Discrimination between prion-infected and normal blood samples by protein-misfolding-cyclic-amplification.

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Running Head : Detection of prion infection in blood.

Abstract

Background

Diagnosis of prion disease from blood samples requires the detection of minute quantities of misfolded protein (PrP^{Sc}) against a high background of correctly folded material (PrP^{C}). Protein Misfolding Cyclic Amplification (PMCA) is a technique which can amplify small amounts of seed PrP^{Sc} to a level detectable by conventional methods. Application of PMCA to the testing of whole blood samples enhances the ability to detect PrP^{Sc} and allows ante-mortem detection of prion infection and could facilitate blood screening.

Study Design and Methods

The PMCA methodology was used to detect prion infection in blood samples obtained from mice experimentally infected with prion disease. Mice were culled at various time points throughout the incubation period for disease and subjected to serial PMCA (sPMCA). Amplified samples were then analysed by western blotting to confirm the presence or absence of infection.

Results

After sPMCA, blood samples from RML-infected mice showed amplification of PrP^{Sc} to levels readily detectable by western blotting. Control samples obtained from mice mock inoculated with sterile PBS did not yield any amplification products.

Conclusion

Serial PMCA performed on small volumes of whole blood gave amplification of PK resistant material to a level detectable by standard methods. Discrimination between infected and control samples was achieved without the need for processing or fractionation of whole blood. The use of whole blood as an analyte circumvents the need to identify the optimal blood compartment for analysis and guarantees the totality of misfolded PrP will be available for detection.

Keywords: Variant CJD, Prion, PMCA, PrP

Introduction

Prion diseases are fatal neurodegenerative disorders that included bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and Creutzfeldt-Jakob disease (CJD) in humans[1,2]. The crucial step in the transmission and propagation of prion diseases, according to the protein-only hypothesis[3] is the conversion of the host's normal, cellular form of the prion protein (PrP^{C}), via a post-translational process to a protease-resistant, aggregated form (PrP^{Sc})[4]. Detection of disease-associated PrP^{Sc} is the most widely used and specific criterion for the diagnosis of prion disease in humans and animals[1,2].

Recent studies have shown that significant prion infectivity is present in non-neuronal or lymphatic tissues, such as blood and urine [5,6] which suggests the potential risk of prion infection via a range of different tissues. In fact, current evidence suggests that blood transfusions may provide an effective route of transmission for prion infection in humans as well as animals[7-10]. Taken together with the extended length of the pre-clinical period of vCJD, transmission of vCJD via transfusion or other iatrogenic means has become an issue of public concern. Therefore a diagnostic test which can be applied to the rapid screening of transfusion samples as well as blood products is highly desirable.

However, the diagnostic detection of PrP^{Sc} in blood is not a trivial matter. Very little is known about the nature and distribution of infectious material in blood, and what is know is largely derived from rodent models not human disease states. Infectivity has been detected in buffy coat fractions as well as plasma from rodents,[11,12] with as little as 10 infectious units present in 1ml of whole blood. As well as the extremely low

concentration of disease associated PrP^{Sc} there is a very high background of PrP^{C} present in white blood cells, red blood cells and platelets. A combined methodology of enrichment and detection of PrP^{Sc} by high sensitivity ELISA have shown potential as a blood based test for infection (Tattum *et al.* Submitted).

Further significant increases in the sensitivity of detecting prion infection in blood can be achieved by exploiting the ability of prions to replicate *in vitro* to achieve both amplification of abnormal PrP and a decrease in the ratio of background PrP^{C} to PrP^{Sc}. Several methods have been developed, including those that use recombinant prion proteins as a substrate [13,14]. However, the highest fidelity and the replication of authentic prions are limited to Protein Misfolding Cyclic Amplification (PMCA) *in vitro*. PMCA is a technique developed by Soto and colleagues which allows the amplification of minute quantities of PrP^{Sc} to levels which can be detected by conventional methods[15]. PMCA is a cyclical process analogous to PCR where there are periods of seeded conversion of PrP^{C} from the substrate homogenate by small amounts of PrP^{Sc} interspersed with bursts of sonication which disrupt PrP aggregates allowing continued recruitment and conversion of PrP^{C}. The only limiting factor to the level of amplification is the exhaustion of the substrate PrP^{C} or other factors necessary for amplification. To overcome this serial PMCA (sPMCA), in which samples after amplification are diluted into fresh substrate and subjected to further rounds of PMCA, has been developed[16]. PMCA has been used to detect PrP^{Sc} in Buffy coat fractions from rodent blood[17,18]. More recently PrP^{Sc} was amplified from blood leukocyte preparations taken from scrapie infected sheep[19]. However, the use of buffy coat in the kind of rapid, large-scale blood

test required is likely to be unfeasible due the time-consuming nature of the separation of blood components. Also, further processing of buffy coat fractions is required before it can be used as a seed in PMCA reactions. Despite buffy coat being shown to contain the highest concentration of prions[11,12,20-22] the total infectivity partitioned into this fraction is only about one third of the total in whole blood.

With this in mind we set out to adapt PMCA to use whole blood as the seed for our amplification, as this is the most practical material to use for blood screening and will contain the infectivity associated with all fractions. Substrate homogenates for amplification were generated from the brains of transgenic Tg20 mice which overexpress PrP^{C} [23]. Here we report the first amplification of proteinase-K resistant PrP (PrP^{RES}) from PMCA reactions seeded with prion infected whole blood. Using a small volume of whole blood from RML infected CD1 mice, PrP^{RES} was amplified to a level detectable by Western blotting after 4 rounds of serial PMCA. PrP^{RES} was not observed in control normal whole blood after four sPMCA cycles, suggesting that this represents the amplification of genuine PrP^{Sc} present in the animals blood rather than the *de novo* spontaneous generation of PrP^{RES} that has been reported elsewhere[19,24]. The sensitivity of the technique allowed the diagnosis of prion infection in late-stage pre-clinical animals and represents an important step forward in the simplification of blood-based assays utilising prion propagation *in vitro*.

Methods

Tissues samples

All procedures were carried out in a microbiological containment level III facility with strict adherence to safety protocols. The Rocky Mountain Laboratory (RML) strain of prions[25], a mouse adapted prion strain isolated by serial passage from an original inoculum of sheep scrapie, was used to establish experiment rodent infections. Normal brains from the outbred CD1 strain of wild-type mice and transgenic Tg20[23] mice, as well as brains from CD1 mice which were experimentally infected with the RML scrapie prion strain were homogenized in Dulbecco's PBS (DPBS) lacking Ca^{2+} or Mg^{2+} ions by serial passage through needles of decreasing diameter or the use of tissue grinders (Anachem) to give a final concentration of 10% (w/v)[26,27]. Tissue homogenates were stored as aliquots in eppenforf tubes at -80°C. Preparation of PMCA substrate homogenates is detailed below. Whole normal blood was collected from uninfected CD1 mice.

Time course of RML infection in CD1 mice

RML time course of infection was performed by inoculating CD1 mice inter-cortically (i.c.) with 30µl 1% (w/v) RML infected brain homogenate made up in PBS. Groups of 20 mice were culled at 0, 20, 40, 60, 80, 100, 120, 140 days after infection and animals succumbed to disease 148 days post- infection. Brains and bloods were collected at each time point for analysis. The presence of PrP^{Sc} in brain tissue was determined by western blotting and infectivity quantified by automated Scrapie Cell Assay[28] (SCA), which is a highly sensitive and quantitative assay for the presence of RML prions.

Western Blotting

Samples were analyzed by electrophoresis and immunoblotting following Proteinase K digestion (200 μg/ml final protease concentration) as described previously [27,29]. Blots were blocked in PBS containing 0.05 % (v/v) Tween-20 (PBST) and 5 % (w/v) non-fat milk powder and probed with a biotinylated anti-PrP monoclonal antibody ICSM 18 (D-Gen Ltd, London, UK) at 200 ng/ml final antibody concentration in PBST. Blots were developed using a Streptavidin-Alkyline Phosphatase conjugate (Dako, Denmark) diluted 1:10,000 in PBST and chemiluminescent substrate CDP-Star (Tropix Inc, Bedford, MA, USA). Blots were visualized on Biomax MR film (Kodak; Anachem Ltd, UK) as described [27] and signal intensity of bands was quantified using Imagequant software (Molecular Dynamics).

Protein Misfolding Cyclic Amplification

Protein Misfolding Cyclic Amplification (PMCA) was performed as described previously [16]. Briefly, 10% (w/v) PMCA substrate homogenates were prepared from Tg20 mice brains which had been perfused with PBS containing 5 mM EDTA at the time of death. Brains were homogenised in cold conversion buffer (PBS containing 150mM NaCl, 1.0% Triton X-100, 4mM EDTA and Complete Protease Inhibitor Mixture (Roche Applied Biosciences)). Substrate homogenates were clarified by centrifugation at 1000 g for 45 seconds and then stored at -70°C without freeze-thawing until required. RML infected homogenate was diluted into 100 μl of substrate homogenate in 0.2 ml PCR tubes and 40 μl retained as minus PMCA controls. Samples were subjected to 140 cycles of PMCA

consisting of a 20 second pulse of sonication at 75% power output using a Misonix S3000 sonicator with a microplate horn (Misonix, Farmingdale, USA) followed by incubation for 30 minutes at 35^{o}C. In serial PMCA experiments, samples were subjected to successive rounds of 140 PMCA cycles followed by 1:10 dilution into fresh substrate homogenate.

For PMCA of RML time course and normal blood samples, 1µl of pooled whole blood from 140 day post-infection and uninfected animals was diluted 1:100 into PMCA substrate homogenate. After mixing, 40 µl was removed and stored at -70°C as a PMCA minus control. Samples were subjected to serial PMCA as described above. All samples were analysed by PK digestion (200 µg/ml final protease concentration for 60 minutes at 37°C) and Western blotting.

Results

Serial PMCA of RML infected brain homogenates

To determine the extent of amplification within our PMCA experiments we performed serial PMCA on RML infected CD1 brain homogenates diluted into transgenic Tg20[23] substrate homogenate. RML homogenate was diluted 400 fold and subjected to 140 cycles of PMCA. Further dilutions (10, 100 and 200 fold) were performed into fresh substrate and two subsequent rounds of sPMCA were undertaken (Figure 1). In all cases PrP^{Sc} was amplified efficiently, with a 20 to 40 fold increase in PK resistant material at each sPMCA step when compared to the PK resistant PrP in samples which were not subjected to PMCA. Overall the total amplification achieved was > 16000 fold at the highest dilution, confirming our PMCA system using transgenic murine brain homogenate as the substrate is an efficient means of amplifying mouse PrP^{RES}.

Amplification of PrP^{Sc} spiked into normal whole blood

To establish the efficacy of our Tg20 system to amplify PrP^{RES} out of whole blood, we prepared samples containing RML homogenate diluted into either normal CD1 homogenate or whole blood. These were then used to seed Tg20 PMCA substrate homogenates (1 µl into 100 µl) giving a final dilution of seed RML of 400 and 800 fold (Figure 2). Amplification of PrP^{RES} spiked into whole blood was consistently obtained in all samples over a single PMCA experiment. However, there was a reduction of amplification capability of approximately 50% due to the inhibitory nature of whole blood, which could not be overcome by further optimisation.

Discrimination between whole bloods from normal and pre-clinical RML infected animals

A time course of RML infection after intracerebral inoculation previously performed was monitored by western blot detection for PrP^{Sc} accumulation in the brain and associated infectivity by cell-culture assay (SCA)[28,30]. Brains from 20 animals at each time point were homogenised and analysed independently with PK resistant PrP^{Sc} first detected in brain tissue at 60 days post-infection (pi) (9 out of 20) and gave uniformly positive results 100-120 days pi (Figure 3). However, infectivity measured by SCA was not observed in brain tissue until 100 days pi, reaching a maximum at 140 days pi with a mean titre of 2.6×10^8 $TCLD_{50}$ units while animals were still symptom free. Clinical onset was at 148 days and all animals were sacrificed at this time point.

Blood samples were collected and pooled from animals at all time points. Whole blood from pre-symptomatic animals 140 days post-infection was used to seed serial PMCA reactions. Normal uninfected blood was used as a negative control and RML brain homogenate spiked into whole blood used as a positive control of amplification. Great care was taken when handling the controls to eliminate the likelihood of contamination to the blood spiked samples. For that reason, control samples were handled using clean gloves and pipettes separately from the experimental samples. By sPMCA round 3, PrP^{RES} could be detected in 1 out 3 reactions spiked with blood obtained from infected animals at 140 days post infection. After a further cycle of sPMCA, PrP^{RES} was observed in all three infected blood reactions (Figure 4), whereas no detectible PrP^{RES} resulted

from sPMCA of the normal blood controls at either round 3 or 4, indicating that spontaneous generation of PrP^{RES} is unlikely to have occurred during the period of the experiment[19,24].

Reliable amplification of PrP^{RES} could only be achieved from whole blood obtained 140 days post infection and later. This finding appears to correlate with the sharp rise in infectious titre in the brains of affected animals (Figure 3) which occurs between 100 and 140 days post infection before plateauing prior to the onset of a clinical syndrome.

Discussion

Here we detail the first amplification of PrP^{RES} from whole blood and the clinical discrimination between normal and prion infected pre-symptomatic animal bloods. Although PrP^{RES} has been successfully amplified from blood leukocyte preparations in rodents and sheep[17-19] by PMCA, buffy coat is unlikely to be a plausible analyte for a blood screening assay that can be utilised on a large scale due to the prohibitive time and complexity for preparation. Whole blood represents a more practical material for a blood test as well as being guaranteed to contain the entirety of PrP and prion infectivity in the sample. These results suggest that with carefully optimisation for human PMCA it should be possible to distinguish between normal blood and that from vCJD patients.

Despite these promising results, PMCA still remains a technically challenging procedure. As well as obvious problems such the time consuming nature of serial PMCA and the possibility of *de novo* generation of PrP^{RES} [19,24], choosing the ideal substrate for testing is crucial. In our experiments there is a significant inhibition of amplification (~50%) in the presence of whole blood (Figure 2). Careful study of the effect on amplification efficiency by individual blood components may reveal and allow the exclusion of the inhibitory species. Removal of any such species would greatly increase the sensitivity of PMCA as a method of detecting PrP^{Sc} in infected blood samples.

Another issue of concern limiting the use of PMCA in its current state is the adaptation of the technique to different species. Finding a suitable substrate for amplification of human PrP^{Sc} remains a problem. It is unlikely that there will be a readily available supply of

normal human brains in the quantities necessary for large scale screening. Therefore alternative amplification substrates must be investigated. The use of human platelets has recently been shown to be effective in the amplification of human PrP^{Sc} [31], although there is concern over their widespread usefulness due to codon 129 compatibility problems between substrate and seed. Transgenic animals that express the human prion protein have also been suggested as a conceivable source of PMCA substrate. The simplest and most easily attainable substrate for amplification is recombinant prion protein, which can be cheaply produced in large quantities. Recent reports show the promise of using recombinant protein as a substrate for the diagnostic distinction between normal and prion infected cerebrospinal fluid[13,32]. However, unlike PMCA these reactions do not result in the replication of prion infectivity, questioning their fidelity and they may be significantly more susceptible to false positive results from other amyloidoses.

The detection of PrP^{Sc} in blood is considerably more challenging than other tissues with as little as 10 infectious units present in 1ml of whole blood. In order to achieve the high levels of sensitivity required a blood-based CJD diagnostic assay will need to exploit the unique ability of prions to replicate and incorporate an *in vitro* amplification step in the methodology. However, amplification alone does not provide a solution to blood based diagnosis due in part to the low abundance of PrP^{Sc} requiring large volumes of blood to be sampled to obtain certainty that at low titres during pre-clinical stages of infection a prion particle is certain to be present. In addition the experimental complexities and time scales of amplifying from low titre analytes would preclude the use of amplification alone as a screening assay. It is likely that a combination of enrichment, amplification

and high sensitivity detection will be required to reduce the time scales involved and robustly provide the very high levels of sensitivity and specificity required of a blood screening assay.

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Figure Legends

Figure 1. Serial PMCA of PrP^{RES} using transgenic Tg 20 substrate homogenate

As a proof of concept, 10% (w/v) RML infected brain homogenate was diluted 1:400 into PMCA substrate containing brain homogenate from uninfected CD1 mice and subjected to 140 rounds of amplification (sPMCA #1). Samples subjected to PMCA contained more than 40 times thte level of PrP^{RES} in unamplified controls. Amplified material was then diluted into fresh substrate and PMCA was repeated for another 140 cycles (sPMCA #2 and #3). Each serial cycle of dilution and amplification yielded levels of PrP^{RES} in the region of 20-40 fold higher than control dilutions which were not subjected to PMCA, giving a total amplification of ~16,000x (40x20x20) from the original RML at the highest dilutions.

Figure 2. Amplification of PrP^{RES} spiked into whole blood

RML infected brain homogenate was diluted (1:4 and 1:8) into either normal CD1 brain homogenate or uninfected whole blood. 1 µl of each dilution was added to 100 µl Tg 20 substrate homogenate and subjected to 140 cycle of PMCA. PrP^{RES} was amplified to detectable levels from whole blood as well as normal brain. The efficiency of amplification was reduced by ~50% due to the inhibitory nature of whole blood, an inhibition that could not be overcome by further optimisation of reaction conditions.

Figure 3. RML infection time course in CD1 mice

A large cohort of CD-1 mice were inoculated i.c. with 30µl of a 1% (w/v) RML-infected brain homogenate. Groups of 20 mice were culled at 0, 20, 40, 60, 80, 100, 120, 140 days post-infection and succumbed to clinical disease at 148 days pi. Brain were homogenised and analysed independently for the presence of PrP^{Sc} by western blotting and infectivity quantified by Scrapie Cell Assay. Western blotting of brain homogenates gave uniformly positive results from 120 days post infection and mean levels of infectivity reached a maximum at around 140 days. Blood from 20 mice at each time point was pooled to provide a single sample per time point.

Figure 4. Differentiation between normal whole blood and blood from pre-symptomatic RML infected mice

Whole blood from infected RML CD-1 mice collected 140 days post infection and normal, uninfected whole blood from CD1 mice were diluted 1:100 into PMCA substrate homogenate. Whole blood spiked with RML (1:10) was also diluted 1:100 into PMCA substrate positive control for amplification. Serial PMCA was performed on all samples. After 4 consecutive rounds of sPMCA, amplification of PrP^{RES} could be detected in all of the RML infected blood samples. No PrP^{RES} was detectable in the normal uninfected blood samples.

Figures

Figure 1

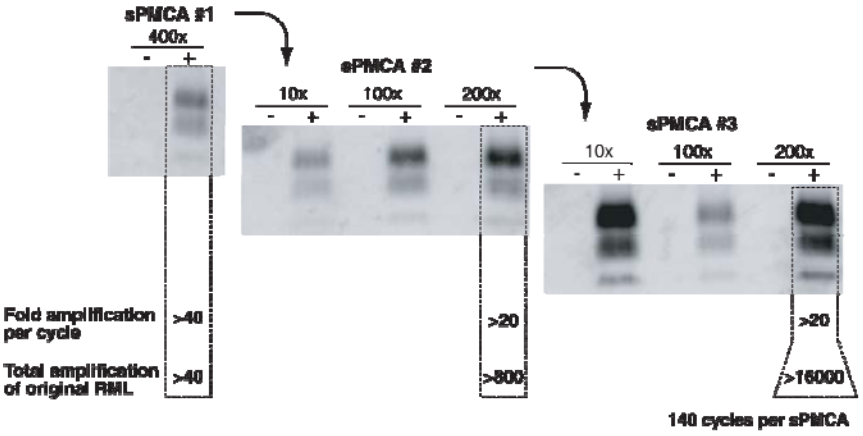

Figure 2

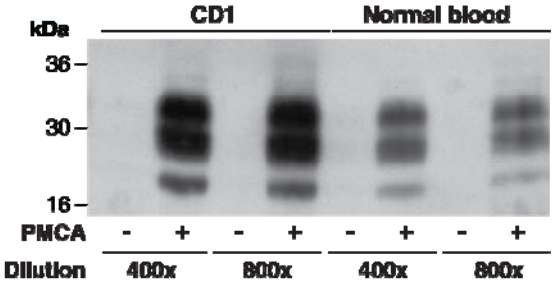
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Figure 3

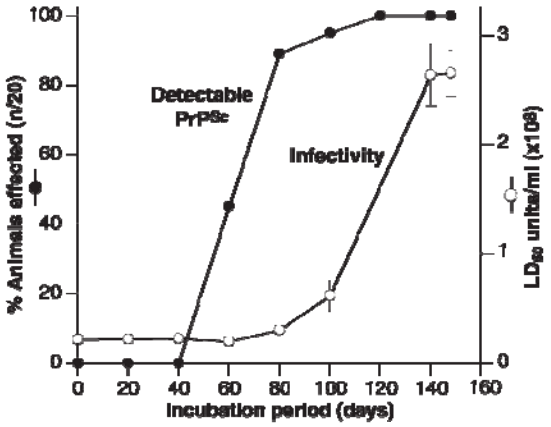
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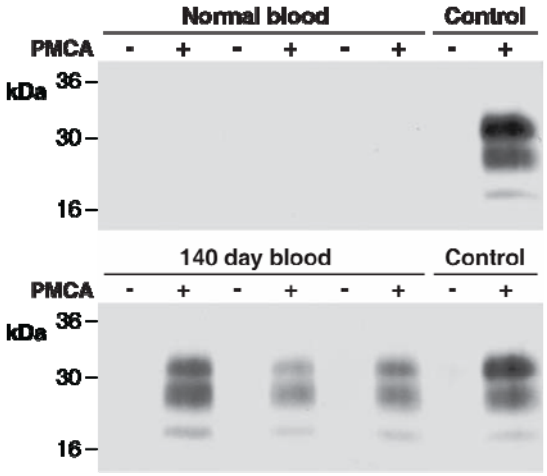
Figure 4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