is due to a defective recognition site on a precursor of the $\beta$-galactosidase molecule.[34] These two diseases are allelic and the phenotypic heterogeneity within each is believed to be due to variable residual activities of the mutant enzymes.[17,35-37] This ovine disease is not similar to Morquio B due to its marked neurologic and its lack of skeletal involvement. Furthermore, patients with $GM_1$ gangliosidosis have normal lev-

els of $\alpha$-neuraminidase activity whereas sheep affected with this condition have diminished (approximately 20% of normal) activity of this enzyme. Therefore, this ovine disease is not similar to $GM_1$ gangliosidosis either.

Galactosialidosis does not belong to the same complementation group as $GM_1$ gangliosidosis and Morquio B.[17,24,38,39] The enzyme deficiencies in galactosialidosis are believed to be due to enhanced degradation of the normal enzymes because of the absence of a protein protective factor.[33,40–42] The enzyme deficiencies in this ovine disease are reversed when compared with galactosialidosis. In galactosialidosis, $\alpha$-neuraminidase is profoundly deficient (less than 2% of normal) and $\beta$-galactosidase has approximately 15% residual activity.[27–29] Conversely, this ovine disease has approximately 20% residual $\alpha$-neuraminidase activity and a profound deficiency of $\beta$-galactosidase (less than 5% of normal).[8] Also, the enzyme deficiencies of this ovine disease are not due to enhanced degradation of enzyme as incubation of fibroblasts in the presence of protease inhibitors does not elevate enzyme activities as it does in galactosialidosis.[8,33,40–42] Clinically and pathologically, this ovine disease appears to have similarities to both $GM_1$ gangliosidosis and galactosialidosis. It is possible that these sheep have a primary mutation in the $\beta$-galactosidase structural gene which is causing a secondary deficiency of $\alpha$-neuraminidase. More work is necessary to determine the relationship of this ovine lysosomal storage disease to these human diseases and to further characterize the primary mutation.

In conclusion, this disease is a lysosomal storage disease with both neuronal and visceral storage. The neuronal storage is more severe and is responsible for clinical signs. Studies in progress to further elucidate the characteristics of this disease and its relationship to human lysosomal storage diseases include lectin histochemistry and ultrastructural evaluation of neuronal and visceral tissue, and interspecific complementation studies with ovine fibroblasts and human fibroblasts with the conditions discussed. Superovulation and embryo transfer techniques are also being applied to carrier ewes to increase numbers of affected and carrier sheep.

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