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Immunohistochemical Detection of Prion Protein in Lymphoid Tissues of Sheep with Natural Scrapie

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The scrapie-associated form of the prion protein (PrP^{Sc}) accumulates in the brain and lymphoid tissues of sheep with scrapie. In order to assess whether detecting PrP^{Sc} in lymphoid tissue could be used as a diagnostic test for scrapie, we studied the localization and distribution of PrP^{Sc} in various lymphoid tissues collected at necropsy from 55 sheep with clinical scrapie. Samples collected from the spleen, palatine tonsil, ileum, and five different lymph nodes were immunohistochemically stained for PrP^{Sc}. PrP^{Sc} was found to be deposited in a reticular pattern in the center of both primary and secondary lymphoid follicles. In addition, granules of PrP^{Sc} were seen in the cytoplasm in macrophages associated with the lymphoid follicles. In 54 (98%) of the 55 scrapie-affected sheep, PrP^{Sc} was detected in the spleen, retropharyngeal lymph node, mesenteric lymph node, and the palatine tonsil. However, only in the palatine tonsils was PrP^{Sc} present in a consistently high percentage of the lymphoid follicles. PrP was not detected in any of the lymphoid tissues of 12 sheep that had no neurohistopathological signs of a scrapie infection. We conclude that the tonsils are the best-suited lymphoid tissue to be biopsied for the detection of PrP^{Sc} in the diagnosis of clinical scrapie in living sheep.

A converted form of the normal cellular prion protein (PrP) accumulates in the brains of sheep with natural scrapie. This so-called scrapie-associated prion protein (PrP^{Sc}) is identical in its primary structure to the cellular prion protein (PrP^{C}) which is normally present in sheep (1). PrP^{Sc} differs, however, from PrP^{C} because it is partially resistant to proteolysis and strongly associated with scrapie infectivity (3, 14, 25).

The detection of PrP^{Sc} in the brains of scrapie-affected sheep has become an important tool in the confirmation of scrapie diagnosis. Western blot (immunoblot) analysis and demonstration of PrP^{Sc} aggregates (scrapie-associated fibrils) by electron microscopy can be used to detect PrP^{Sc} in extracts of scrapie-affected brains (21, 23). If only fixed brain tissue is available, PrP^{Sc} can be detected by immunohistochemical staining, provided that tissue sections are adequately pretreated (16, 26). The major drawback of PrP^{Sc} detection in brain tissue, however, is that it can be done only at the postmortem examination of the animal.

Recently, Western blot analysis has shown that PrP^{Sc} also accumulates in lymphoid tissues of sheep in the preclinical and clinical stage of scrapie infection (10, 17, 18, 21). This means that PrP^{Sc} could be detected in biopsies of lymphoid tissues, which would give a diagnostic test for scrapie in the living sheep. However, until now it has not been known where and to what extent PrP^{Sc} is distributed in the various lymphoid tissues and, thus, which lymphoid tissue would be best suited for biopsy.

In this study, we used PrP^{Sc} immunohistochemistry to determine the distribution and localization of PrP^{Sc} in lymphoid tissues collected at necropsy from sheep clinically affected with scrapie. The results of this study provide the necessary knowledge to evaluate the feasibility of PrP^{Sc} detection in tissue biopsies as an antemortem test for scrapie.

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MATERIALS AND METHODS

Sheep. Sixty-seven sheep with nervous disorders resembling those of a scrapie infection were purchased. Fifty-five sheep were diagnosed with scrapie by histopathological and immunohistochemical examination of the brain (26). One animal suffered from both a scrapie infection and a concurrent meningoencephalitis probably caused by *Listeria monocytogenes*. Scrapie-positive sheep originated from 30 different flocks. The group consisted of 54 females and 1 male ranging in age from 2 to 5 years and comprised eight different breeds and crossbreds. Twelve sheep did not show any histopathological signs of a scrapie infection, nor did they display any PrP^{Sc} immunostaining in the brain. Five of these sheep were diagnosed with meningoencephalitis, one had intramyelinic edema of unknown cause, and six sheep showed no histopathological abnormalities. Scrapie-negative sheep were all females from 10 different flocks and two different breeds and crossbreds, ranging in age from 1 to 5 years.

Necropsy. Necropsy was performed within 36 h after natural death or immediately after killing the animal by intravenous injection of sodium pentobarbital and exsanguination. The brain was removed from each sheep for scrapie diagnosis as described previously (26). Samples were taken from several lymphoid tissues including spleen, palatine tonsil, superficial cervical lymph node (prescapular lymph node), subiliac lymph node (prefemoral lymph node), medial retropharyngeal lymph node, tracheobronchial lymph node, mesenteric lymph node, and ileum.

Histological and immunohistochemical procedures. Tissue samples were immediately immersed for 24 h in periodate-lysine-paraformaldehyde fixative (PLP) containing 2% paraformaldehyde (Merck, Darmstadt, Germany) (15). Samples were then trimmed to a maximum thickness of 2 mm and fixed for another 24 h in freshly prepared PLP. After fixation, tissue samples were washed in water, routinely dehydrated, and embedded in paraffin. Three sections of 5 μm were cut, mounted on 3-aminoalkyltriethoxysilane-coated glass slides (Sigma, St. Louis, Mo.), dried for at least 48 h at 60°C, and deparaffinized. The first section was stained with hematoxylin-eosin. Second and third sections were immunostained with antipeptide serum directed against the ovine prion protein and preimmune serum, respectively, according to the following procedure. After 30-min immersion in 98% formic acid (Merck), sections were washed and autoclaved (immersed in water) for another 30 min at 121°C in a pressure cooker. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol (Merck). Incubation at room temperature for 1 h with antipeptide antiserum or preimmune serum, diluted 1:1,500 in phosphate-buffered saline (pH 7.2) containing 1% bovine serum albumin (Sigma), was followed by incubation, first with biotin-conjugated goat anti-rabbit immunoglobulin G and then with streptavidin-peroxidase for 10 and 5 min, respectively (Dakopatts, Glostrup, Denmark). As substrate we used aminoethylcarbazole (Zymed Laboratories Inc., San Francisco, Calif.) because its red color could easily be differentiated from the yellow-brownish ceroid/lipofuscin and hemosiderin pigment which was often present in the lymphoid tissues. Between the various steps, sections were thoroughly rinsed in phosphate-buffered saline containing 0.05% Tween 20 (Merck). Sections were counterstained with Mayer's hematoxylin for 30 s and mounted in Glycergel

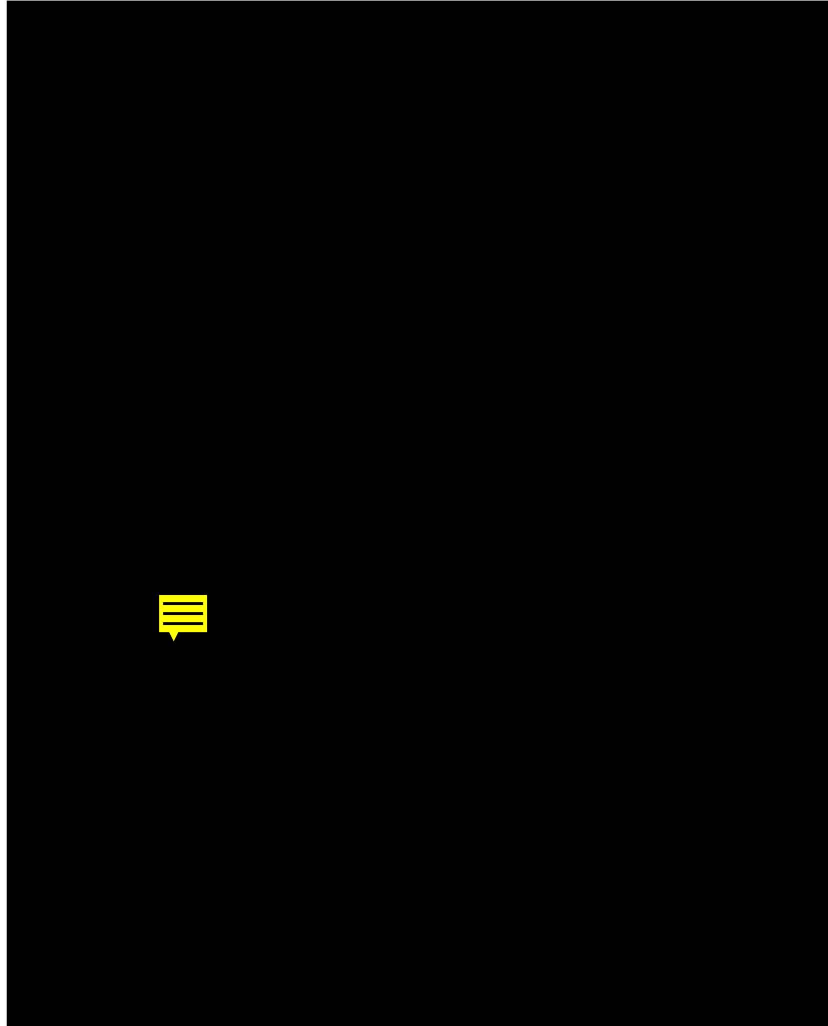

FIG. 1. Accumulation of PrP^{Sc} in the lymphoid follicles of the spleen (A), lymph node (B), and tonsil (C and D). Note the reticular immunolabelling pattern in the center of the lymphoid follicles. Macrophages associated with the lymphoid follicles contain granular deposits of PrP^{Sc} sometimes combined with ceroid/lipofuscin pigment (arrow). Peroxidase-labelled streptavidin-biotin and Mayer's hematoxylin counterstain were used. Bar, 50 μm.

(Dakopatts). With every immunohistochemical staining, a section of the medulla oblongata of a confirmed scrapie-affected sheep was simultaneously stained for PrP to check correct immunostaining procedures.

Peptide synthesis and antipeptide antisera. Five peptides with sequences derived from the ovine prion protein (PrP 94-105, 100-111, 126-143, 145-177, and 223-234) were synthesized and used to raise antipeptide antisera (R521, R505, R568, R532, and R524, respectively) in rabbits by previously published procedures (26). Antisera were confirmed to be specific for PrP (both undigested and after proteinase K treatment) on Western blots of partially purified prion protein from scrapie-affected sheep brain according to established procedures (9). Preimmune sera were collected before immunization and served as negative-control sera.

RESULTS

Immunohistochemical testing of antipeptide antisera. An identical and distinct immunolabelling pattern was detected with all antipeptide antisera in the lymphoid tissues of scrapie-affected sheep. Because the five antisera were directed against different epitopes of the PrP protein, cross-reactivity of the antipeptide antisera with another protein can be excluded. We therefore classified the immunolabelled protein as PrP. We further defined this PrP as scrapie-associated PrP (PrP^{Sc}), because no PrP immunoreactivity was seen in any of the lymphoid tissues of scrapie-negative sheep. Replacing the antipeptide antisera with preimmune sera did not result in any immunolabelling.

Localization of PrP^{Sc} in the lymphoid tissues. PrP^{Sc} was located within the primary and secondary lymphoid follicles of the spleen, palatine tonsil, lymph nodes, and solitary follicles or Peyer's patches of the ileum (Fig. 1A to C). The PrP^{Sc} immunolabelling pattern consisted of a reticular network in the center of the lymphoid follicle which varied in staining intensity. Apart from this network, fine to coarse granules of PrP^{Sc} were seen in the cytoplasm of nonlymphoid cells within the follicle. Several of these cells were identified as macrophages because of the simultaneous presence of ceroid/lipofuscin pigment in their cytoplasm (Fig. 1D). No immunolabelling of the B lymphocytes in the lymphoid follicle was seen.

Occasionally, additional immunolabelling was found in specific cells and regions of the lymphoid tissues. In the spleen, individual cells in the periarterial lymphatic sheath and the marginal zone surrounding the splenic corpuscles contained granules of PrP^{Sc} sometimes combined with ceroid/lipofuscin pigment within the cytoplasm. No PrP^{Sc} was seen in the red

TABLE 1. Immunohistochemical detection of PrP^{Sc} in lymphoid tissues of scrapie-affected sheep

Tissue (no. of sheep[a])	% Sheep with PrP^{Sc}					
	In the tissue	In the following percentage of lymphoid follicles:				
		<20	20–40	40–60	60–80	>80
Spleen (53)	98	30	15	23	19	11
Palatine tonsil (55)	98	0	0	0	5	93
Lymph nodes						
Prescapular (51)	88	27	24	14	15	8
Prefemoral (52)	87	54	17	12	4	0
Retropharyngeal (55)	98	30	24	24	11	9
Tracheobroncheal (54)	93	46	17	17	6	7
Mesenteric (55)	98	24	22	31	12	9

[a] The number of sheep does not always equal 55 because some tissue sections repeatedly got detached from the glass slides after pretreatment.

pulp of the spleen. In the palatine tonsil and ileum, branches or granules of PrP^{Sc} were found interspersed between the lymphocytes of the dome area between the follicles and the crypt epithelium. In the lymph nodes, granules of PrP^{Sc} were seen between the lymphocytes of the paracortex.

Distribution of PrP^{Sc} in lymphoid tissues. PrP^{Sc} was detected in 54 (98%) of the 55 scrapie-affected sheep in the spleen, tonsil, retropharyngeal lymph node, and mesenteric lymph node. In the tracheobronchial, prefemoral, and prescapular lymph node, PrP^{Sc} was seen in a slightly lower percentage of the sheep (Table 1). PrP^{Sc} was found in solitary lymphoid follicles or Peyer's patches of the ileum in 24 (89%) of the 27 sheep in which lymphoid tissue was present in the sections of the ileum. In only 1 of the 55 scrapie-affected sheep, was PrP^{Sc} not detected in any of the lymphoid tissues.

The percentage of lymphoid follicles that contained PrP^{Sc} was estimated for the sections of the spleen, tonsil, and lymph nodes (Table 1). In the palatine tonsil of 98% of the scrapie-affected sheep, over 60% of the lymphoid follicles contained PrP^{Sc}. In the tonsils of 93% of the sheep with scrapie, the percentage of PrP^{Sc}-positive lymphoid follicles even exceeded 80%. In the spleen or lymph nodes, PrP^{Sc} accumulation in more than 60% of the lymphoid follicles was present only in less than 30% of the sheep.

DISCUSSION

Previous studies have shown the close association of PrP^{Sc} and scrapie infectivity in experimental and natural scrapie (3, 14, 25). In mice infected with scrapie, high infectivity titers have been measured outside the central nervous system in the spleen, peripheral lymph nodes, and thymus during the clinical and preclinical stages of infection (5–7). In these tissues, PrP^{Sc} has also been detected by Western blot analysis (4, 20, 22, 24). Mcbride et al. were the first to describe the immunohistochemical localization of PrP in nonneural tissues of mice infected with scrapie or bovine spongiform encephalopathy. Their study showed that PrP was present on follicular dendritic cells (FDCs) of the lymphoid follicles in the spleen, lymph nodes, and Peyer's patches in both scrapie or bovine spongiform encephalopathy-infected mice and uninfected mice (13). In mice infected with Creutzfeldt-Jakob disease, infection-specific prion protein (PrP^{CJD}) has been detected by Western blot analysis in the spleen, lymph nodes, and thymus (11). On immunohistochemical examination, PrP^{CJD} was seen in cells suggested to be FDCs in the white pulp of the spleen and in the germinal centers of the lymph nodes and Peyer's patches. Very weak or no PrP immunostaining was seen in tissues of uninfected mice (12, 19).

In sheep with natural scrapie, infectivity in nonneural tissues is known to be present in the spleen, lymph nodes, tonsil, and ileum during the preclinical and clinical stages of the disease (8). In experimentally and naturally infected scrapie sheep, PrP^{Sc} has been detected by Western blotting in the spleen and lymph nodes (10, 17, 18, 21). However, to our knowledge no previous studies have been undertaken to determine the localization and distribution of PrP^{Sc} in lymphoid tissues of sheep with natural scrapie. We showed that PrP^{Sc} accumulation in lymphoid tissues is confined largely to the lymphoid follicles in the spleen, lymph nodes, palatine tonsil, and ileum. As in murine scrapie, PrP^{Sc} formed a reticular network in the center of the lymphoid follicles which could be associated with accumulation of PrP^{Sc} on FDCs. However, because specific antibodies to ovine FDCs are not yet available, we can only presume this association on the basis of similarities between the PrP^{Sc} immunolabelling and the morphology and localization of FDCs in lymphoid follicles. In contrast to the reports on murine scrapie, we also detected granules of PrP^{Sc} in phagocytic cells of the lymphoid follicles, presumably tingible body macrophages. We did not detect PrP in any of the lymphoid tissues of the sheep without a scrapie infection.

We detected PrP^{Sc} in the spleen, palatine tonsil, retropharyngeal lymph node, and mesenteric lymph node in 54 of the 55 sheep with clinical scrapie. In only one sheep was PrP^{Sc} not detected in the lymphoid tissues. It is interesting to note that after analyzing the PrP gene configuration, this particular sheep turned out to be of a special genotype. In a study to identify the relationship between PrP gene polymorphisms and the occurrence of scrapie in sheep, Belt and coworkers (2) showed that, on the basis of the triplet sequences present at codons 136, 154, and 171 of the PrP gene, the PrP^{VRQ} and the PrP^{ARR} alleles act as antagonists in determining scrapie susceptibility. The PrP^{VRQ} and PrP^{ARP} alleles were associated with a high incidence and low incidence, respectively, of natural scrapie (2). The particular sheep in our study which showed PrP^{Sc} in the brain but not in the lymphoid tissues was the only animal in the scrapie-affected group which carried both the PrP^{VRQ} allele and the PrP^{ARR} allele. After completion of our study we identified another sheep with scrapie which showed PrP^{Sc} deposition in the brain tissue but not in the lymphoid tissues. Again, this animal turned out to be of the PrP^{VRQ}/PrP^{ARR} genotype (1a). Whether the presence of the PrP^{ARR} allele is in any way related to the absence of PrP^{Sc} in the lymphoid tissues of these sheep remains to be established.

In conclusion, PrP^{Sc} was detected immunohistochemically in the spleen, palatine tonsil, retropharyngeal lymph node, and mesenteric lymph node in 98% of the sheep with scrapie. However, in the palatine tonsil PrP^{Sc} was seen in a much higher percentage of the lymphoid follicles than in the spleen and lymph nodes. In sheep, the palatine tonsil is composed of a large number of aggregated lymphoid follicles which are readily accessible via the tonsillar sinus. We therefore conclude that the palatine tonsil is the best-suited lymphoid tissue to biopsy in developing a diagnostic test for scrapie that is based on the detection of PrP^{Sc}.

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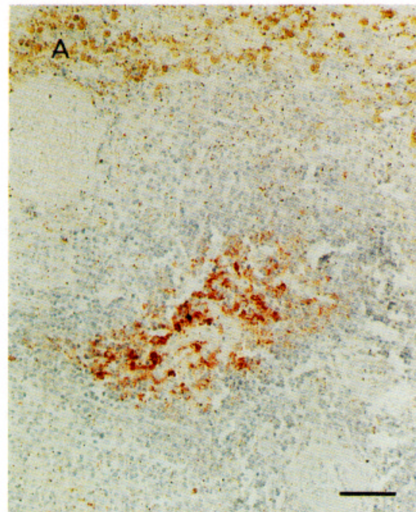
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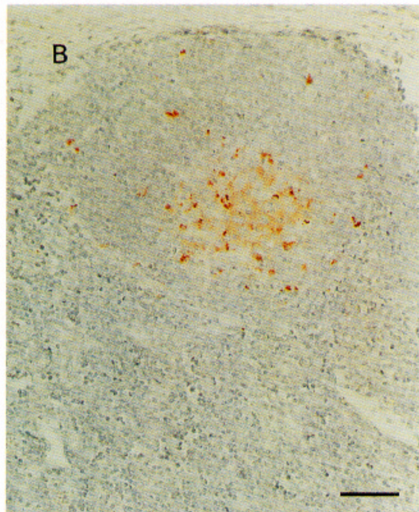
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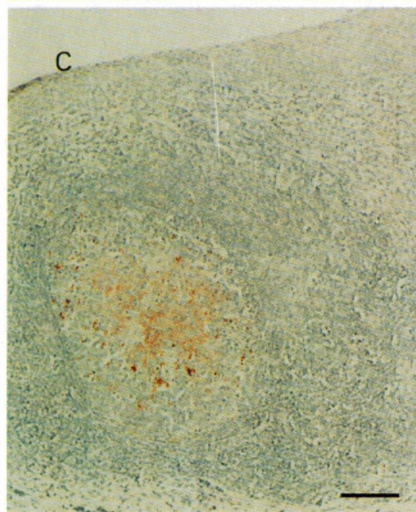
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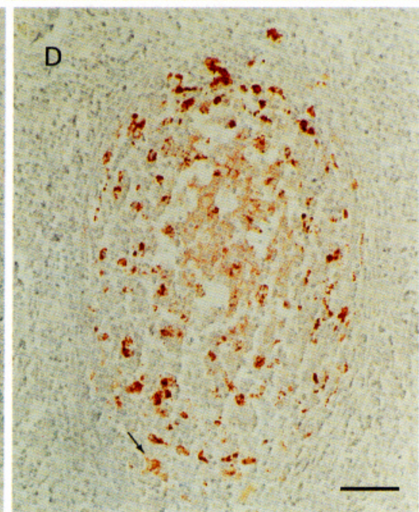
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