れた．このスクリーニング検査において陽性あるいは擬陽性のウシについては，ウェスタンブロッティング(WB)法によるPrP^{Sc}の検出および病理組織学的診断に基づく確定検査が行われる．確定検査によりBSE陽性と判定されたウシは焼却処分され，市場に流通することはない．なお2005年にスクリーニング検査の対象を21か月齢以上とする改正が行われたが，各都道府県では21か月齢以下のウシについても自主的に検査を継続しており，他国に比べより厳しい監視体制が維持されているといってよい．

また，24か月齢以上の死亡牛における検査も行われ，これまでに陽性と判定されたウシは，と畜場で21頭，死亡牛で14頭，最初の1頭と合わせて計36頭である(図2)．2009年2月以降に陽性ウシは確認されていない．これらの陽性ウシを誕生年別に分類すると，1996年および2000年を誕生年とするコホートの2つのピークがあることが分かる(図3)．この時期に特異的に多量もしくは高頻度のBSEプリオンへの曝露があったのか否かについては究明できていないが，2002年2月以降に出生したウシにBSEは見つかっておらず，同年以降に国内飼料での汚染は起きていないと推測される．また，後に述べる2例の非定型BSE例を除く34例では，WB法および病理組織学的解析の特徴が英国で流行したBSE例と同一であることから，過去に英国または欧州から輸入した肉骨粉飼料，あるいは輸入した感染ウシから製造された肉骨粉飼料が感染源であろうとの見解が示されている．[7]

なお，日本におけるvCJDの報告は1例のみである．[8] 英国への渡航歴があったことから，日本での感染ではなく英国滞在中に感染した可能性が高いと考えられている．

5 非定型BSEの発見

タンパク質性の感染因子であるという点で他の病原体と異なるプリオンであるが，細菌やウイルスと同様に，"株"が存在する．株の違いはWB像や病理組織学的特徴の違いとなって表れる．当初，各国で確認されていたWB像や病理組織象の特徴が一様であったことから，BSEプリオンは単一株であると考えられていた．しかし近年，我が国のBSE検査の過程において，従来のBSE株とは異なる新たなBSE株の存在が明らかとなった．従来型の株を"定型BSE"，新規の株を"非定型BSE"と称して区別する．日本で最初に非定型BSEが発見されたのは2003年であるが，[9] 同時期にイタリアから，[10] そして現在では他の欧州諸国や米国などでも少数ながらその存在が報告されている．BSEプリオンを構成するPrP^{Sc}は，タンパク質分解酵素であるプロテナーゼKに対して部分的な抵抗性を示す．

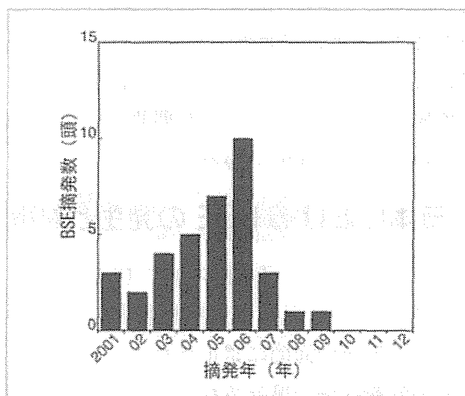

図2 日本におけるBSE陽性ウシの摘発状況

と畜場および死亡牛等での検査により摘発されたBSE陽性ウシの合計数を摘発年ごとに示した．2009年2月以降，陽性ウシは摘発されていないが，これまでに合計36頭がBSE陽性と判定された(2012年8月現在)．厚生労働省ホームページより改変．

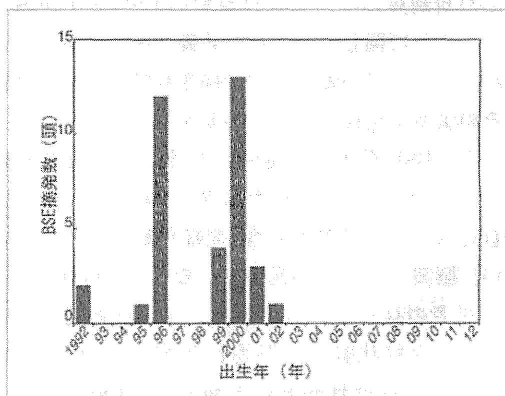

図3 BSE陽性ウシの出生年ごとの分布

日本で摘発されたBSE陽性ウシを出生年ごとに分類すると，不連続な分布パターンを示し，1996年と2000年の出生コホートにBSE感染ウシが多いことが分かる．2002年2月以降に出生したウシからはBSE陽性ウシは確認されていない(2012年8月現在)．厚生労働省ホームページより改変．

したがってプロテナーゼKでプリオンを処理した後，電気泳動と抗PrP抗体を用いたWB法によりPrP[Sc]断片を検出することができる．この際，PrPには2か所の糖鎖付加部位があるために，糖鎖の付加しない無糖鎖型，1か所のみに糖鎖が付加した一糖鎖型，そして2か所ともに糖鎖が付加した二糖鎖型の3本のバンドが観察される(図4A)．電気泳動上で無糖鎖型のバンドが定型BSEのものに比べ低分子側にシフトしている非定型BSEをL-BSE，また高分子側にシフトしている非定型BSEをH-BSEと分類する[11,12]．

日本ではH-BSEの報告はないが，2例のL-BSEが摘発されている．このうち2例目のL-BSE(2006年に摘発，JP 24)[13]では，無糖鎖型の分子量だけではなく糖鎖の付加比も定型BSEのそれと異なり，JP 24では一糖鎖型の割合が多い(図4A，B)．このような生化学的性状の相違とともに，両者の感染性に違いがあるのかを調べるためにマウス，ウシ，霊長類等を用いた伝達実験が進められた．[14~16] その結果，L-BSE(JP 24)は定型BSEとは異なる病原性を持つことが明らかになりつつある．同様の結果が，他国で摘発されたL-BSEについても報告されている．なおサルへの伝達実験において，L-BSEは定型BSEより短い潜伏期間で発症に至った．[16] この結果から，L-BSEは定型BSEよりもヒトに対して感染性が強いのではないかと懸念されるが，[17,18] 定型BSEに比べて発生頻度が極めて少ない非定型BSEに関するデータは不足しており，現時点で結論付けるのは難しい．また，世界における非定型BSEの発生が散発的であることから，非定型BSEは孤発性ではないか(つまり，汚染肉骨粉による感染が原因ではない)との仮説も立てられているが，[12] 確証は未だ得られていない．

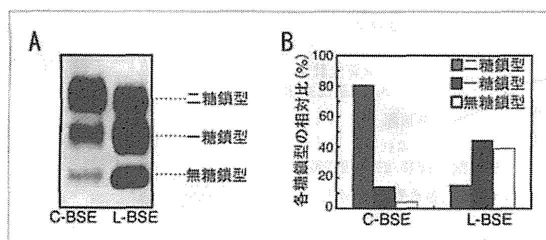

図4　日本で確認された非定型BSEの特徴

A；日本で摘発された定型BSE(C-BSE)，および非定型であるL-BSE(JP 24)のWB像．プロテナーゼK処理後に残存するPrP[Sc]を検出している．L-BSEでは無糖鎖型のバンドがより低分子側に検出される．
B；日本で摘発されたC-BSEおよびL-BSE(JP 24)の糖鎖型の比較．AのWB像を基に，各糖鎖型のバンドのシグナル強度を比較した．L-BSEでは全体に占める一糖鎖型の割合が多いことが分かる．

6 おわりに

プリオンについてはまだ謎が多く，科学的に直接証明されていないことも多い．しかし，科学的知見が限られていても，予測可能なリスクへの対策を適切に講じることが重要であることを，一連のBSE対策に見ることができるように思う．

現在，厚生労働省の諮問を受け，食品安全委員会のプリオン専門調査会がBSEの国内措置等の変更に関し評価を行っている．その中で，我が国のBSE検査対象を現行の21か月齢以上から30か月齢以上へ引き上げた場合，また特定危険部位の一部についても現行の全月齢から30か月齢超に変更した場合のリスクの差は，“あったとしても非常に小さく，人への健康影響は無視できる”と答申している．今後も科学的知見の蓄積とともにそのリスク管理の在り方は適宜慎重に再検討される必要があり，科学的な知見をどのようにリスク管理に活かしヒトの健康を守るのか，引き続き問われることと思う．

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Properties of L-Type Bovine Spongiform Encephalopathy in Intraspecies Passages

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Abstract

The origin and transmission routes of atypical bovine spongiform encephalopathy (BSE) remain unclear. To assess whether the biological and biochemical characteristics of atypical L-type BSE detected in Japanese cattle (BSE/JP24) are conserved during serial passages within a single host, 3 calves were inoculated intracerebrally with a brain homogenate prepared from first-passaged BSE/JP24-affected cattle. Detailed immunohistochemical and neuropathologic analysis of the brains of second-passaged animals, which had developed the disease and survived for an average of 16 months after inoculation, revealed distribution of spongiform changes and disease-associated prion protein (PrP^{Sc}) throughout the brain. Although immunolabeled PrP^{Sc} obtained from brain tissue was characterized by the presence of PrP plaques and diffuse synaptic granular accumulations, no stellate-type deposits were detected. Western blot analysis suggested no obvious differences in PrP^{Sc} molecular mass or glycoform pattern in the brains of first- and second-passaged cattle. These findings suggest failures to identify differences in mean incubation period and biochemical and neuropathologic properties of the BSE/JP24 prion between the first and second passages in cattle.

Keywords

atypical bovine spongiform encephalopathy, L-type, immunohistochemistry, prion, transmission

Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disorder manifested as one of a group of transmissible spongiform encephalopathies, including scrapie in sheep and goats, chronic wasting disease in deer, and Creutzfeldt-Jakob disease in humans. The infectious agent responsible for these diseases is an abnormal isoform prion protein (PrP^{Sc}), currently thought to be a posttranslationally modified form of a host-encoded membrane glycoprotein (PrP^{C}). The uniform nature of the pathology and biochemical features of the proteinase K–resistant PrP^{Sc} (PrP^{res}) profile obtained for cattle infected with BSE and mice in BSE transmission studies suggests that BSE is caused by a single prion strain.

Recently, variants of BSE (denoted atypical BSE) have been detected in cattle in Japan, Europe, and North America.[2] Currently, atypical BSE cases are classified into at least 2 groups, namely the L-type and the H-type, according to the molecular weight of the PrP^{res} in the case compared to that of classical (C-type) BSE cases. Histopathologic as well as immunohistochemical analyses have confirmed that atypical H- and L-type forms of BSE are transmissible to mice and clearly differ from C-type BSE in terms of incubation period, PrP^{res} profile, and pathology.[1] The L-type BSE recognized in Italy, which has been further characterized by the presence of PrP^{Sc}-positive amyloid plaques in the brain, has been termed *bovine amyloidotic spongiform encephalopathy* (BASE).[5]

Western blot analysis of one 14-year-old Japanese black beef cow (BSE/JP24)[7] suggested a glycoform ratio similar to that of BASE-infected cattle, while PrP^{Sc} immunohistochemistry analysis suggested the presence of PrP^{Sc}-positive amyloid plaques in the brain similar to those found in BASE-infected cattle.[5] Additional studies have demonstrated that the BSE/JP24 isolate can be transmitted to cattle[6] and that BSE/JP 24 and BASE have nearly the same incubation period during the first passage in Holstein cattle.[6,8]

Research has also suggested that during subsequent passages, the incubation period of the disease may be shorter or more stable than that of the first passage and that its characterization may vary. The primary objective of this study was to further investigate the characteristics of the second passage of the

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disease by clinicopathologic analysis of BSE/JP24-infected cattle. The secondary objective was to fill a significant research gap by further identifying and describing the topographic distribution and patterns of immunolabeled PrP^{Sc}, thereby redressing the paucity of immunohistochemical data that have been collected regarding atypical BSE cases.

All the experiments involving animals were performed in the biosafety level 3 areas with the approval of the Animal Ethical Committee and the Animal Care and Use Committee of the National Institute of Animal Health. Three Holstein female calves aged 2 to 3 months old were inoculated through the intracerebral route with 1 ml of 10% brain homogenate prepared from the brainstem of a first-passaged BSE/JP24-affected cow (case No. 1A; code 8515) used in a previous experiment.[6] The brain material was confirmed positive by Western blot and immunohistochemical analysis. Two uninoculated calves served as controls and were sacrificed 24 months after inoculation.

Between 12 and 13 months after inoculation, the animals began to exhibit signs of fear or anxiety, which are initial clinical signs of BSE. The animals were observed to exhibit ataxia of the hind limbs before astasia and were subsequently killed at 421 (case No. 2A), 504 (case No. 2B), and 559 (case No. 2C) days after inoculation. The results of unpaired *t* testing (InStat3, GraphPad Software, La Jolla, CA) failed to identify a significant difference in mean incubation period between the first- and second-passaged cattle (486 $\pm$ 11 days[6] and 495 $\pm$ 69 days, respectively).

After the brain was cut longitudinally at necropsy, the left half, including the brainstem and cerebellum, was fixed in 10% neutral buffered formalin containing 10% methanol, while the right half was frozen at $-80°C$ for Western blot analysis. Formalin-fixed coronal slices of the brain and tissue samples from the entire body were trimmed into 3- to 4-mm thick sections, immersed in 98% formic acid for 60 minutes to reduce infectivity, rinsed, dehydrated, embedded in paraffin wax, sectioned at 4 μm, and stained with hematoxylin and eosin. A vacuolar lesion profile of the hematoxylin and eosin–stained sections was obtained using the method described by Simmons et al.[10] Selected sections were stained with phenol Congo red and examined under a polarizing microscope, and the presence of amyloid was confirmed by observation of its characteristic dichroism.

For the demonstration of immunolabeled PrP^{Sc}, dewaxed sections were placed on a silanated slide, treated with 3% hydrogen peroxide at room temperature for 10 minutes, incubated with 10 μg/ml of proteinase K (0.03 U/μg; Nakarai, Kyoto, Japan) in phosphate-buffered saline containing 0.1% Triton-X at room temperature for 10 minutes, and immersed in 150mM sodium hydroxide at 60°C for 10 minutes.[6,9] The sections were then incubated with monoclonal antibody F99/97.6.1 (1 μg/ml; VMRD Inc., Pullman, WA), which reacts with a conserved epitope located at amino acid residues 228–233(QYQRES) of the bovine prion protein when using a horseradish peroxidase–labeled polymer detection system (Nichirei Histofine Simple Stain MAX-PO (M), Nichirei, Tokyo, Japan). The sections were then visualized using 3′-3 diaminobenzidine tetrachloride as the chromogen in accordance with the manufacturer's instructions and slightly counterstained with hematoxylin. The intensity and extent of PrP^{Sc} accumulation were subjectively scored from 0 to 4 (0, negative; 1, apparent at high magnification; 2, apparent at moderate magnification; 3, apparent at low magnification and moderate amounts of accumulation; 4, large amounts of accumulation). Negative controls were incubated with nonimmune mouse or rabbit IgG (1:30 dilution; Dako) and phosphate buffered saline instead of the primary antibody. Schematic representation of immunolabeled PrP^{Sc} distribution in the brain was drawn with Adobe Photoshop. Proteins were extracted from the frozen samples and subjected to Western blot analysis of PrP^{res} according to a previously described method.[6] The blotted membrane was then incubated with anti-PrP-specific monoclonal antibody T2 and the signals detected using a chemiluminescent substrate (SuperSignal; Thermo Fisher Scientific Inc., Rockford, IL). The signal intensity and glycoform ratio of PrP^{Sc} were calculated with Fluorochem software (Alpha Innotech, San Leandro, CA). Brains of 2 sham-inoculated Holstein cattle served as controls for immunohistochemical and Western blot analyses.

The results of the analysis suggested that the neuropil but not the neuronal vacuolation was consistent in most nuclei of the thalamus and brainstem structures, including the midbrain, pons, and medulla oblongata, but not in the dorsal motor nucleus of the vagus nerve. The vacuolar lesion score in the second-passaged animals was similar to that in first-passaged cattle, as previously described (data not shown).[6] The variability in PrP^{Sc} immunolabeling intensity and distribution pattern was found to be nearly identical. Seven varieties of PrP^{Sc} immunostaining patterns—including intraneuronal, perineuronal, intraglial, linear, fine particulate, coarse granular, and plaques—were identified throughout the brain of first- and second-passaged animals. As can be observed in Figure 1, which shows the topographic distribution of the PrP^{Sc} identified, significant quantities of PrP^{Sc} were detected in the brainstem and thalamus. The most conspicuous pattern of PrP^{Sc} was that of fine particulate and coarse granular deposits in the neuropil of the thalamus and midbrain, particularly in the periaqueductal grey matter, and to some extent in the cerebral cortices and cerebellar nuclei (Fig. 2). The major feature of the plaques was a spherical structure with a unicentric core up to 20 μm in diameter. The plaques were sparsely detected in the thalamus, basal ganglia, midbrain, pons, deeper layers of the cerebral cortices, and subcortical white matter and were not detected to any extent in the olfactory bulb, medulla oblongata, cerebellum, or spinal cord. These plaques exhibited a variable morphology; although most plaques that had a uniformly dense core were noncongophilic and less than 15 μm in diameter, some that had a lucent core were congophilic, larger than 15 μm in diameter, irregularly outlined, and showed distinctly intense surrounding immunolabeling (Fig. 3). The stellate-type of PrP^{Sc} deposition usually detected in the cerebral and cerebellar cortices of C-type BSE-affected cattle was undetectable in any sections of any of the animals, while less intraneuronal deposition of PrP^{Sc} was found in the cerebral cortex compared to the thalamus and brainstem (Fig. 2). PrP^{Sc} immunolabeling

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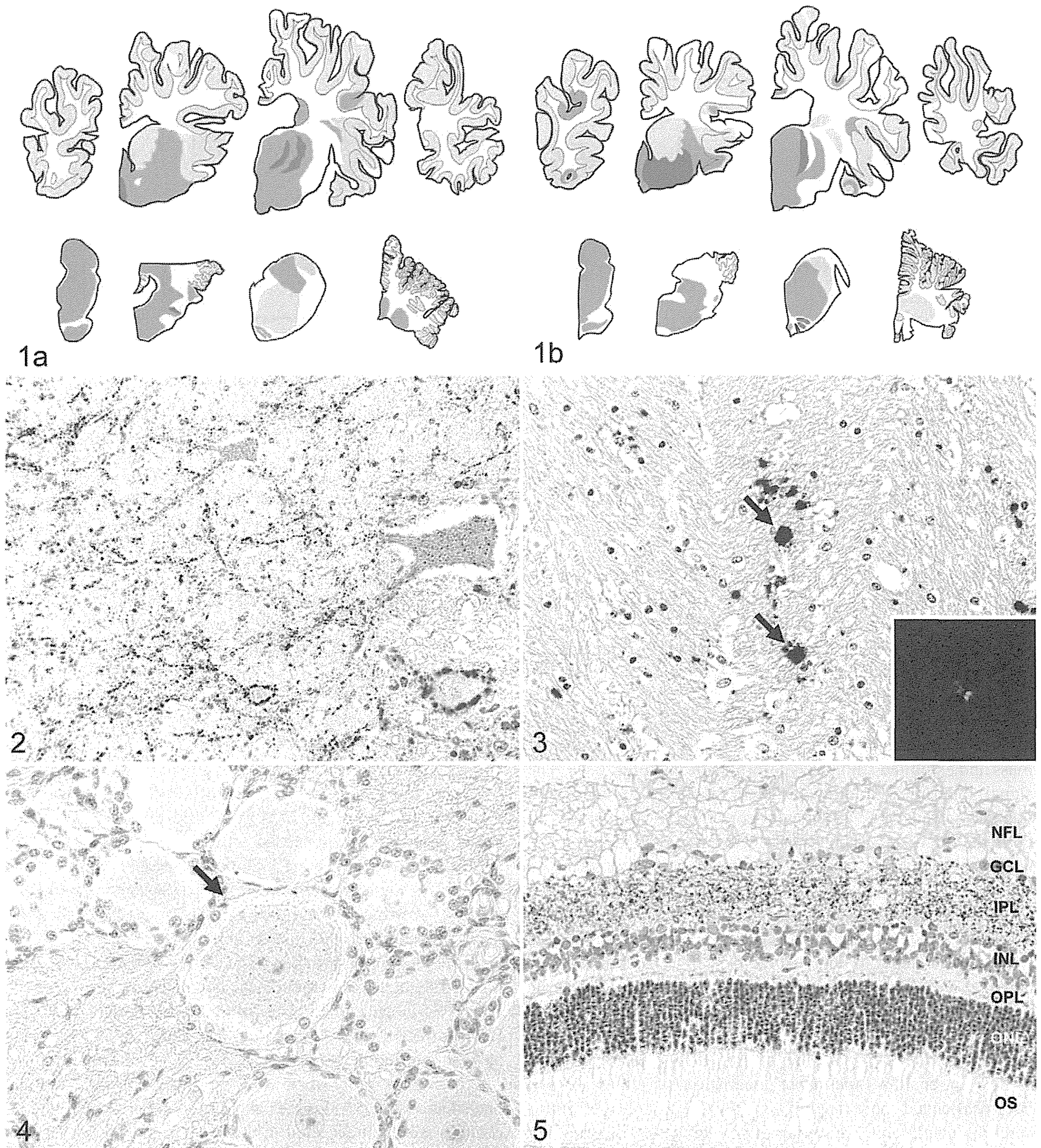

Figure 1. Immunolabeled PrP^{Sc} distribution in the central nervous system of (a) first- and (b) second-passaged cattle (respectively, case Nos. 1A and 2C). Immunolabeling intensity of PrP^{Sc} deposition was scored as none (white), scanty (pale yellow), mild (dark yellow), moderate (orange), and heavy (red). Eight brain areas can be observed, at the level of the frontal lobe, striatum, thalamus, occipital lobe, midbrain, pons, medulla oblongata at the obex, and the cerebellum, from the upper left to the lower right. **Figure 2.** Brain, cow, second passaged (case No. 2C). Granular, linear, intraneuronal, and perineuronal patterns of PrP^{Sc} can be observed in the motor nucleus of trigeminal nerve of pons immunolabeled with monoclonal antibody F99/97.6.1 and counterstained with hematoxylin. The insets in the right corner are higher magnification images of intraneuronal (upper box) and perineuronal labeling of PrP^{Sc} (lower box). **Figure 3.** Brain, cow, second passaged (case No. 2C). Plaque deposits (arrows) can be observed in the white matter of the thalamus immunolabeled with monoclonal antibody F99/97.6.1 and counterstained with

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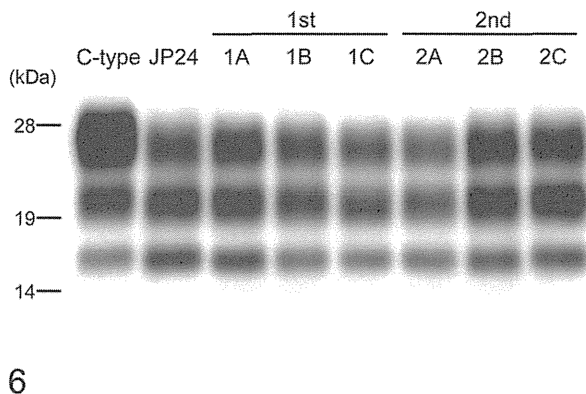

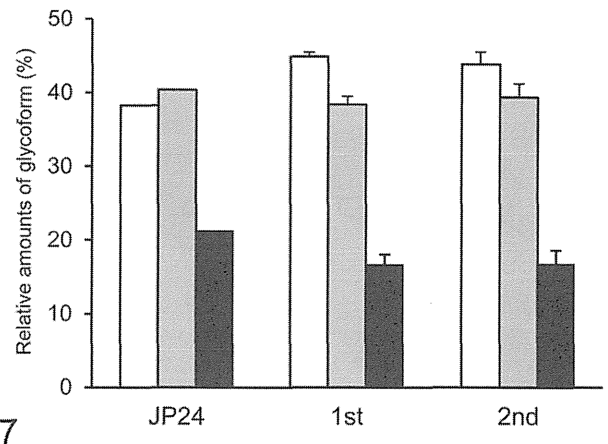

Figure 6. Western blot analysis of proteinase K–digested prion protein (PrP[res]) from the medulla oblongata at the obex level of C-type bovine spongiform encephalopathy (BSE) and BSE/JP24 first- and second-passaged cattle with monoclonal antibody T2. All samples were digested with 50 μg/ml of proteinase K at 37°C for 1 hour. Molecular markers are shown on the left.

Figure 7. The relative amount of the diglycosylated, monoglycosylated, and unglycosylated form of PrP[res] in the BSE/JP24, first-passaged, and second-passaged prion-affected individual with monoclonal antibody T2. The results are shown as mean ± standard deviation in triplicate experiments. Bar diagram indicates diglycoslated form (black column), monoglycosylated form (gray column), and unglycosylated form (white column).

was detected in extracerebral tissues, including the trigeminal and dorsal root ganglia (Fig. 4), neurohypophysis, and retina (Fig. 5). PrP[Sc] accumulation was prominent in the ganglion cell layer as well as the inner and outer plexiform layers of the retina (Fig. 5). No histopathologic changes were present in the peripheral tissues, including the skeletal muscles.

Western blot analysis of PrP[res] suggested that the brains of first- and second-passaged cattle had a similar molecular mass and exhibited similar glycoform patterns (Figs. 6, 7). The signal intensities in diglycosylated, monoglycosylated, and unglycosylated PrP[res] fragments were found to be similar in both the first- and second-passaged cattle. In addition, the unglycosylated PrP[res] fragment detected in both groups showed a lower molecular weight than that in cattle affected with a C-type BSE agent, suggesting that quantitative studies of PrP[res] molecular mass and glycoforms proportions in Western blots will be more useful to demonstrate the difference between the classical and different passages of atypical BSEs. The mean incubation period of the first- and second-passaged Holstein cattle challenged with the BSE/JP24 prion was approximately 16 months, the same as that of BASE-inoculated Holstein cattle.[8] In contrast, the mean incubation period of C-type BSE-challenged cattle ranges from 17 to 22 months after intracerebral inoculation.[8] In studies of bovine PrP-overexpressing transgenic mice, the incubation period was also shorter in L-type BSE-inoculated mice than in C-type.[3,4,9] These results suggest that in cattle, L-type BSE agents have a shorter incubation period compared to that of C-type BSE agents and that the biochemical properties of PrP[Sc] from an original BSE/JP24 isolate have no evident differences in BSE/JP24-infected cattle. The incubation period and molecular profiles of PrP[Sc] suggest that the isolate of BSE/JP24 is clearly distinct from that of C-type BSE while closely resembling that of BASE isolate.[5,6,8]

Immunohistochemical analysis revealed that the patterns of PrP[Sc] deposition in BSE/JP24 prion-affected cattle were characterized by the presence of amyloid PrP plaques and the absence of stellate-type PrP[Sc] deposits. However, except for the detection of higher quantities of PrP[Sc] in the cerebral cortices of BSE/JP24-affected cattle, no striking differences were identified between the topographic distribution of PrP[Sc] in the C-type BSE and BSE/JP24 prion-affected cattle.[6] The incubation periods and neuropathologic and immunohistochemical characteristics of the BSE/JP24-inoculated Holstein cattle closely resemble those of BASE-affected cattle,[8] indicating that both L-type BSE prions have similar biological properties.

Although atrophy of both type I and II muscle fibers had been detected in BASE cases in previous experiments,[8] evident changes were not detected in the skeletal muscle of the cases examined in this study. This discrepancy may have been due

Figure 3. (continued). hematoxylin. The inset in the right corner is a higher-magnification image of the apple-green birefringence of PrP plague stained with phenol Congo red under polarized light. **Figure 4.** Trigeminal ganglion, cow, second passaged (case No. 2B). Faint granular PrP[Sc] depositions can be observed in the ganglionic cell (arrow) immunolabeled with monoclonal antibody F99/97.6.1 and counterstained with hematoxylin. **Figure 5.** Retina, cow, second-passaged (case No. 2C). Diffuse granular deposition of PrP[Sc] can be observed in the ganglion cell layer, as well as in the inner and outer plexiform layers of the retina immunolabeled with monoclonal antibody F99/97.6.1 and counterstained with hematoxylin. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nucleus layer; OPL, outer plexiform layer; ONL, outer nucleus layer; OS, outer segments.

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to the fiber-type grouping used in the previous studies, which had resulted in neurogenic atrophy of the innervated muscles, indicating that the muscular lesions in the BASE cases might not be associated with a neurogenic disorder. The discrepancy might also be due to the use of different technical protocols in tissue processing—specifically, freezing of sections versus formalin fixing and paraffin embedding of sections.

Although L-type BSE cases have been detected throughout Europe, it remains unclear whether the BSE/JP24 prion is identical to other L-type BSE prions. Although the results of this study may accord with those obtained in studies of cattle intracerebrally inoculated with BASE, further research is required to determine if they truly do so. In conclusion, the results of the detailed immunohistochemical and neuropathologic analysis conducted in this study suggest the failure to identify differences between the first- and second-passaged cattle regarding the variables of survival period, molecular properties of PrP^{res}, and other immunohistochemical or neuropathologic features; this suggests that the BSE/JP24 isolate is a biologically and biochemically stable prion in cattle. The findings of the present study will aid future transmission experiments on different L-type BSE isolates.

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Declaration of Conflict of Interest

The authors declare that they have no conflicts of interests with respect to their authorship or the publication of this article.

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ORIGINAL ARTICLE

Ultrasensitive detection of scrapie prion protein derived from *ARQ* and *AHQ* homozygote sheep by interspecies *in vitro* amplification

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ABSTRACT

Prions, infectious agents causing TSEs, are composed primarily of the pathogenic form (PrP^{Sc}) of the PrP^{C}. The susceptibility of sheep to scrapie is determined by polymorphisms in the coding region of the *PRNP*, mainly at codons 136, 154, and 171. The efficiency of *in vitro* amplification of sheep PrP^{Sc} seems to be linked also to the PrP genotype. PrP^{Sc} derived from sheep with $V^{136}R^{154}Q^{171}$-associated genotypes can be amplified efficiently by PMCA in the presence of additional polyanion such as poly A, but there are no reports that cite ultrasensitive detection of PrP^{Sc} derived from sheep of other PrP genotypes. We report here that sheep PrP^{Sc} derived from *ARQ* and *AHQ* homozygotes was amplified efficiently by serial PMCA using mouse brain homogenate as PrP^{C} substrate. *ARQ/ARQ* PrP^{Sc} was detected in infected brain homogenates diluted up to 10^{-10} after five rounds of amplification, and *AHQ/AHQ* PrP^{Sc} was detected in samples diluted up to 10^{-8} after four rounds of amplification. On the other hand, amplification of PrP^{Sc} from *VRQ/ARQ* sheep seemed to be less efficient under the experimental conditions used. The interspecies PMCA developed in this study may be useful in the detailed analysis of PrP^{Sc} distribution in classical scrapie-infected *ARQ* and *AHQ* homozygote sheep.

Key words interspecies amplification, prion gene polymorphism, protein misfolding cyclic amplification, scrapie prion protein.

Transmissible spongiform encephalopathies, commonly known as prion diseases, such as scrapie in sheep, CWD in deer and elk, BSE, and CJD in humans are fatal neurodegenerative disorders (1). One of the hallmarks of prion diseases is the accumulation of protease-resistant, misfolded isoforms of prion protein (PrP^{Sc}) in the central nervous system of infected hosts (2). PrP^{Sc} appears to propagate itself by the conformational conversion of the cellular form of PrP^{C} into a misfolded form (2).

Prion protein gene (*PRNP*) is known to be polymorphic in humans (3) and animals (4, 5). In sheep, susceptibility to natural and experimental scrapie is determined by amino acid polymorphisms, mainly at positions 136, 154 and 171 of the prion protein (6–8). There are five commonly occurring alleles: $V^{136}R^{154}Q^{171}$, *ARQ*, *ARR*, *AHQ* and *ARH*. *ARR/ARR* sheep seemed to be highly resistant to scrapie infection, whereas *VRQ/VRQ* sheep have been found to be most susceptible (9–12). In addition to classical scrapie,

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List of Abbreviations: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; CWD, chronic wasting disease; HRP, horseradish peroxidase; PK, proteinase K; PMCA, protein misfolding cyclic amplification; *PRNP*, prion protein gene; PrP^{C}, host-encoded cellular prion protein; PrP^{res}, protease-resistant PrP; PrP^{Sc}, scrapie form of PrP^{C}; TSE, transmissible spongiform encephalopathy; WB, western blot; WT, wild-type.

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atypical scrapie has been reported in recent years (13–15). Sheep carrying the *ARR* and *AHQ* alleles, known to be less susceptible to infection with classical scrapie, have been affected with the atypical form, while the *VRQ* allele seems to induce partial resistance to atypical scrapie (13,16–20).

PrP^{Sc} can be amplified *in vitro* by PMCA (21, 22), by using brain homogenate as the PrP^{C} substrate. Efficient amplification of PrP^{Sc} has been demonstrated in rodents (23, 24), deer (25), sheep (26), cows (27), and humans (28). In sheep, classical scrapie PrP^{Sc} derived from *VRQ*-associated genotypes could be amplified efficiently in all cases examined by using brain homogenates of healthy sheep with a *VRQ/VRQ* genotype as PrP^{C} substrates in the presence of additional polyanions such as poly A, whereas PrP^{Sc} from the *ARQ/ARQ* genotype only produced occasional amplification even with genotype-matched brain homogenate as PrP^{C} substrate (26). The efficiency of amplification of PrP^{Sc} derived from classical scrapie-infected sheep therefore seemed to be linked to PrP genotype, and detailed analysis of PrP^{Sc} distribution in bodily fluids using PMCA (29–31) were carried out using mainly sheep with *VRQ*-associated genotypes.

Recently, it was shown that *ARQ/ARQ* PrP^{Sc} derived from pooled brain tissues of eight sheep experimentally transmitted with classical scrapie was amplified efficiently without additional polyanions by the conventional PMCA that involves the use of homogeneous brain homogenate as the PrP^{C} substrate (32). Amplification efficiency of PrP^{Sc} varied among sheep with different PrP genotypes, and *ARQ/ARQ* and *ARQ/AHQ* PrP^{Sc} showed higher amplification factor than PrP^{Sc} of *ARH*- or *ARR*-associated genotypes. However, individual differences in the amplification efficiency of PrP^{Sc} of each genotype were not sufficiently analyzed in such study, and it remains to be determined whether the detection sensitivity of the method is high enough for the diagnosis of naturally scrapie-infected sheep.

PMCA can overcome the species barrier in several xenogeneic combinations of PrP^{Sc} seed and PrP^{C} substrate (25, 33–36). In some instances, interspecies PMCA has resulted in efficient amplification and sensitive detection of heterogeneous PrP^{Sc}. These observations suggest that the amplification efficiency of PMCA is governed by the structural compatibility between the PrP^{Sc} seed and PrP^{C} substrate rather than the amino acid sequence identity of prion proteins. Considering that amplification efficiency varies with PrP genotype in sheep, PrP^{Sc} derived from certain PrP alleles may be amplified more efficiently by interspecies PMCA than by homogeneous PMCA.

To confirm this possibility, we examined the efficiency of sheep PrP^{Sc} amplification by interspecies PMCA using several animal substrates. We report here that PrP^{Sc} derived from classical scrapie-infected sheep with an *ARQ/ARQ* ($n = 20$) or *AHQ/AHQ* ($n = 3$) genotype was amplified efficiently when mouse brain homogenate was used as the PrP^{C} substrate, and PrP^{Sc} derived from naturally scrapie-infected sheep with these genotypes could be amplified ultra-efficiently by serial PMCA.

Table 1. Maximum dilution of 10% brain homogenate yielding PrP^{res} signal in the WB analysis

Genotype	Scrapie sheep†	Breed	Maximum dilution (log) of brain homogenate‡
ARQ/ARQ	1	Finnish Dorset × Milk	5
	2	Swaledale	2
	3	Finnish Dorset	4
	4	Finnish Dorset	5
	6	Finnish Dorset	5
	12	Finnish Dorset	5
	14	Finnish Dorset	4
	16	Finnish Dorset	5
	17	Finnish Dorset	4
	23	Finnish Dorset	4
	24	Finnish Dorset	4
	25	Finnish Dorset	5
	26	Finnish Dorset	5
	27	Finnish Dorset	5
	28	Finnish Dorset	5
	29	Finnish Dorset	5
	3571	Suffolk	3
	294	Corriedale	5
	3574	Suffolk	3
	2314	Suffolk	5
AHQ/AHQ	10	Swaledale	5
	19	Finnish Dorset	5
	22	Finnish Dorset	3
VRQ/ARQ	7	Swaledale	<2
	9	Swaledale	<2
	11	Swaledale	<2

†Numbers refer to sheep with *ARQ/ARQ* ($n = 20$), *AHQ/AHQ* ($n = 3$), and *VRQ/ARQ* ($n = 3$) PrP genotypes. ‡The 10% brain homogenates of scrapie-infected sheep were serially diluted from 10^{-2} to 10^{-5} with mouse PrP^{C} substrate and subjected to one round of amplification. The maximum dilution of 10% homogenate yielding PrP^{res} signal in the WB analysis is indicated in each animal.

MATERIALS AND METHODS

Scrapie-infected materials

Sheep with *ARQ/ARQ* ($n = 20$), *AHQ/AHQ* ($n = 3$) and *VRQ/ARQ* ($n = 3$) PrP genotypes were examined in this study (Table 1). Brain tissues of scrapie sheep 1 to 29 were kindly provided by Veterinary Laboratories Agency of United Kingdom, and samples of 3571 and 3574 were kindly provided by the National Animal Disease Center

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of United States of America. All sheep examined in this study, except 294 and 2314, were naturally scrapie-infected sheep. Brain homogenate of a scrapie sheep (3571) was intravenously administered to sheep 294 and 2314 in NIAH as described previously (38). The scrapie-infected brains were homogenized in a 10% (w/v) concentration of PBS containing 1% Triton X-100 and 4 mM EDTA. Brain tissues of 294 and 2314 were collected at the terminal stage of disease and stored at −80°C. The study protocol was approved by the Animal Ethics Committee (approval ID: 153, 404 and 520) and Animal Care and Use Committee (approval ID: 04-III-6) of the National Institute of Animal Health.

Genotyping

The sheep PrP genotype was determined by PCR. DNA samples were prepared from tonsils or tissues around tonsils by using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The open reading frame of *PRNP* of each sample was amplified using the −8 forward (5′-AAGTCATCATGGTGAAAAGCC-3′) and the +788 reverse (5′-AAACAGGAAGGTTGCCCCTA-3′) primers. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), and each purified PCR product was directly sequenced using the dye-termination method and ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Preparation of PrP^C substrates

To avoid contamination, normal brain homogenates were prepared in a laboratory that has never contained infected materials. Brains of healthy cow, sheep (*ARQ/ARQ*), goat, mouse (ICR), and Syrian hamster were homogenized in a 20% (w/v) concentration of PBS containing complete protease inhibitors (Roche Diagnostics). The homogenates were stored in small aliquots at −80°C until further use. For use, the homogenates were mixed with an equal volume of elution buffer (PBS containing 2% Triton X-100 and 8 mM EDTA) and incubated at 4°C for 1 hr with continuous agitation. After centrifugation at 4500 *g* for 5 min, the supernatant was used as PrP^C substrate.

PMCA

The scrapie-infected brain homogenates were diluted from 10^{-1} to 10^{-3} with normal brain homogenates from several animal species (total volume, 100 μL) in an electron beam-irradiated polystyrene tube. Amplification was carried out with a fully automatic cross-ultrasonic protein activating apparatus (Elestein 070-CPR, Elekon Science, Chiba, Japan), as previously described (27). PMCA was performed with 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 hr with gentle agitation. For examination of the sensitivity of this method for the detection of sheep scrapie PrP^{Sc}, the 10% infected brain homogenate was serially diluted from 10^{-2} to 10^{-12} with mouse PrP^C substrate. The amplified product from the first round of amplification was diluted 1:5 with the PrP^C substrate, and a second round of amplification was performed. The dilution of the PMCA product and its subsequent amplification were repeated a maximum of four times.

Western blotting

Samples (10 μL) after each round of amplification were mixed with 10 μL of proteinase K (PK) solution (100 μg/mL) and incubated at 37°C for 1 hr. 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 polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking, the membrane was incubated for 1 hr with HRP-conjugated T2 monoclonal antibody at 1:10 000 dilution (39, 40). The T2 antibody, which recognizes a discontinuous epitope in amino acid residues 132–156 in the mouse PrP sequence, also reacts with sheep PrP of the *VRQ/ARQ* or *ARQ/ARQ* genotypes but does not react with *AHQ/AHQ* PrP (Masujin *et al.*, unpubl. data, 2009). To detect AHQ PrP^{Sc}, anti-mouse PrP monoclonal antibody 4E10 (HRP conjugated, diluted 1:5000) was used. The 4E10 antibody recognizes an epitope in amino acid residues 147–158 (RYYRENMYRYPN) of the mouse PrP sequence (41), and it reacts with sheep PrP of all alleles examined. After washing, the blotted membrane was developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore), according to the manufacturer's instructions. Chemiluminescence signals were analyzed with the Light Capture system (Atto, Tokyo, Japan).

RESULTS

Amplification of sheep PrP^{Sc} by interspecies PMCA

We first examined the amplification efficiency of interspecies PMCA by using brain homogenate of sheep 2 (*ARQ/ARQ*, Table 1) as PrP^{Sc} seed. Figure 1a shows the amplification results of interspecies PMCA. Homogeneous PMCA using brain homogenate of *ARQ/ARQ* sheep as PrP^C substrate produced only a weak signal of PrP^{res} as detected by WB analysis. Brain homogenates of other ruminants (cow and goat) were also not suitable for

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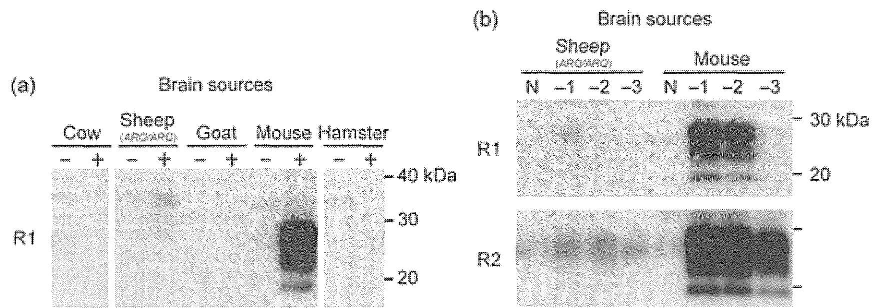

Fig. 1. Amplification of sheep PrP^{Sc} by interspecies PMCA. (a) The PrP^{C} substrate was mixed with 1:10 volume of the 10% brain homogenate of sheep 2 (*ARQ/ARQ*) infected with scrapie (lanes labeled "+"). The lanes labeled "−" are controls in which only the PrP^{C} substrate was treated in the same manner. Samples obtained after one round of amplification (R1) were analyzed by WB using HRP-T2 antibody following digestion with PK. (b) Serial PMCA was performed using sheep (*ARQ/ARQ*) or WT mouse brain homogenate as PrP^{C} substrate. Homogenate (10%) of the scrapie-infected brain (*ARQ/ARQ* sheep 2) was diluted 1:10 (−1) to 1:1000 (−3) with the PrP^{C} substrate. "N" designates the control in which only PrP^{C} substrate was amplified. The amplified product obtained after the first round of amplification (R1) was diluted 1:5 with the PrP^{C} substrate, and a second round of amplification (R2) was performed. The samples were analyzed by WB using HRP-T2 antibody following digestion with PK.

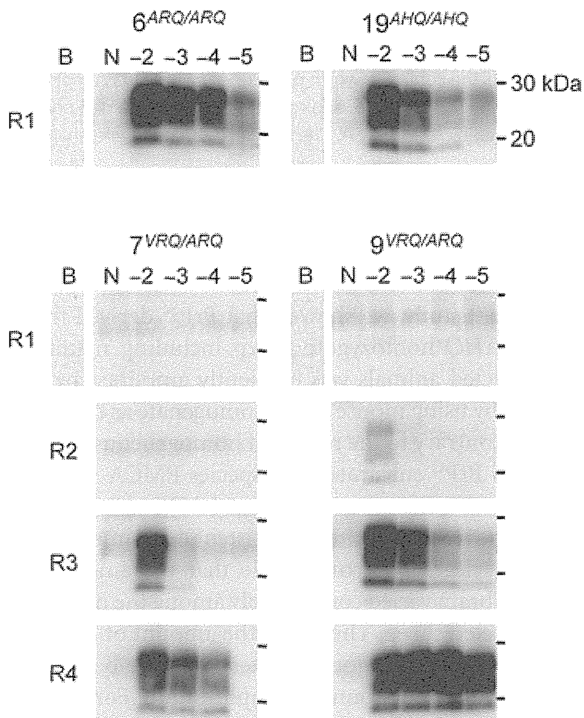

Fig. 2. Amplification of PrP^{Sc} derived from scrapie sheep with *ARQ/ARQ, AHQ/AHQ* and *VRQ/ARQ* PrP genotypes. Homogenates (10%) of the scrapie-infected brains were diluted 10^{-2} (−2) to 10^{-5} (−5) with mouse PrP^{C} substrate. The lanes labeled "N" are controls in which only the PrP^{C} substrate was treated in the same manner. Amplified samples were analyzed by WB with HRP-T2 antibody following PK digestion. Before amplification, no PrP^{Sc} was detected by WB using 4E10 antibody after PK digestion in the respective 10% homogenates (lanes labeled "B"). *VRQ/ARQ* sheep PrP^{Sc} was amplified by serial PMCA. Amplified samples were analyzed after each round of amplification (R1–R4) by WB after PK digestion.

amplification of sheep PrP^{Sc}. On the other hand, a significant amplification of PrP^{res} with the typical three bands was achieved when WT mouse brain homogenate was used as the PrP^{C} substrate. Interspecies PMCA using hamster brain homogenate produced no positive reaction. Figure 1b shows the results of the serial amplification of the sheep PrP^{Sc}. Although no substantial improvement in PrP^{Sc} detection sensitivity was observed with serial homogeneous PMCA, PrP^{Sc} present in 10^{-3} dilution of infected brain homogenate could be detected after two rounds of amplification using mouse PrP^{C} substrate. Therefore, interspecies PMCA using mouse PrP^{C} substrate was more efficient than homogeneous PMCA in the amplification of sheep *ARQ* PrP^{Sc} under the present experimental conditions.

Amplification of PrP^{Sc} derived from sheep of different PrP genotypes

In addition to *ARQ/ARQ* sheep PrP^{Sc}, *AHQ/AHQ* and *VRQ/ARQ* sheep PrP^{Sc} were also examined by interspecies PMCA by using mouse PrP^{C} substrate. The 10% brain homogenates were serially diluted from 10^{-2} to 10^{-5} with mouse PrP^{C} substrate and then amplified. Before amplification, brain PrP^{Sc} was not detected by WB analysis in many cases of naturally infected sheep, as shown in lane "B" of Figure 2. After one round of amplification, PrP^{res} signal was detected in all samples of 20 *ARQ* homozygote sheep (sheep 6 in Fig. 2 and Table 1). In addition, PrP^{Sc} from three *AHQ* homozygote (sheep 19 in Fig. 2 and Table 1) was amplified by interspecies PMCA. The maximum dilution of 10% homogenate yielding PrP^{res} signal in the WB analysis was in the range

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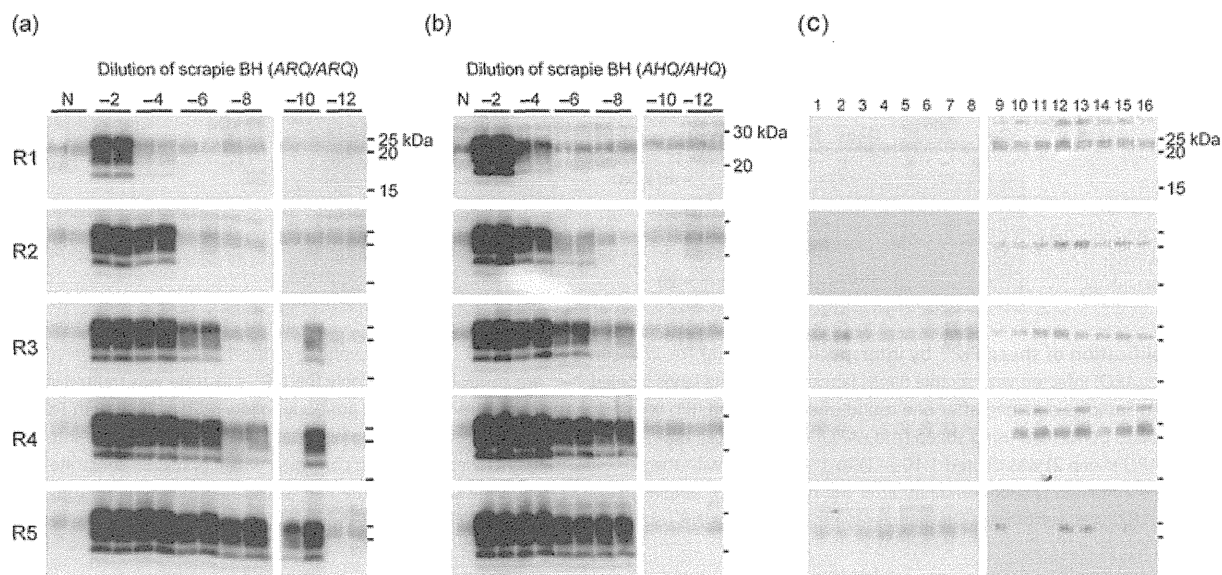

Fig. 3. PrP^{Sc} detection sensitivity in *ARQ* and *AHQ* homozygote sheep. Homogenates (10%) of *ARQ/ARQ* sheep 2 (a) and *AHQ/AHQ* sheep 19 (b) were diluted 10^{-2} (−2) to 10^{-12} (−12) with mouse PrP^{C} substrate, and the samples were serially amplified. The duplicate amplified samples were analyzed after each round of amplification (R1–R5) by WB after PK digestion. "N" represents the control in which only PrP^{C} substrate was amplified. (c) No spontaneous generation of PrP^{res} was observed. Samples 1 to 8 contained only mouse PrP^{C} substrate, and samples 9 to 16 contained normal sheep brain homogenate (*ARQ/ARQ*) diluted 1:100 with mouse PrP^{C} substrate were amplified by serial PMCA. The amplified samples were analyzed after each round of amplification by WB with HRP-T2 antibody after PK digestion.

of 10^{-2} and 10^{-5} in these sheep (Table 1). On the other hand, no significant amplification of PrP^{Sc} from three *VRQ/ARQ* heterozygote sheep (sheep 7 and 9 in Fig. 2 and Table 1) was observed until two or three rounds of amplification.

PrP^{Sc} detection sensitivity in *ARQ* and *AHQ* homozygote sheep

To estimate the detection limit of PrP^{Sc} by interspecies PMCA, 10% brain homogenates of naturally scrapie-infected sheep 2 (*ARQ/ARQ*) and sheep 19 (*AHQ/AHQ*) were serially diluted from 10^{-2} to 10^{-12} with mouse PrP^{C} substrate and then amplified. In the case of *ARQ/ARQ* PrP^{Sc}, PrP^{res} signal was detected in duplicate samples diluted up to 10^{-10} after five rounds of amplification (Fig. 3a). Similarly, PrP^{res} signal was detected in duplicate samples of *AHQ/AHQ* PrP^{Sc} diluted up to 10^{-8} after four rounds of amplification (Fig. 3b). No spontaneous generation of PrP^{res} was observed in samples that contained only mouse PrP^{C} substrate (lane 1–8 of Fig. 3c), and samples that contained normal sheep brain homogenate (*ARQ/ARQ*) diluted 1:100 with mouse PrP^{C} substrate (lane 9–16 of Fig. 3c).

DISCUSSION

In the present study, we showed that PrP^{Sc} derived from *ARQ* and *AHQ* homozygote sheep including naturally scrapie-infected animals was efficiently amplified by serial PMCA by using mouse brain homogenate as the PrP^{C} substrate. Contrary to the results of homogeneous PMCA using sheep PrP^{C} substrate, interspecies PMCA was less effective for amplification of PrP^{Sc} of *VRQ/ARQ* sheep. Although the reason for the inconsistent experimental results is not clear, it is conceivable that the amount of PrP^{Sc} in the brain varied considerably among the naturally scrapie-infected sheep. Therefore, the amount of PrP^{Sc} in these *VRQ/ARQ* brain homogenates might be below the detection limit of one round of amplification. For precise comparison of amplification efficiencies of the different genotypes, standardization of the amount of PrP^{Sc} before amplification is required.

Owing to the advancement of the PMCA technique, polyanions such as RNA and sulfated polysaccharides have been proved to enhance the *in vitro* amplification of PrP^{Sc} derived from hamsters (42), cows (27), and humans (43), as in the case of sheep PrP^{Sc} (26). However, amplification of mouse PrP^{Sc} seemed to be less dependent of such polyanions (44). Polyanion preference for *in vitro* amplification of PrP^{Sc} may differ depending on the sheep

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PrP allele. In fact, amplification of sheep *ARQ* PrP^{Sc} was achieved without additional polyanions (32). Therefore, PrP^{Sc} derived from *ARQ* and *AHQ* alleles could facilitate or stabilize interactions with mouse PrP^{C} substrate in the absence of additional polyanions. On the other hand, the association between sheep *VRQ* PrP^{Sc} and mouse PrP^{C} may be insufficient for propagation of PrP^{Sc}. Further analysis of PrP^{Sc} derived from *VRQ* homozygote sheep is needed to investigate the different genotype compatibility.

Generally, prions are transmitted less efficiently between different species than within species. However, sheep scrapie can be experimentally transmitted to goats, rats, hamsters and mice, and several scrapie prion strains have been established by serial transmission in these animals (45, 46). The sheep PrP genotype also influences the neuropathological characteristics of the disease after primary transmission (47, 48); for example, the survival time of mice inoculated with PrP^{Sc} derived from the *ARQ/ARQ* genotype tended to be shorter than that of mice inoculated with PrP^{Sc} derived from *VRQ*-associated genotypes (47). This finding is consistent with the observed *in vitro* amplification efficiencies of mouse PrP^{res} triggered by sheep PrP^{Sc} derived from the *ARQ/ARQ* or *VRQ/ARQ* genotype. The sheep PrP genotype might also affect the propagation of PrP^{Sc} in mice.

In conclusion, the interspecies PMCA we developed in this study was useful for ultrasensitive detection of PrP^{Sc} in classical scrapie-infected *ARQ* and *AHQ* homozygote sheep. The detection sensitivities of PrP^{Sc} of sheep with these genotypes were comparable with those of sheep carrying *VRQ*-associated genotypes (26). Thus, scrapie diagnosis may be possible in *ARQ* and *AHQ* homozygote sheep by using the xenogeneic PMCA. The advantage of the amplification method developed in this study is that healthy brains are easy to obtain from WT mice, and differences in PrP^{C} substrate (brain homogenate) can be minimized by using such experimental animals.

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DISCLOSURE

The authors declare that no competing interests exist.

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Prion in Saliva of Bovine Spongiform Encephalopathy–Infected Cattle

To the Editor: A definitive diagnosis of bovine spongiform encephalopathy (BSE) in cattle usually relies on Western blot and immunohistochemical testing of samples from the obex region of the brainstem. These conventional diagnostic tests can detect the presence of the abnormal (disease-associated) form of the prion protein (PrP^{Sc}) in brain samples several months before the onset of clinical signs; however, there is no appropriate, universal tool for early preclinical and antemortem diagnosis of BSE. Furthermore, confirmation of the disease is currently only possible by postmortem examination of brain tissues. In this study, we used the serial protein misfolding cyclic amplification (sPMCA) technique to determine the presence of PrP^{Sc} in saliva samples collected from BSE-infected cows before and after the onset of disease (*1*).

In a previous study (*2*), we analyzed the tissue distribution of PrP^{Sc} in cattle up to 66 months after they were orally inoculated with a relatively low dose (5 g) of homogenized brainstem from animals with naturally occurring BSE in England. In 2011, after publication of that study and 83.3 months after the cows were inoculated, clinical signs of BSE developed in 1 cow (no. 5444); necropsy was performed 84.7 months after inoculation. In addition, we used saliva samples from 2 BSE-affected cows (nos. 5413 and 5437) (*2*) to determine the presence of PrP^{Sc}.

We collected saliva samples from animals at 4 monthly intervals, beginning in 2009, 56 months after inoculation. Samples were stored at -80°C until analysis. Using the sodium phosphotungstic acid precipitation method, we concentrated (100-fold) individual 1-mL saliva samples from each time point. We then diluted the concentrated samples 1:10 with the normal isoform of prion protein substrate containing 0.5% potassium dextran sulfate. Using the sPMCA technique as described (*1*), we amplified the samples in 3–8 tubes, and we used Western blot to analyze the proteinase K–treated sPMCA products (*2*).

Using Western blot and immunohistochemical tests, we detected the accumulation of PrP^{Sc} in brains collected at necropsy from the 3 cows examined. In addition, using the sPMCA technique, we detected PrP^{Sc} signal in 1) saliva samples that were concentrated from samples collected from the same 3 cows at necropsy and in 2) concentrated saliva samples that were collected from 2 of the cows (nos. 5413 and 5444) at the early clinical stages of disease.

After saliva samples underwent 3 rounds of amplification, we detected PrP^{Sc} in a saliva sample that was collected from cow number 5437 two months before the clinical onset of clinical symptoms (Figure). For 2 of the cows (nos. 5413 and 5437), the positive ratio of salivary PrP^{Sc} at round 4 of amplification increased as the disease progressed (Figure). Because PrP^{Sc} signal could be detected in BSE-infected brain homogenates diluted up to 10^{-10} after 2 rounds of amplification (*1*), we estimated PrP^{Sc} levels in the nonconcentrated original saliva samples to be lower than those in BSE-infected brain homogenate diluted to 10^{-12}. No PrP^{Sc} signal was detected in samples collected from the 3 cows 3–5 months before the onset of clinical symptoms or from age-matched noninfected controls, even after 4 rounds of amplification.

We demonstrated the presence of PrP^{Sc} in saliva of BSE-affected cows

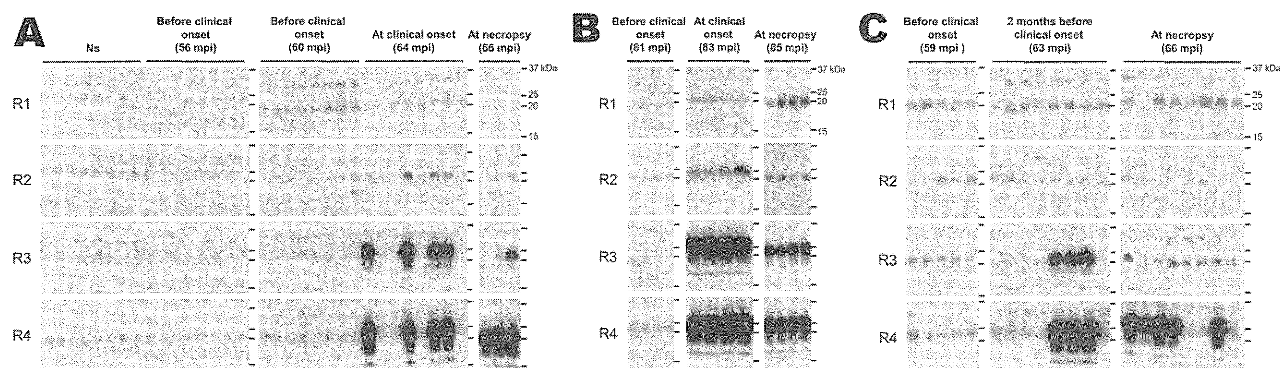

Figure. Western blot detection, using the serial protein misfolding cyclic amplification technique, of the abnormal (disease-associated) form of the prion protein (PrP^{Sc}) in concentrated saliva samples from 3 cows experimentally infected by inoculation with the agent of bovine spongiform encephalopathy: cows 5413 (A), 5444 (B), and 5437 (C). PrP^{Sc} was detected in saliva samples at the initial clinical and terminal stages of the disease (A, B). PrP^{Sc} was also detected in a saliva sample, after 3 rounds of amplification, obtained 2 months before the onset of clinical symptoms in 1 of the 3 cows (C). All saliva samples were concentrated by using the sodium phosphotungstic acid precipitation method. After protein misfolding cyclic amplification, extra bands with a molecular weight higher than that for PrP^{Sc} were occasionally observed, likely corresponding to prion protein aggregates or to residue of the normal isoform of prion protein resulting from incomplete proteinase K digestion. Molecular mass markers (in kDa) are shown on the right. R1–R4, rounds 1–4 of amplification; Ns, no seed control; mpi, months postinoculation.

during the clinical stage of the disease, and in 1 case, at the preclinical or asymptomatic stage. Our findings suggest that PrP^{Sc} is likely to be detected in the saliva of BSE-affected cattle during the clinical stage of disease, after accumulation of PrP^{Sc} in the brain. PrP^{Sc} was found in the salivary glands of BSE-affected cattle at the terminal stage of infection (*1*). Therefore, once the infectious agent reaches the central nervous system, it may spread centrifugally from the brain to the salivary glands through the autonomic nervous system.

Infectivity of saliva and the presence of PrP^{Sc} in saliva have been reported in other ruminants affected with transmissible spongiform encephalopathy. Infectivity of saliva was demonstrated in deer with chronic wasting disease (*3*) and in scrapie-affected sheep (*4*); the immunolabeled PrP^{Sc} accumulated in the salivary glands of scrapie-affected sheep (*5*). A low level of PrP^{Sc} was detected in concentrated buccal swab samples of preclinical scrapie-infected sheep by using sPMCA (*6,7*). These results suggest that small amounts of PrP^{Sc} may accumulate in the salivary glands and are then secreted into saliva.

The presence of infectious prions in saliva may explain the facile horizontal transmission of scrapie in sheep (*4–6*) and chronic wasting disease in deer (*4,8*). There has been no epidemiologic evidence, however, that saliva, milk, blood, and cerebrospinal fluid from BSE-infected cattle are infectious (*9*). Nonetheless, the potential risk for BSE transmission by body fluids or excretions from BSE-infected cattle is cannot be ruled out by the current data.

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Reptile- and Amphibian-associated Salmonellosis in Childcare Centers, United States

To the Editor: *Salmonella* spp. infection represents a major public health problem in the United States; nearly 1.4 million human cases and 600 associated deaths are reported each year (*1*). Reptile and amphibian exposures might cause >70,000 of these cases annually (*2*). Furthermore, children are at increased risk of acquir-

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Anti-Prion Activity of Brilliant Blue G

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Abstract

Background: Prion diseases are fatal neurodegenerative disorders with no effective therapy currently available. Accumulating evidence has implicated over-activation of P2X7 ionotropic purinergic receptor (P2X7R) in the progression of neuronal loss in several neurodegenerative diseases. This has led to the speculation that simultaneous blockade of this receptor and prion replication can be an effective therapeutic strategy for prion diseases. We have focused on Brilliant Blue G (BBG), a well-known P2X7R antagonist, possessing a chemical structure expected to confer anti-prion activity and examined its inhibitory effect on the accumulation of pathogenic isoforms of prion protein (PrPres) in a cellular and a mouse model of prion disease in order to determine its therapeutic potential.

Principal Findings: BBG prevented PrPres accumulation in infected MG20 microglial and N2a neural cells at 50% inhibitory concentrations of 14.6 and 3.2 μM, respectively. Administration of BBG *in vivo* also reduced PrPres accumulation in the brains of mice with prion disease. However, it did not appear to alleviate the disease progression compared to the vehicle-treated controls, implying a complex role of P2X7R on the neuronal degeneration in prion diseases.

Significance: These results provide novel insights into the pathophysiology of prion diseases and have important implications for the treatment.

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Introduction

Prion diseases are fatal, transmissible, and progressive neurodegenerative disorders that include Creutzfeldt–Jakob disease (CJD) and Gerstmann–Straussler–Scheinker syndrome (GSS) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, and chronic wasting disease in deer. The neuropathology is characterized by brain vacuolation, astrogliosis, microglial activation, neuronal loss, and progressive accumulation of a misfolded protease-resistant isoform (PrPres) of the host-encoded protease-sensitive prion protein (PrPsen) [1]. The conversion of PrPsen to PrPres is believed to be the key event in prion pathogenesis. Until date, the precise molecular mechanisms underlying glial activation and neuronal dysfunction remain unknown and there are no effective treatments for prion diseases.

P2X7 ionotropic purinergic receptor (P2X7R) is an ATP-gated ion channel believed to be associated with the regulation of both neuronal death and survival. P2X7R is abundantly expressed in microglia [2] and to a lesser extent in astrocytes [3], oligodendrocytes [4], and the presynaptic terminals of neurons [5]. Accumulating evidence suggests that the P2X7R signal pathways are involved in the modulation of glutamate release from presynaptic terminals of neurons and astrocytes [6–8], resulting in synapse dysfunction and glutamate-mediated excitotoxicity. Furthermore, signaling through P2X7R plays a crucial role in the activation and proliferation of microglia [9]. After activation of P2X7R by ATP, activated microglia release various proinflammatory cytokines (e.g., IL-1β) and other bioreactive substances, leading to neuronal damage [10–12]. In addition to its deleterious effects, P2X7R activation also stimulates the release of γ-aminobutyric acid from nerve terminals [6] and production of endocannabinoids in astrocytes and microglia [13,14], both of which are transmitters with neuroprotective roles. P2X7R activation also involves a neuroprotective effect through activation of ERK1/2 signaling [15]. Thus, P2X7R activation can have both protective and detrimental effects on neurons.

P2X7R expression is upregulated in the brains of patients with and in various animal models of neurodegenerative diseases, including multiple sclerosis and Alzheimer's and Huntington's diseases [16–19]. In addition, we recently reported that P2X7R is upregulated in a mouse model of prion disease [20]. Although it remains debatable whether P2X7R plays a beneficial or detrimental role in these diseases, a number of studies illustrate that the blockade or deficit of P2X7R has neuroprotective effects in animal models of multiple sclerosis [4], Huntington's disease

[19], Alzheimer's disease [21], and spinal cord injury [22]. While data regarding the role of P2X7R in prion diseases is lacking, the simultaneous blockade of this receptor and inhibition of prion replication may alleviate the progression of prion diseases.

One candidate for a therapeutic compound that possess such combined drug actions is Brilliant Blue G (BBG), a well-known P2X7R antagonist; BBG can cross the blood–brain barrier, has low toxicity, and exhibits therapeutic effects in several animal models of neurodegenerative diseases [23]. Furthermore, BBG has a symmetrical bifunctional structure comprising of two moieties joined via a spacer; this molecular framework is expected to confer anti-prion activities [24] as in the case of anti-prion compounds such as Congo red [25], suramin [26], and curcumin [27]. These properties prompted us to assay BBG for its ability to inhibit PrPres accumulation.

In this study, we examined the inhibitory effect of BBG on PrPres accumulation in a cellular and a mouse model of prion disease and we also investigated the therapeutic potentials of BBG due to its P2X7R antagonistic and predicted anti-prion activities. We found that BBG inhibited PrPres accumulation in prion-infected microglial and neural cell lines, possibly via down-regulation of cell-surface PrPsen. BBG prevented PrPres accumulation in the brain of prion-infected mouse. However, BBG did not appear to alleviate the disease progression.

Materials and Methods

Reagents and antibodies

BBG (Ultra Pure Grade) was obtained from AnaSpec (San Jose, CA, USA) and all other reagents were purchased from (Sigma-Aldrich, St. Louis, MO, USA) unless otherwise specified. The following antibodies were used in this study: anti-prion protein (PrP) SAF32 monoclonal antibody (mAb) (SPI Bio, Montigny le Bretonneux, France); anti-PrP mAbs 3H2, 4E10, and T2 [28,29]; anti-P2X7R rabbit polyclonal antibody (Sigma, P8232); anti-glial fibrillary acidic protein (GFAP) mAb (Sigma, G3893); anti-β-actin mAb (Sigma, A2228), anti-synaptophysin 1 mAb (Synaptic Systems, Gottingen, Germany); anti- ionized calcium binding adaptor molecule 1 (Iba1) rabbit polyclonal antibody (Wako Pure Chemical, Osaka, Japan); and HRP-conjugated goat anti-mouse and anti-rabbit antibodies (Calbiochem, San Diego, CA, USA).

Cell culture conditions and inhibition assay of PrPres accumulation

MG20 cells persistently infected with mouse-adapted scrapie ME7 prion (ScMG20 cells) [30] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 10 μM 2-mercaptoethanol, 10 μg/ml insulin,100 units/ml penicillin, 100 μg/ml streptomycin sulfate at 37°C with 5% CO_2. N2a cells were obtained from American Type Culture Collection. N2a cells persistently infected with mouse-adapted scrapie Chandler prion (ScN2a) were cultured in Opti-MEM supplemented with 10% heat-inactivated fetal calf serum at 37°C with 5% CO_2.

The cells were detached by the addition of 5 mM EDTA in phosphate-buffered saline (PBS) with gentle pipetting, and cell density was determined by cell counting using an hemocytometer. ScMG20 and ScN2a cells were plated in 6-well plates (1×10^5 cells/well) and allowed to settle for 1 h or 1day prior to the treatment with an antagonist, respectively. Cells were treated with BBG (0.6–60 μM) or without BBG, oxidized-ATP (25–150 μM; an irreversible inhibitor of P2X7R), or A438079 (4.5–36 μM; an inhibitor of P2X7R) for 3 days, after which the accumulation of PrPres was detected by western blotting of the lyzed cells.

Cell viability

Cytotoxicity was determined using the WST-8 assay (Cell Counting Kit-8; Dojindo Lab, Kumamoto, Japan). ScMG20 cells were plated in the wells of a 96-well plate (3×10^3 cells/well) and incubated with the appropriate concentration of one of the three antagonists for 3 days prior to the WST-8 assay, according to the manufacturer's instructions. The treatment with each substance was performed in triplicate. The percentage cell viability was calculated with reference to that of untreated cells incubated with WST-8 (100%).

SDS-PAGE and western blotting

Cells were washed once with PBS and then lyzed in an ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA with 0.5% Triton X-100, and 0.5% sodium deoxycholate. Frozen mice brain or mice spleen tissue was homogenized in an ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA with 0.5% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS. After removal of insoluble debris by centrifugation at 10,000× *g* for 2 min, the total protein concentrations of the samples were measured by the bicinchoninic acid assay (BCA protein assay; Thermo Fisher Scientific, Rockland, IL, USA) and equal amounts of protein were analyzed.

For PrPres detection, cell lysates and tissue homogenates were digested with proteinase K (20 and 50 μg/ml for the cell tissues and brain tissues, respectively) for 1 h at 37°C. The reactions were stopped by the addition of 4 mM Pefabloc (Roche Diagnostics, Basel, Switzerland). Cell lysates were then ultracentrifuged at 200,000× *g* for 1 h in a TLA 55 rotor (Beckman Coulter, Fullerton, CA, USA) and the resulting pellets were used for further analysis. The samples were solubilized in LDS-sample loading buffer (Invitrogen, San Diego, CA, USA) prior to electrophoresis on 12% NOVEX pre-cast gels (Invitrogen) and electrotransfered onto Durapore (Millipore, Billerica, MA, USA) polyvinylidene fluoride membranes. Chemilumi One Super (Nakarai Chemical, Kyoto, Japan) was used for immunodetection. For quantitation, blots were imaged with a Fluorchem (Alpha Innotech, San Leandro, CA, USA) and analyzed with Image Reader software (AlphaEaseFC; Alpha Innotech) according to the manufacturer's instructions. The values were normalized to β-actin as loading controls.

Degycosylation analysis

For analysis of PrP levels, MG20 cells were incubated with the indicated concentrations of BBG. After 3 days, cell extracts were prepared in lysis buffer, and PrP was deglycosylated by treating the extracts with PNGase F (peptide N-glycosidase F) (New England BioLabs, Beverly, MA, USA) for 2 h at 37°C as described previously [30].

Infectivity assay

The infectivity associated with ScMG20 cells was assayed by intracerebral inoculation into murine PrP-overexpressing (*tga*20) mice. ScMG20 cells were harvested for inoculation at serial passage 5 in the presence or absence of 30 μM BBG. The cells were suspended in sterile PBS(−), freeze-thawed, and sonicated before intracranial inoculation (2.5×10^4 cells/20 μl/mouse). After inoculation, the terminally ill mice were sacrificed for western blotting of the brain tissues. All mice were kept in an air-conditioned room and fed on standard laboratory food pellets and water *ad libitum*.

Detergent solubility assay

Detergent solubility assays were performed as previously described [26]. MG20 cells treated with or without 30 μM BBG were lyzed in cold lysis buffer. Postnuclear lysates were ultracentrifuged for 1 h at 100,000× *g* at 4°C in the presence of Complete proteinase inhibitor cocktail (Roche) and 1% N-lauryl sarcosine. PrPsen in the supernatant and pellet fractions was detected by western blotting.

In vivo BBG treatment

C57BL/6 mice were intracerebrally infected with 20 μl of 10% homogenate prepared from the brains of either healthy C57BL/6 mice or terminally ill C57BL/6 mice with Fukuoka-1 (FK-1) strain of murine GSS. Hundred days after infection, mice were treated intraperitoneally 3 times per week for 3 weeks and 2 times per week for 4 additional weeks with 500 μl of 100 mg/kg BBG diluted with 10% ethanol in saline. A control group of GSS-infected animals was treated with the vehicle only.

Confocal imaging

Cells grown on a 4-well chambered coverglass were incubated with BBG for 3 days. After rinsing with PBS, the cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and then treated with or without 0.5% Triton X-100 in PBS. After a brief wash with PBS, the cells were incubated with blocking solution (1% bovine serum albumin, 5% normal goat serum, and 0.1% Tween 20 in PBS) for 30 min. The primary antibody directed against PrP (SAF32) was used at a 1:500 dilution in blocking solution and incubated for 1 h at room temperature. Cells were subsequently incubated with anti-mouse Alexa Fluor 488-conjugated secondary antibody (Invitrogen) for 1 h. The chambered coverglass was overlaid with a mounting solution [25% glycerol, 10% Mowiol (Calbiochem) in 100 mM Tris-HCl, pH 8.5] and examined with the Zeiss LSM510 inverse laser scan microscope.

Flow cytometric analysis

Cells grown on 100-mm cell culture dishes were incubated with 30 μM BBG for 3 days. After rinsing with ice-cold PBS, cells were harvested by pipetting. Next, 2 μg of several anti-PrP antibodies were pre-labeled using the Zenon PE Mouse IgG Labeling Kit (Invitrogen), according to the manufacturer's instructions. Cells were incubated for 30 min at 4°C with each Zenon-labeled antibody and washed twice in PBS containing 1% BSA. Following fixation with 1% paraformaldehyde in PBS, cells were analyzed with the EPICS XL SYSTEM II flow cytometer (Beckman Coulter, Miami, FL). Fluorescence of 1.5×10^4 cells/antibody was acquired and analyzed using Flowjo software (Tree Star, Ashland, OR, USA) to quantify the surface expression of PrP by calculating the mean fluorescence intensities.

Protein misfolding cyclic amplification (PMCA) assay

Brains from healthy mice and Chandler scrapie-infected mice at the terminal stage of the disease were minced using the BioMasher (Nippi) and suspended in a conversion buffer. After removal of insoluble debris by centrifugation at 4,500× *g* for 5 min, the supernatants were collected. The Chandler brain homogenate was diluted 1:1000, 1:10000, and 1:100000 with healthy brain homogenates. Serial dilutions of BBG in PBS were added to the brain homogenates to obtain final concentrations of 0.6, 6, 60, and 600 μM. The samples were incubated at 37°C with agitation for 30 min and then subjected to a cycle of sonication every hour, as previously described [31].

Quantitative real-time reverse transcriptase polymerase chain reaction (Q-PCR) analysis

The mRNA levels were assayed by Q-PCR. Total RNA was isolated from cells with FastPure RNA Kits and treated on a column with RNase-free DNase I (Takara, Osaka, Japan). Total RNA was reverse transcribed using ReverTra Ace (Toyobo, Osaka, Japan) with random hexamers primers. Diluted reactions were analyzed with SYBR Premix Ex Taq (Takara) and an ABI Prism 7500 detection system (Applied Biosystems). PrP mRNA levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). cDNA levels of PrP and GAPDH were determined relative to standard curves from PCR fragments amplified by RT-PCR using total RNA and a Q-PCR primer pair. The slope of the standard and dissociation curves of the products indicated sufficient PCR quality. The primers used were as follows: mouse PrP, (forward) 5′-ATGGCGAACCTTGGC-TACTG-3′ and (reverse) 5′-CCTGAGGTGGGTAACGGTTG-3′; GAPDH, (forward) 5′-AGGTCGGTGTGAACGGATTTG-3′ and (reverse) 5′-TGTAGACCATGTAGTTGAGGTCA-GATTTG-3′.

Statistical analysis

Values in the figures are expressed as mean ± SD. To determine statistical significance, Welch's *t*-test for unpaired data was applied as appropriate. A value of $p<0.05$ was considered significant.

Ethics Statement

Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee at the National Institute of Animal Health (approval ID: 09-44 and 10-005).

Results

BBG inhibits PrPres accumulation and prion replication in mouse scrapie-infected cells

In the present study, we examined the effects of P2X7R antagonists on PrPres accumulation in cultured microglial cells persistently infected with the ME7 murine strain of scrapie (ScMG20 cells). Cells were treated with one of the three antagonists: BBG, oxidized-ATP, or A438079. BBG inhibited PrPres accumulation in a dose-dependent manner, with a 50% inhibitory concentration (IC50) of approximately 14.6 μM in ScMG20 cells (Figs. 1A, 1B). On the other hand, the levels of PrPres were not reduced following a treatment of cells with oxidized-ATP or A438079. The toxicity of the P2X7R antagonists was assessed using the WST-8 metabolic assay (Fig. 1C). ScMG20 cell viability decreased by approximately 20% at the highest concentration of each antagonist. However, BBG was not toxic at concentrations required for maximal inhibition of PrPres accumulation. These results suggest that anti-prion activity of BBG was derived from its molecular frameworks (Fig. 1D), which are analogous to the anti-prion compounds and not from its P2X7R antagonistic activity.

To determine the effects of BBG on prion replication, bioassays of ScMG20 cells treated with or without BBG were conducted. ScMG20 cells were serially passaged up to 5 times in the presence of 30 μM BBG. The level of PrPres in ScMG20 cells became undetectable after 3 passages with BBG (Fig. 1E insert). Inoculation of *tga20* mice (n = 10) with untreated ScMG20 cells resulted in a mean survival time of 151.5±27.8 days (mean ± SD). On the other hand, mice inoculated with BBG-treated cells showed a prolonged incubation period of the disease, with 6 out of

Figure 1. BBG prevents PrPres and prion accumulation in ScMG20 cells. (A) Western blotting of the levels of PrPres and P2X7 receptor proteins. ScMG20 cells were mock-treated or treated with each tested drug at the indicated concentrations for 3 days. PrPres and P2X7 receptor protein levels in ScMG20 cells were measured by western blotting. Molecular mass standards (kDa) are indicated on the right. Membranes were probed with anti-β-actin to correct any possible deviation in the protein concentration. (B) The densitometric measurement of PrPres and P2X7 receptor protein signals detected in the blots. Band intensity for PrPres and P2X7 receptor proteins was measured, normalized using the values obtained for b-actin intensities, and expressed as percentage relative to the 0 μM concentration points described in the Materials and Methods section. Results are expressed as the mean ± SD of three different experiments. Significant differences at a $p<0.05$ (*) and 0.01 (**) between treated and untreated cells are indicated. (C) The effects of P2X7 antagonists on viability of ScMG20 cells. Cell viability determined by the WST-8 assay is