Effective antigen retrieval method for immunohistochemical detection of abnormal isoform of prion proteins in animals

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Abstract

For immunohistochemistry of the prion diseases, several pretreatment methods to enhance the immunoreactivity of human and animal PrP^{Sc} on the tissue sections have been employed. 121℃ hydrated autoclaving pretreatment method or the combination method of 121℃ hydrated autoclaving with a certain chemical reagent (formic acid or Proteinase K, etc) are now widely used. We found that an improved hydrated autoclaving method at 135℃, more effectively enhanced PrP^{Sc} immunoreactivity for the antibodies recognizing the linear epitope. Also, this method was more effective for the long fixation samples as compared with other previous methods. However, this modified method could not retrieve PrP^{Sc} antigenic epitopes composed of conformational structures or several discontinuous epitopes. We describe the comparative studies between our improved method and other antigen retrieval procedures reported previously. Based on the differences of reaction among the antibodies, we also discuss the mechanisms of the hydrated autoclaving methods to retrieve PrP^{Sc} immunoreactivity.

Key words; Prion protein, Immunohistochemistry, Antigen

retrieval, Autoclaving, Monoclonal antibody

Introduction

Scrapie in sheep and goat, bovine spongiform encephalopathy (BSE), chronic wasting disease in deer and Creutzfeldt-Jakob disease (sporadic, iatrogenic, familial and variant forms) and Kuru in humans are transmissible neurodegenerative disorders belonging to a group of prion diseases. They are characterized by the accumulation of abnormal proteinase-resistant prion protein (PrP^{Sc}), that is an isoform of the cellular, proteinase-sensitive prion protein (PrP^{C}), as a result of post-translational modification with increases of the population of β-sheet conformation in the brain [20]. The pathology is characterized by neuronal cell loss, spongiform change, gliosis and deposition of abnormal amyloid protein.

Immunohistochemistry to demonstrate PrP^{Sc} in tissue sections is now a well-established technique for the diagnosis of prion diseases [2]. It has been reported that PrP^{Sc} immunoreactivity is enhanced by several antigen retrieval procedures such as formic acid [4,10], a combination of formic acid pretreatment and microwave processing [7,14], hydrated or hydrolytic autoclaving [6,11], guanidine thiocyanate [4,19], and combined protocols [1,8,9,15].

The recent disclosure of BSE in Japan has started an active

surveillance for all slaughter cattle since October 2001. Briefly, diagnostic procedure is follows: samples have been taken from the medulla oblongata (obex region) and examined by ELISA test as the primary screening test, and then the ELISA-positive samples have been confirmed by Western blot and/or immunohistochemistry. In starting an active surveillance for BSE in Japan, we applied various pretreatment methods for different antibodies to formalin fixed and paraffin-embedded tissues to enhance PrP^{Sc} immunoreactivity. Although the pretreatment methods reported previously were found to retrieve PrP^{Sc} for antibodies used in this study, we found that an improved hydrated autoclaving method at 135℃, more effectively enhanced PrP^{Sc} immunoreactivity for the antibodies recognizing the linear epitope. However, our modified method could not retrieve PrP^{Sc} antigens well for the monoclonal antibodies recognizing the conformational structures.

In this paper, we describe the comparative studies between our improved method and other antigen retrieval procedures reported previously, and discuss the mechanisms of the hydrated autoclaving methods to retrieve PrP^{Sc} immunoreactivity.

Materials and Methods

Samples

We used the brain tissues that were cut coronally at the level including hippocampus and thalamus from two scrapie–infected and two negative control ICR mice, the medulla oblongata at the level of the obex, and the spinal cord from three scrapie affected and two negative control sheep, and from three BSE affected cattle in Japan and two control cattle. Two mice were inoculated intracerebrally with scrapie G1 strain, which induces amyloid plaque formations in the brain. Affected or non-affected sheep and cattle were diagnosed and confirmed by histological, immunohistochemical, and Western blot methods. These samples were fixed in 15% formalin for 48 to 72 hrs and embedded routinely in paraffin. BSE tissue blocks were treated with 98% formic acid for 1 hr to reduce the infectivity of prion after formalin fixation. Also, we prepared the serial tissue blocks from the medulla oblongata of scrapie-affected sheep, which was immersed in 15% formalin at least for six months.

Immunohistochemistry

Serial tissue sections, 4μm in thickness, were picked up on silane-coated glass slides (Muto Purechemicals Co. Ltd, Japan). After deparaffinization, endogenous peroxidase was blocked by

incubation in 3% H_2O_2 for 5 minutes. We applied six different pretreatment protocols as follows: (1) 98% formic acid for 5 min (designated as FA); (2) hydrated autoclaving at 121℃, 2 atmosphere (atm) for 20 min (with Tomy high-pressure steam sterilizer KS-323. Japan) in distilled water (121DWHA); (3) 121DWHA and 98% formic acid for 5 min (121DWHA/FA); (4) 121DWHA and Proteinase K (0.4mg/ml, DAKO, USA) treatment for 1 min (121DWHA/PK); (5) hydrated autoclaving at 135℃, 3 atm for 20 min in distilled water (135DWHA); (6) 135DWHA and 98% formic acid for 5 min (135DWHA/FA). After applying each pretreatment, tissue sections were incubated with 10% goat or horse normal serum (Nichirei, Japan) for 30 min. In this study, we used the avidin-biotin complex methods (ABC kit; Vector Lab., USA) and the horseradish peroxidase-labeled polymer methods (Envision+ kit; DAKO, USA). Sections were exposed to primary antibodies for overnight at 4℃ or 1 hr at room temperature for ABC kit or Envision+ kit, respectively. As negative controls, the sections were exposed for each primary antibody without any pretreatments. The following steps were performed with second antibodies and others according to the each manufacture's instructions. The signals were detected using diaminobenzidine (Simple stain DAB; Nichirei, Japan).

Sections were counterstained with Mayer's hematoxylin.

The characteristics of the nine primary antibodies used in this study are summarized in Table 1.

For sections prepared from the tissue blocks immersed in 15% formalin for six months, we tested the 121DWHA and 135DWHA methods using B103 and 43C5 antibodies.

Morphometory

Serial sections from BSE were pretreated with 121DWHA, 121DWHA/FA, 121DWHA/PK, 135DWHA, and 135DWHA/FA methods, and immunostained with four antibodies (B103, 43C5, 44B1, and 6H4), respectively. Each of the pretreatment conditions were evaluated on the selected five areas (total μm^2). The Lumina Vision computer analysis system (Mitani Corp., Tokyo, Japan) was used to measure the positive area of PrP immunostaining. The brown-colored chromogen precipitate was selected, digitized images of these areas, and the digital pixels converted into the density area (μm^2)on the software. The highest density measurement was set to 100% and relative density (RD) of immunostaining by the other pretreatments in the same area was calculated.

Results

Histopathology

In the mouse, neuropil vacuolation associated with astrogliosis and microglial proliferation was observed throughout all areas of the brain. Also seen were the amyloid plaque structures, which were often observed in contact with the capillary vessels.

In the obex region of scrapie-infected sheep, neuropil vacuolation and single or multiple intracytoplasmic vacuoles were particularly found in the dorsal motor nucleus of vagus nerve (DMNV), gracile nucleus, nucleus ambiguus and reticular formation [22]. The hypoglossal nucleus, olivary nucleus and nucleus of solitary tract (NST) were only mildly affected. Also, spongiform neuropil lesions were seen in the periphery of the dorsal column of spinal cord and the vertebral column.

In BSE cases, because of the subclinical case, extremely mild spongiform lesions were observed only in DMNV, and periphery of the reticular formation [24].

Immunohistochemistry

Immunohistochemical examination revealed that no prion protein depositions were observed in the sections from the affected animals without pretreatment and from the control animals with and without pretreatment.

PrP^{Sc} immunostaining yielded characteristic patterns in each animals affected with prion disease [21]. The following immunostaining patterns were observed: (1) fine particulate deposition; (2) coarse particulate deposition; (3) perineuronal deposition; (4) glial type deposition; (5) perivascular or perivacuolar deposition; (6) plaque or plaque-like deposition.

In scrapie-infected mice, PrP^{Sc} deposits were observed diffusely in cortex, thalamus, and hippocampus. Also seen were perivascular, perivacuolar PrP^{Sc} deposits and plaque or plaque-like structures in thalamus.

The PrP^{Sc} deposits in scrapie-affected sheep were most intense in DMNV. The hypoglossal nucleus showed also the deposits, but the staining intensity was sparse. Glial, coarse particulate, perineuronal, perivascular and perivacuolar depositions were found in the reticular formation.

In BSE cases, the intense positive reactions of PrP^{Sc} were observed in DMNV, NST and periphery of the reticular formation, which were fine, perineuronal and perivacuolar patterns. The fine or coarse particulate depositions were seen in olivary nucleus. The hypoglossal nucleus showed also positive reactions, but a low intensity of the immunostaining.

Comparison of pretreatment methods

Microscopic examinations in each pretreatments and antibodies were summarized in Table 2. All antibodies used in this study reacted with PrP^{Sc} of all animals under some pretreatments.

Single FA pretreatment gave very weak or no reactions to all antibodies, however, mouse amyloid plaques were positive to some antibodies (mAbs 149, 43C5, 72, 44B1, 44B2, and T2). Among 121DWHA, and a combination of 121DWHA with FA (121DWHA/FA) or PK (121DWHA/PK) pretreatments, 121DWHA/FA was most effective in most antibodies, but 121DWHA/PK was more or equally in the effect as compared with 121DWHA/FA in mAb 43C5 and pAb B103. According to the sensitivity to 135DWHA and 135DWHA/FA pretreatments, the antibodies used in this study were divided into two types, one of which showed the increase in reactivity, and the other significantly reduction or the loss of the reactivity. The mAbs 132, 149, 43C5 and pAb B103 in reacting the linear epitope classified to the former group (Figs. 1a-d), while mAbs 72, 44B1, 44B2 and T2 recognizing the discontinuous epitope referred to the latter group (Figs. 2a-d). The mAb 6H4 against cow recombinant PrP reacted on bovine, ovine and murine PrP^{Sc} with 121DWHA/FA pretreatment, but other pretreatments showed no positive reactions for all animals PrP^{Sc} except for

the murine plaque type of PrP^{Sc} with 121DWHA, 121DWHA/FA and 121DWHA/PK pretreatments.

Effects on the sections from the tissues immersed in formalin for long period

In 121DWHA and 135DWHA pretreatment, both antibodies (B103 and 43C5) gave positive reactions in almost same areas for the sections of scrapie-affected sheep, which were immersed in formalin for six months. However, the sections treated by 135DWHA method yielded intense signal and widespread staining, while the detectable antigens in 121DWHA pretreatment were limited and showed low intensity (Figs. 3a and b).

Morphometry

The comparison of relative density (RD) in each pretreatment method on BSE sections is shown in Fig. 4. This digital morphometry was in good agreement with the observations through the microscope. Although 135DWHA or 135DWHA/FA methods increased the density on the mAb 43C5 and the pAb B103, the effect of these methods was stronger on the former than on the latter (Figs. 4a and b). In mAbs 44B1 and 6H4, 121DWHA/FA protocol was appropriate for the antigen retrieval and the 135DWHA or 135DWHA/FA method were not effective (Figs. 4c and d).

Discussion

In immunohistochemistry for the prion diseases, several pretreatment methods to enhance the immunoreactivity of human and animal PrP^{Sc} on the tissue sections have been reported. 121℃ hydrated autoclaving pretreatment method or the combination method of 121℃ hydrated autoclaving with a certain chemical reagent (formic acid or Proteinase K, etc) are now widely used [5,13]. The precise mechanisms by which pretreatments enhance the PrP^{Sc} immunoreactivity are still unknown. However, the effect of those chemical treatments is considered to make amyloid fibril proteins denature and break down the structure of amyloid fibrils, and expose the buried epitopes [4]. In formic acid pretreatment, microwave irradiation is thought to enable for formic acid penetration in tissue, and to expose the epitope and react with the antigen more efficiently [7]. Also, it is speculated that hydrolytic autoclaving contribute to alter the primary structures of PrP in situ [11].

In this study, we described the new hydrated autoclaving method called 135DWHA method to enhance the immunoreactivity of the PrP^{Sc}, which were compared with the methods reported previously. Generally, 135DWHA or 135DWHA/FA method for the

antibodies reacting with linear epitope showed higher sensitivity than 121DWHA, 121DWHA/FA, or 121DWHA/PK methods, except antibody 6H4. Although prolonged exposure of brain material to aldehydes fixatives usually dramatically decreases the antigenicity of PrP^{Sc} [16], this newly enhancing method revealed more effective for the long fixation samples as compared with other previous methods. On the other hand, our simple modification for the antibodies recognizing discontinuous or conformational epitope could not exert to enhance the prion antigen.

In the immunohistochemical antigen retrieval techniques, breaking crosslinking theory [17] and protein denaturation theory or modification-re-modification hypothesis [3] have been proposed, and thought to have advantage on the basis of observation or supported by several studies, respectively [18,22,23]. Especially, the later theory is based on heat- or chemical-induced modification of the three-dimensional structure of “formalinized” protein, restoring the condition of a formalin-modified protein structure back towards its original structure on the paraffin-embedding tissue sections. Because immunohistochemistry without pretreatment did not give any positive reactions using the pAb B103 and 44B1 in the frozen

sections (data not shown), there are some differences between this theory and our model. However, it seemed probable that the hidden epitope caused by the aggregation of prion proteins exposes in the surface, or the conformational binding sites formed by the other protein molecules disintegrated due to certain conformational changes by the hydrating autoclave methods on the formalin-fixed paraffin sections, assuming that the principle of the antigen retrieval methods is to lead to a re-naturation or partial restoration of the protein structure with re-establishment of the three-dimensional to something approaching its native condition [22,23].

Antibody, especially reacting on discontinuous epitope, recognizes specific epitope localized in a spatial configuration within the protein molecule. The mAb 15B3 recognized discontinuous epitope in the pathological PrP isoform, and a single continuous 15B3 binding site was speculated to be formed either by aggregation of two or several PrP molecules, or by structural rearrangement of a single PrP molecule, or by a combination thereof [12]. Exact mechanisms of the differences between 135DWHA and 121DWHA methods in the antibodies recognizing conformational epitope are still unknown. However, these things can be surmised as follows; Some

of the aggregate proteins or molecules may be loosely arranged and antigenic determinant come to lie on the surface at formic acid or 121℃, 2 atm; Furthermore elevation of the temperature and atmosphere may change certain stereoscopic structure or component of PrP molecules, bring loss of its conformational epitope; Additional formic acid treatment give little change and make some conformational epitope come out.

Further studies of prion antigen retrieval technique, including establishment the exact correlation of this mechanisms and the antibody epitope, may shed new light in the pathology of the prion diseases.

Acknowledgments

This work was supported by a Grant-in-Aid for Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant 14656118) and a Grant from the Ministry of Health, Labour and Welfare of Japan (grant 14240101).

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Legends

Fig. 1. Immunohistochemistry of the prion protein with mAb 43C5 in the thalamus of scrapie-affected mouse; **a** 121DWHA, **b** 121DWHA/FA, **c** 135DWHA and **d** 135DWHA/FA methods. The immunodensity is considerably greater in 135DWHA and 135DWHA/FA methods. *Bars* **a-d** 50 μm

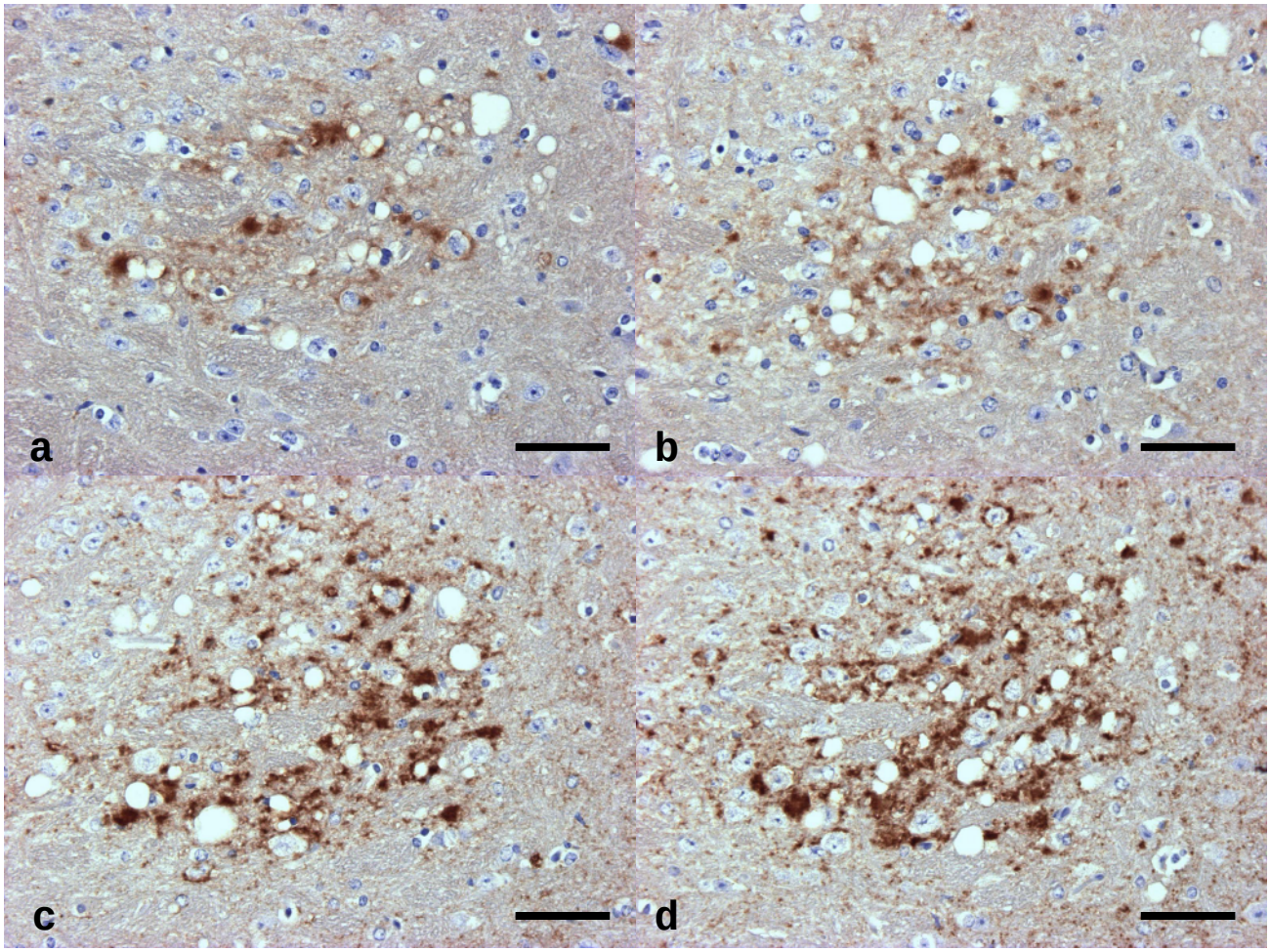
Fig. 2. Immunohistochemistry of the prion protein with mAb 44B1 in the thalamus of scrapie-affected mouse; **a** 121DWHA, **b** 121DWHA/FA, **c** 135DWHA and **d** 135DWHA/FA methods. 121DWHA/FA

method is most effective for enhancing the prion protein and only faint reactivity is observed in 121DWHA method. Although 135DWHA/FA method enhances the prion protein, no reactive deposits are observed in 135DWHA method. *Bars* **a-d** 50 μm

Fig. 3. Immunohistochemistry of the prion protein with pAb B103 in the sections of scrapie-affected sheep from the sample immersed formalin for six months; **a** 121DWHA and **b** 135DWHA methods. *Bars* **a, b** 500 μm

Fig. 4. Comparison of relative density (RD%) under each pretreatment method shows on each antibody for BSE sections; **a** pAb B103, **b** mAb 43C5, **c** mAb 6H4 and **d** mAb 44B1. MAb 44B1 recognizes discontinuous epitope, and others recognize linear epitope. The effect of FA, 121DWHA, 121DWHA/FA, 121DWHA/PK, 135DWHA and 135DWHA/FA is displayed on the bar A, B, C, D, E and F, respectively. **a**. FA displayed 1 RD%. 121DWHA (29 RD%) did not lead to a significant increase. 121DWHA/FA and 121DWHA/PK showed a density of 81, 75 RD%, respectively. Immunodensity was further enhanced by 135DWHA (100 RD%) and 135DWHA/FA (100 RD%). **b.** FA displayed 1 RD%. 121DWHA, 121DWHA/FA and 121DWHA/PK showed 15, 22, 18 RD%, respectively. 135DWHA and 135DWHA/FA resulted in 100 and 94 RD%, which lead to significant increase. **c.** FA (5 RD%), 121DWHA (17 RD%),

121DWHA/PK (3 RD%), 135DWHA (1 RD%) and 135DWHA/FA (2 RD%) did not lead to significant increase in immunodensity. 121DWHA/FA (100 RD%) was the highest increase. **d.** FA showed 22 RD% and 121DWHA 25 RD%. Imunodensity was not enhanced by 121DWHA/PK and 135DWHA. 135DWHA/FA displayed 4 RD%. The best result was obtained by 121DWHA/FA (100 RD%).



Bar:50µm

Figs.1a-d
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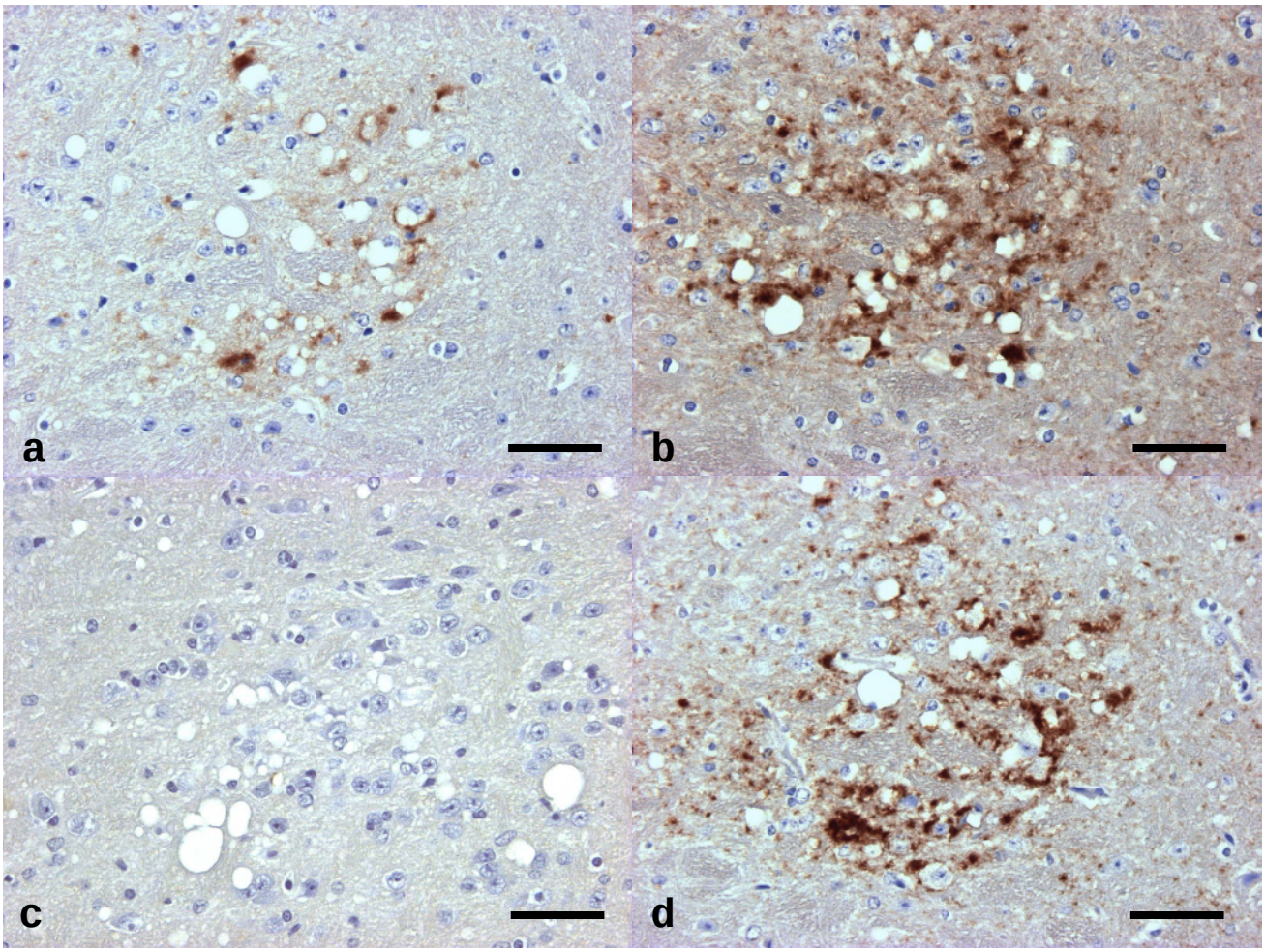

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Figs.2a-d
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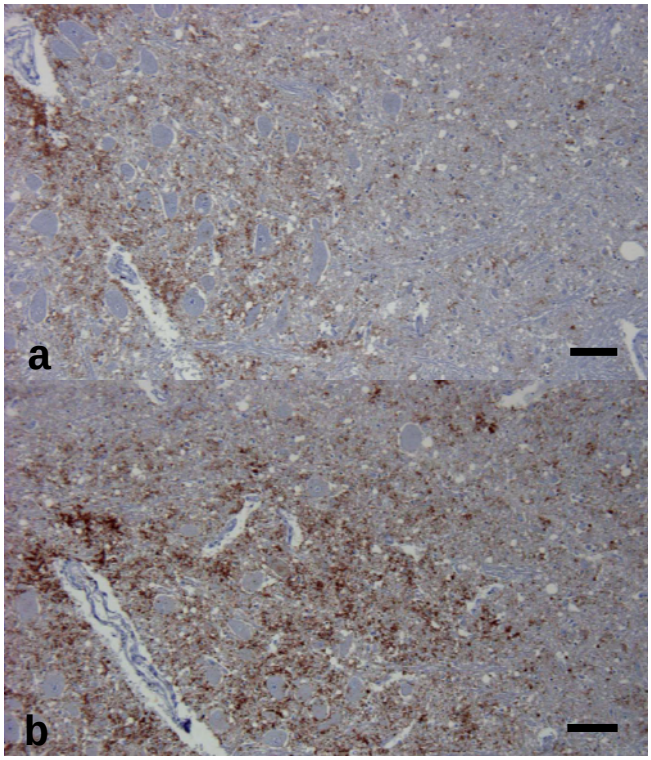

Bar:100µm

Figs.3a,b
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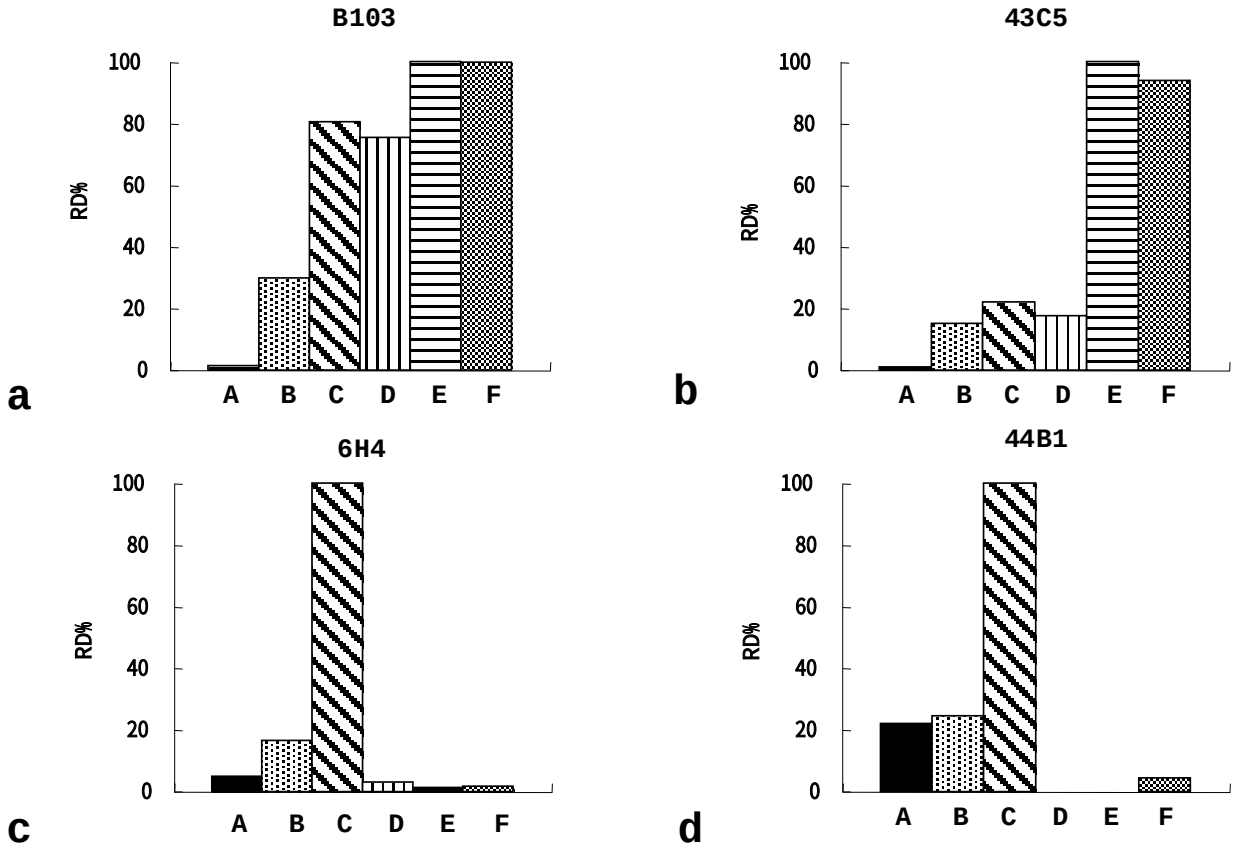
B103
RD%
100
80
60
40
20
0
A B C D E F
a
43C5
RD%
100
80
60
40
20
0
A B C D E F
b
6H4
RD%
100
80
60
40
20
0
A B C D E F
c
44B1
RD%
100
80
60
40
20
0
A B C D E F
d

Fig. 4

Antibodies	Epitope		Clonality	Dilution	Immunogen	Source
	Position	L/DC				
132	119-127	L	mAb	1/200	Mouse recPrP	Horiuchi
149	147-153	L	mAb	1/500	Mouse recPrP	Horiuchi
43C5	163-169	L	mAb	1/10000	Mouse recPrP	Horiuchi
B103	103-121	L	pAb	1/1000	Cow recPrP	Horiuchi
6H4	155-163	L	mAb	1/500	Cow recPrP	Prionics (Zürich, Switzerland)
72	89-231(143-151)	DC	mAb	1/500	Mouse recPrP	Horiuchi
44B1	155-231	DC	mAb	1/200	Mouse recPrP	Horiuchi
44B2	155-231	DC	mAb	1/200	Mouse recPrP	Horiuchi
T2	unknown	DC	mAb	1/500	Mouse recPrP	Tagawa

Table 1. Characteristics of the nine antibodies used in this study.

L: linear epitope; DC: discontinuous epitope; mAb: monoclonal antibody; pAb: polyclonal antibody

Antibodies / Pretreatment	132			149			43C5			B103			6H4		
	M	C	S	M	C	S	M	C	S	M	C	S	M	C	S
	P/D			P/D			P/D			P/D			P/D		
FA	-/-	-	-	2+/+	-	-	+/-	-	-	-/-	-	-	-/-	-	-
121DWHA	2+/+	-	-	+/+	-	+	2+/2+	2+	2+	+/+	+	+	+/+	+	+
121DWHA/FA	2+/2+	+	2+	2+/2+	2+	2+	2+/2+	2+	2+	2+/2+	2+	2+	2+/2+	2+	2+
121DWHA/PK	+/-	-	-	2+/+	-	2+	2+/2+	2+	2+	2+/2+	2+	2+	+/-	-	-
135DWHA	2+/+	3+	2+	3+/3+	+	2+	3+/2+	2+	3+	3+/3+	3+	3+	-/-	-	-
135DWHA/FA	2+/2+	3+	3+	2+/+	3+	3+	3+/2+	3+	3+	3+/3+	3+	3+	-/-	-	-

Antibodies / Pretreatment	72			44B1			44B2			T2		
	M	C	S	M	C	S	M	C	S	M	C	S
	P/D			P/D			P/D			P/D		
FA	2+/-	+	-	+/-	+	-	+/-	-	-	2+/-	-	-
121DWHA	+/-	-	+	+/-	+	-	+/-	-	-	+/-	-	-
121DWHA/FA	3+/+	2+	2+	2+/2+	2+	2+	3+/2+	2+	2+	3+/++	2+	2+
121DWHA/PK	-/-	+	-	-/-	-	-	-/-	-	-	-/-	-	-
135DWHA	-/-	-	-	+/-	-	-	-/-	-	-	-/-	-	-
135DWHA/FA	2+/-	-	-	2+/2+	+	+	2+/+	-	-	+/-	+	-

Table 2. Results of the immunoreactivity for the antibodies under pretreatment methods.

FA: 96% formic acid for 5 minutes; 121DWHA: hydrated autoclaving at 121℃, 2 atmosphere (atm) for 20 minutes in distilled water; 121DWHA/FA: 121DWHA and 96% formic acid for 5 minutes; 121DWHA/PK: 121DWHA and Proteinase K treatment for 1 minute; 135DWHA: hydrated autoclaving at 135℃, 3 atm for 20 minutes in distilled water; 135DWHA/FA: 135DWHA and 98% formic acid for 5 minutes.

M: scrapie-affected mouse; C: BSE-affected cow; S: scrapie-affected sheep; P: plaque type; D: diffuse type

3+: Strongly positive signal; 2+: moderately positive signal; +: faint positive signal; -: negative