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Lectin-histochemistry: glycogenosis in cattle

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ABSTRACT

Ten out of 47 calves that were born in a small Brahman herd from southern Brazil developed progressive muscular weakness and tremors, lethargy and poor body condition. Necropsy was performed on three affected animals. The only gross lesion detected was paleness of the muscles of the trunk and limbs. Multiple cytoplasm vacuoles located in different tissues were the principal microscopic lesions. Vacuoles were particularly evident in skeletal muscles and myocardium. PAS-positive granules were numerous in skeletal muscle fibres and Purkinje fibres of the myocardium, but were also observed in the neurons of the brain and spinal cord, and in the vascular smooth muscle fibres from all the examined tissues. Pretreatment with diastase completely abolished the PAS reactivity. The vacuoles reacted strongly to *Griffonia simplicifolia II* and *Concanavalia ensiformes* lectins, whose biding pattern has been reported as useful for demonstration of glycogen. Examination of the electron micrographs revealed that glycogen was free within the cytoplasm or accumulated in membrane-bound granules of several tissues, especially in striated muscle, liver and neurons of the CNS. These findings were consistent with generalized glycogenosis.

Keywords: Brahman cattle, generalized glycogenosis, lectin histochemistry, lysosomal storage disorder

Abbreviations: CNS, central nervous system; HE, haematoxylin and eosin; PAS, periodic acid–Schiff; PBS, phosphate-buffered saline

INTRODUCTION

There are at least eight types of glycogen storage diseases with differing enzymatic disorders, of which only type II is lysosomal (Summers *et al.*, 1995). The disease has been reported in humans (Pompe, 1932), dogs (Mostafa, 1970), cats (Sandstrom *et al.*, 1969), sheep (Manktelow and Hartley, 1975) and quail (Murakami *et al.*, 1980), and in

Shorthorn (Jolly *et al.*, 1977) and Brahman cattle (O'Sullivan *et al.*, 1981). Two mutations have been identified in Brahmans, and one in Shorthorns, that result in premature termination of translation, leading to generalized glycogenosis (Dennis *et al.*, 2000). Affected calves show loss of condition, poor growth and lethargy, especially after 2–3 months of age. Incoordination, excitement and hyperaesthesia may develop when the animal is driven or handled (O'Sullivan *et al.*, 1981). The main histological features include vacuolation of myocardial, skeletal and smooth-muscle fibres. Vacuolation of neurons, kidneys, thyroid and retinal cells has also been observed (Wisselaar *et al.*, 1993). In cattle, the enzyme deficit can be demonstrated in muscle, lymphocytes (Howell *et al.*, 1981) and liver (O'Sullivan *et al.*, 1981).

The disease affecting Brahman cattle has been well described in Australia (O'Sullivan *et al.*, 1981). Identification of heterozygotes (clinically normal) is the basis of the disease control programmes within herds. A blood mononuclear cell test may be applicable in the detection of cattle heterozygous for this condition (O'Sullivan *et al.*, 1981). However, variation in sample quality, environmental effects and undefined genetic factors compromise the use of this enzyme-based test. For these reasons, DNA-based tests have been developed (Dennis *et al.*, 2002).

While light microscopy and electron microscopy are used to observe the presence of lysosomal storage diseases (Glew *et al.*, 1985), lectin histochemistry performed on paraffin-embedded sections enables the identification of specific sugars and hence aids in the diagnosis of some glycoprotein and glycolipid storage diseases (Alroy *et al.*, 1984). Lectins are carbohydrate-binding proteins of non-immune origin that agglutinate cells and precipitate glycoconjugates (Goldstein and Hayes, 1978; Goldstein *et al.*, 1980) and, when bound to visualizing agents allow localization of complementary carbohydrates (Damjanov, 1987).

The present report describes the clinical, microscopic and ultrastructural features observed in cases of generalized glycogenosis type II in cattle that were born in a Brahman cattle herd in Brazil.

MATERIALS AND METHODS

A small Brahman cattle herd, comprising 20 cows and one bull, that was managed on pastured areas of the Rio Grande do Sul state had been experiencing episodes of weakness in the young animals for the previous three years. Necropsy was performed on three affected animals, and fragments of several organs were collected, fixed in neutrally buffered 10% formalin, prepared by standard histological methods and stained with HE. Additional samples were immediately immersed in 3% glutaraldehyde and prepared for transmission electron microscopy. Selected sections were stained with PAS at times ranging from 3 to 13 min with or without diastase pretreatment (Prophet *et al.*, 1992). Blood smears from affected calves were air-dried, fixed with methanol and stained with PAS.

Paraffin wax embedded sections (5 μm thick) of cerebellum, skeletal muscle and skin were prepared for lectin histochemistry. After deparaffinization, sections were treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature, rinsed

several times in 0.01 mol/L PBS (pH 7.2), and treated with 0.1% bovine serum albumin in PBS for 15 min. The sections were then incubated for 1 h at room temperature in biotinylated lectins (Vector Laboratories, Burlingame, CA, USA), which are presented in Table I. The optimal concentration for each lectin that allowed maximum staining with minimum background was at a dilution of 30 μg/ml in PBS, except for *Arachis hypogaea* lectin, which was applied at a concentration of 10 μg/ml. The slides were incubated with an avidin–biotin–peroxidase complex (Vector Laboratories) for 45 min. The horseradish peroxidase was activated by incubation for 1–2 min with a diaminobenzidine kit (Liquid DAB+ Substrate-Chromogen System, Dako, Carpinteria, USA). Specimens were rinsed in distilled water, dehydrated using graded ethanol solutions, cleared in xylene and mounted in Permount. Additional samples from two unaffected calves were subjected to the same lectin-histochemical staining. Controls for lectin staining included exposure to horseradish peroxidase and substrate medium without lectin and blocking by incubating them with their blocking sugars (0.1–0.2 mol/L in PBS) for 1 h at room temperature before application to the sections.

RESULTS

In the last three calving periods, 10 out of 47 calves developed the condition, as presented in Table II. The clinical signs included loss of condition, poor growth, lethargy, ill-thrift, muscular weakness (Figure 1), muscular tremors and difficulty rising after stumbling. Affected calves were not able to follow their mothers. Antibiotics, vitamins, fluid therapy and anthelmintics were administered to the animals, but no signs of clinical recovery were noted. Because of the progressive impairment of their clinical conditions, the affected animals were euthanized. All diseased animals were sired by the same bull. The only gross lesion detected was paleness of the muscles of the trunk and limbs.

Multiple cytoplasmic vacuoles located in different tissues were the most striking microscopic lesions. Vacuolation was particularly severe and evident in the skeletal muscles and myocardium (Figure 2). Affected neuronal bodies were remarkably enlarged in the brainstem and ventral horns of the spinal cord. In the cerebellum, Purkinje cells were only slightly affected. PAS-positive granules were numerous in skeletal muscle fibres, in Purkinje fibres of the myocardium and in neurons (Figure 3) of the brain and spinal cord. Additionally, PAS-positive material was observed in the hair follicles, sweat glands and monocytes of the blood smears, and in the vascular smooth-muscle fibres from all the examined tissues. Pretreatment with diastase completely abolished the PAS reactivity. The vacuoles reacted strongly to *Griffonia simplicifolia* II and *Concanavalia ensiformes* lectins. In the skin sections, these reactions were seen in the arrector pili muscles, smooth muscle of the blood vessels (Figure 4) and glands. *Concanavalia ensiformes* stained the same elements but less intensively. These two lectins marked the stored material at skeletal muscle and nervous tissues. Neurons were stained in cerebellum, brain and spinal cord. The stored material was not stained with any of the other eight lectins used in this study. Blocking sugars prevented lectin binding in control material derived from affected calves. Lectin

TABLE I
Biotinylated lectins used in the histochemical studies on generalized glycogenosis in Brahman cattle

Lectin	Acronym	Binding specificity[a]
Concanavalia ensiformis agglutinin	Con A	α-D-Man and α-D-Glc
Glycine max agglutinin	SBA	α-D-GalNAc, α-D-GalNAc and α- and β-Gal
Arachis hypogaea agglutinin	PNA	β-D-Gal and (1-3)GalNac
Ricinus communis I agglutinin	RCA- I	β-D-Gal and α-D-Gal
Ulex europaeus I agglutinin	UEA-I	α-L-Fuc
Triticum vulgaris agglutinin	WGA	α-D-GlcNAc and NeuNAc
Succinyl-WGA	sWGA	$(\beta\text{-}(1\text{-}4)\text{-D-GlcNAc})_2$
Lens culinaris agglutinin	LCA	Fucosylated core region of bi- or triantennary
Griffonia (Bandeiraea) simplicifolia agglutinin I	GS-I	α-D-galactose
Griffonia (Bandeiraea) simplicifolia agglutinin II	GS-II	α,β-linked *N*-acetylglucosamine and glycogen

[a]Fuc, fucose; Gal, galactose; GalNac, *N*-acetylgalactosamine; Glc, glucose; GlcNac, *N*-acetylglucosamine; Man, mannose; NeuNac, *N*-acetylneuraminic acid

TABLE II
Generalized glycogenosis in a Brahman cattle herd. Total of the calves born in the three successive calving periods

	Calving period			
	2000	2001	2002	Total
Affected calves	3	5	2	10
Total of calves born	16	19	12	47

Figure 1. Brahman calf atypically recumbent owing to muscular weakness

histochemistry applied to the samples from control unaffected calves stained faintly with all the lectins used here, especially in the sections of skeletal muscle.

Examination of the electron micrographs revealed that stored material was both membrane-bound and free within the cytoplasm in several tissues and also in some mitochondria of the skeletal muscles. These glycogen accumulations were more conspicuous in striated muscle (Figure 5), liver and neurons of the CNS; however, the Purkinje neurons of the cerebellum showed only a few membrane-bounded bodies.

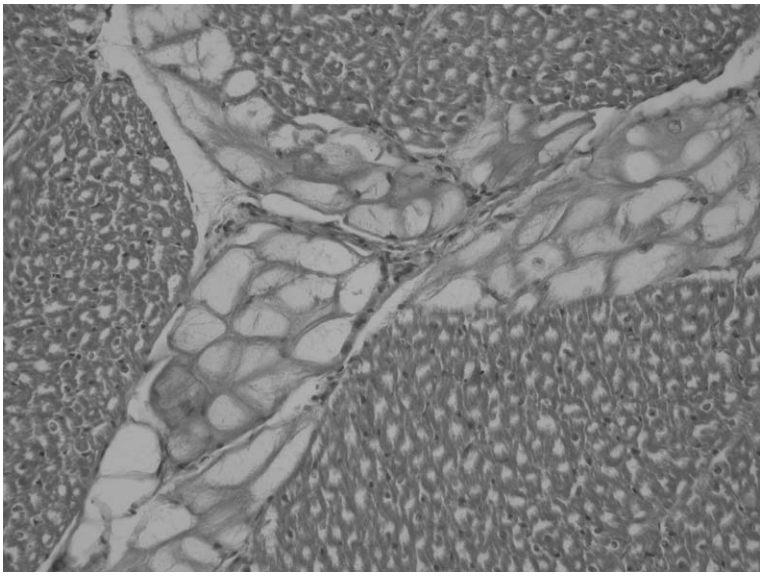

Figure 2. Presence of vacuoles in the myocardium and Purkinje fibres from a Brahman calf affected with generalized glycogenosis. HE; original magnification $\times 200$

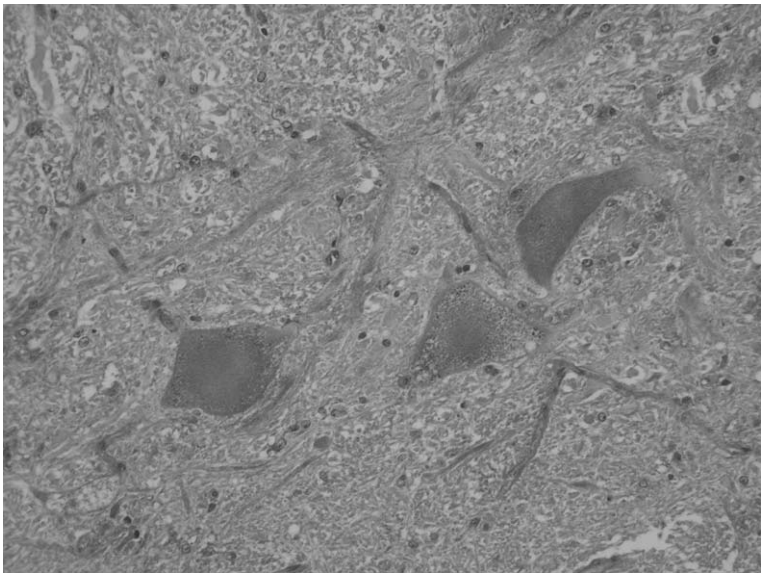

Figure 3. Brainstem from a Brahman calf affected with generalized glycogenosis presenting neuronal vacuoles due to accumulation of PAS-stainable material. PAS staining; original magnification $\times 400$

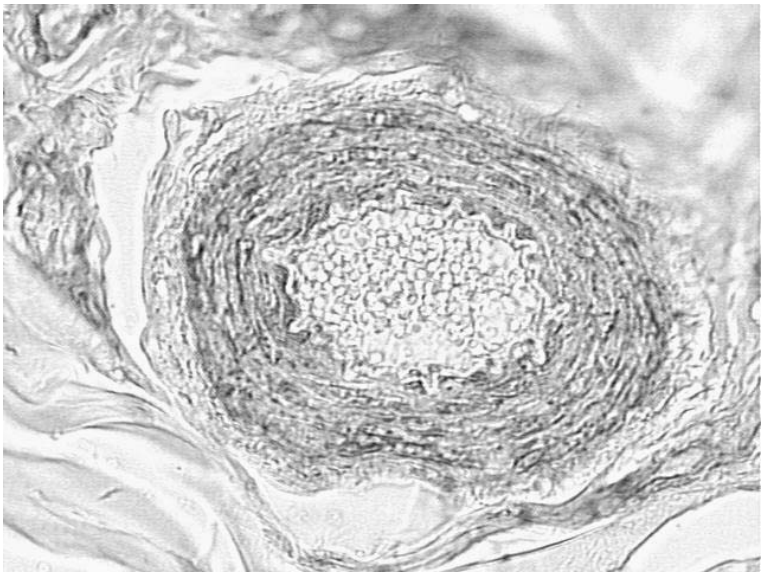

Figure 4. Vascular smooth muscle from a Brahman calf affected with generalized glycogenosis demonstrating clear reaction to *Griffonia simplicifolia* II. Lectin histochemistry staining; original magnification $\times 400$

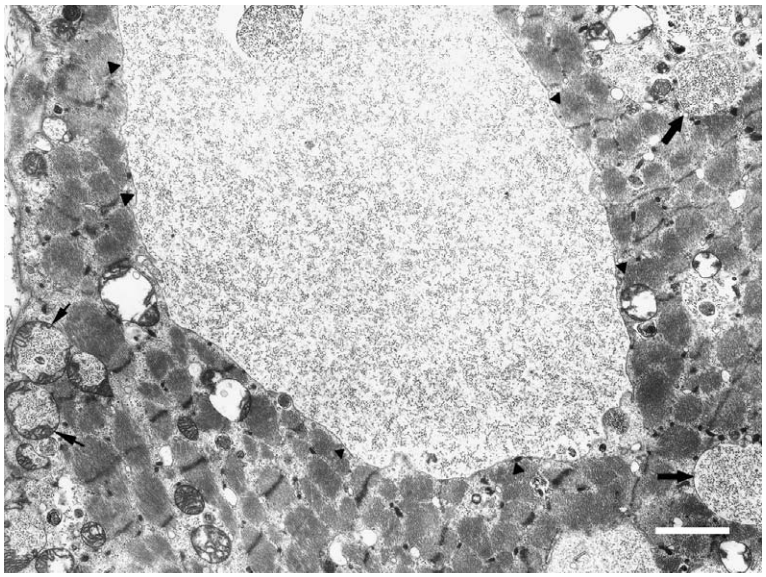

Figure 5. Electron micrograph. Striated muscle of Brahman calf affected with generalized glycogenosis. In the sarcoplasm there is an accumulation of glycogen in the particulated form, delimited by membrane (arrow heads) and glycogenosomes (large arrows). Glycogen particles are seen in the matrix of some swollen mitochondria (small arrows). Bar = 2 μm

DISCUSSION

The clinical and pathological findings observed in these Brahman animals were consistent with previous descriptions of generalized glycogenosis type II in purebred Brahman cattle in Australia (O'Sullivan *et al.*, 1981; Reichmann *et al.*, 1993). Evidence associating these cases with imported animals could not be confirmed. The use of a Nellore bull in the subsequent breeding season solved the immediate problem in this herd.

Type II glycogenosis (Pompe disease) is characterized by the presence of large amounts of glycogen in lysosomes (Cook *et al.*, 1982). The association of this condition with the presence of vacuoles in the cytoplasm of several organs containing stored material stainable with PAS has been reported previously (Reichmann *et al.*, 1993). Additional evidence for the glycogen accumulation observed here was the absence of stainable PAS material after pretreatment with diastase (Prophet *et al.*, 1992). The differences observed in the degree of vacuolation, i.e. severe in the muscule fibres and slight in the cerebellum, may have been associated respectively with the large and small contents of glycogen usually present in those tissues (Brown, 2004).

Electron-microscopic analysis showed a large accumulation of glycogen contained in glycogenosomes. The lectin binding pattern, e.g. positive staining to *Griffonia simplicifolia* II and *Concanavalia ensiformes*, has been reported as useful for demonstration of glycogen *in situ* on fixed paraffin-embedded sections of a variety of tissues (Hennigar *et al.*, 1986; Spicer and Schulte, 1988; Ito *et al.*, 1990), supplying further evidence for glycogen accumulation in the vacuoles observed here. The faintly stained patterns observed in the control samples from unaffected calves subjected to lectin histochemistry may be associated with the normal contents of oligosaccharides in those tissues.

Histological changes observed in bovine glycogenosis are similar to those seen in α-mannosidosis syndromes, including the plant poisonings induced by *Swainsona* and *Astragalus*. However, the evident muscular involvement characteristically present in generalized glycogenosis and the neurological disorder typically reported in α-mannosidosis may differentiate those conditions (O'Sullivan *et al.*, 1981; Reichmann *et al.*, 1993). Furthermore, plants known to induce similar syndromes were not encountered on the farm. Estimation of acidic α-glucosidase activity (Healy *et al.*, 1987) and/or genotyping for loss-of-function alleles within the acidic α-glucosidase gene (Dennis *et al.*, 2002) would have certainly added diagnostic value to this communication; however, the results presented here are strongly suggestive of generalized glycogenosis. Although Brahman cattle herds have been expanding in many countries, this is the first report of the condition from outside the Australian continent.

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