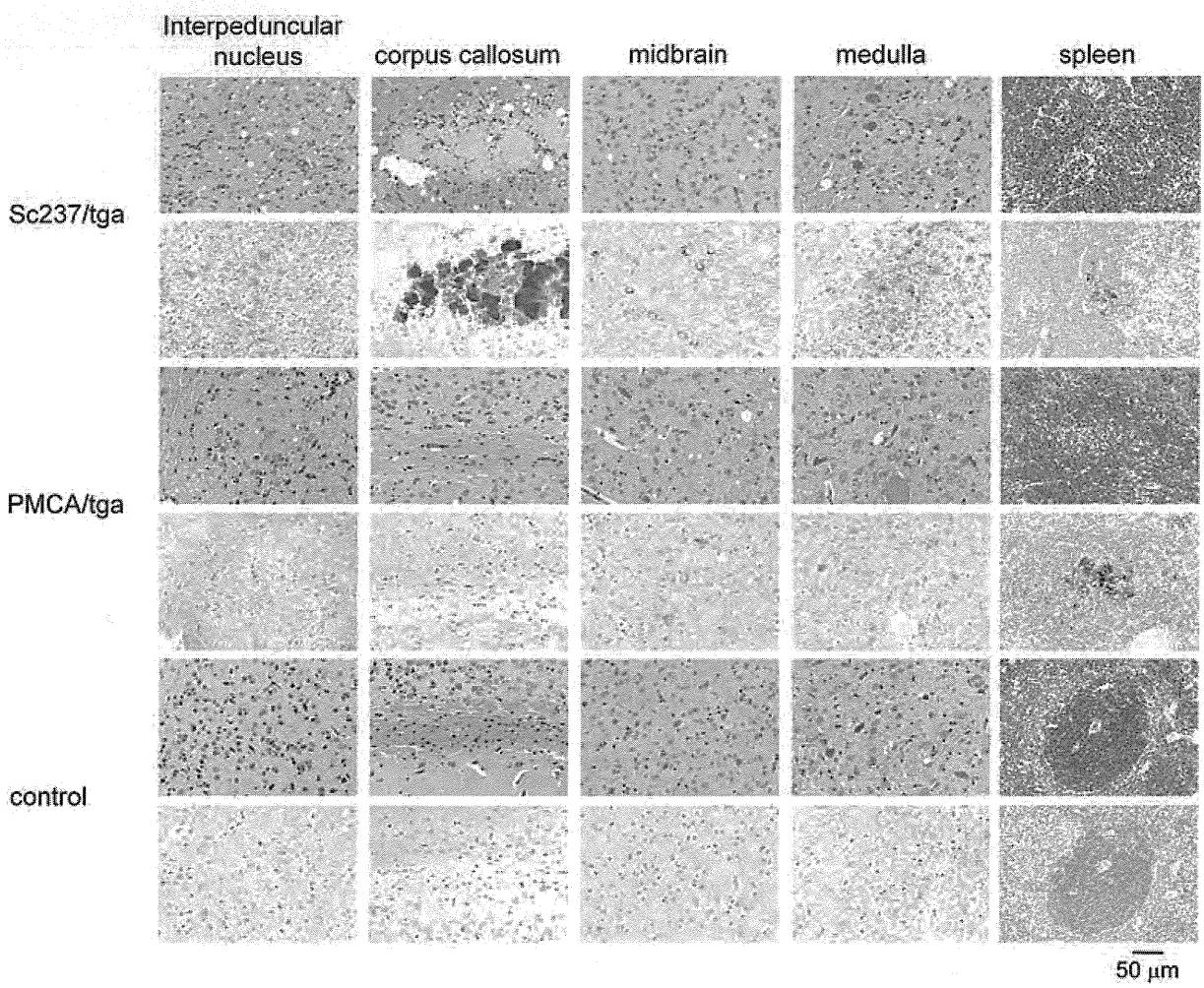

Fig. 4. Vacuolation (upper panels) and PrP^{Sc} accumulation (lower panels) in four different brain areas and spleens of tga20 mice inoculated with Sc237 (Sc237/tga) and PMCA-derived PrP^{Sc} (PMCA/tga). The control for histological analysis was a normal tga20 mouse.

in molecular weight was generally observed between the PMCA/tga and Sc237/tga mice (Fig. 5c). In the secondary passaged mice (PMCA/tga-2 and Sc237/tga-2), this difference was preserved in the WB profiles (Fig. 5b,d).

PrP^{Sc} accumulated in the Sc237/tga and PMCA/tga mice showed similar PK resistance and PK_{50} values (Fig. 6, left panel). In contrast, a distinct difference was observed in the GdnHCl denaturation assay (Fig. 6, right panel). PrP^{Sc} in Sc237/tga and PMCA/tga mice exhibited increased signal intensities after treatment with GdnHCl at 0.5–1.5 M. However, PrP^{Sc} signals in PMCA/tga mice were significantly higher than in Sc237/tga mice (2.0–3.5 M). These results suggest that the PrP^{Sc} that accumulated in the mice inoculated with the PMCA-derived mouse PrP^{Sc} was more resistant to GdnHCl denaturation than the PrP^{Sc} from the mice inoculated with Sc237.

DISCUSSION

Contrary to what has previously been believed, we have demonstrated the development of prion disease in transgenic mice overexpressing mouse PrP in the primary passage of Sc237 transmission after a latent period of over 500 days. Although no clinical sign of disease has previously been observed in wild-type mice after intracerebral inoculation with Sc237 (28), the disease has been found to progress to the subclinical stage of infection in the primary passage in the form of occasional PrP^{Sc} accumulation has in the brain, however pathogenicity has not previously been detected until the secondary passage (29, 30). Because tga20 mice express approximately 10-fold more PrP^{C} in their brains than wild-type mice, the course of disease development might be accelerated by a

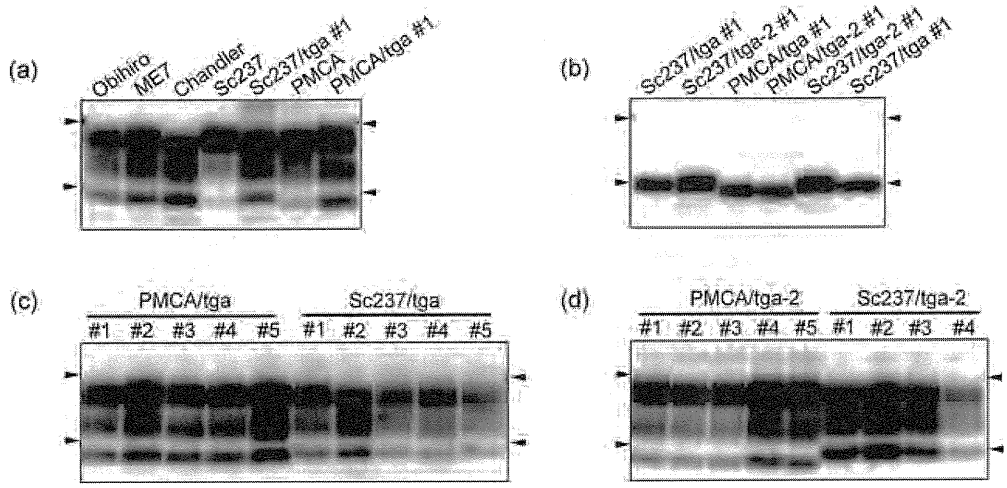

Fig. 5. Comparison of WB profiles of PrP^{Sc} molecules. (a) WB profiles of PrP^{Sc} from mouse scrapie strains (Obihiro, ME7 and Chandler), hamster scrapie strain (Sc237), the brains of tga20 mice inoculated with Sc237 (Sc237/tga #1), the PMCA product obtained in the 22nd round of interspecies amplification (R22 PMCA), and the brains of tga20 mice inoculated with the R22 PMCA products (PMCA/tga #1). After PK digestion, PrP^{Sc} was detected with HRP-T2 antibody. (b) Unglycosylated PrP^{Sc} accumulated in the brains of mice inoculated with Sc237/tga #1, Sc237/tga-2 #1, PMCA/tga #1, and PMCA/tga-2 #1. The PK-digested samples were analyzed after treatment with PNGase F. (c) WB profiles of PrP^{Sc} accumulated in the brains of all affected mice in the primary transmission ($n = 5$ in each group). (d) WB profiles of PrP^{Sc} accumulated in the brains of all affected mice in the secondary transmission ($n = 5$ in PMCA/tga-2 and $n = 4$ in Sc237/tga-2). Arrows indicate the positions of the 30 and 20kDa molecular mass markers.

quantitative effect of PrP^{Sc} molecules accumulated in the brains.

In the homogeneous combination of mouse PrP^{Sc} and PrP^{C} substrate, PMCA products preserve the histopathological and biochemical characteristics of the original PrP^{Sc} in mice (31), suggesting that PMCA is comparable to *in vivo* systems in generating PrP^{Sc}. However, the properties of PrP^{Sc} accumulated in the brains of Sc237/tga and PMCA/tga mice are considerably different. That is, the lesion profiles and PrP^{Sc} accumulation patterns in the brains of PMCA/tga mice are different from those found in Sc237/tga mice, and PrP^{Sc} accumulated in the brains of these mice differs in WB profiles and resistance to GdnHCl denaturation. These results suggest that alteration of mouse PrP^{C} induced by interspecies PMCA is different from that in cross-species transmission *in vivo*. In addition, the results of secondary transmission indicate that Sc237/tga PrP^{Sc} undergoes a significant change in pathogenicity in the mouse brain but PMCA/tga PrP^{Sc} retains its pathogenicity at the level of primary transmission. These observations strongly support the presumption that a different kind of PrP^{Sc} is propagated in the brains of PMCA/tga and Sc237/tga mice. More detailed information on PrP^{Sc} in these mice will be obtained from histopathological and biochemical analysis of brain samples of mice after secondary passage.

Sheep scrapie can be experimentally transmitted to goats, rats, hamsters, and mice and several scrapie prion strains have been established by serial transmission in these animals (32, 33). In these instances, the scrapie prion protein is thought to contain a molecular ensemble of heterogeneous PrP^{Sc} which are maintained in infected sheep (34), and PrP^{Sc} that fit the given environment will be established as a prion strain by selection or mutation in the process of overcoming the "species barrier" (35). According to this idea, a possible explanation for our results is that characteristic selection or mutation of PrP^{Sc} might have occurred during interspecies PMCA with detergents and ultrasonic treatment, and the mouse PrP^{Sc} that was suitable for *in vitro* amplification was able to propagate in the brains of PMCA/tga mice. The observation that unglycosylated PrP^{Sc} of a similar size to that of the PMCA product can be reproduced in PMCA/tga and PMCA/tga-2 mice (Fig. 5a,b) may support the idea that molecular diversity of PrP^{Sc} in a prion strain is changeable by interspecies PMCA.

In conclusion, we have revealed that the biological properties of PrP^{Sc} in mice inoculated with Sc237 are different from those in mice inoculated with its interspecies PMCA product. These observations suggest that *in vitro* amplification of PrP^{Sc} by PMCA is not necessarily equivalent to *in vivo* propagation of PrP^{Sc} in the

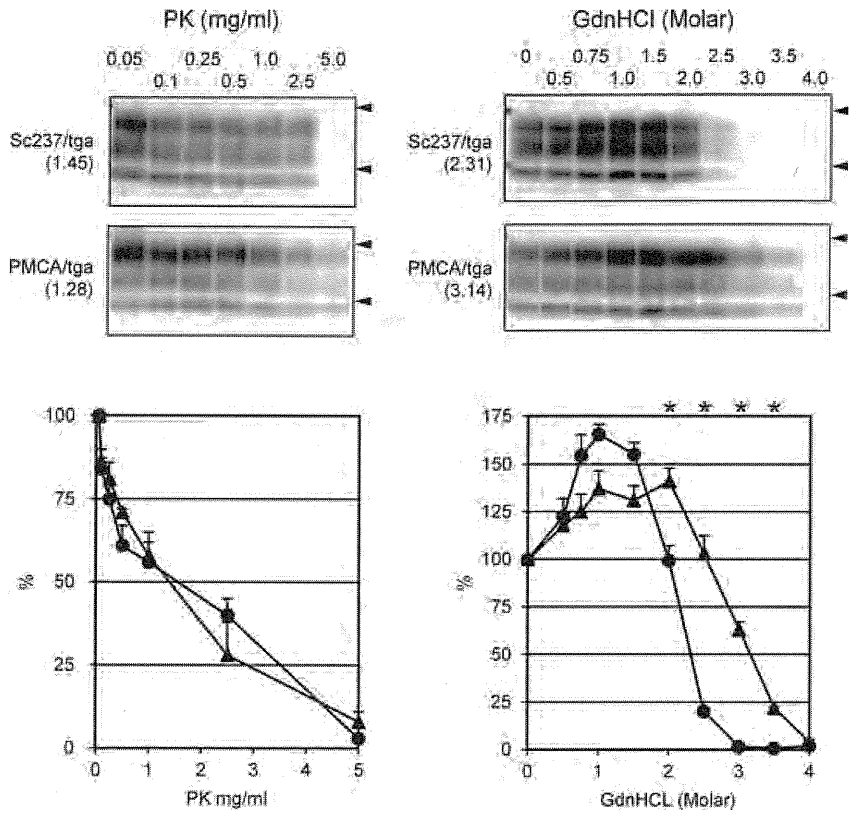

Fig. 6. Biochemical characteristics of PrP^{Sc} accumulated in the brains of Sc237/tga and PMCA/tga mice. Resistance of PrP^{Sc} to treatment with various concentrations of PK (left panels) or GdnHCl (right panels) was compared. The samples were analyzed by WB with HRP-T2 antibody in three independent experiments; typical results are shown in the upper panels. Arrows indicate the positions of the 30 and 20kDa molecular mass markers. The average relative intensity of the PrP^{Sc} signal and SEM of each reagent concentration are represented graphically in the lower panels. The numerals in parentheses in the upper panels indicate the PK_{50} and $GdnHCl_{50}$ values estimated from approximate curves generated from the experimental data. The asterisks at the top of the graph indicate significant differences ($P < 0.05$) between these mice in the average relative intensities of PrP^{Sc} signals at each concentration of GdnHCl. Closed circles, Sc237/tga; closed triangles, PMCA/tga; error bars, SEM.

case of interspecies amplification, and PrP^{Sc} with different biological properties propagate in a heterogeneous environment depending whether that environment is *in vivo* or *in vitro*. Identification of the factors affecting PrP^{Sc} characteristics under artificial conditions may enable the interspecies PMCA technique to contribute to our understanding of the mechanism by which PrP^{Sc} diversity is generated.

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Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Detection of Disease-Associated Prion Protein in the Posterior Portion of the Small Intestine Involving the Continuous Peyer's Patch in Cattle Orally Infected with Bovine Spongiform Encephalopathy Agent

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Keywords:
bovine spongiform encephalopathy; prion; continuous Peyer's patch; cattle; tingible body macrophage

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Summary

Twenty-eight calves were exposed to 5 g of homogenized brainstems confirmed as bovine spongiform encephalopathy (BSE) agents. Two to five animals were sequentially killed for post-mortem analyses 20 months post-inoculation (MPI) at intervals of 6 or 12 months. Samples from animals challenged orally with BSE agents were examined by Western blot and immunohistochemical analyses. Immunolabelled, disease-associated prion protein (PrPsc) was detected in a small portion of follicles in the continuous Peyer's patch from the posterior portion of the small intestine involving the entire ileum and the posterior jejunum but not in the discrete Peyer's patches in the remaining jejunum in preclinical animals at 20, 36, and 48 MPI. The PrPsc-positive cells corresponded to tingible body macrophages on double immunofluorescence labelling. In addition, PrPsc accumulated in 7 of 14 animals in the central nervous system (CNS) after 34 MPI, and five of them developed clinical signs and were killed at 34, 46, 58, and 66 MPI. Two preclinical animals killed at 36 and 48 MPI presented the earliest detectable and smallest deposition of immunolabelled PrPsc in the dorsal motor nucleus of the vagus nerve, the spinal trigeminal nucleus of the medulla oblongata at the obex region, and/or the intermediolateral nucleus of the 13th thoracic segment of the spinal cord. Based on serial killing, no PrPsc was detectable in the CNS, including the medulla oblongata at the obex level, before 30 MPI, by Western blot and immunohistochemical analyses. These results are important for understanding the pathogenesis of BSE.

Introduction

Bovine spongiform encephalopathy (BSE) is a neurodegenerative and fatal disorder that is part of a group of transmissible spongiform encephalopathies (TSEs) or prion diseases, such as scrapie in sheep and goats, chronic wasting disease in deer, and Creutzfeldt-Jacob disease (CJD) in humans. The infectious agent responsible for these diseases is thought to be an abnormally folded isoform (PrPsc) of the normal cellular prion protein (PrPc), which is thought to be a post-translationally modified form of a host-encoded membrane glycoprotein (Prusiner, 1998).

Bovine spongiform encephalopathy (BSE) was first identified in the United Kingdom (UK) in 1986, and the first case in Japan was confirmed in September 2001 (Kimura et al., 2002). Bovine spongiform encephalopathy spread from the UK to European and North American countries and Japan through meat and bone meal, a dietary supplement for cattle, contaminated with infectious prion agent (Wells et al., 1987; Wilesmith et al., 1988) and has affected more than 190 000 cattle in the

world, mostly in the UK. All obex samples over 20 months old from obsolete cattle were examined using specific enzyme-linked immunosorbent assays against the BSE prion protein (PrP) at the Livestock Hygiene Service Center by the active fallen-stock surveillance programme in Japan (Iwata et al., 2006; Onodera and Kim, 2006). Up to now, 12 BSE cases in Holstein-Friesian dairy cows aged 48–102 months old and one case in a female Japanese Black beef cattle aged 89 months old were confirmed in carcasses by Western blot (WB) analysis and histopathological as well as immunohistochemical examinations in our laboratory.

Variant CJD in humans is probably caused by BSE-contaminated foodstuff (Bruce et al., 1997; Hill et al., 1997); therefore, issues about BSE and variant CJD have raised concerns regarding the food safety of beef products (Kimberlin and Wilesmith, 1994). Experimental infection with the BSE agent by an oral route has been accomplished in mice (Maignien et al., 1999), sheep (Jeffrey et al., 2001), cattle (Wells et al., 1994, 2005; Terry et al., 2003; Hoffmann et al., 2007), and non-human primates (Ridley and Baker, 1996; Bons et al., 1999). PrPsc in BSE-affected cattle was thought to be distributed in the brain, spinal cord, eye, dorsal root ganglia, tonsils and distal ileum of the small intestine, which are known as specified risk materials (SRMs) (Wells et al., 1998).

The gut-associated lymphoid tissue (GALT) is mainly comprised of tonsils, Peyer's patches (PPs), appendixes and lymphoid aggregates as isolated lymphoid follicles in the alimentary tract. After being challenged with various tissues from a naturally infected cow at the terminal stage of BSE, the infectivity of transgenic mice expressing bovine PrPc was detected in the distal ileum (Buschmann and Groschup, 2005). In addition, the infectivity of the distal ileum and the detection of PrPsc after oral exposure of cattle to BSE agent have been demonstrated between 6 and 40 months of age at the preclinical stage by using a mouse bioassay and/or PrPsc immunohistochemistry (Terry et al., 2003; Wells et al., 2005). Immunolabelled PrPsc was detected within the follicles of the PPs in the distal ileum, mainly in the tingible-body macrophages (TBMs) after oral BSE challenge in cattle (Terry et al., 2003; Hoffmann et al., 2007). In current models of the TSE propagation pathway after oral exposure, PrPsc spreads from the gut to the peripheral nerve system via microfold cells (M-cell) in the follicle-associated epithelium of the PPs (Aguzzi et al., 2003; Maignien et al., 2005).

Although a high oral dose of inoculum (100 g or more of homogenized BSE brainstem samples) is used in most studies on BSE pathogenesis in cattle (Wells et al., 1998, 2005; Terry et al., 2003; Hoffmann et al., 2007), limited information is available regarding the route of prion spreading from the gut to the brain via peripheral nerves in BSE-affected cattle (Hoffmann et al., 2007). The purpose of the present study is to focus on the distribution of immunolabelled PrPsc in the PP of the ileum and the posterior portion of the jejunum of cattle challenged orally with 5 g of BSE material.

Materials and Methods

Ethics statement

All the experiments involving animals were performed in the biosafety level 3 area of the authors' institution with the approval of the Animal Ethical Committee and the Animal Care and Use Committee of National Institute of Animal Health.

Animal inoculations

Twelve Holstein and one crossbred calves at 3–4 months of age, born in Japan (case no. 1–13), and 15 Holstein female cattle (case no. 14–28) at 9–11 months of age, imported from Australia, were exposed orally to 5 g of pooled brainstems in a 10% (w/v) homogenate (pool code UK11/20) prepared from 10 naturally occurring BSE cases kindly supplied by the Veterinary Laboratories Agency (New Haw, Addlestone, Surrey, UK). The infectivity titre in the inoculums used in this study was $10^{6.7}$ intracerebral LD50/g as determined by end-point titration in TgBoPrP mice, which overexpress the bovine prion protein gene in a null mouse background (Yokoyama et al., 2007). Two calves, one Holstein and one crossbred, born in Japan, were used as uninfected controls. All cattle were clinically monitored throughout the study. At 20, 24, 30, 36, 48 and 60 months post-inoculation (MPI), two to five animals were euthanized for necropsy (Table 1). During the experiment, five animals showing typical clinical signs of BSE were necropsied at 34, 42, 58 and 66 MPI.

Preparation of samples

At necropsy, the posterior portion of the small intestine, mostly ileum containing a continuous PP (CPP) was collected from eight sites at 50-cm intervals from the ileocecal junction towards the anterior jejunum (Fig. 1). The jejunum containing a discrete PP (DPP) was also collected from 12 sites at approximately 2-m intervals from the remaining jejunum. In addition, the left brain, spinal cord samples from the 8th cervical (C8), 4th, 8th, and 13th thoracic (T4, T8, and T13), 2nd and 6th lumbar (L2 and L6), and sacral segments, including the dorsal root ganglia, as well as more than 120 tissue samples from the whole body were collected and fixed in 10% buffered formal saline (pH 7.4) for

Table 1. Summary of the results in cattle exposed orally to bovine spongiform encephalopathy agents

Case	Code	Months post-inoculation	Breed	Sex	PrPsc immunohistochemistry				Clinical signs
					Continuous Peyer's patch	Pharyngeal tonsil	Brain	Spinal cord	
1	5536	18	Holstein	Female	–	–	–	–	–
2	1936	20	Holstein	Bullock	+	–	–	–	–
3	1945	20	Holstein	Bullock	–	–	–	–	–
4	1952	20	Holstein	Bullock	+	–	–	–	–
5	2355	20	Holstein	Bullock	+	–	–	–	–
6	5512	24	Holstein	Female	–	–	–	–	–
7	6416	24	Holstein	Female	–	–	–	–	–
8	8104	24	Holstein	Bullock	–	–	–	–	–
9	8906	24	Holstein	Bullock	–	–	–	–	–
10	9787	30	Crossbreed [a]	Bullock	–	–	–	–	–
11	4008	30	Holstein	Female	–	–	–	–	–
12	7342	30	Holstein	Female	–	–	–	–	–
13	2072	30	Holstein	Female	+	–	–	–	–
14	5499	34	Holstein	Female	–	–	+	+	+
15	5529	36	Holstein	Female	–	–	–	–	–
16	5543	36	Holstein	Female	–	–	–	–	–
17	5451	36	Holstein	Female	–	–	+	+	–
18	5468	42	Holstein	Female	–	–	+	+	+
19	5598	46	Holstein	Female	+	+	–	–	–
20	5581	48	Holstein	Female	–	–	–	–	–
21	5505	48	Holstein	Female	–	–	–	–	–
22	5567	48	Holstein	Female	–	–	–	+	–
23	5475	48	Holstein	Female	–	–	–	–	–
24	5550	58	Holstein	Female	–	–	+	+	+
25	5420	60	Holstein	Female	–	–	–	–	–
26	5413	66	Holstein	Female	–	–	+	+	+
27	5437	66	Holstein	Female	–	–	+	+	+
28 [b]	5444	72	Holstein	Female					–

[a]Crossbreed between Japanese Black and Holstein.
[b]This case is alive without any clinical signs of the disease up to now.

histological and immunohistochemical analyses. At least six blocks were sectioned at 3-mm thicknesses from each intestinal and spinal cord sample. Tissues were treated in 98% formic acid for 60 min to reduce infectivity (Taylor et al., 1997) and then embedded in paraffin wax (Pathoprep580; Wako Pure Chemical, Osaka, Japan) by using an automated embedding machine (Tissue-Tek ETP150; Sakura Finetek Japan, Tokyo, Japan). Serial sections at 4 μm were mounted on silanated glass slides (Immuno-Coat; Muto Pure Chemicals, Tokyo, Japan) and stained with haematoxylin and eosin (H&E) or immunohistochemically. The right hemisphere, remaining spinal cords, and various tissues from the whole body were frozen at −80°C for WB analysis and mouse bioassay.

PrPsc immunohistochemistry

For the detection of PrPsc immunolabelling, antigen retrieval was conducted using a chemical-based method (Okada et al., 2010a) or the combination of enzymatic and chemical pre-treatments (Okada et al., 2010b). After washing in phosphate-buffered saline (PBS, pH 7.4), the sections were placed on an automated immunohistochemical equipment (Dako Cytomation Autostainer Universal Staining System, Carpinteria, CA, USA) and immunostained using a horseradish-coupled polymer method. Critical immunostaining steps included incubation of sections with normal goat serum for 10 min, primary antibody for 30 min, anti-mouse universal immunoperoxidase polymer (Nichirei Histofine Simple Stain MAX-PO (M); Nichirei, Tokyo, Japan) as secondary antibody for 60 min and 3′,3-diaminobenzedine tetrachloride as the chromogen for 7 min. The primary monoclonal antibody (mAb) T1, which was generated in a PrP-deficient mouse by immunization with recombinant mouse PrP 121–231, recognized mouse PrP 137–143 at a concentration of 1 μg/ml (Shimizu et al., 2010).

Negative controls were incubated with non-immune mouse or rabbit IgG (1 : 30 dilution; Dako) and PBS

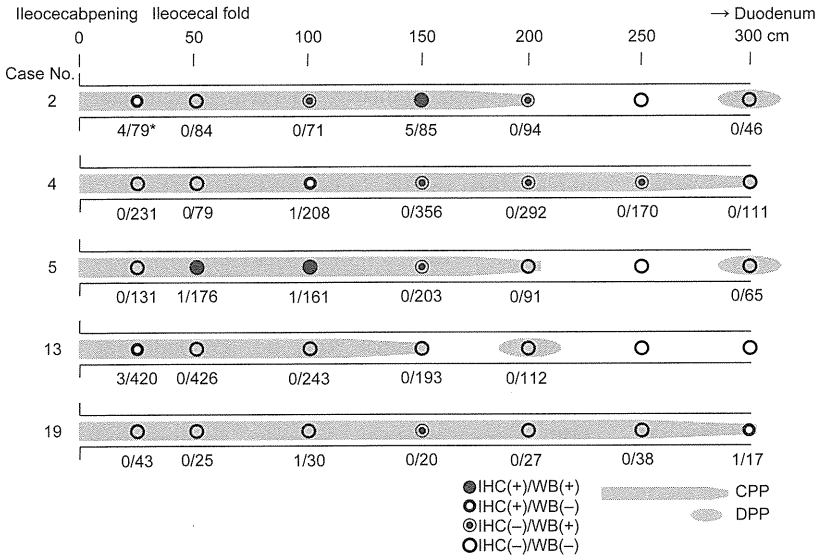

Fig. 1. The presence and proportion of PrPsc-positive follicles in the terminal small intestine of cattle orally dosed with bovine spongiform encephalopathy (BSE) agents as detected by immunohistochemistry with mAb T1. *Number of immunolabelled follicles/total follicles detected by PrPsc immunohistochemistry. CPP, continuous Peyer's patch; DPP, discrete Peyer's patch.

instead of the primary antibody. Finally, all sections were slightly counterstained with haematoxylin.

Double immunofluorescence staining

For double immunofluorescence staining of the CPP in the ileum and pharyngeal tonsil, dewaxed sections were treated with 10 μg/ml proteinase K (PK) in PBS containing 0.01% Triton-X at room temperature (RT) for 10 min and then autoclaved at 121°C for 3 min in citrate buffer (pH 6.0). Sections were incubated with mAb T1 followed by incubation with Alexa 488 goat anti-mouse IgG (1 : 400; Molecular Probes, Portland, OR, USA). PrPsc immunofluorescence was combined with polyclonal antibody (pAb) Iba1 (1 : 300; Wako Pure Chemical) as a macrophage marker (Imai et al., 1996). The rabbit pAb was visualized by incubation with Alexa 546 goat-anti rabbit IgG (1 : 400; Molecular Probes). Immunofluorescence images were evaluated using a Zeiss LSM 510 laser scanning confocal microscope (LSCM; Carl Zeiss, Oberkochen, Germany).

PrPsc western blot

Peyer's patches were examined for the detection of PrPsc by WB using phosphotungstic acid precipitation as described previously (Shimada et al., 2005). 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 $MgCl_2$, 2 mM $CaCl_2$, 20 mg collagenase, and 40 mg DNase I and incubated at 37°C for 2 h. The homogenate was digested in 60 μg/ml of PK for 1 h at 37°C followed by incubation with 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefablock; Roche Diagnostics, Mannheim, Germany). 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 incubated at RT for 1 h with constant rotation; subsequently, it was centrifuged at 9000 *g* for 5 min. Sodium phosphotungstate was added to the supernatant to 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. PrPsc was enriched from the brain according to a method described previously (Hayashi et al., 2005). Western blot analysis was performed as described elsewhere by using horseradish peroxidase–conjugated anti-prion protein mAb T2, and blots were developed with a chemiluminescence substrate (Super Signal; Pierce Biotechnology, Rockford, IL, USA) (Shimada et al., 2005).

Mouse transmission studies

The infectivity of the PPs was investigated using bovine PrP-overexpressing transgenic (TgBoPrP) mice that exhibit an incubation period of <250 days for BSE prion (Scott et al., 1997). TgBoPrP mice ($n = 5$) were inoculated with 20 μl of 10% tissue homogenate and were killed at the terminal stage of the disease. After killing, various tissues were examined by WB and immunohistochemical analyses.

Results

Peyer's patches and tonsils

The properties of DPP and CPP were distinguished from lymphoid follicular appearance in H&E-stained sections (Yasuda et al., 2004; Takanashi et al., 2008). The length of the CPP in the entire ileum and the posterior portion

of the jejunum was 2–3 m and differed among examined animals (Fig. 1). Although the DPP remains completely intact in all examined cattle, the organized lymphoid follicles of CPP almost regressed in cattle more than 5 years old (case no. 25–27).

In five animals killed at 20 (case no. 2, 4 and 5), 30 (case no. 13) and 46 (case no. 19) MPI, immunolabelled PrPsc was detected in the follicles of the CPP from the posterior portion of the small intestine within 3 m of the ileocecal junction (Fig. 1). Intense and dense PrPsc immunolabelling was predominantly confined to the cytoplasm of large mononuclear cells throughout the follicles (Fig. 2). The proportion of PrPsc-positive follicles in the CPP was 2.18% (9/413; case no. 2), 0.07% (1/1447; case no. 4), 0.26% (2/762; case no. 5), 0.23% (3/1282; case no. 13), and 0.5% (1/200; case no. 19) for the samples collected from these five animals. No PrPsc immunolabelling was detectable in DPPs and ileocecal region of these cases and in any PPs of the other animals examined. Similarly, no PrPsc immunolabelling was detectable in the central and peripheral nervous systems, including the medulla oblongata at the obex level without any non-specific immunolabelling. Double immunofluorescence staining revealed co-localization of Iba1 with PrPsc (Fig. 3). Additionally, immunolabelled PrPsc was detected in a follicle of the CPP and follicles of the pharyngeal tonsil but not in the palatine and lingual tonsils in one animal (case no. 19) killed at 46 MPI (Fig. 4).

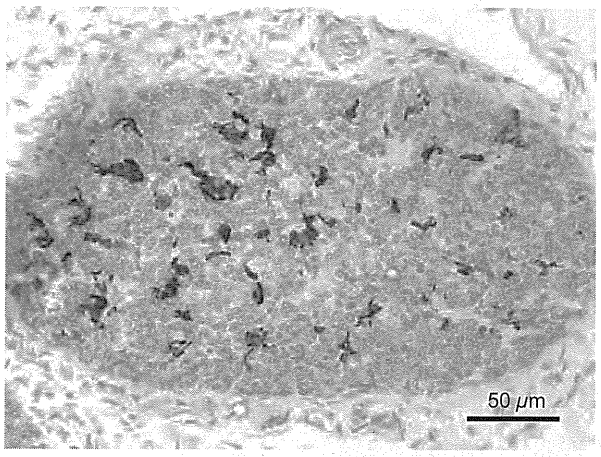

Fig. 2. Ileum 20 cm from the ileocecal junction from case no. 2. PrPsc accumulation in the large pleomorphic mononuclear cells within the follicle of the continuous Peyer's patch of the posterior portion of the small intestine of cattle killed at 20 months post-inoculation. Immunohistochemical labelling with mAb T1 and haematoxylin counterstain.

Moreover, in clinical case no. 26 and 27, killed at 66 MPI, intracytoplasmic, granular immunolabelling was detected in the neurons of the myenteric plexus but not in submucosal plexus of the posterior portion of the small intestine at 1.5–3 m from the ileocecal junction (Fig. 5). However, no PrPsc immunolabelling was detectable elsewhere in the gastrointestinal tract of these two animals.

Western blot analysis showed the typical three bands of PrPsc in samples obtained from the posterior portion of the small intestine involving the CPP but not in that involving the DPPs (Fig. 6).

All TgBoPrP mice inoculated with PrPsc-positive CPP from case no. 2 developed the disease and were killed at 248.9 (14.4) days post-inoculation [mean (SD)]. PrPsc accumulation was detected in the brain with both immunohistochemical and WB analyses (data not shown). On the other hand, TgBoPrP mice inoculated with homogenates prepared from jejunal DPP were alive more than 650 days and showed no clinical signs of the disease.

Central and peripheral nervous tissues in clinical animals

Five animals in the first experiment developed characteristic clinical signs of BSE and were killed 34 (case no. 15),

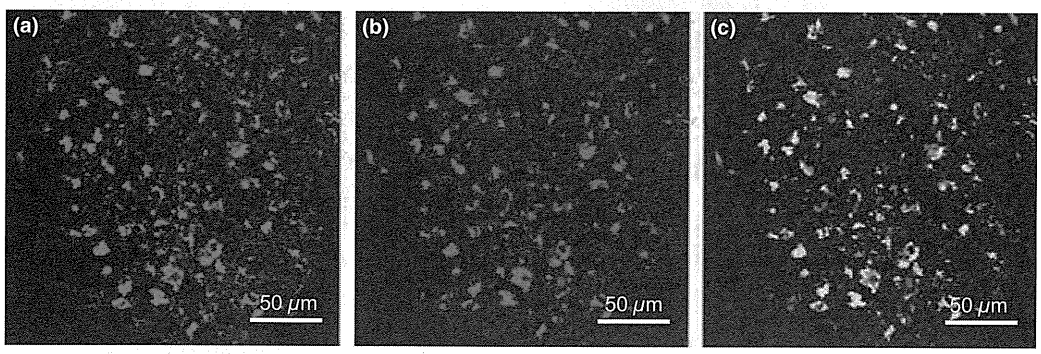

Fig. 3. Ileum 20 cm from the ileocecal junction from case no. 2. PrPsc accumulation in the tingible body macrophages within the follicle of continuous Peyer's patch of the posterior portion of the small intestine of cattle killed at 20 months post-inoculation. Double immunofluorescence images with mAb T1 for PrPsc (a, green) and pAb Iba1 for macrophage (b, red) are merged (c, yellow) under laser scanning confocal microscopy.

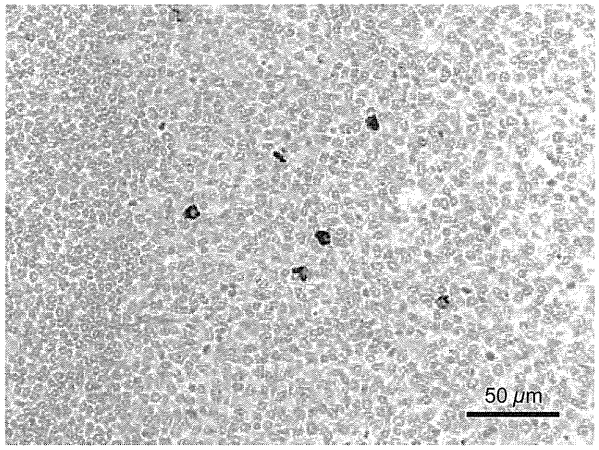

Fig. 4. Pharyngeal tonsil from case no. 19. PrP immunolabelling in the cytoplasm of mononuclear cells of the follicle in the pharyngeal tonsil of cattle killed at 46 months post-inoculation. Immunohistochemical labelling with mAb T1 and haematoxylin counterstain.

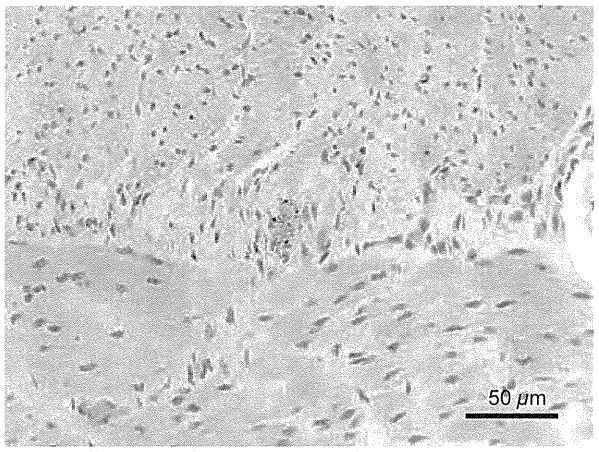

Fig. 5. Jejunum 1.5 m from the ileocecal junction from case no. 26. Granular PrPsc immunolabelling in the cytoplasm of the neuron of cattle killed at 66 months post-inoculation. Immunohistochemical labelling with mAb T1 and haematoxylin counterstain.

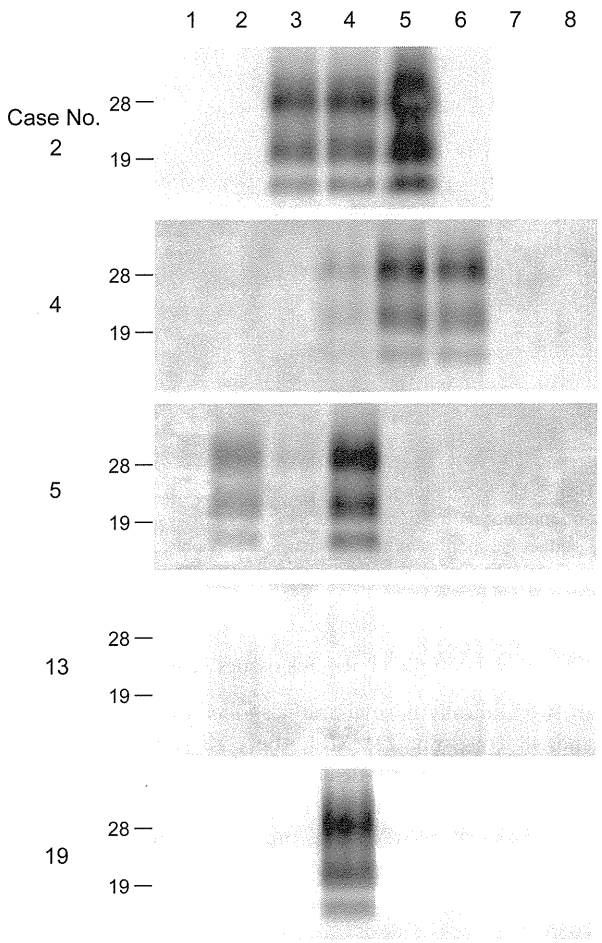

Fig. 6. Western blot analysis. PrPsc in 8 samples from the posterior small intestine of cattle orally dosed with bovine spongiform encephalopathy (BSE) prion of case no. 2, 4, 5, 13 and 19 from top to the bottom. Lane 1, the middle region of ileum; lane 2, ileum at the region of the ileocecal fold; lane 3, jejunum 1 m from the ileocecal junction; lane 4, jejunum 1.5 m from the ileocecal junction; lane 5, jejunum 2 m from at the ileocecal junction; lane 6, jejunum 2.5 m from the ileocecal junction; lane 7, jejunum 3 m from the ileocecal junction; lane 8, jejunum 3.5 m from the ileocecal junction. All samples were digested with 50 μg/ml PK at 37°C for 1 h. Signals were visualized with mAb T2. Molecular markers are shown on the left (kDa).

42 (case no. 18), 58 (case no. 24) and 66 (case no. 26 and 27) MPI. The distribution pattern and severity of vacuolation in the brain were similar among the five animals. The overall severity of vacuolar changes was generally conspicuous in the neuropil of the grey matter of the thalamus and brainstem, but vacuolation was not prominent in the caudal cerebral cortex and cerebellar cortex.

Topographical distribution of PrPsc was similar among the five animals. The intensity and extent of PrPsc immunolabelling was prominent in the thalamus and brainstems, including midbrain, pons, and medulla oblongata, and mild to moderate in the cerebral cortices, basal ganglia and hippocampus. The patterns of PrPsc in the brain consisted of fine and coarse punctuate, coalescing, linear, stellate, intraglial, perineuronal and intraneuronal labelling.

In the extracerebral tissues, such as the trigeminal ganglion, dorsal root ganglia, retina, and neurohypophysis of all animals, positive punctuate immunolabelling was detectable by PrPsc immunohistochemistry. Moreover, PrPsc deposited in the ganglionic and satellite cells of the ganglion nodosum obtained from case no. 27.

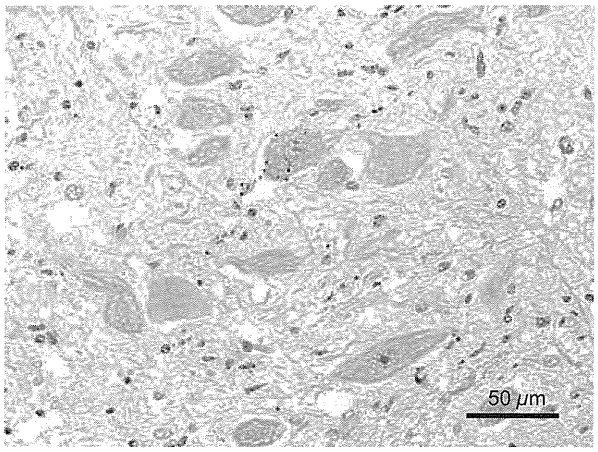

Fig. 7. Medulla oblongata at the obex level from case no. 17. PrPsc accumulation in the dorsal motor nucleus of the vagus nerve of cattle killed at 36 months post-inoculation. Immunohistochemical labelling with mAb T1 and haematoxylin counterstain.

Central and peripheral nervous tissues in subclinical animals

In two clinically normal animals killed at 36 (case no. 17) and 48 (case no. 22) MPI, the earliest immunolabelled PrPsc deposition was detectable in the brainstems and/or spinal cords. In the animal (case no. 17) killed at 36 MPI, immunolabelled PrPsc was present in the dorsal motor nucleus of the vagus nerve, solitary nucleus, and the spinal trigeminal nucleus at the level of the obex, vestibular nucleus of the pons, and the intermediolateral nucleus of T13. In the obex region, immunolabelled PrPsc was sparsely present in the neuropil and neurons (Fig. 7). In the vestibular nucleus of the pons, intaneuronal labelling was detected in some neural cells. In the intermediolateral nucleus of T13, perineural immunolabelling was detected. Western blot analysis showed positive signals in the obex region, pons, and spinal cords of C7, T12 and L5 (data not shown).

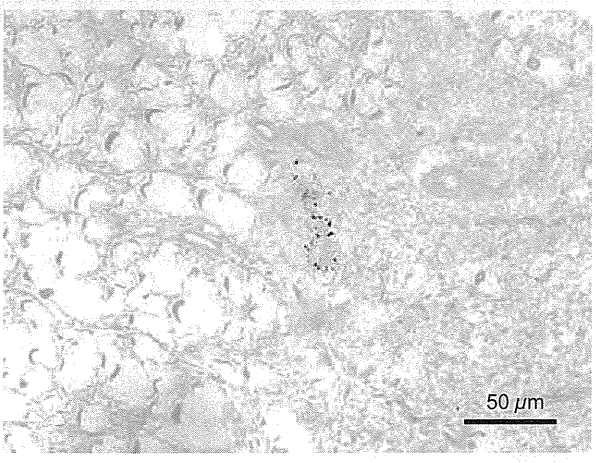

Fig. 8. Spinal cord from case no. 22. PrPsc deposition in neural cells of the intermediolateral nucleus of the 13th thoracic spinal cord of cattle killed at 48 months post-inoculation. Immunohistochemical labelling with mAb T1 and haematoxylin counterstain.

In another animal (case no. 22) killed at 48 MPI, the earliest and minimal immunolabelled PrPsc depositions were detected in a single neural cell that showed perineuronal labelling and weak intraneuronal labelling in the intermediolateral nucleus of T13 (Fig. 8). However, no immunolabelled PrPsc was detectable in the brain including the medulla oblongata at the obex level and other spinal cords such as C8, T4, T8, L2 and L6. Moreover, by WB analysis, PrPsc signal was also not detected in the spinal cords of C7, T12, L1, L5 and the obex region.

Both animals had no histopathological changes, including vacuolations in the brain and no immunolabelling in any ganglia, including the coeliac and mesenteric ganglion complex and other tissues, including those in the peripheral nervous systems.

No PrPsc signal was detectable in the central and peripheral nervous tissues of subclinical animals killed before 30 MPI, using both WB and immunohistochemical analyses.

Discussion

This study demonstrates the accumulation of PrPsc in the follicles of CPP not only in the ileum but also in the posterior portion of the jejunum in 5 of 27 orally BSE-prion exposed cattle killed at 20, 30 and 46 MPI. With the exception of the pharyngeal tonsil of one animal killed at 48 MPI (case no. 19), however, no immunolabelled PrPsc was detected in the myenteric and submucosal plexuses of the enteric nervous system (ENS) of the small intestine or any other tissues, including central and peripheral nervous systems and lymphoid tissues even in gut-associated lymphoid tissues, i.e. DPP scattered throughout the jejunum, ileocecal region, isolated lymphoid follicles of the small and large intestine, and mesenteric lymph nodes. In cattle orally infected with BSE, detection of immunolabelled PrPsc has been reported in the follicles of the distal ileum up to 40 months after exposure and in the neurons of the myenteric plexus in only one clinical animal from each of the groups killed at 38 and 40 MPI (Terry et al., 2003). PrPsc accumulation in the ENS 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) (Masujin et al., 2007). These data also suggest that detectable PrPsc in the neurons of the myenteric plexus is probably caused

by centrifugal spread from the CNS to the ENS via the peripheral nerves during the clinical stage of the disease. An alternative explanation would be that infection reaches the ENS via the blood rather than the centrifugal dissemination from the CNS (Sisó et al., 2009, 2010a).

In most studies on BSE pathogenesis, a massive dose of inoculum (100 g or more of BSE brainstem) was used as an oral challenge (Terry et al., 2003; Wells et al., 2005; Hoffmann et al., 2007). The infection risk of BSE is highest in the first 6 months of life in most cattle (Wilesmith et al., 1992; Ferguson et al., 1997; Arnold and Wilesmith, 2004). The invasion sites of BSE prions are controversial, but the CPP of the ileum seems to be one of most plausible sites because of the early detection of immunolabelled PrPsc at this site following massive oral exposure of cattle to BSE agents (Wells et al., 1998; Hoffmann et al., 2007). PrPsc may be transported from the ileum to the brain and spinal cord via sympathetic, parasympathetic and sensory nerves based on the current understanding of BSE pathogenesis (Hoffmann et al., 2007).

Neuroinvasion could be occurred by blood transfusion of a sheep that had been orally infected with BSE (Sisó et al., 2006, 2009; Houston et al., 2008). PrPsc accumulated in the circumventricular organs (CVOs) which, lacking a blood–brain barrier, of BSE-affected sheep (Sisó et al., 2009, 2010a,b). These results suggest that the CVOs may be the port of entry of infection and the BSE agent can reach the brain through the CVOs by a hematougenous route when the infectivity is present in the blood, either in parallel (peripheral and hematougenous neuroinvasion pathways) or as an alternative to the ascending neuroinvasion routes (Sisó et al., 2009, 2010b). However, immunolabelled PrPsc has not been detected in the area postrema, which is a sensory CVO, of BSE-infected cattle (Sisó et al., 2009). Recently, very small amounts of PrPsc was amplified from the saliva, palatine tonsils, lymph nodes, ileocecal region, spleen, skeletal muscles and cerebrospinal fluid of BSE-affected cattle using the protein misfolding cyclic amplification (PMCA) method (Murayama et al., 2010). However, the presence of PrPsc in the blood including serum, plasma and peripheral blood mononuclear cells was not demonstrated in any BSE-affected cattle with this PMCA technique. Therefore, it is still unclear that the hematogenous neuroinvasion pathway may be a frequent event or have an important role in BSE-infected cattle by the oral route. In addition, M cells on PPs in cattle may be assumed to play a pivotal role in the early uptake and transport of the infectious agent (Heppner et al., 2001; Lwin et al., 2009).

The ileum of ruminants is very distinguishable as it is a very straight portion of the terminal part of the small intestine, which is adherent to the caecum by the ileocecal fold and terminates at the ileocecal junction (Habel, 1975; Dyce et al., 2010). The ileum of cattle is fairly short: 30–45 cm (12–24 inches) in length, depending on the age and size of the animal (Habel, 1975). According to veterinary anatomical terminology, the distal ileum adherent to the caecum can be defined as the lower half of the ileum (Dyce et al., 2010). In cattle and sheep, there is evidence of two types of PP (CPP, the ileal PP, and DPP the jejunal PP) based on morphology, ontogeny, lymphocyte traffic and blood flow (Doughri et al., 1972; Reynolds and Kirk, 1989; Defaweux et al., 2007). The single-large CPP of cattle extends approximately 2–3 m (80–120 inches) along the posterior jejunum and entire ileum, whereas DPPs are distributed throughout the rest of the jejunum as 30–40 lymphoid aggregates (Doughri et al., 1972; Reynolds and Morris, 1983). Thus, the posterior portion of the jejunum contains both CPP and DPP (Takanashi et al., 2008).

As a preventive measure in Japan, the risk of BSE exposure to humans has been minimized by BSE testing of slaughtered cattle of more than 20 months of age and by the removal of SRMs that may contain BSE prions from all slaughtered cattle. Recent studies have shown that peripheral tissues other than the CNS tissue harbour the BSE prion at the clinical stage (Buschmann and Groschup, 2005; Masujin et al., 2007). SRM removal is a useful policy for a control programme to reduce the infectivity risk of BSE to humans. However, the definition of SRMs slightly differs between countries. The EU commission bans the intestine of cattle for human consumption (European Commission, 2000), while Japan and some other countries only classify the distal ileum as SRM. The age of cattle was excluded from the criteria of classifying the intestine or ileum as SRM. This study confirmed early and efficient accumulation of PrPsc only in the CPP of the posterior portion of the small intestine of BSE-exposed cattle, even in the case of a low-dose BSE infection.

Under the LSCM, globular immunolabelled PrPsc overlapped with Iba1 expression in macrophages/microglial cells (Imai et al., 1996). In the present study, this antibody immunostained cells in the CPP that had morphological features of macrophages, suggesting that the majority of the PrPsc was within the macrophages in the follicles of the PP of the small intestine. Intense PrPsc immunolabelling presented in the cytoplasm of large mononuclear cells in the follicles corresponded to previously described PrPsc in the cytoplasm of large mononuclear cells interpreted as TBMs (Jeffrey et al., 2000). TBMs in the germinal centres scavenge apoptotic lymphocytes and are close to antigen-retaining, follicular, dendritic cells (Smith et al., 1998). The decreased number of spleen macrophages before scrapie infection enhances prion susceptibility (Béringue et al., 2000). Macrophages, including TBMs, may either participate in the clearance or transport of prions (Béringue et al., 2000; Jacquemot et al., 2005) or in the affection of

prion transport (Aucouturier et al., 2000; Rezaie and Lantos, 2001; Prinz et al., 2002).

In addition, immunolabelled PrPsc was detected in the macrophages of the pharyngeal tonsil taken from cattle 48 MPI with BSE prions. This is, to our knowledge, the first description of the localization of PrPsc throughout the tonsils of BSE-infected cattle. Unfortunately, this frozen sample was not collected for WB analysis and mouse bioassay. Tonsils in cattle include the following four types: palatine, pharyngeal, lingual and tubal tonsils. The pharyngeal tonsil exists in the rhinopharynx as a part of the skull and, therefore, is removed as SRMs. Infectivity of the palatine tonsil has been reported in cattle killed at 10 MPI after being exposed orally to BSE isolates, but this infectivity failed to be detected by the mouse bioassay (Wells et al., 1998). Therefore, the presence of PrPsc in the tonsils may be the result of experimental error or artefact (Wells et al., 2005). The possibility of this phenomenon is unlikely because of the lack of evidence in early time points of the experiment. Moreover, the potentiality of inoculum residues has been considered to be persistently present in the tonsil by its entrapment. This consideration, however, is also unlikely because of the results from the animal killed 48 months after oral exposure to BSE agents.

In conclusion, the accumulation of PrPsc in the follicles of CPP of the posterior portion of the small intestine and the pharyngeal tonsil, mostly in TBMs, was detected between 20 and 48 MPI in 5 of 22 cattle challenged orally with 5 g of BSE-affected brainstems.

Acknowledgements

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RESEARCH PAPER

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Characterization of Syrian hamster adapted prions derived from L-type and C-type bovine spongiform encephalopathies

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Key words: prion, atypical, L-BSE, PrPcore, hamster, transmission

Abbreviations: BASE, bovine amyloidotic spongiform encephalopathy; BSE, bovine spongiform encephalopathy; C-BSE, classical BSE; H-BSE, H-type of atypical BSE; L-BSE, L-type of atypical BSE; PK, proteinase K; PrP, prion protein; PrP^{C}, cellular isoform of prion protein; PrPcore, PK resistant core fragment of PrP^{Sc}; PrP^{Sc}, abnormal isoform of prion protein

Atypical forms of bovine spongiform encephalopathy (BSE) may be caused by different prions from classical BSE (C-BSE). In this study, we examined the susceptibility of mice overexpressing mouse and hamster chimeric prion protein (PrP) to L-type atypical BSE (L-BSE). None of the transgenic mice showed susceptibility to L-BSE, except mice overexpressing hamster PrP. We also examined the transmission properties of L-BSE in hamsters. The incubation period of hamsters intracerebrally inoculated with L-BSE was 576.8 days, and that of the subsequent passage was decreased to 208 days. Although the lesion and glycoform profiles and relative proteinase K resistant core fragment of the abnormal isoform of PrP (PrPcore) of L-BSE were similar to that of C-BSE, the deposition of the abnormal isoform of PrP (PrP^{Sc}) and the molecular weight of PrPcore of L-BSE was different from than that of C-BSE. In hamster models, some prion strain characteristics of L-BSE were indistinguishable from those of C-BSE.

Introduction

Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disorder that is classified as a prion disease or a transmissible spongiform encephalopathy.[1] BSE and the subsequent identification of variant Creutzfeldt-Jakob disease have raised important food safety issues. The incidence of BSE has decreased because of disease-control programs such as the feed ban;[2] however, different phenotypes of BSE (atypical BSEs) have been identified in several countries.[3] To date, these phenotypes are classified into two forms—L-type atypical BSE (L-BSE) or bovine amyloidotic spongiform encephalopathy (BASE) and H-type atypical BSE (H-BSE)—on the basis of the molecular weight of the proteinase K (PK)-resistant core fragment of the abnormal isoform of the prion protein, PrP^{Sc} (PrPcore).[4,5]

Several studies have indicated that prion strains causing L-BSE and H-BSE are different from those that cause classical BSE (C-BSE).[6-11] The L-BSE prion was experimentally transmissible to cattle,[8,10] bovinized prion protein (PrP)-overexpressing transgenic mice,[6,11] and humanized PrP-transgenic mice[9] with shorter incubation periods and more severe spongiform changes than the C-BSE prion. A transmission study with inbred mice has widely been used for prion strain classification; however, L-BSE could not be transmitted to wild-type mice,[11,12] and subsequent passage in wild-type mice altered the characteristics of L-BSE prions[12] and made comparison of strain characteristics difficult in L-BSE and C-BSE prions. Rodent-adapted L-BSE prions will help analyze L-BSE prion characteristics.

Conversion from cellular isoform of prion protein (PrP^{C}) to PrP^{Sc} is a central event in prion pathogenesis. Amino acid differences between host PrP^{C} and PrP^{Sc} of inoculums result in a species barrier in the interspecies transmission of prions. The mouse and hamster PrPcore regions differ with respect to eight amino acid substitutions.[13] We examined the characteristics of C-BSE by using mice overexpressing mouse and hamster chimeric PrP.[14] The mouse PrP131-188 sequence contributed to the susceptibility of mice with C-BSE prions. L-BSE prions may have a susceptible host range that is different from C-BSE prions, and thus an investigation of the susceptible host range using mice overexpressing mouse and hamster PrP may help reveal the host species susceptible to L-BSE prions. Here, we examined the species barrier to L-BSE prions using a chimeric PrP overexpression mouse model. However, none of the transgenic mice, except those overexpressing hamster PrP, were susceptible to L-BSE prions. We also confirmed this result by carrying out a transmission study in hamsters. Expectedly, the L-BSE prion but not the C-BSE prion

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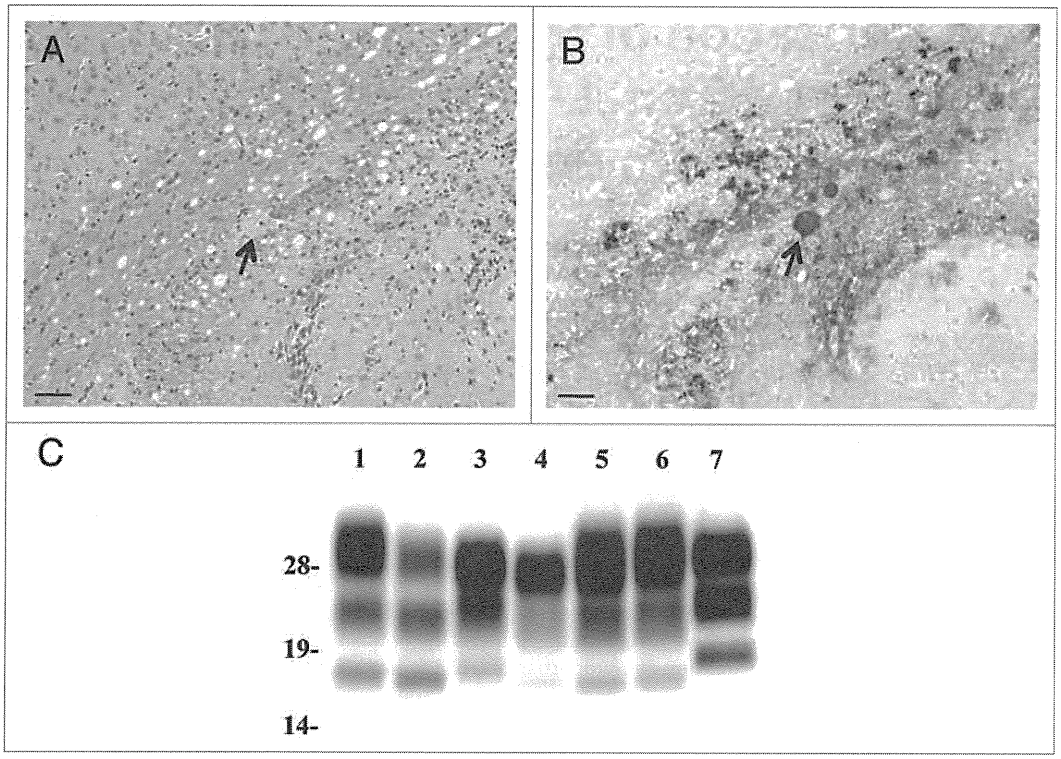

Figure 1. Neuropathology and PrPcore characteristics of L-BSE-affected TgHaNSE mice. (A) hematoxylin and eosin staining of the corpus callosum of mice. (B) PrP^{Sc} deposition was detected in the semiserial sections. PrP-plaque is indicated by an arrow. PrP was detected by mAb SAF-84. Scale bars: 200 mm. (C) PrP^{Sc} in L-BSE affected TgHaNSE mice was detected by western blotting. Lane 1: C-BSE (cattle), lane 2: L-BSE (cattle), lane 3: C-BSE affected hamster, lane 4: L-BSE affected hamster, lanes 5 and 6: L-BSE affected TgHaNSE, lane 7: scrapie Obihiro-affected mouse. PrP was detected by mAb T2.

was transmissible to hamsters. In this study, we also analyzed the disease phenotype and PrPcore characteristics of the L-BSE prion in Syrian hamsters.

Results

No susceptibility of L-BSE to transgenic mice overexpressing chimeric-PrP. As shown in **Table 1**, L-BSE was not transmitted to MHM2, MH2M, wild-type (ICR) or tga20 mice. None of these mice showed clinical signs of L-BSE, and no PrP^{Sc} accumulation was observed. However, L-BSE was transmitted to 2 of 3 TgHaNSE mice incubated for 567 and 853 days. PrP plaque deposition was observed in the brain of L-BSE-affected TgHaNSE mice (**Fig. 1**). The molecular weight of PrPcore of L-BSE affected TgHaNSE was similar to that of L-BSE (**Fig. 1**). However, its glycoprofile was different from that of L-BSE cattle, but similar to that of C-BSE cattle (**Fig. 1**).

BSE transmission to hamsters. C-BSE prions were not transmitted to hamsters. However, mouse-passaged C-BSE prions were transmissible to TgHaNSE mice and prions accumulated in the brains of TgHaNSE mice and were successfully transmitted to wild-type hamsters with an incubation period of 349.5 (6.6) days. A subsequent passage decreased the incubation period to 267 days. The incubation period of the third passage was 271 days (**Table 2**). In contrast, L-BSE was successfully transmitted to hamsters from the first passage. The attack rate with the primary passage and an incubation period of 576.8 days was 75%. The subsequent passage increased the attack rate (100%) and decreased the incubation period to 208 days.

Neuropathology of L-BSE-affected hamsters. Though the L-BSE prion-affected hamster showed a lower vacuolation score of lesion profiling than C-BSE prion-affected hamsters, the targeted regions of the L-BSE prion were similar to those of the C-BSE prion (**Fig. 2A**). However, PrP^{Sc} distribution and patterns of L-BSE differed from those of C-BSE and scrapie (**Fig. 2B**). Most conspicuous pattern of PrP^{Sc} in C-BSE prion-affected hamsters was particulate and stellate deposits in the neuropil of the brain. In Sc237 prion-affected hamsters, plaque-like deposits were visible in the cerebral and cerebellar cortices. In contrast, the pattern of PrP^{Sc} deposition was characterized by the presence of sub-pial accumulation in the cerebral cortex and the absence of stellate and plaque forms in the brains of L-BSE prion-affected hamsters. In addition, PrP^{Sc} accumulation in the cerebellar cortex of L-BSE prion-affected hamsters was less common rather than that of C-BSE or Sc237.

Molecular profile of PrPcore of L-BSE-affected hamsters. PrPcore was detected from both C-BSE- and L-BSE-affected hamsters (**Fig. 3A**). However, the PrPcore glycoform of L-BSE-affected hamsters changed relative to that of the original L-BSE cattle. The PrPcore glycoform of L-BSE-affected hamsters was

similar to that of C-BSE- and scrapie-affected hamsters: dominant in the diglycosylated form (**Fig. 3A** and lanes 4, 5; **3B**). On the other hand, the molecular weight of PrPcore were conserved during hamster passage. After deglycosylation treatment of PrPcore, variations in molecular weight of PrPcore among Sc237-, C-BSE- and L-BSE-affected hamsters were evident (**Fig. 3A** and lanes 6–8). The molecular weight of PrPcore of L-BSE was less than that of C-BSE. C-BSE-affected hamsters harbored the additional truncated PrPcore band (**Fig. 3A** and lane 7),[14] but this band was not present in L-BSE-affected hamsters (**Fig. 3A** and lane 8).

Relative PK resistance of PrPcore of L-BSE. The relative PK resistance of PrPcore of L-BSE in cattle was weaker than that of C-BSE; this has also been observed in the case of TgBoPrP mice.[11] We analyzed the relative PK resistance of PrPcore for C-BSE and L-BSE in hamsters. The signal intensity decreased in 1,000 μg/ml of the PK condition; however, PrPcore was still detected from both C-BSE and L-BSE samples (**Fig. 4** and lanes 4 and 8). PK resistance of PrPcore for L-BSE in hamsters was remarkably different from original L-BSE (**Fig. 4B**).

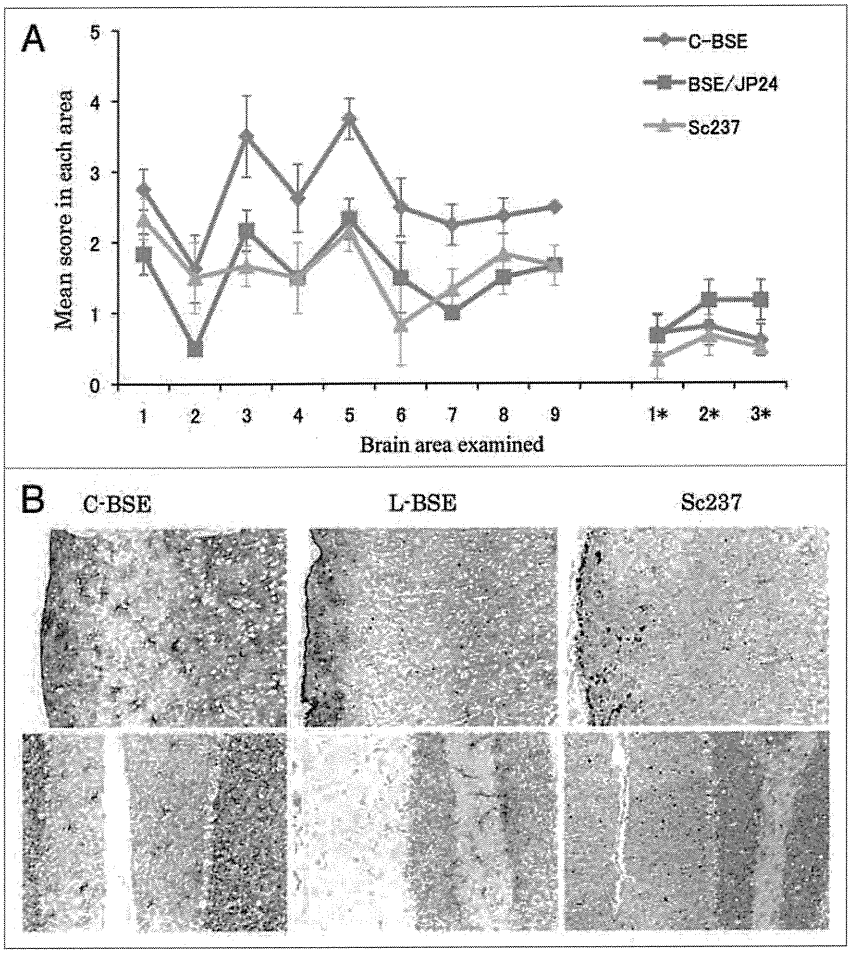

Figure 2. Neuropathological lesion profiling and PrP^{Sc} deposition in hamsters. (A) Vacuolar lesion profiles in Syrian hamster brains as observed for scrapie strains Sc237, C-BSE-affected hamsters (C-BSE) and L-BSE-affected hamsters (L-BSE). Gray matter scoring areas: 1, dorsal medulla; 2, cerebellar cortex; 3, superior colliculus; 4, hypothalamus; 5, medial thalamus; 6, hippocampus; 7, septum; 8, posterior cerebral cortex; 9, anterior cerebral cortex. White matter scoring areas: 1*, cerebellar white matter; 2*, midbrain white matter; 3*, cerebral peduncle. Mean (standard deviation) (n = 4). (B) PrP^{Sc} deposition in the brains of hamsters affected with C-BSE (second passage), L-BSE (second passage) and Sc237 (serial passage). MAb SAF84 was used for immunostaining. Upper, cerebral cortex; lower, cerebellum.

Discussion

This study showed that the host range for L-BSE prions was different from that of C-BSE prions. The C-BSE prion was not transmitted to hamsters (**Table 2**). The mouse and hamster PrPcore subregions differ with respect to 8 amino acid substitutions (**Table 3**). Yokoyama et al.[14] showed that the PrP131-188 subregion contributed to the susceptibility of mice to C-BSE. Once the C-BSE prion was passaged in wild-type mice, it could be transmitted to hamsters; further, its characteristics are similar to the previously reported hamster-adapted C-BSE prion.[27,28] On the other hand, neither MH2M nor MHM2 mice were susceptible to L-BSE prions (**Table 1**). Three amino acid substitutions (V203I, M205I and V215T) at PrP189-231 or in the authentic hamster PrP sequence may be required for conversion of the PrP^{Sc} of the L-BSE prion (**Table 3**).

In order to clarify the characteristics of this prion, an L-BSE prion-adapted rodent model will be a useful tool. On the basis of the result of the transmission study in transgenic mice, we inoculated L-BSE prions into hamsters. L-BSE prion-affected hamsters showed PrP^{Sc} distribution and patterns that differed from those of C-BSE prion-affected hamsters (**Fig. 1B**). The targeted lesions of L-BSE were similar to those of C-BSE; however, the degree of spongiform change differed (**Fig. 2A**).

Glycoform of PrPcore has been used to classify prion strains and/or compare prion characteristics.[29] In cattle, the molecular weight of PrPcore of L-BSE was less than that of C-BSE, and there were glycoprofile differences in their PrPcore (**Fig. 3**). However, the PrPcore of L-BSE in hamsters was different. Although the molecular weight of PrPcore of L-BSE was similar to that of L-BSE-affected cattle, the glycoprofile and relative PK resistance of L-BSE in hamsters differed from those of the original L-BSE (**Fig. 3**). A similar PrPcore glycoprofile within different prion strains was also observed in converted PrP^{Sc} in vitro.[30] These results suggest that the glycoform of PrPcore in

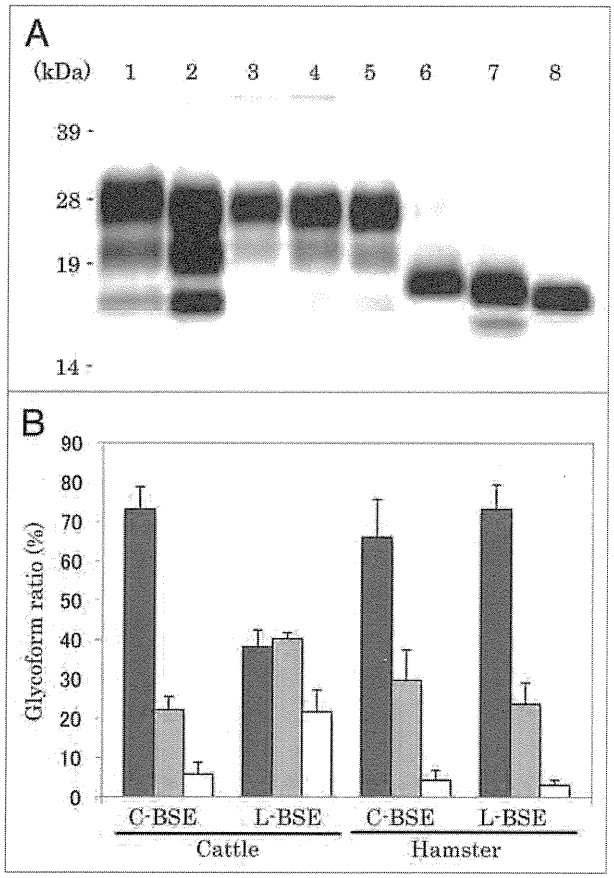

Figure 3. Western blotting analysis of PrPcore from C-BSE- and L-BSE-affected hamsters. (A) Lane 1: C-BSE-affected cattle (natural case); lane 2: L-BSE-affected cattle (natural case); lanes 3 and 6: Sc237-affected hamsters; lanes 4 and 7: C-BSE-affected hamsters; lanes 5 and 8: L-BSE-affected hamsters; lanes 6–8: PNGaseF treatment. (B) The relative amount (%) of di-, mono- and non-glycosylated PrPcore. The results are the mean (standard deviation) of three experiments. Bar diagram: di-(black), mono-(grey) and nonglycosylated form (white).

hamsters may be influenced by host-species characteristics rather than prion-strain characteristics. It has been recently reported that hamsters were susceptible to L-BSE and that, in this experimental model, L-BSE differed from classical BSE by its lower apparent molecular mass, whereas glycoforms proportions were similar.[31] Our results in hamsters and in hamster transgenic mice confirmed these observations.

The biological characteristics of L-BSE and C-BSE prions differed in cattle and TgBoPrP mice.[8,11] In cattle and bovinized mice, L-BSE showed severe spongiform changes, unlike C-BSE. On the other hand, in hamsters, the pathology of L-BSE was less severe than that of C-BSE. L-BSE was transmissible to hamsters (576.8 days), but C-BSE was not (>600 days). Further, the incubation period of C-BSE was approximately 270 days at the third passage, whereas that of L-BSE was shorter (208 days), even in the second passage (**Table 3**). The incubation period was shorter in L-BSE-affected animals than in C-BSE-affected animals; this observation was consistent among several animal species, including hamsters, cattle,[8,10] bovinized PrP-overexpressing mice,[6,11] humanized PrP-overexpressing mice,[9,32] and primates.[7]

Interestingly, L-BSE-affected TgHaNSE mice and hamsters showed a different neuropathology: the former had PrP plaque (**Fig. 1**), but the latter did not (**Fig. 2**). The same neuropathological difference was observed between cattle and TgBoPrP mice.[11] Therefore, we think that the formation of the PrP plaque was not only because of the prion strain and host PrP gene but also a factor that has not yet been identified.

Capobianco et al. reported that serial passage of the BASE prion in wild-type mice showed that the accumulated prion and transmissibility were identical to the C-BSE prion. In wild-type mice, the BASE prion strain was converted to the C-BSE-like prion strain during passages.[12] Further, BASE-inoculated ovine PrP-expressing mice showed indistinguishable phenotypic traits with C-BSE.[33] This study showed that some characteristics (lesion profile, glycoform) of L-BSE in hamsters resembled those of C-BSE, whereas other characteristics such as PrP^{Sc} deposition and molecular weight of PrPcore differed between C-BSE and L-BSE. This partial similarity in hamster-passaged C-BSE and L-BSE may be linked to the results of the previous transmission study, which showed that the subsequent passage of BASE altered its characteristics.[12,27]

In summary, the biochemical nature of L-BSE is modified to some extent after passage in hamsters. This study revealed the unstable phenotypic properties of L-BSE prions in interspecies transmission. The conformational moiety of PrP^{Sc}, which is linked to the N-terminal cleavage site of PrPcore, differed from the moieties linked to the relative PK resistance and glycoprofile of PrPcore.

Materials and Methods

The study protocol was approved by the Animal Ethics Committee and Animal Care and Use Committee of the National Institute of Animal Health, Japan.

Animals and prions. We purchased 3-week-old weanling female Syrian hamsters (SLC). We used transgenic mice that expressed mouse and hamster chimeric PrP (MHM2 and MH2M, respectively),[15] tga20 mice overexpressing mouse PrP,[16] and TgHaNSE mice overexpressing hamster PrP in their neurons.[17] The PrP amino acid sequence of these Tg mice is shown in **Table 3**. PrP sequences are referred from previous reports: mouse,[18] hamster[19] and cattle.[20] In MHM2 mice, amino acid substitutions are present at positions L109M and V112M. In addition to these amino acid substitutions, three other substitutions (I139M, Y155N and S170N) are present in MH2M mice. All mice were maintained by crossing with PrP-deficient mice[21] in a PrP-null background. The expression level of PrP in these transgenic mice was approximately 4–10 times greater than that in wild-type mice.[15-17] Brain samples of natural C-BSE[22] and natural Japanese L-BSE (BSE/JP24),[23] were used in this study.

Transmission experiments. Animals were inoculated with 20 μl of 10% brain homogenate (w/v) of L-BSE. Clinically affected animals were sacrificed and used in the experiments. The hamster-adapted C-BSE prion (described below) was also used.

Hamster-adapted C-BSE prions. For the control, we generated hamster-adapted BSE prions. C-BSE was not transmitted to hamsters; however, C-BSE was passaged in mice once, and this prion was transmissible to TgHaNSE with an incubation period of 153.1 days. The brains of diseased TgHaNSE mice were used as the origin of hamster BSE.

Histopathology and immunohistochemistry. Half brains were fixed in 10% neutral buffered formalin and were then subjected to hematoxylin and eosin staining and immunohistochemical analysis for the detection of PrP^{Sc}, as reported previously in reference 11. The lesion profiles in the brain was determined in nine areas of gray matter and three areas of white matter as described previously in reference 24. A PrP signal was detected with the anti-PrP monoclonal antibody (mAb) SAF-84 (SPI-bio).

Western blot analysis. Brain tissues were homogenized in a buffer containing 100 mM NaCl and 50 mM Tris-HCl (pH 7.6). The homogenate was mixed with an equal volume of detergent buffer containing 4% Zwittergent 3–14, 1% sarkosyl, 100 mM NaCl and 50 mM Tris-HCl (pH 7.6) and then incubated with 0.25 mg collagenase. The homogenate was then incubated with 40 μg/ml PK at 37°C for 30 min. PK digestion was terminated with 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc; Roche Diagnostics). The sample was mixed with 2-butanol:methanol (5:1) and then centrifuged at 20,000x g for 10 min. The extracted PrPcore was subjected to western blot analysis, according to a previously described method in reference 25. The PrP signal was detected with anti-PrP mAb 6H4 (Prionics) or mAb T2.[26]

Band profiles of PrPcore. For band analysis, the relative quantities of the 3 PrPcore bands were measured using the Fluorochem software (Alpha-Innotech), as reported previously in reference 11.

Peptide *N*-glycosidase F digestion. The PrPcore sample was deglycosylated with peptide *N*-glycosidase F (PNGase F; New England Biolabs), according to the manufacturer's instructions.

Relative PK resistance of PrPcore. For comparing the relative PK resistance of PrPcore, the sample was subjected to PK digestion at various concentrations (40–1,000 μg/ml), as reported previously in reference 11.

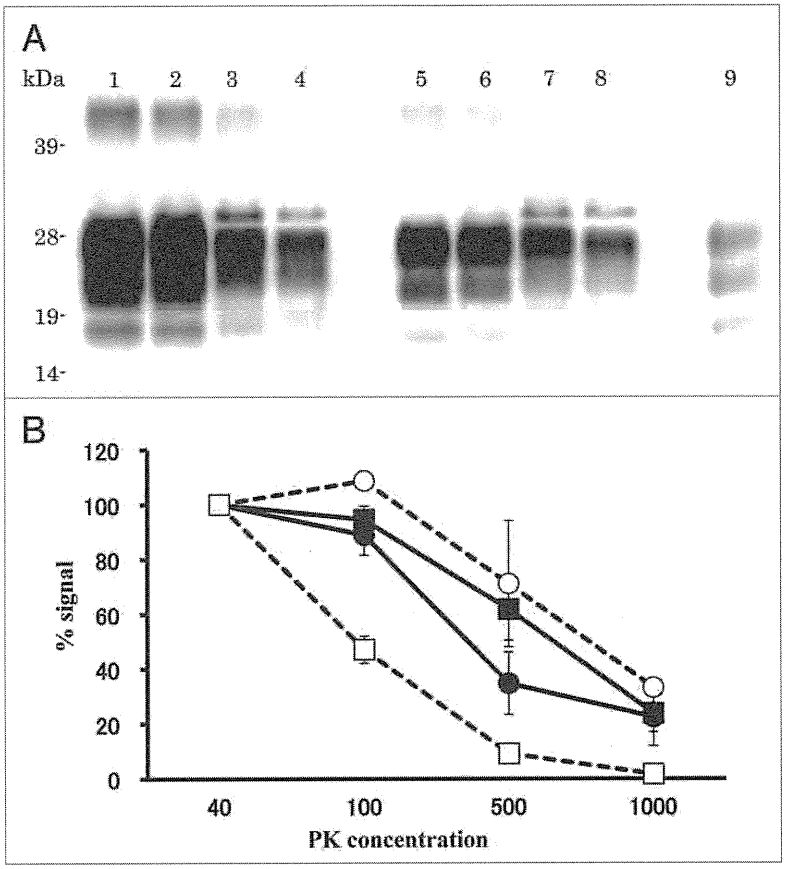

Figure 4. Relative PK resistance of PrPcore in prion-affected hamsters. (A) Western blot results. Lanes 1–4: hamster-adapted C-BSE; lanes 5–8: L-BSE-affected hamster. Lane 9: mouse scrapie prion. The samples were treated with 40 (lanes 1 and 5), 100 (lanes 2 and 6), 500 (lanes 3 and 7) and 1,000 (lanes 4 and 8) μg/ml of PK at 37°C for 1 h. PrPcore was detected with mAb 6H4. Molecular markers are shown on the left (kDa). (B) Relative amount (%) of PrPcore after different PK concentration were indicated. Black circle: C-BSE affected hamster, black square: L-BSE affected hamster, white circle: C-BSE affected cattle, white square: L-BSE affected cattle. Cattle results are obtained from previous study in reference 11.

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