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ORIGINAL PAPER

Pall leukotrap affinity prion-reduction filter removes exogenous infectious prions and endogenous infectivity from red cell concentrates

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Vox Sanguinis

Background and Objectives Three recent probable cases of transmission of a variant of human Creutzfeldt–Jakob disease (vCJD) through blood transfusion suggest that the disease can be transmitted through transfusion of blood components from pre-symptomatic blood donors. In this study, we investigated the performance of a new filter for reducing the levels of infectious prions (PrP^{Sc}) from red cell concentrates (RCC).

Materials and Methods *Endogenous Infectivity:* A pool of 500 ml of whole blood was collected from 263K-strain scrapie-infected hamsters into an anticoagulant, processed into non-leucoreduced RCC (NL-RCC), and then passed through a prion-reduction filter. Pre- and postfiltration samples were tested for PrP^{Sc} by Western blot and infectivity by inoculation of healthy hamsters. Results of the endogenous infectivity study after 200 days post-inoculation are discussed. *Exogenous (Spiking) Study:* Scrapie-infected hamster brain homogenates containing PrP^{Sc} were added to human RCC and then filtered. Levels of PrP^{Sc} were determined by Western blot assay. The effect of prior leucodepletion of 'spiked' RCC on PrP^{Sc} removal by the prion-removal filter was also assessed.

Results In the endogenous infectivity study, at 200-day observation time, the prefiltered RCC transmitted disease to six of the 187 hamsters, whereas the filtered RCC did not transmit disease to any of 413 animals, $P = 0{\cdot}001$. The prion filter also significantly reduced the concentration of leucocytes in the RCC by about 4 logs, $P < 0{\cdot}05$. In the exogenous (spiking) study, the level of PrP^{res} was significantly reduced in RCC $P < 0{\cdot}05$. Prior leucodepletion of the RCC with a leucoreduction filter did not significantly reduce the concentration of exogenously spiked PrP^{Sc}, $P > 0{\cdot}05$.

Conclusion The use of this new prion-reduction filter should reduce the risk of vCJD transmission through transfusion of RCC, the most widely transfused blood component.

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Conflict of Interest Statement

SOS-C, SP, FA and AK are employees of Pall Corporation. The other remaining authors (RBK, RJK, CM and RC) are scientific collaborators on the project and are not employees of Pall Corporation and do not have any financial interest in the product.

Introduction

Prion diseases or transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative diseases that affect both humans and animals. Creutzfeldt–Jakob disease (CJD) is the most common form of human TSE, and although usually sporadic, it has been transmitted from person to person through medical instruments and through transplant of tissues or organs [1,2], but so far as is known, not through the administration of blood or blood products [3–5].

A variant form of CJD (vCJD) appeared in the UK in the mid-1990s as a result of the consumption of tissue or meat products from cattle infected with bovine spongiform encephalopathy (BSE) [6]. As of 6 December 2005, there have been 169 confirmed cases worldwide with the vast majority (159) in the UK [7,8]. Recent animal data [9–13] together with three reported cases of probable transmission of vCJD in humans from transfused blood components [14–16] have raised concerns about the transmission of the causative agent by this means.

Several reports based on experimental models of TSE indicate that the highest concentration of infectivity is associated with leucocytes, and that a lower level of non-cell-associated infectivity circulates in plasma [11,12]. A low level of infectivity in the red cell fraction was thought to be due to contamination by leucocytes and by residual plasma (P. Brown, personal communication). Based on these early studies in rodents, the expected infectivity in blood may be in the range of 2–20 ID_{50}/ml (infectious dose unit ID_{50}/ml of product is defined as the dose that would cause infection of 50% of the exposed population) during the preclinical incubation period, and about 10-fold higher during the clinical phase of disease. Because there are no diagnostic tests with which to identify preclinical infection, precautionary measures have been introduced in many countries to reduce the risk of disease transmission through blood or blood products, including donor deferral and the implementation of universal leucoreduction strategy in the UK [17,18]. There is evidence to indicate that some infectivity present in blood may not be removed with the current standard leucocyte reduction filters [19].

In this study, we describe the performance characteristics of a new leucocyte-reduction filter (leukotrap affinity prion-reduction filter, LAPRF) with prion-reduction capability, in experiments using both exogenously and endogenously infected blood. Because of the limitation of the current leucocyte-reduction filters, this new filter was specifically designed for concurrent reduction in both leucocytes and infectious prions in red cell concentrates (RCC). The polyester fibers that are currently being used in all our leucoreduction filters were modified with specific surface chemistry for prion removal from RCC.

Materials and methods

Exogenous (spiking) study using crude preparation of scrapie-infected hamster brain homogenate (SIHBH)

The objective of this study was to validate the performance of the LAPRF in terms of prion removal under a variety of processing conditions used in the standard processing and storage of red cell units for transfusions. This included lot-to-lot variability of the filter, use of various anticoagulants/additive solutions systems, head heights for filtration and processing temperature. The log removal was evaluated by spiking brain homogenate from infected hamsters into red cell units, which subsequently were filtered. Pre- and postfiltration levels of pathogenic prions were quantified by Western blot analysis.

Preparation of spiking material

Weanling Syrian hamsters (LVG Golden Syrian hamsters, Charles River, MA) were inoculated intracranially with 1% (w/v) high-titre brain homogenate from hamsters infected with scrapie strain 263K. The animals were sacrificed after 70 days at an advanced stage of disease, and the brains were removed to prepare 10% suspensions in phosphate-buffered saline (PBS), pH 7·4, homogenized with Ulter-Turrax T25 homogenizer (IKA Works Inc., Wilmington, NC). The homogenates were then centrifuged at 3000 ***g*** for 3 min at room temperature in a Beckman GPR tabletop centrifuge (Beckman Coulter, Fullerton, CA). The supernatants from this crude brain homogenate preparation that contained both cell-associated and non-cell-associated PrP^{Sc} were removed and used in subsequent studies.

Spiking of SIHBH into human RCC

Part 1: effects of different lots of filter

Units of whole blood (450 ± 45 ml each) were either purchased from an American Association of Blood Banks (AABB)-accredited blood bank or obtained in-house from healthy volunteers (Pall Medical, Blood Donor Program). Each unit was collected into a blood bag containing 63 ml CPD anticoagulant. The units were leucocyte reduced with Pall WBF2 leucocyte-reduction filter according to the manufacturer's instructions for use (Pall Medical, East Hills, NY). Each unit of leucocyte-reduced whole blood was processed into RCC within 8 h of blood collection. Briefly, each unit of leucocyte-reduced whole blood was centrifuged at 5000 ***g*** for 5 min (in Sorvall RC3C centrifuge, Kendro Laboratory Products, Asheville, NC). The supernatant plasma was removed and discarded. The remaining RCC was resuspended in 100 ml SAG-M additive solution. The weight of the RCC suspension was measured with an analytical balance (Sartorius, AG, Germany) and the volume was calculated using a density of 1·07 g/ml for RCC in SAG-M additive solution. Each unit was stored overnight at 4 °C in a blood bank refrigerator. On the day of testing, the RCC was removed from the refrigerator and the volumes of units were again measured as described previously from the weights and calculated density of the RCC. RCC with volumes greater than 270 ml were adjusted to 270 ml by removing the excess RCC. Ten millilitres of 10% (w/v) SIHBH was added to 270 ml RCC so that the final concentration of the SIHBH was 0·36% (v/v) (that is 1 : 28 dilution of the stock suspension of SIHBH). For units with

volumes that were less than 270 ml, appropriate amounts of 10% brain homogenates were added to maintain a final concentration of 0·36% (v/v) SIHBH. Each unit was mixed end-over-end for about 2 min (15–20 rotations) to ensure adequate mixing of the RCC with the brain homogenate. To analyse the level of PrP^{res} in the RCC prior to filtration, 20 ml of RCC was removed and transferred into 50 ml plastic sample tube for analysis using Western blot assay. The blood bag containing the remaining RCC was attached to the Pall LAPRF and then filtered at room temperature (22 ± 2 °C) at a filtration height 76 cm. The concentration of PrP^{res} in the samples before and after filtration was measured with a Western blot assay as described below.

Part 2: effects of different red cell storage solutions

In Part 2 of the validation studies, units of whole blood (450 ± 45 ml) were collected into either CPD or CPDA-1 anticoagulant/storage solution (63 ml per unit) as described in the Part 1. The units were leucocyte reduced according to the standard protocol, and then centrifuged at 5000 ***g*** for 5 min. The supernatant plasma was removed from the RCC and the haematocrit adjusted to between 55% and 60% with plasma. The haematocrits were measured with Cell Dyn 3200 haematology analyser (Abbott Diagnostics, Abbott Park, IL). The weights of the RCC were recorded and the volumes calculated using a density of 1·08 g/ml for RCC in either CPD or CPDA-1. The RCC were stored overnight at 4 °C prior to filtration. On the day of testing, each unit was removed from the refrigerator and the volume adjusted to 270 ml and SIHBH added as described in Part 1. Each unit was filtered at room temperature at a filtration height of 76 cm. The levels of PrP^{res} in the pre- and postfiltration samples were measured using the Western blot assay.

Part 3: effects of filtration height

The protocol, anticoagulant and additive solution are same as in Part 1. Briefly, units of whole blood (450 ± 45 ml) were collected into 63 ml CPD anticoagulant. The units were processed into RCC and resuspended in SAG-M as described in Part 1 for RCC in SAGM. The RCC units were stored at 4 °C for 1–2 days in a blood bank refrigerator prior to testing. On the day of testing, each unit was removed from the refrigerator, the volume adjusted to 270 ml and SIHBH added as described in Part 1. The RCC was attached to the Pall LAPRF and then filtered at room temperature at different filtration heights (38, 76 and 152 cm). The levels of PrP^{res} in the pre- and postfiltration samples were measured using Western blot assay.

Part 4: effect of filtration at 4 °C

Same as Part 2 except that filtration was conducted at 4 °C. Units of whole blood were collected into CPD anticoagulant and then processed into RCC. On the day of testing, each unit was removed from the refrigerator and the volume adjusted to 270 ml. The RCC was attached to the Pall LAPRF and then filtered at 4 °C at a filtration height of 76 cm. The levels of PrP^{res} in the pre- and postfiltration samples were measured using Western blot assay as described in the method section below.

Part 5: effects of leucocytes

Non-leucocyte-reduced RCCs in SAGM additive solution were either prepared in-house from CPD anticoagulated human whole blood or purchased directly from AABB-accredited blood banks. At the time of testing, each RCC was about 1–2 days old. Ten millilitres of 10% (w/v) brain homogenate was added to 270 ml RCC and then filtered with the Pall LAPRF (Pall Medical) at room temperature (22 ± 2 °C) at a filtration height of 76 cm.

Part 6: comparison of prion reduction between standard leucocyte-reduction filter and the Pall LAPRF

The main objective of this part of the exogenous infectivity was to determine the level of prion reduction with a standard leucocyte-reduction filter. Units of whole blood in CPD anticoagulant (450 ± 45 ml each) were either purchased from an AABB-accredited blood bank or obtained in-house from healthy volunteers (Pall Medical, Blood Donor Program) into blood bags containing CPD anticoagulant. Fifty millilitres (50 ml) of 10% (w/v) SIHBH was added to 450 ml of CPD anticoagulated whole blood. Two units of ABO compatible SIHBH-contaminated whole blood were pooled together into a 2-L blood collection bag. The blood bag was placed on a shaker for gentle mixing for 5 min to ensure adequate mixing and uniform distribution of SIHBH. Each SIHBH-contaminated pool of whole blood was divided into 500 ml aliquot in blood bags labelled as A and B (Fig. 1). Units A and B were centrifuged at 5000 ***g*** for 5 min and processed into RCC. The haematocrits of the RCC were adjusted to between 55% and 60% with the supernatant SIHBH-contaminated plasma. RCC from unit A was first filtered with a standard leucocyte-reduction filter (BPF4, Pall Medical, NY) and then with the Pall LAPRF. RCC from unit B was filtered with LAPRF without any prior leucoreduction step (Fig. 1). The concentration of PrP^{res} in the whole blood and in the RCC samples before and after filtration steps were measured with a Western blot assay as described below.

Western blot assays

A high-speed centrifugation protocol in combination with 4% sarkosyl in PBS and phosphotungstic acid (PTA, in 170 mM magnesium chloride pH 7·4) was used to extract and concentrate PrP^{Sc} [20]. In this method, 2·5 ml of RCC was first mixed with 6 ml 4% sarkosyl and then incubated at 37 °C for 10 min to lyse the red blood cells and solubilize cell membranes to release any bound PrP^{Sc}. After incubation, 700 µl of 4% (w/v) PTA solution was added and then incubated

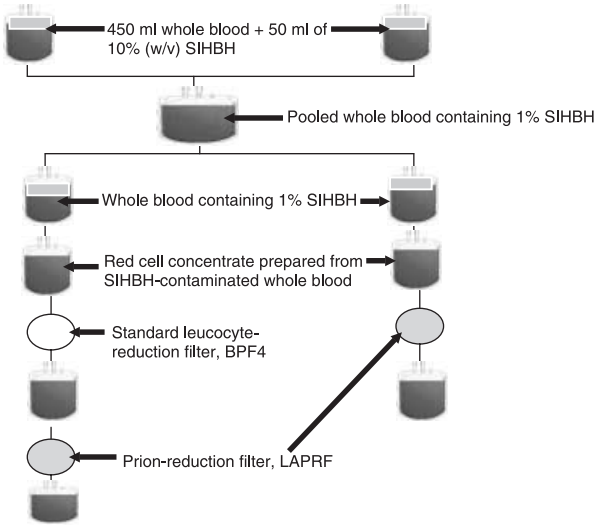

Fig. 1 Schematic of the experimental set designed to compare prion-reduction properties of standard leucocyte reduction and LAPRF. Note that this experimental design only applied to 'Part 6' of this study as described in Methods, as for other parts of the study, spiking was performed on RCC and not on whole blood.

for an additional 30 min to aggregate the PrP^{Sc} and facilitate its recovery from the RCC samples. The red cell lysates were centrifuged at 19 600 ***g*** for 60 min or 14 300 ***g*** for 90 min at room temperature. The supernatant was removed and discarded, and the PrP^{Sc} pellet was resuspended, washed in PSB containing 0·1% sarkosyl (PBS-S), and centrifuged at 19 600 ***g*** for 60 min. After washing and aspirating the supernatant, the pellets were resuspended in 100 µl of PBS-S. From each sample, 40 µl was withdrawn and treated with 10 µl of 250 µg/ml of proteinase K (PK; final concentration was 50 µg/ml) at 37 °C for 60 min to digest any contaminating non-PrP protein. Digestion was terminated by the addition of 12·5 µl of 4× SDS-PAGE sample buffer to 37·5 µl of each sample and heating to 70 °C for 10 min.

SDS gel electrophoresis and Western blots were performed. Briefly, 20 µl (15 µl of sample + 5 µl of sample buffer) of these samples was loaded on 4–12% SDSBis–Tris polyacrylamide gels in NuPage running buffer (MES, Novex-Invitrogen, San Diego, CA) at 200 constant volts for 35 min. The separated proteins were transferred to Immobilon, PVDF membrane (Millipore, Billerica, MA) in Tris–glycine transfer buffer at 125 mA or 25 V for 60 min. Following transfer, the membranes were soaked for 60 min in 5% non-fat milk dissolved in PSB containing 0·2% Tween 20 (PBST). The membranes were then incubated in a hybridization bag with gentle agitation for 2·5 h at room temperature in 1 : 5000 dilution of 3F4 monoclonal antibody (Signet Laboratories, Inc, Dedham, MA) diluted in blocking buffer, PBST, to a final concentration of 0·2 µg/ml. The membranes were washed four times at 5 min per wash with PBST. The washed membranes were incubated at room temperature for 2 h with the secondary antibody, horse radish peroxidase-conjugated goat antimouse IgG (Fab-specific, Sigma-Aldrich, St. Louis, MO) diluted 1 : 2000 in blocking solution. Following incubation with the secondary antibody, the membranes were washed four times at 5 minute each with PBST, and briefly in de-ionized water. The proteins were detected using a chemiluminiscent substrate, SuperSignal West Dura Extended Signal (1 : 1 mixture just before use) as described in the manufacturer's manual (Pierce Biotechnology Inc., Rockford, IL). The signals were developed in an UVP-Chemi-Doc-It portable dark room (UVP, Uppland, CA) and detected using a Hamamatsu Charge Coupled Device (CCD) camera (UVP). The density of each band on the Western blot image was analysed with LABWORKS™ version 4·8 (UVP) densitometry program for analysing 1D gels.

Calibration of Western blot signal with densitometry measurement

In order to determine the concentration of PrP^{Sc} in the samples, we prepared serial dilution on a half log (0·5 log) by mixing 316 µl of 10% (w/v) SIHBH with 684 µl of bovine serum albumin prepared in PBS. From each sample, 80 µl was withdrawn and incubated with 50 µl/ml (final concentration) of PK for 60 min at 37 °C. To terminate the PK reaction, equal volume of 4× SDS sample buffer was added and each sample was heated to 70 °C for 10 min. Twenty microlitres of each sample was loaded onto the 4–12% SDS gel for analysis as described in the preceding section. The density of each band on the Western blot was analysed. We measured the density of each dilution and the linearity between the densitometry measurement and concentration of PrP^{res} was determined using a linear regression analysis of the data generated. In parallel, 10 ml of the undiluted 10%SIHBH was sent to an outside laboratory, BioReliance (BioReliance-Invitrogen, Rockville, MD) a US Food and Drug Administration (FDA)-licensed contract laboratory for prion validation studies for determination of the concentration of PrP^{res} stated as ID PrP^{res}/ml in the stock solution of 10% SIHBH [21].

Negative control for Western blot

In order to determine non-specific staining of the PVDF membranes, RCCs in SAGM were processed exactly in the same manner as the experimental samples, the only difference was that the experimental samples were contaminated with SIHBH while the negative control contained equivalent amount of additive solution.

Calculation of log-reduction factor

The removal of infectious prions from RCC is calculated based on the densitometry readings calibrated using

end-point titration of the 10% (w/v) SIHBH. The formula for calculating the log-reduction factor is shown below:

$$\text{Prion reduction (PR)} = \log_{10} \frac{(\text{input prion titre/volume} \times \text{input volume})}{(\text{output prion titre/volume} \times \text{output volume}).}$$

Endogenous infectivity study: red cell concentrate from scrapie-infected hamsters

Each of 300 weanling normal Syrian hamsters was inoculated intracranially using 40 µl of a 10% (w/v) brain homogenate in PBS with a titre of 9·2 $\log_{10}ID_{50}$/ml. At the onset of the clinical symptoms (wobbling gait and head bobbing), about 65–80 days after inoculation, blood samples were collected from the animals into either CPD or CPDA-1 anticoagulant. A total of 450 ± 45 ml whole blood was collected from the 100 hamsters (about 4–5 ml per hamster) into 63 ml of either CPD or CPDA-1 anticoagulant and processed into a single unit (200–300 ml) RCC by centrifugation at 5000 *g* for 25 min. The non-leucocyte-reduced RCC was then either adjusted to 55–60% haematocrit with the supernatant plasma or resuspended in 100 ml SAGM additive solution in the same manner as single units of human RCC that are prepared from anticoagulated whole blood according to the Council of Europe guidelines. The blood bag containing a full unit (280–300 ml) of scrapie-infected non-leucocyte-reduced RCC was attached to the Pall LAPRF and then filtered at room temperature at a filtration height of 76 cm. The concentrations of leucocytes in the RCC were measured before and after filtration with Cell Dyn 3200 haematology analyser (Abbott Diagnostics) and a flow cytometric method [22]. Aliquots of 40 µl of the pre- and postfiltration RCC samples were injected intracranially into both sides of the brain (total volume injected was 80 µl) of normal Syrian hamsters for each treatment condition (187 hamsters for control unfiltered RCC and 413 hamsters for filtered RCC). The animals were then housed and monitored for clinical signs of scrapie (head bobbing, wobbling gait, weight loss, etc.). Animals that developed clinical symptoms of scrapie were sacrificed, and the brains of all animals (including survivors) were tested for the presence of PrP^{res} by Western blot assay using 3F4 monoclonal antibody.

Leucocyte reduction, red cell rheologic property and membrane integrity

In addition to prion removal, we evaluated leucocyte-removal efficiency of LAPRF and the quality of RCC determined by measurement of RCC deformability. One- to 2-day-old RCC in either SAG-M or CPD additive solutions was filtered with prion- reduction filter at room temperature at a filtration height of either 50 or 76 cm. The concentration of leucocytes in the RCC was measured before and after filtration with Cell-Dyn 3200 haematology analyser and flow cytometric method [22], respectively. Osmotic gradient ektacytometry was used to assess the quality of the filtered and unfiltered RCC after 5 days storage at 4 °C [23]. Red cell membrane integrity was determined by measuring changes in the concentration of red cell cytoskeleton protein, band 3, using a flow cytometric method [24].

Statistical analysis

The difference in the log reduction of PrP^{res} between the different conditions was analysed with a non-parametric statistic, Kruskal–Wallis, with probability level of less than 0·05 being considered significant. The mean, median, standard deviations and the 95% confidence interval were computed with a statistic program, PRISM (GraphPad Inc., San Diego, CA). Linear regression analysis of the data from the calibration study was performed with Excel software program (Microsoft, Seattle, WA). The difference in clinical outcomes between hamsters that received filtered and unfiltered RCC and the relative risk of the hamsters developing scrapie after receiving filtered RCC were analysed with Fisher's exact statistic with probability level of less than 0·05 being considered significant (GraphPad, Inc.).

Results

Calibration of densitometry reading with concentration of PrP^{Sc}

The concentration of the undiluted 10% (w/v) SIHBH was determined by BioReliance to be equivalent to $3{\cdot}0 \times 10^7$ ID PrP^{res} (PK resistance)/ml. The change of the PrP^{res} signal with the 0·5 log dilution of the SIHBH is shown in Fig. 2. The optical density of each protein band on the Western blot membrane was converted to appropriate log titre of the PrP^{res}/ml using a standard calibration curve, Fig. 3. The intercept on the linear regression graph is 2·393, which is the limit of detection of the optical density, which corresponds to 3·77 logs of ID PrP^{res}/ml.

Background non-specific staining – RCC

There was no signal on the Western blot from the RCC samples processed using exactly the same method of extraction as SIHBH spiking study, indicating the specificity of the 3F4 primary monoclonal antibody in the Western blot assay.

Exogenous (spiking) studies – Western blot assay

In order to evaluate the efficiency of removal of PrP^{Sc} and standardize the removal process, brain homogenate containing high titre of PrP^{Sc} was spiked or added into human RCC.

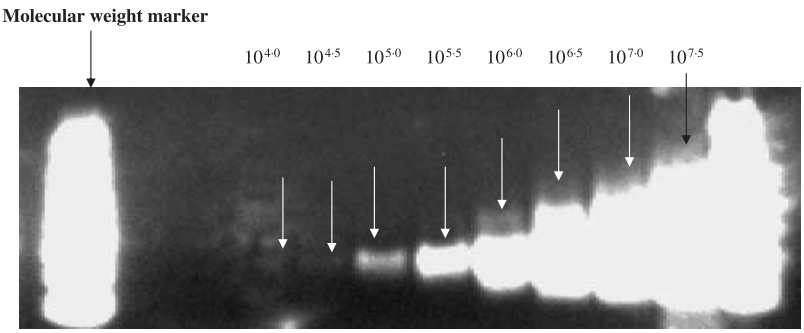

Fig. 2 Western blots images of half-log serial dilutions of 10%(w/v) SIHBH.

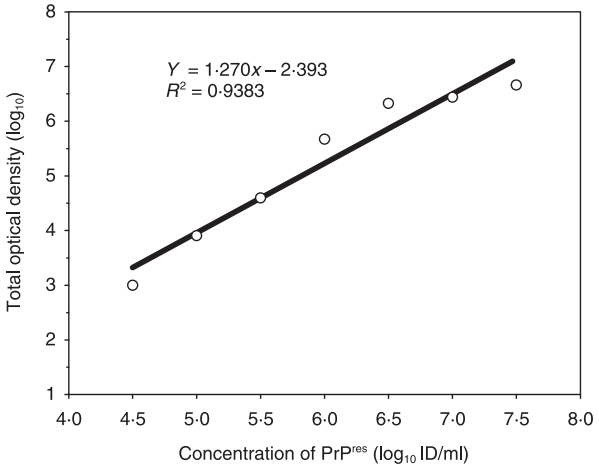

Fig. 3 Relationship between the concentrations of PrP^{res}/ml and the corresponding optical densities.

Treatment of the extracted prions in the pre- and postfiltration samples with PK enzyme resulted in complete digestion of non-Prp protein and conversion of the $PrP^{33–35}$ into $PrP^{27–30}$ PK resistant fraction, PrP^{res}, with molecular weight between 30 and 27 kDa. A very strong PrP^{res} signal was evident in the prefiltration sample and was significantly reduced after filtration with the Pall LAPRF, $P < 0{\cdot}05$, in all the samples processed in the same manner.

The log-reduction factors were calculated from the optical density reading of each band on the Western blots for both pre- and postfiltration samples. Prion reduction was assessed for filters representing three different filter production lots; and, the values were compared by ANOVA. No statistically significant differences were found between the three lots of the Pall LAPRF tested (Table 1). Log prion reduction was measured for units representing each of three storage solutions: CPD ($N = 8$), CPDA-1 ($N = 10$) and CPD/SAGM ($N = 9$). Statistically significant differences were only observed between values for CPDA-1 and CPD as well as CPDA-1 and CPD/SAGM (Table 1); however, CPD did not differ significantly from CPD/SAGM. The haematocrits and the volumes of the residual plasma in all the three preparations were also determined. As expected, the volumes of residual plasma were much higher in CPD (plasma volume = 128 ± 9 ml; hct = 51 ± 4%), and in CPDA-1 (plasma volume = 75 ± 19 ml; hct = 69 ± 9%) than in RCC-SAGM (plasma volume = 38 ± 8 ml; hct = 55 ± 4%). Note that RCC-SAGM also contained 100 ml of SAGM additive solution in addition to the 38 ml of plasma.

The effects of filtration heights on prion reduction were assessed in CPD/SAGM for three different head heights: 38 cm ($N = 6$), 76 cm ($N = 8$) and 101 cm ($N = 8$). Statistically significant differences were only noticed between 38 and 76 cm (Table 1). No statistically significant differences were observed in prion reductions between leucocyte-reduced and non-leucocyte-reduced RCC, $P > 0{\cdot}05$ (Table 1).

Prion reduction was compared in CPD/SAGM for filtration at 4 °C vs. 22 °C. Values for log prion reduction were compared by Mann–Whitney test. In this analysis, a significantly greater reduction in PrP^{res} was noted with filtration at 4 °C, $P < 0{\cdot}05$ (Table 1).

Removal of infectious prion with standard leucocyte-reduction filter

When SIHBH was spiked into whole blood, significant amounts of PrP^{res} were present in the RCC and plasma as demonstrated by the strong signals on the Western blot images (Fig. 4). When the RCCs were filtered with BPF4, there was no significant reduction in the level of exogenously added infectious PrP^{Sc} in any of the red cell units processed ($N = 5$), $P > 0{\cdot}05$. In this study, the exogenously added infectious PrP^{Sc} were not associated with leucocytes, and, thus were not removed with BPF4. This failure of standard leucocyte-reduction filter to remove infectious prions was to be expected since BPF4 was designed for specific removal of leucocytes and not soluble or non-leucocyte-associated infectious PrP^{Sc}. In contrast to BPF4, filtration of the RCC either as a secondary step to leucoreduction or as a primary filtration step resulted in significant reduction in the levels of PrP^{res}, $P < 0{\cdot}05$ (Fig. 4).

Table 1 Effects of different processing and manufacturing variables on the consistency in the log reduction of infectious prions (PrP^{res}) using Pall LAPRF. Significant differences between the variables are discussed in the result section with *P*-value of less than 0·05 being considered significant

Test conditions	Log_{10} prion reduction		*P*-value
	Mean ± SD	95%CI	
Lot-to-lot variation			
Lot 1 (*n* = 10)	2·64 ± 0·82	2·05–3·23	
Lot 2 (*n* = 10)	2·88 ± 0·62	2·42–3·34	
Lot 3 (*n* = 10)	2·74 ± 0·72	2·22–3·26	
Lots 1–3 (*n* = 30)	2·75 ± 0·72	2·49–3·02	*P* > 0·05
Different RCC storage solutions			
CPDA-1 (*n* = 10)	3·34 ± 0·13	3·27–3·45	*P* < 0·05
CPD (*n* = 8)	2·71 ± 0·96	1·91–3·51	
SAGM (*n* = 10)	2·88 ± 0·65	2·42–3·34	
Filtration heights			
38 cm (*n* = 6)	3·33 ± 0·15	3·16–3·49	*P* < 0·05
76 cm (*n* = 11)	2·54 ± 1·04	1·84–3·24	
152 cm (*n* = 8)	2·91 ± 0·32	2·23–3·59	
Leucocyte reduction of RCC			
RCC-SAGM non-leucocyte reduced (*n* = 11)	2·54 ± 1·04	1·84–3·24	*P* > 0·05
RCC-SAGM leucocyte reduced (*n* = 10)	2·88 ± 0·65	2·42–3·34	
RCC-CPDA-1 non-leucocyte reduced (*n* = 9)	3·22 ± 0·23	3·04–3·40	
RCC-CPDA-1 leucocyte reduced (*n* = 10)	3·34 ± 0·13	3·25–3·43	*P* > 0·05
Temperature			
4 °C (*n* = 8)	3·26 ± 0·62	2·74–3·78	*P* < 0·05
22 °C (*n* = 11)	2·54 ± 1·04	1·84–3·24	

SD, standard deviation; CI, confidence interval.

Endogenous infectivity study using scrapie-infected hamster blood

Western blots

The endogenous infectivity study was designed to evaluate the effectiveness of the filter in removing both leucocyte-associated and soluble infectivity that were produced naturally as a result of scrapie infection. This is different from the spiking study using brain homogenates because the prions in the endogenous infectivity study represent the proper configuration of the infectious agent. The levels of PrP^{res} in the pre- and postfiltration RCC from scrapie-infected hamsters were very low and only very weak signals were seen on the Western blot even after a concentration step via high-speed centrifugation. No signal was seen in the samples after filtration with the Pall LAPRF.

Bioassays

Six of the hamsters that were intracerebrally injected with unfiltered RCC developed clinical signs of scrapie (wobbling gait and head bobbing) and died from the disease. To confirm that the animals died from scrapie, their brains were examined for the presence of PrP^{Sc}. The amounts of PrP^{Sc} in the brains were very high as indicated by the strong signals from the Western blot images which were still visible after $1 : 10^4$

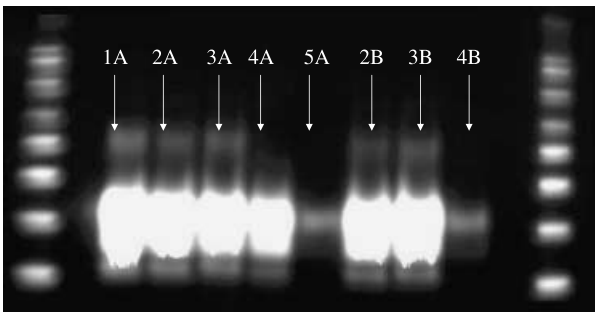

Fig. 4 Effects of prior leucoreduction on prion removal. Western blot images of SIHBH-contaminated whole blood, plasma and CPD-RCC. The levels of PrP^{res} in the RCC were measured before and after filtration using either (1) a secondary filtration protocol – an initial leucoreduction of RCC with BPF4 followed by a secondary filtration step with the Pall LAPRF or (2) a primary filtration protocol – filtration of RCC with the Pall LAPRF without a prior leucoreduction step with BPF4. All PrP^{Sc} in the samples was extracted and treated with PK as described in Materials and Methods section. Note, 1A, 2A, 3A are SIHBH-contaminated whole blood, plasma and unfiltered RCC, respectively, from unit A, while 2B and 3B are SIHBH-contaminated plasma and unfiltered RCC from unit B. Note 4A = after filtration with BPF4, there was no significant reduction in PrP^{res} signal; 5A = after filtration of 4 A with the Pall LAPRF (secondary filtration), there was a significant reduction in PrP^{res} signal. There was a significant difference in prion reduction between BPF4 and LAPRF, $P < 0·05$. Note that 4B = after filtration of 3B RCC with the Pall LAPRF (Primary filtration) without any prior leucoreduction step.

dilution of the initial 10%(w/v) brain homogenates. None of the hamsters that received filtered RCC developed clinical signs of scrapie after 200 days. However, because of the long incubation period of the disease, all animals are being monitored for another 200 days. This additional monitoring period will ensure that all animals that have the potential to develop scrapie are thoroughly investigated until the end of their natural life spans. The difference between hamsters that received unfiltered RCC and subsequently developed scrapie (6/187) and those that received LAPRF-filtered RCC (0/413) was statistically significant, $P = 0{\cdot}001$. Results of the statistical analysis of the data suggest that the risk of normal hamsters developing scrapie after receiving RCC filtered with prion-reduction filter was significantly reduced.

Leucocyte reduction, red cell membrane integrity and rheologic property

Leucocyte concentrations in 18 units of human RCC were significantly reduced from $2{\cdot}54 \pm 0{\cdot}60 \times 10^9$ to $7{\cdot}00 \pm 6{\cdot}40 \times 10^4$ at filtration height of 50 cm, $P < 0{\cdot}05$; and, from $2{\cdot}73 \pm 0{\cdot}96 \times 10^9$ to $5{\cdot}74 \pm 11{\cdot}0 \times 10^5$ at filtration height of 76 cm, $P < 0{\cdot}05$. Similarly, in the endogenous infectivity study using scrapie-infected hamster RCC, the concentrations of leucocytes in the RCC were significantly reduced from $1{\cdot}07 \pm 0{\cdot}08 \times 10^9$ to $3{\cdot}39 \pm 1{\cdot}95 \times 10^5$ ($N = 3$) of leucocytes per unit of RCC after processing with the Pall LAPRF, $P < 0{\cdot}05$.

Measurement of red cell deformability at different osmolalities with the osmotic gradient ektacytometer showed that the osmotic deformability profiles and maximum cell deformability of the Pall LAPRF-filtered red cells were not significantly different from those of control red cells, $P > 0{\cdot}05$ (Fig. 5). Measurement of the red cell cytoskeletal protein band 3 showed no significant difference between the Pall LAPRF-treated red cells and control unfiltered red cells, $P > 0{\cdot}05$, as indicated by the similarity in the mean fluorescent intensity (control = 666 ± 20 compared to 672 ± 25 LAPRF filtered samples, $n = 7$) using antiband 3 antibody labelled with eosin 5-maleimide in a flow cytometric assay.

Discussion

The recent reports of three probable cases of vCJD transmission by blood transfusion support the idea that the causative agent of vCJD can be transmitted to recipients of blood components [14–16]. The donors of the first and third transfusions did not become symptomatic until 3 and 2·5 years respectively after donation, showing that blood may be infectious well before vCJD can be clinically recognized. The recipient of the second donation who was asymptomatic for vCJD (died of unrelated causes) but carried proteinase K-resistant prions (PrP^{res}) in the spleen and lymph node and was heterozygous (methionine/valine) at codon 129 of the *PRNP* (prion) gene, showing that susceptibility to blood-borne vCJD infection is not limited to the methionine homozygous PRNP genotype. These findings have major implications for future epidemiology estimates and surveillance of vCJD especially in the UK, since individuals with this genotype constitute the largest genetic subgroup in the population [15]. In addition, this subgroup might have a different incubation period after exposure to either primary infection by BSE or secondary infection by blood transfusion [15].

Currently, there is no antemortem screening test that can identify potential blood donors who may be carrying the causative agent of vCJD. Since leucocytes have been identified as the major cell type associated with TSE infectivity [11–13],

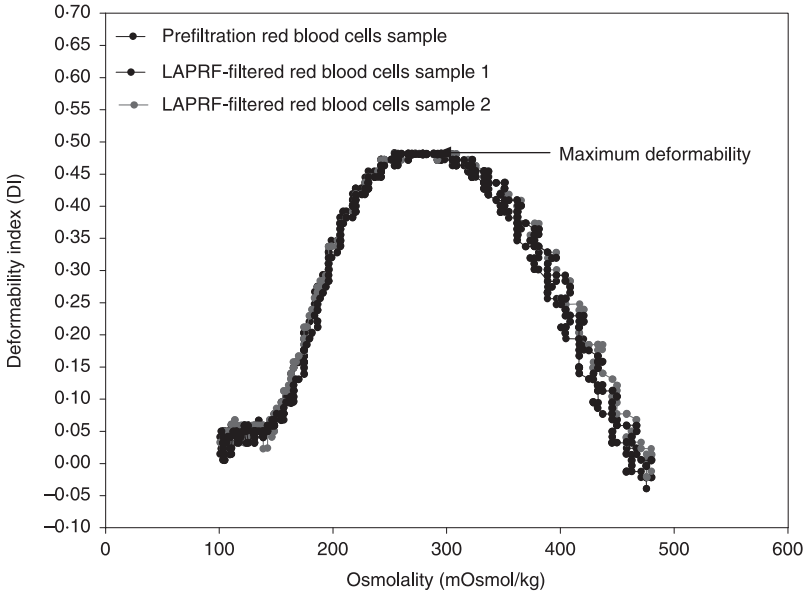

Fig. 5 Osmotic gradient ektacytometry profiles of red blood cells before and after filtration with LAPRF. Both control and filtered red cells had similar deformability profiles at different osmolalities, an indication that LAPRF did not affect the membrane stability and viscoelastic properties. Maximum deformation was observed for control and filtered red cells at about 300–350 mOsmol/kg/water. All samples (both filtered and unfiltered) were tested after 5 day storage at 4 °C.

removal of leucocytes from blood is a prudent and necessary first step to minimize the risk of transmission of vCJD (RCC used for treatment of the two recipient cases were not leucocyte reduced). However, a recent report suggests that the current generation of leucocyte-reduction filters was effective in removing only 42% of the total TSE infectivity in endogenously infected blood [19], and thus the use of current standard leucocyte-reduction may not be fully effective in preventing the transmission of vCJD through blood transfusion.

This study was designed to establish the capacity to remove the causative agent of scrapie from RCC filtration with the Pall LAPRF, a new generation of leucocyte-reduction filters that combines leucocyte reduction with significant reduction in the concentration of infectious prions. Scrapie is a progressive neurodegenerative disease of sheep caused by infectious prions that replicate in the lymphoreticular tissue and central nervous system (CNS). Although scrapie is present mainly in sheep, it can be transmitted to hamsters, inducing a pathology characterized by spongiform degeneration with astrocytic gliosis and the generation of amyloid plaques in the brain of these animals.

The agent used in this study was the hamster-adapted strain (HSc), 263K. Although it would be better to use human-derived brain material for most of the spiking studies or even blood samples from vCJD patients, it is difficult to obtain brain materials from vCJD patients. Blood from vCJD patient is almost impossible to source, particularly in the volumes required for studies such as these, and even if these were achievable, such samples have not been titred in infectivity assays to date. The level of endogenous infectivity in blood is unknown for this type of validation study. Therefore, 263K strain of scrapie from infected hamster brain homogenate has been used by most investigators on prion clearance [25–27] and was used in this study as a source of PrP^{Sc}. Prion protein is well conserved with more than 90% homology between human and hamster protein [28]. In addition, prion proteins (PrP) from different species share similar physicochemical properties, antigenicity and different degrees of resistance to PK digestion [29]. Therefore, these properties of hamster PrP^{Sc} support its use as a suitable model for prion clearance studies. Brain-derived material is one of the most widely used preparations for validating processes or methods that are developed for specific removal or inactivation of infectious prions. Some of the advantages for using brain-derived materials are; high concentration of PrP^{Sc} in 10% brain homogenate and high levels of TSE infectivity (10^9 ID_{50}/ml of 10% brain homogenate), if the validation is based on the bioassay.

The concentration of infectious prions in the RCC was measured using a Western blot assay. This procedure relies on the difference in susceptibility of the infectious PrP^{Sc}, and the non-infectious (PrP^{C}) to protease K digestion. Treatment of PrP^{Sc} with proteinase K produces a smaller protease-resistant molecule of about 142 amino acids; designated PrP 27–30 while under the same condition, PrP^{c} is completely hydrolysed.

The data obtained in this study with a Western blot assay showed that the Pall LAPRF was effective in reducing the concentration of infectious PrP^{Sc} in spiked scrapie-infected brain homogenate by about $2{\cdot}9 \pm 0{\cdot}7$ (mean ± standard deviation) logarithmic units or about 99·0% to 99·9% from RCC contaminated exogenously with infectious prions. The performance of the Pall LAPRF was not altered from lot-to-lot (Table 1) nor by the presence of leucocytes in the RCC (Table 1). There were significant differences between anticoagulants with CPDA-1 having greater prion reduction compared with either CPD or CPD/SAG-M; however, the magnitudes of the differences are small (Table 1). Although there were significant differences in the levels of residual plasma and haematocrits between the three different red cell preparations, the mechanism for the observed difference in prion reduction with CPDA-1 is not known at this time. Both slower flow and cold temperature (Table 1) were associated with better prion reduction and this may be due to the increase in time the blood was in contact with the filtration material. The observation of the failure of the standard leucocyte-reduction filter to remove non-leucocyte-associated infectious PrP is in agreement with previous report [19], and, further demonstrates the additional benefit of a filter that combines leucocyte removal with prion removal.

Data from animal models show that the concentration of pathogenic PrP in blood (at about 10 ID/ml) during the clinical phase of the disease is believed to be several orders of magnitude lower than what is present in brain (about 10^9 ID/ml) [11–13]. Note that an infectious dose is defined as the minimal amount of infectivity with 100% probability of infecting a recipient. It is measured by recording the number of animals infected by a certain volume of test material. For example, if 10 animals were infected from a pool of 1 ml, the infectivity is 10ID/ml. In the preclinical stage of prion disease, the concentration of pathogenic prion is at least 2 orders of magnitude lower than the concentration in the clinical phase [11–13]. Therefore, during the preclinical stage of the disease, the concentration of pathogenic PrP in the blood may be about 0·1ID/ml, which corresponds to about 50ID in a unit of whole blood, distributed mainly between leucocyte and plasma components. During this period when there are no clinical symptoms of the disease, the carrier may still be able to donate blood. Therefore, assuming equal distribution of infectious prions between plasma (25ID) and RCC (25ID), a minimum of 1·4 logs removal of infectivity may be sufficient to prevent the transmission of pathogenic PrP during transfusion of RCC from a blood donor in the preclinical phase of the disease.

In this study, the level of infectious PrP in the RCC prior to filtration was about 10^6ID/ml, which was much higher than

the expected level of 10ID/ml in the clinical stage of prion disease [11–13]. This high titre of infectious PrP was used because the dynamic range of the Western blot was about 3 orders of magnitude and the limit of detection approximates 1000–10 000 ID/ml, representing a difference of about 3 logarithmic units. Overall, prion reduction in the range of 2–3 logs removal may be sufficient to prevent the transmission of prion disease because the levels in blood of patients in the preclinical stage of the disease is unknown but believed to be lower than that in an experimental animal model of prion disease.

Although the log removal based on spiking studies has been correlated with infectivity [25], one must be careful in extrapolating data from exogenous spiking studies to endogenous infectivity because of the known differences in the biophysical properties of different brain homogenates [30]. Therefore, because of the possibility that brain tissue spikes may not accurately reflect the form of infectious agent circulating in the blood of an infected host, and the differences in properties of different preparations of brain homogenates [30], we also evaluated the effectiveness of the filter in removing infectious prions from RCC obtained from the blood of infected hamsters. Results of the statistical analysis of the endogenous infectivity data suggested that the risk of normal hamsters developing scrapie after receiving RCC filtered with the Pall LAPRF was significantly reduced and it is possible that none of these hamsters may ever develop scrapie.

It is important to note that the Pall LAPRF also significantly reduced the quantity of leucocytes in 100% of the RCC processed to less than 5×10^6, which met US FDA/AABB guidelines. However, only 85% of the RCC filtered with the Pall LAPRF contained residual leucocytes of less than 1×10^6, which did not meet Council of Europe guidelines on leucoreduced RCC.

Rheologic properties of the red cells were evaluated after about 5 days storage at 4 °C with an ektacytometer, which combined the deformation of the red cells by hydrodynamic shear forces with laser diffraction to detect the average deformability of the cells. The resulting deformation of the red cells was expressed as deformability index (DI). The osmotic deformability profiles obtained from this type of assay provide information about cell water content, the relationship between surface area and cell volume, membrane stability, and other cellular properties that are essential for physiological functions of the red cells, including effective transportation of oxygen and *in vivo* survival [31,32]. For example, cells with reduced surface area-to-volume ratio deform less extensively than normal cells as seen by a pronounced decrease in DI, with reduction in *in vivo* survival [33]. The Pall LAPRF-filtered red cells had the same osmotic deformability profiles as control unfiltered red cells, which suggest that these cells have normal *in vivo* survival and physiological functions. Single- and double-labelled *in vivo* red cell recovery studies using Technetium 99 m and Chromium 51 show that the Pall LAPRF-filtered red cells have 85% (single labelled) and 83% (double labelled) 24 h *in vivo* survival which are well above the US FDA required mean of 75% [34]. In addition to normal red cell rheologic properties, the integrity of the main red cell membranes was also well maintained as indicated by the normal levels of the main membrane cytoskeletal protein, band 3. Recent report also showed that red cell units that were filtered and then stored for an extended period met all relevant UK and Council of Europe guidelines [35].

In conclusion, the use of the Pall LAPRF, a new generation of filters that combines efficient leucocyte reduction with removal of infectious PrP, should help improve the safety of the blood supply by reducing, and perhaps even eliminating, the risk of transmission of human vCJD and other forms of human prion disease through transfusion of RCC, the mostly widely transfused blood component.

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