NEURONAL CEROID LIPOFUSCINOSIS IN AUSTRALIAN MERINO SHEEP

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SUMMARY

The neuronal ceroid lipofuscinoses (NCLs) are a group of inherited neurodegenerative diseases characterised by the accumulation of autofluorescent lipopigment in a variety of tissues. At least six different forms of NCL occur in humans and the disease has been identified in various animal species, including sheep. Clinical features of NCL are dementia, loss of vision, motor disturbances, and premature death.

Recently NCL was reported for the first time in Merino sheep in Australia. The aim of this project is to characterise the genetic defect in Merino sheep and to compare it to a clinically and genetically well defined form of NCL in South Hampshire sheep. Preliminary results indicate that the NCL gene in Merinos might be located in the same chromosomal region as that in South Hampshires.
Keywords: *Ovis aries*, Merino sheep, neuronal ceroid lipofuscinosis, genetics

INTRODUCTION

The neuronal ceroid lipofuscinoses (NCLs) compromise a group of recessively inherited neurodegenerative diseases characterised by the accumulation of autofluorescent lipopigment in lysosomes in neurons and other cells within the body. The dominant component of the storage material in most of the NCLs is the hydrophobic protein, mitochondrial ATP synthase subunit *c*. The accumulation of this material is associated with brain and retinal atrophy, and a slowly progressive deterioration in cerebrocortical function.

At least 6 human forms of NCL have been identified (CLN1-6) based on age at onset, progression of disease, clinical and histopathological findings, and the genetic loci involved (OMIM: http://www.angis.org.au/Databases/BIRX/). NCL has also been reported in dogs, cats, cattle, goats, mice, and in Finish Landrace (Järplid and Haltia 1993), Rambouillet (Edwards *et al.* 1994) and South Hampshire sheep (Jolly *et al.* 1980). Study of these animal models has been invaluable in the biochemical characterisation of the NCLs (Jolly and Palmer 1995; Palmer *et al.* 1997).

For both human and animal forms of NCL, the identification of genes causing the disease is of major interest. In humans, four different NCL genes have been identified (PPT, CLN2, CLN3 and CLN5). CLN6 has been localized by linkage analysis to human chromosome 15, but no linkage information is available for CLN4 (OMIM). Two mouse models for NCL have been mapped: motor neuron degeneration (mnd) (Bronson *et al.* 1993) and neuronal ceroid lipofuscinosis (nclf) (Bronson *et al.* 1998). In English Setter dogs, linkage between NCL and an unassigned linkage group has been

observed (Lingaas *et al.* 1998). The causal gene for NCL in South Hampshire sheep has not been identified, but has been mapped by linkage analysis to a region on ovine chromosome 7 that is syntenic to the region on human chromosome 15 associated with CLN6 in humans (Broom *et al.* 1998) and on mouse chromosome 9 associated with nclf in mice (Bronson *et al.* 1998).

In May 1997, NCL was diagnosed for the first time in Australia in a flock of fine wool Merino sheep (Cook *et al.* 1998). Our aim is to characterize the genetic defect in Merino sheep and to develop a DNA test for Australian Merino breeders. Initially, NCL in Merino sheep will be compared to the clinically and genetically well defined form of NCL in South Hampshire sheep (Jolly *et al.* 1989; Broom *et al.* 1998). This preliminary investigation will thus show us if we are dealing with the same genetic mutation found in South Hampshire sheep in New Zealand, or if we need to conduct a larger study to characterise a new genetic form of this disorder which may be unique to Merino sheep.

MATERIAL AND METHODS

Animals. A total of at least 19 NCL cases occurred in 1997 and 1998 in the affected commercial Merino flock (500 breeding ewes), and in the stud of origin of the 12 replacement rams used in this flock during the previous five years. At least six cases with similar clinical signs had occurred in 1996. During 1997 and 1998 a number of affected animals were relocated to the Regional Veterinary Laboratory at Wollongbar or the University of Sydney at Camden for clinical observation and necropsy.

Postmortem tissue samples were processed for histopathology and electronmicroscopy. Fresh brain liver and pancreas were processed for isolation of lipopigment storage cytosomes for electronmicroscopy, and for gel electrophoresis and Western blotting for detection of mitochondrial ATP synthase subunit *c*.

Homozygosity mapping. In the absence of appropriate pedigree information for a linkage analysis, we have initiated a homozygosity mapping approach (Lander and Botstein 1987; Krugylak *et al.* 1995; Houwen *et al.* 1995). DNA was extracted from blood or tissue samples from 15 affected sheep (affected group), and from 20 unaffected sheep from the 1997 lamb drop in the commercial flock, 7 of the 12 rams used in the commercial flock, and a putative carrier ram from the stud (control group).

Ovine DNA markers for chromosome 7, that are linked to NCL in South Hampshire sheep, were used initially. Further markers were selected to confirm the initial results. Markers used in this study and their chromosomal location are shown in Table 1. The markers were amplified using a standard touch down PCR protocol, and were analysed on a semiautomated fluorescent DNA sequencer (LI-COR). An association analysis comparing affected sheep and control sheep with respect to allele frequencies was performed using the software CLUMP (Sham and Curtis 1995).

RESULTS AND DISCUSSION

Clinical and pathological findings. First signs were detected in affected animals at about 9 month of age, and included a decreased menace response, and visual impairment. Progressive deterioration in cerebrocortical function occurred during the following months. Signs included repetitive activities (aimless walking, or walking in circles in a confined place), staring into space, abnormal struggling

on normal restraint, episodes of muscle tremors elicited by handling, somnolence, and blindness. At necropsy, the cerebral hemispheres were smaller and firmer than normal and flattened dorsoventrally. Brains weighed 50-80 g (normal is 110-120 g). Microscopic changes comprised severe cerebrocortical atrophy associated with neuronal loss, marked astrocytosis, and the presence of eosinophilic intracytoplasmic inclusions in neurons, hepatocytes and renal cortical tubular epithelium. These inclusions had staining characteristics of 'lipofuscin', and autofluoresced in unstained tissue sections with fluorescent microscopy. Degeneration of the photoreceptor and outer nuclear layers of the retina developed after the cerebrocortical changes. Electronmicroscopic examination of brain, and of storage cytosomes isolated from fresh brain, liver and pancreas revealed electron-dense aggregates or multilamellar profiles, which were confirmed on Western blotting to comprise accumulated mitochondrial ATP synthase subunit *c*.

Homozygosity mapping. Assuming a recessive mode of inheritance, we would expect DNA markers linked to the defect to show homozygosity for one allele in affected animals whereas carriers or unaffected animals will show a variety of different alleles. Genotyping was started with markers on ovine chromosome 7 that were previously reported to be linked to NCL in South Hampshire sheep (BM3033, BMS528, Broom *et al* . 1998; OCLM1.1, OCLM2.1, OCLM8.7, unpublished data). Additional markers on chromosome 7 (McM223 and TGLA444) and control markers on other chromosomes (INRA132, INRA135, MAF64 and TGLA10) were also analysed. The number of different alleles and the degree of homozygosity (%) in the affected and control groups are shown in Table 1. All 15 affected sheep showed homozygosity for the same allele for the markers BMS528, OCLM1.1, OCLM8.7 and MCM223, whereas the 28 unaffected control sheep did not show this trend towards homozygosity.

Table 1. Homozygosity mapping results

Marker	BM 3033	BMS 528	OCLM 1.1	OCLM 2.1	OCLM 8.7	MCM 223	TGLA 444	INRA 135	MAF 64[A]	TGLA 10[A]	INRA 132[A]
Chromosome	7	7	7	7	7	7	7	2	2	1	20
Affected (n=15):											
%Homozygosity	26.7%	100%	100%	93.3%	100%	100%	80%	26.7%	46.7%	28.6%	20%
No. of alleles	3	1	1	2	1	1	3	7	6	6	5
Controls (n=28):											
%Homozygosity	17.9%	64.3%	32.1%	25%	53.6%	39.3%	71.4%	21.4%	22.2%	25%	21.7%
No. of alleles	8	3	7	7	4	6	4	8	6	9	5
CLUMP:											
chi-square	11.07	8.2	31.62	33.58	13.96	14.88	2.91	7.36	1.15	7.61	3.72
p	0.097	0.011	0	0	0.001	0.004	0.482	0.415	0.959	0.496	0.666

[A] = genotyping incomplete: 1, 5 and 10 animals respectively are missing

The results of the CLUMP T1 analysis comparing allele frequencies in the 15 affected and 28 unaffected control sheep are shown in Table 1. Allele frequencies in the affected and control animals differed significantly for the markers BMS528, OCLM1.1, OCLM2.1, OCLM8.7, and MCM223, which are all located close to the NCL gene locus in South Hampshire sheep, but not for markers on

OAR7 that are located further away from the disease locus in South Hampshire sheep (BM3033 and TGLA444), or for markers on other chromosomes (INRA132, INRA 135, MAF64 and TGLA10).

These results strongly suggest that the same chromosomal region is associated with NCL in both Merino and South Hampshire sheep. To confirm these preliminary results, we are generating families suitable for linkage studies. So far, two embryo transfers, using an affected ewe and mixed semen from two putative carrier rams, have resulted in the birth of 7 lambs.

When the causative gene for NCL in South Hampshire sheep is identified, we will be able to test our Merino sheep for mutations in the same gene. NCL in Merinos may be caused by a different mutation in this same gene, and therefore provide an alternative model for NCL in humans. The long term aim of this work is the development of a DNA test to enable Australian Merino breeders to control the disease.

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REFERENCES

Bronson, R.T., Lake, B.D., Cook, S., Taylor, S. and Davisson, M.T. (1993) *Ann. Neurol.* **33**:381

Bronson, R.T., Donahue, L.R., Johnson, K.R., Tanner, A., Lane, P.W. and Faust, J.R. (1998) *Am. J. Med. Genet.* **77**:289

Broom, M.F., Zhou, C., Broom, JE., Barwell, K.J., Jolly, R.D. and Hill, D.F. (1998) *J Med Genet* **35**:717

Cook, R.W., Jolly, R.D., Palmer, N.D., McKinnon, R. and Tammen, I.(1998) *Proc. Ann. Conference of the Australian Society for Veterinary Pathology*, 16-17.May, Sydney, pp51

Edwards, J.F., Storts, R.W., Joyce, J.R., Shelton, J.M. and Menzies, C.S. (1994) *Vet. Pathol.* **31**:48

Houwen, R.H.J., Baharioo, S., Blankenship, K., Raeymaeker, P., Juyn, J., Sandkuijl, L.A. and Freimer, N.B. (1995) *Nature Genetics* **8**:380

Järplid, B. and Haltia, M. (1993) *J. Inher. Metab. Dis.* **16**:274

Jolly, R.D., Janmaat, A., West, D.M. and Morrison, I. (1980) *Neuropathol. Appl. Neurobiol.* **6**:195

Jolly, R.D. and Palmer, D.N. (1995) *Neuropathol. Appl. Neurobiol.* **21**:50

Krugylak, L., Daly, M.J. and Lander, E. (1995) *Am. J. Hum. Genet.* **56**:519

Lander, E.S. and Botstein, D. (1987) *Science* **236**:1567

Lingaas, F., Aarskaug, T., Sletten, M., Bjerkas, I., Grimholt, U., Moe, L., Juneja, R.K., Wilton, A.N., Galibert, F., Holmes, N.G. and Dolf, G. (1998) *Anim Genet* **29**:371

Palmer, D.N., Jolly, R.D., van Mil, H.C., Tyynelä, J. and Westlake, V.J. (1997) *Neuropaediatrics* **28**:45

Sham, P.C. and Curtis D. (1995) *Ann. Hum. Genet.* **59**:97