Immunohistochemical detection of prion protein in sheep with scrapie


Abstract. Prion protein (PrP), which is involved in the pathogenesis of scrapie, occurs in 2 forms. The form extracted from scrapie brain is protease resistant (PrP-res), whereas PrP from normal brain is protease sensitive (PrP-sen). This study examined whether PrP-res could be detected in brains of sheep with scrapie by immunohistochemistry (IHC). A suitable IHC procedure was developed using brain tissue from hamsters that had been inoculated with the transmissible mink encephalopathy agent. Tissue samples were fixed in PLP (periodate, lysine, paraformaldehyde) that contained paraformaldehyde at a concentration of 0.125%. Before application of the IHC technique, tissue sections were deparaffinized and treated with formic acid to simultaneously enhance PrP-res immunoreactivity and degrade PrP-sen. Primary antibody was obtained from a rabbit immunized to PrP-res extracted from brains of mice with experimentally induced scrapie. Brains from 21 sheep with histopathologically confirmed scrapie were examined by IHC. In all 21 brains, PrP-res was widely distributed throughout the brain stem. Staining was particularly intense in neuronal cell bodies and around blood vessels. The IHC technique successfully detected PrP-res in brain samples that had been frozen or that were severely autolyzed before fixation in PLP. Brains from 11 scrapie-suspect sheep that were not considered histologically positive were also examined by IHC. PrP-res was found in 4 of these brains. Sections of brains from 14 clinically normal sheep did not have detectable PrP-res. Results of this study indicate that IHC detection of PrP-res is equivalent, and perhaps superior, to histopathology for the diagnosis of scrapie in sheep. Furthermore, IHC is applicable to tissues that have autolytic changes or processing artifacts that prevent satisfactory histopathologic evaluation for lesions of scrapie.

Scrapie is a natural disease of sheep and goats that was recognized in Europe at least 200 years ago, although the disease was not diagnosed in the United States until 1947. Similar diseases have been described in many other species, including mink, deer, elk, cattle, cats, zoo ruminants, and humans. The primary tissue affected is the central nervous system, resulting in a variety of clinical neurologic signs. The most widely accepted histologic lesion is vacuolation in neuronal cell bodies and gray matter neuropil, leading to the classification of these diseases as spongiform encephalopathies.

The etiology of spongiform encephalopathies is complex. Some forms of the disease in humans appear to be heritable, but others occur sporadically. Most animal and human spongiform encephalopathies (whether heritable or sporadic) are experimentally transmissible to laboratory animals. Scrapie, in addition to being experimentally transmissible, also spreads naturally among sheep and goats, although the exact modes of transmission have not been determined.

The nature of the transmissible agent that causes spongiform encephalopathies is a matter of considerable debate. At present, there is no evidence that a nucleic acid is involved. An altered form of a cellular protein termed prion protein (PrP) has been proposed as the primary initiating factor. The gene that encodes PrP is present in normal and in spongiform encephalopathy-affected brain. However, the protein in diseased brain undergoes a posttranslational, probably conformational, alteration that causes it to be resistant to protease digestion. This abnormal, protease-resistant isoform of PrP is designated PrP-res, whereas the isoform from normal brain, which is protease sensitive, is termed PrP-sen.
Diagnosis of scrapie in sheep and goats depends on the observation of typical clinical neurologic signs and the finding of characteristic histologic lesions. Unfortunately, the intensity of clinical signs does not always correlate with the severity of microscopic lesions. Unless tissue changes are well developed and completely typical of scrapie, the histologic diagnosis may be equivocal, even if the examination is conducted by a person with considerable experience. Furthermore, it has been recognized for many years that sheep with classical clinical signs of scrapie do not always have diagnostic histologic lesions. Therefore, the accuracy of scrapie diagnosis should be improved by use of complementary techniques to detect the scrapie-associated protein, PrP-res.

Several techniques have been developed to detect PrP-res in tissues, including western blot or other immunoblot methods. These procedures rely primarily on the elimination of PrP-sen by incorporation of a protease digestion step in the PrP extraction protocol. Another approach that has proven useful is the in situ detection of PrP by immunohistochemistry (IHC) \( ^{4,5,9,20,24,33} \). In most IHC procedures, the tissue sections are treated with formic acid, rather than protease, to destroy PrP-sen because formic acid also enhances the immunoreactivity of PrP-res. The present paper describes some modifications to previously published methods for IHC detection of PrP-res and demonstrates the applicability of this technique to scrapie diagnosis in sheep. Because sheep with scrapie were not always readily available, we used brain tissue from hamsters with experimentally induced transmissible mink encephalopathy to develop a suitable IHC procedure.

**Materials and methods**

*Hamster tissues.* Hamsters inoculated with the HYPER strain of hamster-passaged transmissible mink encephalopathy were euthanized in terminal stages of the disease, approximately 75 days after inoculation. Brains from noninoculated hamsters were collected for negative control tissue.

To test the effect of tissue fixative, hamster brains were collected in 10% buffered formalin or in McLean’s PLP fixative \( ^{21} \) (periodate, lysine, paraformaldehyde) with either 0.125% or 0.5% paraformaldehyde. Several parameters related to the use of PLP with 0.125% paraformaldehyde were examined. Shelf life of the fixative was examined by fixing half of a brain in freshly prepared PLP and the other half in PLP that had been stored at 4 C for 4 mo. To test the effect of prolonged fixation, half of a brain was held in PLP for 1 wk after collection, whereas the other half was held for 2 mo before being processed. The effect of tissue autolysis was examined by fixing half of a brain immediately after death and holding the other half of the brain at 4 C for 96 hr prior to fixation.

*Sheep tissues.* Brain samples from 33 scrapie-suspect sheep were provided either by pathologists in veterinary medical colleges and state diagnostic laboratories or by personnel from the USDA Animal and Plant Health Inspection Service. One dead sheep was held at 21 C for 48 hr before removal of the brain, but all other sheep were necropsied within 24 hr after death. One half of each brain was fixed immediately after necropsy in 10% buffered formalin for histopathologic examination. Criteria used for the diagnosis of scrapie included intracytoplasmic vacuolation of neurons, neuronal degeneration (pyknosis and shrinkage), vacuolation of gray matter neuropil, and astrocytosis.

The brain samples collected for IHC were handled in several different ways. Tissues from 8 of the 33 sheep were fixed in PLP (0.125% paraformaldehyde) immediately after necropsy. The other 25 samples were either frozen (17) or refrigerated for up to 24 hr (8) before fixation. Frozen specimens usually were placed in PLP without thawing; but in 6 cases, the tissues were thawed before fixation as an adjacent sample could be tested for PrP-res by the western blot assay. Most submissions included the entire brain stem, and multiple areas were examined: obex, midmedulla, pons, mesencephalon, and thalamus. In a few cases when only a portion of the brain stem was submitted, precise anatomic identification of the areas examined was not possible.

Control brains were obtained from 14 clinically normal sheep, 3 of which were in a flock with no known history of scrapie. Fixation procedures were identical to those used for scrapie suspects. In 3 sheep, one half of the brain was frozen before fixation in PLP.

**Immunohistochemistry.** Tissues were held in PLP for 7-14 days, trimmed into cassettes, and then held in 70% ethyl alcohol for 3-5 days before submission to the histopathology laboratory. Tissue processing was continued using standard procedures for further dehydration and paraffin embedding. Paraffin sections were mounted on positively charged glass slides and heated at 80 C for 10 min immediately prior to immunostaining. The staining procedures were carried out with an automated capillary action system. Pairs of slides were positioned with the tissue sections in apposition so that reagents could be applied to both slides simultaneously. Detergents were added to aqueous reagents to maximize the rate of capillary flow.

The clearing used for deparaffinization was prepared by mixing xylene with a dewaxing agent at a ratio of 1:3. \( ^{35} \) The first application of clearing was for 1 min at room temperature and was followed by 2 successive 5-min applications at 45 C. Slides were then immersed twice in clearing agent mixed 1:1 with 100% ethyl alcohol, followed by 3 treatments with alcohol alone. The slide holder then was moved from the automatic stainer to a chemical fume hood for a 30-min exposure to 99% formic acid. After the formic acid was removed, slides were returned to the automatic stainer and rinsed 8 times in distilled water that contained 0.25% Brij 35 and 0.1% Tween 20. Each water rinse was for 1 min. Slides were then rinsed 8 times in 95% alcohol, followed by a rinse in Automation buffer. The next step was a 20-min application of blocking solution (0.45-μm-filtered normal goat serum diluted 1:5 in Automation buffer) at room temper-
ature, followed by application of the primary antibody diluted 1:200 in blocking solution.

Two rabbit antisera were tested. One antibody was generated by injecting a rabbit with antigen derived from sodium dodecyl sulfate-polyacrylamide gel electrophoresis-purified PrP-res from brains of mice inoculated with the ME7 strain of scrapie. The other antibody (R27) was to a synthetic peptide corresponding to residues 89-103 of the complete mouse PrP amino acid sequence. Slides were incubated in primary antibody overnight at 4°C, rinsed 3 times in Automation buffer, then held in the buffer at 37°C for 30 min. Biotinylated anti-rabbit IgG, diluted 1:200 in blocking solution, was applied at 37°C for 30 min. After a rinse in Automation buffer, slides were exposed for 20 min at room temperature to avidin-alkaline phosphatase complex, followed by 3 rinses in 0.1 M Tris-HCl buffer, pH 8.2, with 0.25% Tween 20. This same buffer was used to prepare the substrate: with addition of levamisole. The substrate was centrifuged at 1,000 × g for 5 min, then applied at room temperature in 3 cycles of 5, 5, and 10 min, without intervening washes. After 2 rinses in distilled water, slides were stained for 4 min in Mayer’s hematoxylin with 0.25% Brij 35 and 0.1% Tween 20, followed by 2 water rinses, a rinse in Automation buffer to “blue” the hematoxylin, and 2 more water rinses. Tissues were then dehydrated through the same alcohol and clearant solutions used for hydration. A xylene-compatible permanent mounting medium was used for application of coverslips.

Western blot assay. The method used for detection of PrP-res has been described previously.

Results

Hamster tissues. The first parameter we examined in developing an IHC protocol was primary antibody. Brains from clinically affected hamsters were fixed in formalin or PLP (0.5% paraformaldehyde) and tested with varying dilutions of the rabbit antisera. Staining was seen with both antisera at dilutions up to 1:200. Most of the stained material appeared as fine granules that were diffusely distributed throughout gray matter neuropil. The amount of positively stained material was greater in PLP-fixed than in formalin-fixed brains. Neither antiserum stained negative control brains from noninoculated hamsters.

The effect of paraformaldehyde concentration in PLP was examined using both of the primary antibodies at a 1:200 dilution. The amount of PrP-res in brain fixed with 0.125% paraformaldehyde (Fig. 1) was much greater than that in brain fixed in a 0.5% concentration (Fig. 2). There was a concomitant increase of background staining at the lower paraformaldehyde concentration, and tissue morphology was not as well preserved. Nevertheless, because of the markedly superior retention of PrP-res reactivity, PLP with 0.125% paraformaldehyde was chosen as the fixative for all subsequent IHC tests.

The hamster brain that was refrigerated for 96 hours before fixation in PLP had as much PrP-res as did the brain that was fixed immediately. Similarly, PrP-res reactivity was not reduced in brain fixed in 4-month-old PLP or in brain held in PLP for 2 months prior to embedding in paraffin.

Sheep tissues. Brains from 3 sheep with histopathologically confirmed scrapie and 3 sheep in a scrapie-free flock were tested with both of the rabbit PrP antisera. The antibodies reacted positively with all 3 scrapie brains, causing especially dense staining in neuronal cell bodies (Fig. 3). The anti-peptide antibody also reacted with some structures in normal sheep brain (especially glial cell nuclei), although there was no staining in neurons. Because the antibody that was prepared to gel-purified PrP-res did not stain brains of normal sheep (Fig. 4), it was selected for the examination of all sheep brains reported in this study.

Twenty-one sheep were diagnosed as scrapie positive, based on histologic evaluation of formalin-fixed brain. The IHC test detected PrP-res in all 21 scrapie-positive sheep, a result that was confirmed by western blot assay in the 6 sheep that were examined by both methods. Although the amount of positively stained...
tissue varied from one sheep to another, PrP-res was generally abundant and was present in all parts of the brain stem. The most consistent staining pattern was seen in neuronal cell bodies, where PrP-res appeared as large, brightly stained granules (Fig. 3). Sometimes the granules could be seen extending into a neuronal process. In sections of frozen specimens, it was sometimes difficult to locate neurons that had the characteristic intracytoplasmic stained granules (Fig. 5), but neurons frequently had a ring of stained material around the plasma membrane. All histologically positive brains had small discrete granules of PrP-res in the gray matter neuropil. The amount of neuropil staining differed greatly between sheep and within a sheep from one area of the brain stem to another. In some cases there were large aggregates of stained material that appeared as plaques (Fig. 6). Several brains also had intensely stained accumulations of PrP-res around blood vessels (Fig. 7).

Brains from 11 clinically suspect sheep did not meet the criteria necessary for a histopathologic diagnosis of scrapie. In the IHC test, 4 of these sheep were positive for PrP-res. The positive staining in 1 sheep was restricted to the obex (Fig. 8), even though all parts of the brain stem were examined.

Because the scrapie-suspect brain that was collected 2 days after death had severe autolytic changes, a histopathologic diagnosis was not possible. However, PrP-res was readily detected by IHC in the gray matter neuropil, and some tissue sections had clusters of recognizable neurons with the characteristic granular intracytoplasmic staining pattern. The presence of PrP-res in this autolyzed brain was confirmed by western blot assay (J. Katz, personal communication).

**Discussion**

Probably the most important modification we made to previously published methods for IHC detection of PrP-res was a reduction of the paraformaldehyde concentration in PLP to 0.125%. Although the superiority of PLP over formalin for preservation of PrP had been demonstrated previously, the paraformaldehyde concentration used in that work was 0.5%. The marked improvement in amount of PrP-res detected with our fixative, as compared with either formalin or PLP with 0.5% paraformaldehyde, reflects the propensity of aldehyde-based fixatives to cross-link proteins and “mask” important antigenic epitopes in IHC reactions. This problem is of special concern when tissues are stored in fixative for long periods of time. The immunoreactivity of PrP-res was preserved in PLP with 0.125% paraformaldehyde, even after prolonged fixation of the tissue. Also, the fixative has a reasonably long shelf life. These are desirable characteristics for an IHC fixative that would be used to collect brains under field conditions. Unfortunately, because our PLP formulation does not preserve tissue morphology as well as formalin, brain samples collected for IHC cannot also be used for histopathologic diagnosis of scrapie. Investigation of other fixative formulations might lead to a method that would allow IHC and histopathologic examinations for scrapie to be made on the same specimen.

The IHC technique has been used by many investigators to study PrP in laboratory animal models of scrapie, but this is only the second report describing use of the method on sheep tissues. In the earlier study, antisera to mouse and hamster PrP were used to stain formalin-fixed brains from 2 sheep with natural scrapie and 2 sheep and a goat with experimental scrapie. Specific staining was seen only as plaques and perivascular deposits. Although we confirmed these observations, our work also demonstrated the presence of PrP-res in gray matter neuropil and neuronal cell bodies. We detected more PrP-res probably because we used the low-paraformaldehyde PLP fixative rather than formalin. We have applied our IHC method to formalin-fixed brains from 3 scrapie cases and found little or no PrP-res instead of the amount routinely observed in PLP-fixed brains.

Results of our study support the use of IHC to detect PrP-res in sheep with natural scrapie. Furthermore, for the diagnosis of scrapie in sheep, the IHC method we have developed is equivalent to histologic evaluation (using criteria established by National Veterinary Services Laboratories personnel). The IHC demonstration of PrP-res in sheep that are clinically and histologically diagnosed with scrapie confirms results of studies on other sheep that compared histopathology and the western blot technique.

The IHC detection of PrP-res in 4 of 11 sheep that were not confirmed with scrapie by histopathology suggests that the method may be capable of identifying sheep with preclinical scrapie or sheep that develop clinical signs without concurrent development of characteristic histologic lesions. Both of these possibilities have been suggested by results of other studies that used the western blot method for detection of PrP-res. In these reports, PrP-res was detected in 2 experimentally inoculated sheep before clinical signs or lesions had developed, in 3 sheep that had clinical signs of scrapie with minimal or no histologic lesions, and in 9 sheep that did not meet all of the histopathologic criteria necessary for a positive diagnosis of scrapie.

One limitation to the use of histology for scrapie diagnosis is that an accurate interpretation of lesions may be difficult or impossible unless the brain is fixed in formalin shortly after death. Freezing of formalin-fixed tissue almost invariably renders the specimen useless for scrapie histopathology. In this study, we
were able to detect PrP-res by IHC in a brain that was severely autolyzed and in brains that were frozen prior to PLP fixation. Autolyzed or frozen brain samples also can be tested with the western blot assay.\(^{18,28}\) The availability of methods that can be used on less than optimally collected and preserved tissues will increase the number of questionable scrapie cases that can be confidently diagnosed as positive. The choice of which method(s) to use will depend on many factors, including relative cost, availability of reagents, and technical abilities of personnel.

One of the most important requirements for a successful IHC test is an appropriate primary antibody. With regard to scrapie, western blot studies have shown that antisera to sheep and mouse PrP give different reactivities when tested with ruminant PrP-res. One report described differences in the staining intensity of some bands and also noted that an anti-sheep serum detected a band not recognized by anti-mouse serum.\(^{10}\) Another study concluded that the most reactive epitopes in sheep PrP-res were not the same as those identified in the mouse protein? These observations suggest that antibodies to sheep PrP-res might be better suited for IHC than antibodies to mouse PrP-res, and development of anti-sheep PrP is currently in progress.

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Sources and manufacturers

a. ProbeOn\textsuperscript{TM} Plus, Fisher Scientific, Pittsburgh, PA.
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   d. Sigma Chemical Co., St. Louis, MO.
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