**In vitro amplification of PrPSc derived from the brain and blood of sheep infected with scrapie**

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Scrapie is a fatal, naturally transmissible, neurodegenerative prion disease that affects sheep and goats and is characterized by the accumulation of a misfolded protein, PrPSc, converted from host-encoded PrPc, in the central nervous system of affected animals. Highly efficient in vitro conversion of host PrPc to PrPSc has been achieved in models of scrapie and in natural prion diseases by protein misfolding cyclic amplification (PMCA). Here, we demonstrate amplification, by serial PMCA, of PrPSc from individual sources of scrapie-infected sheep. Efficiency of amplification was affected by the pairing of the source of PrPSc with the control brain substrate of different genotypes of PrP. In line with previous studies, efficiency of amplification was greatly enhanced with the addition of a synthetic polyanion, polyadenylic acid (PolyA), facilitating rapid detection of low levels of PrPSc from body fluids such as blood. To this end PrPSc was amplified, in a 3 day PMCA assay, from blood leukocyte preparations from VRQ/VRQ scrapie-affected sheep at clinical end point. While PolyA-assisted PMCA resulted in spontaneous conversion of PrPc, we were able to distinguish blood samples from unaffected and affected sheep under controlled conditions. This study demonstrates that highly efficient amplification of PrPSc can be achieved for ovine scrapie from both brain and blood from naturally infected sheep and shows potential applications for improvements in current diagnostics and pre-mortem testing.

**INTRODUCTION**

Prion diseases are fatal neurodegenerative disorders that affect a number of species including humans, cattle, sheep and deer. The accumulation of an abnormal conformer (PrPSc) by in vivo conversion of a host protein (PrPc) is likely to be involved in the aetiology of the disease (Prusiner et al., 1982), although the mechanisms of pathological damage are still not well understood. However, the detection of PrPSc is considered to be a reliable diagnostic marker for prion diseases (EFSA, 2005) and PrPSc can be detected predominantly in the central nervous system and lymphoreticular system. The mechanisms of conversion of PrPc to PrPSc are ill-defined, but it is broadly believed that the abnormal conformer acts as a template for conversion of host PrPc (Prusiner, 1991). Reproduction of PrPc conversion in in vitro models has long been an area of investigation (Kocisko et al., 1994). Recently highly efficient conversion has been achieved in hamster models (Castilla et al., 2005a) and also using proteins derived from humans (Jones et al., 2007) and deers (Kurt et al., 2007) using a method involving protein misfolding cyclic amplification (PMCA). PMCA is a procedure first described by Saborio et al. (2001) that facilitates rapid conversion of excess PrPc to PrPSc following incubation in vitro. This is achieved by disruption of newly formed aggregates of PrPSc by sonication into smaller units to act as new templates in subsequent incubations. The ability of PMCA to convert and amplify low initial concentrations of PrPSc in a given tissue has enabled the demonstration of PrPSc in blood (Saa et al., 2006) and urine (Murayama et al., 2007). This has opened up possibilities of studying the pathogenesis of prion diseases, of more sensitive diagnostic tests and of ante-mortem analysis.

In sheep, susceptibility to classical scrapie disease is partly controlled by polymorphisms of the PrP gene. Thus sheep bearing the VRQ residues (at codons 136, 154 and 171) are highly susceptible to the classical form of scrapie, while those bearing ARQ appear to have intermediate susceptibility and homozygous ARR sheep are relatively resistant (Goldmann et al., 1994; Hunter et al., 1993; Laplanche et al., 1993; Maciulis et al., 1992). In addition, there is evidence that the differences in susceptibility between VRQ- and ARQ-bearing sheep is strain- or scrapie-source-dependent. This is exemplified by experimental studies in which VRQ/VRQ and ARQ/ARQ sheep have been found to be differentially susceptible to two sources of scrapie, SSBP1 and CH1641, respectively (Houston et al., 2002; Jeffrey et al., 2006). Here, we apply the in vitro method of serial PMCA to ovine scrapie PrPSc from individual sources and demonstrate highly efficient amplification of PrPSc from VRQ/XXX sources of scrapie in a VRQ/VRQ control brain as a substrate containing PrPc. We were unable to achieve similar levels of amplification from four of the five
scrapie sources in an ARQ/ARQ substrate including those matched at codon 136. Additionally, three of the four ARQ/ARQ sources of scrapie were not amplified in VRQ/VRQ substrate. These data suggest that both the primary sequence of PrP and the individual source of scrapie affect the efficiency of amplification. In addition, we show amplification of PrPSc from the blood leukocyte fraction from scrapie-infected sheep, the first demonstration, to our knowledge, of PrPSc in blood from a naturally infected sheep source.

METHODS

Source and preparation of tissues. Substrate sheep brains were prepared from a New Zealand-derived scrapie-free flock. Whole brains were removed from adult sheep (one VRQ/VRQ, one ARQ/ARQ and one ARQ/VRQ) immediately after euthanasia. Meninges, large blood vessels and blood clots were removed from the brain tissue prior to washing in ice-cold amplification buffer (PBS containing 150 mM NaCl, 4 mM EDTA pH 8.0, 1.0% (v/v) Triton X-100 and mini-complete protease inhibitor (Roche)]. The tissue was then liquidized in ice-cold amplification buffer to give a 10% (w/v) homogenate. Further homogenization was achieved using a Hybaid ribolysr (Power setting 5 for 20 s) in 2 ml tubes containing ceramic beads. Large particulate matter was removed by low-speed centrifugation at 2000 r.p.m. for 20 s (Eppendorf 5804R centrifuge). Final homogenates from each brain were stored in aliquots of 1 ml at 

Sheep naturally infected with scrapie were sourced from the Veterinary Laboratories Agency (VLA) transmissible spongiform encephalopathy (TSE) archive or collected prospectively from confirmed scrapie-infected sheep submitted to the VLA for diagnosis. Individual sources of cerebellum from all scrapie-infected sheep were selected and homogenized as described above.

All samples were confirmed negative or positive by routine diagnosis using Bio-Rad TeSeE ELISA, immunohistochemistry and VLA hybrid Western blot (Stack et al, 2002).

Blood leukocytes were collected from 10 VRQ/VRQ sheep showing full clinical signs at post-mortem and from eight uninfected VRQ/VRQ sheep derived from the scrapie-free flock. All of the naturally infected sheep were derived from the VLA scrapie flock. Blood samples collected in EDTA as anti-coagulant were centrifuged at 2000 r.p.m. for 20 s (Eppendorf 5804R centrifuge). Final homogenates from each brain were stored in aliquots of 1 ml at 80°C.

PMCA. PMCA was performed as described elsewhere (Castilla et al., 2005a). Brain homogenates, prepared as 10% (w/v) as described above, from scrapie-infected sheep or from uninfected sheep, were serially diluted in control brain homogenate (CBH) (genotype as indicated in Results). Pre-amplification dilutions, ranging from 10-3 to 10-9, were either frozen immediately at -80°C to be used as unamplified controls or processed immediately for PMCA. Subsequent dilutions are expressed relative to the undiluted brain tissue. Each dilution (100 µl) was placed into a 200 µl PCR tube and placed in an ultrasonicating water bath (model 3000; Misonix) at 37°C and sonicated for 40 s at power 7.0. This was repeated every 29 min and 20 s. After 24 or 48 sonication/incubation cycles (one PMCA round) the amplified samples were diluted 1:3 with CBH and divided into two; one part was subjected to a further 24 or 48 cycles of PMCA and the other was frozen at -80°C. This step was repeated until termination of the experiment as indicated in the results.

For amplification of blood samples, 106 blood leukocytes were diluted in 1 ml 10% (w/v) CBH, prepared as described above and 100 µl (107 cells) were processed immediately for amplification.

PrPSc analysis by enzyme immunoassay (EIA). The samples were tested as directed by the manufacturers (IDEXX HerdChek BSE-scrapie Antigen Test kit, enzyme immunoassay). Briefly, samples were diluted 1:5 in homogenization buffer, incubated with working plate diluent and mixed gently. Sample was then applied to the test plate provided and incubated at room temperature for 3 h. In each well 16 µl of the final reaction mixture was applied to the plate. After washing, the bound sample was further treated for 10 min with conditioning buffer and then labelled with an anti-PrP horseradish peroxidase conjugate for 75 min. Visualization of binding was achieved with TMB colour substrate and measured at 450 nm using a reference filter at 620 nm (Wallac Victor 1420 reader). Positive and negative classification of samples were interpreted as directed by the manufacturer. The cut-off value was calculated as mean of the negative control plus an AU value of 0.12. Values equal or greater than this value were classified as positive.

Western immunoblot analysis of PrPSc. Samples were denatured and prepared for SDS-PAGE by mixing with 0.4% (w/v) SDS and then digested with 100 µg proteinase K ml-1 (PK; Sigma P2308) at 40°C for 60 min with continual agitation. Equal volumes of digested sample were mixed with Laemmli solution (consisting of Laemmli sample buffer (Bio-Rad) containing 5% (v/v) 2-mercaptoethanol and 2% (w/v) SDS) and heated to 100°C for 5 min. A 3 µl volume of the final amplification reaction mixture was loaded into each lane and SDS-PAGE and immunoblotting were performed as described previously (Terry et al., 2007). Proteins were labelled with anti-PrP monoclonal antibody 9A2 (0.68 µg ml-1, Langeveld et al., 2006) and visualized by chemiluminescence (ECL; Amersham).

Calculation of factor for increase in sensitivity of detection. A factor that represents the increase in the level of detection afforded by serial PMCA was calculated as described previously (Castilla et al., 2005b). The pre-amplification dilution at which the non-amplified sample has an absorbance value of close to the cut-off value was divided by the post-amplification dilution at which the amplified sample had an equivalent absorbance value.

RESULTS

Brain homogenate from a sheep of genotype VRQ/VRQ, with confirmed scrapie, was diluted into a brain homogenate from an unexposed scrapie-free sheep (CBH) with homologous PrP genotype. The infected brain was diluted at serial log dilutions from 10-3 to 10-10 and subjected to PMCA according to the method of Castilla et al. (2005a). Unamplified PrPSc was detected at the 10-3 dilution only (Fig. 1) by HerdChek EIA and Western blot (WB). The reaction mixtures were subjected to serial PMCA for 4 days either as two 48 h rounds (Fig. 1a) or as four 24 h rounds (Fig. 1b). After each round of amplification, consisting of either 96 cycles (48 h) or 48 cycles (24 h) of sonication and incubation, the samples were diluted 1:3 in fresh CBH. Following amplification after each round, PrPSc was
detected by both the Idexx HerdChek BSE/scrapie EIA, a method that does not require PK for discrimination between PrPc and PrPSc, an approved European diagnostic test for scrapie (EFSA, 2005), and by Western blotting for PK-digested PrPSc. Amplification of PrPSc was observed with both PMCA regimes but greater efficiency of amplification, by up to 1000-fold, was achieved when the CBH was replenished more frequently, i.e. after 24 h (Fig. 1b). Thus, PrPSc could be detected following amplification of the initial brain homogenate to a dilution of $10^{-8}$ when four rounds of 48 cycles each were performed. This finding is mirrored by the analysis of the PK-resistant PrPSc by WB (Fig. 1c). Less sample is loaded on the WB than in the EIA and lack of visualization of bands (e.g. after round 1) is not necessarily a result of differences in analytical sensitivity of the two methods of detection. In addition, the banding patterns of the amplified PrPSc were indistinguishable from those of PrPSc in the absence of amplification (0 rounds). PrPSc was not observed after serial PMCA of four 48 h rounds when the reaction was seeded with unexposed CBH of the same genotype (data not shown).

Using the more efficient four 24 h-round regime of serial PMCA, further individual samples of scrapie, derived from sheep of different genotypes, were subjected to amplification in CBH from both a VRQ/VRQ and an ARQ/ARQ scrapie-free sheep. In each experiment, the pairing of the seed and substrate shown in Fig. 1 was added as a positive control for the PMCA. PrPSc from all 11 individual sources of scrapie brain homogenate derived from sheep bearing at least one VRQ allele was amplified (Fig. 2) in the VRQ/VRQ substrate. However, PrPSc was amplified from only one of the four ARQ/ARQ sources tested in the VRQ/VRQ substrate. The ARQ/ARQ CBH appeared to be less able to support the amplification of PrPSc from both ARQ/ARQ and VRQ/VRQ-infected sheep: the single efficiently amplified ARQ/ARQ scrapie source was the same source that amplified in the VRQ/VRQ substrate. Conversely, the VRQ/VRQ positive-control source that reliably amplified in each reaction with homologous substrate (>10 times) did not amplify in the ARQ/ARQ substrate (three times). Variability or lack of amplification was not a result of differences in absolute quantities of PrPSc in the seeded reaction, since end-point dilution of the original brain homogenates showed the ARQ/ARQ sources to contain as much or more PrPSc than the VRQ sources of scrapie (data not shown).

Efficiency of amplification was further increased with the addition of synthetic PolyA RNA. PolyA showed optimal enhancement of amplification at 100 μg ml$^{-1}$ (compared with 10 and 1 μg ml$^{-1}$, data not shown) and, following two rounds of PMCA, detection was increased by up to 5000-fold for the VRQ/VRQ seed amplified in VRQ/VRQ CBH (Fig. 3a) compared with PMCA in the absence of PolyA. Furthermore, amplification of PrPSc was observed following PolyA-assisted PMCA from two further sources of scrapie of different genotypes, all containing at least one VRQ allele (Fig. 3a and b) with equivalent levels of amplification. The bands visualized by Western blotting detected by both the Idexx HerdChek BSE/scrapie EIA

![Fig. 1. Amplification of PrPSc from the cerebellum of a VRQ/VRQ scrapie-affected sheep in a VRQ/VRQ uninfected control whole brain substrate. Brain homogenate was serially diluted in CBH as indicated (x axis) prior to amplification. PrPSc was detected by the Idexx HerdChek BSE/scrapie EIA (a and b) or by WB using the PrP antibody 9A2 (c). PMCA was performed either as two 48 h rounds, with 96 cycles in each round (a) or four 24 h rounds, with 48 cycles in each round (b) and PrPSc was assayed after each round. The total number of cycles in each experiment is the same (192). (c) PK (100 μg ml$^{-1}$)-resistant PrPSc in brain homogenate before (0) and after each round of PMCA as detected by WB. Molecular mass in kDa is indicated to the left of the blots.](http://vir.sgmjournals.org)
did not reveal broad differences in molecular mass compared with unamplified PrPSc (Fig. 3b). However, high-molecular-mass bands were observed in the amplified samples and may represent PrP aggregates or incomplete PK digestion. This was only observed when using PolyA to assist the PMCA reaction in this study but has been reported by others following PMCA without the addition of PolyA (Soto et al., 2005). No amplification of PrPSc was observed in reaction mixtures containing only the CBH (Fig. 3c) following two rounds of PMCA in the presence of PolyA. The equivalent of 10^6 leukocytes were amplified under the same conditions as the brain samples in the presence of PolyA. PrPSc was detected by EIA (Fig. 4a) in all 10 blood samples following PMCA for three 24 h rounds (total 144 cycles) from sheep with terminal scrapie, while signals from blood of the eight scrapie-free sheep were negative (100% sensitivity and specificity). WBs of the same samples indicated positive signals in seven of 10 of the scrapie cases and the amplified PrPSc was not distinguishable from PrPSc from brain before or after amplification (Fig. 4b). The WB procedure accounts for five times less of the sample as loaded onto the gel compared with the EIA and is likely to account for apparent lower sensitivity of detection.

Further rounds of PMCA with PolyA resulted in de novo synthesis of PrPSc (Fig. 5a) as detected by EIA from four of eight blood samples from unexposed sheep. WB analysis showed that the de novo-synthesized PrP could not be distinguished from that amplified from the scrapie-affected sheep in that banding patterns were similar in all cases (Fig. 5b). In the experiment shown, de novo synthesis was apparent after four rounds, but this was variable between experiments as shown in Fig. 3. In addition, the protein was not consistently amplified from all sources of scrapie-free blood. We were unable to repeat these studies in a scrapie-free environment, but were careful to include all eight negative control blood samples and to analyse PrPSc content after each round of amplification to control for these anomalies.

**DISCUSSION**

PMCA has been reported previously to increase the sensitivity of the detection of PrPSc from brains of experimentally scrapie-infected rodents (Saborio et al., 2001; Bieschke et al., 2004; Deleault et al., 2003), cattle and sheep naturally infected with bovine spongiform encephalopathy and scrapie, respectively (Soto et al., 2005), and more recently from humans with Creutzfeldt–Jakob disease (Jones et al., 2007) and deer with chronic wasting disease (Kurt et al., 2007). Furthermore, the application of this technology for the detection of PrPSc in blood, both at terminal stages of disease and in pre-symptomatic animals shown in this figure but see Fig. 5, where de novo synthesis is discussed further).
and in urine and cerebrospinal fluid (Murayama et al., 2007; Atarashi et al., 2007, 2008), is likely to lead to a new generation of diagnostic tools that enable pre-mortem testing for TSEs. We have demonstrated in this study, using PMCA, the efficient amplification of PrPSc from brain and blood from sheep naturally infected with scrapie. Previously Soto et al. (2005) indicated that PMCA could be applied successfully to sheep with natural scrapie with low levels of amplification (8×) after 10 cycles. Here, we show increases of sensitivity up to 650 000-fold after 192 cycles of PMCA, which has facilitated the observation of PrPSc present at very low concentrations. Similar degrees of amplification have been reported by Castilla et al. (2005a) for amplification of hamster prions after equivalent numbers of cycles.

Efficiency of amplification was dependent on the pairing of the CBH substrate with the source of scrapie. The factors that control these events are unclear but previous studies have shown that both the primary sequence of the PrP gene and strain effects may play a role (Bossers et al., 1997; Raymond et al., 1997; Kupfer et al., 2007). The second allele paired with VRQ did not greatly influence the level of amplification obtained in the VRQ substrate although the numbers tested were small. However, the lack of amplification of 75 % of the ARQ/ARQ sources in the VRQ substrate suggests that conversion is dependent in part on the matching at codon 136. The observation that the VRQ/VRQ scrapie source was not converted in the ARQ/ARQ substrate is consistent with this finding. However, matching of the codon at 136 is clearly not always sufficient or necessary, given that one ARQ/ARQ source of scrapie efficiently amplified in both the VRQ and ARQ sources of substrate, and further studies are required to fully understand the restraints of in vitro conversion. Moreover, for a diagnostic test to be broadly applicable to all scrapie sources, it will be essential for these limitations to be eliminated.

This is the first published report, to our knowledge, of immunochemically detectable PrPSc amplified from blood cells from sheep naturally infected with scrapie using PMCA. Detection of PrPSc in blood is consistent with the demonstration of infectivity by blood transfusion from infected sheep (Houston et al., 2000; Hunter et al., 2002) in which disease was transmitted in both the plasma and buffy coat fractions from symptomatic and pre-symptomatic animals. In blood we were able to detect PrPSc amplified after 144 cycles from 10⁶ cells (~0.5 ml of blood) in 100 % of sheep tested. This is many times more efficient than that observed for blood from experimentally infected hamsters (Castilla et al., 2005b) in which 89 % sensitivity was obtained following >800 PMCA cycles. This may be
related to the differences in pathogenesis of the disease in the two species, but is also likely to be due to the increased efficiency afforded by the addition of PolyA, a phenomenon reported by others (Deleault et al., 2005, 2007). However, the observation of apparent spontaneous conversion of PrPc to a PK-resistant form in the absence of PrPSc template is a limitation of the extensive use of PolyA. 

De novo synthesis of PK-resistant forms of PrP from partially purified PrPc in the presence of PolyA has been observed by others (Deleault et al., 2007) and also from recombinant PrPc in the presence of 0.1% SDS (Atarashi et al., 2007). Although environmental contamination in either case cannot be entirely ruled out, previous studies showed that, even in prion-free environments and under strictly controlled conditions, spontaneous PrPSc molecules were formed (Deleault et al., 2007). Methods for eliminating the simultaneous de novo synthesis of PrPSc molecules that could interfere with confident interpretation of assay results are being explored.

We have not yet attempted to amplify PrPSc from plasma samples or from blood samples taken before the onset of disease but these studies are planned or under way. Studies from rodent models of scrapie indicate that PrPSc is present in the blood of pre-symptomatic animals (Saa et al., 2006; Murayama et al., 2007). Moreover, that infectivity is present in blood of naturally infected sheep at least halfway through the course of the disease (Houston et al., 2000) implies that PrPSc should be detectable early in infection. Coupling this amplification technique to conventional

Fig. 4. Amplification of PrPSc from blood leukocytes from scrapie-affected VRQ/VRQ sheep at the terminal stage of the disease, in the presence of 100 μg PolyA ml⁻¹ following three rounds of PMCA (24 h rounds). PrPSc was detected by EIA (a) and WB (b) as described in Fig. 1. Blood leukocytes (10⁷ cells) from eight unexposed sheep (C1–C8) and 10 scrapie-affected sheep at clinical end point (T1–T10) were frozen and thawed once and subjected to PMCA for three rounds (144 cycles) in CBH (VRQ/VRQ). M, Molecular mass markers (Sigma) in kDa. BU and BA, brain homogenates before (U) and after amplification (A) for three rounds (144 cycles) from controls (−) and scrapie-positive sheep (+).

Fig. 5. Spontaneous conversion of PrPc from blood leukocytes from scrapie-free VRQ/VRQ sheep. Following four rounds of amplification (192 cycles), control blood samples from unexposed sheep (C1–C8) and from scrapie-affected animals (T3 and T10) were assayed for the presence of PrPSc by EIA (a) and WB (b) as described in Fig. 4. M, Molecular mass markers (Sigma). BU and BA, brain homogenates before (U) and after amplification (A) for three rounds (144 cycles) from controls (−) and scrapie-positive sheep (+).
rapid TSE tests, as shown here, therefore offers a realistic prospect of a long-sought-after ante-mortem method of screening TSE-susceptible sheep for evidence of infection.

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