Genotype-dependent Molecular Evolution of Sheep Bovine Spongiform Encephalopathy (BSE) Prions in Vitro Affects Their Zoonotic Potential*

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Background: The results of serial passage of BSE in sheep are unknown.

Results: In vitro modeling shows a sheep genotype-dependent switch in prion protein type and loss of the ability to convert human prion protein.

Conclusion: Molecular evolution of sheep BSE prions in vitro is genotype-dependent and affects zoonotic potential.

Significance: Zoonotic risk might be predicted from cell-free modeling.

Prion diseases are rare fatal neurological conditions of humans and animals, one of which (variant Creutzfeldt-Jakob disease) is known to be a zoonotic form of the cattle disease bovine spongiform encephalopathy (BSE). What makes one animal prion disease zoonotic and others not is poorly understood, but it appears to involve compatibility between the prion strain and the host prion protein sequence. Concerns have been raised that the United Kingdom sheep flock may have been exposed to BSE early in the cattle BSE epidemic and that serial BSE transmission in sheep might have resulted in adaptation of the agent, which may have come to phenotypically resemble scrapie while maintaining its pathogenicity for humans. We have modeled this scenario in vitro. Extrapolation from our results suggests that if BSE were to infect sheep in the field it may, with time and in some sheep genotypes, become scrapie-like at the molecular level. However, the results also suggest that if BSE in sheep were to come to resemble scrapie it would lose its ability to affect humans.

Prion diseases are transmissible fatal neurodegenerative disorders of the central nervous system that occur in animals and humans and are caused by unconventional agents termed prions. A hallmark of acquired prion diseases is a prolonged asymptomatic phase followed by the appearance of neurological signs, neuropathological changes, and the accumulation of a misfolded pathogenic isoform (PrPSc) of the cellular prion protein (PrPc) in the central nervous system (1). Prion diseases can be transmitted within and between species, but crossing a so-called “species barrier” often results in prolonged incubation periods (2). It is only after adaptation of the agent to the new host that the incubation period shortens and the disease phenotype stabilizes. This species barrier effect is thought to be determined at least in part by prion protein sequence homology between the species involved (3).

Bovine spongiform encephalopathy (BSE) is the prototypic acquired prion disease of cattle, first recognized in 1986 in the United Kingdom (4) and reaching epidemic proportions before being brought under control (5). The appearance of a new form of Creutzfeldt-Jakob disease (variant Creutzfeldt-Jakob disease (vCJD)) in the United Kingdom during the following decade was strongly suspected to result from dietary exposure of the human population to the BSE agent (6), and subsequent investigations of its transmission properties are consistent with this explanation (7–9). The number of cases of vCJD occurring in the United Kingdom peaked in the year 2000 and has declined since. All cases of definite clinical vCJD are of one prion protein genotypic group (PRNP codon 129 MM). BSE is the only known zoonotic animal prion disease (5).

Scrapie is the most intensively researched animal prion disease. It is endemic in sheep in many countries, including the United Kingdom. Scrapie prion strain diversity can be inferred from differences in incubation period, in PrPSc profile and distribution, and in vacuolar lesions in the brain (11). Scrapie susceptibility and incubation period are largely determined by polymorphic variation in the sheep prion protein gene (Prnp) with those at 136, 154, and 171 and the VRQ, ARQ, and ARR alleles having the greatest effect (12). Depending on the infecting source or strain (11), VRQ/VRQ and ARQ/ARR sheep are the most susceptible to classical scrapie, whereas ARR/ARR is considered to be effectively resistant (13).

It was recognized early on that if BSE were to have infected sheep then its presence could be masked by endemic scrapie with potentially serious and long-term effects for animal and...
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human health (14, 15). Sheep were confirmed to be experimentally susceptible to BSE (16–19). The clinical signs of scrapie and ovine BSE are indistinguishable (20); however, the diseases can be distinguished by careful immunohistochemical analysis of PrPSc accumulation patterns in the brain and lymphoid tissues (21–25) and by the biochemical characteristics of the protease-resistant prion protein (PrPPrres) in the brain (26, 27). Susceptibility to experimental BSE is associated with the ARQ/ARQ genotype, but BSE in VRQ/VRQ sheep can likewise be obtained (28), and infection of ARR/ARR sheep has also been reported (19, 29–31). Discriminatory tests for sheep BSE based on differences in molecular profile were shown to remain effective after three serial experimental passages of the BSE agent in ARQ/ARQ sheep, but there was an indication of a shift in the biochemical properties of PrPPrres during these passages away from that associated with BSE (27). It is therefore possible that adaptation of the BSE agent to an ovine host involves a change in the molecular properties of the BSE prions and that this change may have occurred prior to the introduction of statutory testing. In this scenario, sheep BSE in the field may have become indistinguishable from scrapie, but it may have retained its pathogenicity for humans.

PrP misfolding and prion replication can be mimicked in cell-free PrP conversion assays such as protein misfolding cyclic amplification (PMCA) (32, 33). PMCA can be used to model the transmission and strain adaptation phenomena associated with prion replication in vivo but at an accelerated rate (34–40). The results from these studies suggest that species, strain, and genotypic barriers to prion disease can be modeled in vitro. We have modeled serial passage of the BSE agent in sheep by conducting serial PMCA (sPMCA) using sheep BSE brain homogenates to seed ovine brain substrate of three Prnp genotypes and then testing whether this in vitro adaptation process results in changes in the potential of sheep BSE to convert human PrPSc in a further PMCA reaction.

EXPERIMENTAL PROCEDURES

Ethics Statement—Human tissues were obtained from the CJD Brain and Tissue Bank, which is part of the Medical Research Council Edinburgh Brain Banks. Tissues were collected with consent for research use. Ethical approval for the use of the human tissues in this study was covered by LREC 2000/4/157 (Professor James Ironside). All studies, including experimental inoculations, care of animals, and euthanasia, were carried out in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986. Sheep were obtained from one of two facilities. Experiments performed at the Moredun Research Institute were carried out under licenses from the United Kingdom Government Home Office number 60/2656 (renewed in 2005 with number 60/3646). The remaining sheep were obtained from experiments carried out at the Agricultural Development and Advisory Service facilities at High Mowthorpe under project license number 70/5155. Animals were monitored daily for the presence of neurological signs compatible with TSE and were euthanized once those signs reached a predetermined end point, when showing signs of intercurrent disease unresponsive to treatment, or for welfare reasons. In all cases, euthanasia was performed by intravenous injection of barbiturate overdose followed by exsanguination.

Uninfected Animal Brain Tissues—Nine samples of ovine brain tissue of the three major scrapie-susceptible or –resistant variants, differing in their Prnp polymorphism at codons 136, 154, and 171 (both PBS-perfused or non-perfused; two VRQ/VRQ, three ARQ/ARQ, and four ARR/ARR) were obtained from a scrapie-free flock (ARSU flock) at the Animal Health and Veterinary Laboratories Agency (Weybridge, UK). The bovine (BSE-negative) sample came from cow with limited or no exposure to BSE reared under controlled conditions, and the tissues were provided by the Animal Health and Veterinary Laboratories Agency TSE Archive (Weybridge, UK). All brain tissues were stored at −80 °C immediately after animals were sacrificed. The disease status of these animals was confirmed at source by prion protein immunohistochemistry and Western blot.

Experimental Sheep BSE, Cattle BSE, and Sheep Scrapie Tissues—Brain stem samples from five sheep experimentally infected with BSE (homozygous VRQ/VRQ, ARQ/ARQ, and ARR/ARR BSE-infected sheep), the scrapie-infected sheep, and the BSE-infected cattle brain tissues were produced or collected by Animal Health and Veterinary Laboratories Agency (Lasswade and Weybridge, UK). The BSE-positive cow was a field suspect that had been identified through passive surveillance, and the tissues were provided by the Animal Health and Veterinary Laboratories Agency TSE Archive. The disease status of the animals was confirmed at source by prion protein immunohistochemistry and Western blot.

Prnp Sequencing—Prnp genotyping of the sheep involved in this study was performed on blood samples by PCR amplification and sequencing of the whole open reading frame of the Prnp gene on a 3130 Genetic Analyzer with the BigDye® terminator v3.1 cycle sequencing kit according to the manufacturer’s protocol (Applied Biosystems).

Human Brain Tissues—All tissues were handled exclusively in the category 3ª biosafety containment facility according to stringent health and safety protocols. Human brain tissues (frontal cortex) were sampled from a frozen half-brain collected at autopsy with the appropriate consent for tissue retention and research use. The vCJD specimen was from a patient (PRNP codon 129 MM) with definite variant CJD diagnosis as defined by established criteria. The sCJD sample was from a patient with a diagnosis of sporadic CJD (VV2 subtype). The non-CJD human brain specimens used for PMCA substrate preparation were from frontal cortex from patients with Guillain-Barré syndrome (PRNP codon 129 MM) and dementia with Lewy bodies (PRNP codon 129 VV), and their use as PMCA substrates has been described previously (39).

Preparation of Brain Homogenates—Bovine, ovine, and human tissue homogenates (10%, w/v) were prepared by manual homogenization of brain tissues using glass grinders (Fisher Scientific Ltd.) in PMCA conversion buffer (PBS, 1% Triton X-100, 150 mM NaCl) containing protease inhibitors (Complete Mini EDTA-free, Roche Applied Science). A non-ionic detergent-insoluble nucleocytoskeletal fraction was cleared by centrifugation at 424 g for 40 s. The supernatants were divided into aliquots and stored at −80 °C until use as PMCA substrate.
**Protein Misfolding Cyclic Amplification**—EDTA (Fluka, Sigma-Aldrich) was added to all brain homogenate substrates at a final concentration of 5 mM prior to conducting the PMCA reaction. When human tissue was used as substrate, heparin (LKT Laboratories) (10) was added to the samples at a final concentration 1 mM. Substrates were seeded at 4 °C with a dilution of brain homogenate (previously titrated by Western blotting) such that the seed material contained sufficient PrPSc to be detectable at the lower end of the linear range of a standard Western blot. A negative control aliquot not subjected to PMCA (20 μl) was immediately frozen at −80 °C, and the remainder (sample of 100 μl) was subjected to PMCA using a Misonix model 4000 sonicator. A single round of PMCA comprised 96 cycles of 20-s sonication at 80% total power output and a 29-min 40-s incubation at 37 °C. When serial PMCA reactions were performed, each new round was seeded at a 1:10 dilution with the product of the previous PMCA round (i.e., a 10-fold dilution of the PMCA product in fresh brain homogenate substrate). An aliquot of each sPMCA reaction product was retained for a use as a source for human brain tissue substrate in phase III of the study.

**Sample Preparation for Western Blot Analysis**—Equal volumes of PMCA products and their corresponding negative controls were digested using proteinase K (PK; Novagen®, EMD Millipore) at a final concentration of 50 μg/ml at 37 °C for 1 h. Protease digestion was terminated with the addition of Fabafloc SC (Roche Applied Science) to a final concentration of 1 mM, and the samples were then collected by centrifugation at 20,817 × g for 1 h at 4 °C. Pellets were resuspended in PMCA conversion buffer, and an equal volume of 4× NuPAGE® lithium dodecyl sulfate sample buffer (Invitrogen) was added to each sample aliquot to a final concentration 1×.

**PrP-specific Antibodies**—The primary antibodies used were mouse monoclonal antibodies 6H4 (IgG1; recognizing ovine PrP amino acids 148–155; Prionics), 12B2 (IgG1; recognizing ovine PrP amino acids 89–107) (41), and 3F4 (IgG2a; recognizing human PrP amino acids 106–112; EMD Millipore).

**Western Blot Analysis**—Samples were boiled at 100 °C for 10 min and subjected to electrophoresis for 50 min at 200 V using pre-set gel cassettes (Invitrogen) and NuPAGE MES-SDS 1× running buffer (Invitrogen). A BenchMark prestained protein ladder (Invitrogen) and a MagicMark™ XP Western protein standard (Invitrogen) were run alongside the samples. The gels were then electroblotted onto polyvinylidene difluoride (PVDF) membrane (Hybond-P, GE Healthcare) for 1 h at 30 V using 1× transfer buffer consisting of 4% 20× NuPAGE transfer buffer (Invitrogen), 16% MeOH, 80% diluted H2O. For immunodetection, the PVDF membrane was blocked with a solution of 5% (w/v) nonfat milk powder (Sigma-Aldrich) dissolved in TBS-T (200 mM Tris/HCl, 150 mM NaCl, pH 7.6) containing 0.1% Tween 20 (polyoxyethylenesorbitan monolaurate, Fisher Scientific Ltd.) for 60 min. Then the PVDF membrane was incubated with anti-PrP monoclonal antibody 6H4, 12B2, or 3F4 in TBS-T for 60 min and subsequently with horse-radish peroxidase-conjugated sheep anti-mouse IgG (GE Healthcare) antibody for 60 min. The membranes were developed using ECL Plus (GE Healthcare) and imaged using the ChemiDoc™ XRS+ System (Bio-Rad) following the manufacturer’s instructions.

**Quantitative Densitometry and Statistical Analysis**—The difference in sheep BSE PrPSc amplification efficiency in ovine substrates of different genotype was analyzed from three independent identical experiments. Serial PMCA products were analyzed by Western blotting, and densitometry of the appropriate bands was performed using a volume tool of the XRS+ System Image Lab™ 2.0 software. The background signal values of individual blots were subtracted before data were analyzed and portrayed using GraphPad Prism version 6.01 software.

**Comparison of PrPc Levels in Substrate Brain Homogenates**—The total protein concentration of the brain homogenates was obtained using DC™ Protein Assay kit (Bio-Rad). Equal amounts of protein were analyzed by Western blot using anti-PrP primary antibody 6H4. The PrPc concentration was then obtained by densitometry using a known amount of recombinant PrPc run on the same blot. In experiments designed to normalize PrPc levels in sheep brain homogenates to be used as PMCA substrates, equivalent volumes of 10% brain homogenates were analyzed by Western blotting using the anti-PrP primary antibody 6H4, the PrPc signal was quantitated by densitometry, and the dilution factors to reach equivalence were calculated.

**Deglycosylation of Ovine PrPc**—Ovine brain homogenates of equal PrPc amount (20 μg) were denatured at 100 °C for 10 min and then incubated with 500 units/μl peptide-N-glycosidase F (PNGase F kit, New England Biolabs) for 2 h at 37 °C according to the manufacturer’s instructions. Deglycosylated proteins were precipitated using 4 volumes of ice-cold 80% MeOH and collected by centrifugation at 18,787 × g for 30 min at 4 °C. The supernatant was removed, and the pellets were resuspended in NuPAGE sample reducing agent (Novex®, Invitrogen) and NuPAGE lithium dodecyl sulfate sampling buffer and analyzed by Western blot.

**RESULTS**

**Overall Study Design**—Fig. 1 gives an overview of the study. Phase I established the PMCA conditions that resulted in efficient amplification of cattle PrPSc in normal cattle brain substrates and in normal ovine brain substrates of the VRQ/VRQ, ARQ/ARQ, and ARR/ARR genotypes. Phase II tested whether the BSE-like molecular signature of ovine BSE PrPSc was stable when serially amplified in ovine substrates or whether there was a shift toward a scrapie-like molecular signature. Classical scrapie brain homogenates from VRQ/VRQ and ARQ/ARQ sheep were used to seed all three ovine polymorphic groups, providing a positive control for amplification and PrPSc-type conservation. Phase II determined whether any molecular switch from a BSE-like to a scrapie-like molecular signature altered the ability of the sheep-adapted BSE PrPSc to convert PrPc in human brain substrates. In this phase, the sPMCA products from rounds 1 and 8 produced in phase II were used to seed human brain substrates of the PRNP codon 129 MM and VV genotypes and subjected to a single round of PMCA. The PMCA products were then analyzed by Western blot after PK treatment using the anti-PrP mAb 3F4 (which detects human but not ovine or bovine PrP).
BSE Agent Replication Modeled by Amplification in Ovine Substrates—Sheep BSE brain homogenates and positive control brain homogenates of cattle BSE and classical sheep scrapie gave the expected protein banding pattern corresponding to the three glycoforms of PrPres on Western blot analysis. The diglycosylated form of the protein (top band) exhibited the strongest signal followed by the mono- (middle band) and non-glycosylated (lower band) forms, which exhibited weaker signals (Fig. 2A). The non-glycosylated band of PrPres of cattle BSE and sheep BSE had an apparent molecular mass of around 19 kDa (Fig. 2A, lane 1–6), whereas in classical scrapie, it was around 20–21 kDa (Fig. 2A, lane 7). Next, we established the PMCA conditions needed to amplify BSE PrPres efficiently in normal bovine brain (Fig. 2B) and PrPres from classical scrapie in ovine brain (Fig. 2C). The results confirmed that the PrPres type of PMCA products resembled that of the seed (Fig. 2, B and C). In subsequent PMCA reactions, the initial seed dilution was sometimes adjusted, but the duration and power output of sonication, duration of incubation period, and number of sonication/incubation cycles per round were kept identical for all experiments of this study. We then used these standardized conditions to amplify BSE PrPres in ovine substrates of the three main ovine Prnp polymorphic genotypes (Fig. 2, D and E). Amplification was observed after a single round of PMCA in ovine substrate of the VRQ/VRQ (Fig. 2, D and E, lane 2) and the ARQ/ARQ (Fig. 2, D and E, lane 4) genotypes but not in ARR/ARR (Fig. 2, D and E, lane 6) genotype substrates. Amplification was found to be slightly more efficient using substrates from perfused animals (Fig. 2D, lanes 2 and 4) when compared with the non-perfused brains.
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BSE Agent Adaptation to an ARQ/ARQ Ovine Host Modeled by Serial PMCA—Next, we modeled sheep BSE agent adaptation by conducting sPMCA of experimental sheep BSE brain homogenate seeded in ovine brain substrates of different Prnp genotypes (Fig. 1, phase II). Each round was composed of 96 cycles of sonication and incubation (as described under "Experimental Procedures"). Samples from each round were analyzed for PrPres by Western blotting using the anti-PrP mAb 6H4 (which recognizes both BSE and scrapie PrPres) and 12B2 (which, after protease digestion, detects scrapie but not BSE PrPres) (41). Proce

FIGURE 3. Ovine brain substrates of the VRQ/VRQ and ARQ/ARQ genotypes exhibit higher levels of PrP$^\mathrm{C}$ than the ARR/ARR genotype. A, representative Western blot of the nine ovine brain substrates used in this study. Samples were loaded at 5 µg of total protein/lane, and the Western blot was probed with mAb 6H4. B, graph portraying the levels of PrP$^\mathrm{C}$ in the ovine brain substrates. Data were obtained by densitometry of three independent Western blot analyses (background-subtracted) of identical sample loading, an example of which is shown in A. Data are means ± S.D. (error bars).

BSE Agent Adaptation to a VRQ/VRQ Ovine Host Modeled by Serial PMCA—Next, we modeled sheep BSE agent adaptation by conducting sPMCA of experimental sheep BSE brain homogenate seeded in ovine brain substrates of different Prnp genotypes (Fig. 1, phase II). Each round was composed of 96 cycles of sonication and incubation (as described under "Experimental Procedures"). Samples from each round were analyzed for PrPres by Western blotting using the anti-PrP mAb 6H4 (which recognizes both BSE and scrapie PrPres$^\mathrm{cys}$) and 12B2 (which, after protease digestion, detects scrapie but not BSE PrPres$^\mathrm{cys}$) (41). When the VRQ/VRQ ovine brain substrate was seeded with BSE brain homogenate from either VRQ/VRQ, ARQ/ARQ, or ARR/ARR sheep (Fig. 4A), amplification of sheep BSE PrPres was maintained, and a clear molecular switch of PrPres$^\mathrm{type}$ from a BSE-like to scrapie-like molecular signature occurred at rounds 6–8. The sheep-adapted BSE PrPres$^\mathrm{cys}$ PMCA product exhibited both the non-glycosylated band of higher molecular mass than that of the cattle BSE and was comparable with that of the natural scrapie positive controls (Fig. 4A, marked Sc). This switch from BSE-like to scrapie-like PrPres$^\mathrm{cys}$ was confirmed by analyses using the scrapie PrPres$^\mathrm{cys}$-specific mAb 12B2. The sheep BSE PMCA products were only poorly detected with this anti-

body during rounds 1 to 5 or 6, but a strong PrPres$^\mathrm{cys}$ signal appeared at rounds 6–8 (Fig. 4A, right). The experiment was conducted using identical conditions on three independent occasions (the last time in triplicate), and the switch from BSE-like to scrapie-like PrPres$^\mathrm{cys}$ type was found on each occasion. Semiquantitative densitometric assessment of the PrPres signal in the nine sPMCA reactions of ovine VRQ/VRQ substrate seeded with sheep BSE isolates (n = 9 per substrate genotype) confirmed the visual observations (Fig. 4B). PrPres$^\mathrm{cys}$ was efficiently amplified throughout the sPMCA (Fig. 4B, 6H4 analysis, blue bars), and a clear rise of scrapie-like PrPres$^\mathrm{cys}$ signal detected by 12B2 was evident at rounds 6–8 (Fig. 4B, red bars).

BSE Agent Adaptation to an ARQ/ARQ Ovine Host Modeled by Serial PMCA—The ARQ/ARQ ovine brain substrate seeded with sheep BSE of the VRQ/VRQ, ARQ/ARQ, or ARR/ARR genotype (the same as those used above) and subjected to eight rounds of sPMCA also supported PrPres$^\mathrm{cys}$ amplification throughout sPMCA as seen in Western blotting using the 6H4 mAb (Fig. 4C, left). The sPMCA sheep BSE PrPres$^\mathrm{cys}$ product exhibited the non-glycosylated band of the same molecular mass as the cattle BSE seeds when the 6H4 mAb was used, indicating retention of the BSE-like signature. Analysis of the same samples using the 12B2 mAb (Fig. 4C, right) showed low levels of PrPres$^\mathrm{cys}$ with a molecular mass similar to that of the classical scrapie positive control. This suggests that a minor subpopulation of scrapie-type PrPres$^\mathrm{cys}$ co-amplifies in the samples. This experiment was carried out using the same conditions on three separate occasions (the last time in triplicate), and the results were reproducible, and a semiquantitative densitometric assessment of the Western blots (n = 9 per substrate genotype) is shown (Fig. 4D).

BSE Agent Adaptation to an ARR/ARR Ovine Host Modeled by Serial PMCA—In contrast, despite some evidence of amplification in the firsts PMCA rounds, the ovine ARR/ARR substrate failed to support amplification in subsequent rounds with any of the three sheep BSE seeds used as above (Fig. 4E). The PrPres signal was gradually lost whether the detection mAb was 6H4 or 12B2. The experiment was conducted three times (last time in triplicate), and the loss of PrPres signal during sPMCA using the ARR/ARR was found on each occasion. Densitometric assessment of the Western blots (n = 9 per substrate genotype) is shown (Fig. 4F).

Confirmatory and Control PMCA Experiments Using Additional Seeds and Non-perfused Brain Substrates—The same set of experiments was repeated using non-perfused ovine brain substrates (VRQ/VRQ, ARQ/ARQ, and ARR/ARR), but the PrPres was lost after the third round in all experiments (data not shown). Amplification of scrapie PrPres$^\mathrm{cys}$ in the perfused ovine substrates was also carried out as a control for sheep PrPres$^\mathrm{cys}$-type conservation during sPMCA. The VRQ/VRQ ovine substrate seeded with VRQ/VRQ, ARQ/ARQ, or ARR/ARR sheep scrapie amplified scrapie-like PrPres$^\mathrm{cys}$ throughout sPMCA, whereas the ARQ/ARQ ovine substrate amplified the ARQ/ARR but not the VRQ/VRQ sheep scrapie. The ARR/ARR substrate failed to support scrapie PrPres$^\mathrm{cys}$ amplification. An example of ovine VRQ/VRQ substrates seeded with classical ARQ/ARQ scrapie, subjected to eight rounds of sPMCA, and analyzed by Western blot using mAbs 6H4 and 12B2 is shown (Fig. 5, A and B), demonstrating
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A
Substrate ovine
VRQ/VRQ

mAb 6H4

mAb 12B2

Seed
Sheep BSE
VRQ

Seed
Sheep BSE
ARQ

B
VRQ/VRQ

- r1 r2 r3 r4 r5 r6 r7 r8 Sc BSE

PrP signal (arbitrary units)

- r1 r2 r3 r4 r5 r6 r7 r8 Sc BSE

C
Substrate ovine
ARQ/ARQ

mAb 6H4

mAb 12B2

Seed
Sheep BSE
VRQ

Seed
Sheep BSE
ARQ

D
ARQ/ARQ

- r1 r2 r3 r4 r5 r6 r7 r8 Sc BSE

PrP signal (arbitrary units)

- r1 r2 r3 r4 r5 r6 r7 r8 Sc BSE

E
Substrate ovine
ARR/ARR

mAb 6H4

mAb 12B2

Seed
Sheep BSE
VRQ

Seed
Sheep BSE
ARQ

F
ARR/ARR

- r1 r2 r3 r4 r5 r6 r7 r8 Sc BSE

PrP signal (arbitrary units)

- r1 r2 r3 r4 r5 r6 r7 r8 Sc BSE

mAb 6H4
mAb 12B2

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Western blot analysis using the 6H4 mAb showed that the non-glycosylated band of PrP\textsuperscript{res} in the amplified PMCA product was of molecular mass typical for BSE (Fig. 7B, lanes 2–5) rather than of classical scrapie run alongside the sPMCA samples (Fig. 7, B and C, lane 6, marked Sc) or of the scrapie-like seed (Figs. 4A, r8, and 7A, lane 3) used to seed this reaction. Analysis using the 12B2 mAb confirmed this observation as the PrP\textsuperscript{res} signal detected by 12B2 antibody was lost in the subsequent passages of the sPMCA (Fig. 7C, lanes 2–5). Graphical representation of densitometry data confirmed this visual observation (Fig. 7D).

Controlling Serial Protein Misfolding Cyclic Amplification for de Novo PrP\textsuperscript{res} Formation—In each of the above experiments, unseeded control PMCA reactions were run in parallel with the seeded PMCA reactions to test for de novo PrP\textsuperscript{res} formation. At early stages of the project, PrP\textsuperscript{res} was found in some of these unseeded controls. This only occurred when scrapie-seeded samples were run in the same sPMCA experiment. Results from these experiments were disregarded. After the introduction of stringent liquid handling precautions to avoid cross-contamination, unseeded controls remained PrP\textsuperscript{res}-negative (Fig. 8). We concluded that the appearance of PrP\textsuperscript{res} in the unseeded control reactions at the early stages of the project represented cross-contamination rather than de novo formation.

Competence of the ARR/ARR Substrate to Support PrP\textsuperscript{res} Replication in PMCA—The failure of sheep ARR/ARR substrates to support amplification in any of the above sPMCA experiments might be attributed directly to the presence of arginine at position 171, but other aspects of PrP\textsuperscript{C} expression may also be relevant. The ARR/ARR substrate contained lower levels of PrP\textsuperscript{C} compared with VRQ/VRQ or ARQ/ARQ (Fig. 3). Densitometric analysis of a comparative Western blot indicated that the VRQ/VRQ substrate contained 2.2 times the amount of PrP\textsuperscript{C} found in the ARR/ARR substrate. Hence, we repeated the sPMCA experiment using the ARR/ARR brain homogenate at 20% (w/v) instead of the standard 10%, effectively doubling the PrP\textsuperscript{C} concentration inter alia. However, the results were again negative (Fig. 9, A and B). The reverse strategy was also attempted in which the 10% (w/v) VRQ/VRQ substrate was diluted to match the PrP\textsuperscript{C} concentration of the 10% (w/v) ARR/ARR substrate. Dilution of the VRQ/VRQ substrate reduced the amplification efficiency of VRQ sheep BSE seed, resulting in amplification in sPMCA rounds 1 and 2 that failed to overcome the effect of serial dilution in subsequent rounds (Fig. 9, C and D).

Nevertheless, amplification of the VRQ sheep BSE seed in the ~5% (w/v) VRQ/VRQ sheep substrate was greater than amplification of the same seed in the 10% (w/v) ARR/ARR substrate

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FIGURE 4. VRQ/VRQ and ARQ/ARQ ovine brain substrates support amplification of sheep BSE PrP\textsuperscript{res} in serial PMCA. A, ovine VRQ/VRQ substrate seeded with sheep BSE, subjected to eight rounds (r1–r8) of sPMCA and analyzed by Western blotting using anti-PrP mAb 6H4 (left) and 12B2 (right). C, the ARQ/ARQ substrate seeded and analyzed as above. E, the ARR/ARR substrate seeded and analyzed as above. Ovine brain substrates were seeded with experimental sheep BSE VRQ/VRQ, ARQ/ARQ, and ARR/ARR as indicated in the figure and subjected to eight rounds of sPMCA. –, corresponding controls not subjected to PMCA. Classical scrapie (Sc) and BSE standards were used as controls of PrP\textsuperscript{res} migration, antibody specificity, and blotting procedure. Samples were analyzed after PK treatment using anti-PrP mAbs 6H4 (red bars) and 12B2 (blue bars) and analyzed by densitometry (n = 9 per genotype; three independent but identical experiments). The data were background-subtracted. Means ± S.D. (error bars) are shown. B, sheep BSE PrP\textsuperscript{res} amplification in ovine substrate of VRQ/VRQ genotype. D, sheep BSE PrP\textsuperscript{res} amplification in ovine substrate of ARQ/ARQ genotype. F, sheep BSE PrP\textsuperscript{res} amplification in ovine substrate of ARR/ARR genotype.

FIGURE 5. Ovine substrate amplifies classical scrapie PrP\textsuperscript{res} efficiently in serial PMCA. A and B, representative Western blot analysis of ovine VRQ/VRQ substrate seeded with classical scrapie (ARQ/ARQ genotype) and subjected to eight rounds (r1–r8) of sPMCA. Classical scrapie (Sc) and BSE standards were used as a control for PrP\textsuperscript{res} migration and the blotting procedure. Samples were analyzed after PK treatment using anti-PrP mAb 6H4 (A) and 12B2 (B). Nevertheless, amplification of the VRQ sheep BSE seed in the ~5% (w/v) VRQ/VRQ sheep substrate was greater than amplification of the same seed in the 10% (w/v) ARR/ARR substrate.
Fig. 9, E and F) as shown by densitometric analysis (Fig. 9, D and F). The 10% VRQ/VRQ substrate efficiently supported the sheep BSE PrP\textsuperscript{res} amplification in all four rounds of sPMCA in agreement with our earlier results (Fig. 9, G and H). Additionally, PrP\textsuperscript{C} deglycosylation and Western blot analysis showed that all VRQ/VRQ, ARQ/ARQ, and ARR/ARR (Fig. 10, A, lanes 2, 4, and 6, respectively, and B) homogenates contained full-length PrP\textsuperscript{C} and the C1 fragment at approximately equal relative amounts in all three polymorphic variants, suggesting that differential proteolytic processing is not responsible for the failure of the ARR/ARR samples to support amplification.
Zoonotic Transmission of in Vitro Sheep-adapted BSE Agent Modeled by PMCA Using Human Substrates—In the third phase of the project (Fig. 1, phase III), we first seeded the human brain substrate of PRNP codon 129 MM and VV genotypes with experimental sheep BSE, cattle BSE, and classical scrapie samples and subjected these to a single round of PMCA (Fig. 11). Seeding with human vCJD (MM brain substrate) and sCJD (VV brain substrate) brain samples served as positive controls for the PMCA reactions. Samples were PK-treated and analyzed by Western blotting using anti-PrP mAb 6H4 (A) and 12B2 (B).

DISCUSSION

BSE is a prion disease of cattle that has been shown to be transmissible to other animals and is zoonotic, causing vCJD in humans. If BSE entered the United Kingdom sheep flock and has been maintained since the 1980s and 1990s, it would currently be at its ~20th passage. It is possible that this process could have resulted in an adaptation of the agent to the ovine host that rendered BSE indistinguishable from scrapie at the molecular level. Because of the protracted nature (and the attendant financial and ethical considerations) of modeling such a process in vivo, we have chosen to model this scenario by conducting serial PMCA of ovine brain substrates seeded with experimental sheep BSE brain homogenates. We estimate that conducting eight serial passages in vivo using large animals (sheep) or small animals (appropriate ovine transgenic mouse lines) followed by transmission to non-human primates or humanized transgenic mice would take over 8 years (in mice) and over 20 years (in sheep) to conduct. In contrast, eight serial rounds of ovine PMCA followed by a single round of PMCA in human substrate can be completed in 1 month.

Using this in vitro approach, we found that the major factor influencing the propagation of the sheep BSE agent was the genotype of the host sheep brain substrate (Table 1). The VRQ/VRQ substrate efficiently amplified sheep BSE PrPres during serial rounds of PMCA. Inclusion of appropriate comparable controls (scrapie and BSE) on each Western blot was important for distinguishing between the molecular types of PrPres propagated in the ovine substrates. In addition, a discriminatory antibody comparison method was used (mAbs 6H4 and 12B2) that, in conjunction with Western blotting, allowed us to unambiguously classify the predominant PrPres types present before and after PMCA. The Western blotting results demonstrated a clear and reproducible switch of mobility from that of BSE in sheep to that found in natural scrapie after five to six rounds of sPMCA that was confirmed by the relative 6H4/12B2 antibody binding. Aspects of the agent strain are often preserved for several passages following cross-species transmission (27, 42, 43). It has been proposed previously that a single round of PMCA reaction approximates to one in vivo passage, and a process of adaptation of certain prion strains to a new host was reported to occur following three to six rounds of serial PMCA (36, 37). Our data appear consistent with these observations. The ARQ/ARQ substrate also supported efficient PrPres amplification throughout the serial rounds of PMCA, but in this substrate, a switch to a scrapie-like PrPres was not evident; instead a BSE-like PrPres predominated accompanied by a less readily detectable scrapie-like PrPres component. This suggests a role for valine (as opposed to alanine) at position 136 in favoring a scrapie-like PrPSc conformation (Table 1). The relative genotypic specificities of sheep BSE seeds and the substrate genotype-dependent switch in predominant PrPres type were replicated when cattle BSE was used as a seed in sPMCA instead of the experimental sheep BSE samples.
In an effort to determine whether the VRQ/VRQ substrate-dependent switch from a BSE-like to a scrapie-like PrPres type was reversible, we attempted to reamplify the sheep VRQ/VRQ sPMCA product in a bovine brain substrate. Sustained amplification of PrPres was not achieved for either PrPres type in the bovine substrate. This outcome is consistent with a previous report that sheep scrapie fails to amplify in a bovine PMCA substrate (44) and may reflect the known difficulty of transmitting scrapie to cattle (45).

SPMCA of the classical scrapie agent in the three ovine substrates served as a positive control in our experiments, and we saw conservation of the expected molecular banding pattern of typical scrapie PrPres. These results are in agreement with previous reports of efficient amplification when the donor seed and the host substrate are matched for the amino acid at position 136 (46) and more importantly with observations of scrapie agent propagation in vivo (13).

Our modeling of the sheep BSE agent amplification in ovine substrate is broadly consistent with experimental in vivo observations in which sheep of the ARQ/ARQ genotype propagated the BSE agent for several passages (27). Our findings are also in agreement with recently published observations assessing species and ovine polymorphic barriers to BSE, including the efficiency of amplification of the BSE agent in ovine ARQ/ARQ and VRQ/VRQ substrates by a single round PMCA (40). However, we do note a discrepancy between our and others' PMCA data (40) and the experimental in vivo data concerning BSE and the ARR/ARR genotype that show that sheep of the ARR/ARR genotype are susceptible to experimental BSE infection (19, 31). The explanation for this difference between the in vitro and in vivo...
vivo results is not clear at present, but we have investigated some of the potential causes. We discount proteolytic processing of ovine PrP<sup>C</sup> as an explanation of genotype-dependent amplification potential (47, 48). We found that the ARR/ARR substrates had half the amount of PrP<sup>C</sup> found in the VRQ/VRQ and ARQ/ARQ substrates, but doubling the concentration of the ARR/ARR substrate did not result in amplification. It therefore seems likely that the failure of the ARR/ARR substrate to support sustained amplification of sheep BSE PrP<sup>res</sup> is due to a genuine relative molecular barrier to conversion associated with the substitution of arginine for glutamine at position 171 (Table 1) but one that can be overcome in the more complex process of infection and disease progression in vivo.

Another major objective of our study was to use PMCA to address molecular conversion barriers between potentially zoonotic animal diseases and humans. We have shown previously that brain samples from vCJD (in humans), BSE in cattle, and experimental sheep BSE (ARQ/ARQ genotype) can convert codon 129 MM human PrP<sup>C</sup> in PMCA reactions, whereas ARQ/ARQ scrapie does not do so to a measurable extent (38). This suggests that the known zoonotic potential of the BSE agent is recapitulated in this cell-free prion protein conversion assay irrespective of the host species (cow, sheep, or human). We have also shown that classical BSE cattle brain homogenates have a greater potential to convert human PrP<sup>C</sup> in PMCA than any other tested animal prion disease (39). Here we show that samples of experimental sheep BSE isolates of the VRQ/VRQ, ARQ/ARQ, and ARR/ARR genotypes are all able to convert human PrP<sup>C</sup> during a single round of PMCA and that the preference is for the PRNP codon 129 M over the V allele (Table 1). We also show that there is an association between the PrP<sup>res</sup> type present and the ability to convert human PrP<sup>C</sup>: when sheep BSE of any genotype is amplified in an ARQ/ARQ substrate it retains its molecular signature and its ability to convert human PrP<sup>C</sup>, whereas if the same starting seeds are amplified in a VRQ/VRQ substrate the PrP<sup>res</sup> adapts to a scrapie-like signature and loses its ability to convert human PrP<sup>C</sup>. This provides a more comprehensive data set than previous transgenic animal-based...
studies (49, 50) and points to a decisive role for the interaction of conformation-enciphered agent strain and host genotype in determining prion replication.

Extrapolation from our results using the serial PMCA method would suggest that if BSE were to infect sheep in the field the molecular phenotype of sheep BSE agent might, through time and in certain sheep genotypes, come to resemble scrapie; however, if this were the case, then there may be an associated increase in the molecular barrier of disease transmission to humans. The concepts of Darwinian evolution are increasingly being applied to the epigenetic molecular evolution of prions and amyloids, and the subject is becoming experimentally tractable (51, 52). The accelerated in vitro molecular evolution of BSE prions in sheep shown here strongly indicates that both replicative efficiency and zoonotic potential may be determined in a direct manner by a combination of the PrPSc conformation associated with the agent and aspects of host prion protein genetics that determine key polymorphic residues in the prion protein sequence.

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REFERENCES

1. Prusiner, S. B. (2013) Biology and genetics of prions causing neurodegeneration. Annu. Rev. Genet. 47, 601–623
2. Hill, A. F., and Collinge, J. (2004) Prion strains and species barriers. Contrib. Microbiol. 11, 33–49
3. Moore, R. A., Vorberg, I., and Priola, S. A. (2005) Species barriers in prion diseases—brief review. Arch. Virol. Suppl. 19, 187–202
4. Wells, G. A., Scott, A. C., Johnson, C. T., Gunning, R. F., Hancock, R. D., Jeffrey, M., Dawson, M., and Bradley, R. (1987) A novel progressive spongiform encephalopathy in cattle. Vet. Rec. 121, 419–420

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Human brain homogenate substrate (codon 129-MM) seeded with sPMCA products:

![Diagram showing the seeding of BSE substrates and sPMCA products](image)

**TABLE 1**

The relevant polymorphic residues in the human and ovine prion protein, and the amino acids present at equivalent position in the human, ovine, and bovine proteins

| Species (polymorphisms) | Human 129 | Oxine 129 | Oxine 154 | Oxine 171 |
|--------------------------|-----------|-----------|-----------|-----------|
| Bovine                   | M††       | A††       | R††       | Q††       |
| Human (129M)             | M††       | A††       | R††       | E††       |
| sheep (ARQ)              | M††       | A††       | R††       | Q††       |
| Sheep (VRQ)              | M††       | V††       | R††       | Q††       |
| Sheep (ARR)              | M††       | A††       | R††       | R††       |
| Human (120V)             | V††       | A††       | R††       | E††       |
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17. Foster, J., Parnham, D., Chong, A., Goldmann, W., and Hunter, N. (2001) Clinical signs, histopathology and genetics of experimental transmission of BSE and natural scrapie to sheep and goats. Vet. Rec. 148, 165–171.

18. Foster, J. D., Parnham, D., Hunter, N., and Bruce, M. (2001) Distribution of the prion protein in sheep terminally affected with BSE following experimental oral transmission. J. Gen. Virol. 82, 2319–2326

19. Jeffrey, M., Ryder, S., Martin, S., Hawkins, S. A., Martin, S., Parnham, D., and Hunter, N. (2003) BSE in sheep bred for resistance to infection. Nature 423, 498

20. Stack, M., Jeffrey, M., Gubbins, S., Gummer, S., Gonzalez, L., Martin, S., Chaplin, M., Webb, P., Simons, M., Spencer, Y., Bellerby, P., Hunt, S., Wilesmith, J., and Matthews, D. (2007) Monitoring for bovine spongiform encephalopathy (BSE). 1. Onset and distribution of disease-specific PrP accumulation in brain and viscera. J. Comp. Pathol. 124, 280–289

21. Houston, F., Goldmann, W., Chong, A., Jeffrey, M., Gonzalez, L., Foster, J., Parnham, D., and Hunter, N. (2003) BSE in sheep bred for resistance to infection. Nature 423, 498

22. Stack, M., Jeffrey, M., Gubbins, S., Gummer, S., Gonzalez, L., Martin, S., Chaplin, M., Webb, P., Simons, M., Spencer, Y., Bellerby, P., Hope, I., Wilesmith, J., and Matthews, D. (2006) Monitoring for bovine spongiform encephalopathy in sheep in Great Britain, 1998–2004. J. Gen. Virol. 87, 2099–2107

23. Jeffrey, M., Martin, S., Gonzalez, L., Chong, A., Foster, J., Goldmann, W., Hunter, N., and Martin, S. (2006) Ovine infection with the agents of scrapie (CH1641 isolate) and bovine spongiform encephalopathy: immunohistochemical similarities can be resolved by immunohistochemistry. J. Comp. Pathol. 134, 17–29

24. Gonzalez, L., Martin, S., and Jeffrey, M. (2003) Distinct profiles of PrP immunoreactivity in the brain of scrapie- and BSE-infected sheep: implications for differential targeting and PrP processing. J. Gen. Virol. 84, 1339–1350

25. Gonzalez, L., Martin, S., Houston, F. E., Hunter, N., Reid, H. W., Bellworthy, S. I., and Jeffrey, M. (2005) Phenotype of disease-associated PrP accumulation in the brain of bovine spongiform encephalopathy experimentally infected sheep. J. Gen. Virol. 86, 827–838

26. Thuring, C. M., Erkens, J. H., Jacobs, J. G., Bossers, A., Van Keulen, L. J., Garssen, G. J., Van Zijderveld, F. G., Ryder, S. J., Groushop, M. H., Sweeney, T., and Langeveld, J. P. (2004) Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein. J. Clin. Microbiol. 42, 972–980

27. Stack, M., Gonzalez, L., Jeffrey, M., Martin, S., Macaldowie, C., Chaplin, M., Thorne, I., Sayers, R., Davis, L., Bramwell, D., Grimmer, S., and Bellworthy, S. (2009) Three serial passages of bovine spongiform encephalopathy in sheep do not significantly affect discriminatory test results. J. Gen. Virol. 90, 764–768

28. Bellworthy, S. J., Dexter, G., Stack, M., Chaplin, M., Hawkins, S. A., Simmons, M. M., Jeffrey, M., Martin, S., Gonzalez, L., Martin, S., and Hill, P. (2008) Oral transmission of BSE to VRQ/VRQ sheep in an experimental flock. Vet. Rec. 162, 130–131

29. Andreoletti, O., Morel, N., Lacroux, C., Rouillon, V., Barb, T., Tabouret, G., Sarradin, P., Berthon, P., Bernardet, P., Mathey, J., Lugen, S., Costes, P., Corbiere, F., Espinosa, J. C., Torres, J. M., Grassi, J., Schelcher, F., and Lantieri, F. (2006) Bovine spongiform encephalopathy agent in spleen from an ARR/ARR orally exposed sheep. J. Gen. Virol. 87, 1034–1046

30. Ronzon, F., Bencsik, A., Lema, S., Vulin, J., Kodjo, A., and Baron, T. (2006) BSE inoculation to prion diseases-resistant sheep reveals tricky silent carriers. Biochem. Biophys. Res. Commun. 350, 872–877

31. Gonzalez, L., Chianini, F., Martin, S., Sisio, S., Gibbard, L., Reid, H. W., and Jeffrey, M. (2007) Comparative titration of experimental ovine BSE infectivity in sheep and mice. J. Gen. Virol. 88, 714–717

32. Saborio, G. P., Permanne, B., and Soto, C. (2001) Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 411, 810–813

33. Morales, R., Duran-Anioz, C., Diaz-Espinoza, R., Camacho, M. V., and Soto, C. (2012) Protein misfolding cyclic amplification of infectious prions. Nat. Protoc. 7, 1397–1409

34. Kurt, T. D., Perrott, M. R., Wilusz, C. J., Wilusz, J., Supattapone, S., Telling, G. C., Zabel, M. D., and Hoover, E. A. (2007) Efficient in vitro amplification of chronic wasting disease PrPres. J. Virol. 81, 9605–9608

35. Murayama, Y., Yoshioka, M., Yokoyama, T., Iwamaru, Y., Imamura, M., Masujin, K., Yoshiha, S., and Mohri, S. (2007) Efficient in vitro amplification of a mouse-adapted scrapie prion protein. Neurosci. Lett. 413, 270–273

36. Castilla, J., Gonzalez-Romero, D., Saá, P., Morales, R., De Castro, J., and Soto, C. (2008) Crossing the species barrier by PrP(Sc) replication in vitro generates unique infectious prions. Cell 134, 757–768

37. Green, K. M., Castilla, J., Seward, T. S., Jewell, D. L., Piris, J. E., Soto, C., and Telling, G. C. (2008) Accelerated high fidelity prion amplification within and across prion species barriers. PLoS Pathog. 4, e1000139

38. Jones, M., Wight, D., Barron, R., Jeffrey, M., Manson, J., Prowse, C., Ironside, J. W., and Head, M. W. (2009) Molecular model of prion transmission to humans. Emerg. Infect. Dis. 15, 2013–2016

39. Barria, M. A., Balachandran, A., Morita, M., Kitamoto, T., Barron, R., Manson, J., Knight, R., Ironside, J. W., and Head, M. W. (2014) Molecular barriers to zoonotic transmission of prions. Emerg. Infect. Dis. 20, 88–97

40. Priem, J., Langeveld, J. P., van Keulen, L. J., van Zijderveld, F. G., Andreoletti, O., and Bossers, A. (2014) Enhanced virulence of sheep-passaged BSE is revealed by decreased polymorphism-barriers in prion protein conversion studies. J. Virol. 88, 2903–2912

41. Langeveld, J. P., Jacobs, J. G., Erkens, J. H., Bossers, A., van Zijderveld, F. G., and van Keulen, L. J. (2006) Rapid and discriminatory diagnosis of scrapie and BSE in retro-pharyngeal lymph nodes of sheep. BMC Vet. Res. 2, 19
42. Race, R., Raines, A., Raymond, G. J., Caughey, B., and Chesebro, B. (2001) Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: analogies to bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease in humans. J. Virol. 75, 10106–10112
43. Race, R., Meade-White, K., Raines, A., Raymond, G. J., Caughey, B., and Chesebro, B. (2002) Subclinical scrapie infection in a resistant species: persistence, replication, and adaptation of infectivity during four passages. J. Infect. Dis. 186, S166-S170
44. Murayama, Y., Imamura, M., Masujin, K., Shimozaki, N., Yoshioka, M., Mohri, S., and Yokoyama, T. (2012) Ultrasensitive detection of scrapie prion protein derived from ARQ and AHQ homozygote sheep by interspecies in vitro amplification. Microbiol. Immunol. 56, 541–547
45. Cutlip, R. C., Miller, J. M., Hamir, A. N., Peters, J., Robinson, M. M., Jenny, A. L., Lehmkuhl, H. D., Taylor, W. D., and Bisplinghoff, F. D. (2001) Resistance of cattle to scrapie by the oral route. Can. J. Vet. Res. 65, 131–132
46. Thorne, L., Holder, T., Ramsay, A., Edwards, J., Taema, M. M., Windl, O., Maddison, B. C., Gough, K. C., and Terry, L. A. (2012) In vitro amplification of ovine prions from scrapie-infected sheep from Great Britain reveals distinct patterns of propagation. BMC Vet. Res. 8, 223
47. Westergard, L., Turnbaugh, J. A., and Harris, D. A. (2011) A naturally occurring, C-terminal fragment of the prion protein delays disease and acts as a dominant negative inhibitor of PrPSc formation. J. Biol. Chem. 286, 44234–44242
48. Campbell, L., Gill, A. C., McGovern, G., Jalland, C. M., Hopkins, J., Tranulis, M. A., Hunter, N., and Goldmann, W. (2013) The PrPC C1 fragment derived from the ovine A136R154R171 PRNP allele is highly abundant in sheep brain and inhibits fibrillisation of full-length PrPC protein in vitro. Biochim. Biophys. Acta 1832, 826–836
49. Padilla, D., Beringue, V., Espinosa, J. C., Andreoletti, O., Jaumain, E., Reine, F., Herzog, L., Gutierrez-Adan, A., Pintado, B., Laude, H., and Torres, J. M. (2011) Sheep and goat BSE propagate more efficiently than cattle BSE in human PrP transgenic mice. PLoS Pathog. 7, e1001319
50. Pinston, C., Hart, P., Chong, A., Hunter, N., Foster, I., Piccardo, P., Manson, J. C., and Barron, R. M. (2011) Increased susceptibility of human-PrP transgenic mice to bovine spongiform encephalopathy following passage in sheep. J. Virol. 85, 1174–1181
51. Li, J., Browning, S., Mahal, S. P., Oelschlegel, A. M., and Weissmann, C. (2010) Darwinian evolution of prions in cell culture. Science 327, 869–872
52. Krishnan, R., Goodman, J. L., Mukhopadhyay, S., Pacheco, C. D., Lemke, E. A., Deniz, A. A., and Lindquist, S. (2012) Conserved features of intermediates in amyloid assembly determine their benign or toxic states. Proc. Natl. Acad. Sci. U.S.A. 109, 11172–11177