

Immunohistochemical Detection of Disease-Associated Prion Protein in the Intestine of Cattle Naturally Affected with Bovine Spongiform Encephalopathy by Using an Alkaline-Based Chemical Antigen Retrieval Method

Hiroyuki OKADA¹⁾, Yoshihumi IWAMARU¹⁾, Morikazu IMAMURA¹⁾, Kentaro MASUJIN¹⁾, Takashi YOKOYAMA¹⁾ and Shirou MOHRI¹⁾

¹⁾Prion Disease Research Center, National Institute of Animal Health, 3-1-5 Kan-nondai, Tsukuba, Ibaraki 305-0856, Japan

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ABSTRACT. An alkaline-based chemical antigen retrieval pretreatment step was used to enhance immunolabeling of disease-associated prion protein (PrP^{Sc}) in formalin-fixed and paraffin-embedded tissue sections from cattle naturally affected with bovine spongiform encephalopathy (BSE). The modified chemical method used in this study amplified the PrP^{Sc} signal by unmasking PrP^{Sc} compared with the normal cellular prion protein. In addition, this method reduced nonspecific background immunolabeling that resulted from the destruction of the residual normal cellular form of prion protein, and reduced the treatment time compared with the usual autoclave pretreatment step. Immunolabeled PrP^{Sc} was thereby clearly detected in the myenteric plexus of the ileum in naturally occurring BSE cattle. **KEY WORDS:** antigen retrieval, BSE, cattle, immunohistochemistry, prion.

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Formalin-fixed paraffin-embedded (FFPE) specimens are conventionally used for the histopathological and immunohistochemical diagnosis of transmissible spongiform encephalopathies (TSEs), including bovine spongiform encephalopathy (BSE) [3]. Formalin fixation produces cross-links between proteins; therefore, FFPE tissue sections often require antigen retrieval protocols [18]. Moreover, the retrieval of the appropriate antigen is essential to unmask the immunoreactive disease-associated prion protein (PrP^{Sc}) epitope and to suppress normal cellular prion protein (PrP^C) in FFPE tissue sections from TSE-affected animals [6, 15, 25, 26]. Although PrP^{Sc} antigen retrieval is required to enhance the specific immunolabeling of PrP^{Sc}, nonspecific background immunostaining is observed for proteinase K (PK)-resistant PrP^C detected in the brains of older bovinized transgenic mice [28] and in the enteric nervous system (ENS), i.e., the myenteric and submucosal plexus, of normal animals when using the usual autoclave antigen retrieval method [8]. To the best of our knowledge, there are no antibodies that can distinguish between PrP^C and PrP^{Sc} in FFPE tissues.

The primary objective of this study is to characterize the improved chemical method and to perform comparative studies between the conventionally used antigen retrieval procedures for BSE immunohistochemistry. The secondary objective is to describe the detection of immunolabeled PrP^{Sc} in the intestines of naturally affected BSE cases in Japan using this new method.

MATERIALS AND METHODS

Ethics statement: Experiments involving animals were approved by the Animal Ethical Committee and the Animal Care and Use Committee of National Institute of Animal Health.

Preparation of samples: Four naturally occurring BSE cases (BSE/JP15, 17, 21, and 22) from the fallen stock surveillance program in Japan were confirmed using western blot analysis as well as histopathological and immunohistochemical examinations in our laboratory. After BSE confirmation, the carcasses were stored at 4°C (BSE/JP21, 22) or at –20°C (BSE/JP15, 17) in the fallen stock point. Various tissues and organs, including the brain and spinal cord from 4 BSE animals, were removed 4–10 days after their death. Two BSE-uninfected cattle, confirmed by western blot and immunohistochemistry, were used as controls.

Various tissues from the naturally occurring BSE cases and control animals were collected and fixed in 10% buffered formal saline (pH 7.4) for histopathology and immunohistochemistry, and the corresponding tissues were frozen at –80°C for western blot analysis. Formalin-fixed coronal slices of the brain, spinal cords, and peripheral tissues were then immersed in 98% formic acid for 60 min at room temperature (RT) to reduce infectivity [24]. The samples were routinely embedded in paraffin wax using an automated processing machine (ETP-150C; Sakura Finetek Japan, Tokyo, Japan). Serial sections were cut at 4 µm and mounted on 3-aminopropyltriethoxysilane-coated glass slides (Immuno-Coat; Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The sections were stained with hematoxylin and eosin (HE) or provided for PrP^{Sc} immunohistochemistry.

Pretreatment for PrP^{Sc} immunohistochemistry: Before PrP^{Sc} immunolabeling, we used 3 antigen retrieval pretreatment protocols: (1) Dewaxed sections on the slide were

* CORRESPONDENCE TO: OKADA, H., Prion Disease Research Center, National Institute of Animal Health, 3-1-5 Kan-nondai, Tsukuba, Ibaraki 305-0856, Japan.
e-mail: okadahi@affrc.go.jp

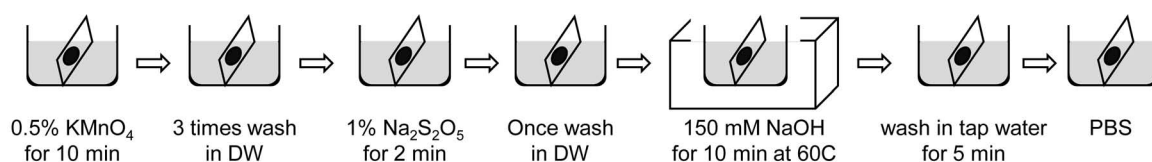


Fig. 1. Flow chart of the chemical pretreatment for PrP^{Sc} immunohistochemistry. KMnO₄, potassium permanganate; DW, distilled water; Na₂S₂O₅, sodium disulfite; NaOH, sodium hydroxide; PBS, phosphate-buffered saline (pH 7.4).

Table 1. Characteristics of the 11 antibodies used in this study and PrP^{Sc} immunolabeling intensity from treatment with 150 mM NaOH at 60°C

Antibody	Epitope location numbering	PrP sequence	Clonality	Immunogen intensity ^{a)}	Signal	Source ^{b)}
N-terminal region	SAF32	Octa-repeat region	Monoclonal	SAF from infected hamster brain	–	SPI-bio (Montigny le Bretonneux, France)
	B103	103–121	Cattle	Cattle recPrP	+	FUJIREBIO (Tokyo, Japan) [9]
Core region	T1	137–145	Mouse	Mouse recPrP	3+	Dr. Tagawa [21]
	12F10	142–160	Human	Hamster recPrP	–	SPI-bio (Montigny le Bretonneux, France)
	SAF54	142–160	Human	SAF from infected hamster brain	2+	SPI-bio (Montigny le Bretonneux, France)
	F89/160.1.5	146–159	Cattle	Cattle recPrP	3+	VMRD (Pullman, WA, U.S.A.)
	44B1	155–231	Mouse	Mouse recPrP	3+	Dr. Horiuchi [11]
	SAF84	160–170	Human	SAF from infected hamster brain	3+	SPI-bio (Montigny le Bretonneux, France)
	43C5	163–169	Mouse	Mouse recPrP	3+	Dr. Horiuchi [11]
C-terminal region	T4	221–239	Cattle	Cattle recPrP	+	Dr. Sata [23]
	F99/97.6.1	228–233	Cattle	Cattle recPrP	2+	VMRD (Pullman, WA, U.S.A.)

a) – None, + weak, 2+ distinct, 3+ strong.

b) Manufacturer and reference number.

treated with 3% H₂O₂ for 10 min, incubated with 10 µg/ml PK at RT for 10 min, and then autoclaved for 3 min at 121°C in 10 mM citrate buffer (pH 6.0). (2) Sections were pretreated with a recently developed alternative chemical method at RT [2] as follows (Fig. 1). In brief, sections were immersed in 0.5% potassium permanganate (KMnO₄, pH 7.0) for 10 min; rinsed in distilled water 3 times; soaked in 1% sodium disulfite for 2 min; briefly rinsed in distilled water; and finally immersed in a solution of 75 mM NaOH, 0.1% *N*-lauroylsarcosine, and 2% NaCl. (3) Sections were immersed in 0.5% KMnO₄ dissolved in phosphate-buffered saline (pH 7.4) for 10 min and soaked in 1% sodium disulfite for 2 min. After a brief wash in distilled water, the sections were immersed in a different concentration of NaOH corresponding to 25, 75, 150, and 300 mM in the absence of *N*-lauroylsarcosine and NaCl from the original formula described above, and incubated at RT, 37°C, and 60°C in each concentration of NaOH. A simple protocol of this method is illustrated in Fig. 1.

PrP^{Sc} immunohistochemistry: After each pretreatment, sections were placed on a DakoCytomation Autostainer Universal Staining System (Dako, Carpinteria, CA, U.S.A.) and incubated with the primary anti-prion protein (PrP) antibodies for 60 min, then with anti-mouse or anti-rabbit universal immunoperoxidase polymer [Nichirei Histofine Simple Stain MAX-PO (M) or (R); Nichirei, Tokyo, Japan] as the secondary antibody for 30 min, and with 3,3'-diaminobenzidine tetrachloride as the chromogen for 7 min. This immunohistochemical staining procedure was carried out at RT. The primary anti-PrP antibodies used in this study were SAF32, SAF54, SAF84, T1, 12F10, F89/160.1.5, F99/

97.6.1, 44B1, and 43C5 as the monoclonal antibody (mAb), and B103 and T4 as the rabbit polyclonal antibody (pAb). The characteristics of the 11 primary antibodies are summarized in Table 1. The concentrations of mAbs and pAbs used for immunohistochemistry were 1 µg/ml and 5 µg/ml, respectively. Finally, sections were lightly counterstained with Mayer's hematoxylin.

PrP^{Sc} western blot: The corresponding tissues from the digestive tracts of the carcasses were examined for PK-resistant PrP^{Sc} by western blotting using the phosphotungstic acid precipitation method as previously described [20].

The samples were homogenized in 50 mM Tris-HCl (pH 7.5), 2% Triton X-100, 0.5% *N*-lauroylsarcosine, 100 mM NaCl, 5 mM MgCl₂, 2 mM CaCl₂, 20 mg collagenase, and 40 mg DNase I, and incubated at 37°C for 2 hr. The homogenate was digested in 60 µg/ml of PK for 1 hr at 37°C followed by an incubation with 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefablock; Roche Diagnostics, Indianapolis, IN, U.S.A.). The sample was centrifuged at 68,000 × *g* for 20 min at RT. The supernatant was discarded, and the pellet was suspended in 6.25% (w/v) *N*-lauroylsarcosine in 10 mM Tris-HCl (pH 7.5) and then incubated at RT for 1 hr with constant rotation. The sample was subsequently centrifuged at 9,000 × *g* for 5 min. Sodium phosphotungstate was added to the supernatant at a final concentration of 0.3% (v/v) and incubated at 37°C for 30 min with constant rotation. Pellets were obtained by centrifugation at 20,000 × *g* for 30 min. PrP^{Sc} was enriched from the sample according to a previously described method [7]. Western blotting was performed as previously described using the horseradish-peroxidase-conjugated anti-

PrP T2 mAb. Blots were developed using a chemiluminescent substrate (SuperSignal; Pierce Biotechnology, Rockford, IL, U.S.A.) [20].

Mouse transmission assay: Tg(BoPrP)-4092HOZ/Prnp^{0/0} (TgBoPrP) mice overexpressing the bovine PrP gene in a null mouse background that is highly susceptible to BSE prion and exhibits an incubation period of less than 250 days [17] were kindly supplied by Dr. S.B. Prusiner. TgBoPrP mice aged 3 weeks old were inoculated intracerebrally with 20 μ l of 10% homogenates prepared from the digestive tracts according to our institutional guidelines. Following inoculation, the clinical status of the mice was monitored daily to assess the onset of neurological symptoms.

RESULTS

Although various degrees of autolysis and artificial histopathological changes were obvious in HE-stained sections from all BSE-affected cattle because of damage by autolysis and/or freezing, PrP^{Sc} immunolabeling was well preserved, and various types of PrP^{Sc}, such as punctuate, intraneuronal, perineuronal, glial, linear, stellate, and coalescing depositions, were detected throughout the brain and spinal cords treated with the chemical and autoclave pretreatment methods.

The most conspicuous type of PrP^{Sc} in the neuropil of the gray matter throughout the brains and spinal cords was the fine and coarse punctuate type. The stellate type of PrP^{Sc} was predominantly detected in the cerebral and cerebellar cortices, basal ganglia, and thalamic nuclei. Intraneuronal and perineuronal types of PrP^{Sc} deposition were more intense in large neurons. Linear and coalescing depositions were more evident in the basal ganglia and thalamus. Intra-glial type of PrP^{Sc} was less common compared to other types of PrP^{Sc} depositions throughout the brain and spinal cords.

The chemically treated sections showed strong and well-contrasted immunolabeling using T1, SAF84, F89/160.1.5, 44B1, and 43C5 mAbs; moderate immunolabeling with SAF54 and F99/97.6.1 mAbs; and weak immunolabeling with B103 and T4 pAbs, compared to those pretreated with autoclaving (Fig. 2 and Table 1). Moreover, the chemical methods failed to detect immunolabeled PrP^{Sc} when using the commercially available SAF32 and 12F10 mAbs. No significant difference in the intensity of PrP^{Sc} immunolabeling and nonspecific background immunostaining was observed for the chemical method in the presence or absence of 0.1% *N*-lauroylsarcosine and 2% NaCl in the denaturing solution of the original formula (data not shown). Immunolabeled PrP^{Sc} pretreated with autoclaving revealed more nonspecific background immunostaining compared to chemically pretreated PrP^{Sc} (Fig. 2b).

Sections treated with each concentration of NaOH at each temperature produced intense immunolabeling; however, there were differences in intensity of PrP^{Sc} immunolabeling between the different NaOH concentrations and between the different temperatures (Table 2). The punctuate PrP^{Sc} signal in the neuropil of the gray matter was increased in a concen-

tration- and temperature-dependent manner without any tissue loss (Fig. 3). However, no significant difference in the intensity of PrP^{Sc} immunolabeling was observed when the sections were treated with 150 or 300 mM NaOH. The intensity of hematoxylin-counterstained sections treated with 300 mM NaOH was weaker than in sections treated with 150 mM NaOH. Therefore, the most enhanced PrP^{Sc} signal detected using mAbs was obtained from treatment with 150 mM NaOH at 60°C.

In addition, neither PrP^{Sc} nor background immunolabeling was detectable in sections prepared from the peripheral nerves and the ENS (submucosal and myenteric plexuses) of the gastrointestinal tract, i.e., duodenum, jejunum containing discrete Peyer's patches, ileum containing a continuous Peyer's patch, ileocecal plica, cecum, colon, and rectum, pretreated with the chemical method (Fig. 4a). Conversely, sections pretreated with the conventional autoclave method were moderately immunolabeled in the ENS and peripheral nerves and had moderate levels of nonspecific immunolabeling (Fig. 4b).

Intraneuronal and perineuronal types of PrP^{Sc} accumulation were detected in the neurons of the myenteric plexus, but not of the submucosal plexus at the ileum in all naturally occurring BSE-affected cases (Table 3). In addition, intraneuronal PrP^{Sc} immunolabeling was detected in the myenteric plexus of the colon in BSE/JP17 (Fig. 5). Specific PrP^{Sc} signals and nonspecific immunostaining were less intense in the chemically pretreated ENS sections (Fig. 4c) compared with the autoclaved sections (Fig. 4d). No PrP^{Sc} immunolabeling was present in sections from the other areas of the digestive tract, i.e., the duodenum, jejunum, ileocecal region, cecum, and rectum, which were chemically pretreated.

Western blot analysis revealed weak positive signals in the ileum of all cases and in the colon of BSE/JP17, but not in the samples from the duodenum, jejunum, cecum, and rectum (Fig. 6 and Table 3). TgBoPrP mice inoculated with BSE/JP17 samples prepared from the terminal ileum and colon developed the disease, and the survival period was 528.7 ± 10.2 days (mean \pm standard deviation, $n=3$) and 421.7 ± 48.2 days ($n=3$), respectively. Thus, the infectivity level of the intestine was lower than that of the brain. Western blot analysis showed a typical molecular mass and glycosylation pattern of PK-resistant PrP^{Sc} in the brains collected from these diseased TgBoPrP mice (data not

Table 2. Intensity of PrP^{Sc} immunolabeling from treatment with various NaOH concentrations and temperatures, determined with mAb T1

NaOH concentration (mM)	RT	37°C	60°C
0	\pm	+	+
25	2+	2+	2+
75	2+	2+	2+
150	2+	2+	3+
300	2+	2+	3+

\pm Barely, + weak, 2+ distinct, 3+ strong.

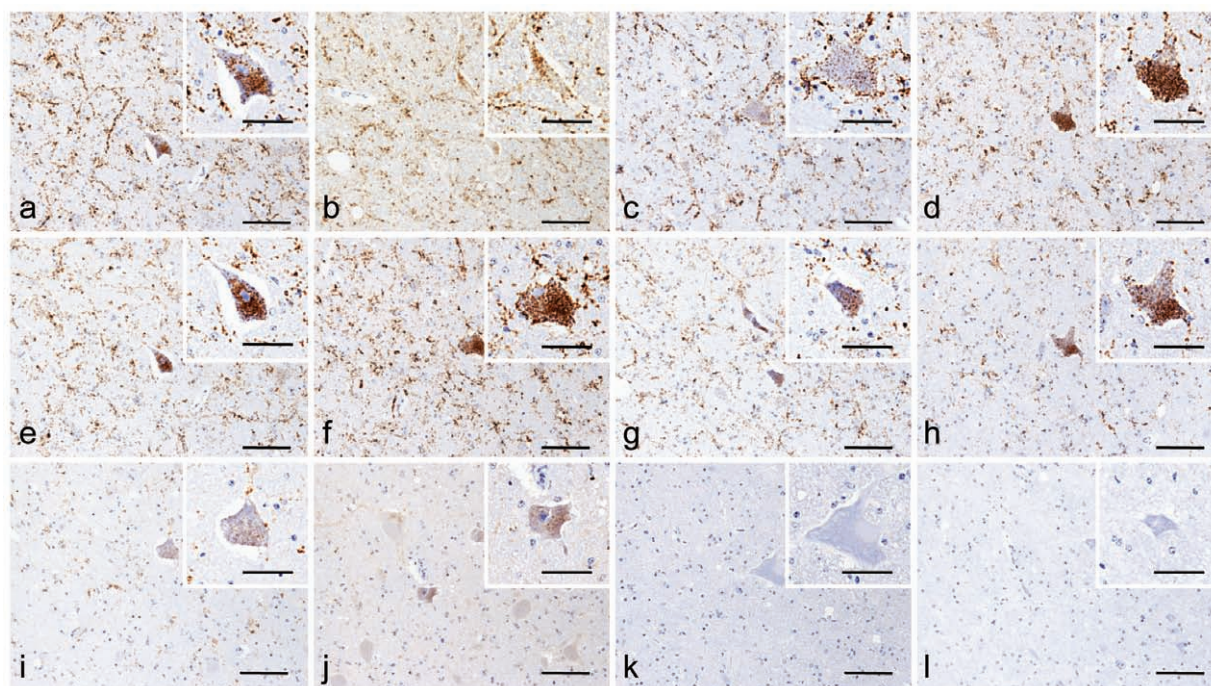


Fig. 2. Immunolabeled PrP^{Sc} in the thalamus of BSE/JP26 using the modified chemical method (75 mM NaOH at 60°C) with various primary antibodies (a, T1; c, SAF54; d, SAF84; e, 43C5; f, 44B1; g, F89/160.1.5; h, F99/97.6.1; i, B103; j, T4; k, SAF32; and l, 12F10) and the usual autoclave pretreatment method with the T1 mAb (b). Bar=100 μ m. The insets at the upper right corner of each figure are the higher-magnification images of a representative neuron. Bar=50 μ m.

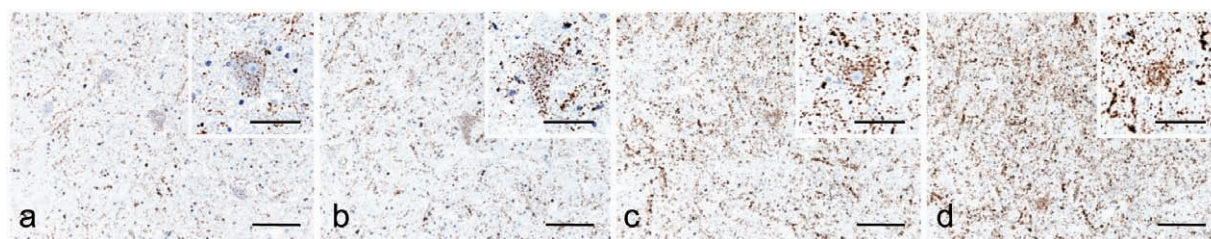


Fig. 3. Immunohistochemical detection of PrP^{Sc} in semiserial sections from the ventral horn of the lumbar spinal cord from BSE/JP22 with the T1 mAb using the modified chemical pretreatment (a, 75 mM NaOH at RT; b, 75 mM NaOH at 37°C; c, 150 mM NaOH at 37°C; and d, 150 mM NaOH at 60°C). Bar=100 μ m. The insets at the upper right corner of each figure are the higher-magnification images of a representative neuron. Bar=50 μ m.

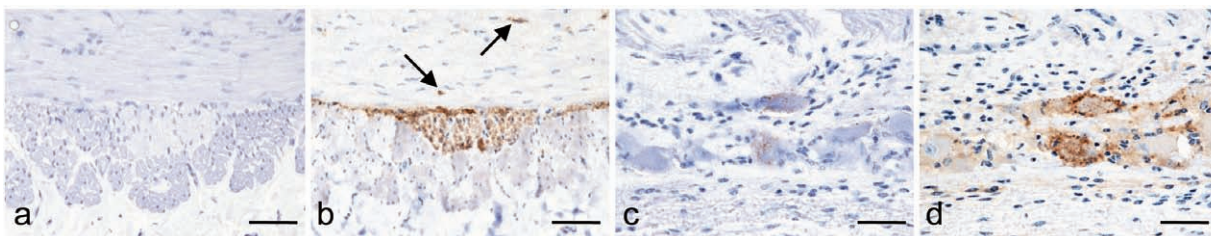


Fig. 4. Detection of immunolabeled PrP^{Sc} and nonspecific immunolabeling using the T1 mAb in the myenteric plexus of the ileum at 30 cm from the ileocecal junction of a control animal (a and b) and BSE/JP17 (c and d) using the chemical pretreatment method (a and c, 150 mM NaOH at 60°C) and the usual autoclaving method (b and d). Nonspecific immunostaining in the myenteric plexus and peripheral nerve fibers (arrows) is generated with the autoclave pretreatment method (b and d), but not with chemical pretreatment (a and c). Bars: a, b=100 μ m; c, d=50 μ m.

Table 3. Detection of PrP^{Sc} in the myenteric plexus by immunohistochemistry and PrP^{Sc} detection from the corresponding tissue by western blot

Tissues	BSE/JP15		BSE/JP17		BSE/JP21		BSE/JP22	
	IHC	WB	IHC	WB	IHC	WB	IHC	WB
Duodenum	—	—	—	—	—	—	—	—
Jejunum with discrete Peyer's patch	—	—	—	—	—	—	—	—
Jejunum without discrete Peyer's patch	—	—	—	—	—	—	—	—
Jejunum of 1 m the part from the ileocecal junction with continuous Peyer's patch	+	+	+	+	+	+	NE	NE
Ileum of 30 cm the part from the ileocecal junction with continuous Peyer's patch	+	+	+	+	+	+	+	+
Ileocecum	—	—	—	—	—	—	—	—
Cecum	—	—	—	—	—	—	—	—
Colon	—	—	+	+	—	—	—	—
Rectum	—	NE	—	NE	—	NE	—	NE

+, Positive; —, negative; NE, not examined.

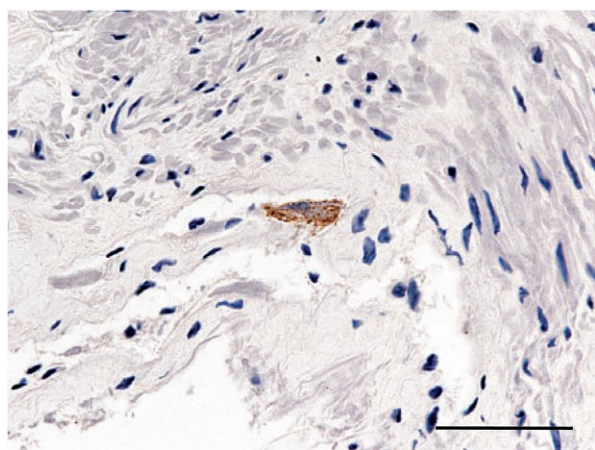


Fig. 5. Detection of immunolabeled PrP^{Sc} in the neural cells of the myenteric plexus at the colon of BSE/JP17 with the T1 mAb using chemical pretreatment (150 mM NaOH at 60°C). Bar=50 μ m.

shown).

DISCUSSION

The present study indicated that this improved chemical pretreatment step using the combination of a high concentration of NaOH and a high temperature amplified PrP^{Sc} immunolabeling compared with the originally reported chemical method [2]. The denaturation step of the original chemical method was performed at RT in a 0.1% *N*-lauroylsarcosine, 75 mM NaOH, and 2% NaCl solution. A combination of strong oxidation using KMnO₄ and protein denaturation using *N*-lauroylsarcosine may frequently affect tissue adherence [2]. Although *N*-lauroylsarcosine is a protein-denaturing agent and therefore was not used in the denaturation solution of the original formula, we detected an almost equal intensity of PrP^{Sc} immunolabeling in its presence as observed with an equal concentration of NaOH (75 mM). Why the chemical method did not work with every anti-PrP antibody tested in the BSE-affected sections is currently unclear. The specific mechanism of interaction

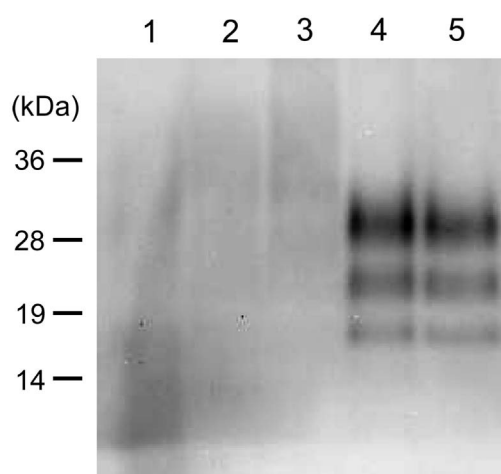


Fig. 6. Detection of PK-resistant PrP^{Sc} in the ileum of BSE/JP17 by western blot analysis. Lanes 1 and 2, jejunum with a discrete Peyer's patch; lane 3, jejunum without a discrete Peyer's patch; lane 4, ileum with a continuous Peyer's patch at 30 cm from the ileocecal junction; and lane 5, jejunum with a continuous Peyer's patch at 1 m from the ileocecal junction. We loaded 100 μ g tissue equivalent in a 10 μ l sample in each lane. All samples were digested with 50 μ g/ml PK at 37°C for 1 hr. Molecular markers are shown on the left (kDa).

between KMnO₄ and the epitope recognized by each antibody may have key events. The merit of chemical pretreatment using KMnO₄ for PrP immunohistochemistry appears to be the complete destruction of PrP^C by strong oxidation [2]. Moreover, autoclaves are very expensive, whereas the present technique is very easy to use and needs only cheap water bath equipment. In addition, autoclaving needs at least 110 min, consisting of endogenous peroxidase quenching for 10 min, enzymatic digestion for 10 min, and heating of the retrieval solution to a boiling point of 121°C and then cooling to less than 60°C for 90 min. On the other hand, the present chemical pretreatment needs only 30 min. Therefore, chemical pretreatment reduces the sample processing time and equipment cost in comparison with autoclaving [16].

Incubation at a higher temperature in the NaOH denatur-

ation step enhanced PrP^{Sc} immunoreactivity without any nonspecific background immunostaining at all concentrations of NaOH (i.e., 25, 75, 150, and 300 mM). The precise mechanism of this enhancement is unclear. The mechanism of unmasking by autoclaving was speculated to alter the three-dimensional structure of the formalin-modified antigen [13, 19]; therefore, the use of heat in the improved chemical method may restore the conformation of PrP by modifying its three-dimensional structure and/or possibly by hydrolyzing the formalin-induced cross-links [19].

The majority of anti-PrP antibodies currently available for immunohistochemistry in FFPE tissue sections have affinities for PrP^C and PrP^{Sc}. The presence of immunolabeled PrP^C in FFPE tissue sections may also depend on the fixation conditions [13, 19]. Therefore, more specific and sensitive immunohistochemical procedures are required to enhance the immunodetection of PrP^{Sc} and to diminish PrP^C immunoreactivity [2, 5, 6, 15, 26]. PrP^C was constantly detected in the intestinal ENS of normal and scrapie-infected lambs [8]. Thus, distinguishing between PrP^{Sc} and PrP^C, and developing antibodies that recognize PrP^{Sc} for immunohistochemical investigations using TSE FFPE tissue sections, are necessary.

This is, to the best of our knowledge, the first description of the localization of PrP^{Sc} within the colon of naturally affected BSE cattle. In the present study, PrP^{Sc} was observed in the myenteric plexus of the ileum and colon, but not in the duodenum and jejunum, using immunohistochemistry, western blot analysis, and mouse bioassay (Table 3). PrP^{Sc} has been detected in the myenteric plexus of the ileum from naturally affected BSE cattle [10, 12], chronic wasting disease in deer [22], and sheep scrapie [1]. Based on the current BSE research, PrP^{Sc} is not found in gastrointestinal tissues other than the distal ileum [27]. BSE infectivity of the peripheral nervous system (PNS) was found in one clinical case [4]. PrP^{Sc} accumulation in the PNS of BSE-affected cattle during the clinical stages of infection may be the rule in BSE rather than the exception; it also may be the main mechanism rather than merely preceding the accumulation in the central nervous system (CNS) [14]. Results from western blotting and mouse bioassay indicated relatively low amounts of PrP^{Sc} deposits in the ileum and colon. These data suggest that detectable PrP^{Sc} in the ileum and colon is probably caused by centrifugal spread from the CNS to the ENS within the peripheral nerves during the clinical stage of the disease. Therefore, to determine whether PrP^{Sc} was present in the ENS, the corresponding tissues should be subjected to western blot analysis by using the highly sensitive protocols described in this study or analyzed with a more sensitive mouse bioassay, or both.

In summary, chemical pretreatment with NaOH may provide a useful procedure for the detection of immunolabeled PrP^{Sc} and the suppression of the remaining PK-resistant PrP^C without any nonspecific background immunostaining in FFPE tissue sections from BSE-affected cattle compared to the usual autoclave pretreatment step. In addition, pretreatment using NaOH at a higher temperature in the alkali-

line-based chemical antigen retrieval method clearly enhanced the strength of the immunolabeled PrP^{Sc} signal.

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