

Original Article

Distribution of PrP^{Sc} in Cattle with Bovine Spongiform Encephalopathy Slaughtered at Abattoirs in Japan

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SUMMARY: Three 80- to 95-month-old Holstein dairy cattle infected naturally with the agent of bovine spongiform encephalopathy (BSE) and slaughtered at abattoirs in Japan were examined for the distribution of disease-specific and protease-resistant prion protein (PrP^{Sc}) by immunohistochemistry (IHC) and Western blot (WB) analyses. The cattle showed no clinical signs or symptoms relevant to BSE but were screened as positive by enzyme-linked immunosorbent assay, a rapid test for BSE. This positive result was confirmed by IHC or WB in a specimen of the medulla oblongata. Histopathologically, these cattle showed no vacuolation in tissue sections from the central nervous system except for the medulla oblongata. Both IHC and WB analyses revealed PrP^{Sc} accumulation in the brain, spinal cord, satellite and ganglionic cells of the dorsal root ganglia, and the myenteric plexus of the distal ileum. In addition, small amounts of PrP^{Sc} were detected in the peripheral nerves of 2 cattle by WB. No PrP^{Sc} was demonstrated by either method in the Peyer's patches of the distal ileum; lymphoid tissues including the palatine tonsils, lymph nodes, and spleen; or other tissues. The distribution of PrP^{Sc} accumulation in the preclinical stage was different between naturally infected cattle and cattle inoculated experimentally with the BSE agent.

INTRODUCTION

Bovine spongiform encephalopathy (BSE), one of a group of transmissible spongiform encephalopathies (TSE) caused by prion infections, was first identified in British cattle that showed unusual neurological signs in 1986 (1). Subsequent epidemiological studies provided evidence that the disease had occurred as a result of the oral exposure of cattle to a scrapie-like agent that had contaminated the meat and bone meal used in concentrated feeds (2). The consumption of cattle-derived food contaminated with BSE prion is believed to cause variant Creutzfeldt-Jakob disease (vCJD) in humans (3,4).

The first case of BSE in Japan was confirmed on September 11, 2001 (5). Since October 18, 2001, all cattle slaughtered at abattoirs for meat consumption were screened for BSE prion at local meat inspection centers by means of enzyme-linked immunosorbent assay (ELISA), a rapid test for BSE. A specimen found to be positive by ELISA would then be reexamined for confirmation by Western blot (WB) and by histological and immunohistochemical (IHC) analyses at authorized laboratories in Japan. Based on these data, the Expert Committee for BSE Diagnosis, Ministry of Health, Labour and Welfare (MHLW) of Japan would confirm BSE. Up to November 30, 2005, 15 BSE-confirmed cases from more than 5 million cattle screened over 4 years were eliminated from the food chain destined for human consumption. The 15 BSE cases found at

abattoirs were aged from 21- to 109-month-old, and consisted of 13 Holstein milk cows and 2 Holstein steers, with the latter cases two being atypical BSE occurring at 23 months of age (6) and young BSE occurring at 21 months of age. Nine of the BSE cases appeared to be healthy while the other 6 showed some clinical signs and symptoms, but not those relevant to BSE. All 15 cattle were found to be positive for disease-specific and protease-resistant prion protein (PrP^{Sc}) in the ELISA and WB analyses, but the 2 young BSE cases were found to be negative for this protein in the IHC analyses and for spongiform changes in the histological analyses. In addition, 5 BSE cases in Holstein milk cows aged from 48 to 102 months old, including the first BSE case, were found through fallen stock surveillance during the past 3 years. This surveillance program has been conducted by the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan on cattle older than 24 months under a system similar to that of the MHLW.

The findings of BSE pathology, particularly in BSE cattle at a preclinical stage in the field and that were slaughtered at abattoirs, are limited in the literature. We obtained various tissues from 3 of the BSE cases and examined the distribution of PrP^{Sc} by WB and IHC in order to characterize the cases of BSE at a preclinical stage in Japan.

MATERIALS AND METHODS

BSE specimens: From abattoirs we obtained various tissue specimens from 3 of the 15 BSE cases, not including the medulla oblongata specimens that were initially utilized for the screening at a meat inspection center and thereafter for the confirmation of BSE at our laboratories. The 3 BSE cattle

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were 80-, 83-, and 95-month-old Holstein dairy cows born between December 1995 and March 1996, and they belonged to a representative cohort of BSE-positive cattle in Japan. There were no clinical signs or symptoms relevant to BSE, such as fear or aggressive behavior, incoordination of gait, hypermetria, falling, or recumbency in any of the cattle, but they did have dysstasia, dislocation of the hip joint, arthritis and mastitis. The cattle were slaughtered in a room separated from the usual slaughter line. Tissue specimens were collected from the liver, spleen, kidney, heart, lung, tongue, stomach, duodenum, jejunum, distal ileum and ileum (taken at 2 or 6 m from the distal end), cecum, colon, rectum, retina, pancreas, adrenal glands, lymph nodes, palatine tonsil, muscle, brain, spinal cord, dorsal root ganglia, and various peripheral nerves approximately 30 cm from the dorsal root ganglia. All tissue specimens were taken within 3 days after slaughter, during which time the carcasses were kept at 4°C. The tissues, both those frozen for WB analyses and those fixed in 15-20% buffered formalin for histological and IHC analyses, were sent to authorized laboratories in the Department of Biochemistry and Cell Biology, and the Department of Pathology at the National Institute of Infectious Diseases, Japan.

Histopathology and IHC: For the histological and IHC examinations, the medulla oblongata specimens for screening and confirmatory tests were divided longitudinally into two parts, with one part either frozen or kept at 4°C, and the other fixed in buffered formalin overnight. The fixed specimens were cut into 3 mm in thickness and placed in a plastic cassette, fixed again in a fixating agent (Yufix; Sakura Finetek Japan, Tokyo, Japan) for 1 h at 60°C with continuous shaking, and then immersed in 98% formic acid to inactivate the abnormal isoform of prion protein for 60 min at room temperature with shaking. The tissue block was washed in tap water for 15 min. To embed them in paraffin, the tissue specimens were processed in an automated processor (SAKURA ETV-150CV; Sakura Finetek Japan) for 4.5 h in total. Each section was cut into 4 µm in thickness, mounted on silan-coated slides (Matsunami, Tokyo, Japan), and dried for 15 min using a dryer. The paraffin section was melted for 30 min at 60°C so it would adhere to a slide glass before being stained with hematoxylin and eosin (H.E.) for histological examination. The other sections were subjected to IHC to detect the antigen of BSE prion. Sections were deparaffinized in xylene 3 times, rehydrated through graded ethanol, then immersed in distilled water (DW) and dried completely to improve adhesion. The antigen was retrieved by hydrolytic autoclaving for 20 min at 121°C in 1 mM HCl. After they cooled down, the sections were immersed in 0.1 M PBS. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in 100% methanol for 5 min. After being washed in PBS, the sections were applied with 10% normal goat serum in PBS for 5 min at room temperature, then anti-prion protein antibody (T4 rabbit polyclonal antibody raised against bovine prion protein peptide corresponding to 221 to 239 amino acids; [7]) or 44B1 mouse monoclonal antibody (8), which recognizes discontinuous epitope located in 155 to 231 amino acids of mouse prion protein (PrP^C), was applied for 45 min at 37°C. Envision+ (Dako, Kyoto, Japan) was reacted after PBS washing for 30 min at room temperature, and labeled peroxidase activity was detected by diaminobenzidine (40 mg/0.05M Tris HCl buffer, pH 7.6) and 100 µl hydrogen peroxide. IHC was carried out with a positive control section of medulla oblongata derived from BSE-positive cattle and a negative control section derived from BSE-negative cattle in

Japan. In addition, omission of the primary antibody was also subjected to negative controls. Nuclei were counterstained with hematoxylin for a short period. The rapid confirmatory IHC described above takes at least 12 h beginning with the dissection of the specimen in our laboratory. The protocol we followed is recommended for use by the Expert Committee for BSE Diagnosis, MHLW of Japan. The other tissues obtained from the 3 BSE cases were fixed in formalin for 3 days and routinely embedded in paraffin after treatment with 98% formic acid for 1 h. H.E. stained sections were subjected to histopathological examination. Other sections were stained with Congo-red in order to detect amyloid fibrils. IHC was carried out as described above. In addition, we used antibodies to glial fibrillary acidic protein (GFAP; Dako) and neuron specific enolase (NSE; Novocastra Laboratories, Newcastle upon Tyne, UK) to characterize the cells in the tissue sections.

WB analyses: The remains of the ELISA sample and tissues dissected from appropriate parts of the medulla oblongata that were frozen or kept at 4°C were subjected to WB analyses, which were performed according to the protocol recommended by the Expert Committee for BSE Diagnosis, MHLW of Japan, as described below.

Two hundred milligrams of the tissue was homogenized for 5 min in a 2-ml plastic tube with 0.8 ml of TN buffer (50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl) and 250 mg of Zirconia beads (Nikkato, Tokyo, Japan) using a Multi-Beads Shocker (YASUI-KIKAI, Osaka, Japan) at 2,500 rpm. Zirconia beads of 1 mm diameter were used for the homogenization of brain tissue, while the other tissues were homogenized with 1.5 mm- or 2 mm-diameter beads.

Aliquots (usually 250 µl) of the homogenate (containing 50 mg tissue equivalent) were mixed with the same volume of detergent buffer (TN buffer containing 4% twittergent 3-14 and 1% salkosyl) and 25 µl of 2-butanol (5% v/v). The mixture was clarified by sonication for 1 min using a bath-type sonicator, BIORUPTOR (COSMO BIO, Tokyo, Japan). Then, 12.5 µl of a 20% solution of collagenase (Wako Chemical, Tokyo, Japan) was added and incubated for 30 min at 37°C. Subsequently, 20 µl of 1% solution of proteinase K (PK; Roche Diagnostics, Tokyo, Japan) was added and incubated for a further 30 min. After PK was inhibited by 10 µl of 1 M Pefa-block SC (Roche Diagnostics), 2 µl of DNase I (1% solution) was added and incubated for a further 5 min at room temperature. PrP^{Sc} was then precipitated by the addition of 250 µl of 2-butanol-methanol mixture (5:1, v/v) and collected by centrifugation at 15,000 rpm for 10 min at room temperature. The precipitate thus obtained was dissolved in 50 or 100 µl of SDS-sample buffer and heated for 5 min at 100°C before electrophoresis. Insoluble materials were removed by centrifugation at 5,000 rpm for 1 min.

PrP^{Sc} was partially purified using a phosphotungstic acid (PTA) precipitation method (9) when needed for further testing. Briefly, the precipitate obtained by the 2-butanol-methanol mixture detailed above was resuspended in 250 µl of PBS (pH 7.4) containing 2% sarcosyl, and insoluble material was removed by centrifugation at 5,000 rpm for 2 min. To the supernatant, PTA stock solution (4% w/v sodium PTA dissolved in 170 mM MgCl₂ and adjusted to pH 7.4 with NaOH) was added to give a final concentration of 0.3% (w/v) PTA, and was agitated for 30 min at 37°C with mild shaking. The precipitate was collected by centrifugation at 15,000 rpm for 15 min at room temperature and dissolved in 50 µl of SDS-sample buffer. This method was successfully applied

to tissue samples such as those from the spleen, lymph nodes and nerve fibers, since they contained large amounts of insoluble materials even after PK digestion.

Usually, a 10-20 μ l sample (containing 10 mg tissue equivalent) was applied to 12% NuPAGE polyacrylamide gel (Invitrogen, Tokyo, Japan) and electrophoresed for 60 min at 200 V using NuPAGE MOPS running buffer (Invitrogen). Proteins were transferred onto PVDF membrane (Invitrogen) for 45 min at a constant voltage of 25 V using NuPAGE blotting buffer containing 0.1% SDS and 10% methanol. The blot was then soaked for 30 min into 50 mM Tris-HCl buffer, pH 7.5, containing 10% defatted milk and 5% fetal calf serum. Subsequently, the blot was incubated for 45 min with anti-prion protein monoclonal antibody 44B1 (0.2 μ g/ml) in 50 mM Tris-HCl buffer, pH 7.5, containing 2% defatted milk and 1% fetal calf serum. After washing 4 times with PBS containing 0.1% Tween 20 for 5 min, the blot was incubated with 1:2,500 dilution of horse radish peroxidase (HRP)-conjugated sheep antibody (Fab')₂ against mouse IgG (NA 9310; Amersham Biosciences, Piscataway, N. J., USA) for 40 min. After washing the membrane with PBS-Tween as described, blots were developed with an ECL detection kit (Amersham Biosciences), and immunoreactive proteins were recorded on X-ray film.

RESULTS

Clinical signs and symptoms: There were no signs or symptoms relevant to BSE. Clinical findings and some laboratory data related to BSE from these cattle are summarized in Table 1.

Histopathology: Histopathological examination of the organs obtained revealed that there were some mature cysts of sarcocysts in the hearts of all of the cattle, and also in the tongues of Case 1 and 3. Case 1 and 2 were found to have slight lymphocytic infiltration in the hepatic triad. Case 3 showed an accumulation of lymphocytes in the interstitium of the kidney. No histopathological lesions were found in the other organs.

Autolysis was found in the brain, except for the medulla oblongata, of Case 1, and frontal, parietal, and temporal cerebral cortex was also lost by severe autolysis. Case 1 was able to have its thalamus, hippocampus, occipital lobe of the cerebrum, midbrain, pons, medulla oblongata and cerebellum examined because formalin fixative was poured into the stunning hole and foramen magnum of the skull at the slaughterhouse. No vacuolation (a mild form of spongiform changes) or histological lesions were detected in the central nervous system (CNS) tissues of Case 1. But mild vacuolar changes were observed in the medulla oblongata of Case 2 and 3. This vacuolation was principally observed in the neuropil,

Table 1. Findings in the medulla oblongata obtained by ELISA, WB, IHC and from histopathology in 3 BSE-confirmed cattle

Case No.	Age ¹⁾ /Sex	Birth date	Breed	Clinical symptoms	ELISA (Plateria kit)	WB	IHC	Spongiform change
1	80F	December 5, 1995	Holstein	dysstasia, dislocation of the hip joint, arthritis, mastitis, heat stroke	>3.5 ²⁾	positive ³⁾	positive	negative
2	83F	February 10, 1996	Holstein	dysstasia	>3.5	positive	positive	positive
3	95F	March 17, 1996	Holstein	dysstasia, dislocation of the hip joint	>3.5	positive	positive	positive

¹⁾: Age shown as months-old.

²⁾: Scale over.

³⁾: Only the specimen at the level of the obex showed positive for PrP^{Sc}, but all specimens taken from the medulla oblongata in another cattle showed positive for PrP^{Sc}.

Table 2. Distribution of PrP^{Sc} in tissue specimens examined by IHC and WB

Tissues	Case 1		Case 2		Case 3	
	WB	IHC	WB	IHC	WB	IHC
Cerebrum						
Frontal lobe	ND	ND	+	+	+	+
Caudate nucleus	ND	ND	ND	+	ND	-
Thalamus	ND	+	+	+	+	+
Corpus striatum	ND	ND	ND	+	ND	+
Hippocampus	ND	-	ND	-	ND	+
Occipital lobe	ND	-	+	-	-	-
Cerebellum	+	+	+	+	+	+
Medulla oblongata	+	+	+	+	+	+
Spinal cord						
Cervical	+	+	+	+	+	+
Thoracic	+	+	+	+	+	+
Lumbar	+	+	+	+	+	+
Dorsal root ganglia	+	+	ND	ND	+	+
Peripheral nerves	+	-	ND	ND	+	-
Distal ileum	+	+	+	-	-	-

ND: not done.

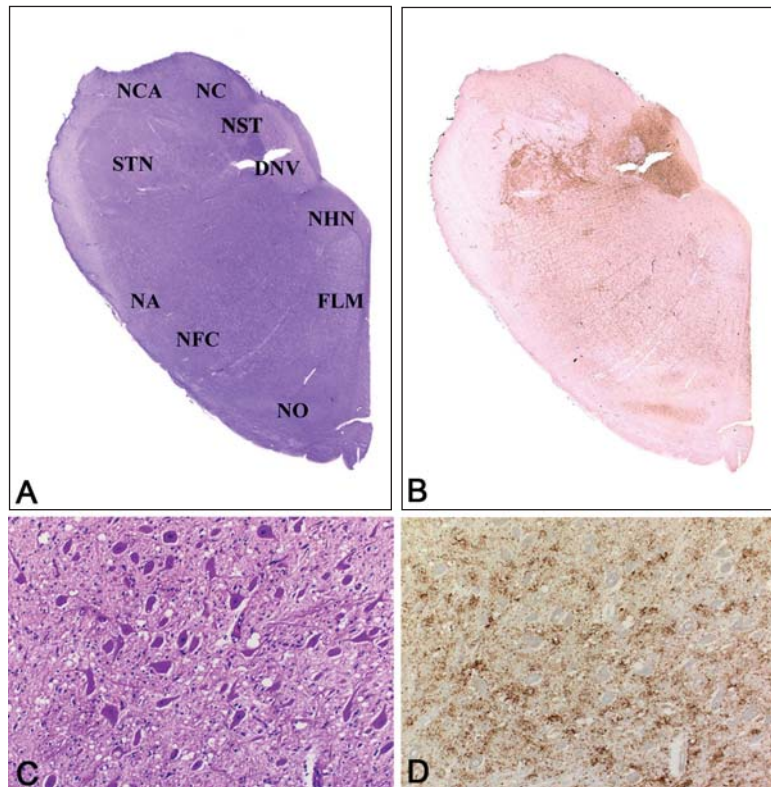


Fig. 1. The section of medulla oblongata at the level of the obex from Case 2 (A-D). (A) left side of medulla oblongata. H.E. DNV, dorsal nucleus of the vagus; NST, nucleus of the solitary tract; STN, spinal trigeminal nucleus; NHN, nucleus of the hypoglossal nerve; NO, nucleus olivaris; NC, nucleus cuneatus; NCA, nucleus cuneatus accessories; NA, nucleus ambiguus; NFC, nucleus fasciculi gracilis; FLM, fasciculus longitudinalis medialis. (B) PrP^{Sc} immunolabelling with T4 rabbit antibody in the same section as in (A). (C) Spongiform change is found in the neuropils and neurons of DNV. H.E. (D) PrP^{Sc} immunolabelling with T4 rabbit antibody showing fine punctuate accumulation of PrP^{Sc} in neuropils of the DNV. Original magnification, (A) and (B) $\times 1$, (C) and (D) $\times 10$.

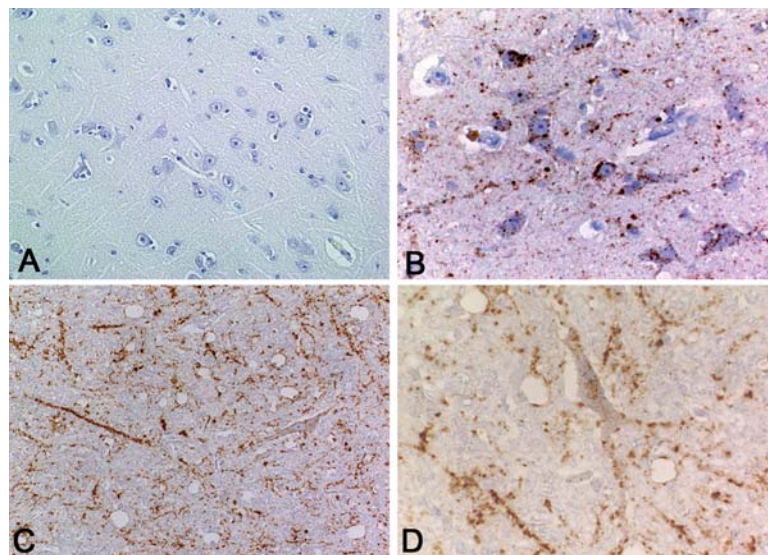


Fig. 2. The detection of PrP^{Sc} in the cerebral cortex from a normal cow used as negative control (A) and Case 1 (B) and in the thalamus from Case 2 (C, D) by immunohistochemistry with T4 antibody. Original magnification, (A), (B) and (D) $\times 40$, (C) $\times 10$.

but was rare in the neurons (Fig. 1). Neuronophagia, neuron loss and gliosis was not observed in the brains of the cattle, as confirmed by IHC for GFAP and NSE. There were no lesions in the spinal cord, and no amyloid plaques were observed in the CNS tissue of all three cases.

IHC: IHC identified PrP^{Sc} in CNS regions of all cases

(Table 2); and PrP^{Sc} was widely distributed in the brain from the medulla oblongata to the frontal cortex, cerebellum, and throughout the spinal cord. In particular, intense PrP^{Sc} depositions were detected in the thalamus (Fig. 2C and 2D), dorsal nucleus of the vagus (DNV), nucleus of the solitary tract (NST), spinal trigeminal nucleus (STN), nucleus of the

hypoglossal nerve (NHN) and nucleus olivaris (NO) in the medulla oblongata at the level of the obex (Fig. 1), cerebral cortex (Fig. 2B) and nuclei of the cerebellum, and in the ventral and dorsal horns of spinal cords in all cattle. In Case 1, in the medulla oblongata at the level of the obex, PrP^{Sc} deposition was clearly detected, but the other part of the medulla oblongata appeared to be negative for PrP^{Sc}, whereas PrP^{Sc} was detected throughout the medulla oblongata in Case 2 and 3. Topical accumulation of PrP^{Sc} in the obex region of Case 1 was also confirmed by WB (Fig. 3). Sparse PrP^{Sc} deposition in the hippocampus was only found in Case 3. Regional distribution of PrP^{Sc} in the cerebrum was represented in the brain maps of Case 2 and 3 (Fig. 4). In addition, PrP^{Sc} was found in some satellite and ganglionic cells of the dorsal root ganglia obtained from Case 1 and 3 (Fig. 5B), but there was no apparent histopathological lesion (Fig. 5A). In the organs except for the CNS, an accumulation of PrP^{Sc} was seen in the myenteric plexus (Auerbach's plexus) at the distal ileum of Case 1 (Fig. 6), but not in the ileum of the part 2 m and 6 m

from the distal end. No PrP^{Sc} deposition was detected in other tissues including the Peyer's patches, lymphoid tissues, spleen and lymph nodes.

WB analyses: The results of WB analyses in the brain are shown in Fig. 7A. Beside the medulla oblongata at the level of the obex, PrP^{Sc} was seen as being heavily accumulated in the midbrain, cerebellum and thalamus. PrP^{Sc} was also detected in the cerebrum, but the amount was estimated to be 1/20-1/100 of that accumulated in the medulla oblongata at the level of the obex of Case 2. PrP^{Sc} was evenly distributed in the cervical, thoracic and lumbar segments of the spinal cord (Fig. 7B), but was not observed in the cauda equine. PrP^{Sc} was detected in most of the dorsal root ganglia of Case 3 (Fig. 8).

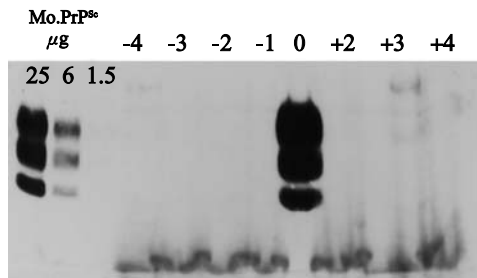


Fig. 3. Topical accumulation of PrP^{Sc} at the level of the obex of the medulla oblongata of Case 1. The tissue specimen of medulla oblongata in Case 1 was dissected to 5 mm in thickness. '0' corresponds to the obex region and the specimens are numbered in the brain direction as + and to the spinal cord as -. 600 µg and 20 mg tissue equivalent was loaded at 0 and the other lane, respectively. Different amounts of mouse PrP^{Sc} were also electrophoresed on the same membrane as a positive control.

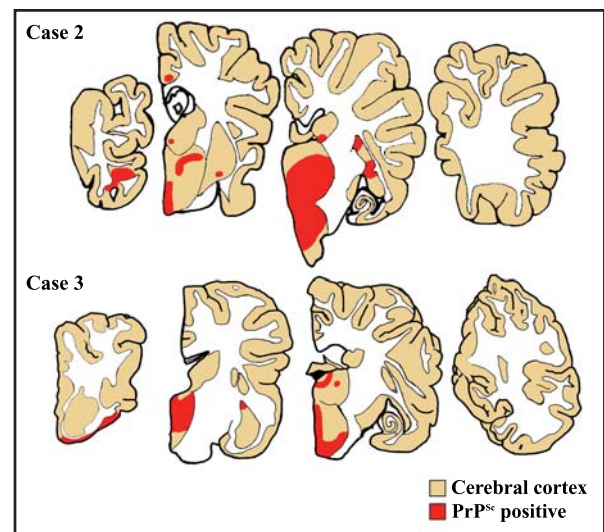


Fig. 4. The distribution of PrP^{Sc} detected by immunohistochemistry (drawn in red) in the cerebrum of Case 2 (top) and 3 (bottom). The schema represents 4 coronal sections at the levels of the frontal lobe, the striatum, thalamus and hippocampus, and the occipital lobe of the cerebrum (from left to right).

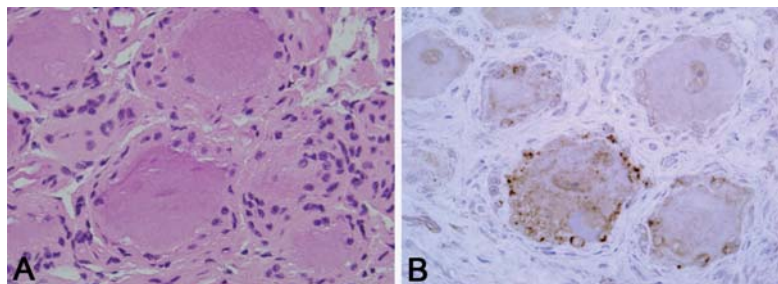


Fig. 5. Histopathological findings (H.E.). (A) and immunohistochemistry for PrP^{Sc} (B) on the dorsal root ganglion from Case 3 reveals punctuate immunolabelling on ganglionic and satellite cells. Original magnification, (A) and (B) ×40.

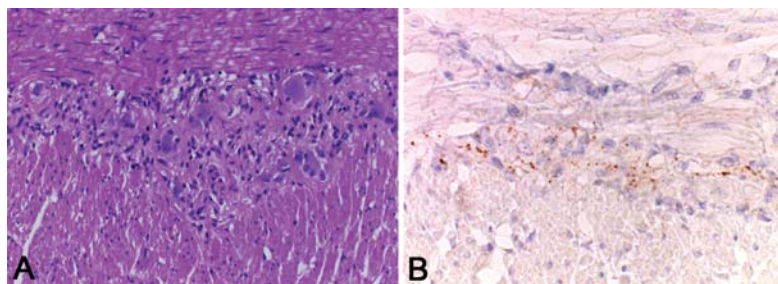


Fig. 6. Histopathological findings (H.E.). (A) and immunohistochemistry for PrP^{Sc} (B) on the distal ileum from Case 1. PrP^{Sc} is detected in the ganglionic cells of the myenteric plexus (Auerbach's plexus). Original magnification, (A) ×20, (B) ×40.

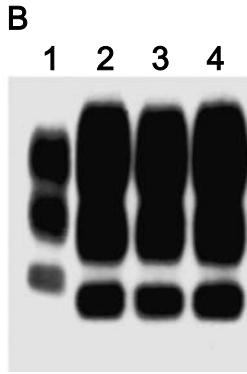
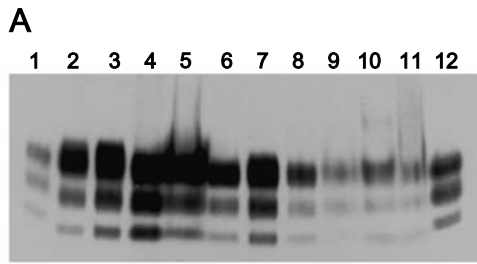


Fig. 7. Distribution of PrP^{Sc} in the CNS of Case 2. (A) Distribution of PrP^{Sc} in the brain of Case 2. Lane 1 and 12, mouse scrapie-infected brain (0.4, 1.5 μ g tissue equivalent [eq.], positive control); lane 2, mid brain (200 μ g tissue eq.); lane 3, dentate nucleus of cerebellum (100 μ g eq.); lane 4, cerebellar cortex (10 mg eq.); lane 5, occipital lobe (10 mg eq.); lane 6, temporal lobe (10 mg eq.); lane 7, thalamus (100 μ g eq.); lane 8, parietal lobe (10 mg eq.); lane 9, corpus striata (10 mg eq.); lane 10, lower part of frontal lobe (10 mg eq.); lane 11, upper part of frontal lobe (10 mg eq.). (B) Distribution of PrP^{Sc} in the spinal cord of Case 2. Lane 1, mouse scrapie-infected brain (0.8 μ g tissue eq.); lane 2, cervical spinal cord (100 μ g eq.); lane 3, thoracic spinal cord (100 μ g eq.); lane 4, lumbar spinal cord (100 μ g eq.).

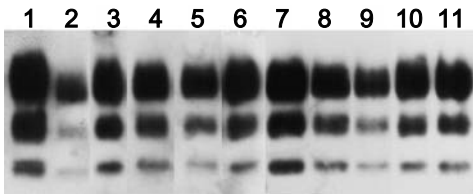


Fig. 8. Accumulation of PrP^{Sc} in dorsal root ganglia (DRG) of Case 3. Lane 1, spinal cord (100 μ g tissue equivalent [eq.], positive control); lane 2-11 DRG (1 mg tissue eq.), lane 2, DRG (C2 at the level of spinal cord); lane 3, DRG (C5); lane 4, DRG (C6); lane 5, DRG (Th 1); lane 6, DRG (Th 6); lane 7, DRG (Th 11); lane 8, DRG (L1); lane 9, DRG (L3); lane 10, DRG (L4); lane 11, DRG (L5).

The amounts of PrP^{Sc} were estimated to be 1/10 of that accumulated in the spinal cord. In these cattle, a very small amount of PrP^{Sc} also accumulated in the femoral and lumbar nerves obtained approximately 30 cm from the dorsal root ganglia. The amount of PrP^{Sc} in these peripheral nerves was estimated to be 1/1,000-1/4,000 of that in the spinal cord (Fig. 9). In this connection, PrP^{Sc} was also scarcely detected in the cranial gluteal nerve of Case 1, but was not detected by IHC, probably due to its low sensitivity. No PrP^{Sc} was detected in homogenates prepared from the palatine tonsils, lymph nodes or spleens of all cattle (Fig. 10). We detected PrP^{Sc} in homogenates prepared from the Peyer's patches taken from the distal ileums of Case 1 and 3. However, IHC studies clearly demonstrated that the deposition of PrP^{Sc} detected in the homogenate by WB was located at the myenteric plexus (Auerbach's plexus), not in the lymphoid cells of the Peyer's

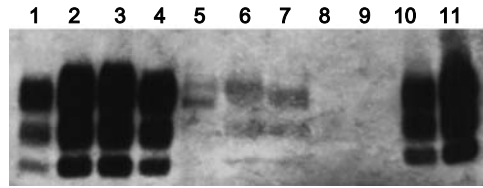


Fig. 9. Accumulation of PrP^{Sc} in the spinal cord (lane 2-4) and peripheral nerves (lane 5-9) of Case 3. Lane 1, positive control of PrP^{BSE} (8 μ g tissue equivalent, thalamus of Case 2); lane 2, cervical spinal cord; lane 3, thoracic spinal cord; lane 4, lumbar spinal cord; lane 5, cervical nerve; lane 6, femoral nerve; lane 7, lumbar nerve; lane 8, tibial nerve; lane 9, sciatic nerve; lane 10-11, mouse scrapie-infected brain (0.4 and 1.5 μ g tissue equivalent). Lane 2-9, 100 μ g tissue equivalent; lane 5-9, 25 mg tissue equivalent.

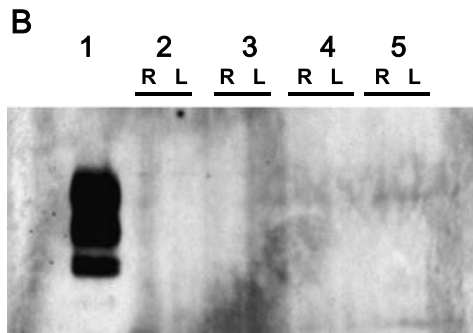
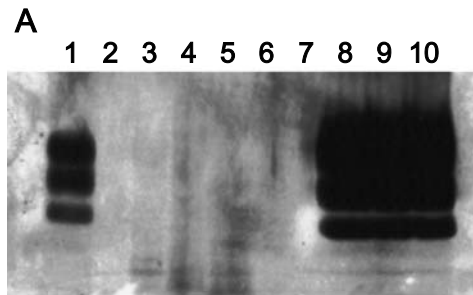


Fig. 10. Detection of PrP^{Sc} in lymphoid tissues (A) and lymph nodes (B) in Case 1. (A) Lane 1, mouse scrapie-infected prion (1.5 μ g equivalent brain tissue) as a positive control; lane 2 and 3, tonsil; lane 4 and 5, spleen; lane 6, ileum 6 m apart from the ileal end; lane 7, ileum 2 m apart from the ileal end; lane 8-10, distal ileum within 2 m from ileal end. (B) Lane 1, mouse scrapie-infected prion (1.5 μ g equivalent brain tissue) as a positive control; lane 2, superficial cervical lymph nodes; lane 3, subiliac lymph nodes; lane 4, axillar lymph nodes; lane 5, inguinal lymph nodes; R, right side; L, left side. 10 μ g tissue equivalent in 10 μ l sample is loaded in each lane.

patches.

DISCUSSION

Three BSE cattle at the preclinical stage that were slaughtered in Japan were examined histopathologically and for PrP^{Sc} distribution by IHC and WB assays. In two of the cattle, mild vacuolations (spongiform changes) in neuropils and neurons were limited in the medulla oblongata around the level of the obex. PrP^{Sc} was detected by IHC and WB in the brain, for example in the thalamus, frontal cortex, cerebellum, medulla oblongata and throughout the spinal cord, dorsal root ganglia, and in the ganglion cells of the myenteric plexus of the distal ileum. WB analyses revealed that PrP^{Sc} was also detectable in the occipital cortex of the brain and in peripheral nerves. Both IHC and WB analyses showed no PrP^{Sc} accumulation

in the ileum from the distal end, in the duodenum, in the jejunum or in other organs and tissues, including the lymphoid system such as the palatine tonsils and spleen.

Vidal et al. have described the PrP^{Sc} distribution in brains from naturally occurring BSE cases using WB and ELISA (10). The highest levels of PrP^{Sc} were detected in the medulla oblongata, spinal cord, pons, and brain including the cerebellar hemisphere. Olfactory bulbs and the temporal and parietal cortices were the areas with a medium to low level of PrP^{Sc} deposition. On the other hand, in recently described atypical BSE cases in the field, the pattern of PrP^{Sc} distribution was markedly different within the brain and was characterized by the presence of PrP^{Sc}-amyloid plaques deposition (11). PrP^{Sc} accumulation in atypical BSE cases was abundant in the olfactory bulbs, thalamus and hippocampus, and a lower level of PrP^{Sc} was detected in the medulla oblongata. In the present study, no PrP^{Sc}-amyloid plaques were detected in the brains of any of the cattle, and a similar PrP^{Sc} distribution pattern was observed in each brain. Abundant PrP^{Sc} deposition was found in the medulla oblongata at the level of the obex, thalamus, cerebellum and spinal cord, whereas the frontal cortex and temporal cortex were areas with weak PrP^{Sc} deposition. Although we could not examine PrP^{Sc} localization in the olfactory bulb in this study, the results of IHC and WB showed that our BSE cases with preclinical stage had a PrP^{Sc} distribution that was somewhat similar to those in reported cases with naturally occurring BSE, not those seen in atypical BSE cases (10,12). In addition, we identified PrP^{Sc} depositions in the myenteric plexus of the distal ileum, but not in the Peyer's patches. These findings in the distal ileum are consistent with those in BSE cattle with a naturally occurring clinical stage, but not in cattle experimentally induced by oral exposure to a BSE agent. These latter cattle show PrP^{Sc} localization in a small proportion of the follicles of the Peyer's patches, mainly in macrophages, but not in the myenteric plexus (13).

In cattle experimentally exposed to the BSE agent via an oral route, a low level of infectivity has been obtained by mouse bioassay in CNS tissues, peripheral ganglia (14), sternal bone marrow (15), distal ileum (13,16) and tonsil (17). In tissues from cattle orally exposed to the BSE agent, infectivity has also been assayed by intracerebral inoculation of the cattle, with the results showing infectivity in the caudal medulla/spinal cord, distal ileum, and tonsils (18). By contrast, the organs, except for the CNS of naturally occurring cases of BSE, have shown no evidence of infectivity by mouse bioassay (19).

In this study, PrP^{Sc} deposition was also observed in the dorsal root ganglia and peripheral nerves in 2 BSE cases. To the best of our knowledge, no IHC for PrP^{Sc} deposition in the dorsal root ganglia of cattle has been reported, but such findings have been reported for sheep (20). Therefore, the results of our IHC and WB analyses indicated new findings regarding the presence of PrP^{Sc} accumulation. In the peripheral nervous system, samples of several nerves obtained 30 cm from the ganglia showed a small amount of PrP^{Sc} according to WB analyses, but not according to IHC. Recently, PrP^{Sc} deposition has also been reported in the peripheral nerves of cattle investigated as part of a fallen stock surveillance program in Japan (21), as well as in sheep (22) and monkey (23) experiments, and in human CJD cases (24-26). Weak infectivity was demonstrated in the sciatic nerve of cattle with naturally occurring BSE at the clinical phase by transmission experiments to highly BSE-sensitive transgenic mice expressing bovine PrP^C (27). Thus, the distribution of infectivity in

naturally occurring BSE cases is mostly compatible with that of PrP^{Sc} deposition as determined by WB analyses. These findings suggest that the risk for exposure to humans could remain even after the complete removal of specified risk materials, although very small amounts of PrP^{Sc} have been detected in peripheral nerves, despite the fact that there may be a variety of barriers to human infection.

On the other hand, both IHC and WB in the present study detected no PrP^{Sc} accumulation in the tissues of the lymphoid system; these results were consistent with reports of natural BSE cases (19). Tonsils have been designated as specified risk materials, but no evidence of PrP^{Sc} deposition was obtained from our studies of 3 BSE cases in Japan. In contrast to other TSE, cattle naturally infected with the BSE agent may show no PrP^{Sc} deposition in the tonsil and lymphoid system (13), although a trace of infectivity was found in the palatine tonsils of cattle after experimental exposure (18), and weak infectivity was shown in the Peyer's patches of naturally occurring cattle (27). Therefore more evidence of PrP^{Sc} distribution in naturally occurring BSE cases is needed to clarify the pathogenesis of BSE cattle in the field.

In this study, PrP^{Sc} was detected by IHC and WB in all parts of the medulla oblongata in 2 cases, but was detected only in the obex region in another case of BSE, suggesting that the obex is confirmed to be an appropriate area for the examination of the postmortem brain. Thus, detailed sampling at the obex region should be required for a reliable diagnosis of BSE. The accumulation of PrP^{Sc} in the occipital cortex of the brain, gut, and peripheral nerves could not be detected by IHC in this study, but it could be detected by WB analysis in some cases, including young BSE cases (21-23 months old), in Japan. It was clearly shown that WB analysis is a more sensitive method than IHC; therefore, WB should be used in combination with IHC, particularly in examinations of naturally occurring BSE cattle in the field.

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