INTRODUCTION

Prion diseases, commonly referred to as transmissible spongiform encephalopathies (TSEs), are neurodegenerative disorders that have been described in humans as Creutzfeldt–Jakob disease, in sheep and goats as scrapie, and in cattle as bovine spongiform encephalopathy (Prusiner, 1991). A characteristic of TSEs is the accumulation of a protease-resistant, misfolded prion protein (PrPSc), which is the pathogenic form of the host-encoded prion protein (PrPC) (Caughey et al., 1991; Pan et al., 1993). PrPSc accumulation occurs in the central nervous system (CNS) of infected subjects through the structural conversion of PrPC. TSE infectivity has also been demonstrated in a variety of peripheral tissues (Prusiner, 1998) and body fluids such as blood (Houston et al., 2000; Taylor et al., 2000; Holada et al., 2002; Hunter et al., 2002; Cervenakova et al., 2003) of infected subjects.

Chronic wasting disease (CWD) is the only prion disease known to affect free-ranging mule and deer (Williams, 2005), and can spread horizontally (Miller et al., 1998, 2000). Therefore, it was suspected that transmission of CWD occurred through contaminated saliva, faeces and urine, and it has been demonstrated that the saliva of infected deer contain the infectious agent (Mathiason et al., 2006). With regard to excrement, a protease-resistant urinary PrP, designated uPrPSc, has been found in the urine of infected animals and humans (Shaked et al., 2001); however, it was reported that the anti-uPrPSc reactivity was primarily due to an immunoglobulin light chain (Serban et al., 2004; Head et al., 2005) or a contaminated bacterial component (Furukawa et al., 2004). There are several reports that demonstrate infectivity in urine during scrapie infection. Urine from scrapie-infected mice with concurrent nephritis was infectious (Seeger et al., 2005). A concentrated sample from pooled urine collected at the terminal stage of the disease exhibited a low level of infectivity in hamsters (Kariv-Inbal et al., 2006). The result obtained in hamsters is important from the viewpoint that the urinary excretion of PrPSc possibly occurred without severe complications; however, the time course and frequency of appearance of urinary PrPSc remain to be determined.

As the PrPSc level in urine is expected to be diminutive when compared with that in the brain tissue, conventional immunoassays might not be applicable to the detection of urinary PrPSc. Recently, it has become possible to perform in vitro amplification of hamster PrPSc by protein misfolding cyclic amplification (PMCA) (Saborio et al., 2001). By repeated sonication and incubation, PrPSc converts PrPSc to the protease-resistant form (PrPRes); this PrPRes then becomes the new nucleus, thereby facilitating PrPRes formation in the cyclic incubation process. PMCA is a highly effective method for detecting minute amounts of hamster PrPSc (Castilla et al., 2005a; Saá et al., 2006a) and enables the detection of PrPSc in the blood of infected hamsters (Castilla et al., 2005b; Saá et al., 2006b).
In the present study, using the PMCA technique, we examined the urinary excretion of PrPSc in hamsters that were inoculated intracerebrally or orally with the scrapie prion strain Sc237. We also investigated PrPSc in the plasma, Buffy coat and urine during the period from latent to terminal stages of the disease and compared PrPSc dynamics among the above-mentioned samples. This paper is the first demonstration of urinary PrPSc in any disease model.

METHODS

Inoculation of the hamster prion. The hamster-adapted scrapie prion strain Sc237 was propagated in hamsters. The brains of hamsters at the terminal stage of the disease, with a titre of 5 × 10⁶.5 LD₅₀ g⁻¹ by bioassay (Murayama et al., 2006), were pooled and homogenized at a 10 % concentration (w/v) in PBS. The homogenate was injected intracerebrally (20 μl per hamster) or administered orally (100 μl per hamster) using a gastric tube. All animal experiments were performed in accordance with National Institute of Animal Health guidelines.

Preparation of urine and blood samples. Urine samples were collected at several time points after inoculation. From noon to about 3 p.m., the hamsters were held with both hands for spontaneous urination in a sterilized Petri dish. To separate urinary exudates such as leukocytes and epithelial cells of renal tubules from urine, the individual or pooled urine sample (20 μl) was diluted 1 : 10 with PBS and passed through a 0.45 μm membrane filter unit (Ultrafree-MC; Millipore). The flow-through in the collection tube was removed and 40 μl of 2 % Triton X-100, 8 mM EDTA in PBS was added to the membrane. The entire surface of the membrane was rinsed with the detergent solution by pipetting, and the membrane unit was incubated for 30 min at room temperature. After centrifugation, the flow-through was recovered and stored at −80 °C until use.

For blood collection, hamsters were sacrificed at several time points after inoculation. From noon to about 3 p.m., the hamsters were held with both hands for spontaneous urination in a sterilized Petri dish. To separate urinary exudates such as leukocytes and epithelial cells of renal tubules from urine, the individual or pooled urine sample (20 μl) was diluted 1 : 10 with PBS and passed through a 0.45 μm membrane filter unit (Ultrafree-MC; Millipore). The flow-through in the collection tube was removed and 40 μl of 2 % Triton X-100, 8 mM EDTA in PBS was added to the membrane. The entire surface of the membrane was rinsed with the detergent solution by pipetting, and the membrane unit was incubated for 30 min at room temperature. After centrifugation, the flow-through was recovered and stored at −80 °C until use.

PMCA and Western blotting. The PMCA and Western blotting procedures have been described in our previous study (Murayama et al., 2006). Briefly, normal hamster brains were homogenized at a 10 % concentration (w/v) in PBS containing complete protease inhibitors (Roche Diagnostics), 1 % Triton X-100, and 4 mM EDTA. To avoid contamination, the PrPSc source was prepared in a laboratory in which infected materials had never been handled. The urine and plasma samples were diluted 1 : 10 and 1 : 100, respectively, in the PrPSc source (total 100 μl). Buffy coat samples were prepared by three freeze-thaw cycles and mixed with 100 μl of the PrPSc source. Amplification was performed using a fully automatic cross-ultrasonic amplification apparatus (ELESTEIN 070-GOT; Elekon Science Corp.), which has a capacity to generate high ultrasonic power (700 W). PMCA was performed by 40 cycles of sonication (a pulse oscillation for 3 s was repeated five times at 1 s intervals), followed by incubation at 37 °C for 1 h with gentle agitation. The amplified product obtained after the first round of amplification was diluted 1 : 10 in the PrPSc source and a second round of amplification was performed. The process was repeated to obtain the amplified products. Samples were treated with proteinase K (50 μg ml⁻¹), separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). After blocking, the membrane was incubated for 1 h with 1 : 10000-diluted alkaline phosphate-conjugated 3F4 monoclonal antibody (mAb; Signet Laboratories). After washing, the blotted membrane was developed using Immobilon Western Chemiluminescent AP substrate (Millipore) according to the manufacturer’s instructions. After exposure for 5 min, chemiluminescence signals were analysed using a Light Capture System (ATTO).

Histological analysis. The urinary PMCA product obtained by 160 amplification cycles (see Fig. 1b, lane f) was diluted 1 : 10 with PBS and injected intracerebrally into Tg52NSE mice (20 μl per mouse), which overexpress hamster PrP in their nervous system (Race et al., 1995). Densitometric analysis of Western blots revealed that the PrPres signal intensity in the urinary PMCA product was approximately one-eighth of that in the 1 % homogenate of Sc237-infected brain. Therefore, the 10 % homogenate of Sc237-infected brain was diluted 1 : 800 and injected into Tg52NSE mice as a control. Tg52NSE mice exposed to the urinary PMCA product and Sc237-infected brain homogenate were sacrificed at 148 and 62 days post-inoculation (p.i.), respectively.

The left hemisphere of the brain was fixed in 10 % buffered formal saline (pH 7.4). Coronal slices of 2 mm thickness were immersed in 98 % formic acid to diminish prion infectivity (Taylor et al., 1997) and embedded in paraffin. Serial sections were mounted on new silane II-coated glass slides (Muto Pure Chemicals Co. Ltd) and routinely stained with haematoxylin and eosin. For the detection of immunoreactive PrPSc, dewaxed sections were immersed in 98 % formic acid for 5 min and then treated with 3 % hydrogen peroxide in methanol to block endogenous peroxidase activity. Sections were treated by autoclaving for 3 min at 121 °C in a low-ionic-strength buffer [2.1 mM Tris/ HCl (pH 7.8), 1.3 mM EDTA, 1.1 mM sodium citrate; Asante et al., 2006]. After washing in distilled water, the sections were placed on an automated immunohistochemical stainer (Autostainer Universal Staining System; DakoCytomation). mAb T1, which was generated in PrP-deficient mice by immunization with recombinant mouse PrP121–231 and which recognizes mouse PrP138–144, was used as the primary antibody. Anti-mouse universal immunoperoxidase polymer (Histofine Simple Stain MAX-PO (M); Nichirei) was used as the secondary antibody, and 3'3'-diaminobenzidine tetrachloride was used as the chromogen. Astrocytes were immunostained with a mouse anti-human glial fibrillary acid protein antibody (GFAP; DakoCytomation) after heat-mediated antigen retrieval. All sections were counterstained with haematoxylin.

RESULTS

Detection of urinary PrPSc in Sc237-infected hamsters

In the Western blot analysis, PrPSc propagation and accumulation in hamster brains became apparent 38 days after intracerebral inoculation (data not shown). The mean incubation time of the inoculated hamsters was 70 ± 2 days (mean ± SD, n = 58) and the onset of clinical signs appeared 4–7 days prior to death. Fig. 1(a) illustrates the results of the amplification of the urine samples. No PrPres was amplified in the uninfected control samples and pooled samples obtained during the period 26–46 days p.i. after 280 amplification cycles. In contrast, PrPres was detected...
after 160 cycles in two different pools of urine collected from five hamsters at 60 days p.i. To examine the incidence of urinary PrPSc excretion at the terminal stage of the disease, urine was collected from seven individual hamsters at 68 days p.i. and amplified (Fig. 1b). PrPres signals were detected in two samples (Fig. 1b, hamsters 'e' and 'f') after 120 amplification cycles. Based on previous data (Murayama et al., 2006), we estimated that the level of PrPSc present in these samples corresponded to that present in a 10^{-9}-diluted infected brain homogenate; such a dilution is well below the level detectable by any other method. PrPres signals were detected in one sample (Fig. 1b, hamster 'c') after 160 cycles, in two samples (Fig. 1b, hamsters 'b' and 'g') after 200 cycles, and in one sample (Fig. 1b, hamster 'a') after 280 cycles of amplification. In contrast, no signals were detected in hamster 'd' after 280 amplification cycles.

We also examined the detection limit of our amplification system (Fig. 2) and confirmed that PrPSc present in an infected brain homogenate diluted 1:10^{10} could be detected after 160 cycles, and that PrPres signals were enhanced in a similar manner during the further amplification process in the duplicated samples. However, in the more extreme dilution range, the number of amplification cycles required to amplify PrPSc to a detectable level varied in the duplicated samples. After 240 amplification cycles, both samples diluted 1:10^{12} became positive for PrPres; however, the PrPres signal remained very weak in one of the samples that was diluted 1:10^{14}, even after 280 cycles. As PrPSc tends to aggregate, the weak detection of PrPres in the reaction may have been due to the near-absence of PrPSc seed, which would have been almost completely diluted out. Therefore, it was assumed that PMCA with 280 cycles could reproducibly detect PrPSc present in the samples at a level equivalent to that present in an infected brain homogenate diluted 1:10^{12}. Taken together, the PMCA analysis results indicated that PrPSc was excreted in the urine in a higher rate (86%, six out of seven hamsters in Fig. 1b) at the terminal stage of the disease, although PrPSc concentrations differed considerably among the urine samples.

---

**Fig. 1.** (a) PMCA results from urine samples obtained from hamsters inoculated intracerebrally with Sc237. The PrPSc source was mixed with 0.1 vols of the pooled urine samples prepared as described in Methods, and one round (40 cycles) of the PMCA reaction was performed. This process of 1:10 dilution of the PMCA product and subsequent amplification was repeated six times. Samples before (−) and after (+) amplification were analysed by Western blotting following digestion with proteinase K. Lanes: a–d, negative controls in which the urine sample from an uninfected hamster was used for PMCA. No signals were detected in these samples. Arrows indicate the positions of molecular mass markers corresponding to 30 and 20 kDa. (b) PMCA results from urine samples obtained at the terminal stage of disease. Urine samples were prepared from seven individual hamsters (labelled ‘a’–‘g’) and used for sequential PMCA. NS, No seed: negative control reaction in which uninfected brain homogenate (PrPSc source only) was treated in the same manner; NT, not tested.
Histological analysis of brain tissue infected with urine-derived PMCA product

Neuropathologically, vacuolar degeneration was detected in the thalamus, mesencephalon and medulla oblongata of Tg52NSE mice inoculated with Sc237 and the PMCA product (Fig. 3). This vacuolation was present mainly in the neuropil but was rare in the neurons. No vacuolation was observed in control mice brains. Granular-type PrPSc accumulation principally associated with the presence of vacuolation was observed in the neuropil and neurons of the thalamus, mesencephalon and medulla oblongata stained with mAb T1. Swollen or hypertrophic astrocytes proliferated in the same lesion that showed PrPSc accumulation. No significant difference was observed in vacuolar change, PrPSc accumulation and astrogliosis between Tg52NSE mice inoculated with Sc237 and those inoculated with the PMCA product.

PrPSc levels in the blood

As it has been demonstrated that PrPSc binds to a blood component (Fischer et al., 2000), we examined PrPSc levels in both the plasma and buffy coat samples collected during the period of detection of urinary PrPSc. Fig. 4(a) shows the results of the amplification of buffy coats obtained at 60 and 68 days p.i. All samples became positive for PrPres signals after 160 (60 days p.i.) or 120 (68 days p.i.) amplification cycles; therefore, we confirmed that PrPSc could be detected in the buffy coat samples by PMCA, as reported previously (Castilla et al., 2005b). With regard to

---

**Fig. 2.** Sensitivity of detection of hamster scrapie Sc237 using sequential PMCA. The PrPSc seed was diluted from $10^{-10}$ to $10^{-14}$ and used as the PrPc source. Lanes a and b represent the results of duplicate samples. Arrows indicate 30 and 20 kDa molecular mass markers. NS, No seed.

**Fig. 3.** Pathology of Tg52NSE mice exposed to urine-derived PMCA product from hamster ‘f’ in Fig. 1(b) (PMCA) and Sc237-infected brain homogenate (Sc237). Thalamus sections of mice that succumbed to scrapie after intracerebral inoculation with the PMCA product or the infected brain homogenate showed similar levels of vacuolation, PrPSc accumulation (labelled with mAb T1) and astrogliosis (labelled with anti-GFAP antibody). Bars, 50 μm.
the plasma samples, PrP^res^ signals were detected after 160 amplification cycles in two samples (‘c’ and ‘d’) collected at 60 days p.i., whereas no signal was detected, even after 280 cycles, in other samples (Fig. 4b). In the three samples collected at 68 days p.i., 80–160 amplification cycles were required to amplify PrP^res^ to a detectable level. These results indicated that PrP^Sc^ was also present in the plasma at symptomatic and terminal stages of the disease. However, the detection sensitivity of PrP^Sc^ in plasma was low compared with that in buffy coats.

**PrP^Sc^ levels in urine and blood after oral administration**

The mean survival time of orally administered hamsters was 171 ± 15 days (mean ± SD, n=18). Fig. 5 illustrates the results of the amplification of urine samples collected between 4 and 155 days p.i. PrP^res^ was detected after 200–240 amplification cycles in two of four samples collected at 4 days p.i., and an additional sample became positive for PrP^res^ after 280 cycles. In contrast, PrP^res^ signals could not

Fig. 4. PMCA of buffy coat (a) and plasma (b) samples obtained at 60 and 68 days p.i. from individual hamsters inoculated intracerebrally with Sc237. The PrP^Sc^ source was mixed with the buffy coat sample prepared as described in Methods or with a 1:100 dilution of the plasma sample. Sequential PMCA was performed on three to five hamsters (labelled ‘a’–‘e’). Arrows indicate 30 and 20 kDa molecular mass markers. NS, no seed; NT, not tested.
be detected in urine obtained between 30 and 147 days p.i. At 155 days after administration, urinary PrP Sc was detected in two of three samples after 240–280 amplification cycles. This observation was in agreement with the results that urinary PrP Sc was detected in the symptomatic and terminal stage of disease after intracerebral inoculation.

Fig. 6 (a) and (b) illustrate the results of amplification of plasma and buffy coats, respectively, obtained from animals sacrificed during the period 30–166 days p.i. PrPSc was not detected in plasma collected at the asymptomatic stage (<75 days p.i.) (Fig. 6a). At 103 days p.i., the propagation and accumulation of PrPSc became apparent in brains (Fig. 6c); at this stage, PrPres signals were detected in the plasma after 160–200 amplification cycles (Fig. 6a). However, no PrPres signal could be detected in one plasma sample (sample b) collected at 103 days, even after 280 amplification cycles. No remarkable PrPSc accumulation was observed in the brain of the plasma donor (Fig. 6c, 103 days p.i., sample b) when compared with the other donor (Fig. 6c, 103 days p.i., sample a). With regard to the buffy coats, PrPres signals were detected after 120–160 cycles in all hamsters examined during the period 75–166 days p.i (Fig. 6b). We could not examine the buffy coat samples at 30 days p.i. because of a technical failure and could not verify the previous observation that PrPSc disappeared in the buffy coat fraction during the period of asymptomatic stage of the disease (Saa et al., 2006b).

**DISCUSSION**

In the present study, using PMCA, we have revealed for the first time the presence of PrPSc in the urine of hamsters infected intracerebrally or orally with scrapie, without any dialysis and concentration procedures. A characteristic property of the urinary PrPSc was its detectability only at the terminal stage of the disease, except for a few days immediately after oral administration (Fig. 7). We also examined PrPSc levels in blood and clarified that PrPSc could be detected in plasma as well as in buffy coat fractions. There was an obvious time lag between PrPSc excretion in the urine and the appearance of PrPSc in the blood, and urinary PrPSc did not increase in proportion to PrPSc accumulation in the CNS. Furthermore, in some cases, urinary PrPSc could not be detected, even during the terminal stage of the disease, using the highly sensitive PMCA technique. Hence, urinary PrPSc appears to be an unsuitable marker for the early diagnosis of prion infection.

With regard to urinary PrPSc amplification, 120 or more cycles were required to amplify PrPSc to a detectable level, indicating that the amount of PrPSc present may be insufficient to induce infection. Although an extremely small amount of PrPSc is excreted in the urine, PrPSc can survive in the natural environment for a long time. In fact, prions remain infectious after incubation for several years in soil (Brown & Gajdusek, 1991), and recent research has demonstrated that PrPSc adsorbs firmly to soil components (Leita et al., 2006) and that the soil minerals–PrPSc complex remains infectious (Johnson et al., 2006). Therefore, it is conceivable that PrPSc is captured and absorbed effectively on soil and is then concentrated. Furthermore, it has been indicated that soil ingestion behaviours have been observed frequently in wild animals including mule deer (Arthur & Alldredge, 1979). A recent study indicated that excrement-borne transmission was not proved in an experimental CWD infection (Mathiason et al., 2006); however, the possibility exists that some prion diseases may spread via contaminated soil in nature.

Where is the urinary PrPSc derived from? PrPres was amplified successfully from urinary material trapped on a filter membrane. The urinary material probably included leukocytes and epithelial cells from the renal tubules. In preliminary experiments, PrPres could not be amplified when the flow-through fraction was concentrated and used as a PMCA seed (data not shown); this suggests that PrPSc was primarily present in the insoluble and solid fraction in the urine samples. Kidney dysfunction or bacterial urinary tract infection might occur following disease progression, and lymphocyturia might arise at the terminal stage of the disease. However, the above elucidation does not provide a reasonable explanation for amplification of PrPRes in urine.
samples collected at 4 days after peroral inoculation. It has been demonstrated that PrPSc can be detected on migrating intestinal dendritic cells (DCs) immediately after oral administration (Huang et al., 2002), and that infected DCs can transmit the PrPSc prion to the CNS in recombination activating gene (RAG) knockout mice (Aucouturier et al., 2001). These observations indicated that DCs could transport PrPSc from the intestinal tract to other tissues and body fluids without any propagation of the prion in the peripheral tissues. DCs also exist in the kidney, and it has been demonstrated that renal DCs form a contiguous network that continuously surveys the tubulointerstitium (Soos et al., 2006). Renal DCs would capture PrPSc from the tubular lumen. Therefore, migrating DC-mediated transport may also be involved in urinary excretion of PrPSc, particularly in the early stage of infection.

Fig. 6. (a, b) PMCA results from plasma (a) and buffy coat (b) samples obtained at 30–166 days p.i. from hamsters inoculated orally with Sc237. Two hamsters (labelled 'a' and 'b') were sacrificed at each time point, and blood and brain samples were prepared. (c) PrPSc accumulation in the brains of hamsters after oral administration of Sc237. Serial twofold dilutions of 10% brain homogenate were prepared and the results analysed by Western blotting. NS, no seed; NT, not tested.

Fig. 7. Time course for the appearance of PrPSc in urine (●), plasma (×) and buffy coat (■) of orally infected hamsters.
The results of PrP<sub>res</sub> amplification in the plasma samples after oral administration provide a possible explanation for the origin of plasma PrP<sup>Sc</sup>. PrP<sub>res</sub> could not be amplified from the plasma samples collected at 30 and 75 days p.i. and from one sample at 103 days p.i.; PrP<sup>Sc</sup> accumulation could not be detected in the brains of these hamsters (Fig. 6). In contrast, PrP<sub>res</sub> could be amplified from all plasma samples that were obtained when PrP<sup>Sc</sup> propagation was apparent in the brains. These observations imply that PrP<sup>Sc</sup> might leak into the plasma from the infected brain, probably due to destruction of the blood–brain barrier. The source of PrP<sup>Sc</sup> in theuffy coats during the asymptomatic stage appeared to be the spleen and other lymphoid organs, as suggested previously (Saá et al., 2006b). A more detailed analysis needs to be performed to confirm whether plasma PrP<sub>res</sub> can serve as an indicator for the propagation of PrP<sup>Sc</sup> in the CNS.

ACKNOWLEDGEMENTS

We are indebted to Dr Yuichi Tagawa for kindly providing mAb T1. We also thank Ms Meihua Wu and Ms Noriko Yaguchi for their excellent technical assistance. This study was funded by a Grant-in-Aid from the BSE Control Project of the Ministry of Agriculture, Forestry and Fisheries of Japan.

REFERENCES


