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Immunohistochemical detection and distribution of prion protein in a goat with natural scrapie

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BRIEF COMMUNICATIONS

Immunohistochemical detection and distribution of prion protein in a goat with natural scrapie

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Abstract. Formalin-fixed, paraffin-embedded tissue sections from a 3-year-old female Angora goat suffering from clinical scrapie were immunostained after hydrated autoclaving using a monoclonal antibody (mAb, F99/97.6.1; IgG1) specific for a conserved epitope on the prion protein. Widespread and prominent deposition of the scrapie isoform of the prion protein (PrP^{Sc}) was observed in the brain, brainstem, spinal cord, retina, postganglionic neurons associated with parasympathetic ganglia of myenteric and submucosal plexuses, Peyer's patches, peripheral lymph nodes, and pharyngeal and palatine tonsils. The goat was homozygous for PrP alleles encoding 5 octapeptide repeat sequences in the N-terminal region of the prion protein and isoleucine at codon 142, a genotype associated with high susceptibility and short incubation times in goats. The results of this study indicate that mAb F99/97.6.1 is useful for detection of PrP^{Sc} deposition, and this is a specific and reliable immunohistochemical adjunct to histopathology for diagnosis of natural caprine scrapie, although precise determination of the diagnostic sensitivity and specificity of the assay as a diagnostic test for scrapie in goats will require examination of a sufficiently large sample size. As with ovine scrapie, prion protein is widely distributed in the central and peripheral nervous systems, gastrointestinal tract, and lymphoid tissues in natural caprine scrapie.

Scrapie is a naturally occurring transmissible spongiform encephalopathy of sheep and goats caused by a proteinaceous infectious particle termed a prion protein (PrP). Prions are unprecedented transmissible pathogenic particles that are devoid of nucleic acid and seem to be composed exclusively of a modified protein.[15] The normal cellular isoform of the protein (PrP^{C}) is converted into the disease-associated pathologic scrapie isoform (PrP^{Sc}) through a posttranslational process, and deposition of PrP^{Sc} in neural tissue results in progressive neurodegeneration.[15]

Detection of PrP^{Sc} is a confirmatory adjunct to histopathologic examination of brain for the diagnosis of scrapie in sheep.[11,16] Immunohistochemical detection of PrP^{Sc} in lymphoid tissues is particularly useful for the identification of scrapie-affected sheep during the early, preclinical stage of the disease.[12] A monoclonal antibody (mAb, F89/160.1.5; IgG1) that binds to residues 142–145 of ovine PrP recognizes a conserved epitope on the prion protein in formalin-fixed tissues from the central nervous system of cattle, sheep, mule deer, and elk with naturally occurring transmissible spongiform encephalopathies.[13] The accumulation of PrP^{Sc} in lymphoid tissue of the nictitating membrane using mAb F89/160.1.5 alone or in combination with mAb F99/97.6.1 (IgG1), which binds to residues 220–225 of ovine PrP,[12] is the basis of an antemortem antigen-based assay for preclinical diagnosis of scrapie in sheep.[12,13] mAb F99/97.6.1 recognizes an epitope conserved on all 4 reported alleles[6] of the caprine PrP gene. Here, the use of mAb F99/97.6.1 for detection and localization of prion protein in formalin-fixed caprine tissues in a goat with natural scrapie is demonstrated.

A 3-year-old nulliparous female Angora goat showing signs of severe clinical scrapie characterized by ataxia, proprioceptive deficits of the hind limbs, hyperexcitability, head tremors, dysphagia, and bloat was euthanized on account of progressive degeneration. The goat's dam had died previously from scrapie, as indicated by histopathologic examination of the brain, after showing similar clinical signs. The dam had been purchased at a public livestock auction and cohoused with sheep on a ranch with no known cases of clinical scrapie in central Wyoming.

Representative tissues, collected on a complete postmortem examination and fixed in 10% buffered formalin, were embedded in paraffin, sectioned at 4.0 μm and stained with hematoxylin and eosin (HE) for routine histopathologic examination. Immunohistochemical staining was performed as described previously.[13] Tissue sections were mounted on positively charged glass slides, deparaffinized, rehydrated, exposed to 98% formic acid for 5 minutes, washed in 0.1 M Tris–HCl (pH 7.6), and autoclaved at 121 C for 25 minutes in 0.1 M Tris–HCl (pH 7.6). The slides were immunostained by automated immunostaining.[a] Primary mAb F99/97.6.1 was applied at a concentration of 3–5 μg/ml for 32 minutes at 37 C followed by application of biotinylated goat anti-mouse IgG for 8 minutes at 37 C and streptavidin–horseradish peroxidase for 8 minutes at 37 C and detection with 3-amino-9-ethylcarbazole–H_2O_2. All slides were counterstained with Mayer's hematoxylin. Negative control procedures consisted of 1) substitution of mAb F99/97.6.1 with a

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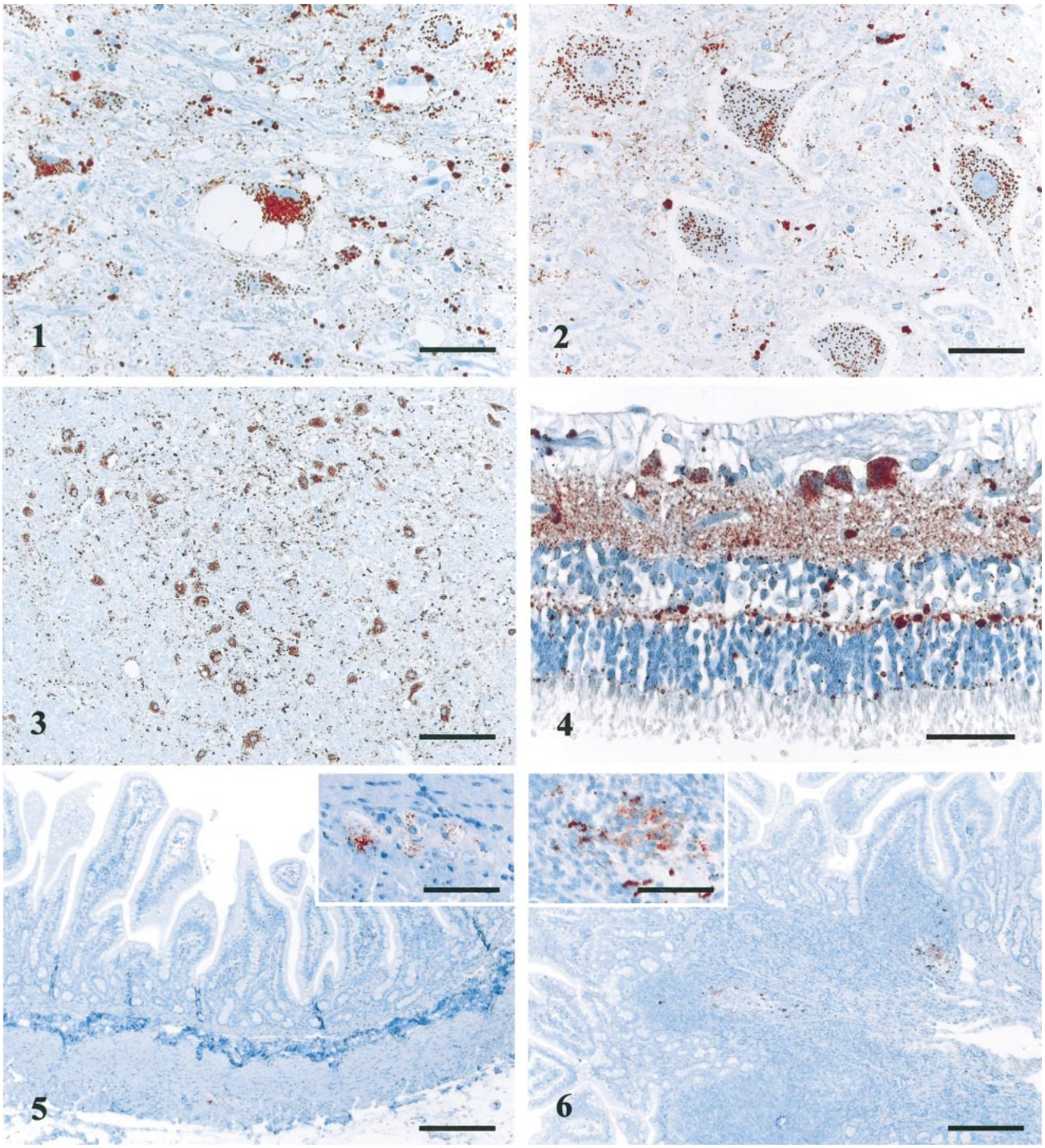

Figure 1. Spinal cord, Angora goat. PrP^{Sc} accumulation in thoracic spinal cord gray matter at the level of the fourth thoracic vertebra. Neuropil vacuolation and intraneuronal vacuoles with neuropil immunoreactivity (red granular/globular foci) surrounding microglia and at the periphery of intraneuronal cytoplasmic vacuoles. Biotin–streptavidin–horseradish peroxidase counterstained with Mayer hematoxylin. Bar = 50 μm.

Figure 2. Spinal cord, Angora goat. PrP^{Sc} accumulation in spinal cord gray matter at the level of the fourth thoracic vertebra. Neuropil immunoreactivity (red granular/globular foci) surrounding microglia and within the cytoplasm of motor neurons. Biotin–streptavidin–horseradish peroxidase counterstained with Mayer hematoxylin. Bar = 50 μm.

Figure 3. Brainstem, Angora goat. PrP^{Sc} accumulation within the nucleus ambiguus. Cytoplasmic immunoreactivity (red granular/globular foci) associated with vacuolated and nonvacuolated neurons. Biotin–streptavidin–horseradish peroxidase counterstained with Mayer hematoxylin. Bar = 200 μm.

similar concentration of an irrelevant control mAb of the same isotype and 2) incubation of mAb F99/97.6.1 with representative tissues from goats with no evidence of scrapie as indicated by histopathologic examination of brain. Positive controls consisted of incubation of mAb F99/97.6.1 with lymph node tissue from a sheep that was confirmed scrapie-positive by Western immunoblotting of Proteinase K–digested preparations from the brain.[13] To assess PrP^{Sc} deposition in Proteinase K–treated tissue sections, replicate tissue sections also were pretreated with Proteinase K before application of the primary antibody. Representative sections of the central nervous system of the Angora goat also were incubated with a primary antibody directed against glial fibrillary acidic protein (GFAP).[b] Tissue sections (4 μm thick) were deparaffinized in xylene and rehydrated through graded alcohols. Endogenous peroxidase was blocked by incubation with 0.5% hydrogen peroxide in methanol at room temperature for 30 minutes. Sections were washed with Tris-buffered saline (0.1 M Tris–HCl, 0.8% sodium chloride, pH 7.6) and then incubated, first with 5% normal goat serum for 10 minutes and next with polyclonal rabbit anti-GFAP (1:3000) for 30 minutes at room temperature. Biotinylated goat anti-rabbit IgG was used as a link antibody for the demonstration of rabbit anti-GFAP, followed by the use of the streptavidin–biotin–peroxidase complex.[c] Negative control procedures for GFAP consisted of substitution of the primary antibody with a similar concentration of rabbit antisera immunoglobulin fraction from normal nonimmunized animals. Canine brain tissue served as a positive control.

For analysis of the PrP gene, DNA was isolated from blood, and genomic DNA was amplified by polymerase chain reaction (PCR) using primers flanking the open reading frame (PCR primer forward: GGC ATT TGA TGC TGA CAC C; PCR primer reverse: TAC AGG GCT GCA GGT AGA C).[20] PCR products were sequenced by dideoxynucleotide chain termination[d] (sequencing primer forward: CTG GGG TCA AGG TGG TAG CC; sequencing primer reverse: TGG TGG TGA CTA TGT GTT GCT TGA).

The most severe microscopic changes involved the gray matter neuropil of the brain and spinal cord. The cerebral cortex had diffuse, moderate to severe gliosis, and astrocytosis as indicated by histopathology and prominent GFAP immunoreactivity. Neuronal degeneration and necrosis and vacuolar degenerative neuronal changes along with spongiosis were widespread throughout the gray matter neuropil of the cerebral cortex and brainstem nuclei and in both dorsal and ventral horns and intermediate substance of the spinal cord. Degenerate neurons were swollen and had central chromatolysis. Necrotic neurons were shrunken and hypereosinophilic and had small, deeply basophilic ovoid nuclei. Necrotic neurons were occasionally surrounded by microglial cells (satellitosis). Numerous randomly scattered neurons contained multilocular or unilocular, clear vacuoles, which often resulted in marked cytoplasmic distension with peripheral displacement of nuclei and the residual perikaryon. There was bilaterally symmetrical involvement of the gray matter regions of the telencephalon, diencephalon (hypothalamic nucleus, mammillary nucleus, medial lemniscus, thalamic nucleus), mesencephalon (brachium of the caudal colliculus, central gray substance, medial lemniscus, motor nucleus of the trochlear nerve, red nucleus, rostral and caudal colliculus, substantia nigra), metencephalon (dorsal nucleus of the trapezoid body, medial, lateral and caudal vestibular nuclei, motor nucleus of the trigeminal nerve, nucleus of the lateral lemniscus, nucleus of the spinal tract of the trigeminal nerve, pontine nucleus), and myencephalon (dorsal motor nucleus of the vagus nerve, facial motor nucleus, hypoglossal motor nucleus, medial and lateral cuneate nuclei, nucleus ambiguus, nucleus gracilis, nucleus of the solitary tract, nucleus of the spinal tract of the trigeminal nerve, olivary nucleus, reticular formation). In the cerebellum, there was moderate vacuolation of randomly scattered Purkinje cells, Purkinje cell necrosis, and Purkinje cell drop-out. Both molecular and granular layers of the cerebellum had mild vacuolar degenerative changes. Throughout the white matter of the spinal cord there was widespread moderate to severe axonal degeneration, characterized by retraction of myelin surrounding degenerate axons, with formation of ellipsoids and digestion chambers that often contained fragments of degenerate axons admixed with microglia.

Positive immunoreactivity to mAb F99/97.6.1 was widespread throughout the central nervous system and was associated with all areas in which degenerative changes were observed on microscopic examination of HE-stained tissues. There was strong PrP^{Sc} cytoplasmic immunoreactivity of both degenerate and morphologically normal neurons scattered throughout the gray matter neuropil of the telencephalon, diencephalon, mesencephalon, metencephalon, myencephalon, and spinal cord. Vacuolated neurons consistently had strong cytoplasmic immunoreactivity, characterized by granular and globular aggregates of PrP^{Sc} around the periphery of intraneuronal vacuoles (Fig. 1). There was also fre-

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Figure 4. Eye, Angora goat. PrP^{Sc} accumulation in the retina. Immunoreactivity (red granular/globular foci) associated with rod and cone processes, outer limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, and efferent fibers. Biotin–streptavidin–horseradish peroxidase counterstained with Mayer hematoxylin. Bar = 200 μm.

Figure 5. Jejunum, Angora goat. PrP^{Sc} accumulation within a myenteric (Auerbach's) plexus. Cytoplasmic immunoreactivity (red granular/globular foci) of postganglionic neurons associated with parasympathetic ganglia. Biotin–streptavidin–horseradish peroxidase counterstained with Mayer hematoxylin. Bar = 200 μm. Inset: Higher magnification of PrP^{Sc} accumulation (red granular/globular foci) within postganglionic neurons. Bar = 50 μm.

Figure 6. Ileum, Angora goat. PrP^{Sc} accumulation within aggregated lymphoid follicles (Peyer's patches). Cytoplasmic immunoreactivity (red granular/globular foci) of individual cells within germinal centers. Biotin–streptavidin–horseradish peroxidase counterstained with Mayer hematoxylin. Bar = 200 μm. Inset: Higher magnification of PrP^{Sc} accumulation (red granular/globular foci) within a single germinal center. Bar = 50 μm.

quent and widespread positive immunoreactivity of morphologically normal neurons and of both white matter and gray matter neuropil in which no significant degenerative lesions were seen (Fig. 2). Throughout the gray matter of the cerebrum and spinal cord, moderate to strong cytoplasmic immunoreactivity was often associated with astrocytes, microglia, and oligodendrocytes. In the cerebellum there was strong and widespread cytoplasmic immunoreactivity of small neurons scattered throughout the molecular layer (stellate and basket cells), small neurons scattered throughout the granular layer (Golgi cells), and Purkinje cells. Positive immunoreactivity of essentially all nuclei located throughout the brainstem was particularly prominent and consisted of strong cytoplasmic immunoreactivity to mAb F99/97.6.1 of nearly all neurons and numerous scattered glial cells in addition to neuropil (Fig. 3). A few ovoid cells, which morphologically resembled oligodendrocytes, scattered throughout the cerebellar white matter folia and white matter of the spinal cord, as well as ependymal cells lining the central canal of the spinal cord, had weak cytoplasmic immunoreactivity.

There was prominent immunoreactivity to mAb F99/97.6.1 throughout the retina, with weak cytoplasmic staining of the rod and cone processes of the photoreceptor cells and outer and inner nuclear layers, as well as strong prominent cytoplasmic staining of the inner and outer limiting membranes, inner and outer plexiform layers, and the ganglion cell layer (Fig. 4). In the optic nerve, few scattered cells that morphologically resembled Schwann cells had weak cytoplasmic immunoreactivity. Randomly scattered individual neurons of spinal ganglia dorsal roots had moderate to strong cytoplasmic immunoreactivity. Postganglionic neurons associated with parasympathetic ganglia of myenteric (Auerbach's) and submucosal (Meissner's) plexuses had uniform moderate to strong cytoplasmic immunoreactivity (Fig. 5). Individual aggregated lymphoid follicles (Peyer's patches) throughout the mucosa of the jejunum and ileum had irregular moderate to strong cytoplasmic immunoreactivity of individual cells primarily associated with germinal centers of individual follicles (Fig. 6). Occasional scattered lymphoid follicles throughout the retropharyngeal and parotid lymph nodes as well as pharyngeal and palatine tonsils had variable numbers of scattered immunoreactive cells within germinal centers of individual follicles. Mesenteric, superficial cervical, prefemoral, submandibular, and popliteal lymph nodes also were examined but lacked positive immunoreactivity to mAb F99/97.6.1. Other tissues that were examined but that lacked positive immunoreactivity to mAb F99/97.6.1 included kidney, thyroid gland, parathyroid gland, adrenal gland, spleen, liver, gall bladder, lung, integument, lymphoid tissue of nictitating membranes (third eyelids), vagus nerve, parotid salivary gland, myocardium, diaphragm, esophagus, omasum, reticulum, abomasum, rumen, and pancreas. Tissue sections from a scrapie-affected sheep (positive control) and a control goat with no histologic evidence of scrapie (negative control) were prepared and immunostained in parallel. There was neither immunoreactivity of negative control goat tissues immunostained with mAb F99/97.6.1 nor immunoreactivity of tissue sections from a scrapie-affected sheep incubated with an isotype control mAb rather than with mAb F99/97.6.1. There was no difference in the distribution of immunoreactivity when tissue sections were pretreated with Proteinase K before application of the primary antibody or when the detection reagents were replaced with those of a different manufacturer.[e]

Sequence analysis of the full PrP open reading frame of the PrP gene and its deduced protein sequences revealed a homozygous genotype at each of the following codons: $(Ala/Ala)_{136}$, $(Leu/Leu)_{141}$, $(Ile/Ile)_{142}$, $(His/His)_{143}$, $(Arg/Arg)_{154}$, $(Glu/Glu)_{171}$, and $(Pro/Pro)_{240}$.

The microscopic changes observed in HE-stained tissues in the central nervous system are similar to those previously reported in goats with natural scrapie.[7,21] The distribution and localization of PrP^{Sc} in the central nervous system of the goat in the present report is similar to the reported distribution of PrP^{Sc} in sheep with natural scrapie.[16] PrP^{Sc} immunoreactivity did not always colocalize with neuronal vacuolation, and prominent positive PrP^{Sc} immunoreactivity was often observed in neurons without evidence of vacuolation, as with the pattern of PrP^{Sc} deposition reported for sheep with natural scrapie.[16] Vacuolation of neurons also was rarely apparent without PrP^{Sc} positive immunoreactivity in the central nervous system of the goat in the present report.

PrP^{Sc} deposition in the retina of goats and sheep with natural scrapie has not been previously reported, although PrP^{Sc} deposition in the retina of a single sheep experimentally infected with scrapie[8] and PrP^{Sc} deposition in a single sheep affected with bovine spongiform encephalopathy[4] following experimental transmission have been recently described. Retinopathy in sheep affected with natural scrapie also has been previously described.[2] The strong and prominent immunoreactivity to mAb F99/97.6.1 is consistent with previous reports describing immunolocalization of prion protein in scrapie-affected retinas of experimentally infected mice and hamsters.[3,9] In experimentally infected hamsters the accumulation of prion protein in the eye has been shown to precede the development of progressive retinal degeneration characterized by destruction and loss of photoreceptor cells and thinning of the inner and outer nuclear and plexiform layers.[10] PrP^{Sc} is reportedly readily detectable in the retina of humans with variant Creutzfeldt-Jakob disease by using a highly sensitive immunoblot method.[19] Although PrP^{Sc} deposition was prominent in the retina of the Angora goat in the present report, there were no significant microscopic retinal lesions.

The detection and localization of PrP^{Sc} deposition within postganglionic neurons associated with parasympathetic ganglia throughout the gastrointestinal tract is similar to reported observations from previous studies in which PrP^{Sc} was found to accumulate in both the myenteric (Auerbach's) and submucosal (Meissner's) plexuses in sheep with natural scrapie, with no apparent morphological alterations of the enteric neurons.[1,18] The accumulation of PrP^{Sc} in Peyer's patches also is similar to that reported for sheep with natural scrapie.[1] PrP^{Sc} deposition was detected in lymphoid follicles of the retropharyngeal lymph node and palatine tonsil of this goat, similar to reported prion protein deposition in lymphoid tissues of sheep with natural scrapie.[17] PrP^{Sc} deposition in the goat of the present report was not detected in the spleen or mesenteric lymph nodes, in contrast to PrP^{Sc} deposition in

some sheep naturally infected with scrapie.[17] PrP^{Sc} deposition also was not detected within germinal centers of nictitating membranes in the goat of this report, in contrast to PrP^{Sc} deposition in third eyelids of sheep with natural scrapie.[12,14] Because PrP^{Sc} deposition was detected in many other tissues of this goat, however, the lack of positive immunoreactivity in mesenteric, superficial cervical, prefemoral, submandibular, and popliteal lymph nodes and in lymphoid tissue of the third eyelid may have been caused by the lack of uniformly distributed prion protein.

Age at disease onset and rate of progression of scrapie are modulated by the host genome, in particular by the PrP gene and its allelic forms.[5,6] The Angora goat of this report was homozygous for caprine PrP alleles[22] encoding 5 octapeptide repeat sequences in the N-terminal region of the prion protein and isoleucine at codon 142, a genotype associated with high susceptibility and short incubations times in goats.[5,6]

In this brief communication, widespread immunoreactivity to mAb F99/97.6.1 and deposition of PrP^{Sc} in caprine tissues from a goat with natural scrapie has been demonstrated. PrP^{Sc} is a marker protein for transmissible spongiform encephalopathies, and detection of PrP^{Sc} by immunohistochemical analysis is a useful adjunct to histopathology for the diagnosis of naturally occurring disease in sheep, cattle, mule deer, and elk.[13] The results presented here validate and demonstrate the utility of mAb F99/97.6.1 as a specific and reliable immunohistochemical adjunct for detection and distribution of prion protein in formalin-fixed caprine tissues, although precise determination of the diagnostic sensitivity and specificity of the assay as a diagnostic test for scrapie in goats will require examination of a sufficiently large sample size. Immunohistochemistry also may be particularly useful early in the course of disease when morphological changes associated with scrapie is subtle or when there is tissue autolysis of a magnitude sufficient to impede a definitive histologic diagnosis of spongiform encephalopathy.

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Sources and manufacturers

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b. Dako, Carpinteria, CA.
c. Signet Pathology Systems, Inc., Dedham, MA.
d. Molecular Genetics Instrumentation Facility, University of Georgia, Athens, GA.
e. IHC/TSE99, VMRD, Pullman, WA.

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