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Studies on neuropathogenesis and neuroanatomical distribution of disease-specific prion protein in cattle experimentally infected with bovine spongiform encephalopathy

牛海綿状脳症実験感染牛における神経病理発生と異常型プリオンタンパク質の神経解剖学的分布に関する研究

Shigeo FUKUDA

CONTENTS

Abbreviations

BAEP	brainstem auditory evoked potential
BASE	bovine amyloidotic spongiform encephalopathy
BSE	bovine spongiform encephalopathy
BSE/JP5	The fifth classical BSE case in Japan
BSE/JP6	The sixth classical BSE case in Japan
BSE/JP24	The second atypical BSE case that was identified in Japan
C-BSE	classical BSE
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
DMNV	dorsal motor nucleus of the vagus nerve
mAb	monoclonal antibody
min	minute
mpi	months post-inoculation
PK	proteinase K
PrP^{C}	cellular prion protein
PrP^{Sc}	disease-specific prion protein
PrP-res	proteinase K-resistant PrP^{Sc}
rt	room temperature
TSEs	transmissible spongiform encephalopathies
UK	United Kingdom

Preface

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders that include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease in deer and Creutzfeldt-Jakob disease (CJD) in human. The key event in the pathogenesis of TSEs is the conformational change of the normal host prion protein (PrP^{C}) into the disease-specific prion protein (PrP^{Sc}), which is thought to be the main, if not only, agent of TSEs.

BSE was first identified in the United Kingdom (UK) in 1986 (Wells et al., 1987) and subsequently, it spread to European, Asian, and North American countries. The first case of BSE in Japan was reported in September 2001 (Kimura et al., 2002), and the most recent case, the 36th, was confirmed in January 2009. BSE is characterized by spongiform changes (Wells et al., 1991) and accumulation of PrP^{Sc} in the central nervous system (CNS) (Prusiner et al., 1982). PrP^{Sc} is commonly accepted as a major component of BSE agent and is thought to be produced by post-translational modification from PrP^{C} (Prusiner, 1991a; Zhong et al., 2013). PrP^{Sc} is the only known disease-specific marker (Bolton et al., 1982; Prusiner, 1991b).

The etiological agent of BSE, BSE prions, is transmissible to different mammalian species. A variant form of CJD 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 (Chazot et al., 1996; Cousens et al., 1997; Will et al., 1998; Will et al., 1996). Therefore, it is important to understand the pathogenesis of BSE in cattle in order to eliminate BSE-contaminated food chain from human food and thereby preserve public health.

A definite diagnosis of prion diseases including BSE is performed by a postmortem biochemical or immunohistochemical detection of PrP^{Sc} using tissues of CNS (Castilla et al.,

2005; Haik et al., 2003; Wells et al., 2007). On the other hand, diagnosis of BSE-affected cattle based on clinical symptoms, particularly in the early clinical phase, is difficult because of the non-specific nature of prodromal clinical signs. Typical clinical signs of BSE in cattle are changes in behavior, abnormality in locomotion and hypersensitivity to stimuli (Konold et al., 2004). For example, cattle affected with BSE become sensitive to noise (e.g., hand clapping or a metallic clank). BSE-affected cattle may also be hypersensitive to touch, sound or light, but these clinical signs are not always observed. Thus, more subjective diagnosis method for BSE-affected cattle is required.

Brainstem auditory evoked potentials (BAEP) are bioelectric waves that can be recorded within 10 ms after an auditory stimulus, and the measurement of BAEP is useful in the objective assessment of auditory function and localization of brainstem lesions (Chiappa, 1997). The abnormality of BAEP has been reported in BSE-affected cattle (Arai et al., 2009). However, the relationship between auditory abnormality and neuropathological features is unclear. Information on the relationship between affected brain regions and clinical manifestation is important to understand pathophysiology of BSE infection.

The uniformity in the pathological features and biochemical characteristics of the proteinase K (PK)-resistant PrP^{Sc} (PrP-res) in BSE-affected cattle suggests that a single prion strain is responsible for BSE. This type of BSE, which originated from UK and thereafter became detected in European countries, North America and Japan, is called classical BSE (C-BSE). Recently, variants of BSE (named atypical BSE) have been disclosed in cattle in Europe (Biacabe et al., 2004; Casalone et al, 2004), North America (Dudas et al., 2010; Richt, 2007), and Japan (Hagiwara et al., 2007; Yamakawa et al., 2003). Those atypical BSEs are characterized by a higher or lower molecular mass of the unglycosylated form of PrP-res (H-type BSE and L-type BSE, respectively) than that in C-BSE (Jacobs et al., 2007). The

second atypical BSE case (BSE/JP24) that was identified in Japan in an aged beef cattle, Japanese Black, showed PrP-positive amyloid plaques in histopathological examination of the brain and the glycoform profile similar to L-type BSE (Hagiwara et al., 2007). These properties seem to be similar to the L-type BSE reported in Italy (this is also called as bovine amyloidotic spongiform encephalopathy [BASE]) (Casalone et al., 2004). However, unlike the BASE prions, shortening of the incubation periods were observed during serial passage of the BSE/JP24 isolate in transgenic mice expressing bovine PrP (Masujin et al., 2008). Thus, further investigation on biological properties and pathogenicity of the BSE/JP24 using experimental infected cattle is required to clarify similarities and dissimilarities among atypical BSEs.

In this thesis, the author investigated the association of the clinical symptoms with the accumulation of PrP^{Sc} and the vacuolar changes of cattle experimentally infected with BSE to understand pathogenesis of BSE. In Chapter 1, the author analyzed the distribution of PrP^{Sc} in the CNS of cattle experimentally infected with BSE by the intracerebral route in order to investigate the relationship between the kinetics of PrP^{Sc} deposition in CNS and the clinical course of the disease. Since BSE-affected cattle showed hypersensitivity to sound, in Chapter 2, the author concentrated on neuropathological investigation particularly in the central auditory pathway of cattle experimentally infected with C-BSE. In Chapter 3, the author described transmission of the BSE/JP24 isolate to Holstein/Friesian cattle to assess its risk against cattle species.

Chapter 1

Neuroanatomical distribution of disease-specific prion protein in experimental bovine spongiform encephalopathy in cattle after intracerebral inoculation

Introduction

Due to the limitation of the experimental infection of BSE, current knowledge of the pathogenesis of BSE in cattle is not enough to understand the pathogenesis of BSE and to assess the risk of further spread of BSE to cattle as well as human being. A mouse bioassay showed that in cattle, the infection was limited to the brain, spinal cord, eyes, dorsal root ganglia, and distal ileum (Wells et al., 1994, 1998). Occasionally, infectivity has also been detected in the bone marrow and tonsils of experimentally infected cattle (Hoffmann et al., 2007; Wells et al., 1999), and recent studies have shown that peripheral tissues other than the CNS may harbor PrP^{Sc} at the clinical stages of the disease (Buschmann and Groschup, 2005; Wells et al., 2005). The distribution of PrP^{Sc} in the brain has been mapped in both naturally occurring BSE and orally infected cattle (Masujin et al., 2007; Vidal et al., 2006; Okada et al., 2011). However, it is still unclear the relationship between the kinetics of PrP^{Sc} deposition in the CNS and the clinical course of the disease. To clarify this point, in Chapter 1, the author extensively analyzed the distribution of PrP^{Sc} in cattle intracerebrally inoculated with C-BSE prions by immunohistochemical and Western blotting analyses.

Materials and Methods

Ethical considerations

All animal experiments 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 in phosphate-buffered saline) were prepared from the brainstems of 3 C-BSE-affected cattle: the first cattle was naturally infected with C-BSE in UK (BSE/UK, provided by the Veterinary Laboratory Agency, UK). The second cattle infected with C-BSE was disclosed in Kanagawa prefecture through BSE screening at 80 months of age (BSE/JP5). The last cattle infected with C-BSE was disclosed in Wakayama prefecture at 83 months of age (BSE/JP6) (Iwata et al., 2006; Yokoyama et al., 2007). Three-month-old sixteen female Holstein/Friesian calves 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 with 1ml of brain homogenate in the right side of the midbrain 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.

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	886	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	astasia	22	+	+	+
11	5523	BSE/JP6	19	nervous	ataxia	23	+	+	+
12	4437	BSE/UK	18	ataxia	astasia	23	+	+	+
13	1479	BSE/JP5	20	gait abnormality	astasia	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	+	+	+

Neuropathology

At necropsy, the brains and cerebella were removed and hemisected at the midline. Samples of various tissues, including those of the left hemisphere and spinal cord at the levels of cervical (C8) and lumbar enlargement (L6), were fixed in 10% neutral buffered formalin (pH 7.4) for 3 days at 37^{o}C. The contralateral side was frozen at -80 ^{o}C for the detection of PrP^{Sc} by Western blotting. 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 minute (min) at room temperature (rt) to reduce prion infectivity (Taylor et al., 1997). The tissues were automatically processed through a graded series of alcohol to xylene and then paraffinembedded (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 (HE) 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. (1996). 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 cattle, 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 (Bencsik et al., 2005). 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 disulfide 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 2z 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, CA, USA). They were then incubated sequentially with 1 μg/mL anti-PrP monoclonal antibody (mAb) T1, goat anti-mouse Fab' universal immunoperoxidase polymer (Histofine SimpleStain MAXPO (M); Nichirei, Tokyo, Japan), and 3–3' diaminobenzidine tetrachloride as the chromogen. The T1 anti-PrP mAb was raised against mouse PrP amino acid residues 121–231 but cross-reacts with bovine PrP (Shimizu et al., 2010). Finally, the sections were counterstained with hematoxylin. All the steps in the immunohistochemical staining procedure were carried out at rt.

PrP^{Sc} mapping and profiling

For each cattle, the topographical distribution of PrP^{Sc} deposition was mapped at 14 different areas of 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 (Debeer et al., 2003; Vidal et al., 2006). 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 (Debeer et al., 2003; Vidal et al., 2006). 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) (González et al., 2002, 2005). It was then topographically mapped to the different CNS areas as 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 the 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 and electroblotted onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA., USA). The blotted membrane was incubated with horseradish-conjugated anti-PrP mAb T2 (Shimizu et al., 2010) at rt for 60 min. Signals were developed with a chemiluminescent substrate (SuperSignal; Pierce Biotechnology, Rockford, IL., USA).

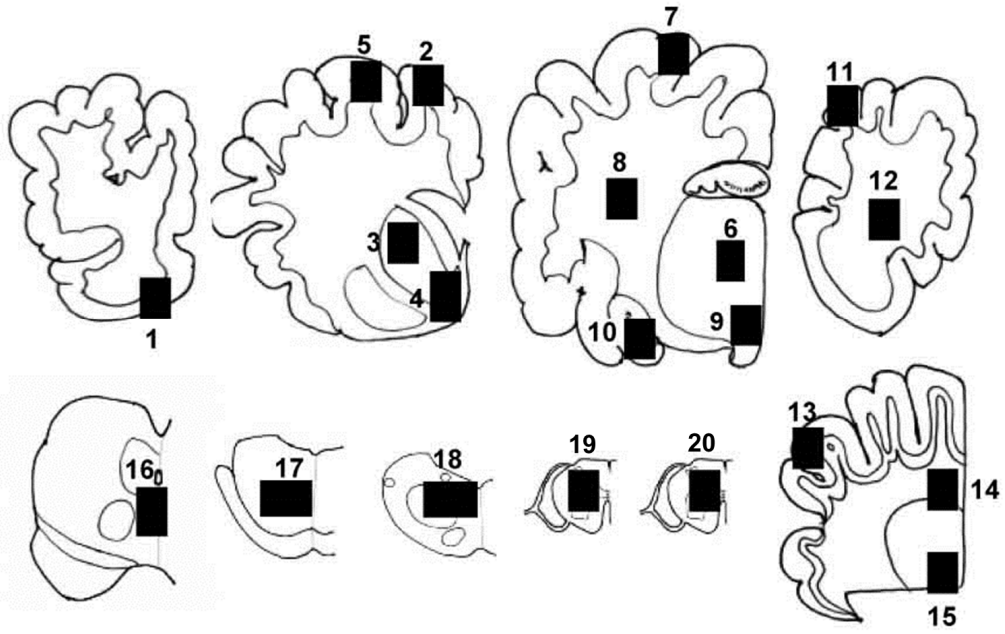

Fig. 1. Schematic representation of brain and spinal cord areas dissected for the Western blotting analyses.

Ten coronal slices of the brain and spinal cord are as follows (from upper left): at the levels of 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; 19, spinal cord (C7); 20, spinal cord (L5)

Results

Clinical signs

Of the 16 cattle 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 cattle (Cases 8–16) exhibited the initial clinical signs of the disease between 18 and 22 mpi (19.7 ± 1.6, mean ± standard deviation [SD]); 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 cattle 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 cattle inoculated with the 3 different C-BSE isolates.

Histopathology

The severity of vacuolation in the brain was scored as described in the Materials and 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 cattle. One cattle (Case 7) showed no clinical signs of the disease and was determined to be at the preclinical stage of the disease when euthanized at 19 mpi. This cattle 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 cattle that exhibited clinical signs and were euthanized between 20 and 24 mpi (Cases 8–16) than in the others cattle 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 cattle with clinical signs of the disease.

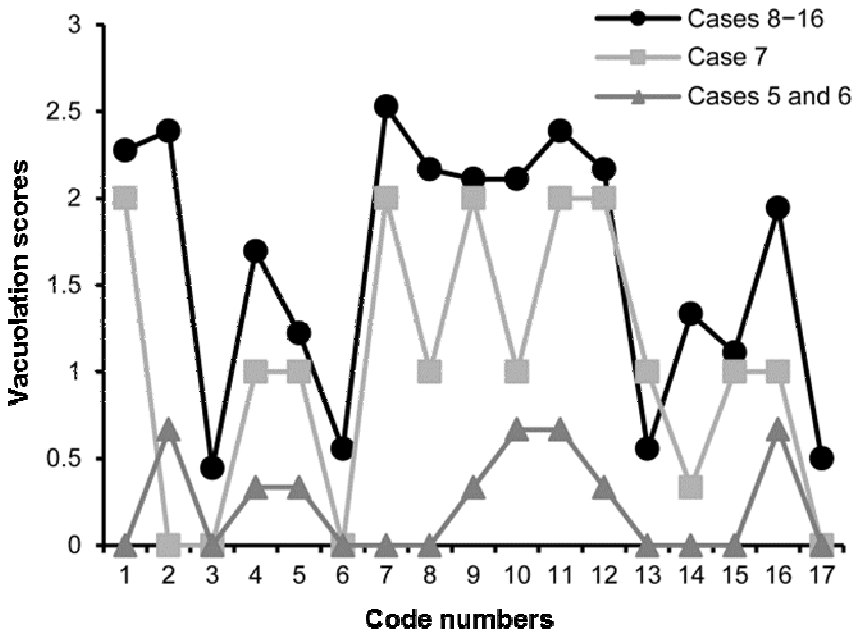

Fig. 2. Vacuolation scores in C-BSE challenged cattle at preclinical and clinical stages of the disease.

Points from Cases 5 and 6 represent the means of 2 cattle 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 ; 13, frontal cortex; 14, accumbens; 15, caudate nucleus; 16, putamen; and 17, claustrum.

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 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 and medulla oblongata (mostly spinal trigeminal nucleus) of the cattle 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 2 cattle 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 was detected in the brain sections of the cattle euthanized at 3 mpi (Case 1). The cattle (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 cattle 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 cattle was more

apparent than that in the cattle (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 cattle that showed clinical signs (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 (data not shown). 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.

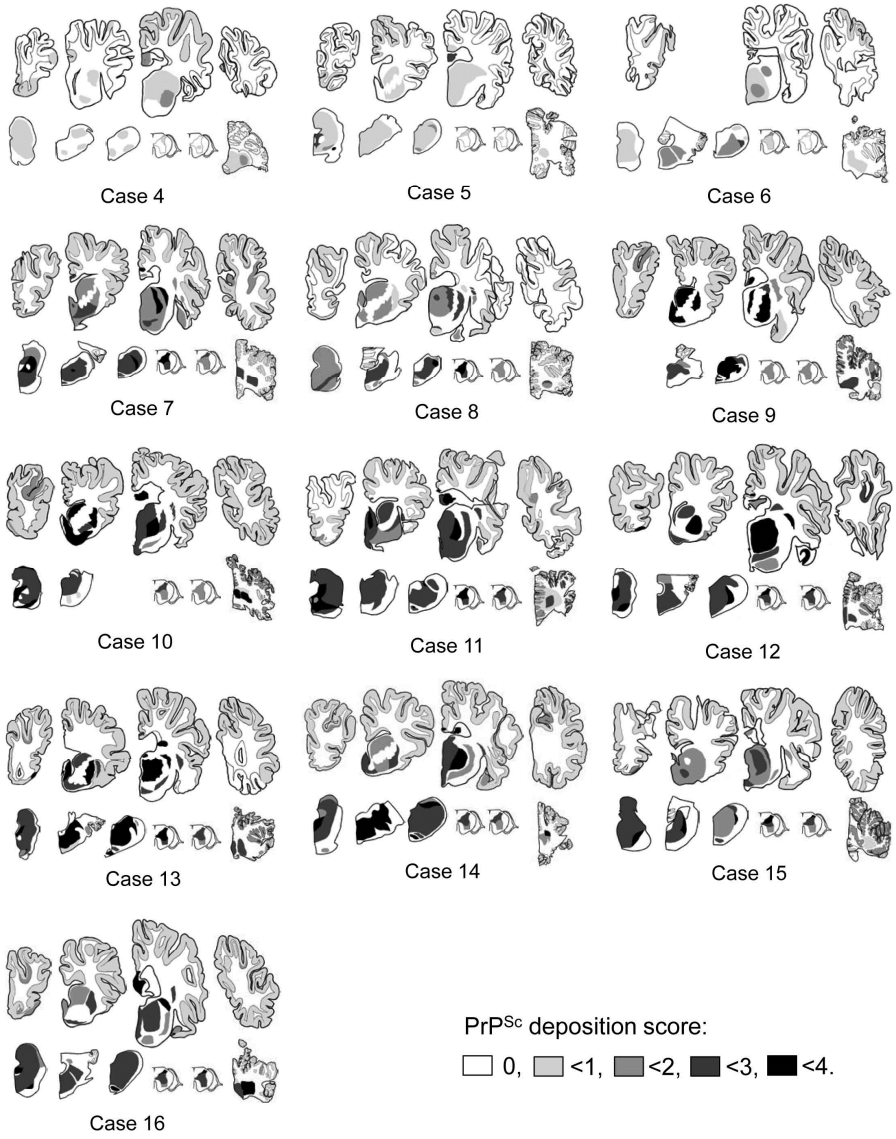

Fig. 3. Schematic representation of PrP^{Sc} distribution.

Distribution 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.

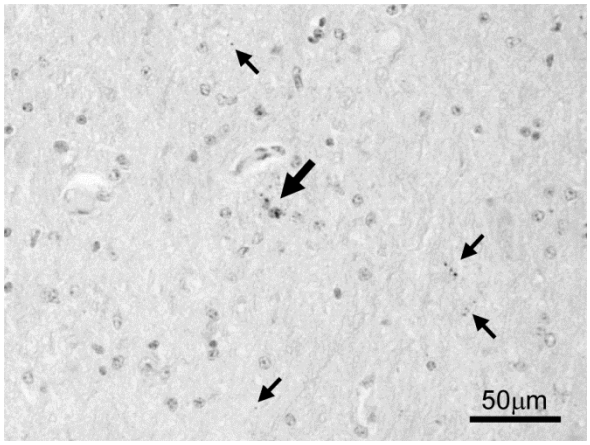

Fig. 4. Immunohistochemistrical analysis of PrP^{Sc} in thalamus of Case 2.

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

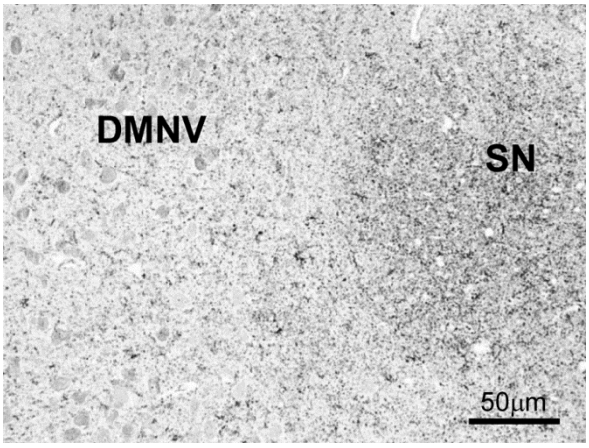

Fig. 5. Immunohistochemical analysis of PrP^{Sc} in the medulla oblongata at the obex level in Case 12.

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.

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 cattle killed at 10 mpi (Case 2; Fig. 6). The signal intensities of the extracts from different cattle varied. For example, the signals obtained in Cases 4 and 7, in which the cattle were killed at 12 and 19 mpi, respectively, were slightly stronger than those obtained in Cases 3 and 6, in which the cattle 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.

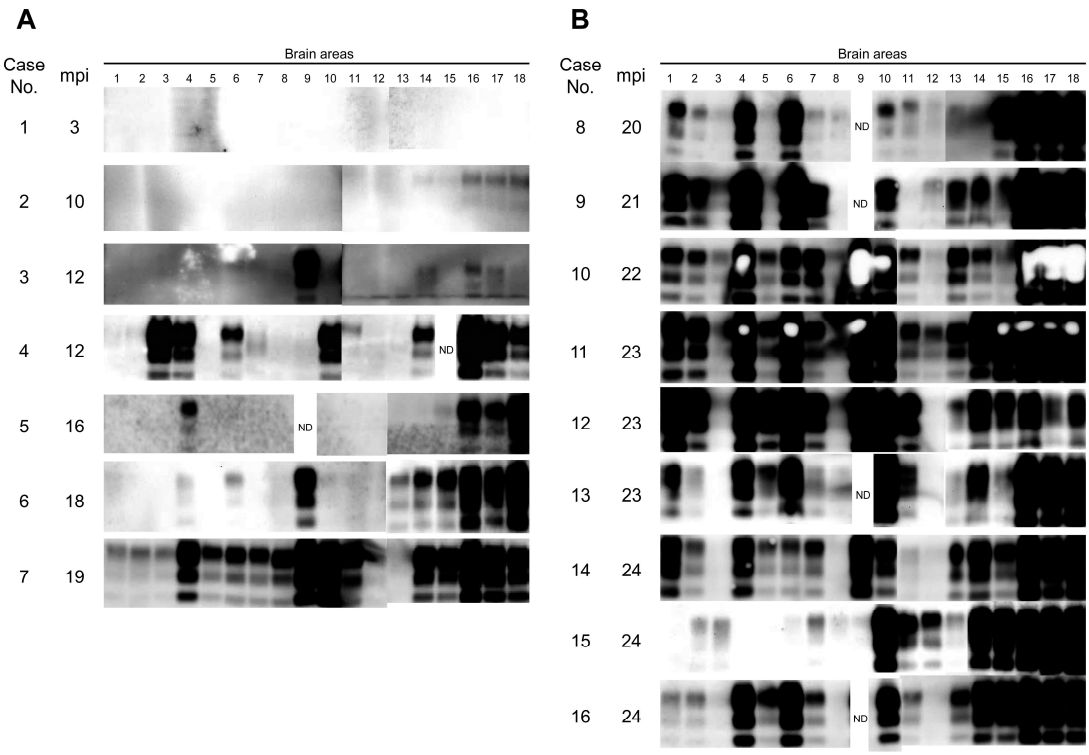

Fig. 6. Presence of PrP-res in CNS of C-BSE prion-infected cattle.

PrP-res in the CNS extracts from cattle at the preclinical (A) or clinical stages of disease (B) was analyzed by Western blotting. Lanes are numbered according to the 18 different CNS regions shown in Figure 1. Each lane was loaded with 20 mg tissue equivalent. Blots were probed with mAb T2 to detect PrP-res. ND, not done.

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

	Status	Preclinical							Clinical								
	Case No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Brain areas[1)] mpi	3	10	12	12	16	18	19	20	21	22	23	23	23	24	24	24
1	Frontal cortex	–[2)]	–	–	–	–	–	+	++	++	++	++	++	++	++	–	+
2	Parietal cortex	–	–	–	–	–	–	+	++	++	++	++	++	++	++	+	+
3	Caudate nucleus	–	–	–	++	–	–	+	+	+	+	+	+	–	–	+	–
4	Accumbens	–	–	–	++	++	+	++	++	++	++	++	++	++	++	–	++
5	Parietal cortex	–	–	–	–	–	–	++	+	+	++	++	++	++	++	–	++
6	Thalamus	–	–	–	++	–	+	++	++	++	++	++	++	++	++	–	++
7	Parietal cortex	–	–	–	–	–	–	++	+	++	++	++	++	++	++	+	++
8	White matter at the level of the 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	–	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++
19	Spinal cord (C7)	ND	ND	ND	ND	++	++	++	+	ND	++	++	++	++	++	++	++
20	Spinal cord (L5)	ND	ND	ND	ND	++	++	++	++	ND	++	++	++	++	++	++	++

1) Numbers correspond to the 18 different brain areas as shown in Figure 1

2) ++, strongly positive (compared to positive control of mouse scrapie-infected brain 1.6 μg tissue equivalent); +, positive; –, none; ND, not done.

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 prions. Although this 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 (Lombardi et al., 2008). In addition, although the number of study cattle was small, the cattle inoculated with the 3 different C-BSE isolates did not differ in terms of the vacuolation 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 C-BSE isolates used in this study may be identical and originate from a single origin. The author detected an early accumulation of PrP^{Sc} in the brainstem of infected cattle: the reasons for this were that the structure lies in the intracerebral inoculation path or because the brainstem is a target site for the C-BSE prions (Vidal et al., 2005). 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 cattle 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 (Hamir et al., 2002). PrP^{Sc} might be widely distributed in a nonuniform manner from the inoculated area to other targeted brain areas, suggesting that the C-BSE prions had a strong regional tropism for the brainstem and thalamus (Vidal et al., 2006). This possibility was not ruled out because the author 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 (Hamir et al., 2005, 2004). In addition to the vacuolar lesion profiles, the author 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 C-BSE (Debeer et al., 2003; Iwata et al., 2006; Orge et al., 2000; Sisó et al., 2004; Vidal et al., 2005; Vidal et al., 2006; Wells et al., 1995). 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, the author observed differences in the PrP-res signal intensity on Western blotting between cattle sacrificed at the same point after inoculation. For example, the intensity differed between Case 3 (inoculum: BSE/UK) and Case 4 (inoculum: BSE/JP6), wherein the cattle were sacrificed at 12 mpi, and between Case 6 (inoculum: BSE/UK) and Case 7 (inoculum: BSE/JP6), wherein the cattle were sacrificed at 18 and 19 mpi, respectively. These differences may be attributed to the low number of experimental cattle used, variations in the prion titers of the inoculums, breeding conditions, or additional biological, neuropathological or biochemical factors associated with prion propagation in the brain. The vacuolation scores of symptomatic cattle in this study were considerably higher than those of asymptomatic cattle, and they were consistent with those previously described for C-BSE-affected cattle that had been naturally or experimentally infected (Breslin et al., 2006; Lombardi et al., 2008; Simmons et al., 1996). According to the current models of peripheral pathogenesis in orally infected transmissible spongiform encephalopathies (TSEs), C-BSE prions probably reaches the medulla oblongata and then spreads along the parasympathetic efferent fibers of the autonomic nerve system, i.e., the vagus nerves (McBride et al., 1999; van Keulen et al., 2000). In one study, cattle receiving a high-dose oral challenge of C-BSE prions 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 (Masujin et al., 2007). The DMNV was also the first region of PrP^{Sc} deposition in the brain of cattle naturally affected by C-BSE (Schulz-Schaeffer et al., 2000) and those with experimentally affected by oral inoculation of C-BSE prions (Masujin et al., 2007). Therefore, the discrepancy between the findings of our study and those reported for naturally occurring C-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, the author 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.

Brief summary

To evaluate the relationship between the kinetics of PrP^{Sc} deposition in the CNS and the clinical course of the disease, temporal and special distribution of PrP^{Sc} was analyzed in the CNS of Holstein/Friesian cattle inoculated intracerebrally with 3 sources of C-BSE isolates. Cattle euthanized at 10 mpi showed PrP^{Sc} deposits in the brainstem and thalamus, but no vacuolation. This suggested that the C-BSE prions 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 indicate that PrP^{Sc} accumulation was detected approximately 10 months before the clinical onset. In addition, the 3 sources of C-BSE prions 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. These results suggest that the 3 C-BSE isolates used in this study may be identical and originate from a single origin.

Chapter 2

Neuropathological changes in auditory brainstem nuclei in cattle with experimental bovine spongiform encephalopathy

Introduction

TSEs are fatal neurodegenerative disorders that include BSE in cattle, scrapie in sheep and goats, chronic wasting disease in deer and CJD in human. The histopathological features of BSE are spongy vacuolation of the neuropil and vacuolation of neurons in the grey matter with mild astrogliosis and activation of microglia (Wells et al., 1991).

It is difficult to determine the clinical onset of BSE-affected cattle because of the non-specific nature of prodromal clinical signs. Typical clinical signs of C-BSE in cattle are changes in behavior and locomotion and hypersensitivity to stimuli (Konold et al., 2004). C-BSE-affected cattle may be hypersensitive to touch, sound or light, but usually not to all of these types of stimuli. For example, cattle with C-BSE can become sensitive to noise (e.g., hand clapping or a metallic clank).

BAEP are bioelectric waves that can be recorded within 10 ms after an auditory stimulus, and the measurement of BAEP is useful in the objective assessment of auditory function and localization of brainstem lesions. The abnormality of BAEP in C-BSE-infected cattle has been reported as prolonged peak latency of wave III and V as well as the I-V inter-peak latency (Arai et al., 2009). The origin of wave III of the BAEP is the cochlear nucleus and superior olivary complex (Chiappa et al, 1997). The cochlear nucleus is the origin of the ascending

central auditory pathway. Wave V arises in the inferior colliculus, the nucleus of which is the largest structure in the central auditory pathway and the focus for processing of both ascending and descending information.

Although C-BSE-affected cattle showed the abnormality of BAEP as well as hyperacusia, little neuropathological information other than vacuolation in the cochlear nucleus described in a lesion profile, is available for the auditory brainstem of C-BSE-affected cattle (Breslin et al., 2006; Simmons et al., 1996). To investigate any relationship between neuropathological lesions and auditory disfunction, in Chapter 2, the author focused on the neuropathological changes and PrP^{Sc} accumulation in the central auditory pathway of cattle exposed experimentally to C-BSE.

Materials and methods

Animals

Sixteen cattle experimental inoculated with C-BSE as described in Chapter 1 were used in this study (Table 1).

Neuropathology

At the time of necropsy examination, the left brain, including the brainstem and cerebellum, was fixed in 10%neutral buffered formalin (pH 7.4). Coronal slices of the formalin-fixed samples (4 μm) were immersed in 98% formic acid for 60 min to reduce infectivity, embedded in paraffin wax, sectioned (4 μm) and stained with haematoxylin and eosin (HE). The severity of vacuolation in the brain was scored according to the method of Simmons et al. (1996).

Immunohistochemistry

The IHC for PrP^{Sc} was carried out as described in Chapter 1.

PrP^{Sc} profiling

The intensity and extent of each morphological type of PrP^{Sc} deposition were scored subjectively from 0 to 4 (0, negative; 1, apparent at high magnification; 2, apparent at moderate magnification; 3, apparent at low magnification and moderate accumulation; 4, marked accumulation) as described by González et al. (2002, 2005). Scored data from lesion profiles and immunohistochemical profiles were analyzed by one-way analysis of variance (ANOVA) using the statistical program Instat3 (GraphPad Software, San Diego, CA, USA) and were expressed as mean ± SD.

Results

C-BSE-infected cattle as described in Chapter 1 were sacrificed at 3, 10, 12, 16, 18 and 19 mpi prior to developing signs of clinical disease (Table 3). One cattle was sacrificed at each time point, except at 12 months when two cattle were sacrificed. Nine cattle, which demonstrated signs of clinical disease, were killed between 20 and 24 mpi. Two negative control cattle were sacrificed at 24 mpi. In C-BSE-challenged cattle, initial clinical signs (e.g., abnormal posture and changes in locomotion) appeared between 18 and 20 mpi and advanced clinical signs (e.g., tremor and difficulties in rising) developed within 22-24 mpi. No clinical abnormalities were observed in the control cattle. The four cattle that were sacrificed at 3, 10

and 12 mpi had no vacuolar change in the brain, but some PrP^{Sc} deposits were present in the brainstem nuclei and medial geniculate body of cattle sacrificed at 10 or 12 mpi (Figs. 7 and 8). PrP^{Sc} was mostly detected in the perikarya (Fig. 8) and often in the neuropil of the grey matter of each auditory brainstem nucleus. In the cattle sacrificed at 16 mpi, minimal vacuolation was present in the nucleus of the inferior colliculus, but not in the cochlear nucleus, superior olivary complex or medial geniculate body (Fig. 9). Moreover, low to moderate amounts of PrP^{Sc} accumulated in the auditory brainstem nuclei (Fig. 10). In the two cattle without clinical signs that were killed at 18 and19 mpi, mild vacuolar changes and moderate PrP^{Sc} accumulation were observed in the nuclei of the auditory brainstem and medial geniculate body (Figs. 7 and 8, Table 3).

All nine cattle sacrificed between 20 and 24 mpi showed clinical signs and spongy change was evident in the nucleus of the inferior colliculus. Prominent PrP^{Sc} accumulation was apparent in the auditory brainstem nuclei and medial geniculate body of nine cattle (Figs. 9 and 10). Consistent PrP^{Sc} types in these areas were granular, punctate and intraneuronal. Other types of PrP^{Sc} deposition (e.g. linear, stellate, coalescing, intraglial or perineuronal) were not evident in the nuclei of the auditory brainstem and medial geniculate body. The severity of vacuolation, as well as PrP^{Sc} accumulation in the nucleus of the inferior colliculus, of cattle showing clinical symptoms between 20 and 24 mpi was significantly higher than those of subclinical and preclinical cattle prior to 19 mpi (Fig. 9). In cattle with advanced neurological signs associated with C-BSE between 22 and 24 mpi, spongiosis and PrP^{Sc} deposits were evident in the nuclei of the auditory brainstem and medial geniculate body and were greatest in the nucleus of the inferior colliculus (Figs. 7 and 8, Table 3). The level of vacuolar change in the nucleus of the inferior colliculus at the clinical stage was significantly higher than levels in the cochlear nucleus, the superior olivary complex and the nucleus of

medial geniculate body (Fig. 9), while the severity of PrP^{Sc} accumulation showed no significant difference among each nucleus at the same stage (Fig. 10). In addition, slight PrP^{Sc} deposition was detected in the lateral lemniscus and auditory cortex.

Table 3. Individual animal data on clinical status, vacuolar lesion score, and intensity of PrP^{Sc} accumulation

Case No.	*Code No.*	*Inoculum*	*Post inoculation period (months)*	*Clinical signs*	*Cochlear nucleus*		*Olivary nucleus*		*Lateral lemniscus*		*Inferior colliculus*		*Medial geniculate body*		*Auditory cortex*	
					Lesion score	*PrP^{Sc} score*	*Lesion score*	*PrP^{Sc} score*	*Lesion score*	*PrP^{Sc} score*	*Lesion score*	*PrP^{Sc} score*	*Lesion score*	*PrP^{Sc} score*	*Lesion score*	*PrP^{Sc} score*
1	0801	BSE/UK	3	–[1)]	0	0	0	0	0	0	0	0	0	0	0	0
2	9066	BSE/UK	10	−	0	0	0	0	0	0	0	0	0	0.5	0	0
3	9385	BSE/UK	12	−	0	0	0	1	0	0	0	2	0	1	0	0
4	3962	BSE/JP5	12	−	0	1	0	1	0	0	0	1	0	1	0	0
5	2601	BSE/UK	16	−	0	2	0	2	0	0	1	2	0	2	0	0
6	886	BSE/UK	18	−	2	3	1	3	0	0	1	1	0.5	1	0	0.5
7	3955	BSE/JP5	19	−	2	2.5	2	2.5	0	0	2	2	1	2.5	0	1
8	4394	BSE/UK	20	+	2	3	2	2	0	0.5	2	2	2	3	0	0.5
9	3728	BSE/JP6	21	+	NA[2)]		NA		0	1	NA		2	3.5	0	1
10	5426	BSE/JP6	22	+	2	4	2	3	0	1	3	4	2	3	0	0.5
11	5523	BSE/JP5	23	+	2	3	2	3	0	1	4	4	2	3.5	0.5	1
12	4437	BSE/UK	23	+	3	3	3	3	0	1	4	3	2.5	3.5	0	0
13	1479	BSE/JP6	23	+	2	3	2	3	0	1	4	3	2.5	3	0.5	1
14	5087	BSE/UK	24	+	NA		2	3	0	1	3	3.5	2	3.5	0.5	1
15	3321	BSE/JP6	24	+	2	3	2	3	0	1	2	3.5	2	3	0	0.5
16	4612	BSE/JP5	24	+	1	4	2	3.5	0	1	4	3	2.5	4	1	1

1) +, positive; −, negative.

2) NA, tissue not available.

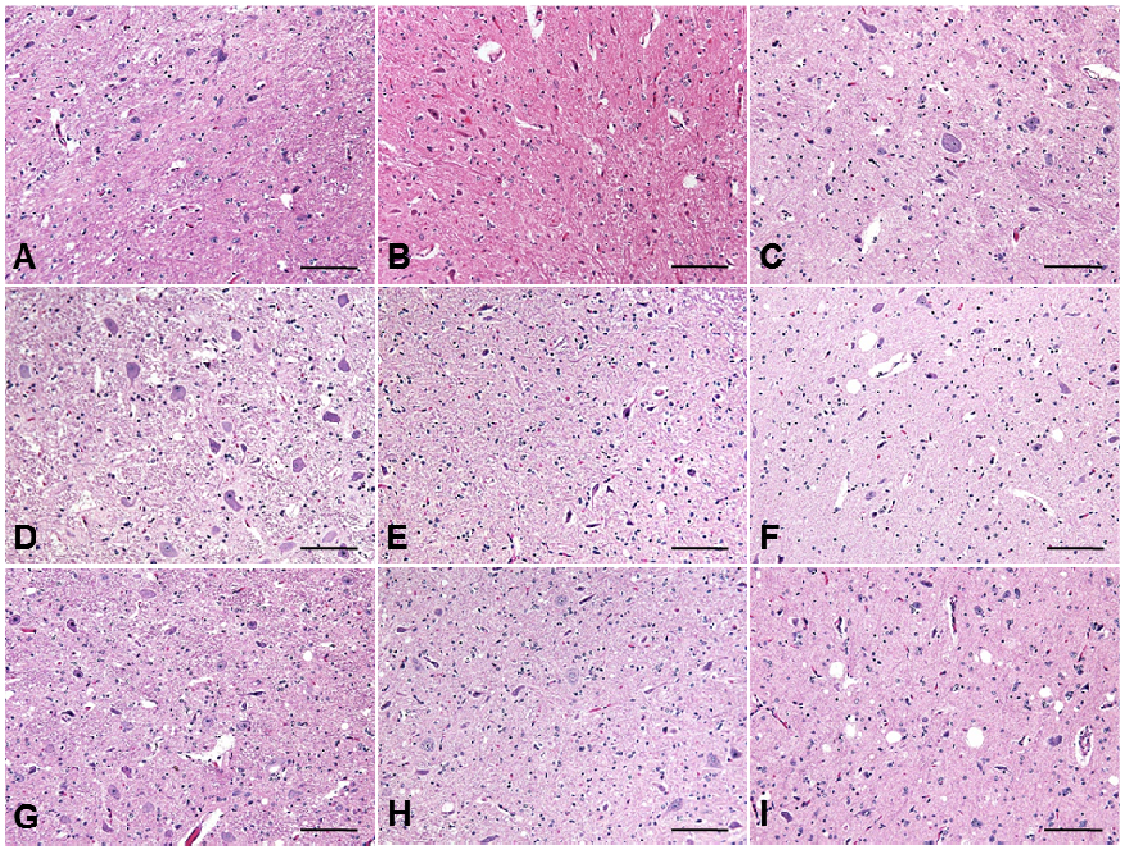

Fig. 7. Vacuolation of the auditory brainstem nuclei of BSE-challenged cattle.

HE sections of the auditory brainstem nuclei of BSE-challenged cattle sacrificed at 12 (A-C), 19 (D-F) and 24 mpi (G-I). Images show the cochlear nucleus (A, D, G), nucleus of the trapezoid body (B, E, H) and nucleus of the inferior colliculus (C, F, I). No vacuolar changes were present at 12 mpi (A, B, C). Mild vacuolation was observed in the nucleus of the inferior colliculus at 19 mpi (F) and in the cochlear nucleus at 24 mpi (G). Moderate neuropil vacuolation was seen in the nucleus of the inferior colliculus at 24 mpi (I). Bar, 100 µm.

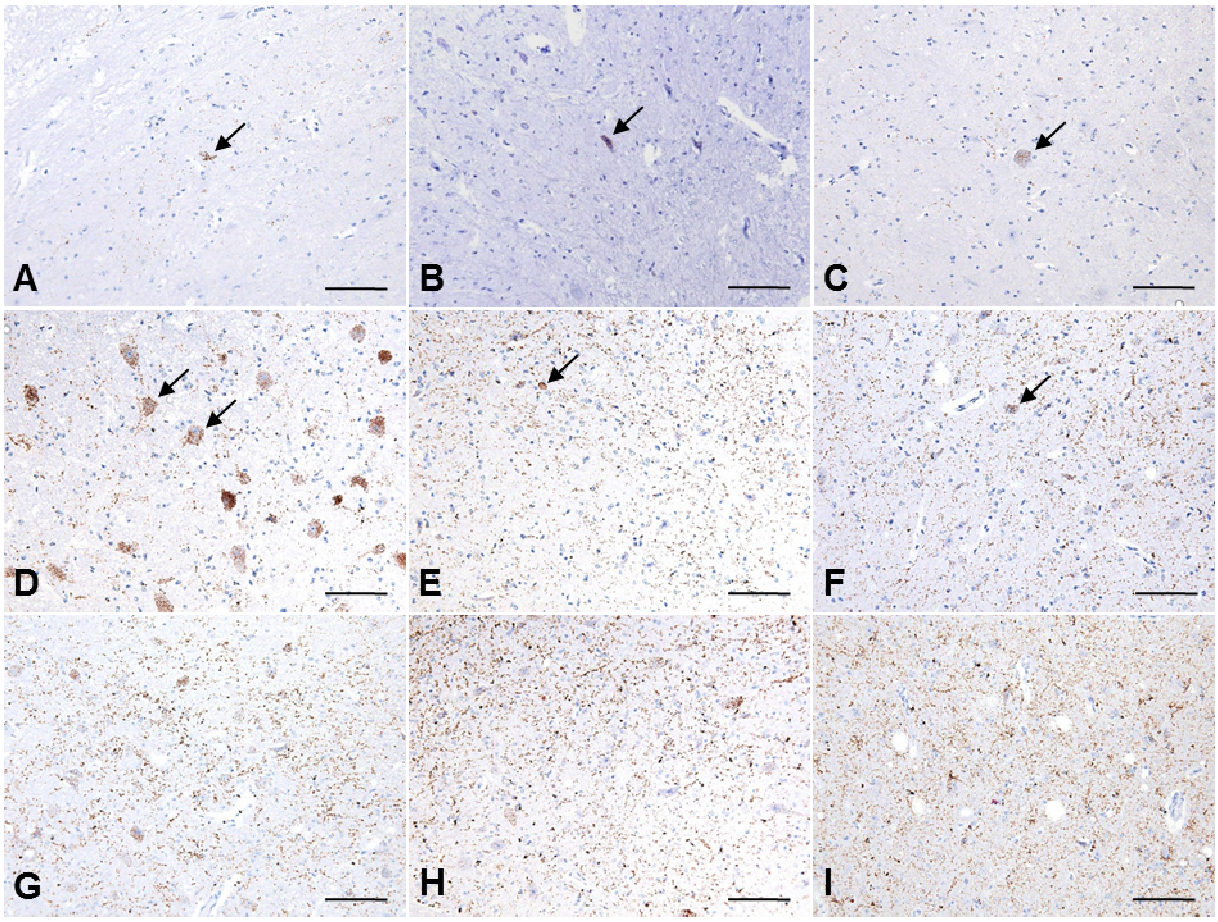

Fig. 8. PrP^{Sc} accumulation in the auditory brainstem nuclei of BSE-challenged cattle

Immunohistochemical staining of PrP^{Sc} accumulation in the auditory brainstem nuclei of BSE-challenged cattle sacrificed at 12 (A-C), 19 (D-F) and 24 mpi (G-I) using each semi-serial section shown in Fig. 1 (D-F). Images show the cochlear nucleus (A, D, G), nucleus of the trapezoid body (B, E, H) and nucleus of the inferior colliculus (C, F, I). Intraneuronal PrP^{Sc} accumulation (arrow) is observed in each auditory brainstemnucleus at 12 mpi (A-C). Intraneuronal (arrows) and granular accumulation of PrP^{Sc} is apparent in auditory brainstem nuclei at 19 (D-F) and 24 mpi (G-I). IHC. Bar, 100 μm.

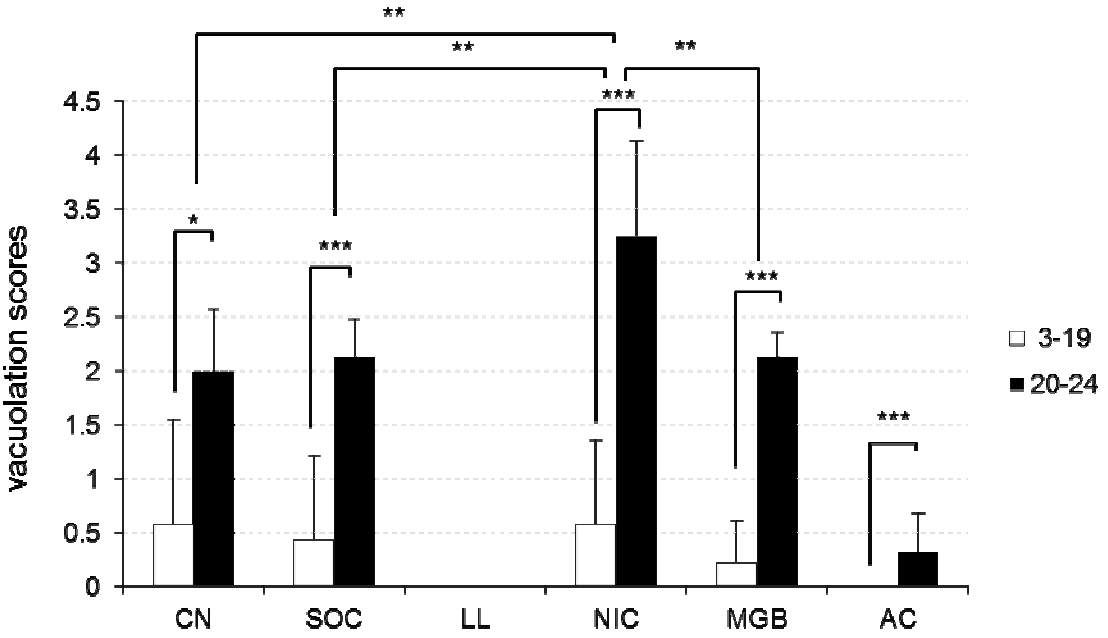

Fig. 9. Vacuolation scores in the auditory brainstem nuclei of BSE-challenged cattle.

Vacuolation scores in the auditory brainstem nuclei of BSE-challenged cattle sacrificed prior to 19 mpi (white bars) and after 20 mpi (black bars) were compared. Vacuolar change in the nucleus of the inferior colliculus after 20 mpi is significantly greater compared with changes in the cochlear nucleus, superior olivary complex and nucleus of medial geniculate body. CN, cochlear nucleus; SOC, superior olivary complex; LL, lateral lemniscus; NIC, nucleus of inferior colliculus; MGB, medial geniculate body; AC, auditory cortex. ***$p < 0.0001$; ** $p < 0.001$; * $p < 0.05$. All data are expressed as mean ± SD.

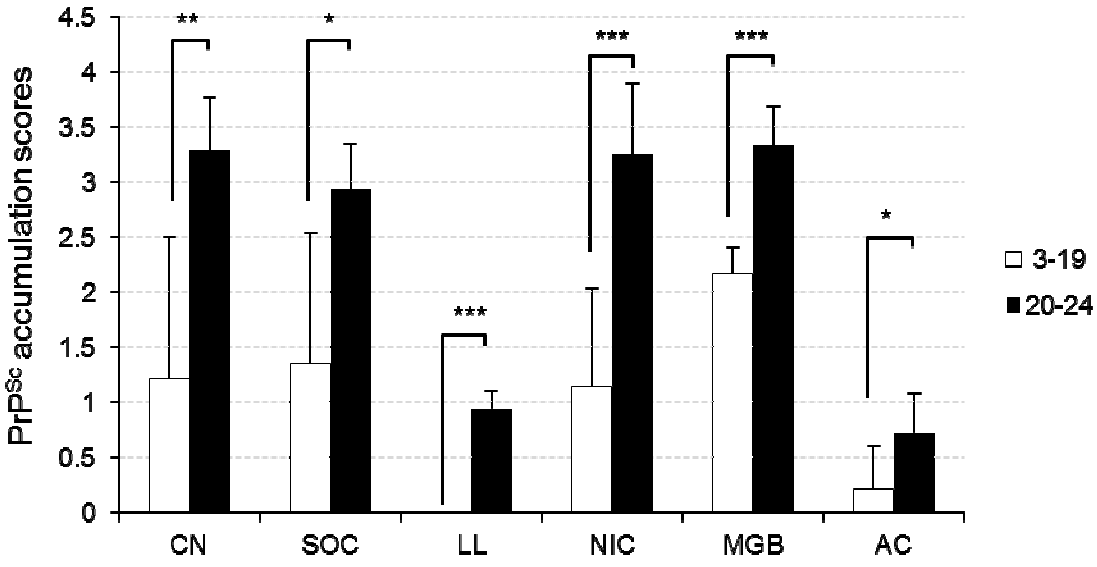

Fig. 10. Scoring of PrP^{Sc} staining in the auditory brainstem nuclei of BSE-challenged cattle.

PrP^{Sc} immunolabelling in the auditory brainstem nuclei of BSE-challenged cattle sacrificed prior to 19 mpi (white bars) and after 20 mpi (black bars) were compared. No significant differences in PrP^{Sc} accumulation were present in the auditory brainstem nuclei at the same stage. CN, cochlear nucleus; SOC, superior olivary complex; LL, lateral lemniscus; NIC, nucleus of inferior colliculus; MGB, medial geniculate body; AC, auditory cortex. ***$p < 0.0001$; ** $p < 0.001$; * $p < 0.05$. All data are expressed as mean ± SD.

Discussion

Overall, typical spongiosis and PrP^{Sc} accumulation were mainly present in the neuropil of most brainstem nuclei of all C-BSE-challenged cattle with clinical signs. However, intraneuronal vacuolation was minimal in most clinical cases. The various types of PrP^{Sc} deposition (Debeer et al., 2003; Siso et al., 2004) were widely detected in the brain. Cattle affected with C-BSE show progressive degeneration of CNS (Wells et al., 1991). Affected cattle display many clinical signs that depend on the stage of the disease, which may worsen with time. It is difficult to diagnose C-BSE before clinical onset because early clinical signs of C-BSE are not always typical (Konold et al., 2004). C-BSE-infected cattle generally appear disturbed, anxious and nervous and are sensitive to loud noise or metallic sounds. These neurological signs usually appear at later stages of the disease. In the nine cattle showing clinical signs of C-BSE that were killed between 20 and 24 mpi, moderate vacuolar changes and abundant PrP^{Sc} accumulation were evident in all auditory brainstem nuclei. Clinical signs may appear after the presence of spongiform changes (Siso et al., 2004). Vacuolation was most prominent in the nucleus of the inferior colliculus compared with other auditory brainstem nuclei and the medial geniculate body. Lesion scoring of the cochlear nucleus has been reported to be very low compared with that of other brainstem nuclei (Breslin et al., 2006; Simmons et al., 1996). Topographical distribution of PrP^{Sc} deposits has been shown to correspond with vacuolar lesions (Wells and Wilesmith, 1995), but PrP^{Sc} accumulation in some nuclei may not be accompanied by any vacuolation (Debeer et al., 2003; Kimura et al., 2002). Neuropathological appearance and topographical PrP^{Sc} immunolabelling in the brain of these two preclinical cattle killed at 18 and 19 mpi were similar to those described previously in naturally-occurring C-BSE cases (Debeer et al., 2003; Kimura et al., 2002; Siso

et al., 2004). To our knowledge, there is no appropriate tool currently available for ante-mortem diagnosis of C-BSE, and confirmation of the disease is only possible following post-mortem examination of brain tissue. In human prion diseases, the combination of auxiliary examinations based on an electroencephalogram and magnetic resonance imaging is used for clinical diagnosis (Cataldi et al., 2000; Wieser et al., 2006). In human, the audiometry of BAEP is a neurological test of auditory brainstem function in response to auditory stimuli, therefore BAEP changes may be accompanied by damage to the auditory pathways in the brain or auditory nerve indicative of underlying neuropathological alterations (Cascino et al., 1988). Results of the present study suggest that spongy changes in the auditory brainstem nuclei, especially in the nucleus of the inferior colliculus of C-BSE-affected cattle, may be related to prolonged BAEP latency and reflect the dysfunction of auditory stimuli rather (Arai et al., 2009).

Brief summary

Since the C-BSE-affected cattle showed auditory abnormality such as hypersensitivity to sound, this study focused on neuropathological changes observed in the auditory brainstem of the C-BSE-challenged cattle. Sixteen cattle experimentally inoculated with C-BSE as described in Chapter 1 were used in this study. Before the appearance of clinical signs (i.e., at 3, 10, 12 and 16 mpi), vacuolar change was absent or mild and PrP^{Sc} deposition was minimal in the auditory brainstem nuclei. The two cattle sacrificed at 18 and 19 mpi without clinical signs showed mild vacuolar degeneration and moderate amounts of PrP^{Sc} accumulation in the auditory brainstem pathway. In the cattle showing clinical manifestation (i.e., after 20 mpi), spongy changes were more prominent in the nucleus of the inferior colliculus compared with the other nuclei of the auditory brainstem and the medial geniculate body. These pathological findings suggest that neuropathological changes characterized by spongy lesions accompanied by PrP^{Sc} accumulation in the auditory brainstem nuclei may be associated with hyperacusia in C-BSE-affected cattle.

Chapter 3

Experimental transmission of L-type atypical bovine spongiform encephalopathy detected in Japan to cattle.

Introduction

The C-BSE was first recognized in UK in 1986 (Wells et al., 1987), and has subsequently spread to other European countries, Japan and North America. Until recently, it was believed that the BSE agent was a single strain based on biological, neuropathological and biochemical characteristics in field BSE cases (Bruce et al., 1996; Kuczius et al., 1998; Simmons et al., 1996). However, since 2003, atypical BSE cases that show different neuropathological and molecular phenotypes have been reported (Ducrot et al., 2008). At present, atypical BSE are classified as the H-type and L-type according to the higher and lower molecular masses of the unglycosylated form of PrP-res in Western blotting analysis, respectively, compared with those from C-BSE cases (Jacobs et al., 2007). Among the L-type BSE cases reported from various countries (Ducrot et al., 2008), the Italian L-type BSE cases were further characterized by the presence of PrP-positive amyloid plaques in the brain (Casalone et al., 2004) and is termed as BASE. BASE prions were experimentally transmitted to cattle, and the phenotypes of the BASE prions have been partly characterized (Lombardi et al., 2008). However, it remains to be determined whether the L-type BSE prions detected in other countries are identical to the BASE prions. Resolving this issue is crucial for future research aimed at exploring the origin of atypical BSE, assessing the risk of atypical BSE and

reviewing of the current administrative measures for BSE control. In Japan, two atypical BSE cases have been identified to date. The first case showed an L-type-like electrophoretic mobility of the unglycosylated PrP-res on Western blotting analysis (Yamakawa et al., 2003). The second atypical case (BSE/JP24) was identified in an aged beef cattle, Japanese Black, and showed PrP-positive amyloid plaques in histopathological examination of the brain and a distinct glycoform profile of the L-type BSE (Hagiwara et al., 2007). Such properties seem to be similar to those reported in the BASE cases (Casalone et al., 2004). However, the lesion profile of the BSE/JP24 prion-inoculated transgenic mice expressing bovine PrP (TgBoPrP) was slightly different from that of the BASE prion-inoculated another line of transgenic mice expressing bovine PrP (TgBov XV) (Capobianco et al., 2007). In addition, a serial passage of the BSE/JP24 in the TgBoPrP shortened the incubation periods from 197.7 ± 3.4 days at the first passage to 152.2 ± 3.1 days at the second passage; however, this characteristic was not reported in the European L-type BSE (Masujin et al., 2008). Thus, it remains controversial whether the BSE/JP24 isolate is identical to the BASE or L-type BSE prions.

To characterize properties of the BSE/JP24 prions, in Chapter 3, the author carried out experimental transmission of the BSE/JP24 isolate to Holstein/Friesian cattle and examined the clinical course of the disease. Furthermore, biochemical and neuropathological properties of the BSE/JP 24 isolate were compared to those of the C-BSE prions.

Materials and Methods

Ethical considerations

This study was approved by the Animal Ethical Committee and the Animal Care and Use Committee of National Institute of Animal Health and Hokkaido Animal Research Center, respectively.

Animals

Three Holstein/Friesian calves aged 2–3 months were intracerebrally inoculated with 1 ml of 10% (w/v) brain homogenates prepared from the medulla oblongata of L-type BSE (BSE/JP24) (n = 3). Three cattle experimentally inoculated with C-BSE prions from UK were used in this study (Case 8, 12 and 15 in Table 1 of Chapter 1). These cattle were clinically monitored and sacrificed at the terminal stage of disease.

PrP-res detection

Western blotting analysis for PrP-res from PK-treated brain homogenates was carried out as described previously (Iwata et al., 2006). To detect PrP-res, anti-PrP mAbs 6H4 (Roche Diagnostics, Basel, Switzerland) and T2 (Hayashi et al., 2004) were used for Western blotting. The signal intensities in di-, mono-, and non-glycosylated fragments of PrP-res were measured and semiquantified by densitometric analysis.

Scoring of vacuolation and immunohistochemistry for PrP^{Sc}

Vacuolation profile was determined using HE-stained sections as described previously (Simmons et al., 1996). For the immunohistochemical detection of PrP^{Sc}, dewaxed sections

were pretreated with the chemical solutions as described previously (Bencsik et al., 2005) and immunolabeled with mAbs F99/97.6.1 (VMRD, Pullman, WA, USA) and T1 (Furuoka et al., 2007) using the tyramide signal amplification system (NEN Life Science Products, Boston, MA, USA).

Results

Clinical manifestation

Cattle inoculated with the C-BSE or the BSE/JP24 prions appeared to show clinical signs indicative of BSE, such as mild anxiety and/or hyperesthesia evoked by sudden loud noises or waving of clipboard at 548 ± 25 and at 344 ± 14 days post inoculation (dpi) (mean ± SD), respectively. Clinical durations were 128 ± 46 and 141 ± 25 days for C-BSE and BSE/JP24 inoculated cattle, respectively. The BSE/JP24-inoculated cattle were inactive and displayed little aggression during the clinical stage. Periods to the terminal stage of the diseases were 675 ± 57 and 486 ± 11 dpi for C-BSE- and BSE/JP24- inoculated cattle, respectively (Table 4).

Table 4. Incubation period and clinical duration in BSE and BSE/JP24 prion-inoculated cattle

Code	Breed	Inoculum	Period to terminal stage of disease (days)	Incubation priod (days)	Clinical duration (days)
4394	Holstein	C-BSE	610	533	77
4437	Holstein	C-BSE	700	533	167
5087	Holstein	C-BSE	716	577	139
			Mean: 675 ± 57[1)]	Mean: 548 ± 25[1)]	Mean: 128 ± 46[1)]
528	Holstein	BSE/JP24	497	337	160
1061	Holstein	BSE/JP24	476	363	113
5566	Holstein	BSE/JP24	484	333	151
			Mean: 486 ± 11[1)]	Mean: 344 ± 14[1)]	Mean: 141 ± 25[1)]

1) Mean ± SD

PrP-res glycoform

The unglycosylated fragments of PrP-res derived from the original BSE/JP24 cattle and the BSE/JP24-affected cattle migrated slightly faster than those of PrP-res from the C-BSE-affected cattle (Fig. 11A and B). The relative amounts of di- mono-, and non-glycosylated PrP-res fragments from the BSE/JP24-affected cattle resembled those from the original BSE/JP24 cattle. These glycoform properties are similar to those of the Italian L-type BSE (Casalone et al., 2004). The glycoform ratios of PrP-res from the BSE/JP24-affected cattle were distinguishable from those observed in C-BSE affected cattle (Fig. 11A and C): ratio of di- and mono-glycosylated PrP-res were nearly comparable to PrP-res from the BSE/JP24-affected cattle, whereas di-glycosylated PrP-res is abundant in PrP-res from the C-BSE-affected cattle.

Vacuolation lesion and PrP^{Sc} distribution

Vacuolation was more severe in the midbrain, thalamus, hypothalamus and frontal cortex (Fig. 12 A) of the BSE/JP24-affected cattle compared to that of the C-BSE-affected cattle. The PrP^{Sc} deposition in the BSE/JP24-affected cattle was characterized by diffuse synaptic-punctuate staining, low-grade stellate-type PrP^{Sc} deposits, and amyloid PrP plaques by immunohistochemistry (Fig. 12B). However, no striking differences were observed in the topography of PrP^{Sc} deposition between the C-BSE prion- and the BSE/JP24-affected cattle (data not shown). PrP^{Sc} deposits were pronounced in the neuropil of the thalamus and midbrain, particularly in the periaqueductal gray matter of the brains of cattle affected with the C-BSE and the BSE/JP24 prions (data not shown). Western blotting analysis also showed that there were no marked differences in the PrP^{Sc} distribution, except for high PrP^{Sc} levels in the frontal cortex of the BSE/JP24-affected cattle (Fig. 12C).

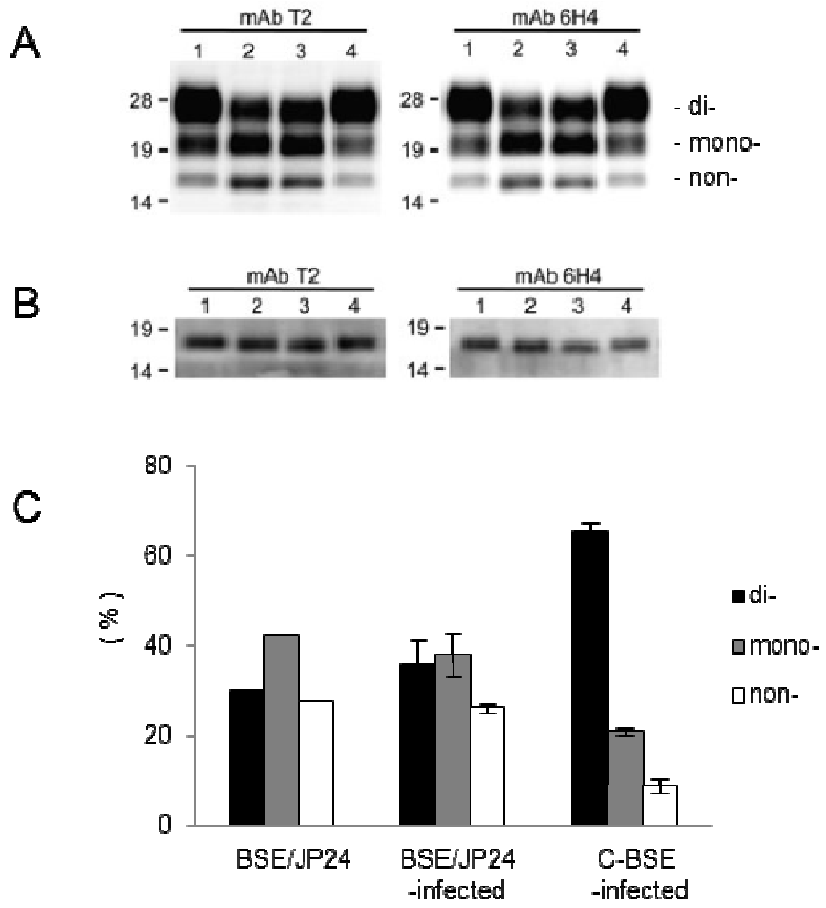

Fig. 11. Western blotting analysis of PrP-res.

(A) Western blotting of PK-treated brain homogenates from the original BSE/JP24 cattle (lane 2), the C-BSE prion- (lanes 1 and 4) and the BSE/JP24 prion-challenged cattle (lane 3). Blots were probed with mAbs T2 and 6H4. (B) Samples after deglycosylation by PNGase treatment. Molecular mass standards (kDa) are indicated on the left. (C) Ratios of the di-, mono- and non-glycosylated forms of PK-treated PrP^{Sc}. Error bars indicate standard deviation (SD).

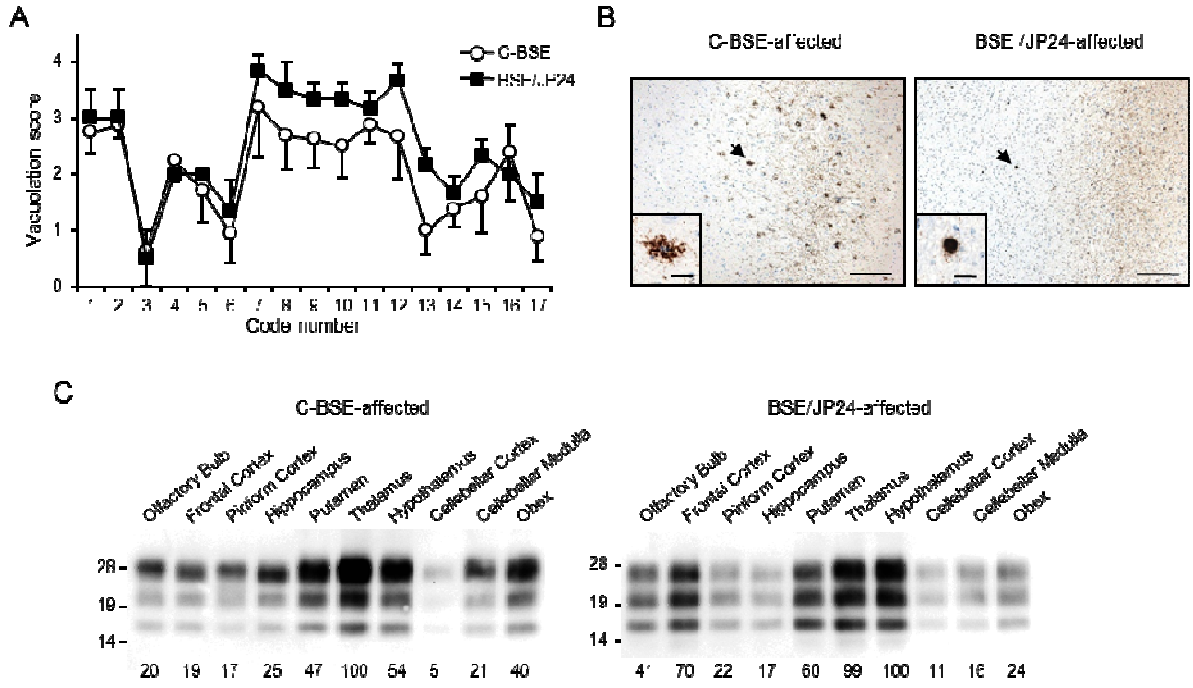

Fig. 12. Neuropathological and biochemical comparison between C-BSE or the BSE/JP24-affected cattle.

(A) Lesion profile of the C-BSE and the BSE/JP24-affected cattle. The mean scores for the C-BSE-affected cattle (C-BSE; open circles, n = 3) and the BSE/JP24-affected cattle (BSE/JP24; closed squares, n = 3) are shown. Error bars indicate SD. The neuroanatomical regions are as follows: 1, nucleus of the solitary tract; 2, nucleus of the spinal tract of V; 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, septal nuclei; 15, caudate nucleus; 16, putamen; 17, claustrum. (B) PrP^{Sc} deposition in the frontal lobe of the C-BSE- (left panel) and the BSE/JP24 prion-affected (right panel) cattle. Stellate-type PrP^{Sc} deposit and PrP-plaque are indicated by arrows and insets, respectively. Bars in the main panels = 200 μm; bars in the insets = 20 μm. (C) Comparison of regional PrP^{Sc} deposition in the brain between the C-BSE and the BSE/JP24-affected cattle. A representative Western blot of PrP^{Sc} is shown. The levels of PrP^{Sc} relative to the thalamus (C-BSE-affected cattle) or hypothalamus (BSE/JP24-affected cattle) are indicated below the panels.

Discussion

Atypical BSE cases were usually detected in aged cattle over 8-year old with few exceptions and thus, it is conceivable that atypical BSE might occur sporadically in aged cattle like as sporadic CJD in human and that atypical BSE might be an origin of C-BSE (Biacabe AG et al., 2008). Therefore, it is of interest to know the disease phenotype after the transmission of atypical BSE to cattle. This study demonstrated the successful transmission of the BSE/JP24 prion to cattle. Molecular properties of PrP-res in cattle experimentally infected with the BSE/JP24 isolate unchanged. In addition, although most of brain regions except for the medulla oblongata of the original BSE/JP24 case were unable to be investigated due to inadequate specimen collection, neuropathological features such as severe vacuolation in the medulla oblongata at the obex level and the presence of PrP^{Sc} plaques closely resembled each other. Based on molecular properties of PrP-res and immunohistochemical and neuropathological properties, the disease phenotype of the BSE/JP24 case was reproduced in cattle inoculated with the BSE/JP24 isolate and was obviously different from that of the C-BSE cases. This suggests that atypical BSE, at least L-type BSE like the BSE/JP24 case, may not simply be an origin of the C-BSE. Feeding of meat-and-bone meal and change in the rendering process that produces meat-and-bone mead around 1980s are believed to be a major cause of the BSE emergence in UK (Wilesmith et al., 1997). Thus, it cannot be denied that properties of atypical BSE prions would change under harsh condition during the rendering process.

The time of clinical onset in the BSE/JP24 prion-inoculated cattle was apparently earlier than that in cattle inoculated with the C-BSE prions in this study or than that in a previous study (Wells et al., 2005). A major clinical symptom of the BSE/JP24-affected cattle was

depression, and being different from the C-BSE cases, no aggressiveness was observed in clinical phase. With disease progression, both the C-BSE and BSE/JP24-affected cattle showed an ataxic gait, which appeared to be due to uncoordinated hind limbs and had difficulty in rising in the terminal stage. Recently, Lombardi et al. (2008) reported that a mean period to the terminal stage of disease was 470 days in experimental BASE infection in cattle and major clinical signs were dullness and amyotrophy, which are similar to the observation on BSE/JP24-affected cattle in this study.

Incubation period of the TgBoPrP transgenic mice challenged with the BSE/JP24 isolate (199.7 ± 3.4 days) was reported to be shorter than that of the TgBoPrP transgenic mice challenged with the C-BSE prions (223.5 ± 13.5 days) (Masujin et al., 2008). Similarly, the transmission studies of BASE and L-type BSE prions emerged in European countries to another transgenic mice line TgBov XV revealed that BASE (228 ± 10 days) and L-type BSE prions (212 ± 15 days) showed shorter incubation period than the C-BSE prions (298 ± 7 days) (Beringue et al., 2007). Taken together with similarities of the BSE/JP24 isolate to BASE in experimental infection in cattle, the similarity of the BSE/JP24 isolate to BASE and L-type BSE isolated in transmissibility to transgenic mice expressing bovine PrP, suggests that the BSE/JP24 prion shares common features of BASE and L-type BSE prions disclosed in European countries.

In this study the author showed that the BSE/JP24 prion, L-type like BSE prion disclosed in Japan, was apparently different from the C-BSE prions, but that the BSE/JP24 prion appears to be rather similar to the BASE prions (Lombardi et al., 2008). Of interest, experimental transmission of the BSE/JP24 prion to cattle induced a shorter incubation period and more severe neuropathological changes compared to the C-BSE prions, similar to the transmission study of the BASE prions to cattle (Lombardi et al., 2008). These findings

suggest that the L-type BSE prions, such as BASE and the BSE/JP24 prions, might be more virulent in cattle species than C-BSE prion. However, this speculation conflicts with reports that atypical BSE field cases have been mainly found in adult and aged cattle without noticeable clinical symptoms under an active surveillance scheme (Ducrot et al., 2008). The reasons for this discrepancy between L-type BSE field cases and cattle of experimental transmission, i.e., difference in incubation periods and clinical manifestations, are unknown. Route of infection and/or prion titer may account for the differences.

One public concern is that atypical BSE is a sporadic form of BSE and is able to spread from cattle to cattle. If this is the case, risk managements against atypical BSE have to be carefully assessed and have to be continued at certain level to prevent re-emergence of BSE. Further transmission studies on atypical BSE using naturally occurring route, for instance, oral route, should be addressed to provide scientific evidence for a risk analysis of atypical BSE.

Brief summary

It has been assumed that the causative agent of the C-BSE in cattle is a single strain that first emerged in UK. However, atypical BSE cases that show different neuropathological and molecular phenotypes have been recently reported in European countries, North America, and Japan. To clarify the characteristic of L-type like atypical BSE case that was disclosed in Japan, BSE/JP24, the author inoculated brain homogenates from cattle of the BSE/JP24 case into Holstein/Friesian cattle and examined biochemical and neuropathological properties as well as the clinical course of the disease. The BSE/JP24 isolate successfully transmitted to Holstein/Friesian cattle. Based on the incubation period, neuropathological hallmarks, and molecular properties of PrP^{Sc}, the BSE/JP24 prions were apparently distinguishable from the C-BSE prions and closely resemble BASE prions that were found in Italy.

Conclusion

Bovine spongiform encephalopathy (BSE) was first identified in the United Kingdom in 1986. Subsequently, it spread to European, Asian, and North American countries. A variant form of Creutzfeldt-Jakob disease that was reported in 1996 is thought to be caused by the consumption of BSE-contaminated beef products. Therefore, it is important to understand the pathogenesis of the classical BSE (C-BSE) in cattle that first emerged in UK and spread all over the world, in order to reduce the C-BSE risk to public as well as to animals. However, due to the difficulty in using cattle for experimental infection, our current knowledge of the pathogenesis of the C-BSE in cattle is still inadequate. Therefore, the author investigated the relationship among the clinical manifestation, the accumulation of disease-specific prion protein (PrP^{Sc}) and the vacuolar changes of cattle experimentally infected with the C-BSE in order to clarify neuropathogenesis of the C-BSE. Furthermore, different type BSE cases from the C-BSE cases, called atypical BSE, disclosed in 2003, and thereafter, the presence of atypical BSE cases was reported in many countries including Japan. In this thesis the author also analyzed properties of the BSE/JP24 isolate, the second L-type-like atypical BSE disclosed in Japan, by experimental infection in cattle.

In Chapter 1, to evaluate the relationship between the kinetics of PrP^{Sc} deposition in the central nervous system and the clinical course of the disease, temporal and special distribution of PrP^{Sc} was analyzed in the central nervous system of Holstein/Friesian cattle inoculated intracerebrally with 3 sources of C-BSE isolates. Cattle euthanized at 10 months post-inoculation (mpi) showed PrP^{Sc} deposits in the brainstem and thalamus, but no vacuolation. This suggested that the BSE prions 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 indicate that PrP^{Sc} accumulation was detected approximately 10 months before the clinical onset.

Since the C-BSE-affected cattle showed auditory abnormality such as hypersensitivity to sound, in chapter 2, the author focused on neuropathological changes observed in the auditory brainstem of the C-BSE-challenged cattle. Before the appearance of clinical signs (i.e., at 3, 10, 12 and 16 mpi), vacuolar change was absent or mild and PrP^{Sc} deposition was minimal in the auditory brainstem nuclei. The two cattle sacrificed at 18 and 19 mpi without clinical signs showed mild vacuolar degeneration and moderate amounts of PrP^{Sc} accumulation in the auditory brainstem pathway. In the cattle showing clinical manifestation (i.e., after 20 mpi), spongy changes were more prominent in the nucleus of the inferior colliculus compared with the other nuclei of the auditory brainstem and the medial geniculate body. These pathological findings suggest that neuropathological changes characterized by spongy lesions accompanied by PrP^{Sc} accumulation in the auditory brainstem nuclei may be associated with hyperacusia in BSE-affected cattle.

It has been assumed that the causative agent of the C-BSE in cattle is a single strain that first emerged in UK. However, atypical BSE cases that show different neuropathological and molecular phenotypes have been recently reported in European countries, North America, and Japan. In Chapter 3, to clarify the characteristic of L-type like atypical BSE case that was disclosed in Japan, BSE/JP24, the author inoculated brain homogenates from cattle of the BSE/JP24 case into Holstein/Friesian cattle and examined biochemical and neuropathological

properties as well as the clinical course of the disease. The BSE/JP24 isolate successfully transmitted to Holstein/Friesian cattle. Based on the incubation period, neuropathological hallmarks, and molecular properties of PrP^{Sc}, the BSE/JP24 prions were apparently distinguishable from the C-BSE prions and closely resemble bovine amyloidotic spongiform encephalopathy prions that were found in Italy.

In conclusion, this study clarified a part of the C-BSE pathogenesis by analyzing the association of the clinical symptom, the accumulation of PrP^{Sc} and the vacuolar changes of cattle experimentally infected with the C-BSE. In addition, this study clarified biochemical and neuropathological features of the BSE/JP24, the L-type like atypical BSE isolate in Japan. Results of this study provide important information on the risk analysis and management on BSE to reduce the risk of BSE to public as well as the risk of re-emergence of BSE.

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References

Arai, S., Matsui, Y., Fukuda, S., Okada, H., and Onoe, S. Brainstem auditory evoked potentials in experimentally-induced bovine spongiform encephalopathy. *Res Vet Sci* 87:111-114 (2009).

Bencsik, A.A., Debeer, S.O., and Baron, T.G. An alternative pretreatment procedure in animal transmissible spongiform encephalopathies diagnosis using PrP^{Sc} immunohistochemistry. *J Histochem Cytochem* 53:1199-1202 (2005).

Biacabe, A.G., Laplanche, J.L., Ryder, S., and Baron, T. Distinct molecular phenotypes in bovine prion diseases. *EMBO Rep* 5:110-115 (2004).

Biacabe, A.G., Morignat, E., Vulin, J., Calavas, D., and Baron, T.G. Atypical bovine spongiform encephalopathies, France, 2001-2007. *Emerg Infect Dis* 14:298-300 (2008).

Bolton, D.C., McKinley, M.P., and Prusiner, S.B. Identification of a protein that purifies with the scrapie prion. *Science* 218:1309-1311 (1982).

Breslin, P., McElroy, M., Bassett, H., and Markey, B. Vacuolar lesion profile of BSE in the Republic of Ireland. *Vet Rec* 159:889-890 (2006).

Brown, P. McShane, L.M., Zanusso, G., and Detwile, L. On the question of sporadic or atypical bovine spongiform encephalopathy and Creutzfeldt-Jakob disease. *Emerg Infect Dis* 12:1816-1821 (2006).

Bruce, M., Chree, A., McConnel, I., Brown, K., and Fraser, H. Transmission and strain typing studies of scrapie and bovine spongiform encephalopathy. In: Court L., Dodet B. eds. *Transmissible Subacute Spongiform Encephalopathies: Prion Diseases*. Elsevier, Paris, France, 259-62 (1996)

Buschmann, A., and Groschup, M.H. Highly bovine spongiform encephalopathy-sensitive transgenic mice confirm the essential restriction of infectivity to the nervous system in clinically diseased cattle. *J Infect Dis* 192:934-942 (2005).

Buschmann, A., Gretzschel, A., Biacabe, A.G., Schiebel, K., Corona, C., Hoffmann, C., Eiden, M., Baron, T., Casalone, C., and Groschup, M.H. Atypical BSE in Germany--proof of transmissibility and biochemical characterization. *Vet Microbiol* 117:103-116 (2006).

Capobianco, R., Casalone, C., Suardi, S., Mangieri, M., Miccolo, C., Limido, L., et al. Conversion of the BASE prion strain into the BSE strain: the origin of BSE? *PLoS Pathog* 3:e31 (2007).

Casalone, C., Zanusso, G., Acutis, P., Ferrari, S., Capucci, L., Tagliavini, F., Monaco, S., and Caramelli, M. Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. *Proc Natl Acad Sci* 101:3065-3070 (2004).

Cascino, G.D., Ring, S.R., King, P.J., Brown, R.H., and Chiappa, K.H. Evoked potentials in motor system diseases. *Neurology* 38:231-238 (1988).

Castilla, J., Saá, P., and Soto, C.. Detection of prions in blood. *Nat Med* 11:982-985 (2005).

Cataldi, M.L., Restivo, O., Reggio, E., Restivo, D.A., and Reggio, A. Deafness: an unusual onset of genetic CreutzfeldteJakob disease. *Neurological Sciences* 21: 53-55. (2000)

Chazot, G., Broussolle, E., Lapras, C., Blattler, T., Aguzzi, A., and Kopp, N. New variant of Creutzfeldt-Jakob disease in a 26-year-old French man. *Lancet* 347:1181 (1996).

Chiappa, K.H., Evoked potentials in clinical medicine. In: Chiappa KH., *Brainstem Auditory Evoked Potentials, third edition*, Lippincott-Raven, NY, USA, 199-249 (1997)

Cousens, S.N., Vynnycky, E., Zeidler, M., Will, R.G., and Smith, P.G. Predicting the CJD epidemic in humans. *Nature* 385:197-198 (1997).

Debeer, S., Baron, T., and Bencsik, A. Neuropathological characterisation of French bovine spongiform encephalopathy cases. *Histochem Cell Biol* 120:513-521 (2003).

Ducrot, C., Arnold, M., de Koeijer, A., Heim, D. and Calavas, D. Review on the epidemiology and dynamics of BSE epidemics. *Vet Res* 39:15 (2008).

Dudas, S., Yang, J., Graham, C., Czub, M., McAllister, T.A., Coulthart, M.B., and Czub, S. Molecular, biochemical and genetic characteristics of BSE in Canada. *PLoS One* 5:e10638 (2010).

Furuoka, H., Yabuzoe, A., Horiuchi, M., Tagawa, Y., Yokoyama, T., Yamakawa, Y., Shinagawa, M., and Sata, T. Species-specificity of a panel of prion protein antibodies for the immunohistochemical study of animal and human prion diseases. *J Comp Pathol* 136:9-17 (2007).

González, L., Martin, S., Begara-McGorum, I., Hunter, N., Houston, F., Simmons, M., and Jeffrey, M. Effects of agent strain and host genotype on PrP accumulation in the brain of sheep naturally and experimentally affected with scrapie. *J Comp Pathol* 126:17-29 (2002).

González, L., Martin, S., Houston, F.E., Hunter, N., Reid, H.W., Bellworthy, S.J., and Jeffrey, M. Phenotype of disease-associated PrP accumulation in the brain of bovine spongiform encephalopathy experimentally infected sheep. *J Gen Virol* 86:827-838 (2005).

Hagiwara, K., Yamakawa, Y., Sato, Y., Nakamura, Y., Tobiume, M., Shinagawa, M., and Sata, T. Accumulation of mono-glycosylated form-rich, plaque-forming PrP^{Sc} in the

second atypical bovine spongiform encephalopathy case in Japan. *Jpn J Infect Dis* 60:305-308 (2007).

Haïk, S., Faucheux, B.A., Sazdovitch, V., Privat, N., Kemeny, J.L., Perret-Liaudet, A., and Hauw, J.J. The sympathetic nervous system is involved in variant Creutzfeldt-Jakob disease. *Nat Med* 9:1121-1123 (2003).

Hamir, A.N., Miller, J.M., Stack, M.J., and Chaplin, M.J. Failure to detect abnormal prion protein and scrapie-associated fibrils 6 wk after intracerebral inoculation of genetically susceptible sheep with scrapie agent. *Can J Vet Res* 66:289-294 (2002).

Hamir, A.N., Miller, J.M., O'Rourke, K.I., Bartz, J.C., Stack, M.J., and Chaplin, M.J .Transmission of transmissible mink encephalopathy to raccoons (Procyon lotor) by intracerebral inoculation. *J Vet Diagn Invest* 16:57-63 (2004).

Hamir, A.N., Kunkle, R.A., Cutlip, R.C., Miller, J.M., O'Rourke, K.I., Williams, E.S., Miller, M.W., Stack, M.J., Chaplin, M.J., and Richt, J.A. Experimental transmission of chronic wasting disease agent from mule deer to cattle by the intracerebral route. *J Vet Diagn Invest* 17:276-281 (2005).

Hayashi, H., Takata, M., Iwamaru, Y., Ushiki, Y., Kimura, K.M., Tagawa, Y., Shinagawa, M., and Yokoyama, T. Effect of tissue deterioration on postmortem BSE diagnosis by immunobiochemical detection of an abnormal isoform of prion protein. *J Vet Med Sci* 66:515-520 (2004).

Hoffmann, C., Ziegler, U., Buschmann, A., Weber, A., Kupfer, L., Oelschlegel, A., Hammerschmidt, B., and Groschup, M.H. Prions spread via the autonomic nervous system from the gut to the central nervous system in cattle incubating bovine spongiform encephalopathy. *J Gen Virol* 88:1048-1055 (2007).

Iwata, N., Sato, Y., Higuchi, Y., Nohtomi, K., Nagata, N., Hasegawa, H., Tobiume, M., Nakamura, Y., Hagiwara, K., Furuoka, H., Horiuchi, M., Yamakawa, Y., and Sata, T. Distribution of PrP(Sc) in cattle with bovine spongiform encephalopathy slaughtered at abattoirs in Japan. *Jpn J Infect Dis* 59:100-107 (2006).

Jacobs, J.G., Langeveld, J.P., Biacabe, A.G., Acutis, P.L., Polak, M.P., Gavier-Widen, D., Buschmann, A., Caramelli, M., Casalone, C., Mazza, M., Groschup, M., Erkens, J.H., Davidse, A., van Zijderveld, FG., and Baron, T. Molecular discrimination of atypical bovine spongiform encephalopathy strains from a geographical region spanning a wide area in Europe. *J Clin Microbiol* 45:1821-1829 (2007).

Kimura, K.M., Haritani, M., Kubo, M., Hayasaka, S., and Ikeda, A. Histopathological and immunohistochemical evaluation of the first case of BSE in Japan. *Vet Rec* 151:328-330 (2002).

Konold, T., Bone, G., Ryder, S., Hawkins, S.A., Courtin, F., and Berthelin-Baker, C. Clinical findings in 78 suspected cases of bovine spongiform encephalopathy in Great Britain. *Vet Rec* 155:659-666 (2004).

Kuczius, T., Haist, I., and Groschup, M.H. Molecular analysis of bovine spongiform encephalopathy and scrapie strain variation. *J Infect Dis* 178:693-699 (1998).

Lombardi, G., Casalone, C., D'Angelo, A., Gelmetti, D., Torcoli, G., Barbieri, I., Corona, C., Fasoli, E., Farinazzo, A., Fiorini, M., Gelati, M., Iulini, B., Tagliavini, F., Ferrari, S., Caramelli, M., Monaco, S., Capucci, L., and Zanusso, G. Intraspecies transmission of BASE induces clinical dullness and amyotrophic changes. *PLoS Pathog* 4:e1000075 (2008).

Masujin, K., Matthews, D., Wells, G.A., Mohri, S., and Yokoyama, T. Prions in the peripheral nerves of bovine spongiform encephalopathy-affected cattle. *J Gen Virol* 88:1850-1858 (2007).

Masujin, K., Shu, Y., Yamakawa, Y., Hagiwara, K., Sata, T., Matsuura, Y., Iwamaru, Y., Imamura, M., Okada, H., Mohri, S., and Yokoyama, T. Biological and biochemical characterization of L-type-like bovine spongiform encephalopathy (BSE) detected in Japanese black beef cattle. *Prion* 2:123-128 (2008).

McBride, P.A., and Beekes, M. Pathological PrP is abundant in sympathetic and sensory ganglia of hamsters fed with scrapie. *Neurosci Lett* 265:135-138 (1999).

McBride, P.A., Schulz-Schaeffer, W.J., Donaldson, M., Bruce, M., Diringer, H., Kretzschmar, H.A., and Beekes, M. Early spread of scrapie from the gastrointestinal tract to the central nervous system involves autonomic fibers of the splanchnic and vagus nerves. *J Virol* 75:9320-9327 (2001).

Okada, H., Iwamaru, Y., Imamura, M., Masujin, K., Matsuura, Y., Shimizu, Y., Kasai, K., Takata, M., Fukuda, S., Nikaido, S., Fujii, K., Onoe, S., Mohri, S., and Yokoyama, T. Neuroanatomical distribution of disease-associated prion protein in cases of bovine spongiform encephalopathy detected by fallen stock surveillance in Japan. *J Vet Med Sci* 73: 1465-1471 (2011).

Orge, L., Simas, J.P., Fernandes, A.C., Ramos, M., and Galo, A. Similarity of the lesion profile of BSE in Portuguese cattle to that described in British cattle. *Vet Rec* 147:486-488 (2000).

Prusiner, S.B., Bolton, D.C., Groth DF, Bowman KA, Cochran SP, and McKinley MP Further purification and characterization of scrapie prions. *Biochemistry* 21:6942-6950 (1982).

Prusiner, S.B. Molecular biology of prion diseases. *Science* 252:1515-1522 (1991a).

Prusiner, S.B. Molecular biology of prions causing infectious and genetic encephalopathies of humans as well as scrapie of sheep and BSE of cattle. *Dev Biol Stand* 75:55-74 (1991b).

Richt, J.A., Kunkle, R.A., Alt, D., Nicholson, E.M., Hamir, A.N., Czub, S., Kluge, J., Davis, A.J., and Hall, S.M. Identification and characterization of two bovine spongiform encephalopathy cases diagnosed in the United States. *J Vet Diagn Invest* 19:142-154 (2007).

Schulz-Schaeffer, W.J., Tschoke, S., Kranefuss, N., Drose, W., Hause-Reitner, D., Giese, A., Groschup, M.H., and Kretzschmar, H.A. The paraffin-embedded tissue blot detects PrP(Sc) early in the incubation time in prion diseases. *Am J Pathol* 156:51-56 (2000).

Shimizu, Y., Kaku-Ushiki, Y., Iwamaru, Y., Muramoto, T., Kitamoto, T., Yokoyama, T., Mohri, S., and Tagawa, Y. A novel anti-prion protein monoclonal antibody and its single-chain fragment variable derivative with ability to inhibit abnormal prion protein accumulation in cultured cells. *Microbiol Immunol* 54:112-121 (2010).

Simmons, M.M., Harris, P., Jeffrey, M., Meek, S.C., Blamire, I.W., and Wells, G.A. BSE in Great Britain: consistency of the neurohistopathological findings in two random annual samples of clinically suspect cases. *Vet Rec* 138:175-177 (1996).

Siso, S., Ordonez, M., Cordon, I., Vidal, E., and Pumarola, M. Distribution of PrP(res) in the brains of BSE-affected cows detected by active surveillance in Catalonia, Spain. *Vet Rec* 155:524-525 (2004).

Taylor, D.M., Brown, J.M., Fernie, K., and McConnell, I. The effect of formic acid on BSE and scrapie infectivity in fixed and unfixed brain-tissue. *Vet Microbiol* 58:167-174 (1997).

van Keulen, L.J., Schreuder, B.E., Vromans, M.E., Langeveld, J.P., and Smits, M.A. Pathogenesis of natural scrapie in sheep. *Arch Virol Suppl* 16:57-71 (2000).

Vidal, E., Marquez, M., Ordonez, M., Raeber, A.J., Struckmeyer, T., Oesch, B., Siso, S., and Pumarola, M. Comparative study of the PrP^{BSE} distribution in brains from BSE field cases using rapid tests. *J Virol Methods* 127:24-32 (2005).

Vidal, E., Marquez, M., Tortosa, R., Costa, C., Serafin, A., and Pumarola, M. Immunohistochemical approach to the pathogenesis of bovine spongiform encephalopathy in its early stages. *J Virol Methods* 134:15-29 (2006).

Wells, G.A., Scott, A.C., Johnson, C.T., Gunning, R.F., Hancock, R.D., Jeffrey, M., Dawson, M., and Bradley, R. A novel progressive spongiform encephalopathy in cattle. *Vet Rec* 121:419-420 (1987).

Wells, G.A., Wilesmith JW, and McGill IS Bovine spongiform encephalopathy: a neuropathological perspective. *Brain Pathol* 1:69-78 (1991).

Wells, G.A., Dawson, M., Hawkins, S.A., Green, R.B., Dexter, I., Francis, M.E., Simmons, M.M., Austin, A.R., and Horigan, M.W. Infectivity in the ileum of cattle challenged orally with bovine spongiform encephalopathy. *Vet Rec* 135:40-41 (1994).

Wells, G.A., and Wilesmith, J.W. The neuropathology and epidemiology of bovine spongiform encephalopathy. *Brain Pathol* 5:91-103 (1995).

Wells, G.A., Hawkins, S.A., Green, R.B., Austin, A.R., Dexter, I., Spencer, Y.I., Chaplin, M.J., Stack, M.J., and Dawson, M. Preliminary observations on the pathogenesis of

experimental bovine spongiform encephalopathy (BSE): an update. *Vet Rec* 142:103-106 (1998).

Wells, G.A., Hawkins, S.A., Green, R.B., Spencer, Y.I., Dexter, I., and Dawson, M. Limited detection of sternal bone marrow infectivity in the clinical phase of experimental bovine spongiform encephalopathy (BSE). *Vet Rec* 144:292-294 (1999).

Wells, G.A., Spiropoulos, J., Hawkins, S.A., and Ryder, S.J. Pathogenesis of experimental bovine spongiform encephalopathy: preclinical infectivity in tonsil and observations on the distribution of lingual tonsil in slaughtered cattle. *Vet Rec* 156:401-407 (2005).

Wells, G.A., Konold, T., Arnold, M.E., Austin, A.R., Hawkins, S.A., Stack, M., Simmons, M.M., Lee, Y.H., Gavier-Widén, D., Dawson, M., and Wilesmith, J.W. Bovine spongiform encephalopathy: the effect of oral exposure dose on attack rate and incubation period in cattle. *J Gen Virol* 88:1363-1373 (2007).

Wieser, H.G., Schindler, K., and Zumsteg, D. EEG in CreutzfeldteJakob disease. *Clinical Neurophysiology* 117: 935-951. (2006)

Wilesmith, J.W., Wells, G.A., Ryan, J.B., Gavier-Widen, D., and Simmons, M.M., A cohort study to examine maternally-associated risk factors for bovine spongiform encephalopathy. *Vet Rec* 141:239-243 (1997).

Will, R.G., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A., Mitrova, E., de Silva, R., D'Alessandro, M., Delasnerie-Laupretre, N. Zerr, I., and van Duijn, C. Descriptive epidemiology of Creutzfeldt-Jakob disease in six European countries, 1993-1995. EU Collaborative Study Group for CJD. *Ann Neurol* 43:763-767 (1998).

Will, RG., Ironside, J.W., Zeidler, M., Cousens, S.N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A., and Smith, P.G. A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* 347:921-925 (1996).

Yamakawa, Y., Hagiwara, K., Nohtomi, K., Nakamura, Y., Nishijima, M., Higuchi, Y., Sato, Y., and Sata, T. Atypical proteinase K-resistant prion protein (PrPres) observed in an apparently healthy 23-month-old Holstein/Friesian steer. *Jpn J Infect Dis* 56:221-222 (2003).

Yokoyama, T., Masujin, K., Yamakawa, Y., Sata, T., Murayama, Y., Shu, Y., Okada, H., Mohri, S., and Shinagawa, M. Experimental transmission of two young and one suspended bovine spongiform encephalopathy (BSE) cases to bovinized transgenic mice. *Jpn J Infect Di*s 60:317-320 (2007).

Zhang, Z., Zhang, Y., Wang, F., Wang, X., Xu, Y., Yang, H., Yu, G., Yuan, C., and Ma, J. De novo generation of infectious prions with bacterially expressed recombinant prion protein. *FASEB J* (in press)

要　約

牛海綿状脳症（BSE：Bovine spongiform encephalopathy）は、1986年に英国で初めて確認された。その後、BSEは、ヨーロッパ、アジアおよび北アメリカ諸国に広がった。1996年に報告された変異型クロイツフェルト・ヤコブ病は、BSEプリオンが混入した牛肉製品を消費したことに起因すると考えられている。従って、英国で発生して世界各地に広がった定型BSE (C-BSE: classical BSE) の病理発生を解明することは重要である。しかしながら、牛を用いた実験感染の実施が難しいため、牛におけるC-BSEの病理発生に関する知見は、未だ十分でない。それゆえ、著者はC-BSEの神経病理発生を明らかにするため、C-BSE実験感染牛の臨床症状、PrP^{Sc}の蓄積および空胞病変の関連を調査した。さらに、2003年以降、非定型BSEと呼ばれる、C-BSEとは病型の異なるBSEの存在が日本を含め多くの国で報告された。そこで本論文では、日本で見つかったL型様非定型BSEであるBSE/JP24の特徴を、牛への実験感染により解析した。

第 1 章では、中枢神経の PrP^{Sc} 蓄積の経時変化と病気の臨床経過の関連性を評価するため、3 つの C-BSE 分離株を脳内接種したホルスタイン種牛の中枢神経系における PrP^{Sc} の分布を解析した。接種 10 ヶ月後の牛では、PrP^{Sc} の沈着は脳幹部および視床で検出されたが、空胞病変は認められなかった。接種 16 および 18 ヶ月後では、わずかな空胞病変が脳幹部と視床に検出されるが、大脳皮質には見られなかった。臨床症状を示す接種 20 から 24 ヶ月後において、強い PrP^{Sc} の沈着が脳および脊髄の至る所で認められた。臨床症状が現れる平均月数は接種後 19.7 ヶ月であり、平均生存期間は接種後 22.7 ヶ月であった。これらの知見は、BSE の臨床症状が明らかになる約 10 ヶ月前に PrP^{Sc} の蓄積が検出されることを示している。

C-BSE 発症牛は音への過敏症状のような聴覚異常が見られることから、第 2 章では、著者は C-BSE 接種牛の聴性脳幹部における神経病理学的変化に焦点をあてて解析し

た。臨床症状が現れる前（接種 3、10、12 および 16 ヶ月後）では、聴性脳幹神経核における空胞変化は無いか軽度であり、PrP^{Sc} の沈着はわずかであった。同じく臨床症状を呈する前の接種後 18 および 19 ヶ月後に安楽殺した 2 頭の牛では、聴性脳幹経路において、軽度の空胞変性と中程度の PrP^{Sc} の蓄積が見られた。臨床症状を示した牛（接種 20 ヶ月以降）では、他の聴性脳幹神経核および内側膝状体に比較し、下丘核ではスポンジ状変化が顕著であった。これらの病理学的発見は、PrP^{Sc} の蓄積が付随するスポンジ状病変に特徴づけられる神経病理学的変化は、聴覚過敏と関連しているかもしれない。

牛の BSE 病原因子は英国に端を発する一つの株と考えられてきた。しかしながら、神経病理学的および分子表現型の異なる非定型 BSE が近年欧州諸国、北アメリカおよび日本で報告されている。第 3 章では、日本で確認された L 型様非定型 BSE である BSE/JP24 の特徴を明らかにするため、著者は BSE/JP24 症例の脳乳剤をホルスタイン種に接種し、病気の臨床経過に加え、生化学的および神経病理学的特徴を調査した。BSE/JP24 分離株はホルスタイン種牛に伝達した。潜伏期間、神経病理学的特徴および宿主の異常プリオンタンパク質の分子的性質に基づき、BSE/JP24 プリオンの性質は、従来型 BSE プリオンから明らかに区別でき、イタリアで見つかった牛アミロイド性海綿状脳症に酷似している。

結論として、本研究は、脳内接種した牛を用いて、臨床症状、PrP^{Sc} の蓄積および空胞病変を解析することで、C-BSE の病理発生の一部分を明らかにした。さらに、本研究は、日本の L 型様非定型 BSE である BSE/JP24 の生化学的および神経病理学的特徴を明らかにした。本研究の成果は、BSE の再発生のリスクだけでなく、国民への BSE のリスクを低減するための BSE のリスク解析とリスク管理に重要な情報を提供する。