Species-specificity of a Panel of Prion Protein Antibodies for the Immunohistochemical Study of Animal and Human Prion Diseases

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Running title: Species-specificity of PrP antibodies.

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Summary

Monoclonal antibodies to the prion protein (PrP) have been of critical importance in the neuropathological characterization of PrP-related disease in men and animals. To determine the influence of species-specific amino-acid substitutions recognized by monoclonal antibodies, and to investigate the immunohistochemical reactivity of the latter, analyses were carried out on brain sections of cattle with bovine spongiform encephalopathy, sheep with scrapie, mice infected with scrapie, and human beings with Creutzfeldt-Jakob disease (CJD) or Gerstmann-Sträussler-Sheinker disease (GSS). Immunoreactivity varied between the antibodies, probably as the result of differences in the amino-acid sequence of the prion protein in the various species. Some monoclonal antibodies against mouse recombinant PrP gave strong signals with bovine, ovine and human PrP^{Sc}, in addition to murine PrP^{Sc}, even though the amino-acid sequences determined by the antibody epitope are not fully identical with the amino-acid sequences proper to the species. On the other hand, in certain regions of the PrP sequence, when the species-specificity of the antibodies is defined by one amino-acid substitution, the antibodies revealed no reactivity with other animal species. In the region corresponding to positions 134-159 of murine PrP, immunohistochemical reactivity or species-specificity recognized

by the antibodies may be determined by one amino acid corresponding to position 144 of murine PrP. Not all epitopes recognized by a monoclonal antibody play an important role in antigen-antibody reactions in immunohistochemistry. The presence of the core epitope is therefore vital in understanding antibody binding ability.

Keywords: BSE; cattle; CJD; GSS disease; man; mouse; scrapie; sheep

Introduction

Sheep and goat scrapie, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob disease (CJD) (sporadic, iatrogenic, familial and variant forms), Gerstmann-Sträussler-Sheinker disease or syndrome (GSS) and Kuru disease in man are all referred to as prion diseases. A common feature of these diseases is the accumulation of abnormal proteinase-resistant prion protein (PrP^{Sc}), an isoform of the cellular proteinase-sensitive prion protein (PrP^{C}), which occurs as a result of post-translational modification leading to increases in the population of β-sheet conformation in the brain (Prusiner, 1998).

Monoclonal antibodies raised against the prion protein (PrP) have been of critical importance in the neuropathological characterization of PrP-related disease in man and animals (Bodemer, 1999). Numerous monoclonal antibodies (mAbs) for detecting prion proteins in tissue sections have been developed and characterized in terms of species-specificity and epitope (Kascsak *et al.*, 1987; Bolton *et al.*, 1991; Piccardo *et al.*, 1998; Zanusso *et al.*, 1998; Van Everbroeck *et al.*, 1999a; Privat *et al.*, 2000). For example, mAb 3F4, whose epitope is mapped between amino acids 109 and 112 of the human prion protein, has been widely used in immunohistochemistry and immunoblotting experiments in human prion

disease (Kascsak *et al.*,1987; Zanusso *et al.*, 1998). This antibody detects the prion protein in man and the hamster but not in the mouse, cow, sheep, Capuchin monkey or squirrel (Zanusso *et al.*, 1998). On the other hand, mAb F89/160.1.5, raised against a synthetic peptide and representing residues 146 to 159 of the bovine prion protein, reacts with the prion protein in human, ovine and bovine tissue (O'Rourke *et al.*,1998; Van Everbroeck *et al.*, 1999a). Although the prion protein is highly conserved, there is some sequence divergence among species. Possibly a single amino-acid substitution affects epitope recognition by mAb 3F4, in the case of mAb F89/160.1.5 it is possible that the amino-acid sequence recognized is based on the epitope conserved in man and ruminant species.

To determine the effects of species-specific amino-acid substitutions, immunohistochemical analysis with a panel of monoclonal antibodies was undertaken on sections of brain tissue from BSE-infected cattle, scrapie-infected sheep, scrapie-infected mice, and human CJD and GSS cases.

Materials and Methods

Samples

Immunohistochemical analysis was carried out on the following brain tissues, cut coronally at the appropriate level: hippocampus and

thalamus from two ICR mice inoculated intracerebrally with the Obihiro strain of scrapie (Shinagawa *et al.*, 1985) and from two negative control mice; medulla oblongata at the level of the obex and spinal cord from three scrapie-affected and two negative control sheep and from three BSE-affected and two control cattle. The disease status of the cattle and sheep was established by histological, immunohistochemical, and Western blot methodology. These samples were fixed in 15% formalin for 48 to 72 h and embedded in paraffin wax by conventional methods. Tissue blocks containing BSE-affected tissue were treated with 98% formic acid for 1 h to reduce the risk of prion infectivity.

In addition, human post-mortem brain samples were obtained from one patient with sporadic CJD (sCJD) (63-year-old male; codon 129M/M, codon 219E/E) and one with GSS (57-year-old male with PrP P105L mutation; codon 129Val/Val, codon 219E/E). These samples has been fixed in 15% formalin, after which the fixed blocks were immersed in 98% formic acid for 1 h and embedded in paraffin wax. A few tissue sections, including those of the cerebral cortex, prepared from these blocks, were submitted to our laboratory.

Immunohistochemistry(IHC)

Serial tissue sections (4μm) were placed on silane-coated glass

slides (Muto Purechemicals, Tokyo, Japan). After dewaxing, endogenous peroxidases were blocked by incubation in 3% H_2O_2 for 5 min. Six different pretreatment procedures were used, as follows. (1) Pretreatment “FA” (98% formic acid for 5 min). (2) Pretreatment “121DWHA” (hydrated autoclaving at 121℃, 2 atmospheres [atm] for 20 min in distilled water). (3) Pretreatment “121DWHA/FA” (121DWHA and 98% formic acid for 5 min). (4) Pretreatment “121DWHA/PK” (121DWHA and Proteinase K [0.4mg/ml; DAKO, California, USA] treatment for 1 min. (5) Pretreatment “135DWHA” (hydrated autoclaving at 135℃, 3 atm for 20 min in distilled water). (6) Pretreatment “135DWHA/FA (135DWHA and 98% formic acid for 5 min). The last two methods (5 and 6) were improved hydrated autoclaving methods designed to retrieve PrP^{Sc} immunoreactivity and to be more sensitive the previous three methods (2-4) for the antibodies reacting with linear epitopes (Furuoka *et al.*, 2004). Because of the limited number of slide sections, sCJD and GSS cases were pretreated by only one, or at most two, of the six methods. After pretreatment, tissue sections were incubated with 10% normal goat or normal horse serum (Nichirei, Tokyo, Japan) for 30 min. The horseradish peroxidase-labelled polymer method (Envision+ kit; DAKO) was used to “visualize” positive antibody binding. The 13 primary antibodies (12 monoclonal and one polyclonal) used are

listed in Table 1. Sections were exposed to primary antibodies for 1 h at room temperature. As negative controls, further sections were exposed to each primary antibody without any of the pretreatments. The sections were then incubated with the second antibody for 30 min at room temperature. Positive immunoreactive binding signals were detected with diaminobenzidine (Simple stain DAB; Nichirei). Sections were counterstained with Mayer's haematoxylin. The intensity of specific labelling was scored as follows: 3+, strong; 2+, moderate; +, weak; -, nil.

Results

Histopathology and Immunohistochemistry

The typical lesions and patterns described previously in each species (Fraser and Dickinson, 1968; Wells *et al.*, 1992; Privat *et al.*, 2000; Ryder *et al.*, 2001) were seen. In scrapie-infected mice, neuropil vacuolation was associated with astrogliosis, and microglial proliferation was observed throughout all areas of the brain. Immunohistochemical PrP^{Sc} deposits were distributed diffusely in the cortex, thalamus, and hippocampus.

In the obex region of scrapie-infected sheep, neuropil vacuolation and single or multiple intracytoplasmic vacuoles were found particularly in the dorsal motor nucleus of the vagus nerve (DMNV),

which also exhibited particularly intense PrP^{Sc} deposition. Numerous amyloid plaques along blood vessels or plaque-like structures showed PrP^{Sc} accumulations in the reticular formations. Although other nuclei showed immunohistochemical PrP^{Sc} deposits, the intensity was mild. PrP^{Sc} deposition varied in type (fine particulate, coarse particulate, glial-vacuolar, peri-vacuolar).

Due to the subclinical nature of the BSE cases, spongiform lesions (mild) were observed only in the DMNV, and at the periphery of reticular formations. Intense PrP^{Sc} immunoreactivity was also observed at these two locations. Fine or coarse particulate deposits were seen in the olivary nucleus. The hypoglossal nucleus showed positive immunoreactivity, but at a low intensity. In the spinal cord, no obvious vacuolation was observed. Immunohistochemically, however, fine and coarse particulate deposits, and linear and perineuronal labelling were seen in the neuropil of the grey matter. No PrP^{Sc} deposits were observed in untreated sections from the affected animals, or in section (with or without pretreatment) from control animals.

The sCJD case showed mild to moderate spongiform degeneration in the cerebral cortex, associated with neuronal loss and severe astrogliosis. Immunolabelling, which was confined to the cerebral cortex, was diffuse, synaptic granular or coarse particulate. In

the GSS case, the characteristic lesions consisted of numerous multicentric amyloid plaques in the cerebral cortex. Neuronal loss and severe astrogliosis were also observed, but spongiform degeneration was not prominent. All plaques was immunolabelled with PrP antibodies. Diffuse PrP deposits were present in deep layers of the neocortex. Also, large punctate areas of immunoreactivity were observed between nerve cell bodies.

Antibody Reactivity for Different Species

The characteristics of 12 primary monoclonal antibodies and one polyclonal antibody (B103) used in this study are summarized in Table 1. The epitopes of the prion protein recognized by these antibodies are shown in Fig. 1. The immunolabelling results for each pretreatment and antibody are summarized in Table 2. The antibodies used in this study showed pattern of immunoreactive binding that varied with the method of pretreatment. However, the specificity of each antibody in terms of species was well defined, and is shown in Table 1. With all antibodies, FA pretreatment gave weak if any immunolabelling. With most but not all antibodies, 121DWHA/FA pretreatment was more effective than 121DWHA and 121DWHA/PK pretreatment. In general, 135DWHA and 135DWHA/FA were preferable to other methods of pretreatment for antibodies reacting with linear

epitopes.

Of eight mAbs for mouse recombinant PrP, three (mAbs 110, 132, and T1) reacted with PrP^{Sc} in all species under some pretreatments (Fig.2a-d). MAb 149 reacted with murine, bovine and ovine PrP^{Sc}, but showed no immunoreactivity for human PrP^{Sc}. The mAbs 118, 31C6 and 147 exhibited positive immunoreactivity only with murine PrP^{Sc} and none for bovine, ovine or human PrP^{Sc} (Fig.2e-h). The mAb 43C5 revealed strong immunoreactivity in all animals, but none in human samples. MAb F89/160.1.5, against recombinant cow PrP, gave immunolabelling invariably, except for murine PrP^{Sc} (Fig.2i-l). The mAb 6H4 reacted with bovine, ovine, murine and human PrP^{Sc} with 121DWHA/FA or 121DWHA/PK pretreatment, but not with other pretreatments. Polyclonal antibody B103 against recombinant cow PrP gave positive reactions in all species. The mAb 3F4, which recognizes human recombinant PrP, revealed immunoreactivity with PrP^{Sc} in human but not bovine, ovine or murine samples. The mAb 12F10, also raised against human recombinant PrP, showed positive immunoreactivity for human, bovine and ovine PrP^{Sc} but no immunoreactivity for murine PrP^{Sc}. This particular mAb immunolabelled bovine PrP^{Sc} under every pretreatment. However, as with the ovine PrP^{Sc}, pretreatment with 135DWHA and 135DWHA/FA failed to retrieve the antigen.

As shown in table 2, immunolabelling intensity reflecting the detectable PrP^{Sc} varied with the method of pretreatment (Furuoka *et al.*, 2004). However, there were no obvious differences in labelling patterns.

Discussion

Immunohistochemical demonstration of PrP^{Sc} in tissue sections serves to confirm the diagnosis of prion diseases (Bodemer, 1999). However, specific pretreatment of paraffin wax sections is necessary to enhance PrP^{Sc} immunoreactivity. Pretreatment methods based on hydrated autoclaving at 121℃ or a combination of hydrated autoclaving (121℃) with certain chemical reagents (e.g., formic acid or Proteinase K) are now widely used (Kitamoto *et al.*, 1992; Van Everbroeck *et al.*, 1999b; Kovács *et al.*, 2002). Furuoka *et al.* (2004) reported that an improved hydrated autoclaving method (135℃), enhanced PrP^{Sc} immunoreactivity with antibodies recognizing the linear epitope. The effect of chemical treatments may be to break down the structure of amyloid fibrils and expose the buried epitopes (Doi-Yi *et al.*, 1991; Hashimoto *et al.*, 1992). It is further speculated that hydrated autoclaving contributes to alterations in the three-dimensional structures of PrP, and unraveling the buried epitope caused by PrP aggregation (Kitamoto *et al.*, 1992).

MAbs for mouse recombinant PrP reacted with PrP^{Sc} and PrP^{Sc} in other species provided that these species possessed the same amino-acid sequence in the segment recognized by these antibodies (mAbs 132 and 149). However, the immunoreactivity of mAbs 110, T1, 118, 31C6, 147, F89/160.1.5, 6H4, 3F4 and 12F10 varied when, in heterologous species, the species-specific amino-acid sequence differed. The mAb 110 epitope lies within mouse prion protein residues 56-90, with a species specificity conferred via Ser71 and Ser79. The mouse amino-acid sequence recognized by the mAb T1 epitope showed an overlap with the ovine PrP sequence, but did not fully overlap with the bovine PrP sequence. The mAbs 110 and T1 gave strong immunoreactive signals with bovine, ovine and human PrP^{Sc}, as well as with murine PrP^{Sc}, even though the amino-acid sequence in the epitopes of these antibodies was not completely identical.

On the other hand, mAbs 118 and 31C6 exhibited no immunoreactivity with bovine, ovine or human PrP^{Sc}. The murine amino-acid sequence recognized by mAb 118 recognizes three and two distinct amino-acid sequences in human beings and cattle, respectively, and one such sequence in sheep. One distinct amino-acid region is mapped by mAb 31C6. The mAb F89/160.1.5, which recognizes the 146-157 bovine sequence (O'Rourke *et al.*, 1998), detected bovine, ovine and human PrP^{Sc} but not murine PrP^{Sc}. Similarly, mAb 12F10, raised against human

recombinant PrP (which recognizes protein residues 142-160 in the human sequence), produced strong immunolabelling of bovine and ovine PrP^{Sc} but not murine PrP^{Sc}. It appeared that the species-specific epitope in each antibody was defined by one amino-acid substitution, as follows: tryptophan at position 144 of murine PrP in mAbs 118 and 31C6; tyrosine at position 156 of bovine PrP in mAb F89/160.1.5; and tyrosine at position 145 of human PrP in mAb 12F10. Thus, in the region recognized by these antibodies (T1, 31C6, 118, 149, F89/160.1.5, 6H4 and 12F10; corresponding to positions 134-159 of murine PrP), immunohistochemical reactivity or species-specificity associated with the antibodies may be determined by one amino acid corresponding to position 144 of murine PrP. In addition, the results suggested that the relative immunoreactivity of the antibodies was characterized by the core epitope present in the region of amino-acid sequences recognized by the antibody; i. e., the antibody epitope contained a region of high specificity consisting of only a few epitopes, or unable to recognize different conformations.

The mAb 6H4, produced against cattle recombinant PrP and recognizing protein residues 155-163 in the bovine sequence (Korth *et al.*, 1997) (corresponding to positions 143-151 of murine PrP, recognized by mAb 31C6), produced strong labelling of murine and

ovine PrP^{Sc} as well as bovine PrP^{Sc}. The mAbs 31C6 and 6H4 shared certain features. For instance, the partial or complete loss of immunoreactivity resulting from PK, 135DWHA or 135DWHA/FA pretreatment suggested that the region recognized by mAbs 31C6 and 6H4 may includes the labile conformation affected by these three pretreatments. However, the immunohistochemical core epitope of mAb 6H4 appears not to be defined by the substitution of tyrosine for tryptophan, while mAb 31C6 would seem to be species-specific because of the epitope defined by tryptophan at position 144 of murine PrP. The findings suggested that sequence variations recognized by the antibody were not solely responsible for its immunohistochemical specificity. Equally important was the location of the amino-acid substitution in the epitope.

The mAb KG9 against cow recombinant PrP, which recognizes the bovine sequence HRYPN at positions 166-170 (corresponding to positions 154-158 of murine PrP), reacted with bovine PrP^{Sc} but not sheep PrP^{Sc} (Laffling *et al.*, 2001). On the other hand, mAb L42 raised against an ovine synthetic peptide between positions 145-163 (corresponding to positions 141-159 of murine PrP) reacted with PrP of both cattle and sheep in Western blotting studies (Harmeyer *et al.*, 1998; Vorberg *et al.*, 1999). It is possible that the presence of tyrosine at position 166 of ovine PrP^{Sc} influences the conformation

of the relocated KG9 sequence in ovine PrP^{Sc} at the conversion of PrP^{C} to PrP^{Sc} and prevents reaction with the antibody, because mAb KG9 showed similarly low immunoreactivity with neurons of normal sheep and cattle (Laffling *et al.*, 2001). Although it could not be ruled out that the antigen retrieval method failed to unmask the KG9 epitope in PrP^{Sc} in ovine sections as effectively as in bovine sections (Laffling *et al.*, 2001), the existence of the core epitope may explain the differences between the reactivity of mAbs KG9 and L42.

In conclusion, this study demonstrated the immunohistochemical properties (e. g., species-specificity) of newly developed antibodies, and the existence of a core epitope responsible for antibody specificity. Western blotting reactivity and biochemical analysis of the antibodies used in this study will be described elsewhere.

Acknowledgments

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Legends

Fig. 1. The locations of the prion protein epitopes recognized by the antibodies used in this study.

Amino-acid sequences are based on National Center for Biotechnology Information (NCBI) Protein Databases. MoPrP: amino-acid sequence of murine prion protein (Accession Number NP_035300). BoPrP: amino-acid sequence of bovine prion protein (Accession Number NP 851358. ShPrP: amino-acid sequence of ovine prion protein (Accession Number NP_001009481). HuPrP: amino-acid sequence of human prion protein (Accession Number AAR21603).  ,Antibody against recombinant mouse PrP;  ,antibody against recombinant cow PrP;  ,antibody against recombinant human PrP.

Fig. 2a-l. Immunolabelling of murine, bovine, ovine and human PrP^{Sc} with different antibodies. (a, e and i) Thalamus of scrapie-affected mouse. (b, f and j) DMNV of BSE-affected cow. (c, g and k) DMNV of scrapie-affected sheep. (d, h and l) Cerebral cortex of a GSS case. (a-d) mAb T1. (e-h) mAb 118. (i-l) mAb F89/160.1.5. The mAb T1 reacts with murine, bovine, ovine, and human PrP^{Sc}. The mAb 118 reacts only with murine PrP^{Sc}. The mAb F89/160.1.5 reacts with bovine, ovine and human PrP^{Sc} but not with murine PrP^{Sc}. IHC. Bars, 200µm.

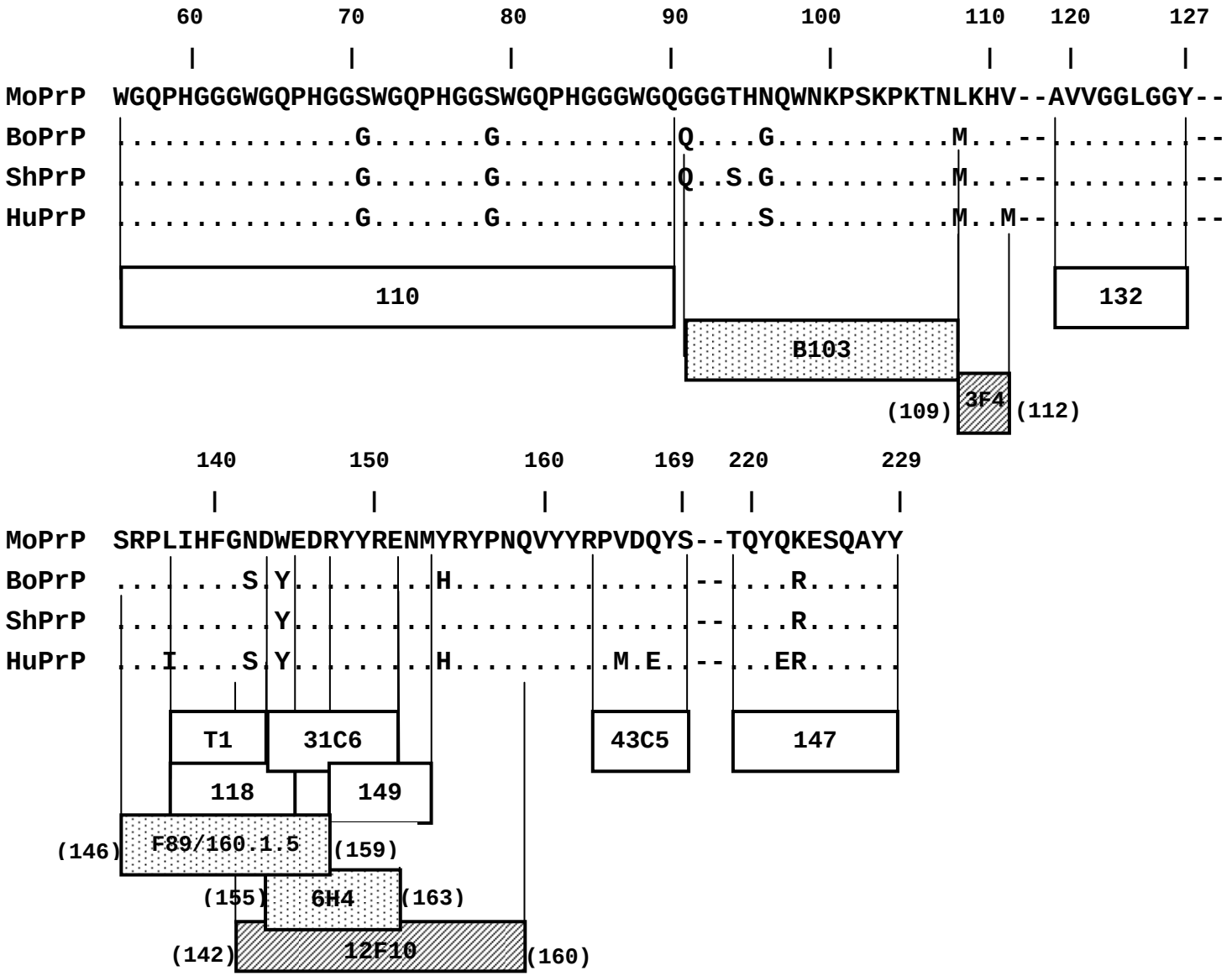
60
70
80
90
100
110
120
127
MoPrP WGQPHGGGWGQPHGGSWGQPHGGSWGQPHGGGWGQGGGTHNQWNKPSKPKTNLKHV--AVVGGLGGY--
BoPrP .............G.....G.........Q....G.........M...--.........--
ShPrP .............G.....G.........Q..S.G.........M...--.........--
HuPrP .............G.....G.............S.........M..M--.........--
110
132
B103
(109)
3F4
(112)
140
150
160
169
220
229
MoPrP SRPLIHFGNDWEDRYYRENMYRYPNQVYYRPVDQYS--TQYQKESQAYY
BoPrP .....S.Y.....H.....--...R.....
ShPrP .....Y.....--...R.....
HuPrP ..I....S.Y.....H.....M.E..--...ER.....
T1
31C6
43C5
147
118
149
(146)
F89/160.1.5
(159)
(155)
6H4
(163)
(142)
12F10
(160)

Fig.1

Fig.2

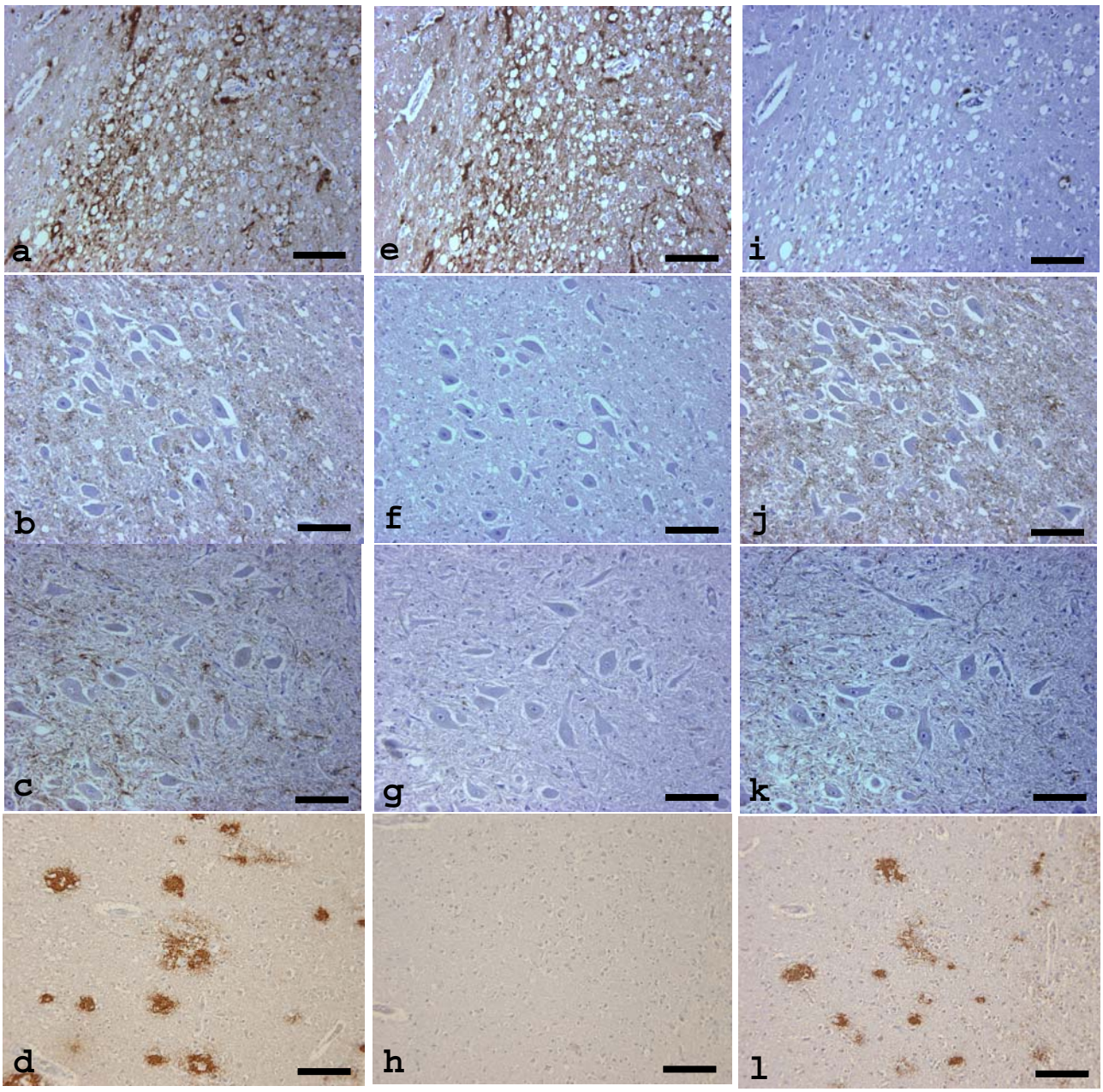

Table 1. Characteristics of the twelve monoclonal and one polyclonal (B103) antibodies used in this study.

Antibodies	Epitope	Dilution	Immunogen	*Species-specificity				Source
				mouse	cattle	sheep	human	
110	56-90	1/500	Mouse recPrP	+	+	+	+	Horiuchi
132	119-127	1/200	Mouse recPrP	+	+	+	+	Horiuchi
T1	137-143	1/200	Mouse recPrP	+	+	+	+	Tagawa
118	137-145	1/500	Mouse recPrP	+	-	-	-	Horiuchi
31C6	143-151	1/500	Mouse recPrP	+	-	-	-	Horiuchi
149	147-153	1/500	Mouse recPrP	+	+	+	-	Horiuchi
43C5	163-169	1/10000	Mouse recPrP	+	+	+	-	Horiuchi
147	219-229	1/200	Mouse recPrP	+	-	-	-	Horiuchi
6H4	155-163	1/500	Cow recPrP	+	+	+	+	Prionics (Zürich, Switzerland)
F89/160.1.5	146-159	1/200	Cow recPrP	-	+	+	+	Affinity Bio Reagents (CO, USA)
3F4	109-112	1/100	Human recPrP	-	-	-	+	Chemicon (CA, USA)
12F10	142-160	1/200	Human recPrP	-	+	+	+	Cayman Chemical(MI, USA)
B103	91-108	1/500	Cow recPrP	+	+	+	+	Horiuchi

*Species-specificity of antibody reveals the summary of the immunohistochemical reactivity shown in Table 2.

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

Antibody	Type of specimen	Results obtained with the stated pretreatment					
		1	2	3	4	5	6
110	M	-	+	2+	3+	3+	3+
	C	-	+	+	2+	3+	2+
	S *P/D	+/-	+/+	+/+	2+/2+	3+/3+	3+/3+
	GSS P/D	NE	NE	NE	NE	3+/3+	3+/3+
	CJD	NE	NE	NE	NE	+	+
132	M	-	+	2+	-	2+	2+
	C	-	-	+	-	3+	3+
	S P/D	-/-	+/-	2+/+	-/-	3+/3+	2+/2+
	GSS P/D	NE	NE	NE	NE	2+/+	2+/+
	CJD	NE	NE	NE	NE	+	+
T1	M	-	+	2+	2+	3+	2+
	C	-	+	2+	-	3+	2+
	S P/D	-/-	2+/+	2+/2+	2+/2+	3+/3+	3+/3+
	GSS P/D	NE	NE	NE	NE	3+/3+	3+/3+
	CJD	NE	NE	NE	NE	+	+
118	M	-	-	+	+	+	+
	C	-	-	-	-	-	-
	S P/D	-/-	-/-	-/-	-/-	-/-	-/-
	GSS P/D	NE	NE	-/-	NE	-/-	-/-
	CJD	NE	NE	-	NE	-	-

Antibody	Type of specimen	Results obtained with the stated pretreatment					
		1	2	3	4	5	6
31C6	M	-	-	2+	+	2+	2+
	C	-	-	-	-	-	-
	S P/D	-/-	-/-	-/-	-/-	-/-	-/-
	GSS P/D	NE	-/-	-/-	-/-	-/-	NE
	CJD	-	NE	-	-	-	-
149	M	-	+	2+	+	3+	+
	C	-	-	2+	-	+	3+
	S P/D	-/-	+/-	2+/+	3+/2+	3+/2+	3+/3+
	GSS P/D	NE	NE	-/-	NE	-/-	NE
	CJD	NE	NE	-	NE	-	NE
43C5	M	-	2+	2+	2+	3+	3+
	C	-	2+	2+	2+	3+	3+
	S P/D	-/-	2+/+	2+/2+	2+/2+	3+/3+	3+/3+
	GSS P/D	-/-	NE	-/-	NE	-/-	NE
	CJD	-	NE	-	NE	-	NE
147	M	-	+	+	-	2+	3+
	C	-	-	-	-	-	-
	S P/D	-/-	-/-	-/-	-/-	-/-	-/-
	GSS P/D	NE	NE	-/-	NE	-/-	NE
	CJD	NE	NE	-	NE	-	NE

Antibody	Type of specimen	Results obtained with the stated pretreatment					
		1	2	3	4	5	6
6H4	M	-	+	2+	-	-	-
	C	-	+	2+	-	-	-
	S P/D	-/-	+/-	2+/2+	-/-	-/-	-/-
	GSS P/D	NE	NE	+/+	-/-	-/-	-/-
	CJD	-	NE	+	-	-	-
F89/160.1.5	M	-	-	-	-	+	-
	C	+	2+	2+	+	2+	3+
	S P/D	-/-	+/+	2+/2+	2+/2+	3+/2+	3+/3+
	GSS P/D	NE	NE	NE	NE	3+/2+	2+/2+
	CJD	NE	NE	NE	NE	+	+
3F4	M	-	-	-	-	-	-
	C	-	-	-	-	-	-
	S P/D	-/-	-/-	-/-	-/-	-/-	-/-
	GSS P/D	-/-	NE	-/-	+/+	-/-	NE
	CJD	-	NE	-	+	-	NE
12F10	M	-	-	-	-	-	-
	C	-	2+	2+	2+	3+	3+
	S P/D	-/-	-/-	2+/2	2+/2	-/-	-/-
	GSS P/D	-/-	NE	-/-	+/+	-/-	NE
	CJD	NE	NE	+	NE	-	NE

Antibody	Type of specimen	Results obtained with the stated pretreatment					
		1	2	3	4	5	6
B103	M	-	+	2+	2+	3+	3+
	C	-	+	2+	2+	3+	3+
	S P/D	-/-	+/+	2+/2+	2+/2+	3+/3+	3+/3+
	GSS P/D	NE	NE	NE	NE	+/+	NE
	CJD	NE	NE	NE	NE	+	NE

1: 96% formic acid for 5 minutes (FA); 2: hydrated autoclaving at 121℃, 2 atmosphere (atm) for 20 minutes in distilled water (121DWHA); 3: 121DWHA and 96% formic acid for 5 minutes; 4: 121DWHA and Proteinase K treatment for 1 minute (121DWHA/FA) (121DWHA/PK); 5: hydrated autoclaving at 135℃, 3 atm for 20 minutes in distilled water (135DWHA); 6: 135DWHA and 98% formic acid for 5 minutes (135DWHA/FA).

M: scrapie-affected mouse; C: BSE-affected cow; S: scrapie-affected sheep; *P/D: P=plaque type, D=diffuse/synaptic type; 3+: Strongly positive signal; 2+: moderately positive signal; +: faint positive signal; -: negative; NE: not examined

Antibodies / Pretreatment	110					132					T1					118				
	M	C	S	GSS	CJD	M	C	S	GSS	CJD	M	C	S	GSS	CJD	M	C	S	GSS	CJD
			P/D	P/D				P/D	P/D				P/D	P/D				P/D	P/D	
FA	−	−	+/−	NE	NE	−	−	−	NE	NE	−	−	−/−	NE	NE	−	−	−/−	NE	NE
121DWHA	+	+	+/+	NE	NE	+	−	+/−	NE	NE	+	+	2+/+	NE	NE	−	−	−/−	NE	NE
121DWHA/FA	2+	+	+/+	NE	NE	2+	+	2+/+	NE	NE	2+	2+	2+/2+	NE	NE	+	−	−/−	−/−	−
121DWHA/PK	3+	2+	2+/2+	NE	NE	−	−	−/−	NE	NE	2+	−	2+/2+	NE	NE	+	−	−/−	NE	NE
135DWHA	3+	3+	3+/3+	3+/3+	+	2+	3+	3+/3+	2+/+	+	3+	3+	3+/3+	3+/3+	+	+	−	−/−	−/−	−
135DWHA/FA	3+	2+	3+/3+	3+/3+	+	2+	3+	2+/2+	2+/+	+	2+	2+	3+/3+	3+/3+	+	+	−	−/−	−/−	−

Antibodies / Pretreatment	31C6					149					43C5					147				
	M	C	S	GSS	CJD	M	C	S	GSS	CJD	M	C	S	GSS	CJD	M	C	S	GSS	CJD
			P/D	P/D				P/D	P/D				P/D	P/D				P/D	P/D	
FA	−	−	−/−	NE	−	−	−	−/−	NE	NE	−	−	−/−	−/−	−	−	−	−/−	NE	NE
121DWHA	−	−	−/−	−/−	NE	+	−	+/−	NE	NE	2+	2+	2+/+	NE	NE	+	−	−/−	NE	NE
121DWHA/FA	2+	−	−/−	−/−	−	2+	2+	2+/+	−/−	−	2+	2+	2+/2+	−/−	−	+	−	−/−	−/−	−
121DWHA/PK	+	−	−/−	−/−	−	+	−	3+/2+	NE	NE	2+	2	2+/2+	NE	NE	−	−	−/−	NE	NE
135DWHA	2+	−	−/−	−/−	−	3+	+	3+/2+	−/−	−	3+	3+	3+/3+	−/−	−	2+	−	−/−	−/−	−
135DWHA/FA	2+	−	−/−	NE	−	+	3+	3+/3+	NE	NE	3+	3+	3+/3+	NE	NE	3+	−	−/−	NE	NE

Antibodies / Pretreatment	6H4					F89/160.1.5					3F4					12F10				
	M	C	S P/D	GSS P/D	CJD	M	C	S P/D	GSS P/D	CJD	M	C	S P/D	GSS P/D	CJD	M	C	S P/D	GSS P/D	CJD
FA	−	−	−/−	NE	−	−	+	−/−	NE	NE	−	−	−/−	−/−	−	−	−	−/−	NE	NE
121DWHA	+	+	+/−	NE	NE	−	2+	+/+	NE	NE	−	−	−/−	NE	NE	−	2+	−/−	NE	NE
121DWHA/FA	2+	2+	2+/2+	+/+	+	−	2+	2+/2+	NE	NE	−	−	−/−	−/−	−	−	2+	2+/2+	2+/+	+
121DWHA/PK	−	−	−/−	−/−	−	−	+	2+/2+	NE	NE	−	−	−/−	+/+	+	−	2+	2+/2+	NE	NE
135DWHA	−	−	−/−	−/−	−	+	2+	3+/2+	3+/2+	+	−	−	−/−	−/−	−	−	3+	−/−	+/−	−
135DWHA/FA	−	−	−/−	−/−	−	−	3+	3+/3+	3+/2+	+	−	−	−/−	NE	NE	−	3+	−/−	NE	NE

Antibodies / Pretreatment	B103				
	M	C	S P/D	GSS P/D	CJD
FA	−	−	−	NE	NE
121DWHA	+	+	+/+	NE	NE
121DWHA/FA	2+	2+	2+/2+	NE	NE
121DWHA/PK	2+	2+	2+/2+	NE	NE
135DWHA	3+	3+	3+/3+	+/+	+
135DWHA/FA	3+	3+	3+/3+	NE	NE

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/synaptic type; 3+: Strongly positive signal; 2+: moderately positive signal; +: faint positive signal; -: negative; NE: not examined