1998

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Short Communications

Preclinical detection of PrP^{Sc} in nictitating membrane lymphoid tissue of sheep

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Veterinary Record (1998) **142,** 489-491

SHEEP scrapie is the prototype of a heterogeneous group of transmissible spongiform encephalopathies (TSEs) which occur in humans, cattle, cats, mink and cervids. TSEs are characterised by the deposition of prion proteins (PrP-Scrapie or PrP^{Sc}) in the central nervous system of affected individuals (Prusiner 1982). PrP^{Sc} and infectivity have also been detected in the lymphoreticular system of sheep well before symptoms occur (Hadlow and others 1982, Race and others 1992). Detection of PrP^{Sc} in lymphoid tissue, such as tonsils or peripheral lymph nodes, has been proposed as a preclinical diagnostic test for scrapie (Ikegami and others 1991, Schreuder and others 1996). This short communication reports the use of a non-invasive diagnostic assay based on biopsy of lymphoid nodules in the nictitating membrane of sheep. The nictitating membrane or third eyelid (palpebra tertia) of ruminant animals consists of a cartilaginous sheet with superficial lymphoid follicles and a seromucinous secretory gland beneath the conjunctiva of the bulbar surface (Slatter and Mills 1981). The lymphoid nodules are easily biopsied under local anaesthesia.

Sheep were pretreated by topical administration of proparacaine hydrochloride (Ophthetic; Allergan) and the nictitating membrane was everted with disposable 1 x 2-toothed, 5½-inch forceps (Sklar 78055-1). Typically two clusters of lymphoid tissue were visualised above the more pale glandular tissue. A biopsy of one cluster of lymphoid tissue was collected using disposable Metzenbaum scissors (Sklar 30455) and a second set of forceps. Biopsied tissue was fixed in formalin and paraffin embedded using conventional methods. Sections typically contained four to seven lymphoid follicles.

PrP^{Sc} was detected in sheep tissues using a monoclonal anti-

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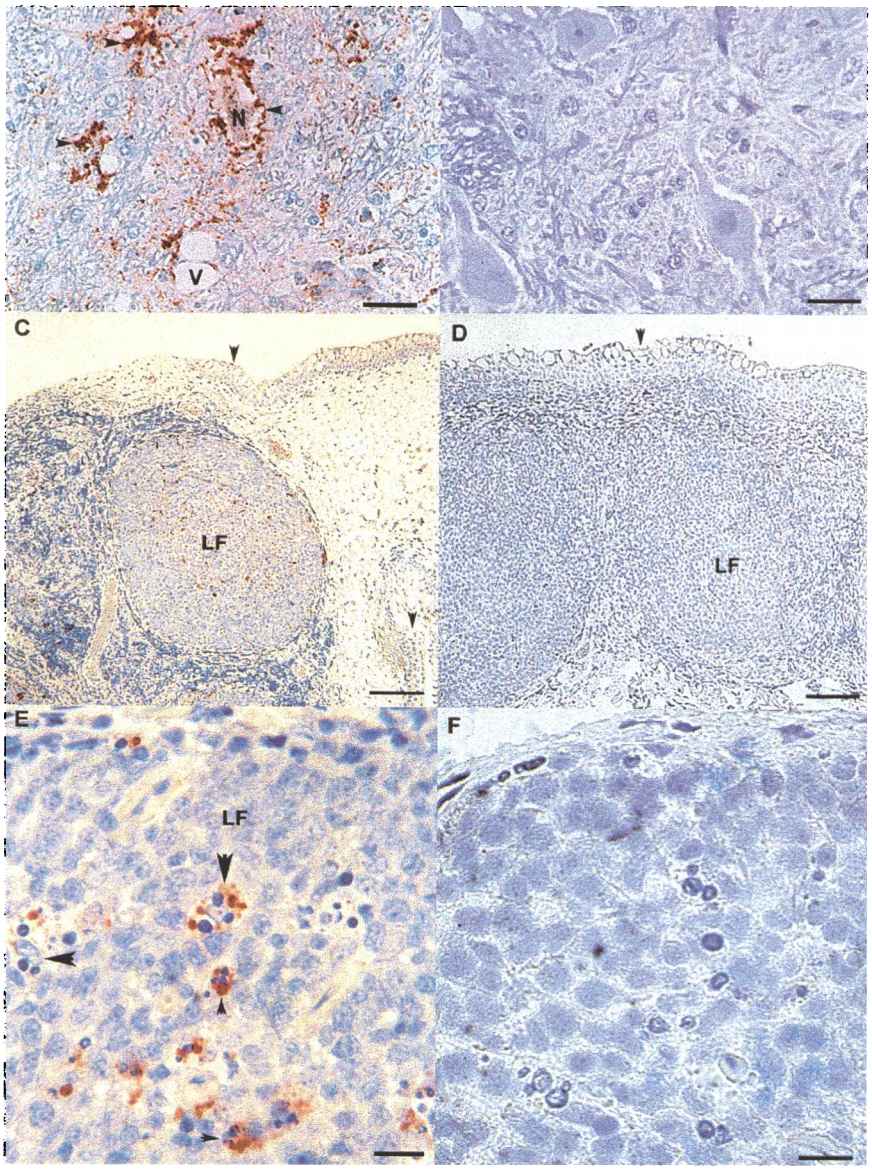

FIG 1: Immunohistochemical detection of PrP^{Sc} in nictitating membrane-associated lymphoid tissue and brain of sheep using MAb F89/160.1.5. (A) Brain (obex) with spongiform encephalopathy from scrapie-affected sheep. PrP^{Sc} immunostaining in brainstem (red) rimming neurons and as plaques around glial cells in the neuropil (arrowheads). N Neuron perikaryon, V Neuropil vacuolar change. Bar = 30 µm. (B) Negative tissue control for Fig 1A. No immunostaining with MAb F89/160.1.5 in brainstem of clinically normal control sheep from a flock with no known exposure to scrapie. Bar = 30 µm. (C) Nictitating membrane lymphoid follicles from clinically normal sheep from a flock with a history of scrapie. Lymphoid follicle germinal centre (LF) with punctate multifocal PrP^{Sc} immunostaining (red). Arrowheads indicate the conjunctiva epithelium. Bar = 115 µm. (D) Negative tissue control for Fig 1C. No immunostaining with MAb F89/160.1.5 in nictitating membrane lymphoid follicles (LF) of clinically normal control sheep from a flock with no known exposure to scrapie. Arrowhead indicates the conjunctiva epithelium. Bar = 115 µm. (E) Nictitating membrane lymphoid follicle from a clinically normal sheep from a flock with a history of scrapie. Coarse and fine granular cytoplasmic PrP^{Sc} immunostaining (red) within dendritic-like cells that have open-faced nuclei and multiple cytoplasmic processes sometimes closely associated with mature lymphocytes (large arrowheads); and macrophage-like cells with multilobed nuclei and phagocytosed cytoplasmic debris (small arrowheads) LF Lymphoid follicle germinal centre. Bar = 18 µm. (F) Negative tissue control for Fig 1E. No immunostaining with MAb F89/160.1.5 in nictitating membrane lymphoid follicle of clinically normal control sheep from a flock with no known exposure to scrapie. Bar = 18 µm

body, F89/160.1.5, which recognises a conserved epitope on cattle, sheep, mule deer and elk PrP^{Sc} (O'Rourke and others 1998). This MAb binds PrP^{Sc} in formalin-fixed, paraffin-embedded ruminant brain tissue pretreated by hydrated autoclaving. Because PrP^{Sc} is less abundant in lymphoid tissue than in brain, the sensitivity of the assay was increased by several modifications, with all variations performed using a paraffin block of tissue from the obex of a scrapie affected ewe. After rehydration and hydrated autoclaving (Haritani and others 1992), non-specific sites on the tissue were blocked by incubation for 30 minutes in 5 per cent normal horse serum (NHS) (Vector Laboratories) in 0·1 M Tris-HCl containing 0·05 per cent Triton X-100 (TTB). Sections were incubated overnight with primary antibody (MAb F89/160.1.5) or an isotype control, adjusted to 3 µg/ml in NHS-TTB. Bound antibody was detected by two cycles of incubation with secondary antibody (biotinylated horse anti-mouse IgG [Vector Laboratories] diluted 1/200 in NHS-TTB, 40 minutes incubation) and avidin-biotin-horseradish peroxidase complex (ABC-HRPO) (Vector Laboratories) (prepared as a 1/250 dilution of each reagent in TTB, 40 minutes incubation). Chromagen/substrate (AEC; Dako Corporation) was applied twice for five minutes each with no intervening washes. Slides were counterstained with Mayer's haematoxylin and coverslips were mounted in Gel-Trol (Lipshaw Immunon). This protocol resulted in an increase in the intensity of the immunostaining in brain tissue from scrapie-affected sheep (Fig 1a) with no development of background when tissue from unaffected sheep was assayed (Fig 1b) or when the primary antibody was replaced with an isotype control. Controls for each subsequent run included brain (obex) from scrapie-positive and scrapie-negative sheep, and each tissue section was prepared in duplicate for incubation with MAb F89/160.1.5 and an isotype control MAb.

The modified assay was applied to lymphoid tissues, including the retropharyngeal, superficial cervical, mesenteric and prefemoral lymph nodes, from a scrapie-affected ewe and healthy control ewes. Samples fixed in buffered formalin, pretreated by hydrated autoclaving and stained with this technique were compared with paired samples fixed in paraformaldehyde and pretreated with formic acid and hydrated autoclaving (Miller and others 1994, van Keulen and others 1996). The samples had a similar intensity and pattern of PrP^{Sc} immunoreactivity, demonstrating that the fixation and pretreatment methods were equivalent when ovine lymphoid tissue was immunostained by this technique.

The immunohistochemistry assay was then applied to nictitating membrane-associated lymphoid tissue from sheep from 11 flocks (all Suffolk, Hampshire and Southdown breeds) with a history of clinical scrapie in one or more sheep, confirmed by histology and/or immunohistochemistry of midbrain. The nictitating membrane lymphoid tissue from nine clinically affected sheep and from 16 clinically normal flockmates showed distinct, multifocal PrP^{Sc} deposition within lymphoid follicle germinal centres (Fig 1c), whereas lymphoid tissue from a negative control sheep showed no

staining (Fig 1d). The mean (sd) percentage of immunoreactive follicles in positive samples was 89 (19) per cent. Most immunoreactivity within germinal centres was located in the cytoplasm of multipolar cells that were sometimes rimmed by mature lymphocytes, characteristics of follicular dendritic cells (Fig 1e). Immunoreactivity was also present in the cytoplasm of cells that contained phagocytosed debris and had a reniform nuclear morphology consistent with resident macrophages (Fig 1e). Staining intensity varied among individual sheep but did not correlate with age or clinical status. No staining was observed in lymphoid follicles of high-risk sheep when MAb F89/160.1.5 was replaced by an isotype control. Negative tissue controls were nictitating membrane biopsies, each containing at least five lymphoid follicles, collected from 20 Suffolk sheep and two Cheviot sheep, two to eight years old, with no known exposure to scrapie. Immunostaining with MAb F89/160.1.5 failed to show staining in these negative tissue controls (Fig 1d and Fig 1f). Ten of the Suffolk sheep and both Cheviot sheep were euthanased; they had no lesions consistent with scrapie and immunohistochemistry failed to show immunostaining in brain, superficial cervical lymph node, tonsil or retropharyngeal lymph node. The other 10 Suffolk sheep remain clinically normal.

Of the 16 clinically normal sheep with positive immunostaining, six subsequently developed clinical signs two to seven months after sampling and scrapie was confirmed by histological examination and/or immunohistochemical analysis of the brain (obex) following necropsy. Four of these six sheep were offspring of ewes with confirmed scrapie; histories were not available for the other two. Of the 10 clinically normal sheep still under observation, one is the offspring of a scrapie affected ewe, three are offspring of healthy ewes with positive immunostaining, and six have no known bloodline relationship with a clinically affected or biopsy positive ewe. All sheep were in the flocks of origin when scrapie-affected ewes produced lambs.

PrP genotypes were determined for 17 of the 25 sheep with PrP^{Sc} positive staining in the nictitating membrane lymphoid tissue. All 17 carried the susceptible PrP genotypes 136AA, 171QQ (n = 15) or 136AV, 171QQ (n = 2) (Goldman and others 1994, Hunter and others 1994, Westaway and others 1994, O'Rourke and others 1996). Sixty-three sheep in these flocks were negative by nictitating membrane lymphoid biopsy and remain clinically normal. They are still under observation.

Neither the source of scrapie, the year(s) in which it was introduced into these flocks, nor the routes of transmission are known. Therefore the optimal sampling intervals cannot be determined by observations on naturally infected flocks alone. Studies of lambs born to ewes with PrP^{Sc} positive lymphoid tissue and raised without further exposure to scrapie will provide more accurate data on sampling protocols. Longitudinal studies are also being conducted on healthy, PrP^{Sc} positive sheep to determine whether all sheep with positive staining in nictitating membrane-associated lymphoid tissue progress to clinical disease. Immunohistochemistry assay of nictitating membrane-associated lymphoid tissue provides a practical method for early detection of scrapie infected sheep. Although infectivity has not been detected in lymphoid tissues of cattle naturally infected with BSE, the retina has been shown to be infectious. The suitability of nictitating membrane lymphoid tissue as a diagnostic site in cattle is under investigation.

Acknowledgements. – The authors would like to thank W. Harwood, P. Dilbeck and W. Labrousse for technical assistance, and P. Steiner and R. Horn for animal care. M. Bulgin, J. Tyler, A. Jenny and numerous United States sheep producers provided access to scrapie-affected and high-risk sheep.

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