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Characterization of antibodies raised against bovine-PrP-peptides

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To analyze the antigenicity of peptides derived from bovine prion protein (PrP) cDNA, we immunized rabbits with four synthetic peptides and compared the immunoreactivity of antibodies to PrPs from various species by immunoblotting and immunohistochemistry. Two of the antibodies reacted strongly with all PrPs. The other antibodies, raised against overlapping peptides close to two glycosylation sites, did not recognize PrPSc-mouse but did recognize PrPSc-sheep which contains two sugar residues and PrPCJD with or without a sugar residue. Our results suggest that these antibodies may have species-specificity for both glycosylation status and amino acid sequences of the protein. In conclusion, we identified two regions in bovine-PrP which appear suitable for raising antibodies that detect various kinds of PrPs, and one region (Ab103–121) which appears suitable for raising antibodies that detect several species of PrPs. These antibodies may be useful for diagnosing prion diseases and for researching their pathogenesis.

Keywords: prion protein; scrapie; BSE; CJD; antibodies; synthetic peptides

Introduction

Transmissible spongiform encephalopathies (TSE) are neurodegenerative diseases of the central nervous system caused by unidentified agents and include scrapie (Sc), Creutzfeldt Jakob disease (CJD), Kuru, Gerstmann-Sträussler-Scheinker syndrome (GSS) and bovine spongiform encephalopathy (BSE). The mechanism of infection has not been elucidated (Bolton and Bendheim, 1991), but the fundamental lesion underlying prion diseases is the conversion of cellular PrP (PrPc) into the scrapie isoform (PrPSc) (Bolton *et al*, 1984; Borchelt *et al*, 1990), and PrPSc is known to accumulate in the affected brain (Hadlow *et al*, 1982; Kitamoto *et al*, 1989).

Among these diseases, BSE in Great Britain was most recently recognized (Wells *et al*, 1987) and is presumed to have arisen from the ingestion of contaminated feed (Bruce *et al*, 1995). In addition, a new form of human prion disease has been reported, also the so-called new variant CJD (vCJD), which usually affects young people. Surprisingly, BSE could be the source of this new disease (Collinge *et al*, 1996; Ironside, 1998).

The diagnosis of TSE relies on the histopathological presence of spongiform lesions, the electron microscopic detection of fibrils (Marsh and Kimberlin, 1975; DeArmond *et al*, 1985), or on the immunological detection of PrP (Kitamoto *et al*, 1986; Roberts *et al*, 1986), the latter of which is the most specific diagnostic marker. PrP genes are highly conserved within many species; amino acids sequences differ about 5% among the PrP from mice, hamsters, sheep and cattle (Basler *et al*, 1986; Locht *et al*, 1986; Oesch *et al*, 1985; Robakis *et al*, 1986). Therefore, cross-reactivity between antibodies raised against PrP is likely.

The purpose of this study was to develop a panel of antibodies that can recognize the known epitopes of bovine PrPs by immunizing the synthetic peptides. We prepared four peptides corresponding to predicted hydrophilic amino acid sequences of bovine PrP and compared their reactivity by immunoblot against PrPs of scrapie-infected mouse (Sc-mouse), scrapie-infected sheep (Sc-sheep), CJD and BSE. We found that antibody Ab103-121

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reacted strongly with all these PrPs, whereas the other antibodies recognized onlyPrPSc-sheep and PrPCJD or PrPCJD alone.

Results

Reactivity of immune sera against synthetic peptides

In initial studies we tried to detect PrPc in the enriched bovine-brain-tissue by immunoblot analysis using antisera raised against synthetic peptides. PrPc reacted with all antisera, unlike the monoclonal antibody 15B3 which reacts only with PrPsc (Korth *et al,* 1997). As expected from previous studies (Horiuchi *et al*, 1995), Ab103–121 showed high specificity and sensitivity for bovine PrPc. Ab221–239 showed relatively weak sensitivity; however, specificity was high (Figure 2). Ab153–176 and Ab166–185 appeared to recognize PrPc; however, they had a tendency to react with non-specific proteins in samples without protease treatment. Ab103–121 and Ab221–239 reactions to normal human PrPc were similar to bovine PrPc as they showed specific reactivity (data not shown). As the amino acid sequences of different species of PrP, shown in Figure 1b show very high homology, we speculate reactivities of these antibodies to PrPc of mouse or sheep show similar tendencies to human and bovine samples, in the sense that Ab103–121 and Ab221–239 show high specificity and Ab153–176 and Ab166–185 show non-specific reaction. Ab103–121 displayed excellent antibody binding to homologous peptides with detection cut-off values of $>1:100\,000$ and plateau levelling-off margins of approximately 1:3000. Half-maximal absorbance levels were achieved with serum dilutions of $>1:25\,800$ for Ab153–176, Ab166–185 and Ab221–239 (data not shown).

Characterization of antibodies against PrP by immunoblot

Each blot signal of the samples from scrapie mouse, scrapie and CJD brain represents about 100 mg of tissue. These abnormal PrPSc, which have been known to be produced from PrP 33–37 by the removal of amino-terminal 67 residues by proteinase K, were shown as smaller molecules of PrP 27–33 (Bolton *et al*, 1982). Ab103–121 and Ab221–239 reacted clearly with PrPSc-mouse and

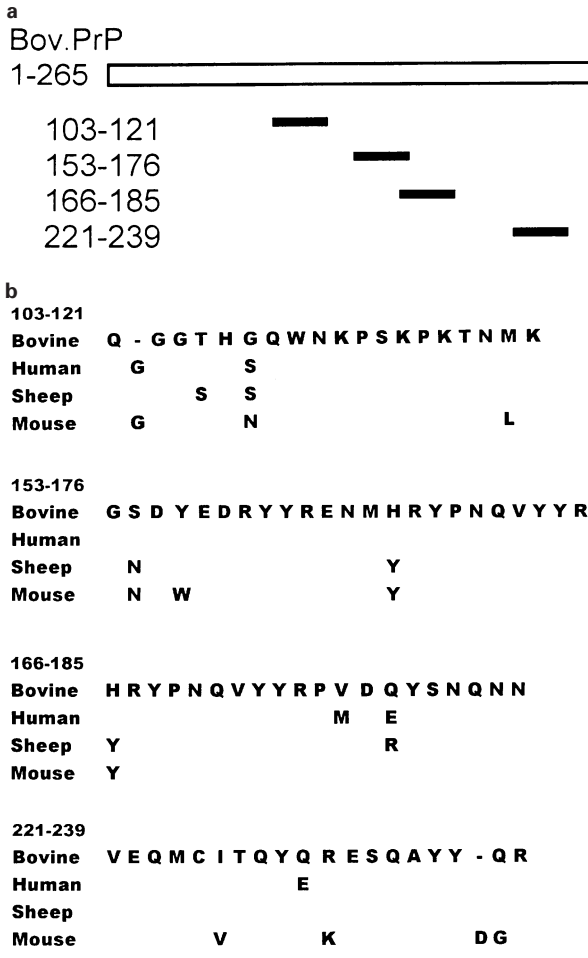

Figure 1 Bovine PrP and synthetic peptides. (**a**) The position of the synthetic PrP peptides are shown as broad bars. (**b**) Amino acid alignments of bovine, human, sheep and mouse used for peptide synthesis.

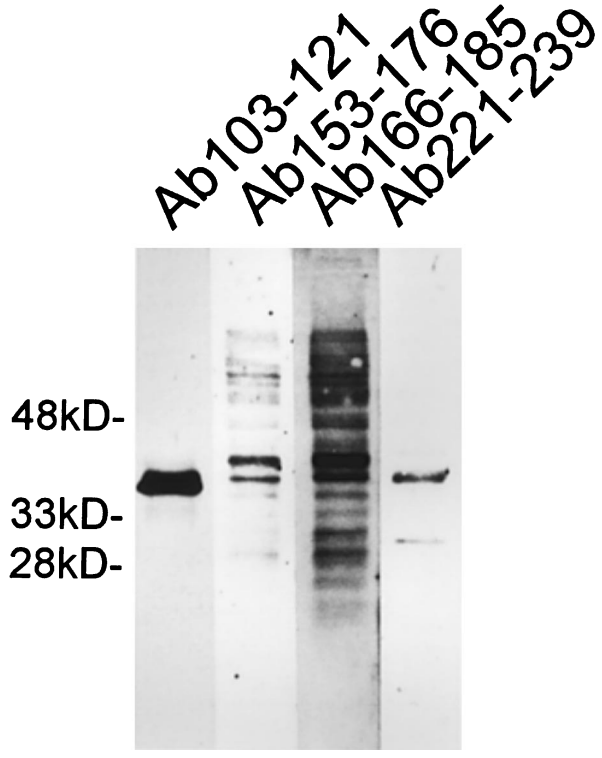

Figure 2 Reactivities of synthetic peptide-induced antisera against PrPc of bovine brain tissues. Crude membrane fractions were extracted and subjected to immunoblot analysis. The antisera used for immunostaining are indicated at the top.

PrPSc-sheep at a dilution of 1 : 5000 (Figure 3). In contrast Ab153 – 176 and Ab166 – 185 showed comparably weak reactions against PrPSc-sheep and did not recognize PrPSc-mouse. Interestingly these latter antibodies showed reactivity to the highest of the three bands (Figure 3), which is considered to be PrPSc-sheep containing two sugar moieties (Parchi *et al*, 1996). Proteinase K completely digested PrPc of both mouse or sheep, and consequently no reactivity was obtained to those antibodies (data not shown).

Strong signals were observed for the PrPCJD with Ab103 – 121 and Ab221 – 239 (Figure 4a). However, different results were obtained for the PrPCJD with Ab153 – 176, where the immunoblot showed a relatively strong signal and Ab166 – 185 could recognize all the forms of PrPCJD relatively weakly. Ab103 – 121 recognized both PrPBSE and PrPSc-sheep of the British strain very well (Figure 4b). However, Ab221 – 239 did not detect either PrPBSE or PrPSc-sheep of the British strain. Again human PrPc, after proteinase K treatment, did not show any reactivity to those antibodies (data not shown).

Detection of paraffin embedded PrP with Ab103 – 121

Kuru-type spherical plaques observed in the cerebellum of a CJD case with codon 129 Met/Val genotype (Figure 5a) were stained after a formic acid pre-treatment method (van Keulen *et al*, 1995; Bell *et al*, 1997) with Ab103 – 121 at a dilution of 1 : 2000 and with Ab221 – 239 at a dilution of 1 : 5000. Positive plaques were revealed predominantly in the granular layer with occasional plaques in the molecular layer. Kuru-type plaques of the Gerstmann-Sträussler-Scheinker syndrome (GSS) with Pro-Leu mutation at codon 102 were also intensely labeled with Ab103 – 121. Synaptic type PrPCJD was demonstrated in two sporadic cases of CJD (CJD-1 used for immunoblotting) as a diffuse fine deposition around the remaining neurons in the affected cortex, similar to those stained with the antibody to synaptophysin (data not shown). Two normal brains (lung cancer and myocardial infarction) and two diseased brains (Alzheimer's disease and herpes encephalitis) were not stained with the antibody. In the BSE affected brain, diffuse particulate PrP immunolabeling of neuropile was seen with Ab103 – 121 at a dilution of 1 : 2560 and with Ab221 – 239 at a dilution of 1 : 10 240 (Figure 5b) (Wells *et al*, 1991). No reaction was detected in the tissues of normal cows.

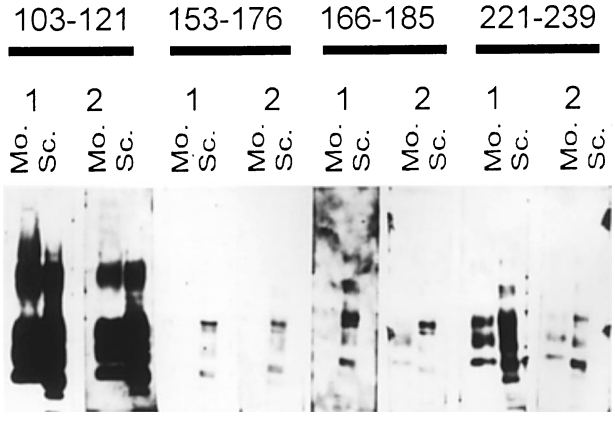

Figure 3 Reactivities of antibodies. PrPSc-enriched fractions (Mo: mouse-scrapie, Sc: scrapie), prepared with proteinase K digestion, were subjected to SDS – PAGE and transferred onto PVDF membranes. Abs used for immunostaining are indicated at the top of each panel. (1) rabbit 1, (2) rabbit 2.

Discussion

We analyzed the antigenicity of synthetic peptides from bovine prion protein. Four peptides were used

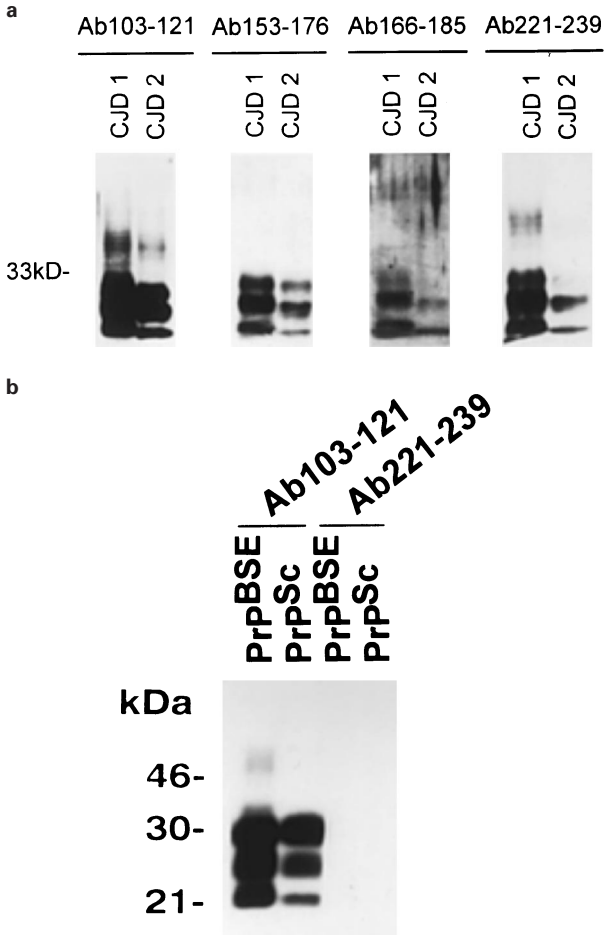

Figure 4 Reactivities of antibodies. (**a**) PrPCJD-enriched fractions prepared with proteinase K digestion, were subjected to SDS – PAGE and transferred onto PVDF membranes. Abs used for immunostaining are indicated at the top of each panel. (CJD 1: CJD patient 1, CJD 2: CJD patient 2). (**b**) Reactivities of Ab103 – 121 and Ab221 – 239 against PrPBSE and PrPSC-sheep of British strain after proteinase K treatment.

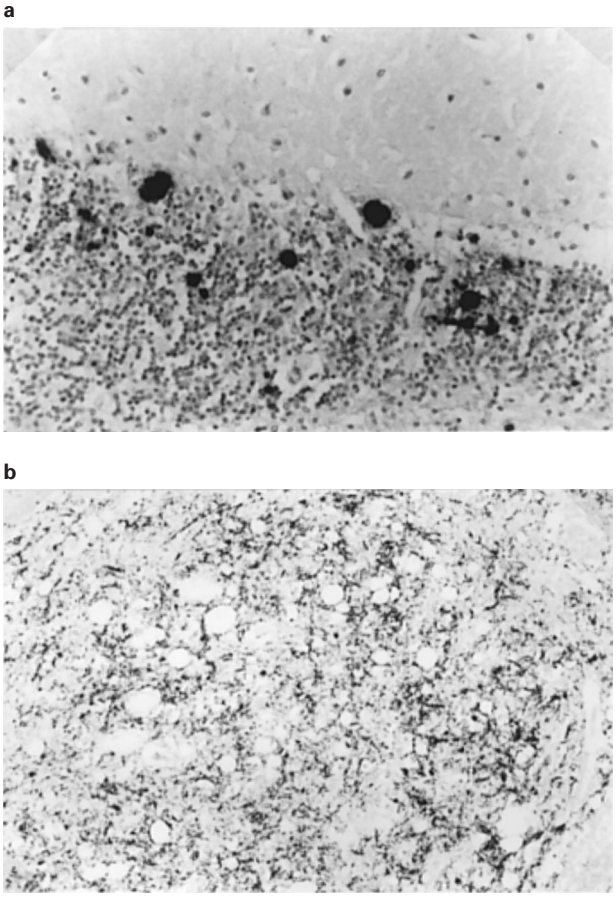

Figure 5 Immunohistochemical findings. (**a**) Kura-type spherical plaques of the cerebellum from CJD patient (Nagashima *et al*, 1998) with Met/Val polymorphism at codon 129 were intensely stained with Ab103–121 ×200. (**b**) Diffuse particulate deposition of PrP were seen in solitary tract nucleus of BSE affected brain with Ab221–239. ×200.

for immunization and each serum was affinity-purified. We first examined whether the synthetic peptide-induced antisera could detect bovine PrPc. Immunoblots demonstrated that the sensitivity and affinity of Ab103–121 and Ab221–239 was very high. However, two antisera raised against the 153–176 and 166–185 regions showed less sensitivity for the samples without protease-treatment.

The monoclonal antibody named 3F4 (Kascsak *et al*, 1987) has been used worldwide to diagnose CJD by immunoblot (Collinge *et al*, 1996; Parchi *et al*, 1996; Beekes *et al*, 1995; Hill *et al*, 1997). 3F4 recognizes the human PrP residues 109–112 (Bolton *et al*, 1991); the number corresponds to codon 120–123 in the bovine system, and they are Met–Lys–His–Met. Hamster PrP has the same sequence at this position and is recognized by 3F4. However, 3F4 does not detect PrPSc from mice, rats, sheep, cattle and rabbits, probably because in these animals the last Met is changed to Val. Although all antisera described here react with PrPc in immunoblotting, another monoclonal antibody named 15B3 (Korth *et al*, 1997) could discriminate the difference between the normal and disease-specific forms of PrP. 15B3 recognizes three different epitopes of PrP (15B3-1, 15B3-2 and 15B3-3), and 15B3-2 and 15B-3 shared with our 166–185 and 221–239, respectively. 15B3-1 and 15B3-2 are associated with beta sheets which are accumulated in PrPSc, and 15B3-3 recognizes amino acid residues near the C-terminus. 15B3-2 and our counterpart of 166–185 reacted well to PrPSc in immunoblotting, which suggested the detection of a newly formed beta sheet.

Synthetic peptides have been used as immunogens to raise antibodies which can recognize isoforms of PrPs and related breakdown products. Shinagawa *et al* (1986) used the synthetic pentadecapeptides, corresponding to the N-terminal regions of scrapie PrP codon 102–116. The generated antibodies reacted well with all three forms of PrP from scrapie-infected mouse brain as well as proteins in the proteinase-untreated fractions. Similarly, Barry *et al* (1988) raised antibodies using a peptide from codon 102–117 and showed that these reacted with both cellular and pathologic hamster PrP. Groschup and Pfaff (1993) and Oberdieck *et al* (1994) used synthetic peptides specific to species of codons 101–115 and 101–116, respectively. Both antibodies reacted with pathologic PrP in a species-specific manner, although they cross-reacted with cellular PrP beyond the species barrier, probably due to conformational similarity of the selected region. Horiuchi *et al* (1995) used the synthetic peptide corresponding to bovine PrP codons 103–121. Although they could not raise monoclonal antibodies against this region, the polyclonal rabbit sera reacted with cellular PrPs from bovine, sheep and mouse. Structurally, this region of PrP is unique in that the region contains the last amino acid of the last octarepeats which forms the new N-terminus after the partial degradation of pathological PrP by proteinase K treatment (Oberdieck *et al*, 1994). Recently, Piccardo *et al* (1997) have shown that out of the various synthesized human PrP peptides antibodies against codon 95–108 recognized CJD, new variant CJD (Hill *et al*, 1997), BSE, sheep with natural scrapie and hamsters with experimental scrapie. The human PrP codon 95–108 is equivalent to bovine codon 106–119, and the alignment of 14 amino acid residues are identical to each other except for only one codon 108, where bovine G is substituted to S in human PrP. For a variety of mammalian species, this region seems to be a highly antigenic site. Thus, our Ab103–121 including codon 108–119 could clearly detect all PrPs tested, including PrPSc-mouse, PrPSc-sheep, PrPCJD and PrPBSE on immunoblot. In addition on immunohistochemistry, Ab103–121 could intensely label both

PrPCJD and PrPBSE. As such this would appear to be very useful for prion-disease diagnosis.

We have also attempted to detect regions of species-specificity in the prion proteins. Our data show that two antibodies raised against overlapping peptides distinguished PrPSc-mouse, PrPSc-sheep, and PrPCJD. Typically, three bands are seen on immunoblots of PrPSc. The two larger-molecular-mass bands represent the main glycosylated forms of PrP and the smaller band represents the unglycosylated prion. We found that Ab153–176 and Ab166–185 did not recognize PrPSc-mouse, but recognized specifically PrPSc-sheep with two sugar residues, and all the forms of PrPCJD, suggesting that these antibodies had a certain species-specificity which could be related to both the level of glycosylation and/or the amino acid sequence.

In addition Ab221–239 was shown to recognize bovine PrP, PrPSc-mouse and PrPSc-sheep. Horiuchi *et al* (1995) showed that rabbit antibodies raised by bovine PrP codon 225–241 recognized PrPc of bovine, sheep and mouse. Thus, this region was shown to be useful for diagnosis of PrP disease of these animals. However, Ab221–239 showed a different reaction compared to Ab103–121, especially against PrPBSE and PrPSc-sheep of the British strain. The latter inconsistency could be explained by a strain difference between British and Japanese scrapie. The discrepancy of Ab221–239, which recognized bovine PrPc, but failed to detect PrPBSE, could be ascribed to the conformational changes that occurred in this region during transition from PrPc to pathologic form. Thus, Ab221–239 will be useful to analyse the protein conformation by narrowing the antigenic site.

Although only 5% of the amino acids differ between species, patterns of PrP transmission are very different. The transmission of prions from one species to another is accompanied by a prolonged incubation time during the initial passage to the host, with subsequent passages being considerably shorter (Manuelidis *et al*, 1978). To explain this observation, a simple mechanism of PrPSc formation has been proposed based on nucleation-dependent protein polymerization (Muramoto *et al*, 1992; Brown *et al*, 1991; Jarrett and Lansbury, 1993; Kocisko *et al*, 1994, 1995). In this scenario, scrapie strains can adapt alternative conformations or packing arrangements of PrPSc polymers, analogous to the alternative crystal forms observed for many proteins. Pre-existing PrPSc can determine the structure of the newly formed PrPSc. In support of this scenario, Collinge's *et al* (1996) identified three forms of the prion protein in BSE or vCJD cases, and prions with two sugar residues were commonly observed.

If the above nucleation scenario is correct, the antigenicity of the PrPs must be retained, even when transmitted to different species. The antibodies we have developed to regions close to two glycosylation sites should recognize the same form of PrP after transmission. These antibodies might play an important role in clarifying the mechanism of prion diseases, or in determining the contribution of species-specificity to the development of prion disease.

Materials and methods

Preparation of synthetic peptides
Four synthetic peptides (Figure 1) corresponding to codons 103–121, 153–176, 166–185 and 221–239 of the bovine PrP gene (Goldmann *et al*, 1991) were synthesized with an automated peptide synthesizer (Applied Biosystem Industry). An additional cysteine residue was added at the carboxy-terminal of each peptide to conjugate the peptides to a carrier protein, bovine serum albumin.

Immunization of rabbits
Five hundred mg of each peptide was emulsified in complete Freund's adjuvant (Wako Chemical), and the emulsion was injected subcutaneously into rabbits. Inoculations were repeated at 1-week intervals with 200 mg of peptide in incomplete Freund's adjuvant.

Antibody response to peptides
Antibody response to the peptides was determined by ELISA. In brief, 96-well ELISA plates were coated with 0.5 mg peptide per well, blocked with 5% non-fat dry milk and incubated with the primary rabbit antibody for 2 h. Binding was detected by a second incubation with horseradish peroxidase-conjugated goat antibody against rabbit IgG (DAKO) for 1 h and was visualized by the conversion of o-phenylenediamine substrates (SIGMA) in the presence of excess hydrogen peroxide. The plates were read with a microplate reader (Sanko Junyaku Co. Ltd.) at a wavelength of 405 nm.

Affinity purification
Immunoaffinity antibody purification was carried out by coupling 5 mg of synthetic peptide to 5 ml of agarose gel. After deactivation of the remaining activated sites, the gel was equilibrated in a physiological buffer at pH 7.5 at room temperature. The crude serum was applied to the gel-containing column. After washes, the absorbed protein was eluted with a glycine-HCl buffer at low pH and collected in a Tris buffer designed to neutralize the elution buffer. The purified antibody was concentrated and diafiltrated against PBS containing 0.1% NaN_3 using an Amicon stirred cell concentrator. Crude sera and purified antibodies were analyzed by ELISA and SDS–PAGE (data not shown).

PrP extraction
In brief (Grathwohl *et al*, 1996), brain tissues were homogenized in a buffer (600–700 μl/100 mg

tissue), containing 2% (vol/vol) Triton X-100, 0.5% (wt/vol) Sarkosyl, 100 mM NaCl and 5 mM $MgCl_2$. Collagenase (0.5 mg/100 mg tissue) and DNase I (40 μg/100 mg tissue) were added and samples were incubated with constant rotation at 37°C for 12 h. Proteinase K was then added (40 μg/100 mg tissue), and incubation was continued for 2 h as described above. Incubation was stopped with phenylmethylsulfonyl fluoride (PMSF; 1 mM). Centrifugation at 15 000 r.p.m. for 20 min at 20°C yielded a pellet that was resuspended in eight times the original tissue weight of 3% SDS-5% mercaptoethanol-sample buffer. To prepare enriched PrPc from the bovine brain, a crude membrane fraction (CSF) was extracted as described below. Samples of bovine brain tissue (4 g) were homogenized to 10% (w/w) CSF buffer (0.25 M sucrose, 10 mM Tris HCl, pH 7.5, 2.5 mM EDTA, 1 mM dithiothreitol (DTT), containing a cocktail of protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 2 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml aprotinin, 2 mM E-64 and 2 mM bestatin). The homogenates were centrifuged at 2000 × g for 15 min and the supernatants were centrifuged at 100 000 × g for 1 h.

Western blot analysis
For immunodetection of PrP and screening of antibodies, material was tested by SDS–PAGE with Western blotting. Samples used were bovine brain tissue, brains from scrapie-infected mouse and scrapie-infected sheep in Japan (Shinagawa *et al*, 1986). They were solubilized in sample buffer and electrophoresed on 12% SDS–PAGE according to Laemmli (1970). The separated proteins were electroblotted onto PVDF membranes (Immobilon). The membrane was blocked with 5% non-fat dry milk in phosphate buffered saline (PBS) for 60 min, washed and then incubated at room temperature with primary rabbit antibodies in PBS containing 0.3% Tween 20 for 1 h. After washing three times each for 5 min, the blot was incubated with horseradish peroxidase-conjugated secondary antibody (DAKO). After 1 h at room temperature the blot was washed again and developed with ECL (Amersham).

Immunohistochemical examination
Four cases of CJD brain samples, consisting of two sporadic cases of CJD, one Gerstmann-Sträussler-Scheinker disease, and one unusual case of CJD with codon 129 Met/Val genotype (Nagashima *et al*, 1998), two normal brains taken from the patient died of lung cancer and myocardial infarction, and two diseased brains of Alzheimer's disease and herpes encephalitis were examined. The brain samples and those from the BSE affected and normal cows were fixed with 10% formalin, dehydrated in alcohol, and embedded in paraffin wax. These samples were sectioned, and served to a formic acid pretreatment method (van Keulen *et al*, 1995; Bell *et al*, 1997), and they were incubated with the rabbit antibodies. They were then incubated with second antibodies and visualized by the avidin-biotin-peroxidase complex (ABC) method with a commercial kit (Vectastain).

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References

Barry RA, Vincent MT, Kent SH, Hood LE, Prusiner SB (1988). Characterization of prion proteins with monospecific antisera to synthetic peptides. *J Immunol* **140:** 1188–1193.

Basler K, Oesch M, Scott M, Westaway D, Walchli M, Groth DF, McKinley MP, Prusiner SB, Weissmann C (1986). Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell* **46:** 417–428.

Beekes M, Baldauf E, Cassens S, Diringer H, Keyes P, Scott AC, Wells GAH, Brown P, Gibbs CJ, Gajdusek DC (1995). Western blot mapping of disease-specific amyloid in various animal species and humans with transmissible spongiform encephalopathies using a high-yield purification method. *J Gen Virol* **76:** 2567–2576.

Bell JE, Gentleman SM, Ironside JW, McCardle L, Lantos PL, Doey L, Lowe J, Fergusson J, Luthert P, McQuaid S, Allen, IV (1997). Prion protein immunocytochemistry – UK five centre consensus report. *J Neuropathol Applied Neurobiology* **23:** 26–35.

Bolton DC, McKinley MP, Prusiner SB (1982). Identification of a protein that purifies with the scrapie prion. *Science* **218:** 1309–1311.

Bolton DC, McKinley MP, Prusiner SB (1984). Molecular characteristics of the major scrapie prion protein. *Biochemistry* **23:** 5898–5906.

Bolton DC, Seligman SJ, Bablanian G, Windsor D, Scala LJ, Kim KS, Chen CMJ, Kascsak RU, Bendheim PE (1991). Molecular location of a species-specific epitope on the hamster scrapie agent protein. *J Virol* **65:** 3667–3675.

Bolton DC, Bendheim PE (1991). Purification of scrapie agents: how far have we come? *Curr Top Microbiol Immunol* **172:** 39–55.

Borchelt DR, Scott M, Taraboulos A, Stahl N, Prusiner SB (1990). Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells. *J Cell Biol* **110:** 743–752.

Brown P, Goldfarb LG, Gajdusek DC (1991). The new biology of spongiform encephalopathy: infectious amyloidoses with a genetic twist. *Lancet* **337:** 1019–1022.

Bruce M, Chree A, McConnell I, Foster J, Pearson G, Fraser H (1995). Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. *Phil Trans R Soc London – B: Biological Sciences.* **343:** 405–411.

Collinge J, Sidle KC, Meads J, Ironside J, Hill AF (1996). Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* **383:** 685–690.

DeArmond SJ, McKinley MP, Barry RA, Braunfeld MB, McColloch JR, Prusiner SB (1985). Identification of prion amyloid filaments in scrapie-infected brain. *Cell* **41:** 221–235.

Goldmann W, Hunter N, Martin T, Dawson M, Hope J (1991). Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon. *J Gen Virol* **72:** 201–204.

Grathwohl KU, Horiuchi M, Ishiguro N, Shinagawa M (1996) Improvement of PrPSc-detection in mouse spleen early at the preclinical stage of scrapie with collagenase-completed tissue homogenization and Sarkosyl-NaCl extraction of PrPSc. *Arch Virol* **141:** 1863–1874.

Groschup MH, Pfaff E (1993). Studies on a species-specific epitope in murine, ovine and bovine prion protein. *J Gen Virol* **74:** 1451–1456.

Hadlow WJ, Kennedy RC, Race RE (1982). Natural infection of Suffolk sheep with scrapie virus. *J Infect Dis* **146:** 657–664.

Hill AF, Desbruslais M, Joiner S, Sidle KCL, Gowland I, Collinge J, Doey LJ, Lantos P (1997). The same prion strain causes vCJD and BSE. *Nature* **389:** 448–449.

Horiuchi H, Yamazaki N, Ikeda T, Ishiguro N, Shinagawa M (1995). A cellular form of prion protein (PrPc) exists in many non-neuronal tissues of sheep. *J. Gen Virol* **76:** 2583–2587.

Ironside JW (1998). New-variant Creutzfeldt-Jakob disease. *Neuropathology* **18:** 131–138.

Jarrett JT, Lansbury PJ (1993). Seeding 'one-dimensional crystallization' of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell* **73:** 1055–1058.

Kascsak RJ, Rubenstein R, Merz PA, Tonna-Demasi M, Fersko R, Carp RI, Wisniewski HM, Diringer H (1987). Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. *J Virol* **61:** 3688–3693.

Kitamoto T, Tateishi J, Tashima T, Takeshita I, Barry RA, DeArmond SJ, Prusiner SB (1986). Amyloid plaques in Creutzfeldt-Jakob disease stain with prion protein antibodies. *Ann Neurol* **20:** 204–208.

Kitamoto T, Mohri S, Tateishi J (1989). Organ distribution of proteinase-resistant prion protein in humans and mice with Creutzfeldt-Jakob disease. *J Gen Virol* **70:** 3371–3379.

Kocisko DA, Come JH, Priola SA, Chesebro B, Raymond GJ, Lansbury PT, Caughey B (1994). Cell-free formation of protease-resistant prion protein. *Nature* **370:** 471–474.

Kocisko DA, Priola SA, Raymond GJ, Chesebro B, Lansbury Jr PT, Caughey B (1995). Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier. *Proc Natl Acad Sci USA* **92:** 3923–3927.

Korth C, Stierli B, Streit P, Moser M, Schaller O, Fischer R, Schulz-Schaeffer W, Kretzchmar H, Raeber A, Braun U, Ehrensperger F, Hornemann S, Glockshuber R, Riek R, Billeter M, Wuthrich K, Oesch B (1997). Prion (PrPSc)-specific epitope defined by a monoclonal antibody. *Nature* **390:** 74–77.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Locht C, Chesebro B, Race R, Keith JM (1986). Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent. *Proc Natl Acad Sci USA* **83:** 6372–6376.

Manuelidis EE, Gorgacz E, Manuelidis L (1978). Interspecies transmission of Creutzfeldt-Jakob disease to Syrian hamsters with reference to clinical syndromes and strains of agent. *Proc Natl Acad Sci USA* **75:** 3432–3436.

Marsh RF, Kimberlin RH (1975). Comparison of scrapie and transmissible mink encephalopathy in hamsters. II. Clinical signs, pathology and pathogenesis. *J Infect Dis* **131:** 104–110.

Muramoto T, Kitamoto T, Tateishi J, Goto I (1992). Successful transmission of Creutzfeldt-Jakob disease from human to mouse verifies by prion protein accumulation in mouse brains. *Brain Res* **599:** 309–316.

Nagashima T, Ishihara Y, Oda M, Ohkawa M, Nagashima K (1998). Fatal insomnia and sleep apnea in Creutzfeldt-Jakob disease with antecedent Wernicke encephalopathy. *J Neurol Sci:* in print.

Oberdieck U, Pocchiari M, Diringer H (1994). Characterisation of antisera raised against species-specific peptide sequences from scrapie-associated fibril protein and their application for post-mortem immunodiagnosis of spongiform encephalopathies. *Arch Virol* **136:** 99–100.

Oesch B, Westaway D, Walchli M, McKinley MP, Kent SB, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, Prusiner SB, Weissmann C (1985). A cellular gene encodes scrapie PrP 27-30 protein. *Cell* **40:** 735–746.

Parchi P, Castellani R, Capellari S, Ghetti B, Young K, Chen SG, Farlow M, Dickson D, Sima AA, Trojanowski JQ, Petersen RB, Gambetti P (1996). Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. *Ann Neurol* **39:** 767–778.

Picardo P, Tagliavini E, Dlouhy SR, Young K, Seiler C, Rossi G, Hill AF, Bugiani M, Bugiani O, Collinge J, Ghetti B (1997). An antiserum to residues 95-108 of human PrP detects PrPres in a variety of human and animal prion diseases. *J Neuropathol Exp Neurol* **56:** 589.

Robakis NK, Sawh PR, Wolfe GC, Rubenstein R, Carp RI, Innis M (1986). Isolation of a cDNA clone encoding the leader peptide of prion protein and expression of the homologous gene in various tissues. *Proc Natl Acad Sci USA* **83:** 6377–6381.

Roberts GW, Lofthouse R, Brown R, Crow TJ, Barry RA, Prusiner SB (1986). Prion-protein immunoreactivity in human transmissible dementias. *New Engl J Med* **315:** 1231–1233.

Shinagawa M, Munekata E, Doi S, Takahashi K, Goto H, Sato G (1986). Immunoreactivity of a synthetic pentadecapeptide corresponding to the N-terminal region of the scrapie prion protein. *J gen Virol* **67:** 1745–1750.

van Keulen IJ, Schreuder BE, Meloen RH, Poelen-van den Berg M, Mooij-Harkes G, Vromans ME, Langeveld JP (1995). Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. *Vet Pathol* **32:** 299–308.

Wells GA, Scott AC, Johnson CT, Gunning RF, Hancock RD, Jeffrey M, Dawson M, Bradley R (1987). A novel progressive spongiform encephalopathy in cattle. *Vet Rec* **124:** 419–420.

Wells GA, Wilesmith JW, McGill IS (1991). Bovine spongiform encephalopathy: a neuropathological perspective. *Brain Pathol* **1:** 69–78.