Administration of L-Type Bovine Spongiform Encephalopathy to Macaques to Evaluate Zoonotic Potential

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We administered L-type bovine spongiform encephalopathy prions to macaques to determine their potential for transmission to humans. After 75 months, no clinical symptoms appeared, and prions were undetectable in any tissue by Western blot or immunohistochemistry. Protein misfolding cyclic amplification, however, revealed prions in the nerve and lymphoid tissues.

Worldwide emergence of classical bovine spongiform encephalopathy (C-BSE) is associated with variant Creutzfeldt-Jakob disease in humans (*1*). Two other naturally occurring BSE variants have been identified, L-type (L-BSE) and H-type. Studies using transgenic mice expressing human normal prion protein (PrP^{C}) (*2*) and primates (*3*–*5*) have demonstrated that L-BSE is more virulent than C-BSE. Although L-BSE is orally transmissible to minks (*6*), cattle (*7*), and mouse lemurs (*5*), transmissibility to cynomolgus macaques, a suitable model for investigating human susceptibility to prions, remains unclear. We orally inoculated cynomolgus macaques with L-BSE prions and explored the presence of abnormal prion proteins (PrP^{Sc}) in tissues using protein misfolding cyclic amplification (PMCA) along with Western blot (WB) and immunohistochemistry (IHC). PMCA markedly accelerates prion replication in vitro, and its products retain the biochemical properties and transmissibility of seed prion strains (*8*).

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The Study

Two macaques orally inoculated with L-BSE prions remained asymptomatic and healthy but were euthanized and autopsied at 75 months postinoculation. WB showed no PrP^{Sc} accumulation in any tissue (Table), IHC revealed no PrP^{Sc} accumulation, hematoxylin and eosin staining revealed no spongiform changes in brain sections, and pathologic examination revealed no abnormalities.

We next attempted to detect PrP^{Sc} using PMCA, performed as previously described (*9*), with minor modifications (Appendix, https://wwwnc.cdc.gov/EID/article/31/5/24-1257-App1.pdf). First, we evaluated the sensitivity of PMCA. Using serial amplification with 10-fold stepwise dilutions of prion-infected brain homogenates as seeds, we amplified PrP^{Sc}-like proteinase K (PK)–resistant prion protein (PrPres) from a 10^{-7} dilution of 10% brain homogenate (BH) obtained from macaque intracerebrally inoculated with L-BSE prions in the fifth amplification round (Figure 1, panel A). This method also enabled propagation of PrPres from a 10^{-8} dilution of BH from C-BSE–affected cattle during the second amplification round (Figure 1, panel B), suggesting PMCA's higher efficiency and sensitivity for detecting C-BSE prions than macaque L-BSE prions.

We attempted to detect prions in the lymphoid and nervous systems, among other tissues, of the 2 orally inoculated macaques using refined PMCA (Figure 2; Appendix Figure, Table). In lymphoid tissue samples prepared using sodium phosphotungstic acid precipitation (Appendix), we amplified PrPres in the inguinal and mesenteric lymph nodes, ileum, and tonsils of both macaques (Figure 2, panels A, B), as well as in the spleen of 1 macaque (#18) and the thy-

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Table. Detection of PrPres in tissue samples obtained from macaques orally challenged with L-BSE prions in study of oral transmission of L-type bovine spongiform encephalopathy in macaques to evaluate zoonotic potential*

	PMCA, no. positive/no. total					WB, orally challenged animals		IHC, orally challenged animals	
	Orally challenged animals		Negative controls						
Sample location	18	19	9086	1004	9040	18	19	18	19
Nerve tissues									
Cerebral cortex, frontal lobe; PTA, PBS	0/2, 0/2	0/2, 0/2	ND	0/2, 0/2	0/2, 0/2	Neg	Neg	Neg	Neg
Cerebral cortex, temporal lobe	ND	ND	ND	ND	ND	Neg	Neg	Neg	Neg
Cerebral cortex, parietal lobe	ND	ND	ND	ND	ND	Neg	Neg	Neg	Neg
Cerebral cortex, occipital lobe	ND	ND	ND	ND	ND	Neg	Neg	Neg	Neg
Hippocampus	ND	ND	ND	ND	ND	Neg	Neg	Neg	Neg
Thalamus	ND	ND	ND	ND	ND	Neg	Neg	Neg	Neg
Cerebellum	ND	ND	ND	ND	ND	Neg	Neg	Neg	Neg
Cervical cord; EtOH, PBS	0/2, 0/2	0/2, **1/2**	0/2, 0/2	0/2, 0/2	0/2, 0/2	Neg	Neg	Neg	Neg
Thoracic cord; EtOH, PBS	0/2, **1/2**	0/2, **1/2**	0/2, 0/2	0/2, 0/2	0/2, 0/2	Neg	Neg	Neg	Neg
Lumbar cord; EtOH, PBS	0/2, 0/2	0/2, 0/2	0/2, 0/2	0/2, 0/2	0/2, 0/2	Neg	Neg	Neg	Neg
Median nerve, PTA	**2/2**	**1/2**	ND	ND	ND	Neg	Neg	Neg	Neg
Sciatic nerve, PTA	0/2	0/2	ND	ND	ND	Neg	Neg	Neg	Neg
Secondary lymphoid tissues									
Spleen, PTA	**1/2**	0/2	ND	ND	0/2	Neg	Neg	Neg	Neg
Tonsil, PTA	**1/2**	**2/2**	0/2	0/2	0/2	ND	ND	ND	ND
Submandibular lymph node, PTA	0/2	0/2	0/2	ND	0/2	Neg	Neg	Neg	Neg
Inguinal lymph nodes, PTA	**1/2**	**2/2**	ND	ND	ND	ND	ND	ND	ND
Mesenteric lymph node, PTA	**1/2**	**1/2**	0/2	0/2	0/2	ND	ND	ND	ND
Primary lymphoid tissues									
Thymus, PTA	0/2	**2/2**	ND	ND	ND	ND	ND	ND	ND
Others									
Submaxillary gland, PTA	**2/2**	**1/2**	ND	ND	ND	ND	ND	ND	ND
Ileum, PTA	**2/2**	**2/2**	0/2	0/2	0/2	Neg	Neg	Neg	Neg

*Bold indicates positive results. EtOH, ethanol precipitation; IHC, immunohistochemistry; ND, not determined; neg, negative; PBS, suspension in phosphate-buffered saline; PMCA, protein misfolding cyclic amplification; PrPres, PrP^{Sc}-like proteinase K–resistant prion proteins; PTA, sodium phosphotungstic acid precipitation; WB, Western blot.

mus of the other (#19), in the second or third amplification round of PMCA (Figure 2, panel C). We observed no PrPres in the submandibular lymph nodes (Appendix Figure 1). Examining the central nervous system, we observed no PrPres amplification in the cerebral cortex (Figure 2, panel C), whether seeded with phosphate-buffered saline homogenates or phosphotungstic acid precipitates. The spinal cord showed no PrPres amplification upon ethanol precipitation. However, PrPres was amplified in the cervical spinal cord of macaque #19 and in the thoracic spinal cord of both macaques with phosphate-buffered saline homogenates (Figure 2, panel D). We also confirmed PrPres in the median nerve of both

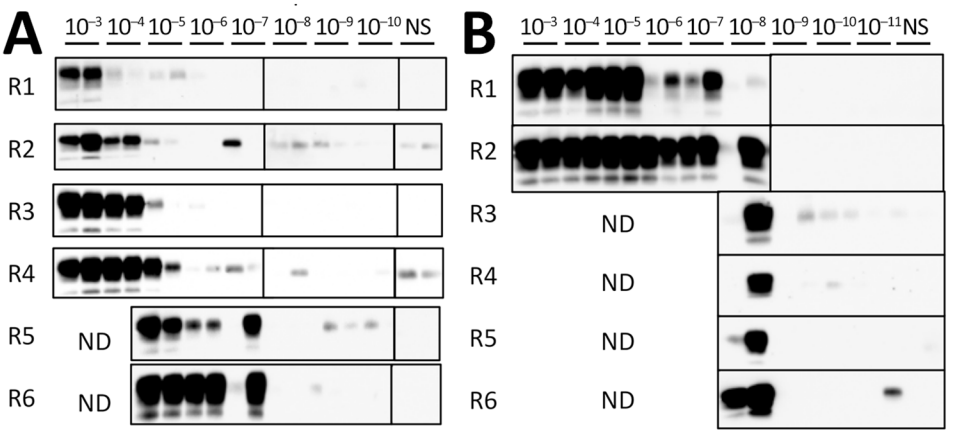

Figure 1. Sensitivity of modified protein misfolding cyclic amplification (PMCA) to detect abnormal prion protein in study of oral transmission of L-type bovine spongiform encephalopathy (L-BSE) in intracerebrally inoculated macaques to evaluate zoonotic potential. We evaluated in macaques intracerebrally inoculated with L-BSE prions (A) and cattle intracerebrally inoculated with classical BSE prions (B) (Appendix, https://wwwnc.cdc.gov/EID/article/31/5/24-1257-App1.pdf). We serially diluted (10^{-3}–10^{-11}) brain homogenates (10% weight by volume) in 50 µL of human normal prion protein (PrP^{C}) substrate and performed PMCA. We further diluted the initial PMCA product to 1:5 with a fresh PrP^{C} substrate for subsequent rounds. We conducted 6 rounds of PMCA in duplicate. In macaques, PMCA propagated PrPSc-like proteinase K–resistant prion protein (PrP^{res}) from a 10^{-7} dilution in the fifth amplification round; in cattle, PMCA propagated PrPres from a 10^{-8} dilution during the second amplification round. We performed Western blot for each PMCA product (2.5 µL) after proteinase K digestion using the T-2 antibody (*10*). ND, assays not done; NS, nonseeded control; R, round.

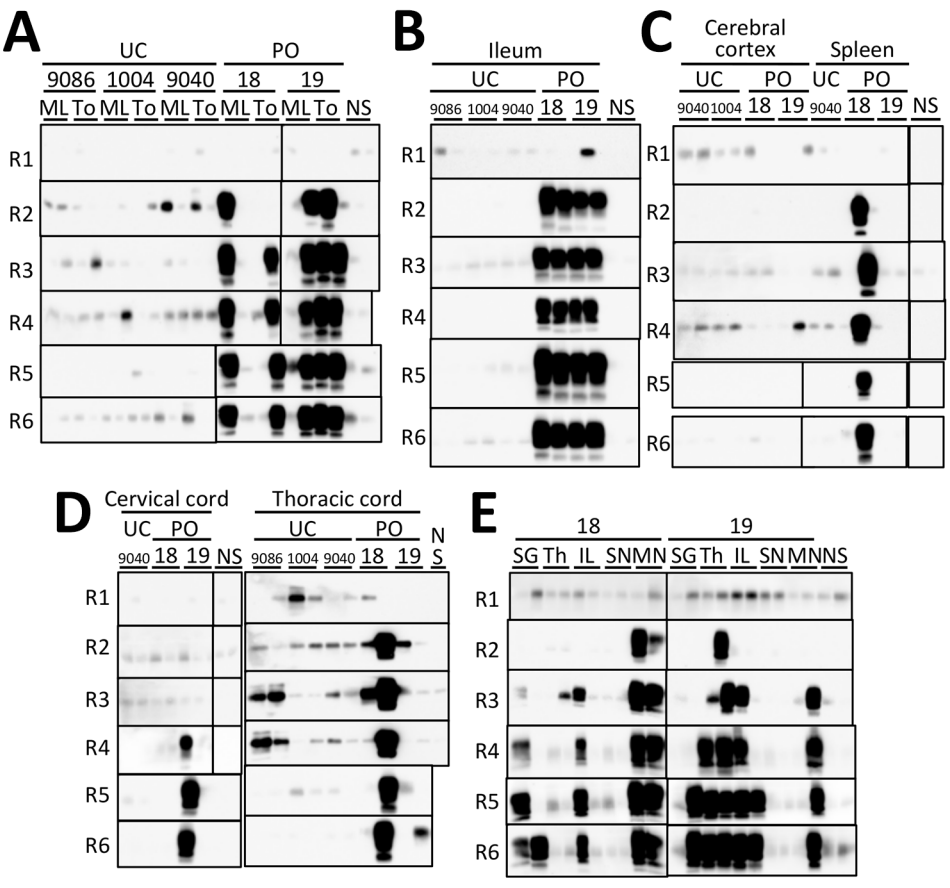

Figure 2. Sensitivity of modified protein misfolding cyclic amplification (PMCA) to detect abnormal prion protein in study of oral transmission of L-type bovine spongiform encephalopathy (L-BSE) in orally inoculated macaques to evaluate zoonotic potential. We performed 6 rounds of PMCA in duplicate in the tissues of 2 macaques (#18 and #19) orally inoculated with L-BSE prions, primarily in the lymphoid and nervous system tissues. A) ML nodes and To tissue; B) ileum; C) cerebral cortex and spleen; D) cervical and thoracic cords; and E) SG, Th, IL nodes, SN, and MN tissues. We prepared PMCA seeds, equivalent to 6.25 mg of tissue, obtained from lymphoid tissues, peripheral nerves, submaxillary glands, and ileum by using a standard sodium phosphotungstic acid precipitation method. For brain tissues, we used 10% homogenates in phosphate-buffered saline (250 µg) and phosphotungstic acid precipitates as seeds. For spinal cords, we used 10% homogenates in phosphate-buffered saline and ethanol precipitates (625 µg equivalent) as seeds. Tissues obtained from 3 uninfected macaques (#9068, #1004, and #9040) served as negative controls and were processed identically to those obtained from inoculated animals. For the thoracic spinal cord of macaque #19, we performed amplification for 7 rounds to determine whether the round 6 signal was positive. IL, inguinal lymph; ML, mesenteric lymph; MN, median nerve; NS, nonseeded control; PMCA, protein misfolding cyclic amplification; PO, L-type bovine spongiform encephalopathy orally inoculated macaques; SG, submaxillary gland; SN, sciatic nerve; TH, thymus; To, tonsil; UC, uninoculated control.

macaques but not in the sciatic nerve (Figure 2, panel E). We noted PrPres signals in the submandibular glands of both animals. In contrast, we found no PrPres amplification in any tissues from uninoculated control macaques.

PrPres obtained from the orally inoculated macaques exhibited diverse banding patterns distinct from those generated by PMCA using L-BSE–affected cattle BH and L-BSE intracerebrally inoculated macaque BH as seeds (Figure 3, panels A–C). Of note, the lowest-molecular-weight PrPres variants from the ileum, spleen, inguinal lymph nodes, thoracic cord, submaxillary gland, and mesenteric lymph nodes of orally inoculated macaques exhibited remarkable PK resistance similarity and banding patterns indistinguishable from those of PrPres generated by PMCA with C-BSE–affected cattle BH as a seed (Figure 3, panel C, Appendix Figures 2 and 3). In contrast, the higher-molecular-weight PrPres variants from the ileum of macaque #18 exhibited a unique banding pattern distinct from those of L-BSE, C-BSE, and H-type BSE prions (Figure 3, panel C). Banding patterns and PK resistance of PrPres amplified from L-BSE–affected cattle BH and L-BSE intracerebrally inoculated macaque BH were notably similar.

This PMCA method was initially designed for the high-sensitivity detection of L-BSE intracerebrally inoculated macaque PrP^{Sc} but was even more efficient and sensitive in detecting bovine C-BSE PrP^{Sc} (Figure 1, panel B). Therefore, we believe that this method enabled the detection of both C-BSE–like PrP^{Sc} and potentially novel PrP^{Sc} variants.

Conclusion

We noted no detectable evidence of PrP^{Sc} by WB or IHC in any tissues of L-BSE orally inoculated macaques. Nevertheless, PMCA successfully amplified PrPres from lymphatic and neural tissues. The PrPres

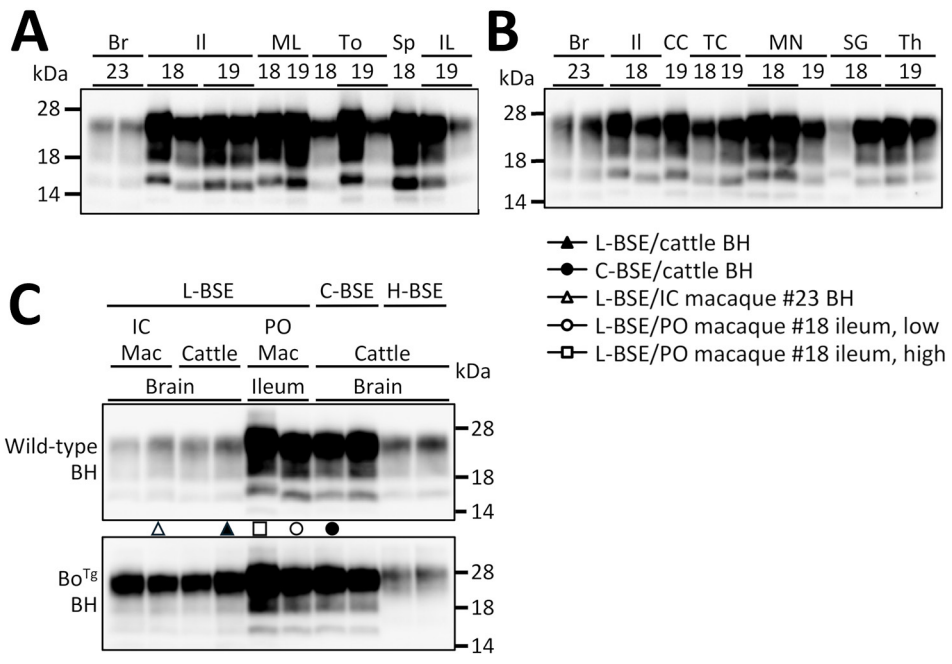

Figure 3. Gel electrophoresis and Western blot testing of tissues obtained from macaques orally inoculated with L-BSE prions in study of oral transmission of L-BSE in macaques to evaluate zoonotic potential. We loaded each 6th-round protein misfolding cyclic amplification (PMCA) product, seeded with tissues obtained from the 2 macaques (#18 and #19), onto 2 gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blot. A) PMCA products amplified from ML, To, Sp, and Il; B) PMCA products amplified from CC, TC, MN, SG, and Th; C) Western blot analysis. For comparison, PMCA products amplified using BH obtained from macaque #23, intracerebrally inoculated with L-BSE, were run on both gels together with products amplified from the ileum of macaque #18. Abnormal prion protein (PrP)-like proteinase K–resistant prion proteins (PrPres) from L-BSE orally inoculated macaques exhibited a few distinct banding patterns, which differed from those of PrPres from L-BSE intracerebrally in macaque #23. For Western blot, we compared 6th-round PMCA products, seeded with IL tissue obtained from macaque #18 and Br tissue obtained from macaque #23, L-BSE–affected cattle, C-BSE–affected cattle, and H-BSE–affected cattle. Banding results represent the products of PMCA using the BHs of wild-type mice (upper image) and bovine normal prion protein–expressing transgenic mice (lower image) as substrates, with identical cofactors. Among the PrPres amplified from the ileum of L-BSE/PO macaque #18, the banding pattern of PrPres with a small molecular weight (low) was very similar to that of PrPres amplified from the brain of cattle inoculated intracerebrally with C-BSE. BH, brain homogenate; Bo^{Tg}, bovine normal prion protein–expressing transgenic; Br, brain; C-BSE, classical bovine spongiform encephalopathy; CC, cervical cord; H-BSE, H-type bovine spongiform encephalopathy; IC, inoculated intracerebrally; Il, ileum; IL, inguinal lymph nodes; L-BSE, L-type bovine spongiform encephalopathy; Mac, macaque; ML, mesenteric lymph nodes; MN, median nerve; PO, inoculated orally; SG, submaxillary gland; Sp, spleen; TC, thoracic cord; Th, thymus; To, tonsil.

exhibited electrophoretic patterns distinct from those detected by PMCA using L-BSE–affected cattle BH as the seed (Figure 3, panel C), indicating that the PrP^{Sc} used as the template for PrPres amplification in orally inoculated macaques did not originate from the bovine L-BSE prions used as inoculum. Instead, PrP^{Sc} were newly generated by the conversion of macaque PrP^{C} by bovine L-BSE prions. Our results provide strong evidence that L-BSE can infect macaques via the oral route.

We found no evidence that PrP^{Sc} reached the brain in orally inoculated macaques; however, the macaques euthanized 6 years postinoculation might have been in the preclinical period. At low infection levels, lymph nodes play a vital role in prion spread to the central nervous system (*11*). Therefore, had the macaques been maintained for a longer period, they might have developed prion disease. Retrospective surveillance studies using the appendix and tonsil tissues suggested a considerable number of humans harboring vCJD in a carrier state (*12*). Thus, we cannot exclude that L-BSE orally inoculated macaques could similarly remain in a potentially infectious state.

The brain of L-BSE intracerebrally inoculated macaque accumulated prions with biochemical properties resembling bovine L-BSE prions (Figure 3, panel C; Appendix Figure 2); however, we observed no PrP^{Sc} accumulation in lymphoid tissues by WB or IHC (*4*). In contrast, macaques orally inoculated with C-BSE prions showed PrP^{Sc} accumulation in lymphoid tissues, including the spleen, tonsils, and mesenteric lymph nodes by WB and IHC (*13*). In our study, L-BSE orally inoculated macaques harbored C-BSE–like prions in their lymphoid and neural tissues. Interspecies transmission of L-BSE prions to ovine PrP transgenic mice can result in a shift toward C-BSE–like properties (*14*,*15*). Our data suggest that L-BSE prions may alter biophysical and biochemical properties, depending on interspecies transmission and inoculation route, acquiring traits similar to those of C-BSE prions. This transformation might result from structural changes in the L-BSE prion to C-BSE–like prions and other

lymphotropic prions within lymphoid tissues or from the selective propagation of low-level lymphotropic substrains within the L-BSE prion population.

The first limitation of our study is that the oral inoculation experiment involved only 2 macaques and tissues collected at 6 years postinoculation, before disease onset. Consequently, subsequent progression of prion disease symptoms remains speculative. A larger sample size and extended observation periods are required to conclusively establish infection in orally inoculated macaques. Furthermore, we performed no bioassays for PMCA-positive samples, leaving the relationship between PMCA results and infectious titers undefined. Considering that PrPres amplifications from tissues from the orally inoculated macaque tissues required 2 rounds of PMCA, the PrP^{Sc} levels in positive tissues might have been extremely low and undetectable in the bioassay.

Previous studies have demonstrated that L-BSE can be orally transmitted to cattle (*7*) and might have caused prion disease in farm-raised minks (*6*), indicating that L-BSE could naturally affect various animal species. Our findings suggest that L-BSE can also be orally transmitted to macaques. Therefore, current control measures aimed at preventing primary C-BSE in cattle and humans may also need to consider the potential risk of spontaneous L-BSE transmission.

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Oral Transmission of L-type Bovine Spongiform Encephalopathy in Macaques to Evaluate Zoonotic Potential

Appendix

Methods

Animal Experiment

The animal experiments were approved by the Animal Welfare and Animal Care and Use Committee (approval ID: DS23–41) and the Animal Ethics Biosafety Committee (approval ID: BSL3-R-10.04, BSL3-R-11.09, and BSL3-R-12.07) at the National Institutes of Biomedical Innovation, Health, and Nutrition, Japan. Two macaques, ≈1.2-year-old (#18 and #19), were orally inoculated with 8 g (5 mL of 20% homogenate for 8 times) of brain tissue from a natural L-BSE case in Japan (case #24).

The two macaques remained asymptomatic and healthy but were euthanized and autopsied at 75 months post-inoculation. For ethical considerations, we established a 75-month endpoint for the prion infection study in cynomolgus macaques. This decision was informed by prior findings that cynomolgus macaques inoculated with brain homogenates (BHs) from L-BSE-infected cattle manifested clinical signs approximately 2 years post-inoculation (*3*) and aimed to balance scientific inquiry with animal welfare considerations.

Uninoculated macaques (#9068 and #1004, #9040) were euthanized at 7 years and 6 months, 7 years and 2 months, and 22 years and 3 months, respectively, served as negative controls.

Western Blot (WB) Analysis of Tissues from Macaques Orally Inoculated with L-BSE Prion

Brain homogenates (50 µL, 20% w/v in PBS) of macaques #18 and #19 were mixed with 50 µL of a buffer containing 4% zwittergent 3–14, 1% lauroylsarcosine, 0.5 mg/mL collagenase, 60 unit/mL benzonase, 100 mM NaCl, and 50 mM HEPES-NaOH (pH 7.4), and incubated at 37°C for 30 minutes. Proteinase K (PK) was added to a final concentration of 50 µg/mL, and samples were incubated at 37°C for 45 minutes. After adding 50 µL of 2-butanol/methanol (5:1, v/v) with 10 mM PMSF, the mixture was centrifuged at 18,000 xg for 15 minutes at 18°C. Pellets were air-dried and stored at −75°C.

Non-brain tissues were similarly treated, then resuspended in 100 µL of buffer (2% zwittergent 3–14, 0.5% lauroylsarcosine, 100 mM NaCl, and 50 mM HEPES-NaOH, pH 7.4) and digested with 2.5 µg/mL PK at 37°C for 30 minutes before PMSF treatment and centrifugation.

PK-digested samples were analyzed by sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) and western blot using Invitrolon PVDF membranes. Membranes were blocked with PBS containing 0.1% Tween 20 and 4% fetal calf serum, incubated with the anti-PrP 3F4 antibody, and detected using HRP-conjugated anti-mouse IgG and Immobilon Forte substrate. Chemiluminescent signals were visualized using an LAS-3000 mini imaging system.

Sodium phosphotungstic acid (Na-PTA) precipitation

Tissues (e.g., lymph nodes) were sectioned into 50 mg portions using 18G needles and homogenized in 1.5 mL Nippi Biomasher II tubes (Nippi, Tokyo, Japan). A digestion solution

containing 50 mM Tris-HCl (pH 7.5), 2% Triton X-100, 0.5% Sarkosyl, 100 mM NaCl, 5 mM MgCl2, 2 mM CaCl2, 0.5 mg collagenase, and 10 µg DNase I was added to each sample. Homogenates were incubated at 37°C for 2 hours with periodic vortexing. Following centrifugation at 20,000 x g for 30 minutes at 20°C, the supernatant was discarded, and the pellet was resuspended in 6.25% lauroylsarcosine. After centrifuging at 5,000 x g for 5 minutes, the supernatant was treated with Na-PTA to a final concentration of 0.3% and incubated at 37°C for 30 minutes with rotation. The sample was then centrifuged, washed with PBS, and resuspended in 20 µL of PBS for protein misfolding cyclic amplification (PMCA). To monitor potential contamination, tissue samples from both inoculated and uninoculated macaques were simultaneously prepared under identical conditions, provided uninoculated tissues were available.

Ethanol precipitation

A 10% spinal cord homogenate (50 µL) was mixed with 450 µL of ethanol, vortexed, and left to stand at room temperature for 5 minutes. The mixture was then centrifuged at 20,000 x g for 5 minutes, and the supernatant was transferred to a fresh tube. The pellet was resuspended in 500 µL of ethanol, vortexed, and incubated for another 5 minutes at room temperature. After a second centrifugation at 20,000 x g for 5 minutes, the supernatant was combined with the first. The pellet was air-dried and resuspended in 20 µL of PBS. The suspension was used for PMCA. To monitor potential contamination, spinal cord samples from both inoculated and uninoculated macaques were simultaneously prepared under identical conditions.

PMCA

The PMCA protocol was adapted from a previous report (*8*), with minor modifications. Ten percent (w/v) wild-type mouse BHs, containing 1 × PBS, 4 mM EDTA, and 1% Triton X-

100, were supplemented with digitonin (0.05%), heparin (300 μg/ml), arginine ethyl ester (10 mM), and 4-sulfotetrafluorophenyl ester (0.01%). This mixture served as the PrP^C substrate. PMCA was performed using an automatic cross-ultrasonic protein-activating apparatus (ELESTEIN 070-GOT; Elekon Science Corp., Chiba, Japan). Amplification was performed using 32 cycles of sonication (pulse oscillation for 3 seconds, repeated five times at the intervals of 0.1 seconds), followed by incubation at 37°C for 30 minutes. The initial PMCA product was further diluted to 1:5 with a fresh PrP^C substrate for subsequent rounds. PMCA products (2.5 μL) were digested with 40 μg/mL proteinase K at 37°C for 1 hour. Samples were boiled in SDS sample buffer for 5 minutes, separated via SDS-PAGE using 15% Tris-glycine gels, and electronically transferred onto polyvinylidene fluoride membranes. The membranes were probed with anti-PrP horseradish peroxidase-conjugated monoclonal antibody T2 (*9*).

We used brain homogenates from macaques intracerebrally inoculated with L-BSE (L-BSE IC macaque #23) and cattle intracerebrally inoculated with C-BSE, L-BSE, and H-BSE (C-BSE, L-BSE, and H-BSE IC cattle) as seeds for PMCA. The L-BSE IC macaque was second-generation macaques obtained by intracerebrally inoculating brain homogenates from a macaque that developed prion disease following inoculation with brain homogenates from natural Japanese L-BSE-affected cattle (JP24) (*3*). The C-BSE, L-BSE, and H-BSE IC cattle were experimentally inoculated with brain homogenates from C-BSE, L-BSE, and H-BSE-affected cattle at the National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki, Japan. To prevent contamination, PMCA substrates were prepared under rigorous prion-free conditions. To monitor potential contamination and the *de novo* generation of prions, negative controls containing uninoculated macaque tissues and non-seeded samples were included in each PMCA round. Additionally, to avoid the cross-contamination bovine L-BSE and C-BSE BHs, PMCA using macaque tissue samples as seeds

was performed separately from PMCA using bovine and macaque L-BSE and C-BSE BHs as seeds.

PK degradation assay

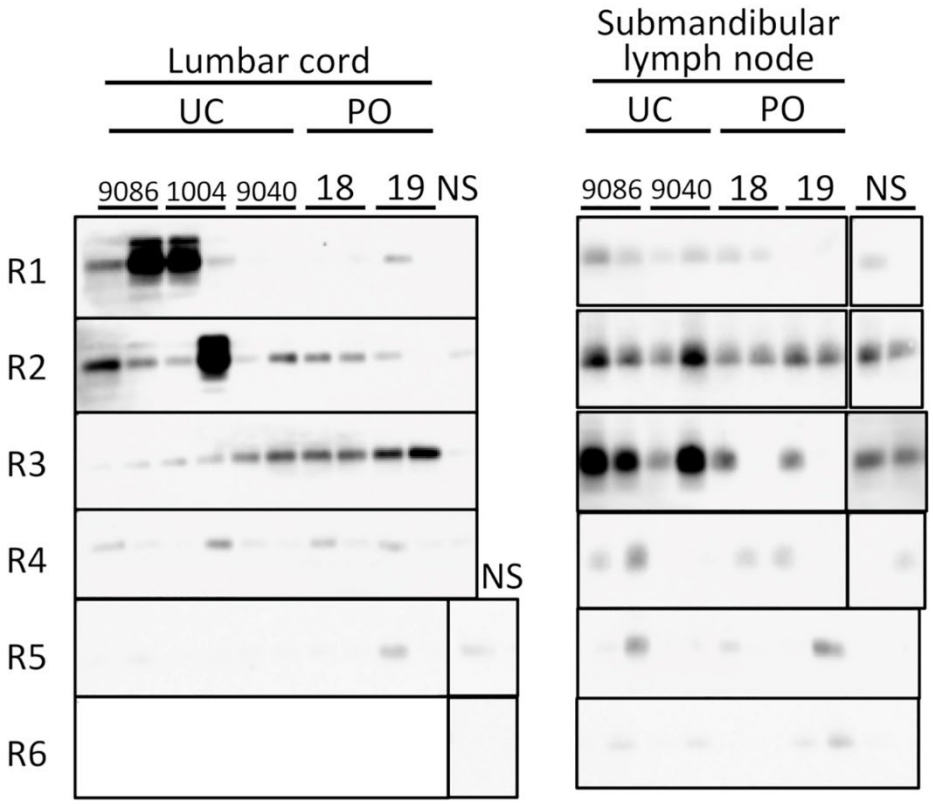
To compare the resistance of PMCA products to PK digestion, the products from six rounds of serial PMCA were digested with various concentrations of PK (50 to 5000 μg/ml) at 37°C for 1 hour. WB analysis was performed in four or three independent experiments. The average intensity of the PrPres signal in each sample was expressed as a percentage relative to that in the sample digested with 50 μg/ml of PK.

Results and Discussion

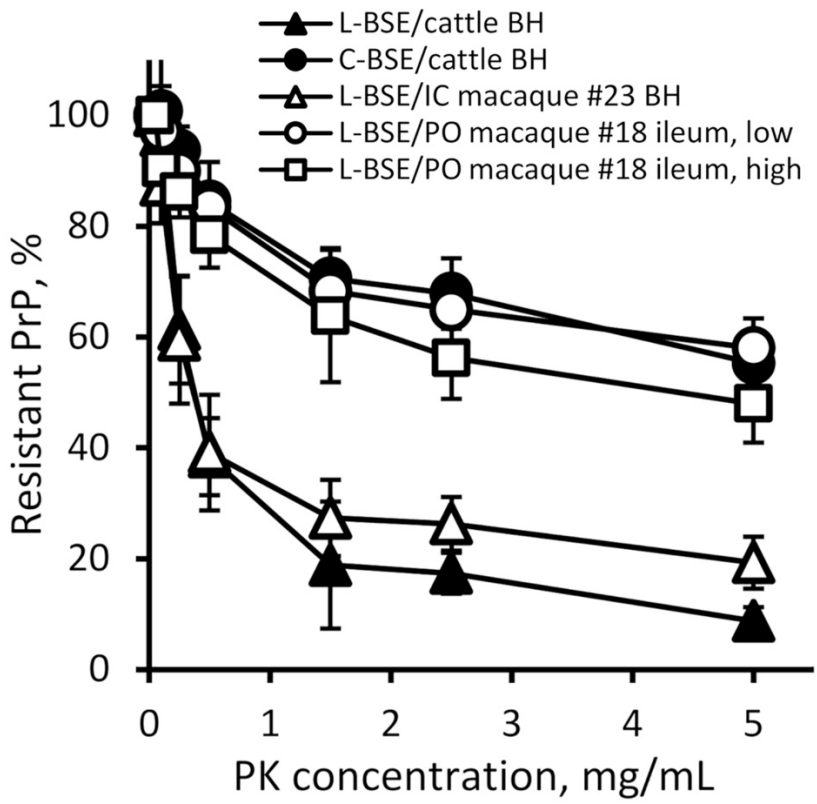
Among the PrPres with banding patterns similar to those of C-BSE-seeded PrPres propagated in various L-BSE-inoculated macaque tissues (Appendix Figure 3, panel A), those derived from the thymus and median nerve of macaque #19, as well as the thoracic cord of macaque #18 exhibited significantly reduced PK resistance compared to other PrPres and bovine C-BSE-seeded PrPres (Appendix Figure 3, panel B), suggesting that these PrPres are distinct from C-BSE-like PrPres. Each tissue of L-BSE PO macaques may produce multiple prion strains with distinct properties.

When the ileum sample from macaque #18 was used as a seed in PMCA using wild-type mouse BH as a substrate, distinct banding patterns of PrPres were amplified within the same sample (Figures 2 and 3). Therefore, it is suggested that two distinct PrP^{Sc} variants may exist in the ileum of #18 in minute amounts, and the amplification of either strain likely occurred by chance during PMCA, resulting in the selective propagation of one prion strain. In contrast,

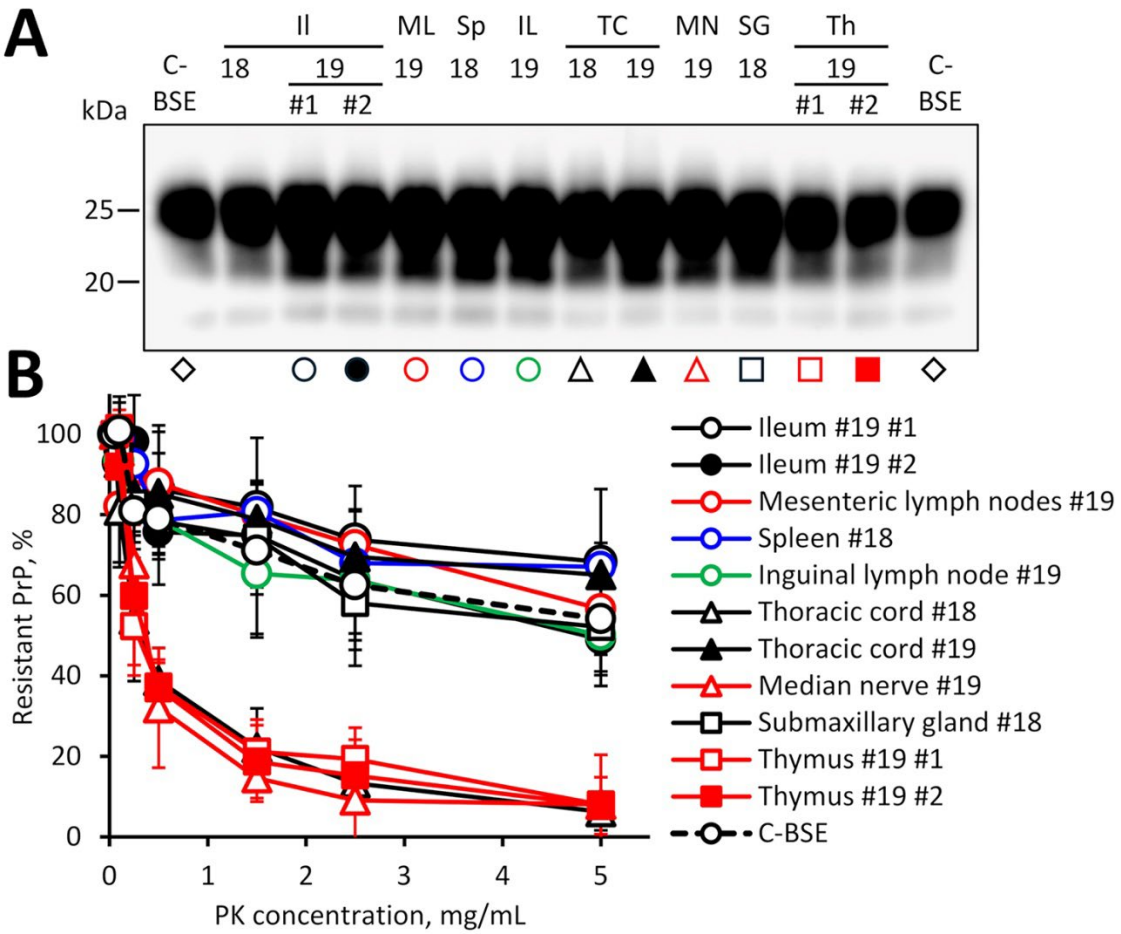
when TgBo BH was used in PMCA, the C-BSE-like prion in the ileum of #18 appeared to be preferentially amplified as the seed.



Appendix Figure 1. Proteinase K-resistant PrP^{Sc}-like prion protein was not amplified by PMCA in the lumbar cord or submandibular lymph nodes of both macaques (#18 and #19) orally inoculated with L-BSE prions. Sample preparation, PMCA, and western blotting were conducted as described in Figure 2. UC, uninoculated control; PO, L-BSE orally inoculated macaques.



Appendix Figure 2. PK resistance of the 6th round PMCA products, seeded with ileum obtained from an L-BSE PO macaque #18 (low-type = open circle; high-type = open square), was compared with PK resistance of brains obtained from L-BSE/IC macaque #23 (open triangle), L-BSE–affected cattle (closed triangle), and C-BSE–affected cattle (closed circle). A PK degradation assay was performed with PK concentrations of 50 µg/mL–5 mg/mL. The PrPres signal intensity after each PK treatment is expressed relative to the western blot signal intensity of PrPres treated with 50 µg/mL PK, which is set as 100%. Error bars represent standard deviation. BH, brain homogenate, C-BSE, classical bovine spongiform encephalopathy; IC, inoculated intracerebrally; L-BSE, L-type bovine spongiform encephalopathy; PK, proteinase K; PrP, prion proteins; PO, inoculated orally.



Appendix Figure 3. (A) Among the PrPres (proteinase K–resistant PrP^{Sc}-like prion proteins) amplified from various tissues of macaques orally inoculated with L-BSE, those exhibiting a banding pattern similar to that of PrPres obtained from PMCA using the brain homogenates of C-BSE-affected cattle as the seed were subjected to western blotting. The symbols correspond to the markers in panel B. In panel B, a PK degradation assay was conducted on the PrPres from panel A, following the method described in the Appendix. Remarkably, the PK resistance of the PrPres amplified from the thymus of macaque #19, the thoracic cord of macaque #18, and the median nerve of macaque #19 was significantly weaker than that of other PrPres, suggesting that these PrPres are distinct from C-BSE–like PrPres. Il (ileum), ML (mesenteric lymph nodes), Sp (spleen), IL (inguinal lymph nodes), PrP^{Sc}, abnormal prion proteins; TC (thoracic cord), MN (median nerve), SG (submaxillary gland), Th (thymus).