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Enhancement of immunohistochemical staining of scrapie proteins and immune cells within lymph nodes of early scrapie-infected sheep

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Abstract

Transmissible spongiform encephalopathies (TSE) are a group of fatal neurodegenerative diseases that affect animals as well as humans. The oldest of these diseases is Scrapie seen in sheep. Scrapie is caused by an altered form (PrP^{Sc}), capable of inducing “self-replication” of the normal host prion protein (PrP^C). There is currently no universal standard for antigen retrieval when using immunohistochemistry to simultaneously stain the PrP^C protein and other cellular markers. The use of formalin-fixed tissue creates a challenge by concealing the antigenic sites where an antibody would bind, and lengthy antigen retrieval methods must be applied in order to facilitate staining. Further complicating sheep tissue immunohistochemistry is a significant lack of commercial antibodies to sheep cell markers available in research. Here we developed a novel immunohistochemical technique using trypsin, formic acid, and hydrated autoclaving using citraconic anhydride buffer to increase sensitivity of staining for scrapie proteins and immune cell subsets. This allowed us to stain and identify cells within lymphoid tissue associated with early lymphoid pathogenesis in scrapie.

Keywords

Immunohistochemistry; Scrapie; prions; citraconic anhydride; antigen retrieval

1. Introduction

Scrapie is a transmissible spongiform encephalopathy (TSE) in sheep caused by a variant form of the ubiquitous cellular protein PrP^C known as protein PrP^{Sc}, or prions (Prusiner 1998). Scrapie is invariably fatal causing degenerative neurological dysfunction and paralysis. PrP^{Sc}, a conformational variant of normal self PrP^C, is not recognized by the immune system due to self-tolerance. However, many data suggest that the lymphoid system acts as a site of congregation and propagation of infectious prion protein (Aguzzi *et al* 2006, Aucouturier and Carnaud 2002, Ersdal *et al* 2005). Lymphoid tissue is involved in the early stages of prion disease and is a location of prion replication and proliferation (Andreoletti *et al*. 2000). Specifically, follicular dendritic cells (FDCs)¹ and B lymphocytes appear to be

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most involved in early disease. It is unclear at this time whether the immune cells are directly infected or are simply utilized by the disease as a site of congregation and/or replication, but it is evident that the immune cells are involved in the manifestation of the disease. FDCs within germinal centers (GCs) of lymph nodes retain infectious PrP^{Sc} in murine models, and mice genetically rendered deficient in B cell populations fail to develop prion infections (Brown *et al.* 1999, Davies *et al.* 2004, Mabbott and Bruce 2002, Sukumar *et al.* 2006). In addition, scrapie does not accumulate in the secondary lymphoid tissue without mature FDCs expressing the PrP^C self-protein or lymphotoxin-secreting B cells (Mabbott and Bruce 2002). These findings offer strong evidence for the involvement of B cells and FDCs in scrapie infection. Other research offers convincing evidence of the involvement of several other immune cells including macrophages and dendritic cells (Mabbott and Bruce 2002, Aguzzi and Heikenwalder 2005, Aguzzi and Heikenwalder 2006). Immunohistochemistry remains the most reliable means to uncover the complex relationships between PrP^{Sc} and specific immune cells in lymphoid organs during scrapie infection.

While it is generally accepted that FDCs are a source of infectious prion protein in lymph nodes and lymphoid follicles, it remains unclear as to how the infectious agent disseminates throughout lymphoid system from the original point of infection. It is also unclear which immune cells are responsible for transport of the prion proteins from the lymphoid system to the central nervous system (CNS), which ultimately leads to the endstage neuropathology associated with TSEs. Therefore, characterizing the association of PrP^{Sc} with multiple immune cells and their subsequent location in the lymph node of scrapie-infected tissue will be vital to a better understanding of the pathogenesis of prion disease.

Detection of leukocytes and the prion protein present a unique challenge in immunohistochemistry. Frozen sections offer optimal staining with most monoclonal antibodies, but the morphology of the tissue being tested is significantly compromised. Therefore, formalin-fixed, paraffin-embedded tissue is preferable because it offers superior morphological preservation of the tissue. However, formalin-fixed, paraffin-embedded tissue presents a considerable challenge, as the process of fixing the tissues in formalin creates chemical linkages between the formalin and proteins in the fixed tissue, masking the epitopes on the tissue targeted by the antibodies and results in an inability to label individual proteins (Ramos-Vara 2005). In addition, antibodies capable of differentiating between PrP^C and PrP^{Sc} by immunohistochemistry do not exist. Common methods therefore rely upon identification of PrP^{Sc} using PrP^C-specific antibodies after first chemically or enzymatically eliminating PrP^C in target tissues. This must be done by enzymatic treatment or high heat exposure, which is part of the antigen retrieval process.

Several methods of antigen retrieval have been designed to expose immunoreactive epitopes and combat this problem. The most effective of these methods appears to be a heating step preceded by exposure to formic acid or other enzymatic treatment (Furuoka *et al.* 2005, Van Everboeck *et al.* 1999, Andreoletti *et al.* 2002). Adding to the challenges of immunohistochemistry in studying scrapie and immune cell interaction is the limited availability of lymphocyte antibodies specific for sheep antigens. Very few antibodies interact with sheep cells and even fewer antibodies interact with formalin-fixed, paraffin-embedded sheep tissue. Therefore, effective antigen-retrieval techniques are vital.

Here we developed a new technique by determining the optimal conditions (heating, buffers, enzymatic treatment) for antigen retrieval in sheep tissue using a panel of antibodies specific

¹**Abbreviations:** TSE, Transmissible spongiform encephalopathies; FDC, Follicular dendritic cells; CNS, Central nervous system; CWD, Chronic wasting disease; GC, Germinal center;

for sheep leukocytes and PrP^{Sc}. There is currently no universal standard for this procedure, so it was necessary to test a range of methods to find the optimal procedure for each antibody.

We injected scrapie-positive brain homogenate subcutaneously into the lymph nodes of a sheep 4, 6 and 8 weeks prior to sacrifice. Samples of brain and lymph nodes were harvested and used for these studies. Formalin-fixed and paraffin-embedded tissues were immunohistochemically stained to determine scrapie accumulation and immune cell location within the tissues. Various antigen retrieval methods were tested to determine the optimal staining conditions for the range of antibodies used. We also developed a novel antigen retrieval method for prion immunohistochemistry.

2. Materials and methods

2.1 Animals

Six Cheviot sheep less than one year old were used in this experiment. At the time intervals indicated in the text, 0.5 ml of 10% scrapie-positive brain homogenate (w/v) in PBS was injected subcutaneously into one side of the animal 4, 6, and 8 weeks prior to sacrifice, and the other side was injected with a similar volume and concentration of scrapie-negative brain homogenate. Care was taken to ensure that no more than 200ul was injected into any one skin site to limit the effects of non-specific inflammation (Borgs and Hay, 1986). This experimental inoculation method has previously been documented to duplicate the lymphoid and neurological progression of natural scrapie (Goldmann et al., 1994; Gonzalez et al., 2002; Houston et al., 2002). Additional tissues were obtained from a previously verified scrapie-positive sheep brain to use as a positive control. To assess species cross-reactivity, we also used tissue from 4 laboratory-confirmed positive CWD cases and experimentally infected clinical deer to confirm the presence of prions following antigen retrieval methods described here across species. All animal protocols were approved by the South Dakota State University's Institutional Animal Care and Use Committee (IACUC).

2.2 Tissue Fixation

Tissue sections (6 µm thick) were immersed in water containing poly-L-lysine, collected on slides, dried for 12 hours at room temperature, then incubated at 37° C overnight. Slides were deparaffinized in xylene and decreasing gradients of ethanol then subjected to a variety of antigen retrieval methods.

2.3 Antigen retrieval steps

Prior to immunohistochemical staining several antigen retrieval methods were tested to determine the optimal method. Specific combinations used are outlined in Table I.

Hydrated microwave treatment—Slides were placed in 10 mM citric acid buffer 0.05% Tween 20 (pH 6.0) and microwaved at 20% power 3 times for 5 minutes at a relatively constant temperature of 98° C then cooled for 20 minutes and washed 2 times in Tris-buffered saline and distilled water.

Hydrated autoclaving—Slides were autoclaved for 10 min at 121° C in either 10 mM citric acid buffer 0.05% Tween 20 (pH 6.0) or 0.05% citraconic anhydride buffer (pH 7.4). Slides were cooled for 20 minutes and washed 2 times with Tris-buffered saline for 5 minutes.

2.4 Monoclonal antibody production

Production and characterization of monoclonal antibodies 2–137 and 2–165 was as previously described (Hein et al., 1998). Antibody 2–104 has been previously described (Mathiason et al., 2010). Antibody AY1-39 was produced following immunization of mice with B cells sorted from sheep peripheral blood. Supernatants were screened based on recognition of sheep B cells as assessed by flow cytometry and immunohistochemical staining of sheep ileal PP, jejunal PP, and lymph nodes.

2.5 Immunohistochemistry

Following antigen retrieval, immunohistochemistry was performed on peripheral ovine lymph nodes and brain. Endogenous peroxidase was blocked by incubating slides with 0.3% H₂O₂ in distilled water or methanol for 20 minute then washing 2 times with Tris-buffered saline for 5 minute. Non-specific binding sites were blocked by incubating slides with 10% goat serum (Invitrogen) in Tris-buffered saline with 0.05% Tween 20 for 30 minute. Excess goat serum was then removed from the slides. Specific primary antibodies were added to slides at various dilutions (Table I), incubated overnight at 4° C, then washed with Tris-buffered saline with 0.05% Tween 20. Secondary biotinylated goat anti-mouse antibodies were added to the slides and incubated for 30 minutes at room temperature, then washed with Tris-buffered saline with 0.05% Tween 20. A streptavidin-peroxidase complex (Vector labs) was added to the slides and incubated 20 minutes. Primary staining was performed using diaminobenzidine (DAB) (Vector labs). Slides were counterstained with hematoxylin (Richard Allan Scientific) and bluing reagent (Richard Allan Scientific), rehydrated in ethanol and xylene, then coverslipped using Vectamount (Vector labs). For morphological comparisons, all tissues were sectioned and then routinely stained with hematoxylin & eosin.

Positive and negative controls- Positive controls were obtained by using sections of obex from a known scrapie positive sheep. Negative controls were done by replacing normal serum in place of the primary antibody for each Ig class of secondary antibody to check for background staining.

3. Results

3.1 Antigen retrieval and Immunohistochemistry (Table I)

Serial sections of all tissues and brain were stained in succession to compare staining patterns among different antigen retrieval techniques. Slides were visually compared and given a ranking of significant, moderate, or no staining observed based on intensity of staining as well as number of locations stained between the sections. Antigen retrieval steps that did not utilize a heat step resulted in a complete lack of staining with all seven antibodies tested. Hydrated autoclaving showed a marked increase in staining of the antibodies when compared to microwave treatment, however, the major factor regarding hydrated autoclaving appears to be the type of buffer used. When using citric acid buffer we observed significant staining with one of the seven antibodies and moderate staining with five of the seven antibodies. The use of citraconic anhydride buffer in combination with hydrated autoclaving resulted in significant staining with six of the seven antibodies, and moderate staining of the remaining antibody. Results of the immunohistochemistry staining procedures are summarized in Table I.

3.2 Sheep lymph node staining

All of the cell-marker antibodies (Table II) showed significant staining when the citraconic anhydride hydrated autoclaving antigen retrieval method was utilized (Figure 1A–D). Staining of PrP^{Sc} was observed in all lymph nodes that were injected with infected brain

homogenate. Most staining was localized within GCs and secondary follicles within lymph nodes (Figure 2A–D). All staining within GCs and follicles demonstrated a reticular staining pattern and staining was limited to the light zone of GCs. Some staining was seen in the medullary sinusoids within the lymph nodes as well as within multiple cells within the lymph nodes.

3.3 Deer lymph node staining

Staining of PrP^{Sc} was observed in all lymph nodes in the clinically-confirmed CWD positive deer. Both PrP^{Sc} –specific antibodies F89 (Figure 3A & C) and 6H4 (Figure 3B) showed significant staining patterns. Like the sheep lymph nodes, stained PrP^{Sc} was localized within GCs and secondary follicles within lymph nodes and demonstrated a reticular staining pattern. A clinically confirmed CWD-negative deer lymph node was stained with F89 to ensure that the staining was not due to background or nonspecific staining (Figure 3D).

4. Discussion

The described antigen retrieval technique that used a combination of trypsin, formic acid, and citraconic anhydride hydrated autoclaving allowed us to simultaneously visualize both PrP^{Sc} and conventional cell-surface markers of leukocytes in sheep. The ability to retrieve antigens and use antibodies that were previously unsuccessful in formalin-fixed, paraffin-embedded tissues allows the use of a wider variety of antibodies and permits simultaneous analysis of PrP^{Sc} and immune cell subsets. Furthermore, the improved clarity and intensity of this staining procedure provides better visualization of prions in lymph nodes and brain and allows for earlier detection of scrapie infection. It is also important to note that the antigen retrieval method described here works on deer tissue as well, and effectively stains PrP^{Sc} in species other than sheep. This is vital to prion studies due to the fact that many different species are utilized as laboratory models and it is important that the same set of standards are effectively applied to all research models. This allows for a more cohesive and reliable set of studies relating to prions.

The reticular staining pattern of PrP^{Sc} observed in the GCs and follicles of the lymph nodes indicates distribution concurrent with FDC distribution within these structures. Furthermore, prior research supports a role of FDCs in prion infection (Brown *et al.* 1999, Davies *et al.* 2004, Sukumar *et al.* 2006). The distribution of PrP^{Sc} within the light zone of GCs also supports possible involvement of B cells in prion infection as well.

The presence and distribution of PrP^{Sc} in the lymph nodes demonstrates early involvement of lymphoid tissue in scrapie infection. Sheep with clinical signs of scrapie show a similar, though more dramatic, pattern of staining in lymphoid tissue. Collection and retention of PrP^{Sc} in GCs as early as four weeks after injection implies a continual maintenance of PrP^{Sc} in lymphoid tissue from time of infection to death. Infection in the medullary sinusoids also supports this conclusion. In recent research prion accumulation in medullary sinusoids was observed a year or more after initial scrapie infection (Ersdal *et al.* 2005).

Examination of suspected scrapie cases utilizing this method of antigen retrieval would be beneficial in a diagnostic setting. This technique capitalizes on the morphological preservation offered by formalin-fixed, paraffin-embedded tissue coupled with improved staining comparable to frozen tissue. This method could also lead to a more selective antemortem test utilizing lymphoid tissues, and could accelerate diagnosis of early scrapie cases. From a research perspective this method will allow detection of PrP^{Sc} in tissues at very low concentrations, and allow detection of PrP^{Sc} shortly after infection so that cellular interactions at these critical early timepoints can be observed.

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Figure 1. Immunohistochemical staining of sheep tissue using the citraconic anhydride antigen retrieval method

A) Popliteal lymph node stained with 2-104 antibody at a magnification of 20X. B) Prescapular lymph node stained with 2-137 antibody at a magnification of 20X. C) Popliteal lymph node stained with 2-165 antibody at a magnification of 10X. D) Prescapular lymph node stained with AY-139 antibody at a magnification of 10X.

Figure 2. Immunohistochemical staining of PrP^{Sc} deposition in sheep tissue using the citraconic anhydride antigen retrieval method

A) Retropharyngeal lymph node stained with 7D9 antibody at a magnification of 20X. B) Retropharyngeal lymph node stained with 6H4 antibody at a magnification of 20X. C) Prescapular lymph node stained with F89 antibody at a magnification of 20X. D) Prescapular lymph node stained with F89 antibody at a magnification of 20X.

Figure 3. Immunohistochemical staining of PrP^{Sc} deposition in deer tissue using the citraconic anhydride antigen retrieval method

A) Retropharyngeal lymph node stained with F89 antibody at a magnification of 20X. B) Retropharyngeal lymph node stained with 6H4 antibody at a magnification of 20X. C) Prescapular lymph node stained with F89 antibody at a magnification of 20X. D) Negative control- Prescapular lymph node of a CWD-negative deer stained with F89 antibody at a magnification of 20X.

Table I

antigen retrieval methods used on sections of lymph node and brain.

ANTIBODIES TESTED	BUFFER/PH	PRIMARY ANTIGEN RETRIEVAL STEP	HEATING METHOD/ TEMPERATURE/LENGTH OF TIME
6H4, 7D9, F89, 2– 137, AY1-39, 2–165, 2–104	Citric acid pH 6.0	98% formic acid	Hydrated autoclaving 121° C for 10 min ¹
6H4, 7D9, F89, 2– 137, AY1-39, 2–165, 2–104	Citric acid pH 6.0	98% formic acid	Citric acid pH 6.0, 98% formic acid, microwave for 20 min ²
6H4, 7D9, F89, 2–137, AY1-39, 2–165, 2–104	Citraconic anhydride pH 7.4	0.1 % trypsin digestion, 98% formic acid	Hydrated autoclaving 121° C for 10 min
6H4, 7D9, F89, 2–137, AY1-39, 2–165, 2–104	-	98% formic acid	-

¹ Ersdal 2003

² Andréoletti *et. al* 2002

Table II

Monoclonal antibodies used for isolation and identification of immune cells and PrP^{sc} in immunohistochemistry.

Clone	Dilutions tested	Antigen	Isotype	Source/Reference	Recognition site
2-137	Stock solution	FDCs	IgM	This Paper	Follicular Dendritic Cells
AY1-39	Stock solution	CD35	IgG1	This Paper	Erythrocytes, neutrophils, monocytes, eosinophils, B cells, Follicular Dendritic Cells
2-165	Stock solution	FDCs	IgM	This Paper	Follicular dendritic cells
2-104	Stock solution	CD72	IgM	Mathiason et al., 2010	B cells
7D9	1:200-1:500	PrP ^{sc}	IgG1	Abcam	Fragment corresponding to amino acids 23-237 of PrP
F89	1:200-1:2000	PrP ^{sc}	IgG1	Abcam	Conserved region amino acids n-IHFG-c of PrP ^{sc}
6H4	1:200-1:500	PrP ^{sc}	IgG1	Prionics	Amino acids 93-99 of ovine PrP

Table III

Results of various staining methods and antibodies.

	6H4	7D9	F89	2-137	AY1-39	2-165	2-104
Citric acid pH 6.0, 98% formic acid, microwave	-	-	-	+	-	-	-
Citric acid pH 6.0, 98% formic acid, hydrated autoclaving	+	+	++	+	+	-	+
Citraconic anhydride pH 7.4, 98% formic acid, hydrated autoclaving	++	+	++	++	++	++	++
98% formic acid	-	-	-	-	-	-	-

Staining patterns are indicated as: significant staining ++, moderate staining +, no staining -.