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 (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 filtration run, by comparing the total amount of PrP^{Sc} present in samples before and after filtration. All studies involving the use of WB1 and 2, and the quantitative bioassays, were performed in facilities in compliance with current GLP regulations. Studies involving the determination of average particle size in normal MF preparations, the use of WB3, and the qualitative bioassay, were performed as non-GLP studies.

3. Results

3.1. Influence of MF preparation method on particle size distribution

Ideally, to represent a "worst case" challenge for a filter, the smallest form of prion protein, or infectious agent, should be used. Studies to investigate the optimum method for preparing the prion spike material were therefore performed. In these studies, changes in the average particle size in normal MF were investigated, as 263K-infected brain material could not be handled within our facility. Although prion particles in MF derived from 263K-infected brain material were not investigated directly, we tried to optimize the design of our experiments by minimizing the size of particles in normal MF, as particle size may influence filtration performance (both with respect to filter blockage, and removal of PrP^{Sc}). The results are shown in Figs. 1 and 2.

Treatment of normal MF with sarkosyl or lysolecithin reduced the average size of particles to approximately 100 nm, when 0.1% or higher concentrations of the detergents were used. However, below that concentration, the particle size did not change significantly, with the exception of 0.01% lysolecithin which reduced the average particle size to approximately 300 nm (Fig. 1A,B). Treatment with Triton X-100 did not result in a significant change in particle size, even at 1% (Fig. 1C). Treatment with 0.3% TNBP or 1% Tween 80 alone was not able to reduce the particle size below 200 nm. However, when combined, one of the conditions generally used for viral inactivation ("SD treatment"), 0.3% TNBP and 1% Tween 80 reduced the average particle size to below 200 nm (Fig. 1D). These results suggest that the reduction in average particle size in normal MF depends on the choice of detergent(s), and the concentration and combination of detergent(s) used.

We also studied the effect of "super-sonication" on the particle size in normal MF. The results showed that "super-sonication" could reduce the average particle size to a very fine level in a short time, without the need to change the composition of the normal MF material (Fig. 2A). Since "super-sonication" is a temporary physical procedure, reversal of the particle size reduction may possibly occur. To exclude this possibility during the experiments, we conducted a stability study on the particle size in normal MF after "super-sonication". There was no significant change in the particle size up to 24 h after "super-sonication", with the size remaining at approximately 100 nm (Fig. 2B).

The results showed that the particle size of normal MF preparations could be reduced significantly by treatment

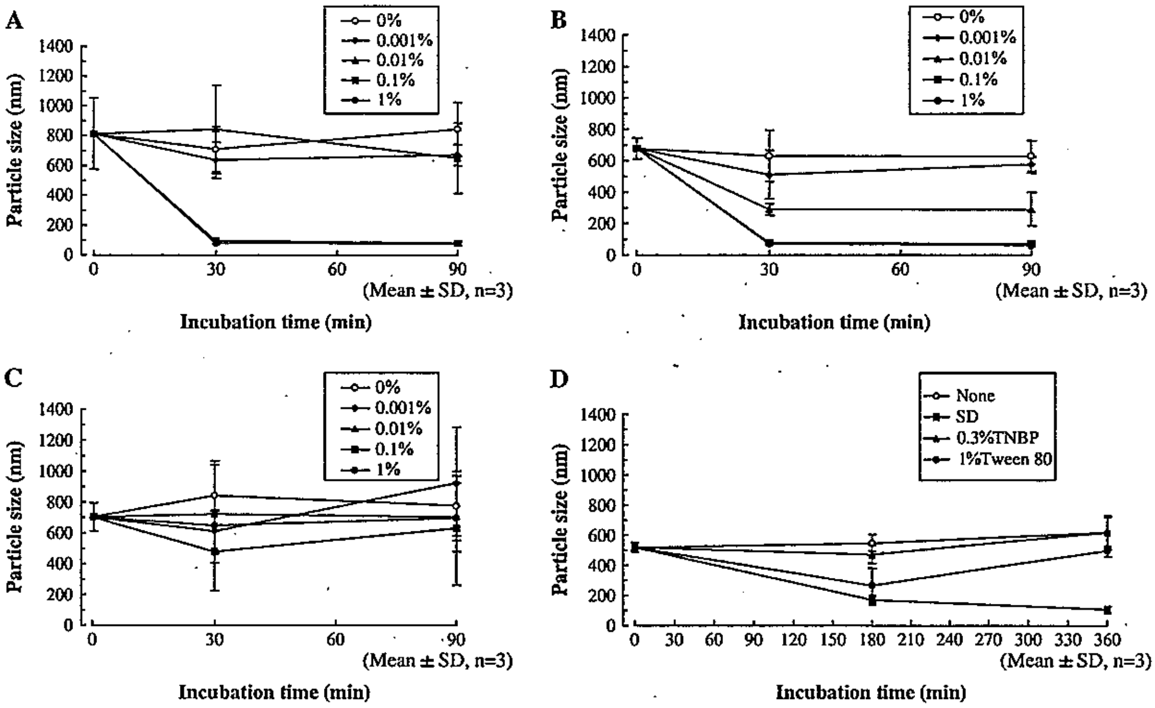

Fig. 1. Change of particle size in normal MF following treatment with various detergents. To normal MF, sarkosyl (A), lysolecithin (B), or Triton X-100 (C) was added to a final concentration of 1%, 0.1%, 0.01%, and 0.001%, respectively. The change in the average particle size was then monitored at room 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

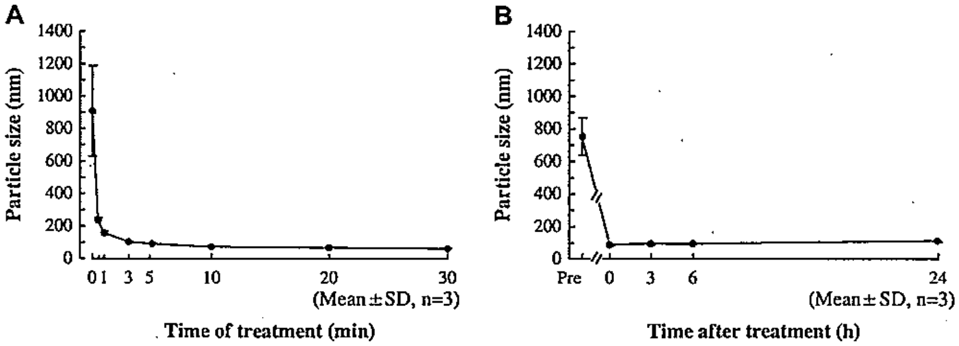

Fig. 2. Change of particle size in normal MF following intense sonication ("super-sonication"). Normal MF in a test tube equipped with a resonance chip (20 kHz, 200 W) was sonicated for 1 min in an ice bath. After 1 min, the sonication step was repeated. The change in average particle size was monitored 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 more severe challenge for a filter step. An approximately 5-fold higher $\log_{10}$ reduction factor was observed for "non-super-sonicated" stocks, for the 100 nm and P-75N filters, for both stocks tested. No significant loss of PrP^{Sc} was observed with either spiking material with 220 nm filtration, and no PrP^{Sc} was detected in the filtrates following P-35N and P-15N filtration.

Previously, we have observed some removal of PrP^{Sc} in some lots of "non-super-sonicated" 263K MF by 220 nm filtration. Strict control of the methodology used to generate the 263K MF stocks appeared to prevent this, suggesting that the method of preparing the 263K MF itself may influence the particle size distribution (data not shown).

3.4. Removal of PrP^{Sc} by Planova filters in the presence of plasma preparations

Removal of PrP^{Sc} by P-15N, P-20N, and P-35N filters was evaluated in the presence of a number of different plasma preparations, under conditions designed to mimic the relevant manufacturing process. The design of the experiments was similar to that of virus clearance studies. Samples were analyzed by WB, and the $\log_{10}$ reduction factor (LRF) was calculated for each filter step. The results are shown in Table 3.

Under all the experimental conditions tested, PrP^{Sc} was not detected by WB after filtration through P-15N. The LRF values were ≥ 2.8. In contrast, PrP^{Sc} was detected by WB in samples following filtration through P-20N and P-35N filters, in three out of the four processes tested, giving LRF values in the order of 2 logs. In one study, P-35N/haptoglobin, using "super-sonicated" 263K MF, PrP^{Sc} was not detected in the filtrate. However, the sensitivity of this study was low, giving a LRF of ≥ 1.4, and therefore the robustness of this filtration process was not evaluated. In the initial studies (Table 2), PrP^{Sc} was not detected in the fractions after P-35N filtration of either "super-sonicated" or "non-super-sonicated" 263K MF in PBS, resulting in log reduction factors in the order of 3 logs. The variance in the results obtained for these filters could be due to a combination of factors, including how the scrapie spike material was prepared, the composition of the starting material, and the precise filtration conditions used.

3.5. Removal of prion infectivity by Planova filters in the presence of plasma preparations

P-15N filtration was shown in these studies to be able to remove PrP^{Sc} to levels below the detection limit of the WB assays used, regardless of the method used to prepare the spike material, the composition of the start material, or the filtration conditions. However, a bioassay study for samples generated in a P-15N/AT study using 220 nm-filtered "super-sonicated" 263K MF, demonstrated that infectivity was recovered following filtration, as clinical signs appeared in all hamsters inoculated with the filtrate, and analysis of hamster brain material confirmed the clinical results. PrP^{Sc} was detected in the brain homogenates from all clinically positive hamsters by WB, and scrapie-associated lesions were observed in all the

Table 4
Scrapie infectivity in samples generated during 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 WB3	Detected	Detected	Detected	Detected	Detected	Detected
Lesions by histopathology	+ve	+ve	+ve	+ve	+ve	+ve
Medulla (oblongata)	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P
Cerebellum (cortex)	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
Paraterminal body	D,P	D,P	D,P	NR	D,V,P	P
Cerebral cortex (posterior midline)	D,P	D,P	D,P	D,P	D,V,P	D,V,P
Cerebral cortex (anterior midline)	D,P	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P

Abbreviations used: +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 infectious 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 ("supersonication") 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.

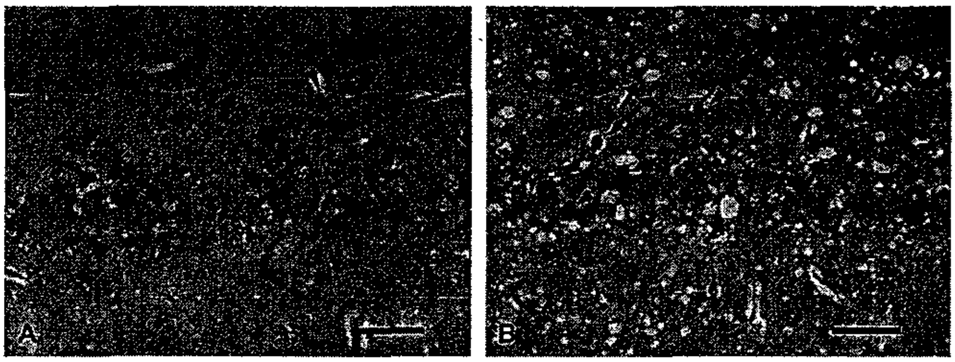

Fig. 3. Typical nerve lesions in the hippocampus of a hamster brain, taken from an animal inoculated with a P-15N-filtered sample (B), in comparison with the corresponding region from an uninfected animal (A). Arrows, vacuolation; Arrowheads, degeneration of nerve cells; scale bar = 50 μm; HE staining 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 processing conditions, there may be incomplete removal of prion contaminants.

Although infectivity was detected in the filtrate fraction from the one process step studied, longer and more variable incubation periods were observed in the animals inoculated with the filtrate sample (Table 4), suggesting a lower prion titer following filtration. However, it was not possible to estimate the relative levels of prion infectivity present in the input and filtrate samples, as no data was available to correlate incubation periods and prion titers for this study. Based on the titers typically observed for 263K MF stocks, the bioassay used could theoretically detect reductions in prion infectivity in the order of 4 logs for this process step. Detection of infectivity in the filtrate fraction by bioassay is therefore not necessarily incompatible with the WB results obtained (LRF $\geq$2.8 logs), and may simply reflect a difference in sensitivity between the two assays used.

As discussed above, uncertainties about the nature of the infectious agent in plasma, and during the manufacturing process, raise concerns about the design and interpretation of prion clearance studies. No single spike preparation is likely to contain all potential forms of the infectious agent. Infectivity is associated with membranes and protein aggregates. In addition, it has recently been shown that the GPI anchor is not required for infectivity, suggesting that endogenous proteolytic release of PrP^{Sc} from cell surfaces may also contribute to the spread of the infectious agent *in vivo* [19,20]. Whether significant levels of infectivity in human plasma are associated with GPI-anchorless prion protein is not yet clear. These different forms of infectivity, with different biophysical properties, could show different partitioning properties through the same manufacturing process [7]. Furthermore, different forms of the agent may differ in their level of infectivity. For example, it was recently reported that particles in the order of 17–27 nm appeared to have the highest relative level of infectivity, in comparison to levels of PrP^{Sc} [21]. Therefore, a better understanding of the nature and forms of the infectious agent is essential to allow the design of more accurate models for prion clearance studies, and a more confident evaluation of the safety of manufacturing processes with respect to potential TSE contamination.

In summary, we used methods intended to reduce the size of particles present within MF preparations in an effort to present a worst-case (smallest) prion challenge during nanofiltration. Using such preparations, P-15N filtration consistently reduced the level of PrP^{Sc} to below the limits of detection of the Western blot assays used, suggesting that this process step is effective for the removal of prions. However, data from a single process step studied suggested that infectivity could be recovered following P-15N filtration, and thus even P-15N filtration may not result in complete removal of prions, at least when used under some conditions.

Acknowledgements

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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 process: Application to a therapeutic immunoglobulin solution (Lymphoglobuline®)

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Abstract

The characteristic of transmissible spongiform encephalopathies (TSE) is an accumulation of partially protease resistant (PrP^{res}) abnormal prion protein (PrP^{sc}). This pathological prion protein is very resistant to conventional inactivation methods. The risk of transmission of TSE, such as Creutzfeldt–Jakob disease (CJD), by biopharmaceutical products prepared from human cells must be taken into account. The nanofiltration process has been proved to be effective in removing viruses and scrapie agent. The major advantages of this technique are flexibility and efficacy in removing infectious particles without altering biopharmaceutical characteristics and properties.

This study focused on the removal of human PrP^{sc} by means of a nanofiltration method after spiking a Lymphoglobuline® solution with a CJD brain homogenate. Lymphoglobuline® equine anti-human thymocyte immunoglobulin is a selective immunosuppressive agent acting mainly on human T lymphocytes. The therapeutic indications are:

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

In our study, CJD homogenate was spiked at three different dilutions (low, moderate and high) in the Lymphoglobuline® product. The nanofiltration process was performed on each sample. Using the western blot technique, the PrP^{res} signal detected in nanofiltrates was compared to that obtained with a reference scale (dilution series of CJD brain homogenate in Lymphoglobuline® detected by western blot and elaborated on 3.3 log). After nanofiltration, the PrP^{res} western blot signal was detected with a significant reduction in the less dilute sample, whereas the signal was undetectable in the two other samples.

These are the first data in CJD demonstrating a clearance between 1.6 and 3.3 log with a Lymphoglobuline® recovery of over 93%. The nanofiltration process confirms its relative efficacy in removing human CJD PrP^{sc}.

Keywords: TSE; CJD; Prion protein; Nanofiltration

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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 90 children died in France and young adults are reported to show clinical progression of iatrogenic CJD.

Products of human origin have generally been withdrawn from therapeutic protocols but some human products such as blood cells can be used as reagents needed in different purification steps for biopharmaceutical products. Though infectivity has never been detected in human red blood cells, a safety process able to decrease prion infectivity significantly to the same extent as infectivity transferred by conventional agents (viruses, bacteria, etc.) could be of great interest.

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