'ME7 spiked supernatant' was taken for bioassay, leaving a volume of 315 ml for further processing.

Cold ethanol at ≤ −5 °C was added to achieve a final ethanol concentration of 7·5–8·5% v/v, and Fraction I (fibrinogen) precipitate was separated by centrifugation at 20 000 *g* for 10 min. Cold ethanol was added to the SNI to give a final ethanol concentration of 18·5–22·5% v/v. The mixture was centrifuged at 20 000 *g* for 10 min at −5 ± 1 °C and the Fraction II + III precipitate (immunoglobulin plus lipoprotein) was collected. Sufficient ethanol at ≤ −5 °C was then added to achieve an ethanol concentration of 20·0% v/v, to precipitate immunoglobulins while leaving albumin in solution. Fraction II + III(W) precipitate was separated by centrifugation at 20 000 *g* for 10 min at −5 ± 1 °C. The fraction III precipitate (lipoprotein and IgM) was separated by centrifugation at 20 000 *g* for 10 min at −5 ± 1 °C. Filter aid Diacel 150 (CFF, Gehren, Germany) was added to the fraction III supernatant and filtered through Seitz EK1 disks (Pall, East Hills, NY). The filtrate was adjusted to pH 4·0 and diafiltered at this pH using 10 kDa ultrafiltration membranes. The sample 'ME7 IgG Cohn' was taken for bioassay.

Bioassay

Samples collected from one control run and the TSE partitioning run were used for intracerebral (IC) inoculation of mice. The test materials were subjected to tenfold dilutions in PBS, and weanling C57 black mice (Animal Resources Center, Perth, WA) were IC inoculated with 30 µl of test dilution in sets of five mice per cage. As shown in results in the tables, some dilutions were inoculated into more than one cage, to improve sensitivity when low prion infectivity was expected (given Western blot study results). The study period for the bioassay was 18 months. Mice showing clinical symptoms of scrapie [22] throughout the study or that died within incubation periods consistent with TSE were harvested for TSE evaluation by haematoxylin and eosin staining to detect spongiform change. Further testing using MAb 6H4 (Prionics, Schlieren, Switzerland) for immunohistology and MAb SAF83 (Cayman, Ann Arbor, MI) for Western blot was performed if required. Mice were scored as scrapie positive when clinical signs were confirmed by two or more methods. At the end of 18 months, histology was performed on all surviving mice in dilutions from which scrapie mice had been culled. Histology was also performed on all mice in the lowest dilution for which there were no scrapie cases recorded.

Negative mouse controls within the bioassay component were deemed to be satisfactory when they showed no signs of toxicity over the period of the study or did not contract scrapie over the full study. The 50% end point for infectious dose (ID_{50}) for the bioassay titration was calculated using the Spearman Kärber method [23]. When no infectivity was present in a sample, a 95% probability formula was used to estimate residual infectivity in the sample [24]. The log reduction factor (LRF) of infectious scrapie over the processes was determined by subtracting the scrapie log load of the final concentrated eluates from the log load of the spiked starting material [24].

Results

Scale-down validity

Protein intermediates from control runs showed that the processes were scaled down accurately and were representative of production processes with regard to protein purity, concentration and chemical composition. Chromatographic profiles as shown for the scrapie ME7 spiked scale-down runs accurately represented those obtained from the industrial-scale production process [25]. All buffers and column eluates achieved the same HETP, pH, and conductivity limits as production processes.

Experiments using microsomal scrapie 263K with Western blot detection

Log reduction factors and recovery of PrP^{sc} are shown for the ion exchange columns used for the production of albumin and IVIG (Table 1). All eluate streams from the columns were assayed for PrP^{sc} using Western blot. Substantial partitioning of PrP^{sc} away from the target proteins was achieved in all ion exchange steps examined. The log reductions across the DEAE Sepharose and CM Sepharose for albumin were ≥ 4·0 and ≥ 3·0, respectively. The log reductions across the DEAE Sepharose and Macro-Prep for immunoglobulin were 3·3 and ≥ 4·1, respectively.

Summation of all the PrP^{sc} recovered from all eluates of each column shows that the overall percentage recovery of PrP^{sc} for the DEAE Sepharose, CM Sepharose and Macro-Prep columns are ≤ 0·34, ≤ 1·84 and ≤ 0·03%, respectively. Mass balance was therefore not achieved in all three ion exchange columns up to the final wash with 1 M NaCl. The 1 M NaOH sanitation washes were not studied as NaOH renders PrP^{sc} sensitive to digestion by proteinase K [26], and could lead to aberrant results. The results indicate that some PrP^{sc} was eluted from the DEAE Sepharose and CM Sepharose, but most of the PrP^{sc} was either not recovered or bound to the chromatography gel prior to the NaOH sanitation step.

Scrapie ME7 spike with bioassay detection

Limiting dilution bioassay was used to determine the titre of the spiked supernatant I starting material and the final concentrated eluates from the CM Sepharose and Macro-Prep columns (Table 2). The control mice for all studies remained normal throughout the observation period, indicating that the inocula were non-toxic and that there was no cross-contamination from cages housing TSE-positive mice.

Table 1 Partitioning of PrP^{sc} microsomal fraction during albumin and immunoglobulin purification across ion exchange columns as determined by Western blot

Step/Fraction	Total PrP^{sc} (log_{10})	% PrP^{sc} in fraction	Reduction (log_{10})[b]
DEAE Sepharose™ FF			
Inoculum	3·80		
Column load	4·30	100·00	
Unbound IgG[a]	0·98	0·05	3·3
Transferrin peak	≤ 0·84	≤ 0·03	≥ 3·5
Wash – 10 mM NaAc	≤ 0·92	≤ 0·04	≥ 3·4
Eluted albumin[a]	≤ 0·32	≤ 0·01	≥ 4·0
Wash – 150 mM NaAc	1·63	0·20	2·7
0·5 M NaCl	≤ 0·11	≤ 0·01	≥ 4·2
CM Sepharose™ FF			
Inoculum	3·11		
Column load	3·64	100·00	
Unbound protein	≤ −0·34	≤ 0·03	≥ 4·0
Wash 110 mM NaAc	≤ 0·41	≤ 0·06	≥ 3·2
Eluted albumin[a]	≤ 0·68	≤ 0·13	≥ 3·0
Wash 400 mM NaAc	≤ 0·23	≤ 0·04	≥ 3·4
0·5 M NaCl	1·83	1·56	1·8
Macro-Prep High Q			
Inoculum	3·80		
Column load	4·18	100·00	
Purified IgG (unbound)[a]	≤ 0·08	≤ 0·01	≥ 4·1
Wash 10 mM NaAc	≤ −0·19	≤ 0·01	≥ 4·4
1 M NaCl	≤ −0·07	≤ 0·01	≥ 4·2

[a]Eluates shown in bold are main column eluents used for ongoing processing of albumin or immunoglobulin. All other eluates are waste streams.
[b]If PrP^{sc} (proteinase K-resistant scrapie prion protein) could not be detected in the neat sample, the PrP^{sc} log reduction was recorded as '≥'.

Table 2 Bioassay of test materials from albumin and immunoglobulin chroma[a]

Sample	Parameter	Sample dilution 10^{-2}	10^{-3}
Control	Mice infected/inoculated	0/5	
SN1	incubation period (days)[a]		
Control	Mice infected/inoculated	0/10	
Albumin	incubation period (days)		
Control	Mice infected/inoculated	0/5	
IgG	incubation period (days)		
ME7	Mice infected/inoculated	5/5	5/5
spiked SN1	incubation period (days)	194 ± 0	193 ± 7
ME7	Mice infected/inoculated	5/5	5/5
Albumin	incubation period (days)		
ME7	Mice infected/inoculated		
IgG	incubation period (days)	0/20	0/5

[a]Mean ± standard deviation.

Table 3 Bioassay of test materials from Normal Immunoglobulin 2VI (Cohn) process

Sample	Parameter	Sample dilution							
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Control cryosupernatant	Mice infected/inoculated incubation period (days)[a]	0/8							
Control IgG (Cohn)	Mice infected/inoculated incubation period (days)	0/9							
ME7 spiked cryosupernatant	Mice infected/inoculated incubation period (days)	5/5 186 ± 6	5/5 235 ± 26	5/5 223 ± 8	4/5 279 ± 67	1/5 279	1/5 347	1/5 230	0/4
ME7 IgG (Cohn)	Mice infected/inoculated incubation period (days)	0/20	0/5	0/5	0/5				

[a]Mean ± standard deviation.

Step/Fraction	Infectivity (log_{10} ID_{50}/ml)	Volume (log_{10})	Total infectivity (log_{10})	Reduction (log_{10})
Chromatography				
ME7 spiked SN1	5·4 (4·5–6·3)[a]	2·1	7·5	
ME7 albumin	≤ 0·7	1·2	≤ 1·8	≥ 5·6
ME7 IgG	≤ 0·7	1·4	≤ 2·1	≥ 5·4
Cohn				
ME7 spiked cyrosupernatant	5·4 (4·4–6·5)[a]	2·5	7·9	
ME7 IgG (Cohn)	≤ 0·7	1·6	≤ 2·3	≥ 5·6

Table 4 Calculation of infectivity in spiked process starting materials and final materials of chromatography and Cohn process

[a]95% confidence interval of Spearman Kärber estimate of ID_{50}.

Discussion

The potential risk of vCJD transmission has led producers of plasma products to examine the prion clearance capacity of their fractionation processes. Whereas it is an accepted principle to add viral log reduction factors attained by mechanistically complementary steps [27], different approaches are needed to establish overall prion removal. The European Agency for the Evaluation of Medicinal Products (EMEA) guidance [28] advises that validation studies of removal/inactivation procedures for TSEs are difficult to interpret due to the necessity to take into consideration the nature of the spiked material and its relevance to the natural situation, the design of the study (including scaling-down of processes) and the method of detection of the agent.

This study programme looked initially at PrP^{sc} removal capacity of individual process steps then examined the potential of two or more combined process steps to remove prion infectivity. The study programme used scrapie 263K and ME7 as models for human prions, an approach that is supported by the finding that partitioning of human prions is similar to that observed in the hamster scrapie model [29]. The studies used different spiking materials (microsomal 263K and 10 000 g supernatant ME7) because the ME7 study sought to use an infectious spike which would consist not only of microsomal infectious units, but of smaller units of infectivity as soluble PrP^{sc} [17]. The study programme found substantial partitioning of prions away from the product streams of chromatographic albumin and immunoglobulin, and for immunoglobulin produced by Cohn fractionation. Importantly, this investigation shows that removal of infectivity from immunoglobulin preparations is similar whether chromatographic or Cohn purification processes are used.

The study with microsomal scrapie 263K showed substantial partitioning of PrP^{sc} away from the target proteins in all ion exchange steps examined. The log reductions across the anion exchange DEAE Sepharose and cation exchange CM Sepharose for the albumin process were ≥ 4·0 and ≥ 3·0, respectively. The log reductions across the DEAE Sepharose and anion exchange Macro-Prep for the immunoglobulin process were 3·3 and ≥ 4·1, respectively.

At the loading pH buffer ranges used for this experiment (pH 5·2 for DEAE Sepharose, pH 4·5 for CM Sepharose, and pH 6·2 for Macro-Prep), scrapie should be below its isoelectric point (pI) of pH 5·4–9·3 [30] on the DEAE and CM Sepharose columns, and hence would be positively charged. While pH 6·2 is within the pI range for scrapie, it is likely that scrapie is predominantly positively charged when loaded onto Macro-Prep. If scrapie bound to chromatography columns purely based on charge, it would be predicted that more binding should occur with the cation exchanger CM Sepharose, and less to the anion exchangers DEAE Sepharose and Macro-Prep. The substantial

removal of PrP^{sc} by the anion and the cation exchange gels, and lack of substantial amounts of PrP^{sc} in the wash fractions indicated that PrP^{sc} removal was more dependent on adsorption to the gel matrix than to the exchange group. There was a partitioning of 0·05% of loaded PrP^{sc} in the unbound IgG eluted from DEAE Sepharose. However, as the Macro-Prep column removed ≥ 4·1 logs, there is a level of confidence that this remaining PrP^{sc} would be removed from the product stream.

Similar results were reported using murine bioassay of BSE 301 V over Toyopearl DEAE-650 M [31], in which LRFs of 2·9 and 2·7 were found in eluted fibrinogen and factor VIII, leading to the conclusion that over 99% of BSE infectivity remained bound to the ion-exchange column. A 2 M NaCl wash removed 5·75% of this infectivity, and infectivity could not be detected in eluates following a 0·1 M NaOH wash.

In our study, new chromatography gels were used, as opposed to production gels that had been exposed to previous cycles. The possibility of infectivity binding to chromatography gels has led to further experimental work examining prion removal and or inactivation of infectivity from chromatography gels, in which it was ascertained that infectious prions did bind to DEAE Sepharose, and the cleaning cycle was able to remove or inactivate this infectivity [32].

A LRF of ≥ 5·6 across the DEAE and CM Sepharose ion exchange columns in the albumin process and a LRF of ≥ 5·4 across the DEAE Sepharose and Macro-Prep ion exchange columns in the immunoglobulin process was achieved. Both processes include a final concentration/diafiltration step using 30 kDa ultrafiltration, with the retentate containing either albumin or immunoglobulin. It is unlikely that substantial prion infectivity would be lost in the permeate stream, as infectious units are believed to have a minimum molecular weight of approximately 55 kDa [33]. Conversely, it is possible that some infectivity is adsorbed to the ultrafilter membrane surface; however, the membrane types used (polyethersulphone for albumin and regenerated cellulose for immunoglobulin) are both specified as low protein binding by the respective manufacturers.

If the starting titre for the ME7 bioassay study had been higher it may have been possible to show removal capacity equal to the addition of removals attained for each column in the Western blot study. Previous studies using scrapie 263K for validation of prion removal in bovine serum albumin production with sequential columns [34] have shown a 5·2 log removal of scrapie 263K over the first ion exchange column, and ≥ 6·2 when the second ion exchange column is included. This implies that the result is limited by the starting titre, and the question of additivity cannot be resolved without a higher infectivity spike. In our study programme, the spike material preparations and the method of detection (Western blot vs. bioassay) were different between the two studies. Higher titre spiking material would be needed to further elucidate the additive vs. non-additive nature of prion removal over sequential columns.

The LRF [illegible] was ≥ 5·6, [illegible] clearance [illegible] detected by [illegible] precipitation [illegible] depth [illegible] overall removal [illegible] additive nature [illegible] was established [illegible] suggests that [illegible] cipitation [illegible] followed by [illegible]

Conclusion

Using both [illegible] experiment[illegible] graphic ste[illegible] munoglobu[illegible] 5·4 logs, re[illegible] munoglobu[illegible] capacity to [illegible] Importantly [illegible] from immuno[illegible] matographic [illegible] the bioassay s[illegible] below the limit[illegible] tined challe[illegible] needed to f[illegible] process step[illegible] removal by in[illegible] to the gel matrix[illegible] of PrP^{sc} was [illegible]

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Referenc

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Prion removal by nanofiltration unde[illegible]

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Abstract

Manufacturing processes used in the production of biopharmaceutica[illegible] potential contaminants, including TSE agents. In the present study, we ha[illegible] different starting materials, using virus removal filters of different pore [illegible] Western blot (WB) analysis when a "super-sonicated" microsomal frac[illegible] used as the spike material. In contrast, no PrP^{Sc} was detected when an un[illegible] ner designed to optimize the particle size distribution within the preparat[illegible] of detection of the WB assays used under all the experimental conditio[illegible] under one experimental condition. The results obtained suggest that th[illegible] ability of filters to remove prions, and that procedures designed to mini[illegible] sonication" or detergent treatments described herein, should be used f[illegible]

Keywords: Prion; Removal; Filter; Clearance study; Spike material

1. Introduction

The transmission of variant Creutzfeldt–Jakob disease (vCJD) through blood transfusion has been of increasing concern, since a fourth possible transmission case was reported [1]. In addition, prions have been detected in the buffy coat separated from the blood of hamsters infected with scrapie, using a biochemical assay (protein misfolding cyclic amplification, or PMCA) [2]. Infectious prions are [illegible]

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consider the effect of the prion spike material when evaluating process steps for prion clearance. A rationale for the choice of the spike preparation used for such evaluation studies should be provided [4].

Several prion strains have been used to evaluate manufacturing processes for their ability to remove TSE agents, including hamster scrapie prion protein (PrP^{Sc}, 263K or [illegible]), and mouse PrP^{BSE} (301V). In a polyethylene glycol (PEG) fractionation process, hamster PrP^{Sc} and human PrP^{CJD}, prepared using the same methodology, were reported to behave in a very similar manner [6]. Different prion spike preparations have been used to investigate prion removal, including crude brain homogenate (BH), microsomal fraction (MF), caveolae-like domains (CLDs), and purified PrP^{Sc}. Of these materials, purified PrP^{Sc} was reported to behave differently from the other preparations in an 8% ethanol fractionation step [7]. This result suggests that the methods used to prepare the prion spike material may be a critical factor in prion clearance studies. Furthermore, these reports are useful in providing a rationale for the choice of the prion source and spike preparation used for such evaluation studies [8].

Tateishi et al. reported that sarkosyl influenced the ability of BMM40 filters to remove prions, using BH derived from CJD-infected mice [9]. The presence of sarkosyl was also shown to significantly reduce the capacity of Planova (P)-35N to remove the scrapie agent ME7, while filtration with P-15N resulted in the complete removal of infectivity, to below the limit of detection of the bioassay used, in both the presence and absence of sarkosyl [10]. Van Holten et al. evaluated the capacity of Viresolve 180 membranes (designed for virus removal from proteins of <180 kDa) to remove prions by using BH which was lysolecithin-treated, sonicated, and subsequently passed through a 100 nm filter (SBH), and demonstrated removal of PrP^{Sc} down to the limit of detection of the Western blot assay used. They argued that by using a better defined spike material, where the size of the scrapie particles was limited, the results may be more relevant with respect to the removal of potential TSE infectivity in plasma than previous studies that used a less well-defined BH [11].

Aggregation of the prion protein is a critical parameter when evaluating nanofiltration steps. The actual form of the infectious agent present in plasma in natural infection is not known. In addition, nanofiltration is typically performed late in the downstream processing, after protein purification steps, which may result in removal of larger or aggregated prion forms. Therefore, use of a spike preparation containing large aggregates may result in an over-estimate of the prion removal capacity of a filter. Although the reports described above, and others, have shown excellent prion removal ability for a number of filters, most reports have not described the particle size distribution of the prion protein in the spike preparations used. Therefore, in this study we have investigated the prion removal capacity of P-35N, P-20N and P-15N filters under diverse conditions, considering the particle size distribution of the MF preparations used.

2. Materials and methods

2.1. Preparation of microsomal fraction (MF)

Brains removed from hamsters infected with scrapie strain 263K [12] (originally obtained from the Institute for Animal Health, Edinburgh, UK), were homogenized in phosphate buffered saline (PBS) until homogeneous, to a final concentration of 10% (w/v). The homogenate was clarified by low speed centrifugation, to remove larger cell debris and nuclei, and the supernatant material was then further clarified by centrifugation at 8,000 × *g* for 10 min at 4 °C, before being ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, to concentrate the scrapie fibrils, and small membrane vesicles and fragments. The pelleted material was resuspended in PBS, aliquoted, and stored at −80 °C. This material was designated 263K MF. Prior to use, stocks were thawed at 37 °C, and sonicated 2 × 4 min on ice water (Ultrawave ultrasonic bath model #U100, 130 W 30 kHz, Ultrawave Ltd., Cardiff, UK). Six independent batches of 263K MF were used in this study. These batches are designated 263K MF preparation lots A–F (Tables 1–3). Normal MF, derived from normal (i.e. uninfected) hamster brain material, was also prepared as described above.

Since we were unable to measure the particle size distribution of contaminated materials in our facility, we used normal MF, and investigated changes in the particle size distribution following strong sonication or treatment with detergent. Various concentrations of sarkosyl (*N*-lauroylsarcosine sodium salt, Nacalai Tesque, Inc., Kyoto, Japan), lysolecithin (L-α-lysophosphatidylcholine, Sigma-Aldrich Corp., St. Louis, USA), Triton X-100 (polyethylene glycol mono-*p*-isooctylphenyl ether, Nacalai Tesque, Inc.), TNBP (tri-n-butyl phosphate, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and/or 1% Tween 80 (Nacalai Tesque, Inc.) were added to normal MF. Changes in the particle size distribution were then monitored by dynamic light scattering method using volume-weighted gaussian analysis using a submicrometer particle sizer (NICOMP Type 370, Particle Sizing Systems, Inc., Santa Barbara, USA). To evaluate the effect of strong sonication, normal MF was sonicated using a closed system ultrasonic cell disruptor (Bioruptor UCD-200T, CosmoBio Co. Ltd., Tokyo, Japan) with a resonance chip set in the tube. Sonication was performed for 1 min at 20 kHz, 200 W in a cold water-bath. Ten cycles of sonication were performed, with a 1 min interval between each sonication treatment. During the treatment cycle, the particle size distribution was monitored. We named this treatment cycle "super-sonication".

Different preparations of 263K MF, treated with various combinations of detergent, ultracentrifugation and/or "super-sonication", were used as the spiking agent in the process evaluation studies, and are described in the relevant methods sections below.

Table 1
Scrapie infectivity in different 263K MF preparations[a]

	Log_{10} LD_{50}/ml	SE at 95% probability
Non-super-sonicated 263K MF lot C	5.7	0.44
Super-sonicated 263K MF lot C	6.0	0.53
Super-sonicated 263K MF lot D	5.3	0.69
SD-treated, ultracentrifuged, super-sonicated and 220 nm-filtered 263K MF lot C	6.9	0.69

[a] This bioassay study was performed in accordance with GLP regulations.

Table 2
Removal of PrP^{Sc} from PrP^{Sc}-inoculated PBS

	PVDF filter				Planova filter					
	220 nm		100 nm		P-75N (72 ± 2 nm)		[illegible] (35 ± 2 nm)		[illegible]	
Super-sonicated	+	−	+	−	+	−	[illegible]	[illegible]	[illegible]	[illegible]
Before filtration	4.2/3.5[a]	3.5/4.2	4.2/3.5	3.5/4.2	4.2/4.2	3.5/4.2	[illegible]	[illegible]	[illegible]	[illegible]
Filtered	3.8/3.8	3.1/3.8	3.8/3.1	2.4/3.1	2.4/2.4	<1.0/<1.0	[illegible]	[illegible]	[illegible]	[illegible]
LRF[b]	0.4/−0.3	0.4/0.4	0.4/0.4	1.1/1.1	1.8/1.8	≥2.5/≥3.2	[illegible]	[illegible]	[illegible]	[illegible]

Data represents total PrP^{Sc} present in samples, expressed as log_{10} arbitrary units, following Western [illegible] in accordance with GLP regulations.

[a] Two independent batches of 263K MF were used: lot C (left) and lot D (right), respectively.

[b] LRF, log reduction factor = total PrP^{Sc} in input/total PrP^{Sc} in filtrate, expressed as a log_{10} [illegible]

2.2. Detection of PrP^{Sc} by Western blotting (WB)

To determine the relative levels of PrP^{Sc} present in different samples, WB assays were performed. Three slightly different WB methodologies were applied over the course of the studies, all of which are based on detection of the disease-associated, protease-resistant form of the prion protein (PrP^{Sc}), using the monoclonal antibody 3F4 (Signet Laboratories, Inc., Dedham, USA) [13]. WB methods 1 and 2 were developed independently, and use different approaches to calculate the titer of PrP^{Sc}. As these assays were performed as part of GLP studies intended for regulatory [illegible] sion, the [illegible] in these st[illegible]

2.2.1. Method [illegible]

Samples [illegible] ntrols were either [illegible] ultracentrifuged at 141,000 × *g* for [illegible] the pelleted material resuspended in [illegible] was performed to concentrate the [illegible] ume samples and to remove soluble [illegible] nents that might interfere with the [illegible] digested with proteinase K (Roche [illegible] berg, Germany) for 60 min at 37 °C. [illegible] tion of proteinase K, to [illegible] interfere with the detection of PrP^{Sc} [illegible] covery of the [illegible] protein [illegible] each sample [illegible] samples were mixed [illegible] sample buffer [illegible] mM Tris-HCl [illegible] erol, 2% [illegible], and [illegible] BioRad Laboratories, Inc., Hercules, [illegible] β-mercapt[illegible] After [illegible]

Table 3
Removal of PrP^{Sc} from PrP^{Sc}-inoculated plasma preparations[a]

Filter	P-35N (35 ± 2 nm)		P-20N (19 ± 2 nm)		[illegible] (15 ± 2 nm)			
Preparation	IVIG	Haptoglobin	IVIG	Haptoglobin	[illegible]	[illegible]	[illegible]	[illegible]
Spike material	263K sMF[c]	263K sMF[c]	263K sMF[d]	263K dsMF[e]	[illegible]	[illegible]	[illegible]	[illegible]
MF preparation lot.	C/D	B	E/F	E/F	[illegible]	[illegible]	[illegible]	[illegible]
Spike ratio	1/100	1/200	1/20	1/200	[illegible]	[illegible]	[illegible]	[illegible]
Detection method[b]	WB1	WB3	WB2	WB2	[illegible]	[illegible]	[illegible]	[illegible]
Before filtration	3.2/2.5	2.4	6.8/6.8	6.7/6.1	[illegible]	[illegible]	[illegible]	[illegible]
Filtered	0.8/0.8	<1.0	4.8/4.3	4.8/4.7	[illegible]	[illegible]	[illegible]	[illegible]
Log reduction factor	2.4/1.7	≥1.4	2.0/2.5	1.9/1.4	[illegible]	[illegible]	[illegible]	[illegible]

Abbreviations used: 263K MF, microsomal fraction derived from hamster adapted scrapie strain [illegible] intravenous [illegible] sonicated" 263K MF; WB, Western blotting; 263K dsMF, detergent treated and "super-sonicated" [illegible] 263K [illegible] assay; +ve, scrapie positive.

[a] Scaled down conditions were designed according to current guidelines. However, in a study using [illegible] and the filtration was subsequently terminated.

[b] WB1, WB2, and WB3 mean Western blotting methods 1, 2 and 3, respectively. The studies involving the use of WB1 [illegible] with GLP regulations; the studies involving the use of WB3 and the qualitative BA shown in this [illegible] performed [illegible]

[c] 263K MF was "super-sonicated" then 220 nm-filtered prior to spiking.

[d] 263K MF was ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended in buffer equivalent to the starting material [illegible] and 220 nm-filtered prior to spiking.

[e] 263K MF was "SD-treated", ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended [illegible] "super-sonicated". These materials were 220 nm-filtered prior to spiking.

[f] 263K MF was treated with 0.1% sarkosyl for 30 min at room temperature.

the sample were then prepared and subjected to electrophoresis using 12% (w/v) SDS-polyacrylamide gels. Proteins were transferred from the gels to 0.45 μm PVDF membranes (Immobilon-P, Millipore Corp., Billerica, USA), and non-specific binding sites on the membranes were then blocked by overnight incubation in buffer containing dried milk and Tween 20. The blocked membranes were incubated with monoclonal antibody 3F4, washed extensively, and then incubated with a secondary horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Sigma-Aldrich Corp.). After further extensive washing, bound antibody was detected using an ECL-Plus detection system (GE Healthcare UK Ltd, Buckinghamshire, UK) and exposure to blue-light sensitive film.

The level of PrP^{Sc} present in each sample was calculated based on the end-point dilution after analysis by WB. The end-point dilution for each titration was taken as the first dilution at which the 28 kDa PrP^{Sc} protein could not be detected. The reciprocal of this dilution was then taken as the titer of the agent, and expressed in arbitrary units/ml.

2.2.2. Method 2 (WB2)

WB was performed essentially as described by Lee et al. [14]. Briefly, samples were digested with proteinase K at approximately 6 U/ml for 60 min at 37 °C and centrifuged at approximately 20,000 × *g* for 60 min at 4 °C. The pellet was then resuspended and denatured in a 1:1 mix of supernatant and sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS and 0.025% (w/v) bromophenol blue, Invitrogen Corp., Carlsbad, USA), by heating at approximately 100 °C. Serial 3.2-fold (0.5 $\log_{10}$) dilutions of the sample were prepared, and loaded onto 12% (w/v) SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Invitrogen Corp.), and the membranes blocked using buffer containing dried milk and Tween 20 for 1–2 h at room temperature. The blocked membranes were then incubated with monoclonal antibody 3F4, washed extensively, and incubated with a secondary alkaline phosphatase (AP)-conjugated anti-mouse antibody (Cambridge Biosciences Ltd., Cambridge, UK). After further extensive washing, bound antibody was detected using a CDP Star/Nitroblock II detection system (Applied Biosciences, Bedford, USA) and exposure to blue-light sensitive film.

The titer of PrP^{Sc} present in each sample was calculated slightly differently from WB1 and WB3. The end-point dilution for each titration was taken as the last dilution at which the 28 kDa PrP^{Sc} protein could be detected. The reciprocal of this dilution was then taken as the amount of agent in the sample volume tested, and was adjusted for the volume tested and any concentration factors, to give a titer/ml for the original process sample.

2.2.3. Method 3 (WB3)

Samples were ultracentrifuged twice at 150,000 × *g* for 1 h. The samples in the precipitates were then resuspended in PBS at 1/1 or 1/10th volume of the original. Resuspended samples were treated with proteinase K at a final concentration of 10–100 μg/ml. After incubation at 37 °C for 60 min, samples were treated with 10 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride (AEBSF) at room temperature for 10 min, then mixed with 5× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (300 mM Tris–HCl, 12% (w/v) SDS, 25% (v/v) glycerol, and 0.025% (w/v) bromophenol blue, pH 6.8, with 25% (v/v) β-mercaptoethanol) and heated at 100 °C for 5 min. Samples were serially 5-fold diluted with 1× PAGE dilution buffer (60 mM Tris–HCl, 2.4% (w/v) SDS, 5% (v/v) glycerol, and 0.005% (w/v) bromophenol blue, pH 6.8). SDS-PAGE was performed at 30 mA per gel for approximately 42 min. The proteins in the gel were transferred to 0.45 μm PVDF membranes. After treating with blocking buffer (5.0% (w/v) skimmed milk in PBS, 0.05% (v/v) Tween 20), the membrane was incubated with monoclonal antibody 3F4 at 4 °C overnight, then incubated with HRP-conjugated sheep anti-mouse IgG (Sigma-Aldrich Corp.). Bound antibody was visualized by chemiluminescence (ECL-Plus) on X-ray film. The titer of PrP^{Sc} present in the samples was calculated as described for method 1 in Section 2.2.1.

2.3. Evaluation of PrP^{Sc} removal by filtration

A 10% (v/v) concentration of "super-sonicated" 263K MF was prepared in PBS, and 10 ml aliquots were then filtered through a 220 nm or a 100 nm 4 cm^2 PVDF filter (Millex-GV or -VV, Millipore Corp.). In addition, 25 ml aliquots of "super-sonicated" 263K MF in PBS were filtered through a 0.01 m^2 P-75N (72 ± 2 nm), P-35N (35 ± 2 nm), or P-15N (15 ± 2 nm) filter (Asahi Kasei Medical Co., Ltd. Tokyo, Japan). Two independent batches of 263K MF were used. WB1 analysis of samples before and after filtration was performed to determine the removal of PrP^{Sc} under the different conditions. Non-sonicated 263K MF (from the same batch of 263K MF) was also filtered as a control.

2.4. Hamster bioassay to determine the infectious titer of 263K scrapie stocks

Three- to four-week-old female specific pathogen-free (SPF) Syrian hamsters were used in these experiments. Serial 10-fold dilutions of each sample or positive control were prepared in PBS. Six hamsters per sample dilution were inoculated intra-cerebrally with 0.02 ml per animal. The inoculated animals were monitored daily for general health, and weekly for clinical evidence of scrapie. Animals were euthanized once advanced signs of scrapie were evident, or at the end of the assay period (200 days). The brain was removed from each hamster following euthanasia: one half was fixed for histopathology and the other half was stored frozen at −70 °C for further analysis if required. For histopathological analysis, sections taken at four standard coronal levels, to cover the nine areas of the brain which are recognized to be mostly infected by the scrapie agent, were stained with hematoxylin and eosin, and scored for the presence or absence of scrapie lesions [15]. Histopathological analysis was performed on samples from around the clinical end-point of the titration assays, to confirm the clinical results. Hamsters that died during the course of the study for reasons other than scrapie infection were not included in the final calculation of infectious titers. Infectious titers were expressed as a 50% lethal dose (LD_{50}) according to the method of Kärber [16].

Samples taken before and after filtration during the P-15N/antithrombin (AT; previously named antithrombin-III) study were tested for the presence of scrapie infectivity using a qualitative hamster bioassay. Syrian hamsters were inoculated with undiluted samples only, as described above, except that only three animals were used per sample.

2.5. Evaluation of PrP^{Sc} removal in the presence of plasma preparations

To investigate whether differences in how the scrapie spike material was prepared influenced our evaluation of prion removal, two different spiked preparations were compared using the manufacturing process for preparing AT (Neuart®, Benesis Corp., Osaka, Japan). Samples taken during the actual manufacturing process, immediately before the Planova step, were spiked with 263K MF treated with 0.1% (w/v) sarkosyl for 30 min at room temperature, or with 220 nm-filtered "super-sonicated" 263K MF. The spiked AT materials were then passed through a P-15N filter. The influence of different filtration conditions on the removal of PrP^{Sc} was compared for the same spike preparations, and for different spike preparations, using heat/PEG-treated intravenous immunoglobulin (IVIG) (Venoglobulin-IH, Benesis Corp.) and haptoglobin (Haptoglobin Injection-Yoshitomi, Benesis Corp.). Samples taken during the actual manufacturing process, immediately before the Planova step, were spiked with: 220 nm-filtered "super-sonicated" 263K MF (IVIG/P-35N and haptoglobin/P-35N); 263K MF ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended in buffer equivalent to the starting material without protein, "super-sonicated" and 220 nm-filtered (IVIG/P-20N); or 263K MF treated with 0.3% (v/v) TNBP/1% (v/v) Tween 80 for 6 h at 30 °C ("SD treatment"), ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended in saline, "super-sonicated", and 220 nm-filtered (i.e., haptoglobin/P-20N). The spiked material was then passed through either a P-35N filter or a P-20N filter (19 ± 2 nm). Although not part of the manufacturing process for haptoglobin, the SD treatment was included for the spiked preparation in an effort to reduce the clogging of the filter that occurs following the addition of a prion spike. Filtration processes for the thrombin preparation (Thrombin-Yoshitomi, Benesis Corp.) were also investigated. For thrombin, a sample taken during the actual manufacturing process immediately before the Planova step was spiked with 263K MF subjected to "SD treatment" followed by ultracentrifugation at 141,000 × *g* for 60 min at 4 °C, resuspended in the starting material, "super-sonicated" and 220 nm-filtered, and the spiked material then passed through a P-15N filter.

The experimental conditions used in the prion removal studies were designed to mimic the conditions used during the actual manufacturing process for the relevant product. For all processes, samples were analyzed by WB. The $\log_{10}$ reduction factor (LRF) for PrP^{Sc} was calculated for each

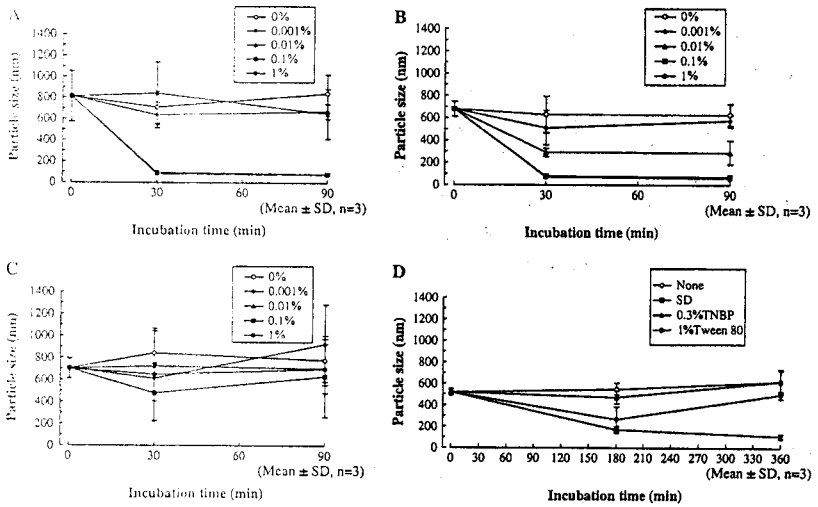

Fig. 1. Change of particle size in normal MF following treatment with various detergents. To normal MF, sarkosyl (A), lysolecithin (B), or Triton X-100 (C) was added to a final concentration of 1%, 0.1%, 0.01%, and 0.001%, respectively. The change in the average particle size was then monitored at room temperature for 90 min. In addition, TNBP or Tween 80 was added to normal MF to a final concentration of 0.3% and 1%, respectively, either alone, or in combination ("SD treatment"). The change in the average particle size was then monitored at 37 °C for 6 h (D).

with 0.1% sarkosyl, 0.1% lysolecithin, "SD treatment", or "super-sonication". The use of detergent or "SD treatment", in combination with "super-sonication", was also shown to effectively reduce the average particle size in normal MF preparations, to comparable levels to the individual treatments alone (data not shown). "Super-sonication" has an advantage over the other treatments in that it can minimize the change of composition of samples taken from the manufacturing process, as it does not require the addition of reagent(s) to the normal MF. For this reason, "super-sonication" is considered to be a useful approach for the treatment of 263K MF for process evaluation. "SD treatment", although slightly less effective, is used in many manufacturing processes, and may therefore be useful alone, or in combination with "super-sonication", for the process evaluation of products whose manufacturing process includes an "SD treatment" step. These approaches, alone or in combination, may also be useful to prevent the clogging of filters that can occur during spiking studies.

3.2. Infectivity of PrP^{Sc} in 263K MF and influence of 263K MF preparation methods on infectivity

The effect of "super-sonication" and "SD treatment" on the infectivity of 263K MF was studied. Infectious titers of

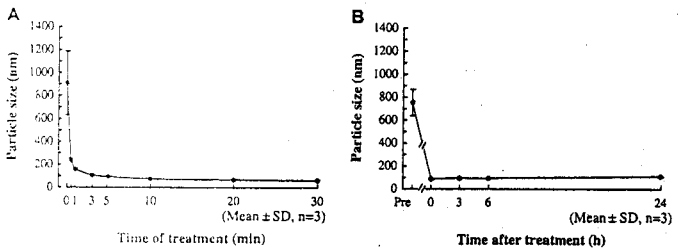

Fig. 2. Change of particle size in normal MF following intense sonication ("super-sonication"). Normal MF in a test tube equipped with a resonance chip (20 kHz, 200 W) was sonicated for 1 min in an ice bath. After 1 min, the sonication step was repeated. The change in average particle size was monitored during 30 cycles of sonication (A). After 10 cycles of sonication ("super-sonication"), normal MF was held at room temperature for 24 h, and the change in particle size was monitored (B).

263K MF, "super-sonicated" 263K MF, and 263K MF subjected to "SD treatment", ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended with thrombin starting material, "super-sonicated", and 220 nm-filtered, were determined using a hamster bioassay. The results are summarized in Table 1.

The titers of two independent batches of 263K MF treated by "super-sonication" were 6.0 and 5.3 $\log_{10}$ LD_{50}/ml, respectively. The titer of the "non-super-sonicated"263K MF used to generate one of these stocks was 5.7 $\log_{10}$ LD_{50}/ml. These results suggest that "super-sonication" does not influence the infectivity of 263K MF. The titer of the 263K MF subjected to "SD treatment", ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended with the thrombin starting material, "super-sonicated", and 220 nm-filtered, was 6.9 $\log_{10}$ LD_{50}/ml, which was approximately 1 log higher than that of the corresponding stock treated by "super-sonication" alone. Whether this difference is significant is unclear. The process to generate the "SD-treated" spike materials included an ultracentrifugation step. We were therefore concerned about recovery of infectivity following centrifugation, as the particle size of 263K MF was highly reduced by the "SD treatment" step. However, these results suggested that the recovery of infectious particles following ultracentrifugation was satisfactory.

Although it is possible that use of a 200 day bioassay may under-estimate the infectious titer of the 263K MF stocks, the use of a relatively short duration bioassay is considered unlikely to affect the main conclusions drawn. At least the last two dilution groups tested showed no animals with evidence of scrapie infection in all four titrations, and only three animals in the study (one in each of three separate titrations) developed clinical symptoms necessitating euthanasia later than day 131 (euthanized on days 160, 183 and 183, respectively), suggesting the titers obtained for all the stocks are close to end-point (data not shown). In addition, as others have demonstrated that treatment with detergent, and exposure to treatments that result in inactivation of the scrapie agent, such as heat or NaOH, may result in extended incubation periods for clinical scrapie, if anything the results may under-estimate the relative titers of the treated stocks [17,18]. Therefore, the bioassay results support the conclusion that "super-sonication" of 263K MF stocks, with or without "SD treatment", does not appear to significantly reduce the infectious titer of the stock, and that these preparations are therefore suitable for use in prion clearance studies.

3.3. Removal of PrP^{Sc} by various filters

To determine whether "super-sonication" influenced the $\log_{10}$ reduction observed for PrP^{Sc} following filtration under defined conditions, "super-sonicated" or "non-super-sonicated" stocks of 263K MF were diluted in PBS, and then filtered through 220 nm, 100 nm, P-75N, P-35N and P-15N filters. Samples were analyzed by WB. The results are summarized in Table 2. The use of "super-sonicated" 263K MF appeared to result in lower $\log_{10}$ reduction values, supporting the idea that "super-sonication" of 263K MF produces a

Table 4
Scrapie [illegible] samples generated using the P-15N/AT study

	Before filtration			Filtrate		
	Animal number			Animal number		
	1	2	3	1	2	3
Appearance of clinical signs (day euthanized)	87	87	87	94	143	105
PrP^{Sc} in brain by WB	Detected	Detected	Detected	Detected	Detected	Detected
Lesions by [illegible]	+ve	+ve	+ve	+ve	+ve	+ve
Medulla [illegible]	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P
Cerebellum [illegible]	D	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P
Midbrain	D,P	D,V,P	V,P	D,P	D,P	D,V,P
Hypothalamus	D,P	D,V,P	D,P	D,V,P	D,P	D,P
Thalamus	D,P	D,V,P	D,P	D,P	D,P	D,P
Hippocampus	NR	D,V	D	D	D,V,P	D,V
Parahippocampal gyrus	D,P	D,P	D,P	NR	D,V,P	P
Cerebral cortex (at lateral midline)	D,P	D,P	D,P	D,P	D,V,P	D,V,P
Cerebral cortex (at dorsal midline)	D,P	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P

Abbreviations: +ve, scrapie positive; NR, no remarkable change; D, degeneration of nerve cell; V, vacuolation; P, proliferation of glial cell.

corresponding hamster brain material on histopathological observation (Table 4). Typical nerve lesions are shown in Fig. 3. Thus, P-15N filtration did not result in the complete removal of infectivity for this process step.

4. Discussion

In this study we have investigated the capacity of P-35N, P-20N and P-15N filters to remove the 263K scrapie prion protein, PrP^{Sc}, under the conditions used for the manufacture of four different plasma-derived products, using spike preparations designed to present a serious challenge to the filters.

Validation studies to evaluate the capacity of manufacturing processes to remove potential contaminants, including prions, are required for biological or biopharmaceutical products intended for human use. When designing these studies, a worst-case challenge should be used wherever possible, to minimize the risk of over-estimating the capacity of the process to remove such contaminants. Virus removal filters (or nanofilters) are designed to remove contaminants predominantly on the basis of size. The worst-case challenge for such steps should therefore be a preparation containing the smallest possible form of the contaminating agent.

TSE clearance studies provide a particular challenge in that the nature of the infectious agent is still uncertain, and the forms of infectious agent present in plasma, and/or during the different stages of a manufacturing process, are not clearly understood. The causative agent of TSE diseases is believed to be strongly associated with, if not solely composed of, the disease-associated prion protein, PrP^{Sc}. Normal cellular PrP is a membrane-bound glycoprotein, which associates with membranes through a glycosylphosphatidylinositol (GPI) anchor. Prion infectivity is associated with heterogeneous particles, including membranes, liposomes and protein aggregates, so called prion rods. Therefore, methods which result in solubilization of membrane proteins, or dispersal of membrane fragments, vesicles and/or protein aggregates, may be expected to reduce the size of particles associated with prion infectivity.

Treatment of MF preparations derived from brains of uninfected (normal) hamsters with either detergent (0.1% lysolecithin or 0.1% sarkosyl) or extensive sonication ("super-sonication") resulted in a rapid reduction in the average particle size, to approximately 100 nm. SD treatment (1% Tween 80 and 0.3% TNBP for 6 h) also resulted in a reduction in particle size, although this was slower and less effective, reducing the average particle size to the order of 200 nm.

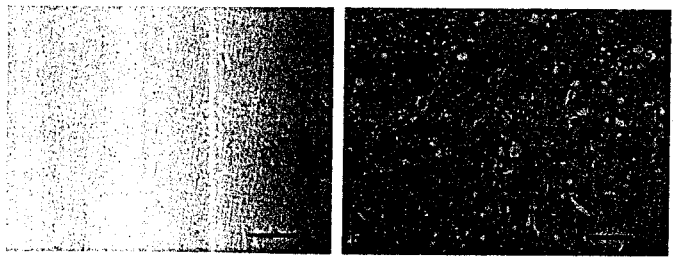

Fig. 3. Typical [illegible] lesions in the hippocampus of a hamster brain, taken from an animal inoculated with a P-15N-filtered sample (B), in comparison with the corresponding region from an uninfected normal (A). Arrows, vacuolation; Arrowheads, degeneration of nerve cells; scale bar = 50 μm; HE staining used.

"Super-sonication" has the advantage that it is a physical disruption process, and does not alter the chemical composition of the spike material, thus minimizing changes to the start material used for nanofiltration. SD treatment is included in many manufacturing processes for plasma-derived products, and therefore, although not as effective as "super-sonication", use of this treatment might be expected to result in a spike material more closely mimicking the form of infectious prion present in the relevant start material during the manufacturing process. Use of these treatments alone or in combination may therefore be useful in reducing the size of infectious particles present in TSE spike materials for prion clearance studies.

The effect of the above treatments was studied using normal MF, as the facility was unable to handle infectious TSE materials. Although some care should be taken in extrapolating these results to TSE-infected brain material, "super-sonication" of 263K MF preparations appeared to reduce the removal of PrP^{Sc} following filtration, while detergent-treated spike preparations have previously been shown to present a more significant challenge to nanofiltration steps than untreated preparations ([9,10] and own unpublished observations). Furthermore, "super-sonication", with or without SD treatment, does not appear to reduce the level of infectivity present within the 263K MF, supporting the use of such preparations for prion clearance studies.

Using 263K MF treated with 0.1% sarkosyl, "super-sonication" or SD plus "super-sonication", we investigated the prion removal capacity of P-15N, P-20N and P-35N filters in the manufacturing processes used for four different plasma products. The results obtained suggest that both the composition of the materials to be filtered and the prion load influences the removal of prions. PrP^{Sc} was recovered in the filtrate fraction from three out of the four processing steps performed for P-20N and P-35N. In contrast, under all conditions tested, P-15N filtration resulted in removal of PrP^{Sc} to below the limit of detection of the Western blot assays used. Thus, P-15N would appear to be a more robust method for the removal of prions, reproducibly giving LRF in the order of 3 logs, under the conditions tested. In practice, however, it is not feasible to incorporate P-15N filtration into the manufacturing process for all plasma derivatives. From the results shown in Table 2, it may also be possible to optimize processing conditions to allow effective removal of PrP^{Sc} using P-20N or P-35N filters.

WB assays were used to monitor the partitioning of PrP^{Sc} during the nanofiltration processes. WB assays are semi-quantitative and serve to provide an indication of the relative levels of PrP^{Sc} present in different samples. However, there are limitations to the sensitivity of available WB assays, and these assays provide only an indirect measure of infectivity. Therefore, to confirm that removal of PrP^{Sc} does reflect removal of infectivity, bioassays need to be performed.

Although PrP^{Sc} was not detected in any of the P-15N filtered samples by WB assay, infectivity was recovered in a filtrate fraction tested by bioassay for one process run. Foster also noted that infectivity was detected in a filtrate fraction after P-15N filtration ([8] reported as personal communication; data not shown). Thus, even with P-15N, depending on the

Planova filters. Some of the data presented in this study has been summarized in a recent review [22].

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CJD PrP^{sc} removal by nanofiltration [illegible] therapeutic immunoglobulin solution [illegible]

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Abstract

The characteristic of transmissible spongiform encephalopathies (TSE) is an acc[illegible] prion protein (PrP^{sc}). This pathological prion protein is very resistant to conventional [illegible] methods. The [illegible] TSE, such as Creutzfeldt–Jakob disease (CJD), by biopharmaceutical products prepared from [illegible] must be ta[illegible] nanofiltration process has been proved to be effective in removing viruses and scrapie agent. [illegible] efficacy in removing infectious particles without altering biopharmaceutical charact[illegible] properties.

This study focused on the removal of human PrP^{sc} by means of a nanofiltration [illegible] a CJD brain homogenate. Lymphoglobuline® equine anti-human thymocyte immun[illegible] mainly on human T lymphocytes. The therapeutic indications are:

- immunosuppression for transplantation: prevention and treatment of graft rejection[illegible]
- treatment of aplastic anemia.

In our study, CJD homogenate was spiked at three different dilutions [illegible] filtration process was performed on each sample. Using the western blot technique, [illegible] that obtained with a reference scale (dilution series of CJD brain homogenate in Lym[illegible] 3.3 log). After nanofiltration, the PrP^{res} western blot signal was detected with a sig[illegible] was undetectable in the two other samples.

These are the first data in CJD demonstrating a clearance between 1.6 and [illegible] Lymphoglobuline® [illegible] nanofiltration process confirms its relative efficacy in removing human CJD PrP^{sc}.

Keywords: TSE; CJD; Prion protein; Nanofiltration

1. Introduction

The safety of biopharmaceutical products used for human therapy has taken on the same importance as the therapeutic effects; this point was highlighted these last years by the contamination of children developing CJD after extractive growth hormone therapy using unsafe lots with respect to prion disease. More than [illegible] children died in [illegible] are reported [illegible] clinical progres[illegible]

Products of human origin have [illegible] from therapeutic [illegible] but some [illegible] blood cells can [illegible] as reagents [illegible] purification steps [illegible] pharmaceutical [illegible] ity has never been [illegible] in human [illegible] process able [illegible] prion infe[illegible] same extent [illegible] transferre[illegible] (viruses, [illegible] be of [illegible]

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Prion infectivity is closely related to neurological disorders called TSE which include human diseases such as Creutzfeldt–Jakob disease (CJD), Fatal Familial Insomnia and Gerstmann–Straussler Scheinker syndrome.

CJD is classified as sporadic, genetic, iatrogenic including the CJD variant associated with bovine spongiform encephalopathy. The infectious agent responsible for this disease was called a "prion" by Prusiner [1]. It is a glycoprotein which is normally present in the physiological form (PrP^c) which becomes pathological in CJD (PrP^{sc}). The transition process from the physiological to the pathological form is complex [2]. Scientists have demonstrated a trans-conformational change between both protein forms [3]. PrP^c primarily contains α helix, though PrP^{sc} contains more β sheets in its three-dimensional structure [4]. CJD is characterized by intra-cerebral accumulation of abnormal prion protein which is partially protease resistant (PrP^{res}). Cleavage of PrP^{sc} by proteinase K results in two types of PrP^{res} after western blot, type 1 and type 2, according to Parchi's classification [5].

Studies on the elimination of viral contamination from biopharmaceutical products (which are prepared from human cells) have been conducted using nanofiltration [6–8]. This nanofiltration method has been effective on many viruses. Albumin solution safety nanofiltration has been conducted on scrapie prion protein [9].

The purpose of this research is to study the efficacy of nanofiltration on CJD PrP^{sc} in order to introduce this method into the manufacturing process of a therapeutic immunoglobulin solution Lymphoglobuline®.

Lymphoglobuline® equine anti-human thymocyte immunoglobulin is a selective immunosuppressive agent acting mainly on human T lymphocytes. It recognizes most of the molecules involved in the cascade of T-cell activation during graft rejection, such as T-cell receptor and CD3, HLA class 1 molecules, CD4 and CD8 co-receptors, co-activation molecules or adhesion molecules CD2, CD5, and CD18. The therapeutic indications are the followings:

- immunosuppression for transplantation: prevention and treatment of graft rejection;
- treatment of aplastic anemia.

During the process of purification of the equine anti-human thymocytes a step of nanofiltration was added for the viral security.

2. Materials and methods

2.1. Biopharmaceutical product

Lymphoglobuline® is an anti-thymocyte equine immunoglobulin that induces immunosuppression as a result of T-cell depletion and immune modulation. It is approved for the prevention and treatment of rejection episodes in kidney, pancreas or liver transplantation. In hematology, Lymphoglobuline® is approved for treatment of aplastic anemia and in the treatment of steroid resistant graft versus host disease.

In the Lymphoglobuline® manufacturing process, human thymocytes, membrane red blood cells and placenta are used. These human elements represent a virtual potential source of contamination of Lymphoglobuline®.

2.2. Human source of pathological prion protein

After the histological, immunohistochemical and biochemical analyses of post-mortem human brains, one case of definite CJD, and one non-CJD were chosen. The anatomic site chosen was the frontal cortex. The CJD case selected was characterized by the presence of PrP^{res} type 1 in western blot analysis according to Parchi's classification and by synaptic deposits of PrP^{sc} with an immunohistochemical technique. The same human cortex was used as source of PrP^{sc} for the reference scale and for the nanofiltration samples.

2.3. Sample preparation

2.3.1. Human brain homogenate

Frontal cortex of CJD and non-CJD cases was spiked in PBS buffer, 1:10 at final dilution. These homogenates were filtered successively with needles of 0.6 mm and 0.5 mm diameter in order to obtain homogenous preparation. After centrifugation at 1000g for 5 min, supernatants were applied to nanofiltration process.

2.3.2. Reference scale

This reference scale was prepared with series of dilutions of CJD brain homogenate in Lymphoglobuline® from 1:10 to 1:20,000. This reference scale was based on the technique used by Lee et al. [10,11].

2.3.3. Nanofiltration samples

These samples were prepared using CJD brain homogenate dilutions in Lymphoglobuline®. Three different samples were produced; samples at a high PrP^{sc} dilution (1:500), samples at a moderate PrP^{sc} dilution (1:100) and samples at a low PrP^{sc} dilution (1:10). Each dilution was prepared for three samples, one non-nanofiltrated (control) and two nanofiltrated. These samples were prepared as a reference scale with an adaptation of the method used by Lee et al. [11].

2.4. Filtration

Small-sized (membrane diameter: 47 mm) Pall® filters (hydrophilic Polyvinylidene fluoride microporous membrane) with mean pore sizes for Pall® DVD of about 0.1 μm, Pall® DV50 of about 50 nm and Pall® DV20 of about 20 nm were used successively in the nanofiltration process. The filtration mode was conducted at a constant membrane pressure of 3 bars. The samples underwent nanofiltration in the following order: negative control, CJD samples at a high PrP^{sc} dilution (1:500), CJD samples at a moderate PrP^{sc} dilution (1:100), CJD samples at a low PrP^{sc} dilution (1:10) and negative control (Fig. 1).

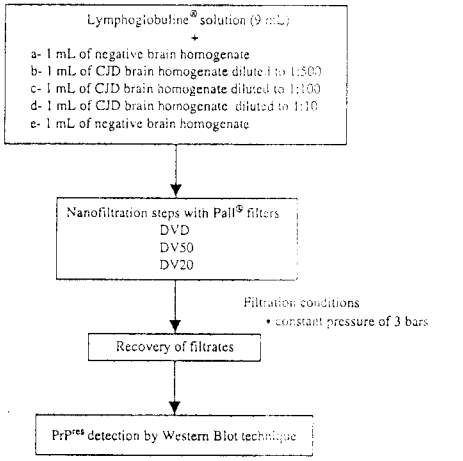

Fig. 1. Nanofiltration process. (a) Negative control sample at 1:10 in Lymphoglobuline®; (b) CJD sample at a high PrP^{sc} dilution (1:500) in Lymphoglobuline®; (c) CJD sample at a moderate PrP^{sc} dilution (1:100) in Lymphoglobuline®; (d) CJD sample at a low PrP^{sc} dilution (1:10) in Lymphoglobuline®; and (e) negative control sample at 1:10 in Lymphoglobuline®, ×2, produced in duplicate.

The nanofiltration material was treated with sodium hydroxide (2 M) for 1 h between each nanofiltration of different PrP^{sc} dilution samples.

2.5. PrP^{res} detection

The western blot technique was used to detect PrP^{res} after proteinase K treatment [12]. The anti-prion protein antibody revealed three strips of a molecular weight between 30 and 22 kDa (Fig. 2) corresponding to the biglycosylated, monoglycosylated and unglycosylated forms. Then, PrP^{res} was revealed by chemiluminescence. This technique was used to detect PrP^{res} in reference scale samples and in samples before and after nanofiltration.

The reference scale samples and samples for nanofiltration were produced and developed by the western blot technique under the same conditions and in the same time.

2.6. Determination of reduction factors

The reduction factors defined as the reduced titer versus the real titer present in the spiked sample were determined by comparing the PrP^{res} signal of samples before and after nanofiltration with the PrP^{res} signal of reference scale. After this comparison, we determined a reduction factor (log) for each sample.

3. Results

The reference scale ranges from 1:10 to 1:20,000 dilutions of CJD brain homogenates. From the 1:10 to 1:2000 dilutions,

processes [14]. Effective methods include for example exposure to 1 M sodium hydroxide during autoclaving at 121 °C. This kind of method using chemical agents (sodium hydroxide, chlorine at high concentrations) and physical treatment by autoclaving is very drastic and it is a real problem to inactivate PrP^{sc} in biopharmaceutical products without modifying their therapeutic properties. The reduction of any risk associated with a pharmaceutical product will be dependent on the physical removal of infective material during product manufacture. Many techniques for plasma-derived products, such as ethanol fractionation, depth filtration and chromatographic processes, may contribute to a significant partitioning of prion protein [10,15–18]. Although early applications of nanofiltration targeted viral removal [7,8,19], new data suggest that it may be a specific removal system for prion proteins as well. Human TSE pathogens in diluted brain homogenate were reported to be removed by a Millipore screen-type 0.025 μm membrane filter employed during production of growth hormone [20]. However, only a small quantity of diluted brain homogenate could pass through the membrane. Planova® cartridges with mean pore sizes from 75 to 10 nm were used to filter brain homogenate from mice infected with human TSE [21]. No infectivity was detected in the 35 nm filtrate. The pathogenic agent was estimated to be approximately 40 nm in size. However, some residual infectivity was found in the 10 nm filtrate when 1% Sarkosyl was added to the homogenate [22]. Recently, removal of scrapie agent ME7, a mouse adapted strain of scrapie used as a model for the BSE or vCJD agents by using nanofiltration of a 2% albumin solution spiked with a brain homogenate [23]. The albumin recovery was over 90%. Extent of removal was influenced by the filter type and by the addition of an anionic detergent (Sarkosyl) to the protein solution. An infectivity of 4.93 and 1.61 log was removed using a 35-nm filter without and with detergent, respectively. Moreover, a reduction of infectivity of >5.87 and 4.21 log was obtained using a 15-nm filter in the absence and presence of detergent, respectively. No residual infectivity was detected in any filtrate when using 15 nm or smaller porosity filters. Studies have shown an efficacy of 35–15 nm filters in achieving some removal of prions from biological solutions with the best removal with a 15-nm filter. The data, although encouraging, should be analyzed more accurately due to the tendency of prion spikes to aggregate under the experimental conditions used and with human prion protein because this removal could be dependent on the "strain" of prion protein.

In our study, we wanted to study the efficacy of nanofiltration on human PrP^{sc} in a biopharmaceutical product (Lymphoglobuline®). We used human PrP^{sc} from CJD patients as the contaminant. This contamination condition was important to study the Lymphoglobuline® nanofiltration technique under conditions as close as possible to a possible contamination by human cells used for the preparation of this product. The extent of removal may be influenced by the aggregation, type [24] and conformation of prion proteins and the physicochemical nature of the solution filtered. These parameters were important to choose the PrP^{res} type for the study. Amyloid plaques or focal deposits of PrP^{sc} still remain after homogenizing the cerebral cortex and the hypothesis was made that this kind of PrP^{sc} aggregation could be the result of a bias in the methodology. For this reason, PrP^{res} type 1 associated with synaptic deposits with an immunohistochemical technique was chosen in order to test the Lymphoglobuline® nanofiltration process under worst conditions to test the filters. In this study, Lymphoglobuline® was spiked with brain homogenate at different dilutions (1:10, 1:100, and 1:500). These PrP^{sc} dilutions can be correlated with World Health Organization (WHO) classification of organ infectivity: the low PrP^{sc} dilution corresponding to 1:10 (brain and spinal cord), moderate PrP^{sc} dilution corresponding to 1:100 (spleen, tonsil, lymph node, intestine, placenta...) and high PrP^{sc} dilution corresponding to 1:500 (brain stem, thymus, liver, pancreas, lungs...).

The comparison of the samples before and after nanofiltration showed a reduction factor between 3.3 and 1.6 log in comparison with the reference scale. The reduction factor of samples at a low PrP^{sc} dilution (1:10) was between 3 and 3.3 log. This dilution could correspond to a brain or a spinal cord PrP^{sc} concentration (WHO). The reduction factors for a very high PrP^{sc} concentration obtained illustrate a very good efficacy of the nanofiltration process.

In samples at a moderate PrP^{sc} dilution (1:100) and samples at a high PrP^{sc} dilution (1:500), the PrP^{res} strips were not detected after nanofiltration, the reduction factor was strictly greater than 2.3 and 1.6 log, respectively. The 1:100 dilution could correspond at a spleen or tonsil or lymph node or intestine or placenta PrP^{sc} concentration (WHO) and the 1:500 dilution could correspond to a brain stem or thymus or liver or pancreas or lungs PrP^{sc} concentration (WHO). In conclusion, the data obtained on both these PrP^{sc} dilutions are encouraging because, after nanofiltration, the PrP^{res} signal was not detected, although they are only indicative with probably underestimated reduction factors. Finally, the reduction factor obtained is 3.3 log and seem to demonstrate the efficacy of the nanofiltration process on human CJD PrP^{sc} with a good protein recovery.

Removal may be based on a sieving mechanism or due to adsorption on the membrane. The potential to use nanofiltration as a dedicated step for prion removal may have a significant impact on the safety of biopharmaceutical products and recombinant proteins, when production involves the use of human or animal derived materials, or medicinal products derived from bovine sources [25,26]. This technique has the ability to extend the concept of sterility of biological products from bacteria to, at least, some viruses. Our results suggest that nanofiltration could be also of interest for the removal of human pathological prion proteins.

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