Prado *et al.*, 2004). Many proteins that cycle between the intracellular compartments and the cell surface are known to accumulate in the ERC (Maxfield & McGraw, 2004). Thus, the presence of PrP^{Sc} at the ERC raised the possibility that PrP^{Sc} in prion-infected cells is intracellularly transported with the membrane trafficking machinery that is associated with endocytic recycling. Although time-lapse imaging of trafficking PrP^{Sc} in cells persistently infected with prions is technically impossible at the moment, the kinetic analysis of PrP^{Sc} distribution in the cells shown in this study provided an interesting insight into the intracellular trafficking of PrP^{Sc} (Fig. 6). PrP^{Sc} granules at the peripheral regions including those at the plasma membrane appeared to increase during incubation at 20 °C, whereas peri-nuclear PrP^{Sc} granules gradually decreased under this condition. Conversely, peripheral PrP^{Sc} granules appeared to decrease as PrP^{Sc} granules accumulated at the peri-nuclear regions once the cells were transferred to 37 °C. This drastic alteration in the distribution of PrP^{Sc} during sequential incubation at 20 and 37 °C suggests that PrP^{Sc} is dynamically transported through compartments that exist at the peri-nuclear regions, at least some of which are thought to be the ERC, by the membrane trafficking machinery of the cell. Combined with the fact that PrP^{Sc} is present at the ERC as described above, it is conceivable that at least some of the PrP^{Sc} cycles between peri-nuclear and peripheral regions including the plasma membrane through the ERC, via the endocytic recycling pathway.

Marijanovic *et al.* (2009) reported that impairment of the transport from early endosomes to the ERC by overexpression of Rab22a reduced the level of PrP^{Sc}, but that impairment of transport from the ERC to the plasma membrane using a dominant-negative mutant of Rab11a increased the level of PrP^{Sc} in GT1 cells infected with prions. These findings suggest that the ERC is possibly one of the sites where PrP^{Sc} formation takes place. Although multiple trafficking pathways may be involved in the intracellular transport of PrP^{Sc} as suggested by the widespread distribution of PrP^{Sc} in prion-infected cells, cycling of PrP^{Sc} between the peri-nuclear and peripheral regions including the plasma membrane through the ERC, rather than accumulation of PrP^{Sc} at specific compartments, may provide a significant advantage for the generation of PrP^{Sc}. For instance, such cycling may provide increased opportunities for PrP^{Sc} to enter a site for conversion, which could then act as a seed for the conversion of PrP^{C} into PrP^{Sc}.

There are a few studies which report that PrP^{Sc} was detected in late endosomes or early endosomes/ERC in the brains of prion-infected animals (Arnold *et al.*, 1995; Godsave *et al.*, 2008). However, intracellular localization of PrP^{Sc} in brains of prion-infected animals is largely unknown. We have confirmed that mAb 132 is also applicable to the PrP^{Sc}-specific detection in brain section of prion-infected animals, and now experiments are under way to extensively analyse the cellular compartments where prions present in the brain of mice infected with prions.

METHODS

Antibodies, reagents and chemicals. Mouse mAbs, 31C6, 44B1, 149 and 132 were used for the detection of PrP. mAbs 31C6, 149 and 132 recognize linear epitopes consisting of mouse PrP aa 143–149, 147–151 and 119–127, respectively, whereas mAb 44B1 recognizes a discontinuous epitope consisting of aa 155–231 (Kim *et al.*, 2004b). Following antibodies were used for IFA: anti-Lamp1 rat mAb (1D4B; Beckman Coulter); rabbit polyclonal antibodies: anti-Tgn38 (ab16059; Abcam), anti-giantin (PRB-114C; Covance), anti-flotillin-1 (F1180; Sigma), anti-Rab5 (#2143), anti-Rab7 (#2094) and anti-Rab11a (#3539) (all three from Cell Signaling Technology), anti-Rab4a (10347-1-AP) and anti-Rab9 (11420-1-AP) (Proteintech Group Inc.). An affinity purified anti-StxB rabbit polyclonal antibody was prepared from polyclonal antiserum raised by immunizing a rabbit with purified StxB1-H and StxB2-H (Shimizu *et al.*, 2007). Alexa-Fluor-488- or 546-conjugated goat $F(ab')_2$ fragment anti-mouse IgG, Alexa-Fluor-555-conjugated goat $F(ab')_2$ fragment anti-rabbit IgG and Alexa-Fluor-555-conjugated goat IgG anti-rat IgG (Invitrogen) were used as secondary antibodies for IFA.

Cell culture. Subclones of mouse neuroblastoma cell line Neuro2a, N2a-3 (Uryu *et al.*, 2007), and of the hypothalamic neuronal cell line GT1, GT1-7 (Schätzl *et al.*, 1997), were used. As prion-infected cells, ScN2a-3-Ch (Uryu *et al.*, 2007), ScN2a-3-22L (Nakamitsu *et al.*, 2010) and ScGT1-7-22L were used. These prion-infected cells maintain PrP^{Sc} without significant loss of PrP^{Sc} more than 50 passages. However, we usually used these cells of passage history between 10 and 30 times. N2a-derived cells were passaged at a 1 : 10 dilution ratio every 3–4 days and were cultured in Dulbecco's modified Eagle's medium (DMEM; ICN Biomedicals) containing 10 % FBS (Gibco), MEM non-essential amino acids (NEAA; Gibco), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) (Gibco) at 37 °C in 5 % CO_2 atmosphere. The GT1-7 cells were passaged at a 1 : 5 dilution ratio every 7 days and were cultured in DMEM containing 5 % FBS, 5 % horse serum (Gibco) and penicillin/streptomycin.

IFA. Cells grown on a Lab-Tek II CC2 eight-well chamber slide (Nunc) were fixed with pre-warmed PBS containing 4 % paraformaldehyde and 4 % sucrose at 37 °C or at room temperature (RT) for 10 min. After removal of the fixation solution, the remaining paraformaldehyde was neutralized with 0.1 M glycine in PBS for 10 min and then the cells were permeabilized with 0.1 % saponin in PBS for 10 min. For the detection of PrP^{Sc}, cells were pre-treated with 5 M GdnSCN for 10 min. The cells were then washed once with PBS and blocked with 5 % FBS in PBS at RT for 30 min. After blocking, the cells were incubated with primary antibodies in PBS containing 0.5 % FBS at 4 °C overnight. Anti-PrP mAbs were used at a concentration of 1 μg ml^{-1}. A mAb P2-284 (1 μg ml^{-1}) against feline panleukopenia virus was used as a negative-control antibody (Horiuchi *et al.*, 1997). After washing five times with PBS, the cells were incubated with Alexa-Fluor-conjugated secondary antibodies (1 : 1000) at RT for 90 min. For counterstaining of cell nuclei, cells were incubated for 30 min with 5 μg DAPI ml^{-1} (Invitrogen) in PBS at RT. The samples were then mounted with ProLong Gold antifade reagent (Invitrogen) and covered with coverslips. The samples were examined using a Nikon C1 laser confocal fluorescence microscope (Nikon) or with a Zeiss laser scanning microscope LSM 700 (Zeiss).

Tfn, CtxB and StxB uptake experiments. For analysis of Tfn uptake, cells grown on Lab-Tek chamber slide were washed twice with Opti-MEM (Gibco) and incubated with 10 μg Alexa-Fluor-555-conjugated Tfn (Invitrogen) ml^{-1} in Opti-MEM at 37 °C for 15 min. The cells were then washed three times with pre-warmed PBS and incubated at 37 °C for up to 30 min. For analysis of the uptake of CtxB, cells grown on a Lab-Tek chamber slide were washed with

Opti-MEM and incubated with 2 μg Alexa-Fluor-488-conjugated CtxB (Invitrogen) ml^{-1} in Opti-MEM at 4 °C for 30 min. For analysis of StxB uptake (Shimizu *et al.*, 2007), cells grown on a Lab-Tek chamber slide were washed with Opti-MEM and incubated with 2 μg StxB ml^{-1} in Opti-MEM at 4 °C for 60 min. The medium was then replaced with pre-warmed fresh DMEM and the cells were incubated at 37 °C for up to 60 min.

Immunoblotting of PrP-res. ScN2a-3-22L cells on 60 mm dishes were washed once with PBS, collected using a cell scraper and the cell number was counted. The collected cells were lysed with lysis buffer (Uryu *et al.*, 2007) at 4 °C for 20 min and cell debris was removed by centrifugation at 2000 *g*. The protein concentration of the lysates was measured using the DC protein assay kit (Bio-Rad) and was adjusted to 1 mg ml^{-1}. The lysates (300 μl) were digested with 20 μg PK ml^{-1} at 37 °C for 20 min. The proteolysis was terminated by the addition of Pefabloc (Roche) to 1 mM. The PK-treated lysates were incubated with 0.3 % phosphotungstic acid at RT for 20 min and then centrifuged at 20 000 *g* for 20 min to precipitate PK-resistant PrP (PrP-res). The pellet was dissolved in SDS sample buffer and boiled for 5 min. A sample volume equivalent to 4×10^5 cells was loaded onto a NuPAGE 12 % Bistris gel (Invitrogen). SDS-PAGE was carried out according to the manufacturer's instructions (Invitrogen). Subsequent Western transfer and chemiluminescence were carried out as described previously (Shindoh *et al.*, 2009).

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Comparative analysis of Japanese and foreign L-type BSE prions

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Key words: prion, atypical BSE, L-type BSE

Abbreviations: BSE, bovine spongiform encephalopathy; BSE/JP8, the 8th BSE case in Japan; BSE/JP24, the 24th BSE case in Japan; BASE, bovine amyloid spongiform encephalopathy; CNS, central nervous system; C-BSE, classical BSE; H-type BSE, high-type BSE; L-type BSE, low-type BSE; mAb, monoclonal antibody; PET blot, paraffin-embedded tissue blot; PK, proteinase K; PNGaseF, N-glycosidase F; PrP, prion protein; PrPcore, proteinase K-digested PrP^{Sc}; PrP^{Sc}, abnormal prion protein; TSEs, transmissible spongiform encephalopathies

L-type bovine spongiform encephalopathy (BSE) is an atypical form of BSE. To characterize the Japanese L-type BSE prion, we conducted a comparative study of the Japanese and foreign L-type BSE isolates. The L-type BSE isolates of Japan, Germany, France and Canada were intracerebrally inoculated into bovinized prion protein-overexpressing transgenic mice (TgBoPrP). All the examined L-type BSE isolates were transmitted to TgBoPrP mice, and no clear differences were observed in their biological and biochemical properties. Here, we present evidence that the Japanese and Canadian L-type BSE prions are identical to those from the European cases.

Bovine spongiform encephalopathy (BSE) is one of the transmissible spongiform encephalopathies (TSEs), or prion diseases, in cattle. TSE is characterized by spongiform changes in the central nervous system (CNS) and the accumulation of an abnormal prion protein (PrP^{Sc}) in the CNS.[1] PrP^{Sc} has been regarded as the major component of TSE pathogens.[2]

BSE was detected in the UK in 1986,[3] and subsequently spread to the other European countries, Japan and North America.[4-6] BSE is thought to be caused by a single prion strain, based on the analyses of its biological and biochemical characteristics.[7] From 2003, however, several atypical neuropathological and molecular phenotypes of BSE (atypical BSE) have been detected in Japan, several European countries and North America.[6,8-17] Currently, based on the molecular size of the proteinase-digested non-glycosylated form of PrP^{Sc}, atypical BSE is classified into two groups (L-type and H-type).[14]

L-type BSE cases have been identified in the European countries, including Italy, France, Germany, Netherland, Poland and in Canada and Japan.[8-15] Two L-type BSE cases have been identified in Japan. One case was detected in a healthy 23-mo-old Holstein steer (BSE/JP8),[8] and the other was detected in a 14-y-old black Japanese beef cattle (BSE/JP24).[9] The latter case was successfully transmitted to bovinized transgenic mice and cattle, and the biological and biochemical properties differed from that of classical BSE (C-BSE).[18,19] However, it is unclear whether Japanese L-type BSE prion is identical to that of L-type BSE isolates from other countries. To characterize the Japanese L-type BSE isolate, we performed a comparative study of the Japanese and foreign L-type BSE isolates.

A transmission study using experimental animals is a useful approach for prion characterization. Therefore, we performed a transmission study of the L-type BSE isolates in bovinized prion protein (PrP)-overexpressing transgenic mice (TgBoPrP).[20] Brain samples of L-type BSE-affected cattle from Japan (BSE/JP24),[9] France,[10] Germany[11] and Canada[12] were used in this study. The brain homogenates were intracerebrally inoculated into TgBoPrP using previously described methods in reference 18. All animal experiments were reviewed by the Committee of the Ethics on Animal Experiment of the National Institute of Animal Health.

All the examined L-type BSE isolates were transmitted to TgBoPrP, and the affected mice developed progressive neurological diseases. Japanese L-type BSE isolate-affected TgBoPrP exhibited a unique clinical sign, the circling behavior. The same phenotype was observed when TgBoPrP were inoculated with German, French and Canadian L-type BSE isolates. On the other hand, in the first passage the incubation period for the Japanese L-type BSE isolate was significantly different from that of the other L-type BSE isolates (**Table 1**). We then performed serial passages of these isolates for further characterization. The incubation periods in the second passage were shorter than those in the first passage. In the third passage, the incubation periods for all the L-type BSE isolates converged at about 145 d. These results suggest that the L-type BSE isolates in the primary passage were not fully adapted to the TgBoPrP mice. Furthermore,

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Table 1. Transmission of L-type BSE isolates in TgBoPrP mice

	Incubation period (days)			
	JPN	CAN	GER	FRA
First passage	197.7 (3.4)†	172.8 (4.0)*	173.3 (3.3)*	175.7 (5.6)*
	(10/10·)	(12/12)	(12/12)	(10/10)
Second passage	152.0 (1.7)	145.7 (1.8)	143.1 (5.7)	143.1 (3.9)
	(24/24)	(23/23)	(18/18)	(18/18)
Third passage	145.1 (3.6)	143.7 (4.6)	145.3 (8.6)	141.6 (4.7)
	(21/21)	(25/25)	(12/12)	(20/20)

†Mean (standard deviation); ·, Number of affected mice/number of inoculated mice; *$p < 0.05$ for Japanese L-type BSE isolate vs. other L-type BSE isolates in the first passage (Student's t-test).

the different incubation periods in the first passage may be caused by the lower titer of the Japanese L-type BSE prion.

Neuropathological examination of the L-type BSE isolate-affected TgBoPrP were performed using previously described methods.[18] Lesion profile analysis revealed that the degree of brain vacuolation due to the Japanese L-type BSE isolate was similar to that caused by the other L-type BSE isolates (**Fig. 1A**). All the L-type BSE isolates caused severe spongiform changes in the hippocampus, septal nuclei of the paraterminal body and cerebral cortex. We next examined the PrP^{Sc} deposition pattern in the brain using paraffin-embedded tissue (PET) blot, as described previously in reference 18. The distributions of PrP^{Sc} deposits in Japanese L-type BSE isolate-inoculated mice were similar to that of mice inoculated with the other L-type BSE isolates; fine punctate and fine granular PrP^{Sc} were predominantly and uniformly distributed in the pons, cerebellar medulla, midbrain, thalamus and corpus callosum (**Fig. 1B**). Furthermore, similar PrP^{Sc} deposits and distribution patterns were observed in the brain in the first and subsequent passages of all the L-type BSE isolates (data not shown).

We further examined the biochemical properties of PrP^{Sc}, such as the glycoform ratio and molecular mass of proteinase K (PK)-digested PrP^{Sc} (PrPcore). PrP^{Sc} were extracted from the brain of L-type BSE isolate-affected TgBoPrP using previously described methods in reference 18. Western blotting analysis revealed that the glycoform patterns and molecular mass of the PrPcore of the Japanese L-type BSE isolate resembled that of the other L-type BSE isolates. In contrast, clear differences were observed between C-BSE and L-type BSE isolates (**Fig. 2A and B**). Next, we examined the relative PK resistance of PrP^{Sc} from L-type BSE isolate-affected TgBoPrP, as described previously in reference 18. The PrP concentration of the sample was adjusted using the signal intensity of western blot. The PK resistance of PrP^{Sc} from the Japanese L-type BSE was similar to that of the foreign L-type BSE isolates. The PrP^{Sc} of C-BSE-affected TgBoPrP was resistant to digestion with 1,000 μg/ml of PK. In contrast to C-BSE, the PrP^{Sc} signal from the L-type BSE isolates decreased when digested with 500 μg/ml of PK (**Fig. 2C**).

The analyses of L-type BSE cases have been performed using different bovinized PrP-overexpressing transgenic mice, such as TgBoPrP,[18] Tgbov XV[11,21] and Tg540.[22] Thus, it has been impossible to compare the properties of L-type BSE isolates in detail. In this study, therefore, we performed a transmission study of the L-type BSE isolates using identical bovinized PrP-overexpressing transgenic mice to further characterize the Japanese L-type BSE prion. All the L-type BSE isolates transmitted to TgBoPrP, and their incubation periods converged at approximately 145 d following serial passages (**Table 1**). Similar degrees of vacuolation and PrP^{Sc} deposition patterns in the brain were observed among the L-type BSE isolates (**Fig. 1A and B**). Besides the biological characteristics, no differences were observed in the biochemical characteristics of PrP^{Sc} from the L-type BSE isolates (**Fig. 2A–C**). These findings suggest that the examined L-type BSE cases were caused by prions with identical characteristics.

Italian L-type BSE cases are called bovine amyloid spongiform encephalopathy (BASE). We could not compare the characteristics of the Japanese L-type BSE with those of the Italian isolates. In a transmission study using transgenic mice, the French L-type BSE isolate and BASE exhibit similar biological characteristics.[22] Our data indicated that the properties of the Japanese L-type BSE prion are identical to those of the French L-type BSE isolate. It has also been reported that the characteristics of Japanese L-type BSE isolate closely resemble those of BASE in an experimental transmission study in cattle.[19]

The origin of L-type BSE prion is unknown. The present study showed that the Japanese and Canadian L-type BSE prions are identical to those from the European cases. The fact that identical L-type BSE prions exhibit a worldwide distribution is important insight for devising atypical BSE control measures.

Acknowledgments

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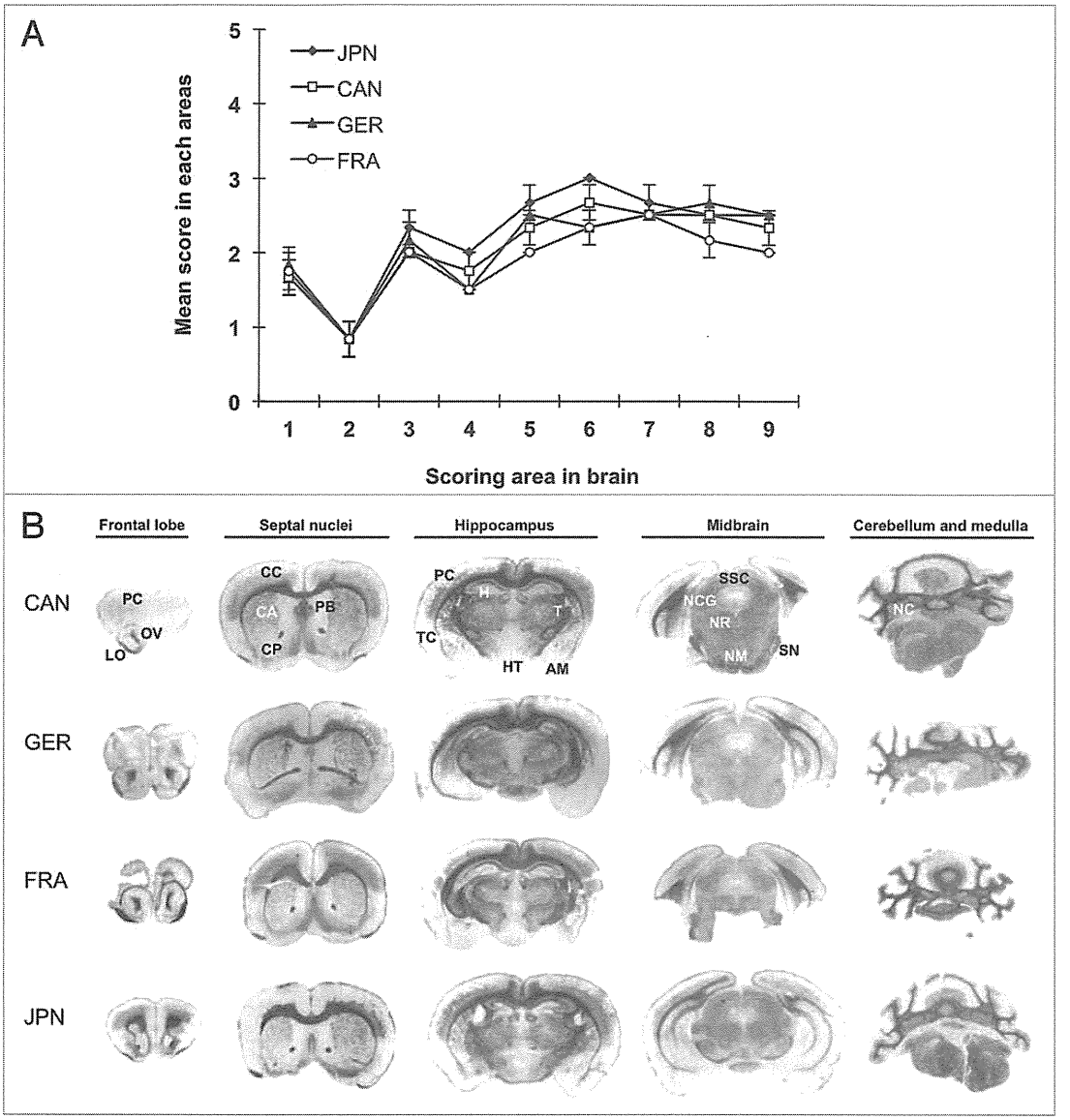

Figure 1. Neuropathological analysis of L-type BSE isolate-affected TgBoPrP. (A) Lesion profile in the first passage. The vacuolation in the following brain regions was scored on a scale of 0–5 (mean values): 1, dorsal medulla; 2, cerebellar cortex; 3, superior cortex; 4, hypothalamus; 5, thalamus; 6, hippocampus; 7, septal nuclei of the paraterminal body; 8, cerebral cortex at the levels of the hypothalamus and thalamus; and 9, cerebral cortex at the level of the septal nuclei of the paraterminal body. The data are presented as mean ± standard deviation (n = 5). ◆, Japanese L-type BSE (JPN); □, Canadian L-type BSE (CAN); ▲, German L-type BSE (GER); ○, French L-type BSE (FRA). (B) The neuroanatomical distribution of PrP^{Sc} in the brain of TgBo-PrP mice infected with Canadian (CAN), German (GER), French (FRA) and Japanese (JPN) L-type BSE isolate by PET-blot analysis. The PET-blot analysis reveals preferential and intense PrP^{Sc} immunolabeling along with periventricular areas, corpus callosum and cerebellar gray matter. Widespread PrP^{Sc} immunolabeling is also detected in the thalamic and brainstem nuclei, while PrP^{Sc} immunostaining in the cerebral and cerebellar cortices and basal ganglia is less conspicuous. Dewaxed membranes were treated with PK (80 μg/mL), followed by denaturation with 3 M guanidine thiocyanate. The monoclonal antibody (mAb) SAF84 was used. Blots corresponding to the brain areas at the level of frontal lob, septal nuclei, hippocampus, midbrain and medulla and cerebellum. FC, frontal cortex; OV, olfactory ventricle; LO, lateral orbital cortex; CC, cingulated cortex; CP, caudate putamen; PB, paraterminal body; CP, caudate putamen; PC, parietal cortex; TC, temporal cortex; H, hippocampus; T, thalamus; HT, hypothalamus; AM, amygdala; SSC, stratum moleculare of the cerebellum; NCG, nucleus corporis geniculati; NR, nucleus rubber; SN, substantia nigra; NM, nucleus mammillaris; NC, deep nuclei of the cerebellum.

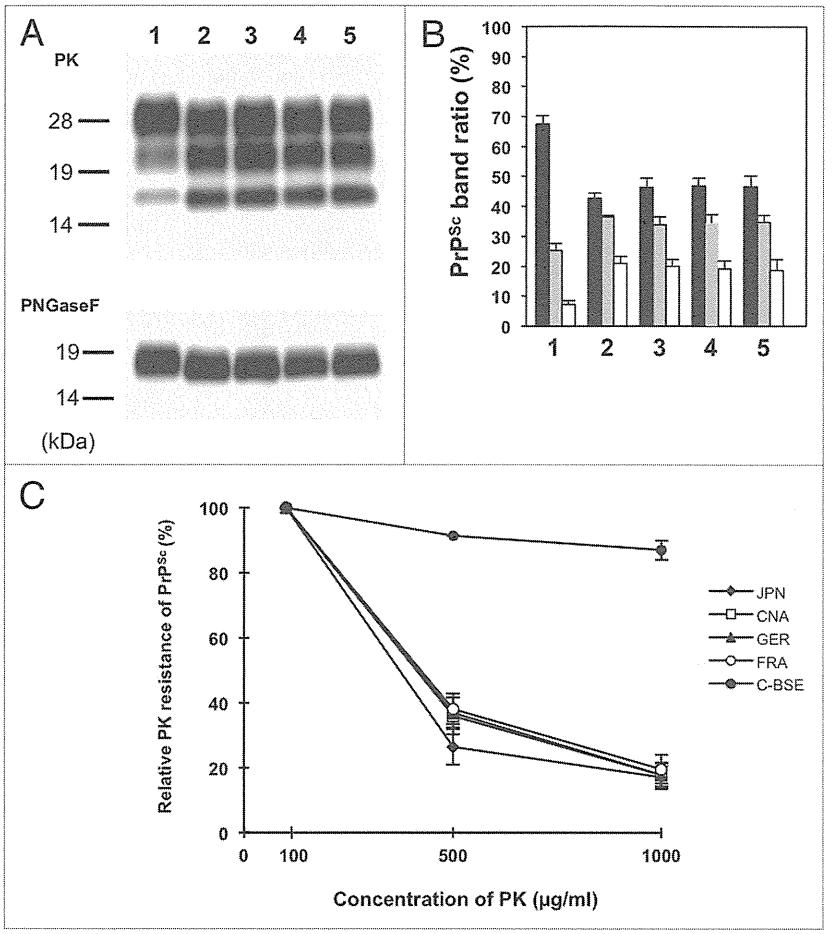

Figure 2. Western blot analysis of proteinase K (PK)-digested prion protein (PrPcore) from the brain of L-type BSE isolate-affected TgBoPrP. (A) Lane 1, Classical-BSE; Lane 2, Japanese L-type BSE; Lane 3, Canadian L-type BSE; Lane 4, French L-type BSE; Lane 5, German L-type BSE. All the samples were digested with 50 μg/ml PK at 37°C for 1 h (upper part), and digested aliquots were treated with N-glycosidase F (PNGaseF), according to the manufacturer's instructions (bottom part). PrPcore was detected with mAb 6H4. Molecular markers are shown on the left (kDa). (B) The relative amounts of the diglycosylated (solid black bar), monoglycosylated (gray bar), and unglycosylated (clear bar) forms in the PrPcore from the brain of L-type BSE isolate-affected TgBoPrP. The lane numbers are as listed in (A). The results are presented as mean ± standard deviation from 5 experiments. (C) Relative PK resistance of PrP^{Sc} from L-type BSE isolate-affected TgBoPrP. The PrP^{Sc} concentration of the sample was adjusted using the western blot signal intensity. The samples were treated with various concentrations of PK (100–1,000 μg/mL). The results are presented as mean ± standard deviation from 3 experiments. PrP^{Sc} was detected with mAb 6H4. ◆, Japanese L-type BSE (JPN); □, Canadian L-type BSE (CAN); ▲, German L-type BSE (GER); ○, French L-type BSE (FRA); ●, Classical-BSE (C-BSE).

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Original Article

Neuroanatomical Distribution of Disease-Associated Prion Protein in Experimental Bovine Spongiform Encephalopathy in Cattle after Intracerebral Inoculation

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SUMMARY: The pathologic disease-associated prion protein (PrP^{Sc}) has been shown to be expressed in the central nervous system of Holstein cattle inoculated intracerebrally with 3 sources of classical bovine spongiform encephalopathy (BSE) isolates. Several regions of the brain and spinal cord were analyzed for PrP^{Sc} expression by immunohistochemical and Western blotting analyses. Animals euthanized at 10 months post-inoculation (mpi) showed PrP^{Sc} deposits in the brainstem and thalamus, but no vacuolation; this suggested that the BSE agent might exhibit area-dependent tropism in the brain. At 16 and 18 mpi, a small amount of vacuolation was detected in the brainstem and thalamus, but not in the cerebral cortices. At 20 to 24 mpi, when clinical symptoms were apparent, heavy PrP^{Sc} deposits were evident throughout the brain and spinal cord. The mean time to the appearance of clinical symptoms was 19.7 mpi, and the mean survival time was 22.7 mpi. These findings show that PrP^{Sc} accumulation was detected approximately 10 months before the clinical symptoms of BSE became apparent. In addition, the 3 sources of BSE prion induced no detectable differences in the clinical signs, incubation periods, neuroanatomical location of vacuoles, or distribution and pattern of PrP^{Sc} depositions in the brain.

INTRODUCTION

Bovine spongiform encephalopathy (BSE), a type of transmissible spongiform encephalopathy (TSE), is a fatal neurodegenerative disease affecting cattle. The disease was first identified in the United Kingdom (UK) in 1986 (1); subsequently, it spread to European, Asian, and North American countries. The first case of BSE in Japan was reported in September 2001 (2), and the most recent case, the 36th, was confirmed in January 2009. BSE is characterized by spongiform changes (3) and accumulation of the disease-associated prion protein (PrP^{Sc}) in the central nervous system (CNS) (4). PrP^{Sc} is commonly accepted as the pathological agent of BSE and is thought to be a post-translationally modified form of the host-encoded membrane glycoprotein (PrP^{C}) (5). PrP^{Sc} is the only known disease-specific marker (6,7).

The pathological agent of BSE is transmissible to different mammalian species. A variant form, i.e., the degenerative brain disease Creutzfeldt-Jakob Disease (vCJD) has been reported in the UK and several other countries, and it is thought that this disease is caused by the consumption of BSE-contaminated beef products (8–11). Therefore, it is important to understand the pathogenesis of BSE in cattle in order to eliminate BSE-contaminated food from human food and thereby preserve public health.

The uniformity in the pathological features and biochemical profile of the proteinase K (PK)-resistant PrP^{Sc} (PrP^{res}) in BSE-affected cattle suggests that a single prion strain is responsible for BSE in these animals. Recently, variants of BSE (named atypical BSE) have been detected in cattle in Europe (12,13), North America (14,15), and Japan (16,17). Currently, atypical BSE cases are classified into 2 groups; those expressing PrP^{res} of lower (L-type BSE) molecular weight and those expressing PrP^{res} with higher (H-type BSE) molecular weight than the PrP^{res} of classical BSE (C-BSE) (18).

Our current knowledge of the pathogenesis of C-BSE in cattle is based on the examination of tissues obtained from orally infected cattle that have been euthanized at different stages of the disease. A mouse bioassay of infectivity showed that in cattle, the infection was limited to the brain, spinal cord, eyes, dorsal root ganglia, and distal ileum (19–21). Occasionally, infectivity has also been detected in the bone marrow and tonsils of experimentally infected cattle (22,23), and recent studies have shown that peripheral tissues other than the CNS may harbor PrP^{Sc} at the clinical stages of the disease (24,25). The distribution of PrP^{Sc} in the brain has been

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mapped in both naturally occurring C-BSE and orally infected cattle (26–28). However, it would be of interesting to evaluate the relationship between the time of detection of PrP^{Sc} in the CNS and the clinical course of the disease. For this purpose, we used the intracerebral inoculation route to infect cattle with 3 different C-BSE strains—one isolated in Kanagawa, Japan (BSE/JP5); one, in Wakayama, Japan (BSE/JP6) (29); and one, in the UK (BSE/UK) (30). We then proceeded to measure the distribution of PrP^{Sc} in the CNS of the infected animals by immunohistochemical and Western blotting analyses.

MATERIALS AND METHODS

Ethical considerations: All experiments involving animals were approved by the Animal Ethical Committee and the Animal Care and Use Committee of both Hokkaido Animal Research Center and National Institute of Animal Health.

Inoculation of cattle with C-BSE agents: Brain homogenates (10% w/v) were prepared from the brainstems of 3 cattle: one had been naturally infected with C-BSE (BSE/UK) provided by the Veterinary Laboratory Agency, UK; one was infected with domestic Kanagawa-1 (BSE/JP5) at 80 months of age; and one was infected with domestic Wakayama (BSE/JP6) at 83 months of age (29,30). Sixteen female Holstein calves aged 3 months were used for this experiment (n = 8 for BSE/UK, n = 4 for BSE/JP5, and n = 4 for BSE/JP6) (Table 1). Each animal was inoculated in the right side of the midbrain and 1 mL of brain homogenate was withdrawn from the brain by using an 18-gauge 7-cm disposable needle (NIPRO, Osaka, Japan). Two uninfected cattle served as controls and were euthanized at 27 months of age.

Neuropathology: At necropsy, the brains and cerebella were removed and hemisected at the midline. Samples of various tissues were fixed in 10% neutral buffered formalin (pH 7.4) for 3 days at 37°C, including those of the left hemisphere and spinal cord at the levels of cervical (C8) and lumbar enlargement (L6). The contralateral side was frozen at −80°C for Western blotting analysis of PrP^{res}. Coronal slices of the brain and various tissues were cut at 3–4 mm thickness and placed in plastic cassettes, which were immersed in 98% formic acid for 60 min at room temperature (RT) to reduce infectivity (31). The tissues were automatically processed through a graded series of alcohol to xylene and then paraffin-embedded (ETP-150C; Sakura Finetek Japan, Tokyo, Japan). Serial sections were cut at a thickness of 4 μm, mounted on silane-coated glass slides (New Silane II; Muto Pure Chemicals Co., Tokyo, Japan) and stained with hematoxylin and eosin or processed for immunohistochemistry, as described below. The distribution and extents of vacuolation in the brain were scored according to the method described by Simmons et al. (32). A vacuolation lesion profile was created by plotting the mean vacuolation score for each neuroanatomical area against the assigned code for that area.

PrP^{Sc} immunohistochemistry: For each animal, tissue samples were examined from at least 8 areas of the brain and 2 spinal cord levels: frontal lobe, striatum, thalamus, occipital lobe, midbrain, pons, medulla oblongata at the obex, and cerebellum, and the C8 and L6 levels of the spinal cord. The paraffin-embedded tissue sections were pretreated at RT for PrP^{Sc} antigen retrieval using a recently developed chemical method (33). Briefly, deparaffinized and rehydrated tissue sections were immersed in a bath of 98% formic acid for 5 min, incubated with 0.5% (w/v) potassium permanganate (in 0.1 M phosphate buffer, pH 7.0) for 10 min, and then washed in distilled water 3 times. The sections were soaked in 1% sodium disulfite for 2 min and then washed in distilled water. The slides were then immersed in a solution of 0.1% *N*-lauroylsarcosine, 75 mM sodium hydroxide, and 2% sodium chloride for 10 min. Next, the sections were washed in tap water for 5 min and then placed in an immunohistochemical autostainer (Dako Cytomation Autostainer Universal Staining System; Dako, Carpinteria, Calif., USA). They were then incubated sequentially with 1 μg/mL anti-PrP primary monoclonal

Table 1. Summary of the clinical and pathological changes in cattle intracerebrally inoculated with the BSE agent

Case	Code	Inoculum	Time of clinical onset (mpi)	Clinical signs at onset	Terminal clinical signs	Time at necropsy (mpi)	Spongiform change	PrP^{Sc} by IHC	PrP^{Sc} by WB
1	0801	BSE/UK	None			3	−	−	−
2	9066	BSE/UK	None			10	−	+	+
3	9385	BSE/UK	None			12	−	+	+
4	3962	BSE/JP6	None			12	−	+	+
5	2601	BSE/UK	None			16	+	+	+
6	0886	BSE/UK	None			18	+	+	+
7	3955	BSE/JP6	None			19	+	+	+
8	4394	BSE/UK	18	gait abnormality	abnormal posture	20	+	+	+
9	3728	BSE/JP5	19	nervous	ataxia	21	+	+	+
10	5426	BSE/JP5	21	ataxia	astaxia	22	+	+	+
11	5523	BSE/JP6	19	nervous	ataxia	23	+	+	+
12	4437	BSE/UK	18	ataxia	astaxia	23	+	+	+
13	1479	BSE/JP5	20	gait abnormality	astaxia	23	+	+	+
14	5087	BSE/UK	19	gait abnormality	ataxia	24	+	+	+
15	3217	BSE/JP5	22	gait abnormality	ataxia	24	+	+	+
16	4612	BSE/JP6	22	abnormal posture	abnormal posture	24	+	+	+

BSE, bovine spongiform encephalopathy; mpi, months post-inoculation; IHC, immunohistochemistry; WB, Western blotting.

antibody (mAb) T1, goat anti-mouse Fab′ universal immunoperoxidase polymer (Histofine SimpleStain MAX-PO (M); Nichirei, Tokyo, Japan), and 3-3′ diaminobenzidine tetrachloride as the chromogen. The T1 primary mAb was raised against mouse PrP amino acid residues 121-231 and cross-reacts with bovine PrP (34). Finally, the sections were counterstained with hematoxylin. All the steps in the immunohistochemical staining procedure were carried out at RT.

Immunohistochemical PrP^{Sc} mapping and profiling: For each animal, the topographical distribution of PrP^{Sc} deposition was mapped at 14 different areas of the CNS: frontal cortex, temporal cortex, parietal cortex, occipital cortex, striatum, hippocampus, thalamus, midbrain, pons, medulla oblongata at the obex, cerebellar cortex, cerebellar medulla, and spinal cord at C8 and L6 segments. The PrP^{Sc} were classified into 8 types, as previously published (27,35). Intracellular PrP^{Sc} were subdivided into intraneuronal and intraglial granular deposits. Reports indicate that the stellate-type of PrP^{Sc} immunolabeling in astrocytes differed from the intraglial-type labeling (27,35). Extracellular PrP^{Sc} depositions in the neuropil were classified as linear, perineuronal, fine particulate, coarse granular, and coalescing.

PrP^{Sc} accumulation was scored subjectively for intensity and extent on a scale from 0 to 4 (0, negative; 1, apparent at high magnification; 2, apparent at moderate magnification; 3, apparent at low magnification and moderate amounts of accumulation; and 4, large amounts of accumulation) (36,37); it was then topographically mapped to the different CNS areas mentioned above.

Western blotting: Tissue samples were obtained from 18 areas of the brain and spinal cord, as shown schematically in Fig. 1. The 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 buffer containing 4% (w/v) Zwittergent 3-14 (Merck, Darmstadt, Germany), 1% (w/v) Sarkosyl, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.6), and incubated with 0.25 mg collagenase, followed by incubation with PK (final concentration, 40 μg/mL) at 37°C for 30 min. PK digestion was terminated by the addition of 2 mM Pefabloc (Roche Diagnostics, Basel, Switzerland). The sample was then mixed with 2-butanol:methanol (5:1) and centrifuged at 20,000 g for 10 min. The extracts were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, Mass., USA). The blotted membrane was incubated with horseradish-conjugated anti-PrP mAb T2 (34) at RT for 60 min. Signals were developed with a chemiluminescent substrate (SuperSignal; Pierce Biotechnology, Rockford, Ill., USA).

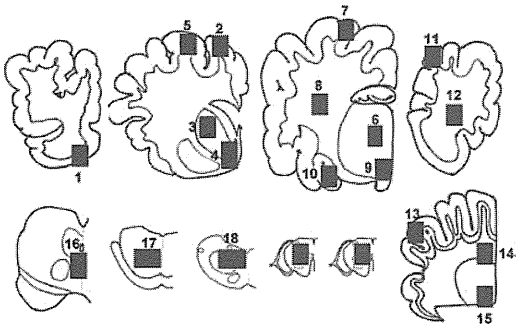

Fig. 1. The 18 areas of brain and spinal cord (black boxes) dissected for the Western blot analyses are schematically represented. They include 10 coronal slices of the brain and spinal cord at the levels of (from upper left) the frontal lobe, striatum, thalamus, occipital lobe, midbrain, pons, medulla oblongata at the obex, spinal cord at the cervical enlargement, spinal cord at the lumbar enlargement, and cerebellum. The brain regions are as follows: 1, frontal cortex; 2, parietal cortex; 3, caudate nucleus; 4, accumbens; 5, parietal cortex; 6, thalamus; 7, parietal cortex; 8, white matter at level of thalamus; 9, hypothalamus; 10, hippocampus; 11, occipital cortex; 12, occipital white matter; 13, cerebellar cortex; 14, cerebellar white matter; 15, cerebellar nucleus; 16, midbrain; 17, pons; and 18, obex.

RESULTS

Clinical signs: Of the 16 animals studied, 7 (Cases 1-7) showed no clinical signs of BSE even as late as 19 months post-inoculation (mpi) (Table 1). The remaining 9 animals (Cases 8-16) exhibited the initial clinical signs of disease between 18 and 22 mpi (19.7 ± 1.6, mean ± standard deviation); these signs included lowering of the head, heightened anxiety, and sensitivity to auditory stimuli. Within 2 to 3 months of the appearance of the initial clinical symptoms, the animals developed ataxia of the hind limbs, which progressed to difficulty in raising them without assistance. C-BSE-infected cattle were euthanized during this stage of the disease between 20 and 24 mpi (Table 1). There was no detectable difference in the clinical signs exhibited by animals inoculated with the 3 different C-BSE isolates.

Histopathology: The severity of vacuolation in the

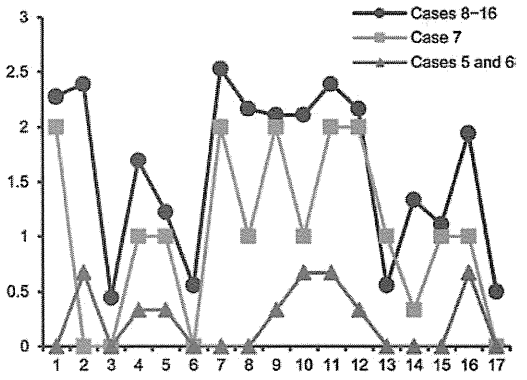

Fig. 2. Vacuolar lesion scores in BSE-challenged cattle at preclinical and clinical stages of the disease. Points from Cases 5 and 6 represent the means of 2 animals euthanized at the preclinical stage of disease at 16 and 18 mpi, respectively. Case 7 was euthanized at 19 mpi. Points for Cases 8-16 represent the mean score of 9 cases with clinical signs, euthanized between 20 and 24 mpi. Scores (y-axis) are plotted against the code numbers (x-axis) for anatomical areas as follows: 1, nucleus of the solitary tract; 2, nucleus of the spinal tract of the trigeminal nerve; 3, hypoglossal nucleus; 4, vestibular nuclear complex; 5, cochlear nucleus; 6, cerebellar vermis; 7, central gray matter; 8, rostral colliculus; 9, medial geniculate nucleus; 10, hypothalamus; 11, nucleus dorsomedialis thalami; 12, nucleus ventralis lateralis thalami; 13, frontal cortex; 14, accumbens; 15, caudate nucleus; 16, putamen; and 17, claustrum.

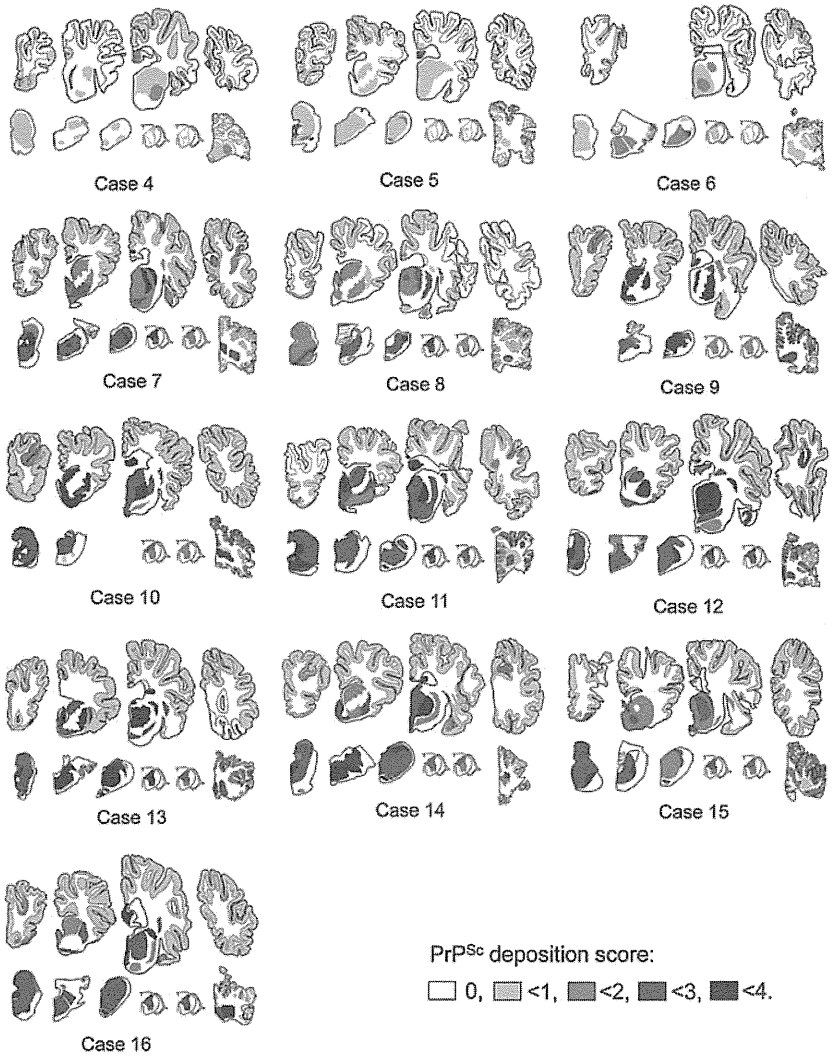

Fig. 3. Schematic representation of PrP^{Sc} in different brain areas of BSE-challenged cattle at preclinical (Cases 4–7) and clinical stages of the disease (Cases 8–16). The severity of PrP^{Sc} deposition is scored on a semi quantitative scale as 0 = no deposition, 1 = scanty, 2 = mild, 3 = moderate, and 4 = severe, with color gradation between white and black as indicated. Topographical brain areas schematically represent 10 coronal slices at the level of (from upper left to lower right): frontal lobe, striatum, thalamus, occipital lobe, midbrain, pons, medulla oblongata at the obex, spinal cord at the cervical enlargement, spinal cord at the lumbar enlargement, and cerebellum.

brain was scored as described in Methods, and the resulting lesion profiles are summarized in Fig. 2. Animals euthanized at 3, 10, and 12 mpi (Cases 1–4) had no vacuolar changes in any regions of the brain. Two cattle (Cases 5 and 6) were euthanized at 16 and 18 mpi, when clinical signs were absent, and they showed a few vacuoles in the neuropil of the thalamic nuclei, hypothalamus, pontine nuclei, nucleus of the spinal tract of trigeminal nerve, and putamen (Fig. 2). However, no vacuolation was detected in the cerebral and cerebellar cortices of these animals.

One animal (Case 7) showed no clinical signs of the disease and was determined to be at the preclinical stage of disease when euthanized at 19 mpi. This animal had a moderate number of vacuoles widely distributed throughout the brain (Fig. 2); vacuolation of the neuropil was evident in the thalamic nuclei, pons, and midbrain, and less frequently, in the cerebral cortices, especially in the caudal cerebrum.

Vacuolar changes of the brain were more frequent in the animals that exhibited clinical signs and were euthanized between 20 and 24 mpi (Cases 8–16) than in the 7 animals without clinical manifestations. The highest mean lesion scores were obtained for the thalamic nuclei and the neuropil of the central gray matter of the midbrain, and the lowest scores, for the caudal cerebral cortices and cerebellar cortex. Moreover, examination of the dorsal motor nucleus of the vagus nerve (DMNV) showed less characteristic vacuolar change. However, spongy change was much more severe and frequent in the trigeminal nucleus and solitary nucleus than in the other nuclei of the medulla oblongata at the obex level. Mild vacuolation was present in the neuropil of the gray matter in the spinal cords of all animals with clinical signs of the disease.

PrP^{Sc} immunohistochemistry: Figure 3 shows brain maps representing the topography and scoring of PrP^{Sc} at the frontal cortex level, striatum level, thalamus and

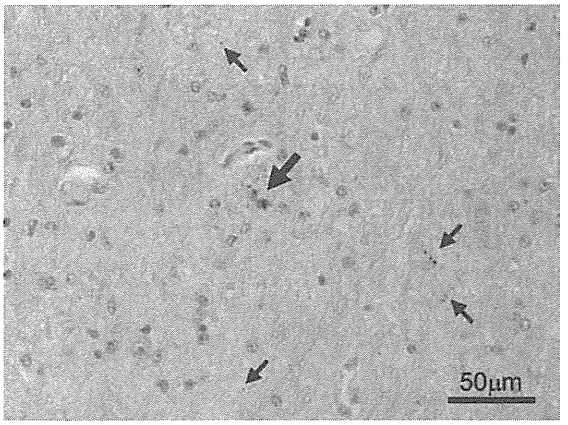

Fig. 4. Thalamus in Case 2. Intraglial (large arrow) and particulate (small arrows) PrP^{Sc} immunolabeling is detected in the dorsolateral thalamic nucleus. Immunohistochemical labeling with mAb T1.

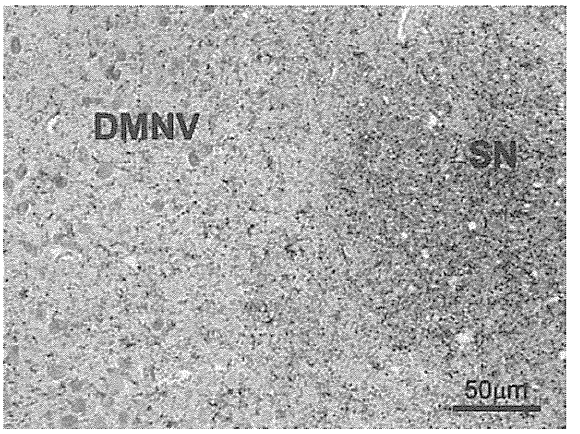

Fig. 5. Medulla oblongata at the obex level in Case 12. Particulate and granular PrP^{Sc} depositions are obvious in the neuropil of the nucleus of the solitary tract (SN). In contrast, PrP^{Sc} accumulation is sparse in the dorsal motor nucleus of the vagus nerve (DMNV). Immunohistochemical labeling with mAb T1 and hematoxylin counterstain.

parietal cortex level, occipital cortex level, midbrain, pons, obex, cerebellum, and spinal cords at the C8 and L6 segments of cattle.

The initial tissue lesion was detected as sparse PrP^{Sc} deposits in the neuronal perikarya and neuropil of gray matter, as neuritic-particulate or granular and linear types in the nuclei of thalamus (mostly ventricular nuclei), midbrain, pons, medulla oblongata (mostly spinal trigeminal nucleus), and septal accumbens of the animal euthanized at 10 mpi (Case 2; Fig. 4). Interestingly, the intraneuronal type of PrP^{Sc} deposit was more frequent than the other types. The neuritic-particulate or granular type of deposition showed neuronal process labeling. Perineuronal labeling was also detected, but less frequently. In the 2 animals euthanized at 12 mpi (Cases 3 and 4), small amounts of particulate or granular labeling in the neuronal cells and particulate or granular neuropil labeling were often present in the gray matter of the C8 and L6 segments of the spinal cord. However, no PrP^{Sc} deposit could be detected in the brain sections of the animal euthanized at 3 mpi (Case 1).

The animals (Cases 5 and 6) that had no clinical signs and were euthanized at 16 and 18 mpi exhibited moderate amounts of intraneuronal and intraglial granular as well as particulate, linear, and coalescing neuropil labeling in the thalamus, midbrain, pons, medulla oblongata, cerebellar medulla, septal accumbens, and spinal cord. Minimal to slight PrP^{Sc} deposition was also present in the cerebral and cerebellar cortices, mostly in the frontal cortex.

Intraneuronal vacuoles were occasionally present in the brainstem and thalamic nuclei of the animal euthanized at 19 mpi (Case 7). PrP^{Sc} deposition was moderately localized in the brainstem, thalamic and septal nuclei, hypothalamus, cerebellar nuclei, and gray matter of the spinal cord, and was sparse in the rostral cerebral cortices and hippocampus. The labeling in the cerebral cortices of this animal was more apparent than that in the animals (Cases 5 and 6) euthanized at 16 and 18 mpi.

In general, the types and topographical distribution of PrP^{Sc} deposits were quite similar among the animals that showed clinical signs of the disease (Cases 8–16; Fig. 3). The different types of immunolabeled PrP^{Sc}, i.e., the particulate or granular neuropil, intraneuronal, perineuronal, glial, linear, and coalescing types, were widely distributed throughout the brain. PrP^{Sc} immunolabeling was most pronounced in the brainstem, thalamus, the white matter of the cerebellum, and the gray matter of the spinal cord (Fig. 3). Small amounts of neuropil labeling were present in the DMNV at the level of the obex. In contrast, large amounts of PrP^{Sc} were evident in the nucleus of the solitary tract and the spinal tract nucleus of the trigeminal nerve (Fig. 5). Strong immunolabeling was conspicuous in both the cervical and lumbar segments of the spinal cord. Slight to moderate amounts of PrP^{Sc} deposits were dispersed in the cerebral and cerebellar cortices. The frontal cortex consistently showed the highest PrP^{Sc} deposition, while the lowest was noted in the occipital cortex. In the cerebellar cortex, PrP^{Sc} accumulation occurred in the granule cell layer, particularly just beneath the Purkinje cell layer.

Western blotting: A PrP^{res} signal was not detected in the brain extracts from the calf euthanized at 3 mpi (Case 1), but a small amount of PrP^{res} was detected in the brainstem and cerebellum of the animal killed at 10 mpi (Case 2; Fig. 6). The signal intensities of the extracts from different animals varied; for example, the signals obtained in Cases 4 and 7, in which the animals were killed at 12 and 19 mpi, respectively, were slightly stronger than those obtained in Cases 3 and 6, in which the animals were killed at similar time points (12 and 18 mpi, respectively). As seen in both Fig. 6 and Table 2, the spread of PrP^{res} throughout the brain and spinal cord correlated with the progression of the disease. The results of the Western blotting analyses are summarized in Table 2.

DISCUSSION

The goal of this study was to investigate the accumulation of PrP^{Sc} in the brain of cattle intracerebrally inoculated with the C-BSE prion agent. Although this

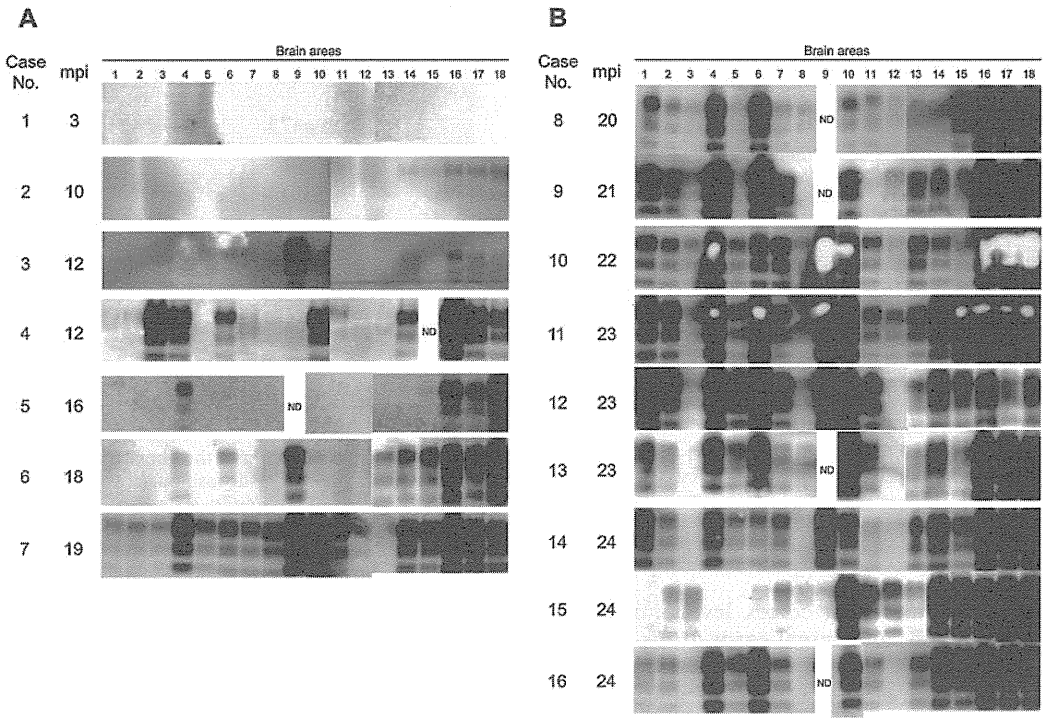

Fig. 6. Western blotting of PrP^{res} in the CNS extracts from animals at the preclinical (A) or clinical stages of disease (B). Lanes are numbered according to the 18 different CNS regions shown in Figure 1. Each lane was loaded with 20 mg of tissue. Western blots were probed with mAb T2 to detect PrP^{res}. ND, not done.

Table 2. Detection of PrP^{Sc} by Western blotting of CNS tissue samples

	Status	Preclinical							Clinical								
	Case no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Months post-inoculation	3	10	12	12	16	18	19	20	21	22	23	23	23	24	24	24
1*	Frontal cortex	−	−	−	−	−	−	+	++	++	++	++	++	++	++	−	+
2	Parietal cortex	−	−	−	−	−	−	+	++	++	++	++	++	++	++	+	+
3	Caudate nucleus	−	−	−	++	−	−	+	+	+	+	+	+	−	−	+	−
4	Accumbens	−	−	−	++	++	+	++	++	++	++	++	++	++	++	−	++
5	Parietal cortex	−	−	−	−	−	−	++	+	+	++	++	++	++	++	−	++
6	Thalamus	−	−	−	++	−	+	++	++	++	++	++	++	++	++	−	++
7	Parietal cortex	−	−	−	−	−	−	++	+	++	++	++	++	++	++	+	++
8	White matter at level of thalamus	−	−	−	−	−	−	++	+	−	+	++	+	+	−	−	−
9	Hypothalamus	−	−	++	−	ND	++	++	ND	ND	++	++	++	ND	++	−	ND
10	Hippocampus	−	−	−	++	−	−	++	++	++	++	++	++	++	++	++	++
11	Occipital cortex	−	−	−	−	−	−	++	++	−	++	++	++	++	+	++	+
12	Occipital white matter	−	−	−	−	−	−	+	+	+	+	++	+	−	−	++	−
13	Cerebellar cortex	−	−	−	−	−	++	−	+	++	++	++	++	++	++	+	++
14	Cerebellar white matter	−	+	+	++	−	++	++	+	++	++	++	++	++	++	++	++
15	Cerebellar nucleus	−	+	−	ND	−	++	++	++	++	+	++	++	++	++	++	++
16	Midbrain	−	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++
17	Pons	−	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++
18	Obex	−	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++
	Spinal cord (C7)	ND	ND	ND	ND	++	++	++	+	ND	++	++	++	++	++	++	++
	Spinal cord (L5)	ND	ND	ND	ND	++	++	++	++	ND	++	++	++	++	++	++	++

−, none; +, positive; ++, strongly positive (compared to positive control of mouse scrapie-infected brain 1.6 μg tissue equivalent); ND, not done.
*Numbers correspond to the 18 different brain areas as shown in Fig. 1.

transmission route does not mimic the natural route of infection, which is most likely the ingestion of infectious material, intracerebral challenge seems to be the most efficient route for the synchronized induction of C-BSE in cattle. In line with this assumption, the incubation periods and disease durations of all the C-BSE-inoculated cattle were consistent with the findings of previous studies (38). In addition, although the number of study animals was small, the cattle inoculated with the 3 different C-BSE isolates did not differ in terms of the vacuolar lesion scores, the PrP^{Sc} topographical distribution, or the extent of PrP^{Sc} accumulation in the brain at the terminal disease stage. These results suggest that the 3 BSE strains used in this study may be identical and originate from a single infectious strain, which we denoted as the C-BSE prion.

We detected an early accumulation of PrP^{Sc} in the brainstem of infected animals; the reasons for this could be that the structure lies in the intracerebral inoculation path or because the brainstem is a target site for the C-BSE agent. Although the PrP^{Sc} detected in the brainstem could thus be attributed to residual material from the inoculation, no PrP^{Sc} was detected in the brainstem of the animal euthanized at 3 mpi (Case 1), either by Western blotting or by immunohistochemistry. This finding is consistent with a previous report of the experimental transmission of sheep scrapie (39). PrP^{Sc} might be widely distributed in a nonuniform manner from the inoculum point to other targeted brain areas, suggesting that the C-BSE prion had a strong regional tropism for the brainstem and thalamus (27). This possibility was not ruled out because we found that PrP^{Sc} was distributed throughout the brain and spinal cord, and not solely localized in the midbrain and cerebrum at the site of inoculation (40,41). In addition to the vacuolar lesion profiles, we found the topographical distribution of PrP^{Sc} in the brains of cattle with clinically evident disease to be consistent with that reported for cattle with naturally occurring BSE (27,29,35,42–45). The results described here also suggest that the accumulation and distribution of PrP^{Sc} in the brain correlated with the disease incubation period.

Although each C-BSE inoculum was prepared from the same brain region (brainstem) of infected cattle, we observed differences in the PrP^{res} signal intensity on Western blots between animals sacrificed at the same point after inoculation. For example, the intensity differed between Case 3 (BSE/UK) and Case 4 (BSE/JP6), wherein the animals were sacrificed at 12 mpi, and between Case 6 (BSE/UK) and Case 7 (BSE/JP6), wherein the animals were sacrificed at 18 and 19 mpi, respectively. These differences may be attributed to the low number of experimental animals used, variations in the infectivity titers of the inoculums, breeding conditions, or additional unknown factors associated with prion propagation in the brain.

The vacuolar lesion scores of symptomatic animals in this study were considerably higher than those of asymptomatic animals, and they were consistent with those previously described for BSE-affected cattle that had been naturally or experimentally infected (32,38,46). According to the current models of peripheral pathogenesis in orally induced TSEs, the BSE prion most probably reaches the medulla oblongata and then spreads along the parasympathetic efferent fibers of the autonomic nerve system, i.e., the vagus nerves (47–49). In one study, cattle receiving a high-dose peroral challenge of the BSE agent showed initial deposition of PrP^{Sc} in the DMNV, celiac and mesenteric ganglion complex, and caudal mesenteric ganglion, as well as in the intermediolateral cell column of the spinal cord, but not in other areas, including the midbrain (26). The DMNV was also the first region of PrP^{Sc} deposition in the brain of cattle naturally affected by BSE (50) and those with experimental BSE induced by oral inoculation (26). Therefore, the discrepancy between the findings of our study and those reported for naturally occurring BSE with regard to the severity of vacuolar changes and PrP^{Sc} accumulation in the DMNV might be attributed to the different routes of infection in the individual studies.

In summary, we found that the earliest accumulation of PrP^{Sc} in intracerebrally inoculated cattle in the brainstem and thalamus occurred at 10 mpi, which was 10 months before the onset of clinical signs. PrP^{Sc} was widely distributed throughout the CNS during this preclinical period and accumulated at the target sites, mostly in the brainstem and thalamus. This study also indicated that clinical signs of the disease might appear after the appearance of vacuolar changes in the brain.

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Conflict of interest None to declare.

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Properties of L-Type Bovine Spongiform Encephalopathy in Intraspecies Passages

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Abstract

The origin and transmission routes of atypical bovine spongiform encephalopathy (BSE) remain unclear. To assess whether the biological and biochemical characteristics of atypical L-type BSE detected in Japanese cattle (BSE/JP24) are conserved during serial passages within a single host, 3 calves were inoculated intracerebrally with a brain homogenate prepared from first-passaged BSE/JP24-affected cattle. Detailed immunohistochemical and neuropathologic analysis of the brains of second-passaged animals, which had developed the disease and survived for an average of 16 months after inoculation, revealed distribution of spongiform changes and disease-associated prion protein (PrP^{Sc}) throughout the brain. Although immunolabeled PrP^{Sc} obtained from brain tissue was characterized by the presence of PrP plaques and diffuse synaptic granular accumulations, no stellate-type deposits were detected. Western blot analysis suggested no obvious differences in PrP^{Sc} molecular mass or glycoform pattern in the brains of first- and second-passaged cattle. These findings suggest failures to identify differences in mean incubation period and biochemical and neuropathologic properties of the BSE/JP24 prion between the first and second passages in cattle.

Keywords

atypical bovine spongiform encephalopathy, L-type, immunohistochemistry, prion, transmission

Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disorder manifested as one of a group of transmissible spongiform encephalopathies, including scrapie in sheep and goats, chronic wasting disease in deer, and Creutzfeldt-Jakob disease in humans. The infectious agent responsible for these diseases is an abnormal isoform prion protein (PrP^{Sc}), currently thought to be a posttranslationally modified form of a host-encoded membrane glycoprotein (PrP^{C}). The uniform nature of the pathology and biochemical features of the proteinase K–resistant PrP^{Sc} (PrP^{res}) profile obtained for cattle infected with BSE and mice in BSE transmission studies suggests that BSE is caused by a single prion strain.

Recently, variants of BSE (denoted atypical BSE) have been detected in cattle in Japan, Europe, and North America.[2] Currently, atypical BSE cases are classified into at least 2 groups, namely the L-type and the H-type, according to the molecular weight of the PrP^{res} in the case compared to that of classical (C-type) BSE cases. Histopathologic as well as immunohistochemical analyses have confirmed that atypical H- and L-type forms of BSE are transmissible to mice and clearly differ from C-type BSE in terms of incubation period, PrP^{res} profile, and pathology.[1] The L-type BSE recognized in Italy, which has been further characterized by the presence of PrP^{Sc}-positive amyloid plaques in the brain, has been termed *bovine amyloidotic spongiform encephalopathy* (BASE).[5]

Western blot analysis of one 14-year-old Japanese black beef cow (BSE/JP24)[7] suggested a glycoform ratio similar to that of BASE-infected cattle, while PrP^{Sc} immunohistochemistry analysis suggested the presence of PrP^{Sc}-positive amyloid plaques in the brain similar to those found in BASE-infected cattle.[5] Additional studies have demonstrated that the BSE/JP24 isolate can be transmitted to cattle[6] and that BSE/JP 24 and BASE have nearly the same incubation period during the first passage in Holstein cattle.[6,8]

Research has also suggested that during subsequent passages, the incubation period of the disease may be shorter or more stable than that of the first passage and that its characterization may vary. The primary objective of this study was to further investigate the characteristics of the second passage of the

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disease by clinicopathologic analysis of BSE/JP24-infected cattle. The secondary objective was to fill a significant research gap by further identifying and describing the topographic distribution and patterns of immunolabeled PrP^{Sc}, thereby redressing the paucity of immunohistochemical data that have been collected regarding atypical BSE cases.

All the experiments involving animals were performed in the biosafety level 3 areas with the approval of the Animal Ethical Committee and the Animal Care and Use Committee of the National Institute of Animal Health. Three Holstein female calves aged 2 to 3 months old were inoculated through the intracerebral route with 1 ml of 10% brain homogenate prepared from the brainstem of a first-passaged BSE/JP24-affected cow (case No. 1A; code 8515) used in a previous experiment.[6] The brain material was confirmed positive by Western blot and immunohistochemical analysis. Two uninoculated calves served as controls and were sacrificed 24 months after inoculation.

Between 12 and 13 months after inoculation, the animals began to exhibit signs of fear or anxiety, which are initial clinical signs of BSE. The animals were observed to exhibit ataxia of the hind limbs before astasia and were subsequently killed at 421 (case No. 2A), 504 (case No. 2B), and 559 (case No. 2C) days after inoculation. The results of unpaired *t* testing (InStat3, GraphPad Software, La Jolla, CA) failed to identify a significant difference in mean incubation period between the first- and second-passaged cattle (486 $\pm$ 11 days[6] and 495 $\pm$ 69 days, respectively).

After the brain was cut longitudinally at necropsy, the left half, including the brainstem and cerebellum, was fixed in 10% neutral buffered formalin containing 10% methanol, while the right half was frozen at −80°C for Western blot analysis. Formalin-fixed coronal slices of the brain and tissue samples from the entire body were trimmed into 3- to 4-mm thick sections, immersed in 98% formic acid for 60 minutes to reduce infectivity, rinsed, dehydrated, embedded in paraffin wax, sectioned at 4 μm, and stained with hematoxylin and eosin. A vacuolar lesion profile of the hematoxylin and eosin–stained sections was obtained using the method described by Simmons et al.[10] Selected sections were stained with phenol Congo red and examined under a polarizing microscope, and the presence of amyloid was confirmed by observation of its characteristic dichroism.

For the demonstration of immunolabeled PrP^{Sc}, dewaxed sections were placed on a silanated slide, treated with 3% hydrogen peroxide at room temperature for 10 minutes, incubated with 10 μg/ml of proteinase K (0.03 U/μg; Nakarai, Kyoto, Japan) in phosphate-buffered saline containing 0.1% Triton-X at room temperature for 10 minutes, and immersed in 150mM sodium hydroxide at 60°C for 10 minutes.[6,9] The sections were then incubated with monoclonal antibody F99/97.6.1 (1 μg/ml; VMRD Inc., Pullman, WA), which reacts with a conserved epitope located at amino acid residues 228–233(QYQRES) of the bovine prion protein when using a horseradish peroxidase–labeled polymer detection system (Nichirei Histofine Simple Stain MAX-PO (M), Nichirei, Tokyo, Japan). The sections were then visualized using 3′-3 diaminobenzidine tetrachloride as the chromogen in accordance with the manufacturer's instructions and slightly counterstained with hematoxylin. The intensity and extent of PrP^{Sc} accumulation were subjectively scored from 0 to 4 (0, negative; 1, apparent at high magnification; 2, apparent at moderate magnification; 3, apparent at low magnification and moderate amounts of accumulation; 4, large amounts of accumulation). Negative controls were incubated with nonimmune mouse or rabbit IgG (1:30 dilution; Dako) and phosphate buffered saline instead of the primary antibody. Schematic representation of immunolabeled PrP^{Sc} distribution in the brain was drawn with Adobe Photoshop. Proteins were extracted from the frozen samples and subjected to Western blot analysis of PrP^{res} according to a previously described method.[6] The blotted membrane was then incubated with anti-PrP-specific monoclonal antibody T2 and the signals detected using a chemiluminescent substrate (SuperSignal; Thermo Fisher Scientific Inc., Rockford, IL). The signal intensity and glycoform ratio of PrP^{Sc} were calculated with Fluorochem software (Alpha Innotech, San Leandro, CA). Brains of 2 sham-inoculated Holstein cattle served as controls for immunohistochemical and Western blot analyses.

The results of the analysis suggested that the neuropil but not the neuronal vacuolation was consistent in most nuclei of the thalamus and brainstem structures, including the midbrain, pons, and medulla oblongata, but not in the dorsal motor nucleus of the vagus nerve. The vacuolar lesion score in the second-passaged animals was similar to that in first-passaged cattle, as previously described (data not shown).[6] The variability in PrP^{Sc} immunolabeling intensity and distribution pattern was found to be nearly identical. Seven varieties of PrP^{Sc} immunostaining patterns—including intraneuronal, perineuronal, intraglial, linear, fine particulate, coarse granular, and plaques—were identified throughout the brain of first- and second-passaged animals. As can be observed in Figure 1, which shows the topographic distribution of the PrP^{Sc} identified, significant quantities of PrP^{Sc} were detected in the brainstem and thalamus. The most conspicuous pattern of PrP^{Sc} was that of fine particulate and coarse granular deposits in the neuropil of the thalamus and midbrain, particularly in the periaqueductal grey matter, and to some extent in the cerebral cortices and cerebellar nuclei (Fig. 2). The major feature of the plaques was a spherical structure with a unicentric core up to 20 μm in diameter. The plaques were sparsely detected in the thalamus, basal ganglia, midbrain, pons, deeper layers of the cerebral cortices, and subcortical white matter and were not detected to any extent in the olfactory bulb, medulla oblongata, cerebellum, or spinal cord. These plaques exhibited a variable morphology; although most plaques that had a uniformly dense core were noncongophilic and less than 15 μm in diameter, some that had a lucent core were congophilic, larger than 15 μm in diameter, irregularly outlined, and showed distinctly intense surrounding immunolabeling (Fig. 3). The stellate-type of PrP^{Sc} deposition usually detected in the cerebral and cerebellar cortices of C-type BSE-affected cattle was undetectable in any sections of any of the animals, while less intraneuronal deposition of PrP^{Sc} was found in the cerebral cortex compared to the thalamus and brainstem (Fig. 2). PrP^{Sc} immunolabeling

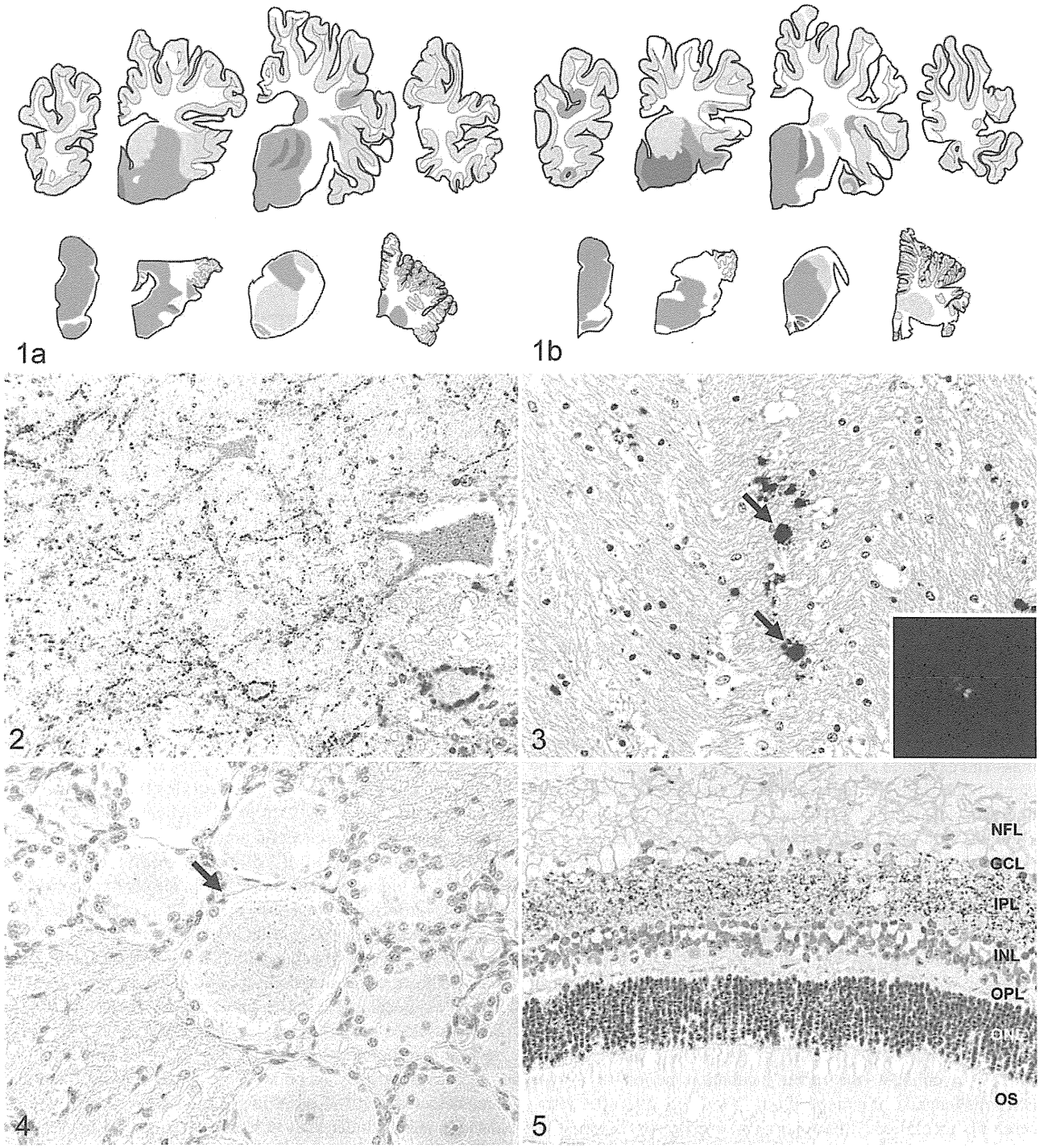

Figure 1. Immunolabeled PrP^{Sc} distribution in the central nervous system of (a) first- and (b) second-passaged cattle (respectively, case Nos. 1A and 2C). Immunolabeling intensity of PrP^{Sc} deposition was scored as none (white), scanty (pale yellow), mild (dark yellow), moderate (orange), and heavy (red). Eight brain areas can be observed, at the level of the frontal lobe, striatum, thalamus, occipital lobe, midbrain, pons, medulla oblongata at the obex, and the cerebellum, from the upper left to the lower right. **Figure 2.** Brain, cow, second passaged (case No. 2C). Granular, linear, intraneuronal, and perineuronal patterns of PrP^{Sc} can be observed in the motor nucleus of trigeminal nerve of pons immunolabeled with monoclonal antibody F99/97.6.1 and counterstained with hematoxylin. The insets in the right corner are higher magnification images of intraneuronal (upper box) and perineuronal labeling of PrP^{Sc} (lower box). **Figure 3.** Brain, cow, second passaged (case No. 2C). Plaque deposits (arrows) can be observed in the white matter of the thalamus immunolabeled with monoclonal antibody F99/97.6.1 and counterstained with

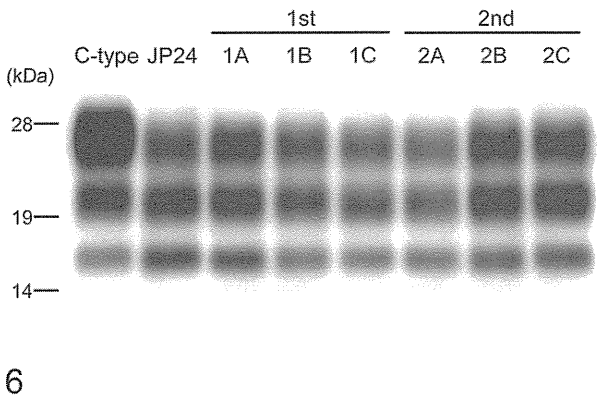

Figure 6. Western blot analysis of proteinase K–digested prion protein (PrP^{res}) from the medulla oblongata at the obex level of C-type bovine spongiform encephalopathy (BSE) and BSE/JP24 first- and second-passaged cattle with monoclonal antibody T2. All samples were digested with 50 μg/ml of proteinase K at 37°C for 1 hour. Molecular markers are shown on the left.

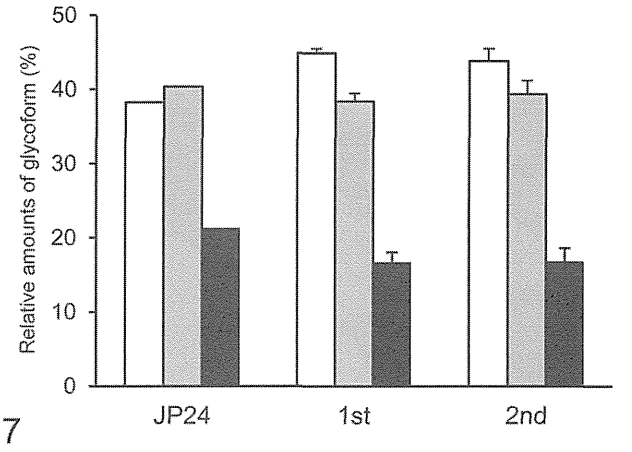

Figure 7. The relative amount of the diglycosylated, monoglycosylated, and unglycosylated form of PrP^{res} in the BSE/JP24, first-passaged, and second-passaged prion-affected individual with monoclonal antibody T2. The results are shown as mean ± standard deviation in triplicate experiments. Bar diagram indicates diglycoslated form (black column), monoglycosylated form (gray column), and unglycosylated form (white column).

was detected in extracerebral tissues, including the trigeminal and dorsal root ganglia (Fig. 4), neurohypophysis, and retina (Fig. 5). PrP^{Sc} accumulation was prominent in the ganglion cell layer as well as the inner and outer plexiform layers of the retina (Fig. 5). No histopathologic changes were present in the peripheral tissues, including the skeletal muscles.

Western blot analysis of PrP^{res} suggested that the brains of first- and second-passaged cattle had a similar molecular mass and exhibited similar glycoform patterns (Figs. 6, 7). The signal intensities in diglycosylated, monoglycosylated, and unglycosylated PrP^{res} fragments were found to be similar in both the first- and second-passaged cattle. In addition, the unglycosylated PrP^{res} fragment detected in both groups showed a lower molecular weight than that in cattle affected with a C-type BSE agent, suggesting that quantitative studies of PrP^{res} molecular mass and glycoforms proportions in Western blots will be more useful to demonstrate the difference between the classical and different passages of atypical BSEs. The mean incubation period of the first- and second-passaged Holstein cattle challenged with the BSE/JP24 prion was approximately 16 months, the same as that of BASE-inoculated Holstein cattle.[8] In contrast, the mean incubation period of C-type BSE-challenged cattle ranges from 17 to 22 months after intracerebral inoculation.[8] In studies of bovine PrP-overexpressing transgenic mice, the incubation period was also shorter in L-type BSE-inoculated mice than in C-type.[3,4,9] These results suggest that in cattle, L-type BSE agents have a shorter incubation period compared to that of C-type BSE agents and that the biochemical properties of PrP^{Sc} from an original BSE/JP24 isolate have no evident differences in BSE/JP24-infected cattle. The incubation period and molecular profiles of PrP^{Sc} suggest that the isolate of BSE/JP24 is clearly distinct from that of C-type BSE while closely resembling that of BASE isolate.[5,6,8]

Immunohistochemical analysis revealed that the patterns of PrP^{Sc} deposition in BSE/JP24 prion-affected cattle were characterized by the presence of amyloid PrP plaques and the absence of stellate-type PrP^{Sc} deposits. However, except for the detection of higher quantities of PrP^{Sc} in the cerebral cortices of BSE/JP24-affected cattle, no striking differences were identified between the topographic distribution of PrP^{Sc} in the C-type BSE and BSE/JP24 prion-affected cattle.[6] The incubation periods and neuropathologic and immunohistochemical characteristics of the BSE/JP24-inoculated Holstein cattle closely resemble those of BASE-affected cattle,[8] indicating that both L-type BSE prions have similar biological properties.

Although atrophy of both type I and II muscle fibers had been detected in BASE cases in previous experiments,[8] evident changes were not detected in the skeletal muscle of the cases examined in this study. This discrepancy may have been due

Figure 3. (continued). hematoxylin. The inset in the right corner is a higher-magnification image of the apple-green birefringence of PrP plague stained with phenol Congo red under polarized light. **Figure 4.** Trigeminal ganglion, cow, second passaged (case No. 2B). Faint granular PrP^{Sc} depositions can be observed in the ganglionic cell (arrow) immunolabeled with monoclonal antibody F99/97.6.1 and counterstained with hematoxylin. **Figure 5.** Retina, cow, second-passaged (case No. 2C). Diffuse granular deposition of PrP^{Sc} can be observed in the ganglion cell layer, as well as in the inner and outer plexiform layers of the retina immunolabeled with monoclonal antibody F99/97.6.1 and counterstained with hematoxylin. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nucleus layer; OPL, outer plexiform layer; ONL, outer nucleus layer; OS, outer segments.