Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disease of cattle and belongs to a family of transmissible spongiform encephalopathies or prion diseases. A proteinaceous pathogen, termed prion, is regarded as the causative agent [12]. Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, and chronic wasting disease in cervids are also included in this category. Since BSE prion is thought to cause variant CJD in humans [2, 4], it has been highlighted as a public health problem.

Prion is considered to consist of an abnormal isoform of the prion protein (PrPSc), as the main component [8]. PrPSc is generated by post-translational modification of the host-encoded normal cellular prion protein (PrP), termed PrPC [10]. PrPSc is known to have unusual resistance to various physicochemical treatments for inactivation [17]. Though PrPC is easily digested by proteinase K (PK), PrPSc partially resists PK and is cleaved to a smaller fragment called PrPcore or PrP27–30 [1], which lacks an N-terminal subregion of 60 to 70 amino acids. Thus, PK digestion is used to distinguish PrPSc from PrPC and is a key to the diagnosis of prion disease.

Currently, diagnosis of BSE is based on postmortem detection of PrPSc in brain tissue, by means of immunological techniques such as enzyme linked immunosorbent assay (ELISA), Western blotting (WB), and immunohistochemistry (IHC), together with histopathology [11]. Several commercial kits have been developed and applied for mass surveillance. With the institution of surveillance programs, BSE has been newly recognized in several countries. Although the occurrence of BSE has decreased in the UK, where it was originally reported [19], it has since spread to other countries.

In Japan, following the occurrence of BSE for the first time in 2001 [7], mass surveillance at abattoirs was started and the Platelia BSE kit (Bio-Rad, France) has introduced as the principal screening test. For confirmation, WB, histopathology and IHC are performed. With this active surveillance, six more BSE cases were detected by the end of June 2003, and these cases were all sampled at abattoirs. To understand the current status of BSE in Japan, examination of fallen stock has been strengthened, and all dead cattle over 24 months old are now required to be subjected to a BSE test. Livestock hygiene service offices that conduct the fallen stock examination are obliged to deal with considerable numbers of deteriorated samples from carcasses that have been left for long periods under poor storage conditions. It has been reported that PrPSc can be detected in autolyzed and/or deteriorated samples with WB and IHC [3, 5, 6, 13, 14], but there is no information about the effects of sample condition on PrPSc detection with the ELISA.

The Platelia BSE kit, which is an antigen-capture ELISA, uses two monoclonal antibodies (mAbs). The mAb used for antigen-capture in this kit recognizes an octarepeat sequence (personal communication with Mr. K. Sugimura from Nippon Bio-Rad Laboratories, Tokyo, Japan), which is located adjacent to (but mostly not included) in the PK-resistant PrPcore region, and therefore the epitope can be digested by PK or other enzymes. The epitope of the other mAb used
for detection is located within PrPcore. Because of this mAb combination, it is critical for this ELISA to control PK treatment precisely. PrPSc has to be digested away, whereas PrPSc has to be conserved with the epitope in the octarepeat region that is PK-sensitive. It is likely that endogenous or secondarily proliferated bacterial enzymes are active in postmortem tissues, which may induce conversion of PrPSc to PrPcore if the carcass is left for a long time before the brain is harvested, especially in warm summer conditions.

To evaluate current BSE surveillance procedures for fallen stock in Japan, we examined PrPSc detection in artificially deteriorated BSE brain samples by means of Platelia-BSE ELISA and WB.

MATERIALS AND METHODS

Preparation and treatment of brain tissue: BSE-affected brain samples were provided courtesy of the Veterinary Laboratories Agency, Weybridge, UK. Brain stem tissues of five BSE-positive cattle were pooled and then minced well to make PrPSc content uniform. Approximately 12 g from the pooled tissue was placed in a 50-ml plastic tube and incubated at 30°C for 4 days. Small aliquots were sampled every day (Experiment [Exp.] 1). In another experiment, minced tissue was divided into 5 small aliquots, followed by incubation at 37°C for 0 to 4 days, respectively (Exp. 2). After incubation, tissue samples were stored at −80°C until use.

ELISA: Twenty-percent brain homogenates were prepared in 5% glucose (Platelia BSE kit homogenization buffer) and subjected to ELISA and WB. The ELISA procedure followed the manufacturer’s protocol. To more efficiently estimate the difference in PrPSc detection depending on the incubation time, samples were serially diluted and subjected to ELISA, and optical density (OD) values at 450/620 nm were compared.

Western Blotting: For WB, 250 µl of the homogenate was mixed with an equal volume of detergent buffer, containing 4% sulphobetaine 3–14 and 1% sarkosyl, and then incubated with 500 µg/ml collagenase, followed by incubation with 40 µg/ml PK at 37°C for 30 min. After PK digestion was terminated with 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF; Pefabloc SC, Roche Diagnostics GmbH, Mannheim, Germany), the sample was mixed with 250 µl of an 2-butanol:methanol (5:1) mixture and then centrifuged at 20,000 × g for 10 min. The pellet was heated at 100°C for 5 min in sodium dodecyl sulfate (SDS)-containing sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and electrical blotting to poly-vinylidene fluoride membrane. The blotted membrane was incubated with peroxidase-conjugated anti-mouse immunoglobulin (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, U.S.A.) at RT for 45 min. Signals were developed with a chemiluminescence substrate (SuperSignal, Pierce Biotechnology, Inc., Rockford, IL, U.S.A.).

RESULTS

Gross findings of deteriorated tissue samples: After the 4-day incubation, the color of the tissue had changed to yellow-brown or grayish-green and had also liquefied. The severity of the gross damage and effluvium increased with longer incubation times except in one sample. The color of the sample incubated for 2 days in Exp. 2 was dark grayish brown; more severe deterioration was seen than the samples incubated for 3 and 4 days.

Detection of PrPSc by ELISA: The ELISA OD values for all samples reached a plateau when samples were diluted at 1:4 or undiluted in Exp. 1. The plateau levels for the samples incubated for 2 days or more were lower than those for fresh and 1-day-incubated samples (Fig. 1A). Although the OD values fluctuated somewhat, a similar tendency was also observed in Exp. 2, with the exception of the 2-day-incubated sample (Fig. 1B). The color of all reaction solutions in the wells of the undiluted samples was dark yellow. One reason for the similar OD values of all undiluted samples and the samples diluted 1:4 seemed to be color quenching. For the unincubated sample in Exp. 1, the OD value was increased by dilution to 1:4 (Fig. 1A). This might have been due to generation of brown precipitates in the reaction solution of the undiluted samples, in additional to color quenching. Accordingly, the values for all undiluted samples diverged from the dose-dependent curve. Therefore, comparison of the OD values among the samples was carried out with the values for samples diluted 1:4 or 1:16, where dose-dependency was observed. The 2-day-incubated sample in Exp. 2 containing the above-mentioned severely deteriorated tissue, had a remarkably lower OD value than did the others at any dilution (Fig. 1B). This sample was omitted from the comparison in the time course of the deterioration.

As shown in Fig. 1A of Exp. 1, incubation at 30°C for 1 day caused no significant change in detection of PrPSc by ELISA. Incubation for one additional day induced a significant reduction in OD values, but no further reduction in OD values was observed with further incubation for up to 4 days. In Exp. 2, where incubation was carried out at 37°C, one-day incubation caused a significant decrease in the OD value. The OD values for the incubated samples diluted at 1:16 were similar, with the exception of the 2-day-incubated sample. This was consistent with the results of Exp. 1, in which the observed decrease in OD values occurred only during the early stage of deterioration. When deteriorated samples were diluted up to 1:16, the OD values remained above the cutoff value for a positive diagnosis (Figs. 1A and B), whereas at a dilution of 1:64 the values for the samples incubated at 30°C for 2 days or more and at 37°C for 1 day
or more, fell below the cutoff value (Fig. 1B). This suggests that the accuracy of diagnosis of BSE based on ELISA testing of deteriorated cattle brain tissues may be unreliable if the suspect tissue contains less than 1/64th the amount of PrP^Sc particles that were present in the BSE brain tissues used here.

Detection of PrP^Sc by Western Blotting: As shown in Fig. 2A, PrP^Sc was consistently detected by mAb T2 in all samples after PK treatment. The intensity of the PrP^Sc signals in respective samples incubated for various time periods remained almost the same, i.e., sample deterioration did not affect the signal intensity. When PK treatment was omitted, the differential signal intensity of two major bands, of 32–36 and 27–30 kDa, shifted, depending on how long the tissue was incubated. The signal of the 32–36-kDa band, which represents full-length PrP, gradually diminished with increasing incubation time, whereas that of the 27–30-kDa band, representing PrP-core, became dominant after incubation for 3 days or more (Fig. 2B). Consistent with this shift, the PrP signal detected by mAb 1H7, which recognizes the octarepeat region, decreased depending on the incubation time of the tissue (Fig. 2C). In Exp. 2, one sample (incubated for 2 days) showed a remarkably lower OD value in the ELISA; nevertheless, the WB result showed that there was no less PrP^Sc in this sample than in the other samples, and that it still harbored the N-terminal sub-region (octare-
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Inhibition of ELISA: The results of WB suggested that the markedly low OD value of the 2-day-incubated sample in Exp. 2 was not caused only by digestion of the N-terminal sub-region of PrPSc during incubation of the brain tissue. It was also noted that the OD values of the samples diluted 1:4 tended to be higher than those of undiluted samples (Fig. 1B). These findings led us to hypothesize that the 2-day-incubated sample with severe deterioration contained some inhibitor(s) for reactivity in the ELISA. Therefore, we investigated the possible presence of an inhibitor in the deteriorated brain samples. Samples were prepared for the ELISA from fresh or artificially deteriorated BSE-negative (normal) cattle brain tissue, and then mixed with an equal volume of a sample prepared from fresh BSE-positive cattle brain tissue. The ELISA procedure was carried out following the manufacturer’s instructions. As shown in Table 1, in the sample incorporating normal brain tissue incubated for 2 days at 37°C, detection of PrPSc by the ELISA was clearly inhibited. For the control sample, containing fresh normal cattle brain tissue, no inhibition of the ELISA was observed, relative to buffer.

In the manufacturer’s protocol, the solubilization step for the preparation of ELISA samples includes a boiling time of 5 min. When the boiling time was prolonged to 30 min, some recovery of ELISA reactivity, relative to inhibited OD values, was noted (Table 1).

**DISCUSSION**

In the present evaluation, PrPSc could be detected with the Platelia BSE kit in all artificially deteriorated samples incubated for up to 4 days, but a reduction in the OD values was observed in the samples incubated for 2 days or more at 30°C, or for 1 day or more at 37°C. It has been reported that, like PK, several other proteases digest PrPSc [9, 16]. As the BSE-positive brain tissues used in this study were not aseptic, it is likely that PrPSc was partially digested by contaminating bacterial proteases in addition to endogenous enzyme(s) and thus lost its N-terminal region during the incubation. The capture antibody used in the Platelia BSE kit recognizes the octarepeat sequence (personal communication with Mr. K. Sugimura from Nippon Bio-Rad Laboratories, Tokyo, Japan), which is adjacent to the N-terminal of PrPcore and therefore sensitive to protease digestion so that loss of the antibody-recognition sequence may cause the reduction in the OD value in the ELISA. This was strongly supported by the WB results that we obtained when PK treatment was omitted from sample preparation (Figs. 2B and C, and 3B).

There was a discrepancy between the ELISA results and those for WB. Although the OD values were significantly decreased after 2 days at 30°C or 1 day at 37°C, prolongation of incubation thereafter did not cause any further decrease, with the aforementioned exception of the 2-day-incubated sample in Exp. 2. On the other hand, WB showed a gradual reduction in the 32–36-kDa band and a reciprocal increase in the 27–30 kDa band depending on the incubation time in both experiments. This discrepancy may have been partly owing to the different antibodies that were used for detection of PrPSc in WB and in the ELISA, but the reason is

**Table 1. Inhibition of ELISA by deteriorated normal brain samples**

<table>
<thead>
<tr>
<th>Incubation time of normal samples (days)</th>
<th>Heating time in preparation (min)</th>
<th>OD value (450/620 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>3.09 ± 0.13</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>0.97 ± 0.06</td>
</tr>
<tr>
<td>Buffer</td>
<td></td>
<td>2.06 ± 0.06</td>
</tr>
</tbody>
</table>

a) Normal brain tissue was incubated at 37°C for 2 days to induce deterioration (2 days), and then mixed with equal volumes of fresh BSE-positive brain samples. Normal brain without deterioration (0 days) was used as the control.

b) Before mixing with the BSE-positive sample, the normal brain samples were boiled for 5 or 30 min.

c) Average ± standard deviation.

- Numbers at the top of each lane indicate days of incubation. PrP signals were detected with mAb T2. (A) Samples treated with PK. (B) Samples prepared without PK treatment. Despite the remarkably low OD value on the ELISA, PrPSc in the 2-day-incubated tissue represented in lane 2 showed no significant loss and no more severe partial digestion than the other samples. The equivalent of 500 µg of wet brain tissue was loaded in each lane. Molecular mass markers (kDa) are shown on the right.

![Fig. 3. Western blotting analysis of PrPSc from tissue incubated at 37°C in Exp. 2. Numbers at the top of each lane indicate days of incubation. PrP signals were detected with mAb T2. (A) Samples treated with PK. (B) Samples prepared without PK treatment. Despite the remarkably low OD value on the ELISA, PrPSc in the 2-day-incubated tissue represented in lane 2 showed no significant loss and no more severe partial digestion than the other samples. The equivalent of 500 µg of wet brain tissue was loaded in each lane. Molecular mass markers (kDa) are shown on the right.](image-url)
not clear.

WB revealed that PrPSc was not always completely digested to PrPcore, even after 4 days of incubation (Figs. 2B and C, and 3B). In this study, we used brain tissues from BSE-affected cattle with typical clinical symptoms. Enough PrPSc was evidently contained in the original tissues to compensate, in the ELISA, for the loss of the N-terminal moiety of PrPSc by deterioration. In the pre-clinical or early stages of infection, the amount of PrPSc in brain tissue might be far less. In such cases, it is probable that deterioration would seriously compromise the ELISA results.

The sample incubated for 2 days in Exp. 2 had an especially low OD value (Fig. 1B), but the PrP band pattern of the sample in WB (Fig. 3B, lane 2) was similar to that of the 2-day-incubated sample in Exp. 1 (Fig. 2B, lane 2). It is difficult to entirely explain this low OD value by partial digestion of PrPSc accompanying the deterioration. Thus, participation of an inhibitor(s), generated by the deterioration of brain tissues, was suspected, as suggested by the data in Table 1. The observed increase in the OD value of the 2-day-incubated sample diluted 1:4, relative to the undiluted sample, suggests that the effect of the inhibitor(s) is decreased by dilution. The inhibitor(s) also seems to be partially thermolabile, since prolonged boiling decreased inhibition in the deteriorated sample, as seen in Table 1. Therefore, the OD value of the ELISA in deteriorated samples seems to be affected not only by partial digestion of PrPSc but also by the presence of some inhibitors of ELISA reaction.

In this study, we pooled brain tissues from several BSE-positive cattle, and thoroughly minced and mixed them before use. In Exp. 2, minced tissues were aliquoted into 5 separate tubes prior to incubation. As the original tissues were not aseptic, and as the minced and mixed tissues were not completely homogenous microbiologically, due to the presence of some small lumps, massive deterioration might have occurred because of the proliferation of contaminating bacteria in the tube of the 2-day-incubated sample. Although it is almost impossible to verify the degree of deterioration of brain tissue within the skull of a cattle carcass, our results seemed to indicate the need to be cautious regarding the possible presence of ELISA inhibitors in severely deteriorated tissue. To avoid or minimize the generation of such inhibitors, tissue specimens must be collected as soon as possible when fallen cattle are found.

Another problem in testing fallen stock for BSE may arise from unequal distribution of PrPSc in BSE-affected brains. Spongiform changes and accumulation of PrPSc are most frequently observed in the obex region [15, 18], but, it could be quite difficult to collect the obex region precisely from extensively deteriorated and liquefied brain tissue. Furthermore, in such cases it would be difficult to perform IHC as a confirmation test.

It has been shown that sample autolysis does not affect detection of PrPSc by means of WB [3, 5, 13]. Our WB results also demonstrated no reduction in the PrPSc signal as a result of deterioration at 30°C or 37°C for up to 4 days, as so far examined (Figs. 2A and 3A). In this study, we showed that several problems undermine the utility of the ELISA with deteriorated samples, whereas WB remains very dependable. Therefore, WB might be the only reliable procedure to detect PrPSc in severely damaged samples from fallen stock.

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REFERENCES


