cence detection after capillary electrophoresis [34], are also being developed as sensitive methods for detection of PrP^{Sc}.

Bieschke and coworkers [35] developed a highly sensitive detection method based on a two-color scanning setup, where intensity analysis is used to detect pathological aggregates of PrP^{Sc} having slow diffusion instead of correlating signals to get size information on the molecule. However, equipment where the samples need to be moved to the focal area by scanning stage seems to be too expensive and sophisticated to use for BSE diagnosis at abattoirs. In this study, we have developed methods for the detection of PrP using conventional FCS and FCCS analyses and attempted to detect PrP^{Sc} using the compact FCCS system produced with the aim of BSE diagnosis at abattoirs.

Although antibodies are essential tools for immunological diagnosis, there are limitations in their use for FCS. Mayboroda and coworkers [36] showed that staphylococcal protein A (PrA, 42 kDa) can be used as a low-molecular weight tag for immune complex. However, PrA is applied not for mouse IgG_1 but for IgG_{2a}, IgG_{2b}, and IgG_3 [37]. Actually, we could not detect rBoPrP with PrA using FCS because there was no difference between the affinity of PrA for immune complexes and that for free anti-PrP antibodies 72 and 44B1. On the other hand, the use of both Fab′ fragment and another antibody binding to independent epitopes is generally applicable for the detection of every antigen using FCS.

Unlike FCS analysis, depending on diffusion time, FCCS is not necessary to prepare a lower molecular weight probe, such as a labeled Fab′ fragment, or to determine the difference of the solution's viscosity for each sample, but the dynamic range of FCCS in this experiment was narrower than that of FCS. This is because nearly identical concentrations of two mAbs labeled with different fluorescence dyes were used in FCCS due to suppression of background fluorescence, whereas sufficient unlabeled antibody was used in FCS to form 44B1–rBoPrP completely. To overcome the limitation of dynamic range, another measurement was carried out with the addition of rBoPrP to all of the samples; namely, by comparing measurements without and with the addition of 4.8 nM rBoPrP, we can determine whether the sample is negative or positive. This technique is generally applicable for other sandwich detection methods with two probes. However, because all samples would need to be measured at least twice given that the majority of all samples in BSE diagnosis are expected to be negative, the problem remains the key issue for the diagnosis using FCCS. The reduction of measuring time in a sample and the development of a totally automated system might be a solution to the problem.

Although the detection and characterization of prion disease [35,38], Alzheimer's disease [39], and Parkinson's disease [40] were reported using confocal fluorescence methods, they were restricted to analyses in transparent media such as reaction buffer and cerebrospinal fluid (CSF). In contrast, we detected PrP using FCCS in turbid samples prepared from tissue. Because the use of both centrifugation and sufficiently labeled antibodies is easy, they are generally applicable for the detection of the target molecule using FCCS in turbid medium.

From infectivity studies in rodent models of TSE [41], it seems that the maximum concentration of infectivity in circulating blood resides in the buffy coat, where the concentration ranges between 5 to 10 infectious units (IU) ml^{-1} and approximately 100 IU ml^{-1} at the onset of symptomatic disease. One picogram of PrP^{Sc} is estimated to contain 100 IU [42]; therefore, the concentrations of PrP^{Sc} in the buffy coat are expected to be 1 pg ml^{-1} (33 fM) and 0.1 pg ml^{-1} (3.3 fM) during the symptomatic and presymptomatic phases, respectively.

In the femtomolar concentration range, a target molecule has a territory of 1 nl, whereas the confocal volume of FCS or FCCS is approximately 1 fl. If the radius of the confocal volume and the diffusion coefficient of particles are 1 μm and 10^{-6} cm^2/s, respectively, the femtomolar concentration is detectable for 15 min measurement using FCS and FCCS [16]. Because this calculation assumes that all of the PrP binds to the fluorescent-labeled antibody, the sensitivity seems to be strongly limited by the K_d values of antibodies. Therefore, high-affinity antibodies to PrP are required for the development of a highly sensitive detection method.

Castilla and coworkers [43] developed an efficient protocol for the amplification of PrP^{Sc}. The combination of our detection method and amplification technique of PrP^{Sc} can improve the detection limit of PrP^{Sc}. A perfect and stable overlap of two laser lines often is difficult, and this makes the reduction of FCF amplitude and sensitivity. Thus, we currently are developing a novel detection method for sensitive improvement with new dyes in FCCS analysis.

All commercial BSE tests currently used detect the PK resistance of PrP, but it has been reported that the pathological state of PrP was not only PK resistant but also PK sensitive [44]. Birkmann and coworkers [45,46] developed methods for detecting both parts of the prion particles with FCS in fluorescence intensity distribution analysis (FIDA) mode, although the limit of sensitivity has not been exploited. On the other hand, our methods demonstrated that denatured PrP and rBoPrP are suitable for the detection of only the PK-resistant form. In the future, the methods need to improve to detect the entire pathological state of PrP.

In conclusion, we have presented methods for the detection of PrP using FCS and FCCS. A combination of a fluorescent-labeled Fab′ fragment and another anti-PrP mAb enabled us to detect rBoPrP using FCS. On the other hand, FCCS detected rBoPrP using two mAbs labeled with different fluorescence dyes. The sensitivity of a compact FCCS apparatus produced with the aim of BSE diagnosis at abattoirs was comparable to that of the ELISA approved by the European Commission for BSE diagnosis. Because

FCS and FCCS analyses require only microliter samples and a single mixing step, the analyses lend themselves to automation for BSE diagnosis.

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Short Communication

Experimental Transmission of Two Young and One Suspended Bovine Spongiform Encephalopathy (BSE) Cases to Bovinized Transgenic Mice

Takashi Yokoyama*, Kentaro Masujin, Yoshio Yamakawa[1], Tetsutaro Sata[1], Yuichi Murayama, Yujing Shu, Hiroyuki Okada, Shirou Mohri and Morikazu Shinagawa

Prion Disease Research Center, National Institute of Animal Health, Ibaraki 305-0856, and [1]National Institute of Infectious Diseases, Tokyo 162-8640, Japan

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SUMMARY: Bovine spongiform encephalopathy (BSE) is caused by a prion that primarily consists of an abnormal isoform of the prion protein (PrP^{Sc}). Since PrP^{Sc} is partially resistant to proteolytic digestion, the routine diagnosis of BSE is based on the immunological detection of the proteinase K (PK)-resistant moiety of PrP^{Sc} (PrP^{core}). However, transmission studies are indispensable in order to demonstrate prion infectivity and to analyze prion characteristics. Transmission experiments were accordingly performed on 2 young BSE cases (BSE/JP8, BSE/JP9) and 1 suspected BSE case (Suspended-1) that were detected by the BSE screening program in Japan. In this study, we attempted to transmit the prion from these 3 animals by using transgenic mice overexpressing bovine PrP (TgBoPrP). In spite of the use of BSE-sensitive transgenic mice, none of the mice developed neurological signs nor accumulated PrP^{Sc} in their brains for more than 600 days post-inoculation, even with subsequent blind passages. The results of a dilution experiment using the classical BSE prion indicated that prion infectivity in these 3 cattle was below the detection limit of $10^{3.0}$ LD_{50}/g.

Bovine spongiform encephalopathy (BSE) is a fatal neurological disease in cattle that was first recognized in the United Kingdom in 1986 (1). The disease belongs to a group of transmissible spongiform encephalopathies (TSEs), or prion diseases (2). The occurrence of the BSE epizootic could be the result of consumption of a BSE prion contaminating proprietary concentrates or feed supplements. Recent cases of BSE have been reported throughout most of Europe, North America, and Japan.

The experimental transmissibility of BSE to cattle and to other animals has been previously demonstrated; however, the nature of the TSE agents has not been fully elucidated. A misfolded isoform of the prion protein (PrP), designated PrP^{Sc}, is considered to be responsible for these diseases. This PrP^{Sc} forms the main component of the prion, and it is partially resistant to proteinase digestion. Several commercial kits are available for BSE diagnosis, and most of which are based on the immunological detection of the proteinase-resistant moiety of PrP^{Sc} (PrP^{core}). The detection of accumulated PrP^{Sc} by immunohistochemistry (IHC) and that of PrP^{core} by Western blot (WB) analysis are routinely performed as confirmatory tests.

The uniform pathology among BSE-affected cattle and the limited results obtained after BSE transmission experiments were conducted in mice have led to the assumption that BSE is caused by a single prion strain (classical BSE). Recently, however, different phenotypes have been reported among BSE cases (atypical BSE) in Japan, Europe, and North America, and the transmissibility of certain atypical BSE cases has been confirmed (3,4). Atypical BSE cases are currently classified into at least two groups, namely, the L-type and the H-type group, in accord with the molecular weight of PrP^{core}. Sheep scrapie prions have been classified into various strains based on their varying incubation periods and/or differences in the lesion profile of spongiform changes observed in inbred mice. The molecular basis for strain variation remains unclear; however, according to the "protein-only" hypothesis, strain characteristics are encoded within different conformations of PrP^{Sc}.

Between September 2001 and March 31, 2007, the presence of BSE infection was confirmed in 32 cattle in Japan as a result of the BSE screening and surveillance programs conducted by the Ministry of Health, Labour and Welfare (MHLW) and the Ministry of Agriculture, Forestry and Fisheries (MAFF), respectively (5). Most of the BSE-diagnosed cattle exhibited a heavy accumulation of PrP^{core} or PrP^{Sc} in the brain, as confirmed by both WB and IHC. Samples from two young healthy Holstein steers yieled weakly positive primary enzyme-linked immunosorbent assay (ELISA) (Platelia BSE; Bio-Rad, Hercules, Calif., USA) results, and revealed an accumulation of unusually small amounts of PrP^{core} detected by WB; the amount of PrP^{core} in the brains of the affected animals was estimated to be as low as 1/1,000 of that in a classical BSE case (BSE/JP6), as estimated by WB (6). Interestingly, one of the cattle (BSE/JP8) accumulated a distinct PrP^{core} with a different glycoform profile and with proteinase K (PK)-resistance properties that differed from those of the classical BSE case, as revealed by WB; this case was classified as atypical BSE (7). In addition to these two young steers, we also detected a faint PrP^{core}-like signal by WB in a sample obtained from an ELISA-weakly positive, 20-year-old Japanese Black cow (Suspended-1). However, due to the faint signal, the diagnosis of this case was equivocal. The specific details regarding these three cattle are listed in Table 1.

No spongiform changes and/or PrP^{Sc} deposition were observed in any of these three cases by histopathological examination. In all three cases, the PrP^{core} accumulation was limited to the obex, which was examined by means of ELISA; it was

*Corresponding author: Mailing address: Prion Disease Research Center, National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan. Tel & Fax: +81-29-838-7757, E-mail: tyoko@affrc.go.jp

not possible to perform a detailed analysis of the remaining brain tissues. Thus, in these cases, we attempted to amplify PrP^{Sc} by using bovine PrP-overexpressing transgenic (TgBoPrP) mice with a null background (kindly provided by Dr. S. B. Prusiner) (8). It has been reported that these mice are 10 times more sensitive than cattle and 1,000 times more sensitive than RIII mice to infection with BSE prions (9). In order to determine the sensitivity of the bioassay using TgBoPrP mice, the brain homogenates of BSE cattle (classical BSE; provided by VLA, Weybridge, UK) were serially diluted, and 20 μl of the dilution were intracerebrally inoculated into the mice. On alternate days, these animals were monitored for the development of clinical signs and stability of health. The mice that were inoculated with the homogenate from the classical BSE infection displayed clinical signs such as behavioral changes, weight loss, and hind limb paresis; the mice that did not exhibit these clinical signs were cared for until they died of natural causes. The results of the end-point titration experiment with classical BSE (97/04417) are shown in Table 2. The infectivity titer for the examined BSE was determined to be $10^{6.7}$ LD_{50}/g. Since incubation period is correlated with prion titer (10), an infective titer of $10^{5.2}$ LD_{50}/g was estimated for the BSE/JP6 sample, the brain homogenate of which was used as an internal standard for the comparison of WB signal intensities. All mouse samples used in this experiment were subjected to WB to determine the presence of PrP^{core} in the brain. Brain homogenate preparation and electrophoresis were performed as described in our previous paper (11). The blotted membrane was incubated with anti-PrP T2 monoclonal antibody (mAb) (12), and the signals were detected using a chemiluminescent substrate (SuperSignal; Pierce Biotechnology, Inc., Rockford, Ill., USA). PrP^{Sc} deposition was also examined by IHC.

Table 1. Summary of examined BSE cases

Case	Age of cattle	ELISA titer[1] (cut off)	WB	IHC	Spongiform changes
BSE/JP8[2]	23 months	0.20, 0.21 (0.23)	+	–	–
BSE/JP9[3]	21 months	0.29, 0.29 (0.24)	+	–	–
Suspended-1[4]	20 years	0.43, 0.44 (0.23)	±	–	–
BSE/JP6[5]	83 months	3.1, 3.3 (0.23)	+	+	+

[1]: Optical density value of Platelia BSE (Bio-Rad).
[2]: Young, atypical BSE case (7), http://www.mhlw.go.jp/houdou/2003/10/h1006-2.html (in Japanese).
[3]: Young, classical BSE case, http://www.mhlw.go.jp/houdou/2003/11/h1104-3.html (in Japanese).
[4]: http://www.mhlw.go.jp/houdou/2003/03/h0327-2.html (in Japanese).
[5]: Classical BSE case (6).

We attempted to transmit the disease from the three cattle (BSE/JP8, BSE/JP9, and Suspended-1) to the TgBoPrP mice. Due to limitations of the available sample, we used the remaining homogenates for the primary ELISA test (homogenate for the grinding buffer of the Platelia BSE kit). The homogenate was diluted four times with phosphate-buffered saline (PBS; final concentration, 5% [w/v]) and was used for the transmission study. As shown in Table 2, none of the TgBoPrP mice that were intracerebrally inoculated with

Table 2. Transmission of BSE to TgBoPrP mice

A. Titration assay of classical BSE[1]

Inoculum	No. diseased /no. inoculated	Mean ± SD (days)
ori	6/6	217.8 ± 3.8
10^{-1}	6/6	257 ± 2.6
10^{-2}	6/6	309 ± 53.4
10^{-3}	6/6	386 ± 13.9
10^{-4}	3/6	479 ± 131.0
10^{-5}	0/6	>495

B. Transmission study of Japanese BSE cases

Inoculum	Mice	No. diseased/ no. inoculated	Mean ± SD (days) or sacrificed days
BSE/JP8[2]			
Primary passage	TgBoPrP	0/5	(600, 786, 788, 788, 860)
2nd passage	TgBoPrP	0/7	>550
2nd passage	ICR	0/7	>550
BSE/JP9[3]			
Primary passage	TgBoPrP	0/6	(505, 577, 704, 881, 927, 927)
2nd passage	TgBoPrP	0/7	>495
2nd passage	ICR	0/7	>495
Suspended-1[4]	TgBoPrP	0/7	(717, 811, 831, 864, 864, 892, 927)
PBS	TgBoPrP	0/5	(432, 475, 534, 609, 717)
BSE/JP6[5]	TgBoPrP	5/5	277.2 ± 12.2

All mice were tested for the presence of PrP^{core} in the brain by WB, and mice that were positive were considered to be diseased.
[1]: BSE sample obtained from the UK. Infectivity titer per gram was $10^{6.7}$.
[2]: Case of atypical BSE in a 23-month-old Holstein steer.
[3]: Case of classical BSE in a 21-month-old Holstein steer.
[4]: BSE diagnosis was suspended in a 20-year-old Japanese Black. The faint unusual PrP^{core}-like band was observed by WB; however, no spongiform change or PrP^{Sc} deposition were detected in pathology.
[5]: Case of classical BSE in a 83-month-old Holstein cow.

the brain homogenates obtained from the two young BSE cases and from the one suspended case displayed any clinical signs associated with BSE. The brain homogenate obtained from TgBoPrP mice that died without clinical signs (600 and 788 days post-inoculation in BSE/JP8 and 505 days post-inoculation in BSE/JP9) were intracerebrally inoculated into additional TgBoPrP mice for a second passage. When observed 500 days post-inoculation, no clinical signs and/or abnormalities were observed after the second passages in these mice. The mice inoculated with PBS, the negative control, died of natural causes between 432 and 717 days post-inoculation.

PrP^{core} was detected in the mouse brains inoculated with BSE/JP6, and its glycoform and molecular size were similar to that of classical BSE (Fig. 1, lanes 5-7). A PrP signal at 25-kD was detected in the PK-treated brain homogenate of some of the mice inoculated with material from both young and suspected BSE cases, although as shown in Fig. 1, the glycoform profile different from that of PrP^{core} in the original inoculum (7, http://www.mhlw.go.jp/houdou/2003/11/h1104-3.html) (Fig. 1, lanes 1-3). The 25-kD signal was not observed in the brains of any young mice examined (6 months old) (lane 4). This observation suggests that the PK-resistant PrP detected in the brains of the older TgBoPrP mice was a product of spontaneous protein misfolding. It was of note that this 25-kD band was also generated in vitro, as determined by protein misfolding cyclic amplification (PMCA) from the normal cattle and mouse brain homogenates; this 25-kD product was designated as $PrP^{C\text{-}res}$ (13). Recently, we confirmed that the $PrP^{C\text{-}res}$ in old TgBoPrP mice was not converted to PrP^{Sc} (data not shown). Furthermore, no PrP^{Sc} signal was detected by IHC in the brains of older TgBoPrP mice (data not shown), and no transmissibility was observed in either TgBoPrP or ICR mice (Table 2). Thus, $PrP^{C\text{-}res}$ may be the result of overexpression of PrP^{C} in TgBoPrP mice. It appeared that the 25-kD band was not a self-propagating product of the prion in the inoculum.

It has been reported that some mice inoculated with a BSE sample did not accumulate PrP^{Sc} in their brains, and that PrP^{Sc} appeared during serial passages following adaptation to the new host (14). We accordingly examined the secondary passage of the two young BSE cases (BSE/JP8 and BSE/JP9) to TgBoPrP and wild-type mice (ICR, SLC/Japan). The blind passage also revealed the non-transmissibility of these samples to the TgBoPrP and wild-type mice (Table 2).

We were unsuccessful at transmitting the three present cases to TgBoPrP mice via intracerebral inoculation. However, given the extremely low content of PrP^{Sc} in these preparations, this does not necessarily mean that the PrP^{core} in the inocula were not infectious. Considering the intensity of the PrP^{core} signal on the WB, it was estimated that the amount of PrP^{core} in the young and suspected BSE cases was as low as approximately 1/1,000 of that of BSE/JP6, for which the prion titer was $10^{5.2}$ LD_{50}/g ($10^{2.2}$ LD_{50}/mg). In this experiment, each mouse received 1 mg (20 μl of a 5% brain homogenate) of brain tissue as an inoculum. If we assume a low content of PrP^{core} in the preparation, the mice received only $10^{-0.8}$ LD_{50} (0.16 LD_{50}) units of prion; this amount would be equivalent to the very limit of sensitivity of the bioassay.

If the susceptibility of the TgBoPrP mice to atypical BSE was similar to their susceptibility to classical BSE, then the amount of PrP^{core} in the cattle brain might have been below the bioassay detection limit. Clearly, the precise susceptibility of the TgBoPrP mice to atypical BSE remains uncertain. The infectivity of BSE/JP8 that is classified as atypical BSE (Table 2) should be carefully considered in this context. It has been reported that the L- and H-types of atypical BSE have different incubation periods in TgbovXV mice (4). This result indicated that several different atypical BSE strains may exist, and that TgBoPrP mice might be less sensitive to certain atypical BSE strains than to the typical strain. Thus, in order to investigate atypical BSE prions, it will be necessary to develop an experimental animal with high susceptibility to atypical BSE prions.

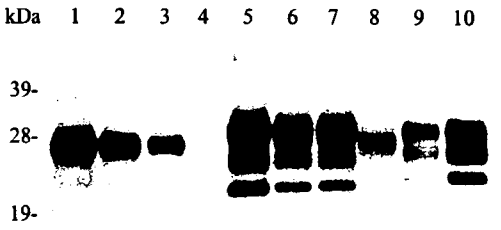

Fig. 1. WB analysis of the brain of the BSE inoculated TgBoPrP mice. Mice brain was homogenized (10%) in detergent buffer and incubated with 50 μg/ml of PK for 30 min at 37°C. The sample was mixed with an equal volume of sodium dodecyl sulfate (SDS) sample buffer and then subjected to WB (11). Lanes 1-3, TgBoPrP mice inoculated with young atypical BSE (BSE/JP8) (860 days post-inoculation); lane 4, mock-infected TgBoPrP mice (6 months old); lanes 5-7, TgBoPrP inoculated with classical BSE (BSE/JP6); lane 8, mock-infected aged TgBoPrP mice (800 days old); and lanes 9-10, PrP^{core} from scrapie-infected mouse brain (0.4 and 1.6 μg brain equivalents). Lanes 1 and 5, 125 μg; lanes 2 and 6, 50 μg; lanes 3 and 7, 25 μg; lanes 4 and 8, 250 μg brain equivalents were examined per lane. Mab.T2 was used for detection.

Most of the atypical BSE cases reported thus far have been considered to be sporadic rather than genetic (4,15). None of the cattle examined in this study harbored the amino acid substitution associated with the PrP gene (Hagiwara et al., unpublished data). The two young cattle with BSE were born in 2001-2002, immediately after the animal feed ban was enforced in Japan following the first reported occurrence of BSE. Therefore, we believe that these cases were a result of cross-contamination of the remaining feed.

Recently, another atypical BSE infection in an old cow (14 years old) was reported in Japan (BSE/JP24); a different phenotype of PrP^{core} was observed by WB, and PrP^{Sc} deposition in this case was detected by IHC (16). Precautions must be taken to account for the possibility of sporadic BSE occurrence in old cattle such as the 20-year-old Suspended-1 and the 14-year-old atypical BSE animals. Unfortunately, the lack of evidence renders it difficult to evaluate the risk of atypical BSE infection in humans.

Along these lines, it is important to consider the sensitivity of the WB technique applied in the BSE screening and active surveillance programs in Japan (http://www.mhlw.go.jp/english/topics/foodsafety/bse/dl/3-1-2-1.pdf). Common practice dictates the detection of PrP^{core} contained in 1 μg of brain tissue of BSE/JP6 confirmed by WB as employed in these programs; thus, the sensitivity of WB is similar to that of the bioassay system using TgBoPrP mice. A highly sensitive WB that can detect small amounts of PrP^{core} would be an extremely advantageous tool for detecting BSE-affected cattle still in the incubation period.

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Prions in the peripheral nerves of bovine spongiform encephalopathy-affected cattle

Kentaro Masujin,[1] Danny Matthews,[2] Gerald A. H. Wells,[2] Shirou Mohri[1] and Takashi Yokoyama[1]

Correspondence
Takashi Yokoyama
tyoko@affrc.go.jp

[1]Prion Disease Research Center, National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

[2]Veterinary Laboratories Agency (VLA), Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, UK

With the use of increasingly sensitive methods for detection of the abnormal isoform of prion protein (PrP^{Sc}) and infectivity in prion diseases, it has recently been shown that parts of the peripheral nervous system (PNS) of bovine spongiform encephalopathy (BSE)-affected cattle may become infected. It has been reported that prions spread to the central nervous system (CNS) via the PNS in sheep scrapie, but the pathogenesis of BSE in cattle is less well understood. To determine whether parts of the PNS other than those implicated directly in the hypothetical pathogenetic spread of agent from the intestine to the CNS become involved before or after the CNS is affected, PrP^{Sc} distribution was investigated by a highly sensitive Western blotting technique in dorsal root ganglia, stellate ganglion, phrenic, radial and sciatic nerves, adrenal gland and CNS of cattle that were inoculated orally with BSE-affected brain and culled sequentially. In experimentally BSE-affected cattle, PrP^{Sc} was first detected in the CNS and dorsal root ganglia; subsequently, PrP^{Sc} accumulation was detected in the peripheral nerve trunks. PrP^{Sc} was also detected in the adrenal glands of cattle that showed clinical signs. No PrP^{Sc} was detected in the PNS of BSE-negative cattle. This study shows that, with respect to dorsal root ganglia, a paravertebral sympathetic ganglion and the somatic nerves examined, PrP^{Sc} is detected in the PNS during the disease course at the same time as, or after, it accumulates in the CNS.

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INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a fatal, neurodegenerative disorder of cattle. It belongs to a group of diseases called transmissible spongiform encephalopathies (TSEs), which also include scrapie in sheep and goats and Creutzfeldt–Jakob disease (CJD) in humans (Prusiner, 1991). TSEs are characterized by spongiform changes in the central nervous system (CNS) (Fraser, 1979; Masters & Richardson, 1978) and by the accumulation, principally in the CNS and lymphoreticular system, of PrP^{Sc}, a pathological, partially protease-resistant isoform of a normal cellular prion protein (PrP^{C}) (Prusiner *et al.*, 1982). The conversion of PrP^{C} to PrP^{Sc} is thought to be central to the pathogenesis of TSEs, and PrP^{Sc} has been recognized as a major component of the pathogen, normally referred to as a prion. PrP^{Sc} is generally, but not exclusively, correlated with infectivity and is the only known disease-specific marker (Bolton *et al.*, 1982; Prusiner, 1991).

A variant form of CJD (vCJD) has been detected in the UK and several other countries, and it is thought that this disease has resulted from the consumption of BSE-contaminated beef products (Chazot *et al.*, 1996; Will *et al.*, 1996, 1998; Cousens *et al.*, 1997). As a consequence, BSE is considered a zoonosis. In most countries where BSE-control programmes have been introduced, the organs that have either been predicted by extrapolation from sheep scrapie data or demonstrated to be infectious in cattle are classified as specified risk materials (SRM). These tissues are excluded from the human diet and destroyed, irrespective of the outcome of post-mortem testing for BSE.

Whilst much has been learned about the pathogenesis of BSE in cattle by examination of tissues from experimentally infected cattle, killed at intervals throughout the disease course, infectivity has been detected consistently only in the distal ileum, the CNS and certain peripheral nervous system (PNS) ganglia: dorsal root ganglia (DRG) and trigeminal ganglion (Wells *et al.*, 1998, 1999, 2005). Rarely, in experimentally infected cattle, infectivity has also been detected in bone marrow and tonsil (Wells *et al.*, 1999, 2005). Until recently, in naturally infected clinical cases of BSE, infectivity had been detected only in the brain, spinal cord and retina by bioassay in wild-type mice (Fraser & Foster, 1994; MAFF, 1995). This apparently restricted tissue distribution of the BSE agent in cattle, compared

with at least some other TSEs, may partially be a reflection of limitations of the assay sensitivity. Although assays have been conducted by intracerebral inoculation of cattle (Wells *et al.*, 2005), evidence of infectivity in tissues to which humans may be exposed, such as muscle, peripheral nerves and lymph nodes, has remained elusive.

Recently, through the use of transgenic mice overexpressing bovine PrP^C (Tgbov XV mice), infectivity was detected in the brain, spinal cord, retina, optic, facial and sciatic nerves and distal ileum of a naturally infected cow at the terminal stage of BSE (Buschmann & Groschup, 2005). In addition, detection of PrP^{Sc} has been reported in the PNS and adrenal glands of a natural case of BSE in Japan (Iwamaru *et al.*, 2005). PrP^{Sc} was also detected in the peripheral nerves of two BSE-positive cows that were detected during surveillance of slaughtered cattle (Iwata *et al.*, 2006). Although Iwata *et al.* (2006) reported that the BSE-positive cattle were not clinically affected, the clinical signs reported at ante-mortem examination were inclusive of those recorded in British BSE-affected cattle. It is therefore probable that these animals were at least in the early stages of clinical disease.

These data indicate that PrP^{Sc} and/or infectivity can be detected outside the CNS and distal ileum, at least in the later stages of disease, and that the presence of agent in the PNS may represent a risk to consumers, as PNS structures are not specifically designated SRM, and are thus not removed from the food chain. In order to facilitate more accurate estimations of risk, in the context of control programmes including the testing of cattle entering the food chain, it was felt to be necessary to investigate whether PrP^{Sc} could be detected in parts of the PNS other than those implicated directly in the hypothetical pathogenetic spread of agent from the intestine to the CNS (McBride & Beekes, 1999; van Keulen *et al.*, 2000; McBride *et al.*, 2001; Wells & Wilesmith, 2004). In particular, it was of interest to determine whether PrP^{Sc} was present before, or only after, detection in the CNS, or after the onset of clinical signs. In this study, we investigated PrP^{Sc} accumulation in the PNS and adrenal gland of naturally infected BSE cases from the UK in order to confirm previous results from cattle in Japan. In addition, using samples from cattle in an experimental time-course study, we investigated the temporal relationship between detection of PrP^{Sc} in the CNS and certain PNS structures following oral exposure to BSE-infected brain material.

In addition, infectivity assays on selected tissues were conducted in transgenic mice expressing the bovine prion protein (PrP) gene (TgBoPrP).

METHODS

Tissue samples from cattle. All samples examined in this study were provided by the TSE Archive of the VLA, Addlestone, UK. For confirmation of earlier Japanese results, we examined the PNS: cervical and thoracic DRG (pooled C3–5 and T7–9), trigeminal, cranial cervical and thoracic ganglia, sciatic, vagus and splanchnic nerves and the adrenal gland from five clinically suspect, BSE-confirmed cases and five clinically suspect animals that were negative after diagnostic examinations of brain. These served as controls, as they presented with neurological signs consistent with BSE.

In the second part of the experiment, we examined the following tissues: brainstem, spinal cord (segments C1–2, or C2–3 and T9–10), DRG (pooled from segments C3–5 and T7–9), stellate ganglion, phrenic and radial nerves and adrenal gland harvested from cattle that were challenged orally at the VLA with either 100 or 1 g BSE-infected brainstem homogenate from clinically affected donors, and culled sequentially. The infectivity titre of the inoculum was determined previously by end-point titration in RIII mice to be $10^{3.1}$ mouse intracerebral and intraperitoneal units $ID_{50}\ g^{-1}$, (M. E. Arnold and others, unpublished data), which is similar to other contemporary titrations in these mice of infectivity of brainstem from clinical cases of BSE (data not shown). Samples collected between 27 and 42 months post-exposure from the 100 g dose group, and between 36 and 51 months post-challenge in the 1 g dose group (Table 1), were selected for testing on the basis of prior knowledge of the time points in the sequential kill studies when PrP^{Sc} was first detected in the CNS (Wells *et al.*, 1998; M. E. Arnold and others, unpublished data). The selection of material was judged to ensure that some samples were available from time points before PrP^{Sc} was detected by immunohistochemistry (IHC) in the CNS. Sample sets were not complete for all animals from the original experiments, as supply was dependent on stocks remaining after use in other studies. In total, 376 tissues obtained from 31 cattle challenged experimentally with a 100 g dose of BSE brainstem homogenate, and 14 challenged with a 1 g dose, were examined. A further seven undosed, control cattle, age-matched approximately to challenged animals killed at 27, 30, 33, 36, 42, 44 and 52 months after dosing, were killed.

Processing of tissue samples. PrP^{Sc} was extracted from peripheral tissues by a method described previously (Shimada *et al.*, 2005). Briefly, the PNS tissues and adrenal glands were suspended in 0.8 ml of a detergent buffer containing 50 mM Tris/HCl (pH 7.5), 2 % (v/v) Triton X-100, 0.5 % (v/v) *N*-lauroylsarcosine, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mg collagenase and 40 μg DNase I, and incubated at 37 °C for 2 h with constant rotation with a metal bead (Metal corn; Yasui Kikai). The homogenate was digested with proteinase K (PK; Roche Diagnostics) (final concentration, 60 $\mu g\ ml^{-1}$) at 37 °C for 1 h. PK digestion was terminated with 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc; Roche

Table 1. Numbers of inoculated cattle from a sequential kill, time-course pathogenesis study providing tissues for examination, according to time killed after inoculation

Dose	Time after inoculation (months)	*n*
100 g	27	5
	30	6
	32	3
	33	3
	35	1
	36	6
	42	7
1 g	36	6
	42	6
	44	1
	51	1

Diagnostics). The homogenate was centrifuged at 68 000 *g* for 20 min (Optima MAX-E/TLA-100.3; Beckman) at room temperature (RT). The supernatant was discarded and the pellet was suspended in 6.25% (w/v) Sarkosyl (Sigma) in 10 mM Tris/HCl (pH 7.5) and incubated at RT for 1 h with constant rotation; subsequently, it was centrifuged at 9000 *g* for 5 min (6200/AF-2730; Kubota). Sodium phosphotungstate was added to the supernatant to a final concentration of 0.3% (v/v) and incubated at 37 °C for 30 min with constant rotation. Pellets were obtained by centrifugation at 20 000 *g* for 30 min (6200/AF-2730; Kubota). PrP^{Sc} was enriched from the brain according to a method described previously (Hayashi *et al.*, 2005). The CNS tissues were homogenized in a buffer containing 100 mM NaCl and 50 mM Tris/HCl (pH 7.6). The homogenate was mixed with an equal volume of detergent buffer containing 4% (w/v) Zwittergent 3-14 (Calbiochem), 1% (w/v) Sarkosyl, 100 mM NaCl and 50 mM Tris/HCl (pH 7.6) and then incubated with 0.25 mg collagenase, followed by incubation with PK (final concentration, 40 μg ml^{-1}) at 37 °C for 30 min. PK digestion was terminated with 2 mM Pefabloc. The sample was mixed with 2-butanol:methanol (5:1) and then centrifuged at 20 000 *g* for 10 min (6200/AF-2730; Kubota).

Western blotting (WB) analysis. The pellets were resuspended in a gel-loading buffer containing 2% SDS and heated at 100 °C for 6 min. The samples were separated by SDS-PAGE (12% gel) and blotted electrically onto a PVDF membrane. The blotted membrane was incubated with anti-PrP monoclonal antibody (mAb) T2 conjugated to horseradish peroxidase at RT for 1 h. Signals were developed with a chemiluminescent substrate (SuperSignal; Pierce Biotechnology) (Hayashi *et al.*, 2004).

Infectivity assays. The transmissibility of infection from brain, vagus nerve and adrenal gland of natural cases of BSE, tissues that were found to contain PrP^{Sc}, was bioassayed in transgenic (Tg) mice expressing bovine PrP [Tg(BoPrP)4092HOZ/$Prnp^{0/0}$; Tg(BoPrP)]. These mice, kindly supplied by Dr S. B. Prusiner, are susceptible to BSE prions and exhibit an incubation period of <250 days (Scott *et al.*, 1997). The tissues were each homogenized in 9 vols PBS by a multi-bead shocker (Yasui Kikai) and centrifuged at 1000 *g* for 5 min (6200/AF-2730; Kubota) at RT; the supernatant was used as the inoculum. Female Tg(BoPrP) mice (3 weeks old) were inoculated intracerebrally with 20 μl supernatant. After inoculation, the clinical status of the mice was monitored daily to assess the onset of neurological signs. Diseased mice were sacrificed and subjected to PrP^{Sc} examination as described previously (Yokoyama *et al.*, 2001).

RESULTS

PrP^{Sc} detection in the PNS and adrenal gland of naturally BSE-affected cattle

Representative results of WB analysis of the vagus nerve and adrenal gland of naturally infected cattle are shown in Fig. 1(a, b). Samples from the diagnostically unconfirmed cattle were negative. The signal intensity from BSE-positive cattle differed between samples and from animal to animal, but typical triple banding was observed in positive tissues, including the adrenal gland and vagus nerve. A weak signal was detected from the sciatic nerve (Fig. 1c). The results of the WB analysis are summarized in Table 2. In addition to the trigeminal ganglion and the mid-cervical and mid-thoracic DRG, PrP^{Sc} was also detected in the peripheral nerves, adrenal gland and thoracic ganglia.

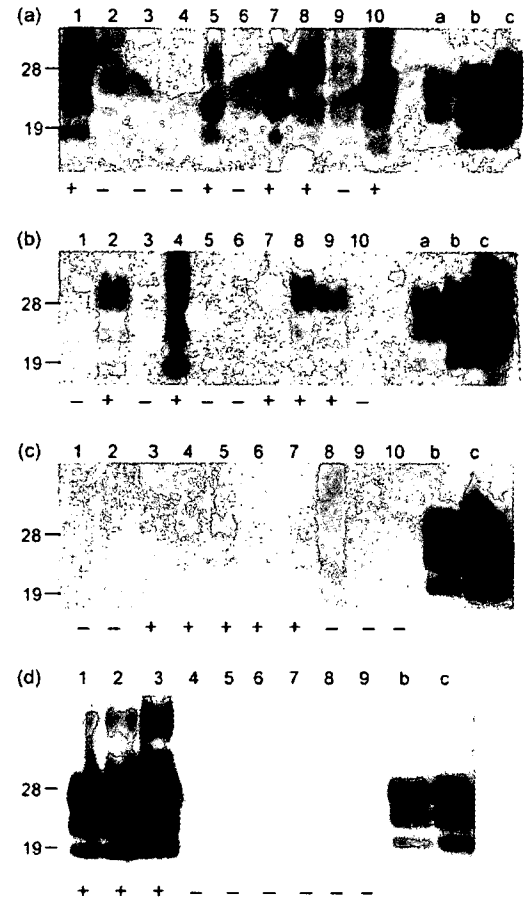

Fig. 1. Detection of PrP^{Sc} in the PNS and adrenal glands of natural BSE cases. (a) Lanes 1–10, adrenal glands [100 mg tissue equivalent (eq.)]. Samples from animals 19591 (lane 1) and 1801 (lane 5) were subjected to transmission study (Table 5). (b) Lanes 1–10, vagus nerve (100 mg tissue eq.). Sample from animal 19591 (lane 4) was subjected to transmission study (Table 5). (c) Lanes 1–10, sciatic nerve (100 mg tissue eq.). (d) Lanes 1–9, midbrain (5 mg tissue eq.). BSE status (shown underneath each gel) was determined by IHC examination of the medulla–obex at VLA, Weybridge, UK: +, BSE-positive cattle; −, BSE-negative cattle. Lanes a–c, mouse scrapie-infected brain was used as the positive control (a, 0.4 μg brain eq.; b, 1.6 μg brain eq.; c, 6.4 μg brain eq.). Molecular markers are shown on the left (in kDa).

PrP^{Sc} detection in the CNS of cattle challenged orally with BSE-infected brainstem

The WB results for individual animals are given in Tables 3 and 4 according to time after inoculation. The results of WB analysis of brainstem and spinal cord were compared with the BSE status of the cattle, as determined previously by IHC examination of the brainstem. The WB results for CNS tissues were completely in accord with the previous diagnoses based on IHC.

Table 2. Numbers of cattle (clinically suspected to be affected with BSE) positive on WB for detection of PrP^{Sc}

Values are no. cattle (clinically suspected to be affected with BSE) positive on WB for detection of PrP^{Sc}/no. animals tested, according to tissue and diagnostic status. Each sample was examined in duplicate and the sample was judged positive on a single positive test result. DRG, Dorsal root ganglia.

Tissue	Diagnostic status	
	BSE confirmed*	BSE unconfirmed†
Cervical DRG	5/5	0/5
Thoracic DRG	5/5	0/5
Trigeminal ganglion	4/4	0/5
Cranial cervical ganglion	4/4	0/5
Thoracic ganglia	2/5	0/4
Sciatic nerve	5‡/5	0/5
Vagus nerve	5/5	0/5
Splanchnic nerve	3/3	0/3
Adrenal gland	5/5	0/5

*Cattle showing neurological signs and diagnosed with BSE on examination of medulla oblongata–obex by histopathology and IHC. †Cattle showing neurological signs, but not diagnosed with BSE on examination of medulla oblongata–obex by histopathology and IHC. ‡A weak signal was detected.

PrP^{Sc} detection in the PNS and adrenal glands of cattle challenged orally with BSE-infected brainstem

PrP^{Sc} was detected in PNS by WB analysis, but only inconsistently in those animals diagnosed as BSE-positive on previous CNS IHC examinations (M. E. Arnold and others, unpublished data) (Tables 3 and 4). Furthermore, irrespective of dose, DRG were positive only in animals in which the corresponding level of spinal cord was positive, and PrP^{Sc} was not always detected at both cervical and thoracic levels of DRG. PrP^{Sc} was detected in the stellate ganglion in three of six animals of the 100 g dose grou that were killed 36 months after inoculation, concurrent with DRG involvement. PrP^{Sc} was detected in the phrenic nerve in the single animal of the 100 g dose group that was analysed 35 months after inoculation, and in one of six animals of the 100 g dose group that were killed 36 months after inoculation, in which CNS and certain ganglia were also positive. PrP^{Sc} was not detected in the radial nerve, but was found in samples of the sciatic nerve (one in the 100 g dose group and two in the 1 g dose group), all in animals with positive CNS and ganglia. Whilst PrP^{Sc} was detected in DRG and stellate ganglion of occasional preclinical animals, PrP^{Sc} was detected in peripheral nerves only in those animals with definite clinical signs at the time of euthanasia. PrP^{Sc} was detected in adrenal gland from three samples from the 100 g dose-group cattle, which were also clinically affected. Detection of PrP^{Sc} was erratic in relation to time post-inoculation (Tables 3 and 4). All tests were negative in challenged and control cattle designated previously as BSE-negative by IHC (Fig. 1d).

PrP^{Sc}-positive vagus nerve and adrenal gland harbour infectivity

The attack-rate and incubation-period data of the mice inoculated with PrP^{Sc}-positive vagus nerve and adrenal gland are shown in Table 5. Typical PrP^{Sc} banding was detected in diseased Tg(BoPrP) mouse brains (Fig. 2). The glycoform pattern and molecular mass of PrP^{Sc} in Tg(BoPrP) mice were identical to those of the BSE-affected cattle brain.

DISCUSSION

This was an opportunistic study, intended to build upon data arising from the Japanese surveillance programme, but also to expand an understanding of the pathogenesis of BSE in cattle in the late incubation period, around the time of initial CNS involvement. The data obtained in this study indicate that PrP^{Sc} accumulates in the PNS and adrenal glands of BSE-affected cattle and that this accumulation appears to coincide with, or follow rather than precede, accumulation in the CNS. Observations reported previously for the PNS and adrenal gland of a BSE case, and for the PNS of two cows slaughtered for human consumption in Japan (Iwamaru *et al.*, 2005; Iwata *et al.*, 2006), have now been repeated in five clinical cases from the UK by using the methods described here. This highly sensitive WB method has also detected PrP^{Sc} accumulation in the PNS and/or adrenal gland of five further natural BSE cases detected during the surveillance of fallen stock in Japan (data not shown). These data reinforce the results of Buschmann & Groschup (2005), which confirmed the presence of infectivity in the PNS of a clinical case of BSE in Germany. Based on these results, it seems likely that PrP^{Sc} accumulation in the PNS during the clinical stages of infection may be the rule in BSE, rather than the exception. PrP^{Sc} accumulation in the PNS has been reported in naturally and experimentally scrapie-affected sheep and hamsters, and in CJD-affected humans (Groschup *et al.*, 1996, 1999; Beekes *et al.*, 1998; McBride & Beekes, 1999; Haik *et al.*, 2003; Favereaux *et al.*, 2004; Head *et al.*, 2004; Ishida *et al.*, 2005). As shown in Fig. 1, the PrP^{Sc} signal intensity varied between samples and among cattle. Based on a comparison of the signal intensity in the WB analysis, the amount of PrP^{Sc} present in the PNS or adrenal glands was estimated to be $<1/120$ of that present in the brain.

By testing cattle from an experimental sequential kill study completed at the VLA, it was proposed to obtain further data on the correlation of CNS and PNS PrP^{Sc} detection relative to time after exposure and thereby extend previous studies (Wells *et al.*, 1998, 2005). Limitations on the availability of certain samples, particularly vagus and splanchnic nerves and abdominal autonomic nervous system ganglia, prevented examinations that would have provided

Table 3. Western blot detection of PrP^{Sc} from cattle challenged orally with a 100 g dose of BSE-infected brainstem, according to time after inoculation

Animal no.	Time after inoculation (months)	Clinical status*	BSE status†	Brainstem	Spinal cord C1–2, C2–3	Spinal cord T9–10	Midcervical DRG	Midthoracic DRG	Stellate ganglia	Phrenic nerve	Radial nerve	Sciatic nerve	Adrenal glands
159	27	−	−	ND	−	−	−	ND	ND	−	−	−	−
175	27	−	−	ND	ND	ND	−	ND	ND	−	−	ND	−
187	27	−	−	−	−	−	−	−	−	−	−	−	−
190	27	−	−	ND	−	ND	−	−	ND	−	−		−
205	27	−	−	−	−	−	−	−	−	−	−	−	−
124	30	+/−	+	ND	ND	ND	−	ND	ND	−	−	ND	−
129	30	−	−	−	−	−	−	−	−	−	−	−	−
130	30	+/−	−	ND	ND	ND	−	−	−	−	−	−	−
132	30	−	−	−	−	−	−	−	−	−	−	−	−
161	30	−	−	−	−	−	−	ND	−	−	−	−	−
197	30	−	−	ND	ND	ND	−	−	−	−	−	ND	−
144	32	−	−	ND	ND	ND	−	ND	−	−	−	−	−
146	32	−	+	+	+	+	+	−	−	−	−	−	−
162	32	−	+	ND	ND	ND	−	−	ND	−	−	ND	−
165	33	−	−	−	−	−	−	−	−	−	−	−	−
168	33	−	+	ND	ND	ND	−	−	−	−	−	−	−
203	33	−	−	ND	−	ND	−	−	−	−	−	−	−
173	35	+	+	+	+	+	+	+	ND	+	−	−	+
115	36	+	+	+	+	+	+	+	+	+	−	−	+
140	36	−	+	ND	+	ND	−	+	+	−	−	−	−
174	36	+	+	ND	+	ND	+	+	+	−	−	+	+
193	36	−	−	−	−	−	−	−	−	−	−	−	−
194	36	−	−	ND	−	ND	−	−	−	−	−	−	−
196	36	−	−	−	−	−	−	−	−	−	−	−	−
120	42	+	+	+	+	+	−	+	ND	−	−	−	−
138	42	+/−	−	−	ND	ND	−	−	−	−	−	−	−
143	42	+/−	+	+	+	+	+	+	−	−	−	−	−
145	42	+/−	+	ND	ND	ND	−	−	−	−	−	ND	−
177	42	−	−	−	ND	ND	−	−	−	−	−	ND	−
178	42	−	−	−	ND	−	−	ND	−	−	−	−	−
180	42	−	−	−	−	−	−	ND	−	−	−	−	−

ND, Not done.

*Clinical status: +/−, probable signs; +, definite signs [for definition and application of clinical categories, see Wells & Hawkins (2004)].

†BSE status (+, positive; −, negative) as determined by IHC examination of midbrain, rostral medulla and medulla–obex (M. E. Arnold and others, unpublished data).

Table 4. WB detection of PrP^{Sc} from cattle challenged orally with a 1 g dose of BSE-infected brainstem according to time after inoculation

Animal no.	Time after inoculation (months)	Clinical signs*	BSE status†	Midbrain	Spinal cord C1–2, C2–3	Spinal cord T9–10	Midcervical DRG	Midthoracic DRG	Stellate ganglia	Phrenic nerve	Radial nerve	Sciatic nerve	Adrenal glands
224	36	–	–	–	–	–	–	ND	ND	–	–	–	–
247	36	–	–	–	–	–	–	ND	–	–	–	–	–
248	36	–	–	–	–	–	–	ND	–	–	–	–	–
259	36	–	–	ND	–	–	–	ND	–	–	–	–	–
271	36	–	–	–	–	–	–	ND	–	–	–	–	–
312	36	–	–	–	–	–	–	ND	–	–	–	–	–
214	42	–	–	–	–	–	–	–	–	–	–	–	–
234	42	–	–	–	–	–	–	–	–	–	–	–	–
236	42	–	–	–	–	–	–	–	–	–	–	–	–
263	42	–	–	–	–	–	ND	–	–	–	–	–	–
266	42	–	–	–	–	–	–	–	ND	–	–	–	–
300	42	–	–	–	–	–	–	–	–	–	–	–	–
313	44	+	+	+	+	+	–	+	ND	–	–	+	–
257	51	+	+	+	+	+	ND	ND	ND	–	–	+	–

ND, Not done.

*Clinical status: +, definite signs [for definition and application of clinical categories, see Wells & Hawkins (2004)].

†BSE status (+, positive; –, negative) as determined by IHC examination of midbrain, rostral medulla and medulla–obex (M. E. Arnold and others, unpublished data).

Table 5. Data on transmission of PrP^{Sc}-positive tissues in Tg(BoPrP) mice

Inoculum	No. diseased/ no. inoculated	Incubation period (days ± SD)	Animal no.
Vagus nerve	5/5	313.6 ± 40.6	19591
Adrenal gland 1	2/5	355.5 ± 23.3	19591
Adrenal gland 2	1/5	425	1801
BSE cattle brain	11/11	223.5 ± 13.5	–
Normal cattle brain	0/8	>506	–

opportunities to investigate potential routes of entry of agent to the CNS. Previous studies have demonstrated that PrP^{Sc} deposition in the CNS of BSE-affected cattle is targeted to certain neuroanatomical areas and is not uniform. In this study, PrP^{Sc} detection in the brainstem and two levels of spinal cord tested by WB analysis confirmed previous results of the BSE status of experimentally infected cattle, based upon IHC examination of three levels of brainstem (M. E. Arnold and others, unpublished data). Interestingly, PrP^{Sc} was detected simultaneously in different parts of the CNS (Tables 3 and 4), even in animals that were only in the earlier stages of clinical disease, suggesting perhaps that, once entry has occurred, spread of agent throughout the CNS is a process involving periods that are shorter than the sequential time intervals in the experimental kill study. In this study, PrP^{Sc} detection in the PNS was an infrequent finding in the late preclinical and the clinical stages of BSE, and the available evidence would be consistent with spread from the CNS to the PNS structures examined (DRG, stellate ganglion, phrenic, radial and sciatic nerves) and adrenal glands. The results did not present inconsistencies that would refute the current understanding of peripheral pathogenesis in models of TSE after oral exposure (McBride & Beekes, 1999; van Keulen *et al.*,

Fig. 2. WB analysis of the brains of Tg(BoPrP) mice. PrP^{Sc} was prepared and dissolved in a gel-loading buffer containing 2% SDS. PrP^{Sc} was detected by using the T2 mAb. Lanes: 1, mouse-adapted scrapie; 2, BSE-affected cattle brain; 3, Tg(BoPrP) mice inoculated with BSE-affected cattle brain; 4, Tg(BoPrP) mice inoculated with PrP^{Sc}-positive vagus nerve; 5, Tg(BoPrP) mice inoculated with PrP^{Sc}-positive adrenal gland; 6, Tg(BoPrP) mice inoculated with normal cattle brain. Molecular markers are shown on the left (in kDa).

2000; McBride *et al.*, 2001). The apparently restricted involvement of lymphoid tissues (Buschmann & Groschup, 2005; Wells *et al.*, 2005) and enteric plexuses (Terry *et al.*, 2003) in BSE pathogenesis in cattle suggests possible differences from events in experimental models of TSE pathogenesis. Such differences can only be investigated by experiments conducted in the natural host species. It is likely from epidemiological data and experimental studies (Wells *et al.*, 2007) that the 1 g dose group in these experimental exposures of cattle approximates the majority of field-case exposures, perhaps suggesting less relevance to risk management of positive results from tissues taken from an animal that has been dosed with 100 g. However, the data here do not suggest marked differences between the dose groups and are insufficient for 1 g-dosed animals to indicate a reduced involvement of PNS tissues relative to dose or timing of positive results in other tissues. Although sparse PrP^{Sc} accumulation was observed in the PNS of experimentally infected cattle, PrP^{Sc} was detected in all PNS tissues examined from natural BSE cases (Tables 2, 3 and 4). This may indicate a progressive involvement of PNS in the clinical phase of disease and has implications for the risk assessment of tissues from such animals should they escape detection at slaughter.

In natural cases of clinically BSE-affected cattle, infectivity, detected by bioassay in wild-type mice, has been found only in the CNS (Fraser & Foster, 1994). In orally inoculated cattle, infectivity has been detected in the brain, spinal cord, distal ileum, DRG and trigeminal ganglion in the late incubation period by wild-type mouse assays (Wells *et al.*, 1994, 1996, 1998). Infectivity was also detected by wild-type mouse assay in bone marrow, but only at a single time point in clinically affected, experimentally infected cattle (Wells *et al.*, 1999). Intracerebral inoculation of calves with tissues collected from experimentally infected cattle indicated further that palatine tonsil, not detected previously as being infective by wild-type mouse assay, was infective at 10 months post-oral infection (Wells *et al.*, 2005). Inoculation of calves has also led to the detection of infectivity in the lymphoid tissues of nictitating membranes (G. A. H. Wells, unpublished data; http://www.seac.gov.uk/papers/seac85-3.pdf) from a pool of nictitating membranes collected from naturally infected cows.

Recently, studies using genetically modified mice (Tgbov XV), shown to be 10 000-fold more sensitive than assay in RIII mice and 10-fold more sensitive than assays by the intracerebral route in cattle, have enabled the detection of amounts of infectivity lower than the previous threshold of detection (Buschmann & Groschup, 2005). The studies also expanded the range of tissues assayed and demonstrated BSE infectivity in brain, spinal cord, retina, optic nerve, distal ileum, peripheral nerves (facial and sciatic nerves) and the semitendinosus muscle. With respect to the latter tissue, it remains unclear whether the small amounts of infectivity present were attributable to muscle tissue per se or to the peripheral nerve or lymphoid tissue content of the muscle.

In the assay study described here, we have confirmed that the detection of PrP^{Sc} is indicative of the presence of infectivity in vagus nerve and adrenal gland of BSE-affected cattle in the clinical stage of disease. This supports the use of PrP^{Sc} detection as a surrogate for detection of infectivity in expanding our understanding of the pathogenesis of BSE. However, tissues without detectable PrP^{Sc} accumulation may harbour prion infectivity as, despite the highly sensitive PrP^{Sc}-detection procedure used in this study, there are clear precedents for the occurrence of infectivity in the absence of detectable PrP^{Sc}. The incubation periods of vagus nerve and adrenal gland suggest that the estimated infectivity in these tissues was 2–2.5 logs lower than that of the CNS (Safar *et al.*, 2002). PrP^{Sc} accumulation in the adrenal gland, vagus nerve and stellate ganglion may result from extension from a primary routeing of BSE prions via sympathetic and parasympathetic pathways to the CNS, or secondary spread from the CNS. The adrenal gland, for example, has a rich sympathetic supply associated with the capsule and the medulla that could be notionally infected primarily or secondarily via the splanchnic nerves. The vagus nerve provides the parasympathetic inervation to the intestine and enters the CNS at the medulla, and the stellate ganglion is part of the paravertebral sympathetic chain of ganglia. Interestingly, there are no precedents for a primary role for sensory neural pathways in the pathogenesis of BSE and, hence, DRG infectivity is considered to have spread from the CNS.

Quite clearly, our data indicate that a consumer-protection policy that is based solely on SRM removal, as currently designated, will not eliminate potential exposure to BSE infectivity completely in the carcass of an animal that is CNS-positive, i.e. clinically affected animals or those close to the onset of clinical disease. For such animals, a positive BSE test at the level of the obex followed by destruction of the carcasses would provide greater consumer protection than the removal of SRM alone. It remains to be determined whether the additional protection is actually significant, taking into account the quantity of infectivity present in amounts of PNS likely to be consumed. Nevertheless, the data provided here can contribute to review of risk assessments in relation to bovine PNS tissues.

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Both host prion protein 131–188 subregion and prion strain characteristics regulate glycoform of PrP^{Sc}

T. Yokoyama[1], K. Shimada[1], K. Masujin[1], Y. Iwamaru[1], M. Imamura[1], Y. K. Ushiki[1,2], K. M. Kimura[1], S. Itohara[3], and M. Shinagawa[1]

[1] Prion Disease Research Center, National Institute of Animal Health, Tsukuba, Ibaraki, Japan
[2] Nippi Research Institute of Biomatrix, Toride, Ibaraki, Japan
[3] Brain Science Institute, RIKEN, Wako, Saitama, Japan

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Summary

Prion proteins (PrPs) contain 2 N-linked glycosylation sites and are present in cells in 3 different forms. An abnormal isoform of prion protein (PrP^{Sc}) has different glycoform patterns for different prion strains. However, the molecular basis of the strain-specific glycoform variability in prions has remained elusive. To understand the molecular basis of these glycoform differences, we analyzed PrP^{Sc} in 2 lines of transgenic mice (MHM2 and MH2M with PrP null background) that expressed a chimeric PrP. Our result indicated that PrP 131–188 (substitutions at I139M, Y155N, and S170N) contributed to both PrP^{C} and PrP^{Sc} glycoform ratios. Furthermore, the PrP^{Sc} glycoform pattern within these transgenic mice showed a subtle difference depending on the inoculated prion. This study indicated that the PrP^{Sc} glycoform ratio was influenced by both host PrP^{C} and the prion strain.

Author's address: Takashi Yokoyama, Prion Disease Research Center, National Institute of Animal Health, Tsukuba, Ibaraki 305-0856, Japan. e-mail: tyoko@affrc. go.jp

Introduction

Transmissible spongiform encephalopathies (TSEs), also called prion diseases, are fatal neurodegenerative diseases that include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob disease (CJD) in humans [13]. The nature of the infectious agent prion has not been fully elucidated. The central event in prion pathogenesis is the conversion of the cellular isoform of a prion protein (PrP^{C}) into an abnormal isoform of the prion protein (PrP^{Sc}). PrP^{Sc} is the only known disease-specific marker and is closely associated with infectivity. Conformational differences are observed between PrP^{C} and PrP^{Sc}. PrP^{Sc} has a large number of β sheets and a diminished α-helical content compared with PrP^{C} [5, 10, 15]; hence, PrP^{Sc} is relatively resistant to protease digestion. The protease resistance of PrP^{Sc} has been widely accepted as the physico-chemical basis for distinguishing between PrP^{C} and PrP^{Sc}.

PrP^{C} is a glycoprotein and contains 2 N-linked glycosylation sites. It is present in cells in 3 different glycosylated forms (diglycosylated, monoglycosylated, and unglycosylated forms). It has been

reported that PrP^{Sc} has different glycoform patterns with different ratios of 3 bands for different prion strains [6]. Analysis of the PrP^{Sc} glycoform ratio has been used to discriminate prion strains and has become increasingly important in the differential diagnosis of human prion diseases. However, the molecular basis of strain-specific glycoform variability in prions has remained elusive. Clarifying the prion protein (PrP) glycosylation mechanism might lead to the understanding of prion strain variations. It has been proposed that the PrP^{C} glycoform ratio differs according to the brain regions, and the differential targeting of neurons by prion strains results in the differences in the PrP^{Sc} glycoform ratio [7, 17]. Furthermore, different scrapie prions can induce the formation of different PrP^{Sc} glycosylation patterns in the same cell line [3, 19]. These observations have raised the possibility that the direct influence of a prion strain on the posttranslational glycosylation modification of PrP^{C} or on PrP^{Sc} itself dictates the strain-specific glycosylation [3, 17, 19].

In this study, we examined the PrP^{Sc} glycoform transition that changes the PrP^{Sc} glycoform ratio, which depends on the adaptation of prions in interspecies transmission. The PrP^{Sc} glycoform ratio was altered depending on the shortening of incubation periods. To understand the molecular basis of this phenomenon, we analyzed mice and hamster chimeric PrP expressed by transgenic mice. The result obtained by using these mice indicated that PrP 131–188 influences the PrP^{C} and PrP^{Sc} glycoform patterns. Furthermore, the PrP^{Sc} glycoform ratio was also modified by the prion strain.

Materials and methods

Scrapie prions and animals

The mouse-adapted scrapie prion Obihiro, and hamster-adapted scrapie prion Sc237 were passaged in CD-1 mice (SLC, Japan) and Syrian hamster (SLC, Japan), respectively [21, 22]. Transgenic mice that expressed mouse and hamster chimeric PrP (MH2M and MHM2) were kindly provided by Dr. S. B. Prusiner [16]. Amino acid substitutions at positions L108M and V111M are present in the MHM2 mice. In addition to these amino acid substitutions, another 3 substitutions (I139M, Y155N, and S170N) are present in the MH2M mice. These mouse lines have been maintained by crossing with PrP0/0 mice [22] as PrP null background. Genotypes of the mice were determined by polymerase chain reaction (PCR) analysis of DNAs prepared from tail samples of the mice. The primers used were 5′-TCGGACGACAA GAGACAATC-3′ and 5′-TAGGGGCCACACAGAAAACA-3′ for chimeric PrP genes, and the primer combination was as previously described for the mouse PrP genotype analysis [22]. These transgenic mice (MH2M and MHM2 with PrP0/0 background) were also used for the transmission experiment.

Transmission experiment

Brains were stored at −80 °C, and brain homogenates (10% w/v) were prepared in phosphate-buffered saline (PBS). The homogenate was centrifuged at 3000 rpm for 5 min, and 20 μL of the supernatant was inoculated intracerebrally into the animals.

Western blot

The brains (brain stem) were homogenized (10%, w/v) in 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, 10 mM EDTA, and 10 mM Tris–HCl (pH 7.5) and then centrifuged at 3000 × *g* for 5 min. The supernatant was incubated with 50 μg/ml of proteinase K (PK) for 30 min at 37 °C. The sample was incubated with 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefablock, Roche; final concentration: 1 mM) for 5 min at 37 °C and then mixed with an equal volume of sodium dodecyl sulfate (SDS) sample buffer. Western blot (WB) analysis was carried out as described previously, and the mouse polyclonal antibody Ab. Tg was used as the primary antibody [22]. PrP signal intensity was calculated using ChemiImager (Alpha Innotech Co.). For PrP^{C} detection, the supernatant of the brain homogenate was subjected to WB without PK digestion, and the detection was carried out with biotinylated Ab. Tg as described previously [22].

Results

PrP glycoform in interspecies transmission

Mouse and hamster PrP^{C} glycoforms were analyzed. The brain homogenates of normal mouse and hamster were subjected to WB without PK digestion. As shown in Fig. 1A, in addition to the major band of diglycosylated PrP, 2 other bands (monoglycosylated and unglycosylated PrPs) were detected from the mouse brain homogenate. However, the diglycosylated PrP band was the main band in hamster PrP^{C} and the other 2 PrP bands were either not observed or were of low intensity.

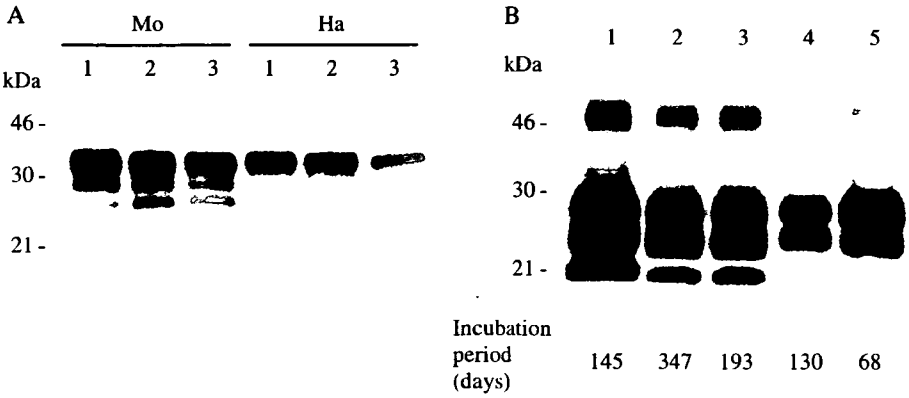

Fig. 1. PrP glycoform analysis in interspecies transmission. **A**, Western blot (WB) analysis of PrP^{C} in mouse (*Mo*) and hamster (*Ha*). Homogenates of cerebrum (*1*), cerebellum (*2*), and medulla (*3*) were subjected to WB without proteinase K (PK) digestion. **B**, WB analysis of PrP^{Sc} in hamsters inoculated with mouse-passaged scrapie prion. *1* Obihiro-strain-inoculated mouse brain; *2* Obihiro-strain-inoculated hamster brain (primary passage); *3* second-passaged hamster brain; *4* third-passaged hamster brain; *5* hamster passaged Sc237 strain inoculated hamster brain. The incubation periods of the examined animals are indicated below. Molecular weights are indicated on the left

This result shows that PrP^{C} glycoform differed depending on the animal species. A difference in the PrP^{C} glycoform ratio was not observed among the 3 brain regions that were examined (Fig. 1A).

Mouse and hamster brains that were inoculated with host-adapted prion showed a different PrP^{Sc} glycoform ratio (Fig. 1B, lanes 1 and 5); it was similar to that of the host PrP^{C}. In interspecies transmission, the glycoform ratio of accumulated PrP^{Sc} was analyzed after adaptation to another host. For this purpose, mouse-adapted Obihiro prion was transmitted intracerebrally into hamsters, and the diseased hamster brain homogenate was subsequently inoculated into other hamsters. At primary and secondary passages, the PrP^{Sc} glycoform ratio was similar to that of the Obihiro-inoculated mouse (Fig. 1B, lanes 1–3). In contrast, at the third passage, the PrP^{Sc} glycoform was altered and was similar to that of the Sc237-inoculated hamster (Fig. 1B, lanes 4 and 5).

Incubation periods and PrP^{Sc} glycoform correlation in interspecies transmission

Widely ranging incubation periods were observed in interspecies transmission, particularly in the primary and secondary passage [9]. The relationship between the PrP^{Sc} glycoform ratio and incubation periods was examined. The incubation periods of Obihiro-inoculated hamsters from primary to third passages were classified into 3 groups – below 200 days, between 200 and 300 days, and over 300 days. PrP^{Sc} glycoform was compared among these 3 groups. As shown in Fig. 2, when the incubation period was below 200 days, the PrP^{Sc} glycoform ratio of the Obihiro-inoculated hamsters was similar to that of the Sc237-inoculated hamsters. In

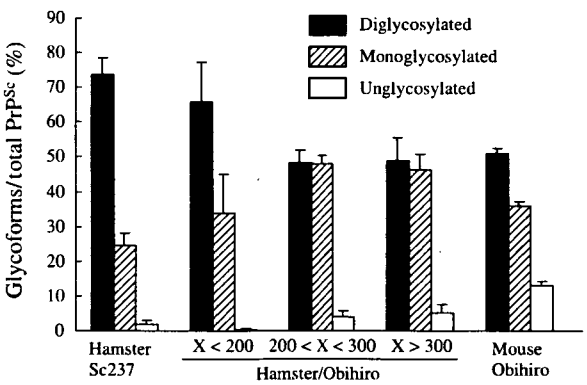

Fig. 2. Correlation between PrP^{Sc} glycoform and incubation periods. Obihiro-strain-inoculated hamsters (from primary to third passage) were categorized by their incubation periods (X; 3 hamsters, below 200 days; 3 hamsters, between 200 and 300 days; and 14 hamsters, over 300 days). Glycoform ratio of PrP^{Sc} was calculated. Means and SDs are shown

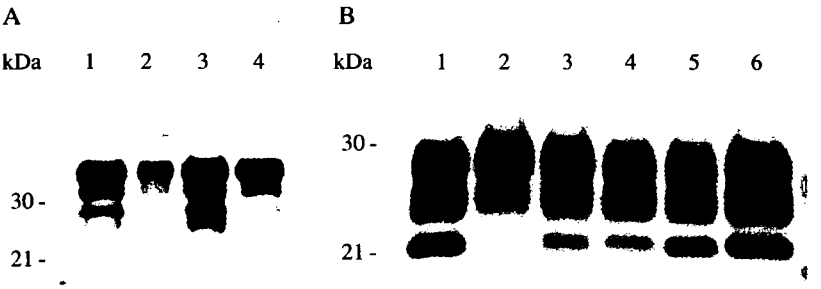

Fig. 3. PrP glycoform analysis in mouse and hamster chimeric PrP expression mice. **A**, Western blot (WB) analysis of PrP^C in mouse (*1*), hamster (*2*), MHM2 mouse (*3*), and MH2M mouse (*4*). Brain homogenate of each animal was subjected to WB without PK digestion. **B**, WB analysis of PrP^{Sc} in Obihiro- and Sc237-affected transgenic mice. *1* Mouse (Obihiro); *2* hamster (Sc237); *3* MH2M (Sc237); *4* Obihiro (MH2M); *5* MHM2 (Sc237); *6* MHM2 (Obihiro). Molecular weight markers are indicated on the left

contrast, in the case of the other 2 groups of longer incubation periods, the PrP^{Sc} glycoform ratios of the Obihiro-inoculated hamsters were similar to that of the Obihiro-inoculated mice. The PrP^{Sc} glycoform ratio was altered depending on the prion adaptation.

PrP glycoform ratio in transgenic mice

To understand the molecular mechanisms of glycoform transition with prion adaptation, we examined the PrP glycoform ratio in transgenic mice that expressed mouse and hamster chimeric PrP (MH2M and MHM2). Three PrP bands were detected in PrP^C of MHM2 mice, and the glycoform ratio of the MHM2 mouse PrP^C was similar to that of the mouse PrP^C (Fig. 3A, lanes 1 and 3). In contrast, the glycoform ratio of MH2M mouse PrP^C was similar to that of the hamster PrP^C (Fig. 3A, lanes 2 and 4). This result showed that these transgenic mice expressed different glycomodified PrP^C.

Mouse- and hamster-adapted scrapie prions were inoculated intracerebrally into both transgenic mice. The incubation periods of these mice are shown in Table 1. The MHM2 mice, which expressed the mouse-type PrP^C, were susceptible to the Obihiro prion; on the other hand, the MH2M mice, which expressed the hamster-type PrP^C, were susceptible to the Sc237 prion. The PrP^{Sc} glycoform ratio of these transgenic mice was analyzed. As shown in Figs. 3 and 4, a difference in the unglycosylated PrP band ratio was observed between the MH2M and MHM2 mice. In the MHM2 mice, there was a resemblance in the PrP^{Sc} glycoform ratio between

Table 1. Incubation periods in prion-inoculated mice and hamsters

Animal	PrP^C glycoform	Prion strain[a]	
		Obihiro	Sc237
Mouse	Mo	146.0 ± 1.0[b]	no transmission
MHM2	Mo	164.7 ± 5.9	420.5 ± 17.2
MH2M	Ha	187.4 ± 7.5	102.3 ± 3.6
Hamster	Ha	385.2 ± 15.7	69.0 ± 1.0

[a] Mice-adapted "Obihiro" strain or hamster-adapted "Sc237" strain was intracerebrally inoculated.
[b] Incubation periods (days); Mean ± SD.

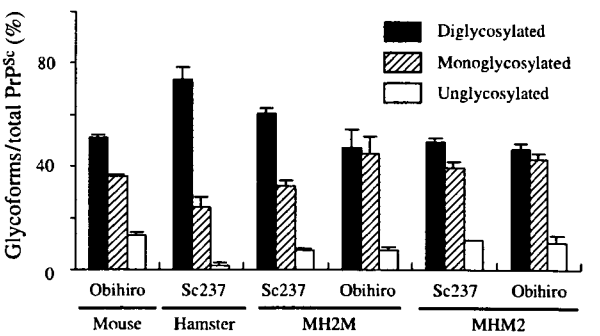

Fig. 4. Glycoform ratio of PrP^{Sc} in transgenic mice. Western blot (WB) signal intensity of diglycosylated, monoglycosylated, and unglycosylated PrP bands are indicated. Means and SDs are shown