Neuroanatomical Distribution of Disease-Associated Prion Protein in Cases of Bovine Spongiform Encephalopathy Detected by Fallen Stock Surveillance in Japan

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ABSTRACT. Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disorder of cattle characterized by accumulation of the disease-associated prion protein (PrPSc) in the central nervous system (CNS). The immunohistochemical patterns and distribution of PrPSc were investigated in the CNS, brains, and spinal cords of 7 naturally occurring BSE cases confirmed by the fallen stock surveillance program in Japan. No animals showed characteristic clinical signs of the disease. Coronal slices of 14 different brain areas in each case were immunohistochemically analyzed using an anti-prion protein antibody. Immunolabeled PrPSc deposition was widely observed throughout each brain and spinal cord. Intense PrPSc deposition was greater in the thalamus, brainstem, and spinal cord of the gray matter than in the neocortices. The topographical distribution pattern and severity of PrPSc accumulation were mapped and plotted as immunohistochemical profiles of the different brain areas along the caudal-rostral axis of the brain. The distribution pattern and severity of the immunolabeled PrPSc in the CNS were almost the same among the 7 cases analyzed, suggesting that the naturally occurring cases in this study were at the preclinical stage of the disease. Immunohistochemical mapping of the PrPSc deposits will be used to clarify the different stages of BSE in cattle.

KEY WORDS: BSE, cattle, immunohistochemistry, prion.
2000 (5 cases) before the official Japanese ban of the import of beef and processed beef products from foreign countries in January 2001 and the use in feeding of ruminant meal and bone meal in September 2001. Two of seven cases (BSE/JP17 and 26) had standing difficulty but none of the animals showed typical clinical signs or symptoms of BSE, including aggressive behavior, gait abnormality, or ataxia. All cases were screened using BioRad Platelia ELISA (Hercules, CA, U.S.A.) and confirmed as C-BSE by western blotting and immunohistochemistry for the detection of PrPSc in the obex-level medulla oblongata. After the confirmation of BSE at our laboratory from the fallen stock surveillance program in Japan, the carcasses were stored in the refrigerator at 4°C (BSE/JP21, 22, 26, 27, and 35) or freezer at −20°C (BSE/JP17 and 29) in the respective fallen stock disposal sites. The brains and spinal cords of the 7 BSE-affected cattle were removed 4–10 days after death.

**Tissue samples and immunohistochemistry:** All removed brains and cerebella were cut sagittally at the midline. The left cerebral hemisphere, cerebellum, and spinal cord at the level of cervical (C8) and lumen (L6) enlargement including frozen brain and spinal cord sections were fixed in 10% buffered formalin (pH 7.4) for 5–10 days at room temperature. Coronal brain slices were cut to 3–4 mm thickness, placed in plastic cassettes, and immersed in 98% formic acid for 5 min. The sections were soaked in 1% sodium disulfite for 2 min and then briefly washed in distilled water 3 times. The sections were soaked in 0.5% potassium permanganate (pH 7.0) for 10 min, and then immersed in 98% formic acid for 5 min, treated in 0.5% dichroism.

**Congo red** [24] and examined under a polarizing microscope to confirm amyloid deposition using its characteristic dichroism.

Pretreatment for PrPSc antigen retrieval was conducted using the recently developed chemical method at RT as described elsewhere [1]. Briefly, dewaxed sections were immersed in 98% formic acid for 5 min, treated in 0.5% potassium permanganate (pH 7.0) for 10 min, and then washed in distilled water 3 times. The sections were soaked in 1% sodium sulfite 2 min and then briefly washed in distilled water. They were then immersed in a solution consisting of 0.1% N-lauroylsarcosine, 0.1% sodium hydroxide, and 2% sodium chloride for 10 min. After washing in tap water for 5 min, the sections were placed on an automated immunohistochemical system (DakoCytomation Autostainer Universal Staining System, Carpinteria, CA, U.S.A.) with normal goat serum for 10 min, anti-PrP primary monoclonal antibody (mAb) for 60 min, anti-mouse universal immunoperoxidase polymer (Nichirei Histofine Simple Stain MAX-PO (M); Nichirei, Tokyo, Japan) as secondary antibody for 30 min, and 3 3’-diaminobenzidine tetra chloride as the chromogen for 7 min. The primary mAb used in this study was T1 raised against mouse PrP amino acid residues 121–231 that recognize mouse PrP amino acid residues 137–143 (1 μg/ml) [25]. Finally, sections were lightly counterstained with hematoxylin.

**Immunohistochemical PrPSc profiling and mapping:** For the immunohistochemical profiling, sections were prepared from 14 different areas: the frontal lobe, the temporal lobe, the parietal lobe, the occipital lobe, the corpus striatum, the thalamus/hypothalamus, the hippocampus, the midbrain, the pons, the obex-level medulla oblongata, the cerebellar cortex at the vermis, the cerebellar medulla at the middle peduncle, and the spinal cord at the cervical (C8) and lumen enlargement (L6). The intensity and extent of each morphologic PrPSc type was subjectively scored from 0 to 4 (0, negative; 1, apparent at high magnification; 2, apparent at moderate magnification; 3, apparent at low magnification and moderate amounts of accumulation; 4, large amounts of accumulation). The average value was calculated as the mean PrPSc accumulation severity score plotted from each animal against the different anatomical brain areas along the caudal-rostral axis of the brain and is shown in the the brain PrPSc immunohistochemical profile [29] and topographically mapped on the coronal slices at the level of the frontal lobe, corpus striatum, thalamus, temporal lobe, parietal lobe, occipital lobe, midbrain, pons, medulla oblongata, cerebellum, and C8 and L6 spinal cord enlargements.

**RESULTS**

**Histopathology:** In most cases, the brain samples were not suitable for analysis of vacuolation distribution and severity, namely the lesion profile [26], because of prominent damage by autolysis and/or freezing. Various degrees of autolysis and artificial histopathological changes were present in all tested HE-stained sections. Histologic damage, such as scarious margins and cleft patterns, was also obvious in frozen brain samples from the BSE/JP17 and BSE/JP29 cases. Therefore, vacuolar lesion profiling was not performed in this study. However, the samples were free of any material and show no accompanying cellular reactions.

**Types and topographical distribution of PrPSc:** A total of 35 transverse medulla oblongata sections from 6 cases (BSE/JP17, 21, 22, 27, 29, and 35) and the cervical spinal cord from 1 case (BSE/JP26) initially utilized to confirm the BSE were intensely positive to mAb T1 with the chemical pretreatment only. Despite the presence of various degrees of autolysis and artificial clefts due to freezing, the configuration and topographical distribution of the immunolabeled PrPSc were well preserved in all brains tested here without nonspecific background staining seen in any of the sections.

Eight different PrPSc deposit types, including fine or coarse particulate, perineuronal, intraneuronal, intraglia, linear, stellate, and coalescing depositions, were identified throughout each cerebral cortex, brainstem, cerebellum, and spinal cord (Fig. 1). No ependymal, subependymal, subpial, perivascular, or vascular PrPSc plaque types that presented in sheep scrapie [9, 10] were detected in any of the brain sam-

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**H. OKADA ET AL.**
The immunohistochemical profiles (Fig. 2) and topographical distributions of PrPSc (Fig. 3) were very similar among the cases. Topographical descriptions of the immunolabeled PrPSc are summarized as follows.

**Cerebral cortex:** The severity of PrP Sc accumulation in the cerebral cortex was generally lower than that in the other brain areas, particularly in the temporal and piriform cortices. Cerebral cortices in gray matter neuropils were characterized by the presence of stellate PrP Sc deposition, which confers a star-like appearance associated with slight to moderate accumulation of the fine particulate PrPSc. Intraneuronal and linear PrPSc deposition was also identifiable in these areas.

**Corpus striatum:** Fine or coarse particulate, intraneuronal, linear, and stellate PrPSc accumulation was conspicuous in the putamen and accumbens. Accumulation of the same PrPSc types was moderate in the caudate nucleus. No PrPSc deposition was detected in the internal capsule.

**Thalamus/hypothalamus:** A large amount of fine and coarse particulate, intraneuronal, linear, and stellate PrPSc accumulation was prominent in the putamen and accumbens. Accumulation of the same PrPSc types was moderate in the caudate nucleus. No PrPSc deposition was detected in the internal capsule.

**Hippocampus:** PrPSc deposition was moderate at this site. Fine and coarse particulate PrPSc deposits were moderate in the molecular, pyramidal cell, and oriens layers and less in the granular layer. Intraneuronal deposits were detected mainly in the pyramidal cells. Stellate and linear PrPSc deposits were often present in the oriens and molecular layers.

**Midbrain:** Large amounts of intense fine or coarse particulate, linear, and coalescing PrPSc deposits were conspicuous in the neuropils of this site. Overall intraneuronal PrPSc deposition was prominent in most nuclei, i.e., the trochlear nucleus, the mesencephalic nucleus of the trigeminal nerve, the oculomotor nucleus, and the interpeduncular nucleus. No PrPSc deposits were present in the cerebral peduncle.

**Pons:** Intraneuronal PrPSc deposition was predominant in all neuronal nuclei in this area. The intraglial PrPSc aggregates and distinctive extracellular PrPSc accumulation in the form of particulate and coalescing types were also prominent.

**Medulla oblongata at the obex:** In the obex-level medulla oblongata, PrPSc aggregates were more common in the DMVN, NST, STN, ON, and the reticular formation than in
other areas. Although immunolabeling patterns of this area were almost same as those of the pons, intraneuronal PrPSc deposition was less involved in the neuronal cytoplasm of the DMVN.

**Cerebellum:** Significant amounts of PrPSc deposition were detected in the medulla at the middle peduncle consisting of the nucleus interpositus, fastigial nucleus, and dentate nucleus, whereas other medullary and cortical areas were less involved. At this site, fine and coarse particulate, linear and intraneuronal PrPSc deposits were obvious. The cortical molecular layer was characterized by the presence of moderate or high stellate PrPSc deposits.

**Spinal cords:** Various PrPSc deposition types, but not stellate or glial, were common in the gray matter of the medulla and the dorsal horns of the spinal cord. Intraneuronal PrPSc accumulation was conspicuous in most neurons. Severe focal PrPSc accumulation characterized by the presence of linear and particulate deposits was particularly present in the bilateral and symmetrical ventral horns.

**DISCUSSION**

Levels and regional tropism of vacuolar changes, namely the lesion profile in the brain, is considered a reliable indicator for histopathological diagnosis of BSE-affected cattle [26, 32]. However, optimal preservation is required to ensure the identification of vacuolar changes in certain areas of the brainstem devoid of artifactual vacuolations induced by a postmortem delay of tissue sampling that led to autolysis. Although autolysis conditions have been usually encountered in field cases of BSE diagnosis, autolysis and the frozen condition do not significantly influence PrPSc detection by immunohistochemistry [4, 6, 7, 18]. Moreover, PrPSc immunohistochemistry is an essential tool and may contribute to our understanding of the pathogenesis of BSE [6, 12].

All cattle in the present study were field BSE cases confirmed by the fallen stock surveillance program in Japan. Despite the limited number of animals and differences in their ages and breeds, PrPSc topographical distributions and types in the brain were remarkably similar among these cases and to those in published reports in BSE cattle [5, 13, 15, 16, 20, 27, 30]. The severity of PrPSc deposition was variable in the brain regions. Large amounts of PrPSc deposits were present in the brainstem and thalamus, whereas the cerebral cortices and hippocampus were less involved, suggesting that PrPSc accumulation in the brain of BSE-affected cattle is related to area-dependent tropism [5, 30]. These results indicate that most BSE cases that occur in Japan have been caused by a single infectious agent strain identical to the C-BSE prion. Moreover, the lack of apparent differences in PrPSc distribution patterns or deposition intensity among the 7 cases in this study might suggest the same clinical stage of the disease. A greater degree of PrPSc accumulation within the thalamus and brainstem of all animals was obvious compared to the slaughtered cases in Japan that seemed to be at the preclinical stage of the disease [13]. The present cases revealed no clinical signs or symptoms relevant to BSE, but some cases exhibited dystasia. These results might indicate that the 2 BSE cases with the locomotive disability were highly likely to be at an early clinical stage of the disease.
Recently, other phenotypes, namely atypical BSE that differs from C-BSE, have been reported among BSE cases in Europe [2, 21], North America [23] and Japan [11, 34]. Atypical BSE cases are temporally classified into 2 types, namely L- and H-types according to the molecular size of the proteinases K-digested nonglycosylated form of PrPSc [14, 21]. The immunolabeled PrPSc distribution patterns and/or types in the brains of cattle affected with L-BSE [3, 17] and H-BSE [23] differed from those with C-BSE. C-BSE was characterized the presence of stellate and intraneuronal immunolabeled PrPSc [17, 27]. Stellate PrPSc deposition was located mainly in the gray matter neuropils of the cerebral and cerebellar cortices [27] and was reported to deposit to astrocytes stained with glial fibrillary acidic protein [30]. Additionally, coarse particulate and coalescing PrPSc deposition was mainly distributed in the brainstem in
the present cases as described in an report [30]. On the other hand, a differential PrP^Sc deposition pattern was detected in atypical BSE cases. L-BSE showed abundant amyloid PrP-plaques and perineuronal PrP^Sc in the brain [3, 17]. PrP-positive amyloid plaques up to 25 μm in diameter were present in an atypical BSE case in Japan (BSE/JP24). In the 7 cases studied here, no PrP-positive plaques were demonstrated in the brain. However, it has been reported that plaque-like PrP^Sc stained with Congo red rarely appeared in the thalamic nuclei of C-BSE–affected cattle [5, 33]. Additionally, in a Swedish H-BSE case, no stellate PrP^Sc deposits were identified [8]. Therefore, immunohistochemical profile and mapping of the pathologic stage of the disease and confirmation of the infectious agent strain, e.g., C-BSE or atypical BSE [30].

In summary, the types and distribution patterns of immunolabeled PrP^Sc in the brain and spinal cords of the 7 naturally occurring BSE cases confirmed by the fallen stock surveillance program in Japan were in accordance with published C-BSE cases, indicating that the BSE in the affected animals reported here was caused by a single infectious agent strain identical to the C-BSE prion.

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