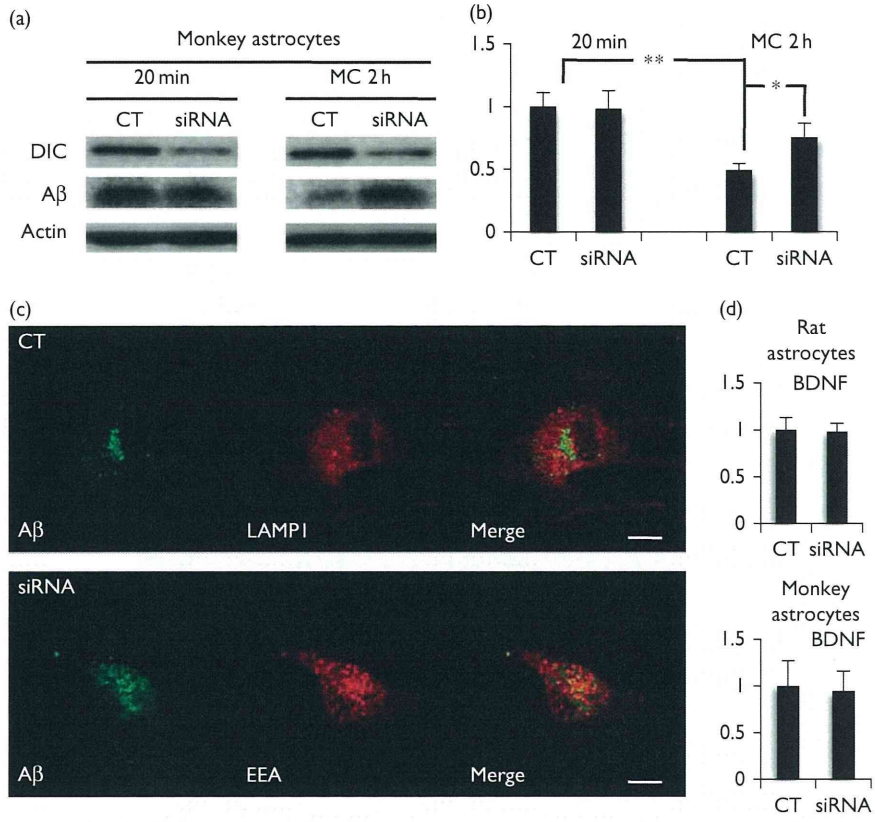
Fig. 3



(a) Western blots showing the amounts of DIC, Aβ, and β-actin in monkey astrocytes 20 min after Aβ treatment (20 min) and 2 h after Aβ depletion from culture medium (MC 2 h). Aβ uptake did not change after dynein depletion. By contrast, Aβ clearance in astrocytes was apparently disturbed in dynein-depleted cells. (b) Histograms showing the effect of dynein depletion on the degree of Aβ uptake in monkey astrocytes. All data were normalized according to β-actin levels. Values are mean±SD (*$P<0.1$, **$P<0.05$). *Y*-axis shows the mean values of the quantified data. (c) Photomicrographs of monkey astrocytes immunostained for Aβ, LAMP1, or EEA1 2 h after Aβ depletion from culture medium. In control siRNA-transfected cells, Aβ taken up was mainly localized to the perinuclear region and colocalized with lysosome marker LAMP1. In contrast, in dynein-depleted cells, Aβ taken up was still distributed in the peripheral region and accumulated in enlarged early endosomes, indicating the disruption of Aβ trafficking to lysosomes (scale bar, 10 μm). (d) Histograms showing the effect of dynein depletion on BDNF secretion from rat and monkey astrocytes. BDNF secreted into culture medium was assessed with ELISA. All data were normalized according to BDNF levels in control siRNA-transfected cells. *Y*-axes show the mean values of the quantified data. Aβ, β-amyloid; BDNF, brain-derived neurotrophic factor; CT, cells transfected with control siRNA; DIC, dynein intermediate chain; ELISA, enzyme-linked immunosorbent assay; siRNA, cells transfected with siDHCp.

endosomes of astrocytes in these brains (Fig. 1). As astrocytes have very little β-secretase activity *in vivo* [20], it is reasonable to conclude that the Aβ accumulated was derived from neurons and was then taken up by astrocytes. This finding led us to hypothesize that aging may disturb Aβ clearance in astrocytes through endocytic disturbances.

In monkey astrocytes, the uptake of Aβ did not change upon the depletion of dynein (Fig. 3a and b). Endocytic uptake is mediated by actin-associated motor proteins, not by microtubule-associated motor proteins such as dynein [21–23]. This may be why the depletion of dynein failed to affect Aβ uptake itself. However, in dynein-depleted monkey astrocytes, the clearance of Aβ was significantly disturbed (Fig. 3a and b). In the present study, we did not use compounds that inhibit the proteasomal or the lysosomal degradation system. It is noteworthy that dynein dysfunction solely disturbed Aβ clearance in astrocytes (Fig. 3a and b). As observed in aged monkey brains, immunocytochemistry confirmed that the Aβ taken up was present in enlarged early endosomes even 2 h after Aβ depletion from the culture medium (Figs 1d and 3c). In control siRNA-transfected astrocytes, Aβ taken up mainly localized to lysosomes at the same time point (Fig. 3c). These findings suggest that dynein dysfunction disrupts astroglial Aβ clearance by disturbing the endocytic system.

In cynomolgus monkey brains, the amount of Aβ increases age dependently and proportionally to endocytic disturbances [6,24]. Thus, age-related endocytic disturbances may cause the age-dependent accumulation of Aβ, ultimately leading to Aβ pathology. Interestingly, we found several areas of CAA near astrocytes showing endocytic pathology, and some of these astrocytes

appeared to be in close apposition to affected cerebral vessels (Fig. 1d). These observations suggest that impaired Aβ clearance in astrocytes may be strongly involved in CAA rather than in SP formation in cynomolgus monkey brains.

However, dynein depletion did not affect BDNF secretion in both rat and monkey astrocytes (Fig. 3d). This finding suggests that dynein dysfunction may not affect ordinary secretion pathways, and the impairment of BDNF signaling during the early stages of AD might be mainly caused by neuronal endocytic disturbances as we have reported previously [6].

Conclusion

We observed endocytic pathology in astrocytes of aged cynomolgus monkey brains. Indeed, Aβ accumulated in the enlarged early endosomes of these astrocytes. RNAi studies showed that dynein dysfunction reproduced endocytic pathology and disrupted Aβ clearance in astrocytes through endocytic disturbances. These findings suggest that dynein dysfunction might be one factor responsible for age-dependent accumulation of Aβ through endocytic disturbances.

Acknowledgements

This study was supported by a grant-in-aid from the Ministry of Health, Labor and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Conflicts of interest

There are no conflicts of interest.

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Journal of General Virology (2014), **95**, 2576–2588 DOI 10.1099/vir.0.066225-0

Ultrasensitive detection of PrP^{Sc} in the cerebrospinal fluid and blood of macaques infected with bovine spongiform encephalopathy prion

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Prion diseases are characterized by the prominent accumulation of the misfolded form of a normal cellular protein (PrP^{Sc}) in the central nervous system. The pathological features and biochemical properties of PrP^{Sc} in macaque monkeys infected with the bovine spongiform encephalopathy (BSE) prion have been found to be similar to those of human subjects with variant Creutzfeldt–Jakob disease (vCJD). Non-human primate models are thus ideally suited for performing valid diagnostic tests and determining the efficacy of potential therapeutic agents. In the current study, we developed a highly efficient method for *in vitro* amplification of cynomolgus macaque BSE PrP^{Sc}. This method involves amplifying PrP^{Sc} by protein misfolding cyclic amplification (PMCA) using mouse brain homogenate as a PrP^{C} substrate in the presence of sulfated dextran compounds. This method is capable of amplifying very small amounts of PrP^{Sc} contained in the cerebrospinal fluid (CSF) and white blood cells (WBCs), as well as in the peripheral tissues of macaques that have been intracerebrally inoculated with the BSE prion. After clinical signs of the disease appeared in three macaques, we detected PrP^{Sc} in the CSF by serial PMCA, and the CSF levels of PrP^{Sc} tended to increase with disease progression. In addition, PrP^{Sc} was detectable in WBCs at the clinical phases of the disease in two of the three macaques. Thus, our highly sensitive, novel method may be useful for furthering the understanding of the tissue distribution of PrP^{Sc} in non-human primate models of CJD.

Received 24 March 2014
Accepted 10 July 2014

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), commonly known as prion diseases, are fatal neurodegenerative disorders that affect both animals and humans (Collinge, 2001). Prion diseases are characterized by the prominent accumulation of a misfolded prion protein, PrP^{Sc}, in the central nervous system (Prusiner, 1991, 1998). PrP^{Sc}, which is rich in beta-sheet structures and resistant to digestion by proteases and various inactivating treatments (Caughey *et al.*, 1991; Pan *et al.*, 1993), is considered to be the infectious agent for TSEs and appears to self-propagate through post-translational modification of the normal prion protein PrP^{C} (Prusiner, 1998).

One type of human prion disease, Creutzfeldt–Jakob disease (CJD), can be aetiologically identified as sporadic, inherited or acquired by infection (Ironside, 1998; Belay, 1999; Glatzel *et al.*, 2002; Geissen *et al.*, 2007). In variant CJD (vCJD), which is a form of CJD caused by consumption of foods contaminated with bovine spongiform encephalopathy (BSE) prions (Will *et al.*, 1996; Hill *et al.*, 1997; Ironside, 2010), small amounts of PrP^{Sc} have been found in a broad range of peripheral tissues, including the lymph nodes, tonsils, spleen, kidneys, portions of the intestinal tract and skeletal muscle (Wadsworth *et al.*, 2001; Hilton *et al.*, 2004; Peden *et al.*, 2006; Notari *et al.*, 2010), as well as in the

Four figures and one table are available with the online version of this paper.

central nervous system. These observations have led to serious concerns that the disease could spread in humans via blood transfusions (Wroe *et al.*, 2006; Knight, 2010) and through the use of contaminated biological and surgical instruments. In order to effectively prevent the spread of this disease, it is important to be able to detect PrP^{Sc} as soon after infection as possible, and then, it is crucial to avoid PrP^{Sc} contamination in human-derived materials. As the concentration of PrP^{Sc} in the tissues or body fluids of infected subjects is predicted to be extremely low until marked clinical signs appear, development of both a sensitive method for detecting PrP^{Sc} and animal models to confirm its validity are necessary.

Several studies have used non-human primates to study the transmissibility of human prion diseases (Gajdusek *et al.*, 1966; Gibbs *et al.*, 1968), and the transmissibility of BSE has specifically been investigated using macaque monkeys (Lasmézas *et al.*, 1996, 2005; Comoy *et al.*, 2008; Ono *et al.*, 2011a, b). These studies have reported a number of advantages of using non-human primate models of prion disease. For example, the pathological feature of florid plaques in the brain tissue of BSE-infected macaques and the biochemical characteristics of the PrP^{Sc} glycoform profile in these macaques have been shown to be identical to those in human subjects with vCJD (Lasmézas *et al.*, 1996). In macaques inoculated with the BSE prion either intracerebrally or orally and in humans infected with vCJD, PrP^{Sc} has been found to be distributed in various peripheral tissues, such as the lymph nodes, spleen, tonsils and muscles. These findings strongly support the possibility that vCJD is caused by an exogenous infection of a BSE prion. Furthermore, BSE can be transmitted via intravenous inoculation (Lasmézas *et al.*, 2001), indicating that macaques can serve as model animals for suspected cases of secondary transmission (via blood transfusion) of vCJD in humans. Therefore, non-human primate models are ideally suited for assessing methods for diagnosis and treatment of prion diseases.

In scrapie-infected rodents (Brown *et al.*, 1998) and sheep (Houston *et al.*, 2008) as well as in deer with chronic wasting disease (CWD), bodily fluids such as the blood, urine, saliva and faeces have been reported to be infectious (Mathiason *et al.*, 2006; Haley *et al.*, 2009b; Mathiason *et al.*, 2010). By using the protein misfolding cyclic amplification (PMCA) technique, which amplifies PrP^{Sc} *in vitro* using normal brain homogenates as the PrP^{C} substrate, PrP^{Sc} has been detected in a variety of bodily fluids, including the blood, cerebrospinal fluid (CSF), urine, faeces, saliva and milk of prion-infected animals (Saborio *et al.*, 2001; Saá *et al.*, 2006; Murayama *et al.*, 2007, 2010; Thorne & Terry, 2008; Terry *et al.*, 2009; Maddison *et al.*, 2009, 2010; Haley *et al.*, 2009a, 2011; Tattum *et al.*, 2010; Gough *et al.*, 2012). Furthermore, several reports have described the successful detection of PrP^{Sc} in bodily fluids of humans with CJD (Orrú *et al.*, 2009; Atarashi *et al.*, 2011; Edgeworth *et al.*, 2011; Peden *et al.*, 2012; Rubenstein & Chang, 2013). For example, PrP^{Sc} in the CSF of patients with sporadic CJD (sCJD) and vCJD has been detected using the quaking-induced conversion technique (Atarashi *et al.*, 2007), which detects PrP^{Sc}-triggered formation of amyloid fibrils of recombinant prion proteins. Similarly, PrP^{Sc} has been detected in the CSF of patients with sCJD using PMCA followed by a sensitive immunoassay termed SOFIA (Rubenstein & Chang, 2013), and bead-captured ELISA has been used to detect blood PrP^{Sc} in patients with vCJD (Edgeworth *et al.*, 2011). Therefore, bodily fluids may have high utility as diagnostic materials for CJD. However, the quantitative changes of PrP^{Sc} in bodily fluids of non-human primate models of CJD has not yet been determined due to a lack of sensitive methods for assessing very small amounts of prions in these animal models.

In the present study, we have developed a highly efficient PMCA method suitable for cynomolgus macaque BSE PrP^{Sc} amplification. This method, which involves amplifying PrP^{Sc} using xenogeneic (mouse) PrP^{C} substrate in the presence of sulfated dextran compounds, is capable of amplifying a very small amount of PrP^{Sc} from the CSF, blood, and peripheral tissue of BSE-infected macaques. We further investigated CSF and blood PrP^{Sc} levels during the period from the latent to terminal stages of the disease and compared PrP^{Sc} dynamics in macaques.

RESULTS

Amplification of cynomolgus macaque BSE PrP^{Sc} by PMCA

We first examined the amplification efficiency of PMCA, using the brain homogenate of BSE-infected cynomolgus macaque no. 7 as the PrP^{Sc} seed. Before amplification, distinct signals of protease-resistant PrP (PrP^{res}) were detected in brain homogenates diluted up to 10^{-2} by Western blot analysis (Fig. 1a). In the absence of potassium dextran sulfate (DSP), brain homogenates derived from the squirrel monkey and cynomolgus macaque were not suitable for amplification of cynomolgus PrP^{Sc} (Fig. 1b, upper panel). Similarly, no significant amplification was observed using cow, TgBoPrP and $PrP^{0/0}$ mice (Fig. 1b, middle panel), or hamster brain homogenates (Fig. 1b, lower panel) as PrP^{C} substrates. On the other hand, amplification of PrP^{Sc} was achieved in samples diluted to 10^{-3} and 10^{-4} when the WT mouse brain homogenate was used as the PrP^{C} substrate (Fig. 1b, lower panel). Furthermore, amplification efficiency of mouse PrP^{Sc} for PMCA was significantly improved in the presence of DSP, and PrP^{res} signals were detected in samples diluted to 10^{-5} after one round of amplification. On the other hand, DSP was less effective in increasing signal intensity of PrP^{res} after amplifications derived using squirrel monkey, cynomolgus macaque, cow, TgBoPrP mouse and hamster brain homogenates. The detection sensitivity for cynomolgus PrP^{Sc} for these PCMAs was lower than for PMCAs conducted using WT mouse brain homogenate. Higher background signal in the no-seed samples was observed after amplification was conducted using macaque brain homogenate in the presence of DSP.

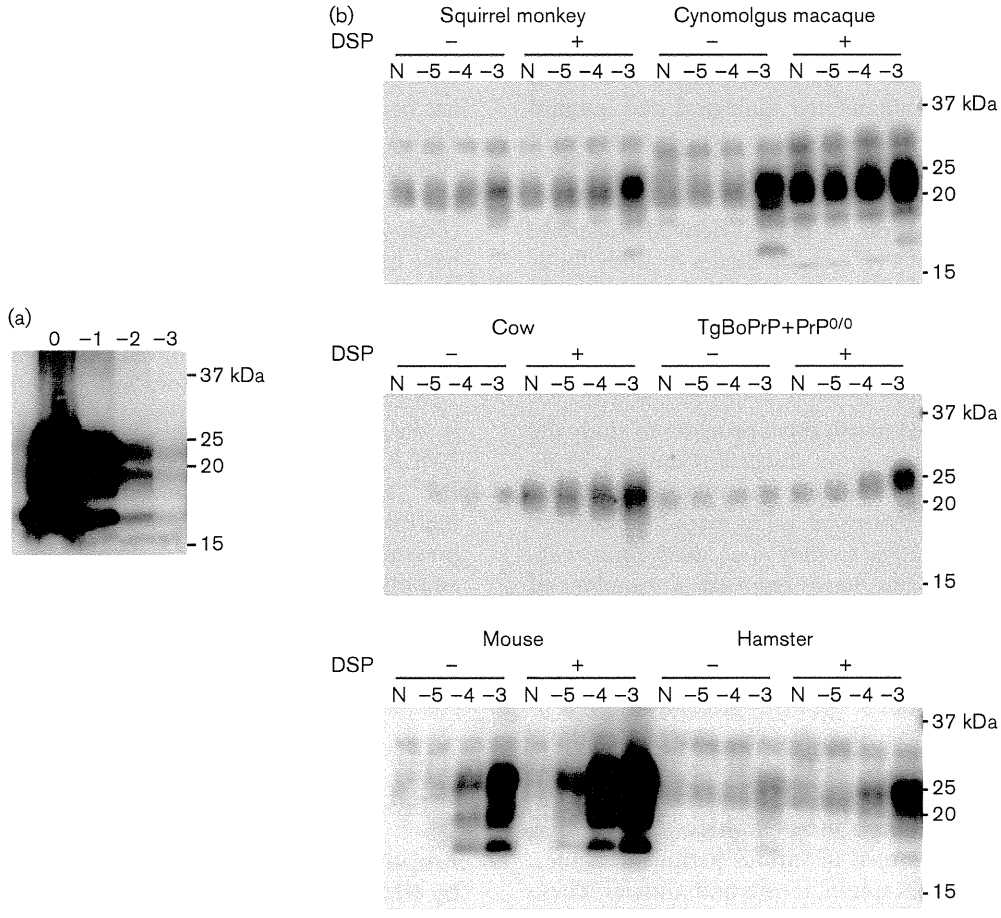

Fig. 1. Amplification of macaque PrP^{Sc} using normal brain homogenates derived from several animal species as PrP^{C} substrates. (a) Ten per cent brain homogenate of BSE-affected cynomolgus macaque was diluted to 10^{-1} (−1) to 10^{-3} (−3) in a normal macaque brain homogenate, an undiluted sample (0) was also included. The diluted samples were analysed by Western blot after digestion with proteinase K (PK). (b) PrP^{Sc} seed (10 % brain homogenate of BSE-affected cynomolgus macaque) was diluted to 10^{-3} (−3) to 10^{-5} (−5) in normal brain homogenates obtained from the squirrel monkey, cynomolgus macaque, cow, mixture of TgBoPrP and $PrP^{0/0}$ (TgBoPrP+$PrP^{0/0}$) mice, mouse and hamster. The diluted samples were amplified in the presence (+) or absence (−) of 1 % (w/v) DSP. After amplification, the samples were digested with PK and analysed by Western blot. 'N' denotes unseeded control samples in which normal brain homogenate that did not receive a PrP^{Sc} seed were processed and analysed in the same manner as PrP^{Sc}-seeded samples. The molecular masses of marker proteins are indicated (kDa).

Detection sensitivity of cynomolgus macaque BSE PrP^{Sc}

PMCA using WT mouse brain homogenate containing DSP as the PrP^{C} substrate was used for amplification of cynomolgus macaque PrP^{Sc}. On the basis of our preliminary experiments, the optimal concentration of DSP was estimated to be 1 % (w/v); therefore, we used 1 % (w/v) DSP for subsequent experiments. We determined the detection limit of the interspecies PMCA technique and confirmed that PrP^{Sc} present in a 10^{-5} dilution of infected brain homogenate could be detected after one round of amplification, and both 10^{-6} and 10^{-7} dilutions were positive for PrP^{Sc} after two rounds of amplification (Fig. 2a). After three rounds of amplification, PrP^{res} signals were detected in the samples diluted to 10^{-8} and 10^{-9}. A PrP^{res} signal was detected in the 10^{-10} dilution samples after four rounds of amplification, but almost no signal was detected in the more extreme dilutions, even after seven rounds of amplification. Thus, compared with no amplification, amplification improved the PrP^{Sc} detection sensitivity by a factor of 10^{8}. No typical PrP^{res} signal was detected in samples that contained normal brain homogenate diluted 1 : 10 with mouse PrP^{C} substrate (Fig. 2b). In addition, the generation of spontaneous PrP^{res}, as has been reported for amplification in the presence of polyanions (Deleault *et al.*, 2007; Wang *et al.*, 2010), was not observed in 16 samples that contained only mouse PrP^{C} substrate following seven rounds of amplification (Fig. 2c).

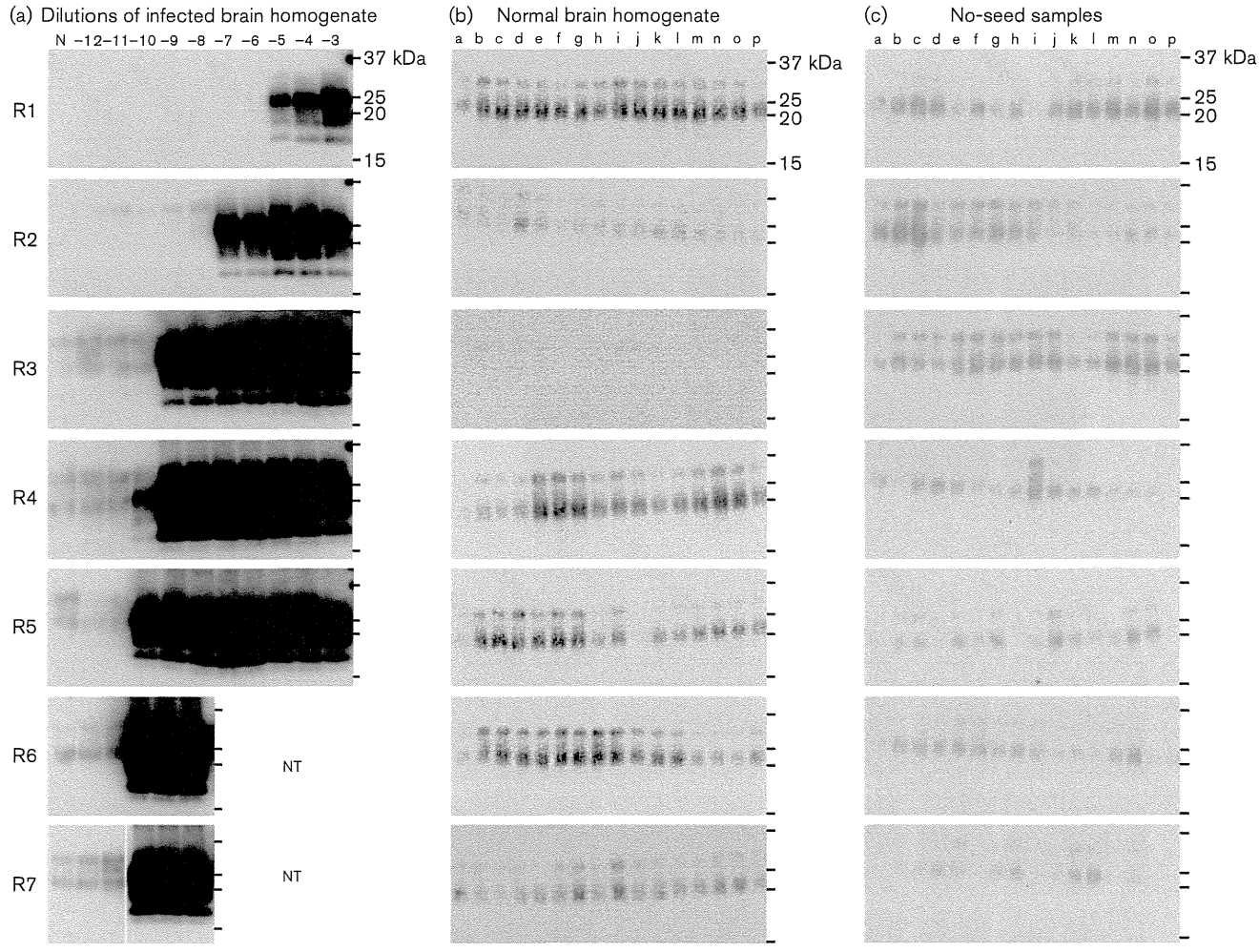

Fig. 2. Detection sensitivity for cynomolgus macaque PrP^{Sc}. (a) PrP^{Sc} seed was diluted to 10^{-3} (−3) to 10^{-12} (−12) with PrP^{C} substrate (10 % normal mouse brain homogenate), and the samples were serially amplified in the presence of 1 % (w/v) DSP. The amplified samples were analysed after each round of amplification (R1–R7) by Western blot after proteinase K (PK) digestion. (b) Normal brain homogenate was diluted to 10^{-1} with the PrP^{C} substrate (lanes a–p), and the samples were serially amplified in the presence of 1 % (w/v) DSP. After amplification, a band with a molecular mass similar to that for PrP^{Sc} was occasionally observed, which likely corresponds to a residue of the normal isoform of prion protein resulting from incomplete PK digestion. (c) No spontaneous generation of PrP^{Sc} was observed in no-seed samples. Lanes a–p contained only PrP^{C} substrate and were amplified in the presence of 1 % (w/v) DSP. Exclusive pipettes, a vortex mixer, and a centrifuge were used for handling unseeded samples. The molecular masses of marker proteins are indicated (kDa). NT, Not tested.

PrP^{Sc} distribution in the peripheral tissues of BSE-affected macaques

We examined PrP^{Sc} distribution in macaques that were intracerebrally administered a brain homogenate prepared from a BSE-infected cow. In BSE-infected macaques, PrP^{Sc} was detected by conventional Western blot analysis in several peripheral nervous tissues and lymph nodes (Table S1, available in the online Supplementary Material). By using serial PMCA, PrP^{Sc} was detected in all examined tissues, including: the peripheral nerves, lymph nodes, spleens, tonsils and adrenal glands (Fig. 3). Most samples were found to be positive for PrP^{Sc} after no more than two rounds of amplification. On the other hand, PrP^{Sc} was detected after three rounds of amplification in four and two of the quadruplicate samples of the tonsil of macaque no. 10 (Fig. 3b) and spleen of macaque no. 11 (Fig. 3c), respectively. No typical PrP^{res} signal was detected in the peripheral nerves, lymph nodes, ileum and glands of an uninfected control macaque (Fig. S1).

PrP^{Sc} levels in the CSF

The amplification results for the CSF samples collected from the three macaques are illustrated in Fig. 4. No typical PrP^{res} signal was observed in samples that contained only

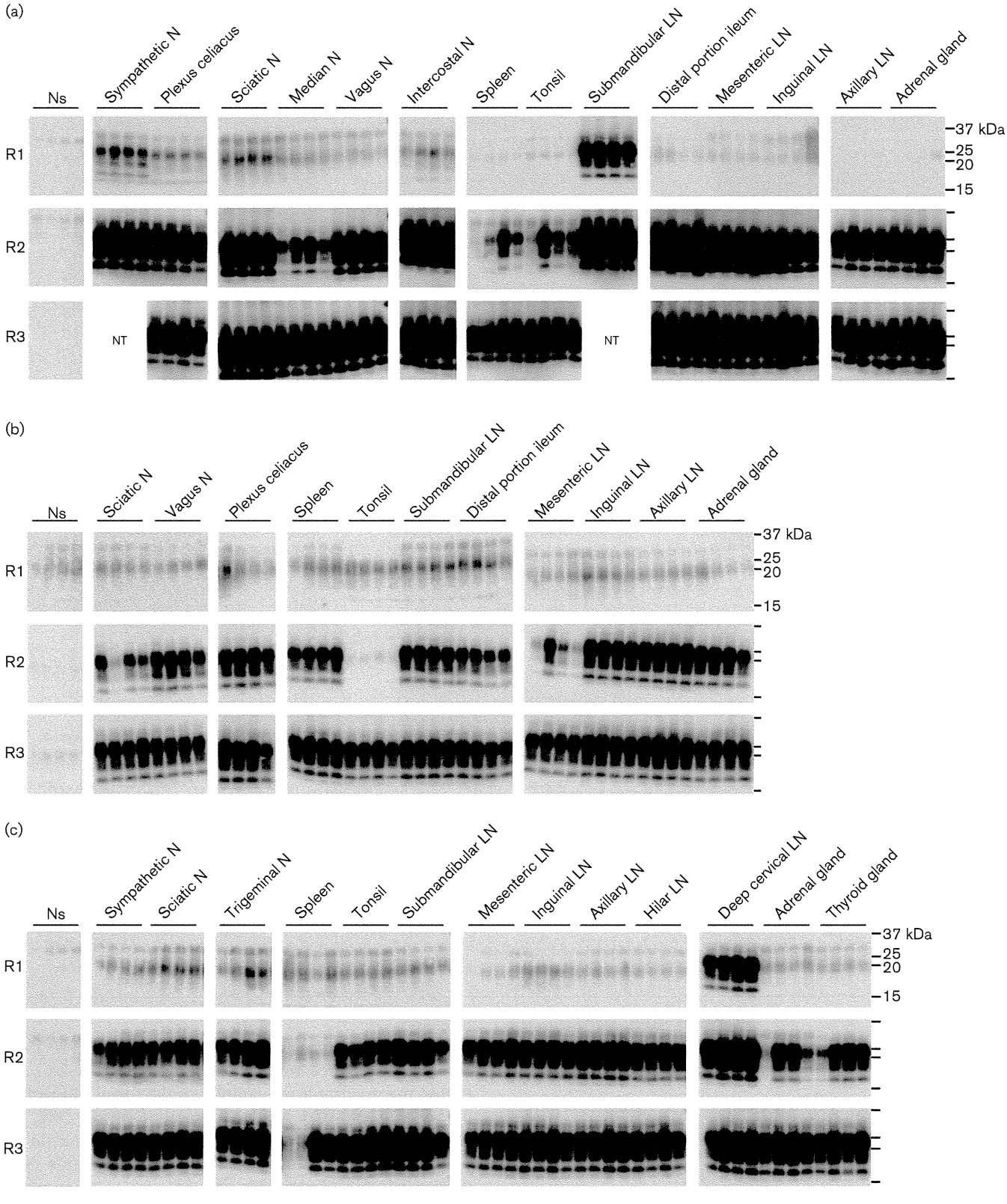

Fig. 3. Tissue distribution of PrP^{Sc} in macaques intracerebrally inoculated with BSE. Tissue distribution of PrP^{Sc} in the terminal disease stage in macaque no. 7 (a), no. 10 (b) and no. 11 (c). Quadruplicate samples of each tissue were serially amplified, and the samples were analysed by Western blot following digestion with proteinase K after each round of amplification (R1–R3). The molecular masses of marker proteins are indicated (kDa). N, Nerve; LN, lymph node; Ns, no-seed samples; NT, not tested.

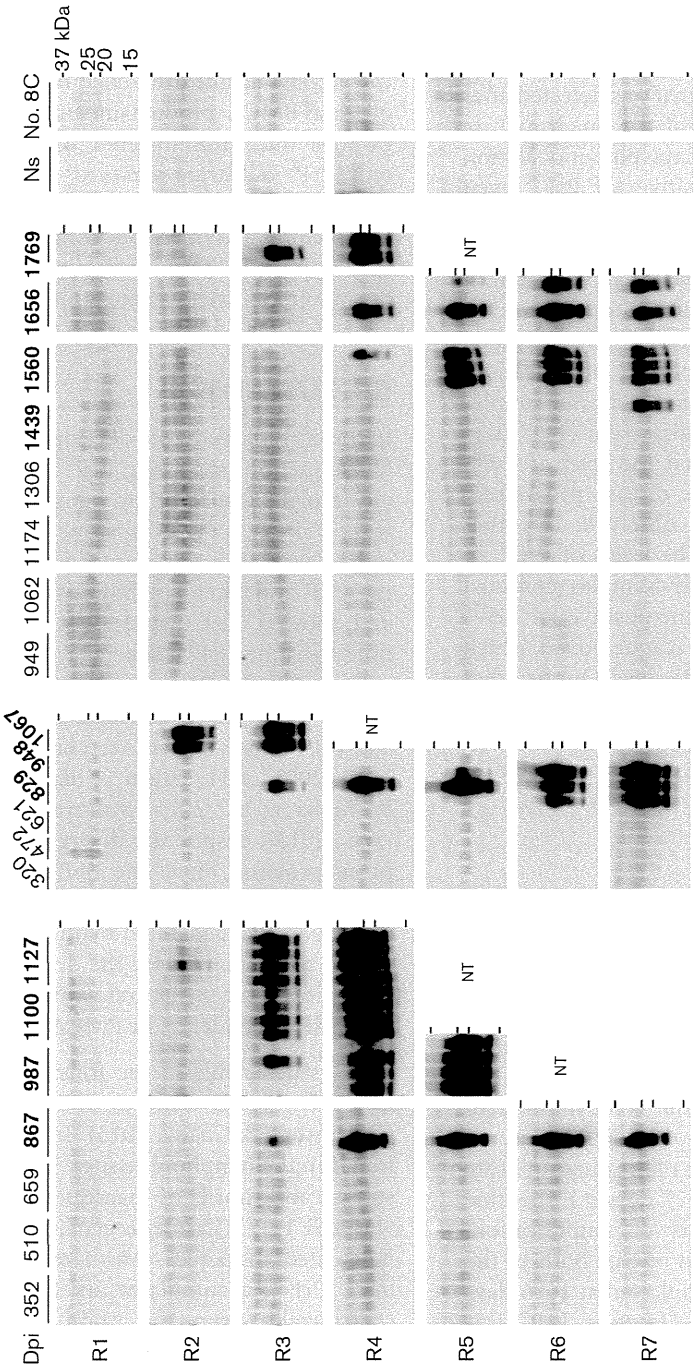

Fig. 4. The appearance of PrP^{Sc} in the cerebrospinal fluid (CSF) of BSE-infected macaques. CSF was collected at several points after intracerebral inoculation. Quadruplicate or duplicate CSF samples from BSE-infected macaque no. 7, no. 10, and no. 11 were analysed by Western blot following digestion with proteinase K after each round of amplification (R1–R7). PrP^{Sc} was also evaluated in CSF samples from an uninfected control macaque (no. 8C). Dpi, Days post-inoculation. Dpi written in boldface represents clinical stages of the disease. The molecular masses of marker proteins are indicated (kDa). Ns, No-seed samples; NT, not tested.

mouse PrP^C substrate (lanes Ns), or samples that contained normal macaque CSF diluted 1 : 10 with mouse PrP^C substrate (Fig. 4, no. 8C and Fig. S2). PrP^{res} signal was not detected in the samples collected 515–208 (macaque no. 7), 509–208 (macaque no. 10) and 490–133 days (macaque no. 11) before disease onset. The existence of PrP^{Sc} in the CSF samples was confirmed after the onset of clinical signs. For example, macaque no. 7 presented with early neurological clinical signs of the disease such as slight tremor, startle response and festinating gait. PrP^{res} signal was detected after four rounds of amplification in one of the quadruplicate samples collected at this time [867 days post-inoculation (p.i.)], but no other sample was positive for PrP^{Sc} even after seven rounds of amplification. Consistent with disease progression, macaque no. 7 presented with ataxia, paralysis of the extremities and rigidity; PrP^{Sc} was detected in all of the quadruplicate samples obtained at 987 days p.i. after five rounds of amplification. The macaque finally developed severe dysstasia, and after three rounds of amplification, PrP^{Sc} was detected in all of the quadruplicate samples obtained at 1100 days p.i. and at the dissection (1127 days p.i.). These observations suggested that the level of PrP^{Sc} tended to increase in the CSF as the disease progressed. Although a similar tendency was observed in other macaques, there were differences in the levels of PrP^{Sc} in the CSF. For example, duplicate CSF samples collected upon dissection (1067 days p.i.) became positive for PrP^{Sc} after two rounds of amplification in macaque no. 10, which showed the shortest latent period of 828 days. On the other hand, the disease developed after a relatively longer latent period of over 1400 days in macaque no. 11, and PrP^{res} signals were detected after four rounds of amplification in both samples collected upon dissection (1769 days p.i.).

PrP^{Sc} levels in the blood

The results of the amplification of white blood cell (WBC) samples collected at several time points after intracerebral administration are illustrated in Fig. 5. No typical PrP^{res} signal was observed in samples that contained only mouse PrP^C substrate (Fig. 5, lanes Ns), or samples that contained normal macaque WBCs (10^4 cells) (Fig. 5, no. 8C and Fig. S2). Furthermore, we confirmed that the WBC matrix had no inhibitory effect on the amplification of PrP^{Sc} by serial PMCA (Fig. S3). In macaque no. 7, one of the quadruplicate samples collected upon dissection (1127 days p.i.) became positive for PrP^{Sc} after five rounds of amplification. Similarly, PrP^{res} signal was detected in one or both of the duplicate samples of macaque no. 11 collected at 1656 days p.i., and at dissection (1769 days p.i.). However, PrP^{Sc} was not detected in the blood of these macaques between the latent and the initial stage of disease onset. In macaque no. 10, PrP^{res} signal was not detected in the WBCs obtained during the experimental period (320–1067 days p.i.) even after seven rounds of amplification. With regard to plasma samples, no PrP^{Sc} was detected in any of the samples collected during the experimental period (data not shown).

Infectivity of the PMCA product

The PMCA product obtained after ten rounds of amplification was diluted 10-fold and inoculated intracerebrally into tga20 mice. The tga20 mice inoculated with the PMCA products derived from the brain or WBC PrP^{Sc} seeds died after an average period of 305 or 310 days, respectively (Table 1). PrP^{Sc} accumulation in the brains of mice was confirmed by Western blot analysis (data not shown). There was no significant difference between the survival periods of these PMCA product-inoculated mice (*t*-test, $P>0.05$). Control mice administered with the product containing only PrP^C substrate survived more than 478 days. These results indicated that both brain- and WBC-derived PrP^{Sc} had seeding activities following the PMCA reactions, and the amplified PrP^{Sc} maintained their infectious ability during *in vitro* xenogeneic amplification.

DISCUSSION

In the current study, we developed an ultra-efficient PMCA technique for amplifying PrP^{Sc} derived from BSE-infected cynomolgus macaques by using mouse brain homogenates with DSP as a PrP^C substrate and a polyanion additive, respectively. We first proved the existence of PrP^{Sc} in the CSF and blood of BSE-infected macaques by PMCA, and showed that cynomolgus macaque BSE PrP^{Sc}, and non-macaque PrP^C, effectively converted mouse PrP^C to a proteinase K (PK)-resistant form. It is well known that PMCA of several xenogeneic combinations of PrP^{Sc} seed and PrP^C substrate can overcome the species barrier (Kurt *et al.*, 2007, 2011; Green *et al.*, 2008; Castilla *et al.*, 2008; Yoshioka *et al.*, 2011; Murayama *et al.*, 2012; Nemecek *et al.*, 2013), despite the divergent amino acid sequence of prion proteins. Since the BSE prion was transmissive to ICR (WT) mice (Masujin *et al.*, 2008), the cynomolgus macaque PrP^{Sc} generated by the cross-species transmission of BSE prion may retain the original characteristics of BSE PrP^{Sc}, including structural compatibility with mouse PrP^C and DSP dependency in PMCA reactions.

PrP^{Sc} is detectable in the tonsil, spleen and lymph nodes in vCJD (Wadsworth *et al.*, 2001) and sCJD patients (Rubenstein & Chang, 2013). In an earlier study, PrP^{Sc} was found in the lymphoid tissues, including: the lymph nodes, spleens and tonsils of macaques intracerebrally inoculated with BSE PrP^{Sc} (Lasmézas *et al.*, 1996), as observed in vCJD-inoculated macaques (Lasmézas *et al.*, 2001). Therefore, once PrP^{Sc} accumulates in the brain, it may spread centrifugally from the brain to the peripheral tissues through the autonomic nervous system. However, in our previous study, we failed to detect PrP^{Sc} in such lymphoid tissues of the BSE-inoculated macaques by conventional Western blotting, except in the submandibular lymph nodes, deep cervical lymph nodes and inguinal lymph nodes (Ono *et al.*, 2011a; Table S1). In the current study, PMCA analysis revealed that PrP^{Sc} was distributed in all lymphoid tissues examined in the BSE-infected macaques.

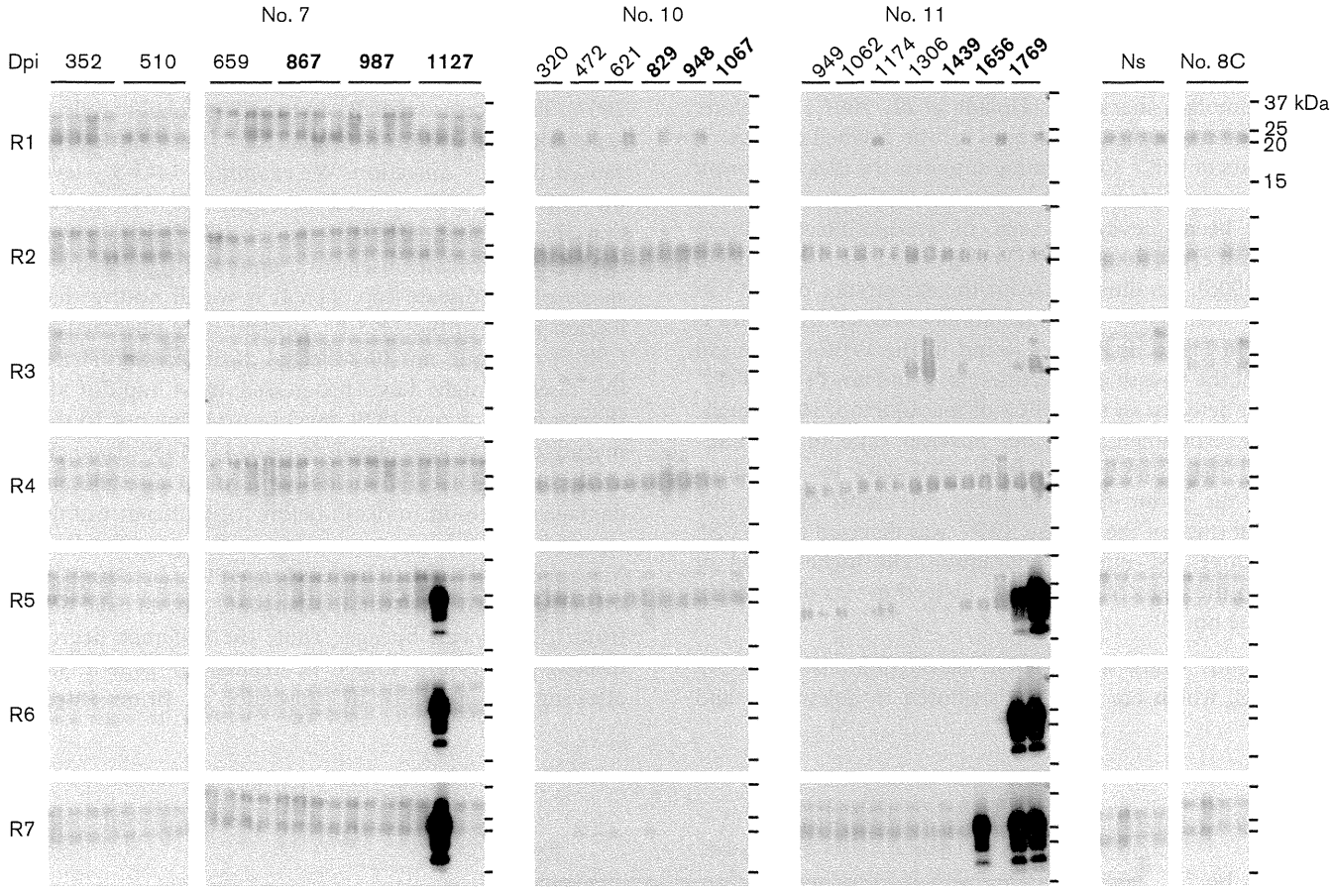

Fig. 5. Appearance of PrP^{Sc} in the WBCs of BSE-infected macaques. WBCs were collected at several points after intracerebral inoculation. Quadruplicate or duplicate WBC samples from BSE-infected macaque no. 7, no. 10 and no. 11 were analysed by Western blot following digestion with proteinase K after each round of amplification (R1–R7). PrP^{Sc} was also evaluated in WBCs from an uninfected control macaque (no. 8C). Dpi, Days post-inoculation. Dpi written in boldface represents the clinical stages of the disease. The molecular masses of marker proteins are indicated (kDa). Ns, No-seed samples.

PrP^{Sc} levels in most of the lymphoid tissues were extremely low, because PrP^{Sc} could only be detected after two or three rounds of amplification. Therefore, significant PrP^{Sc} accumulation in the peripheral non-neuronal tissues might not have occurred in these macaques, and PrP^{Sc} levels in most lymphoid tissues might have been below the detection limit of the conventional Western blot technique used herein, even at the terminal stage of the disease.

Table 1. Mean incubation time following intracerebral inoculation in tga20 transgenic mice

Inoculum (R10 PMCA product)	Transmission rate (total death/total number)	Mean survival time ± SD (days)
Brain seed*	100 % (6/6)	305 ± 10
WBCs seed†	100 % (6/6)	310 ± 23
No seed	0 % (0/4)	>478
10 % Brain homogenate from a BSE-infected cow‡	100 % (20/20)	495 ± 43

R10, Tenth round.

*The final dilution of the infected brain homogenate (macaque no. 7) in the R10 product was 6.4×10^{-11}.

†The PMCA product from the tenth round of amplification of PrP^{Sc}-positive WBCs (macaque no. 7).

‡Classical BSE (c-BSE) prion was inoculated in tga20 mice for comparison of infectivity.

The origin of PrP^{Sc} in WBCs may be the spleen and other lymphoid organs, as suggested previously (Saá *et al.*, 2006). As in humans, PrP^{C} is constitutively expressed in the WBCs of cynomolgus macaques (Holada *et al.*, 2007); therefore, WBCs of cynomolgus macaques can be deemed carriers or reservoirs of PrP^{Sc}. Our finding supports the idea that prion diseases may be transmitted via infected blood in primates, as has been previously seen in scrapie-infected sheep (Houston *et al.*, 2008) and CWD-infected deer (Mathiason *et al.*, 2006). An illustration for the appearance of PrP^{Sc} in the CSF and WBCs of intracerebrally infected macaques is shown in Fig. 6. PrP^{Sc} was found in the WBCs at clinical stages of the disease in macaques no. 7 and no. 11, but PrP^{Sc} was not detected in the WBCs of macaque no. 10 throughout the experimental period. Survival time of the BSE-infected macaques ranged from 1067 days to 1769 days. During the period from the onset of clinical signs to the terminal stage of the disease, PrP^{Sc} was detected in the CSF in all three BSE-infected macaques. The highest level of PrP^{Sc} in the CSF collected upon dissection was observed in macaque no. 10.

A previous study showed that elevated levels of 14-3-3 proteins, which are widely distributed in eukaryotes and play an important role in various signal transduction systems involved in cell proliferation and division, were observed in the CSF of a simian vCJD model (Yutzy *et al.*, 2007). The increase of PrP^{Sc} in the CSF probably reflects the leakage of PrP^{Sc} from neuronal cells after cell destruction caused by PrP^{Sc} infection. We examined 14-3-3 γ levels in the CSF of the BSE-infected macaques (Fig. S4), and found that the signal intensity of the 14-3-3 γ protein became notable after disease onset (no. 7 and no. 10), or in the latter stages of the disease (no. 11). It is worth noting that the highest levels of the 14-3-3 γ protein were observed in the CSF of macaque no. 10 collected at dissection. Therefore, the disease might have progressed most rapidly after a shorter latent period (829 days) in macaque no. 10 than in macaques no. 7 (867 days) and no. 11 (1439 days). Faster accumulation of PrP^{Sc} in the brain may cause acute brain damage and result in death before a significant number of infected WBCs begin circulating in the peripheral blood. Macaques no. 7 and 10 both belonged to a breeding colony introduced from the Philippines, and no. 11 was derived from a Malaysian lineage. Thus, the different degrees of disease progression might be related to genetic factors affecting susceptibility or resistance to prion infection.

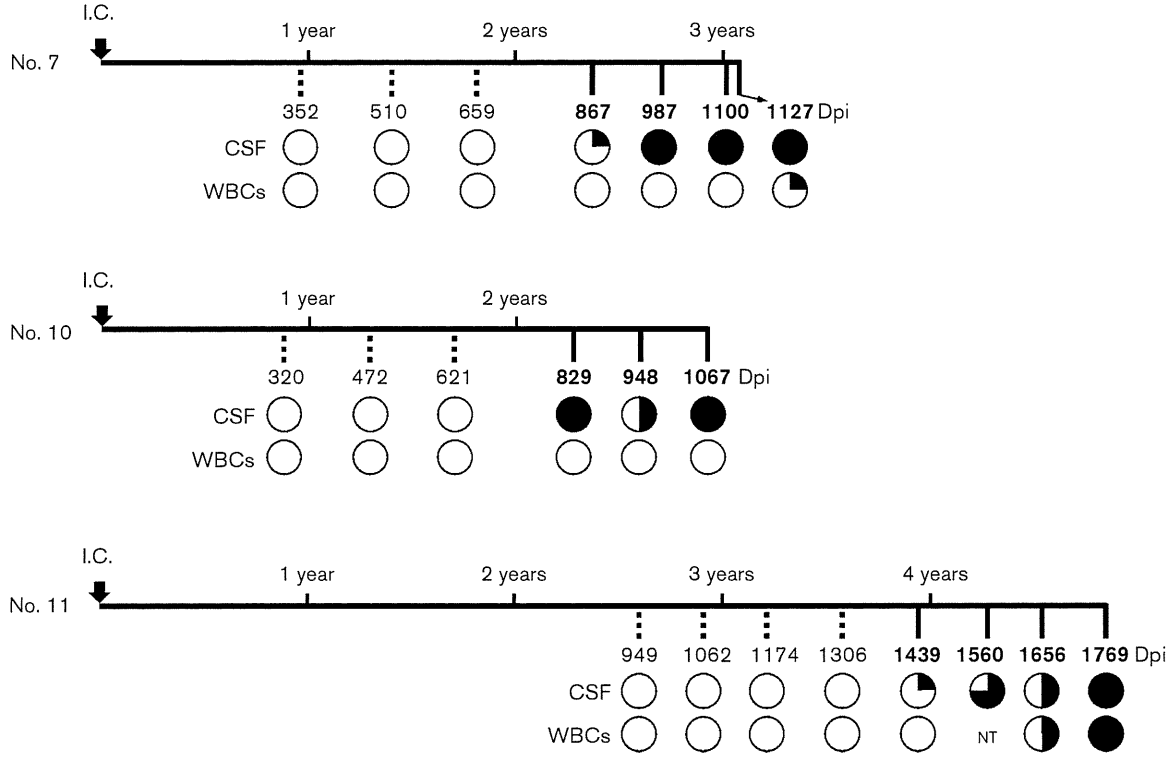

Fig. 6. Schematic illustration for the appearance of PrP^{Sc} in the CSF and WBCs of three BSE-infected macaques. After intracerebral inoculation (I. C.), the presence of PrP^{Sc} in CSF and WBCs was examined by serial PMCA during the asymptomatic (dotted line) and clinical stages (solid lines). Dpi, Days post-inoculation. Dpi written in boldface represents the clinical stages of the disease. Positive ratio of duplicate or quadruplicate samples was shown as open circle (0 %), closed quadrant (25 %), closed semicircle (50 %), closed three quadrants (75 %) and closed circle (100 %). NT, Not tested.

More detailed studies are needed to clarify the above possibility.

In conclusion, we have developed a highly sensitive method that enables a detailed and precise examination of the distribution of PrP^{Sc} throughout the bodies of BSE-infected macaques. We are now conducting experiments analysing oral transmission of the BSE prion and transmission through blood transfusions from BSE-infected macaques. Using our method, PrP^{Sc} could notably be detected in bodily fluids obtained during the latent period of the disease in both primate models. Thus, the method developed in this study may be useful in furthering the understanding of tissue distribution of PrP^{Sc} in non-human primate models of CJD.

METHODS

BSE-infected macaques. This study on non-human primates was conducted according to the rules for animal care and management of the Tsukuba Primate Research Center (Honjo, 1985) and the guiding principles for animal experiments using non-human primates formulated by the Primate Society of Japan (Primate Society of Japan, 1986). The cynomolgus macaques (*Macaca fascicularis*) examined in this study originated from the Philippines (no. 7 and 10) or Malaysia (no. 11), and were bred at Tsukuba Primate Research Center of the National Institute of Biomedical Innovation. Transmission experiments were approved by the Animal Welfare and Animal Care and Use Committee (approval ID: DS18-069R1) and Animal Ethics Biosafety Committee (approval ID: BSL3-R-06.01) of the National Institute of Biomedical Innovation. The brain homogenate (200 µl of a 10 % brain homogenate) derived from a classical BSE (c-BSE)-infected 83-month-old Holstein (Iwata *et al.*, 2006) was intracerebrally administered to three male macaques (no. 7, 10 and 11) that were 24–29 months in age (Ono *et al.*, 2011a). The animals were housed in biosafety level three animal rooms, and their clinical status was monitored daily. After 35–59 months, the animals were euthanized by anaesthesia overdose following evidence of progressive neurological dysfunction, after which the animals were dissected. A healthy macaque (no. 8 or 28) was used as an uninfected control in the PMCA assay of tissues and bodily fluids. All macaques examined in this study were homozygous for methionine at codon 129 (MM) and homozygous for glutamic acid at codon 219 (EE).

Sample preparation. Peripheral nervous and lymphoid tissues were collected upon dissection and stored in small aliquots at −80 °C. Samples from each tissue were homogenized at 10 % (w/v) in PBS. WBCs, plasma and CSF were also collected at several time points after inoculation. The blood samples (1.5 ml) were centrifuged at 1500 *g* for 15 min and the plasma and buffy coat fractions were recovered. Erythrocytes contaminated in the buffy coat fraction were haemolysed in distilled water, and the samples were stored at −80 °C until analysis.

Preparation of PrP^{C} substrates. To avoid contamination, normal brain homogenates were prepared in a laboratory in which infected materials had never been handled. Brains of a healthy cynomolgus macaque, squirrel monkey (*Saimiri sciureus*), cow, PrP^{C}-overexpressing transgenic [Tg(BoPrP) 4092HOZ/$Prnp^{0/0}$, TgBoPrP] mouse (Scott *et al.*, 1997), PrP-knockout ($PrP^{0/0}$) mouse, WT mouse (ICR), and Syrian hamster were homogenized at a 20 % (w/v) concentration in PBS containing a complete protease inhibitor cocktail (Roche Diagnostics). The brain homogenates were stored at −80 °C until further use. For analysis, the homogenates were mixed with an equal volume of the elution buffer (PBS containing 2 % Triton X-100, 8 mM EDTA) and incubated at 4 °C for 1 h with continuous agitation. After centrifugation at 4500 *g* for 5 min, the supernatant was used as the PrP^{C} substrate. When using brain homogenates of TgBoPrP mice, the supernatants were mixed in a 5 : 1 proportion of $PrP^{0/0}$:TgBoPrP, and this mixture was used as the PrP^{C} substrate.

PMCA. For the amplification of brain PrP^{Sc}, the BSE-infected brain homogenate of macaque no. 7 was diluted from 10^{-3} to 10^{-5} with normal brain homogenates from several animal species in an electron beam-irradiated polystyrene tube (total volume, 100 µl). Amplification was performed in the presence or absence of 1 % (w/v) DSP, which has been shown to markedly improve *in vitro* amplification efficiency of bovine BSE PrP^{Sc} (Murayama *et al.*, 2010). Amplification was carried out with a fully automatic cross-ultrasonic protein activating apparatus (Elestein 070-CPR; Elekon Science Corporation), which had the capacity to generate high ultrasonic power (700 W). PMCA was performed by 40 cycles of sonication in which a 3 s pulse oscillation was repeated five times at 1 s intervals, followed by incubation at 37 °C for 1 h with agitation.

To examine the sensitivity of interspecies PMCA using the mouse PrP^{C} substrate for the detection of macaque BSE PrP^{Sc}, the 10 % infected brain homogenate was serially diluted from 10^{-3} to 10^{-12} with mouse PrP^{C} substrate containing 1 % (w/v) DSP (total volume, 80 µl) in an electron beam-irradiated eight-strip polystyrene tube specially designed for PrP^{Sc} propagation (Murayama *et al.*, 2010). To obtain maximum amplification efficiency and reduce non-specific background signal in Western blot analysis, a series of amplification steps were programmed as follows: PMCA was performed with 40 cycles of sonication in which a 15 s oscillation and subsequent incubations at 31 °C for 1 h were repeated 10 times; a 15 s oscillation and subsequent incubations at 33 °C for 1 h were repeated 10 times; an intermittent oscillation (3 s pulse oscillation was repeated five times at 1 s intervals) and subsequent incubations at 35 °C for 1 h were repeated 10 times; and finally intermittent oscillations (3 s pulse oscillation was repeated five times at 1 s intervals) and subsequent incubation at 37 °C for 1 h were repeated 10 times. The amplified product obtained after the first round of amplification was diluted 1 : 5 with the PrP^{C} substrate, and a second round of amplification was performed. This process was repeated for a maximum of six times.

For amplifying PrP^{Sc} in various tissues from BSE-inoculated macaques, the mouse PrP^{C} substrate containing 1 % (w/v) DSP was mixed with a 1/10 volume of homogenized samples or bodily fluids (total volume 80 µl) in eight-strip polystyrene tubes. The WBC pellet (approx. 10^{4} cells) was dissolved in 8 µl of the elution buffer and used as a seed. Serial PMCA was then performed using the four-step amplification programme as described above.

Western blotting. After each round of amplification, samples of 10 µl were mixed with 10 µl of PK solution (100 µg PK ml^{-1}) and incubated at 37 °C for 1 h. The digested materials were mixed with 20 µl of 2× SDS sample buffer and incubated at 100 °C for 5 min. The samples were separated by SDS-PAGE and transferred onto a PVDF membrane (Millipore). After blocking, the membrane was incubated for 1 h with HRP-conjugated T2 mAb (Hayashi *et al.*, 2004; Shimizu *et al.*, 2010) at a 1 : 10 000 dilution. The T2 antibody, which recognizes a discontinuous epitope in amino acid residues 132–156 in the mouse PrP sequence, also reacts with hamster and monkey PrP. After washing, the blotted membrane was developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore), according to the manufacturer's instructions. Chemiluminescence signals were analysed with the Light Capture system (ATTO).

Bioassay. A 10 % brain homogenate from BSE–infected macaque (no. 7) was diluted to 10^{-4} with WT mouse PrP^{C} substrate containing 1 % (w/v) DSP and amplified. The 1 : 5 dilution of the PMCA product

and its subsequent amplification was repeated nine times. The product from the tenth round was diluted 1:10 with PBS and inoculated intracerebrally (20 µl per mouse) into tga20 mice (Fischer *et al.*, 1996) that overexpress mouse PrP^{C}. Infectivity of the PMCA product from the tenth round of amplification of a PrP^{Sc}-positive WBC sample from macaque no. 7 obtained at dissection 1127 days p.i. was also examined. The PMCA product from the tenth round of amplification of no-seed sample was inoculated as negative control. In addition to the PMCA products, 10% brain homogenate of a c-BSE infected cow was also inoculated into tga20 mice to compare infectivity. The bioassay experiments were approved by the Animal Care and Use Committee of the National Institute of Animal Health (approval ID: 09-44) and were conducted in accordance with the guidelines for animal transmissible spongiform encephalopathy experiments of the Ministry of Agriculture, Forestry and Fisheries of Japan.

ACKNOWLEDGEMENTS

We thank the contributions of the animal caretakers. This study was supported by a grant for BSE research from the Ministry of Health, Labour and Welfare of Japan (H20-Shokuhin-Ippan-008), and in part, by a Grant-in-Aid from the BSE and other Prion Disease Control Project of the Ministry of Agriculture, Forestry and Fisheries of Japan.

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革新的医療研究開発で挑む神経変性疾患 ―プリオン病治験体制の確立に向けて―

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桑田 一夫

抄録集

平成27年
日時 2/14(土) 13:00～18:50

場所 名古屋国際会議場 国際会議室
愛知県名古屋市熱田区熱田西町 1－1

主催 国立大学法人　岐阜大学

共催 一般社団法人　ARO 協議会
公益財団法人　先端医療振興財団

後援 文部科学省、厚生労働省

■ 主催者ご挨拶

シンポジウムの開催にあたって

シンポジウム世話人代表・実行委員長
岐阜大学大学院連合創薬医療情報研究科　教授

桑田　一夫

厚生労働省の推計によれば、2025 年には認知症の人は約 700 万人前後になり、65 歳以上高齢者に対する割合は 5 人に 1 人となる見込みです。平成 27 年 1 月 27 日に発表された新オレンジプランでは、認知症の予防法、診断法、治療法の開発を推進することが明記されています。平成 27 年 4 月 1 日には独立行政法人日本医療研究開発機構が発足し、アカデミアが一丸となって医薬品医療機器開発に本格的に取り組みはじめます。本シンポジウムでは、認知症のなかでも、希少疾患であるプリオン病に焦点をあて、その治療薬開発を主要なテーマとして取り上げたいと存じます。

プリオン病やアルツハイマー病などの神経変性疾患は、異常な構造を有する蛋白質が自己複製し、やがて脳の神経細胞が死滅する病気です。その治療には、正常な構造を安定化させ、異常な構造を抑制する薬剤を開発する必要があります。現代における量子サイエンスの発展に伴い、目的に合う分子を論理的に設計・合成することが可能となりました。しかし、それが薬剤となるためには、薬事法及び ICH に基づくレギュラトリーサイエンスを通じて、その効果と安全性が実証されなければなりません。これらの多くの難題を克服し、プリオン病（ヤコブ病などを含む）の治験体制が確立されようとしています。その現況を分かりやすく、市民の皆様にお伝えするのが本シンポジウムの狙いです。

私が医学生の頃（1980 年代）、神経変性疾患は、原因が不明で治療法もありませんでした、1990 年代になると、その原因がそれぞれの疾患特有の蛋白質にあることが分って来ました。1997 年米国の Stanley B. Prusiner 博士が、クロイツフェルト・ヤコブ病（プリオン病）の原因となるプリオン（主にプリオン蛋白質からなる）の発見により、ノーベル賞を受賞しました。これ以降、神経変性疾患に関連する蛋白質の研究が進みました。その結果、2010 年代にはプリオン病以外の神経変性疾患も基本的には、プリオンライクな原因で発症すると考えられるようになりました。神経変性疾患の原因が分かった事により、その治療薬開発が世界的に進められています。プリオン病に対しても、ドキシサイクリンの治験が欧州で行われましたが、効果のない事が分りました。本邦においては、プリオン病に対してヒトにはじめて投与する化合物を対象とする世界初の治験体制が出来上がろうとしています。本シンポジウムの成果としてプリオン病治療薬開発戦略について関係者のコンセンサスが得られ、国際治験に向けての産官学連携が促進されることを願っています。

■ プログラム

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第１部

プリオン病制圧戦略

座長：岐阜大学大学院連合創薬医療情報研究科　教授　**桑田　一夫**　先生

「プリオン病制圧戦略について」

福島　雅典 先生

先端医療振興財団臨床研究情報センター長

■ Profile

1973. 4.23 ～ 1974. 3.31	名古屋第二赤十字病院　医員
1976. 4. 1 ～ 1978. 3.31	浜松医科大学　文部教官助手（生化学第一講座）
1978. 4. 1 ～ 2000. 3.31	愛知県がんセンター病院　内科診療科医長
1980. 8.27 ～ 1980.11.27	Visiting Assistant Professor, Baylor College of Medicine, Dept. of Pharmacology, Houston, TX, USA
1992. 4. 1 ～ 2000. 3.31	京都大学講師、浜松医科大学講師（共に非常勤）
2000. 4. 1 ～ 2001.11.30	京都大学大学院医学研究科　薬剤疫学教授
2001.12. 1 ～ 2009. 3.31	京都大学医学部附属病院 探索医療センター検証部教授（薬剤疫学兼任）
2003. 4. 1 ～ 2009. 3.31	財団法人 先端医療振興財団　臨床研究情報センター 研究事業統括（併任）
2003.10. 1 ～ 2009. 3.31	京都大学医学部附属病院　外来化学療法部長（兼任）
2009. 4. 1 ～	京都大学名誉教授 財団法人 先端医療振興財団・臨床研究情報センター センター長（研究事業統括　兼任）　現在に至る

■ 概　要

私たち医師の使命は、患者さんの予後向上、そして疾病制圧である。ここに集った我々の使命は、プリオン病の制圧である。我々全ての叡智を結集して世界の範となるオールジャパンのプリオン病研究･治療体制を構築したい。

漸く本年７月より発足する日本医療研究開発機構（AMED）は、健康・医療にかかる科学研究費を統合し、強力な PDCA マネジメントのもとに一元管理・一貫管理する。本シンポジウムはまさしくそれを目指して開催されるもので、今後取るべき戦略アプローチの基礎となるものである。

有効かつ安全な医薬品の創出、新たな画期的な医療技術の開発においては、薬事法のもとに綿密なレギュラトリーサイエンスの要求する最高レベルのデータ、知見を積み上げてより深い科学的洞察に到達し、ようやく人に適用することが可能となる。

平成 17 年度より開始された文部科学省による事業によって橋渡し研究拠点の整備が進み、今や薬事法に基づく研究開発は、当然のこととなった。一方、厚生労働省による難治性疾患克服事業（平成 24･25･26 年度）によってほぼ 16 件に達する治験が進行中であり、既にラパマイシンは、LAM（リンパ脈管筋腫症）に対して薬事承認され、近々ロボットスーツ HAL® も神経難病の患者さんの機能回復に承認申請される見込みである。順次こうして難病に対しても、新しい医療技術が患者さんのもとに届けられつつある。プリオン病制圧という目標を見失わずに、我々が叡智を結集してレギュラトリーサイエンスの指示する道を一歩一歩着実に進むなら、必ずやプリオン病に対しても我々は勝利するであろう。