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研究報告の概要

ウイルス不活化方法を検証することは、すでに病因が判明しているエマージング・ウイルス病原体に対し、血漿製剤が安全であるという信頼感を与える。伝達性海綿状脳症（TSE）あるいはプリオン病といったあまり病因が明らかでないもの―ヒトにおいてはクロイツフェルトヤコブ病とその変異型―について、懸念が生じている。現在の研究目標は、アルブミンや免疫グロブリン製剤の製造工程でのTSE因子除去の可能性のため、免疫グロブリンにナノ濾過を利用することに関して検討することである。この調査のために、異なる2つのスクレイピーモデル系が用いられた。第一の系でスパイク用に用いられた感染試料は、ハムスターで測定された感染価のスクレイピー羊脳ホモジェネートであった。第二の系では精製されたスクレイピー因子が用いられ、プロテイナーゼK抵抗性の低下を測定するためにウェスタンブロット法により解析された。データは、両モデル系において製造過程において感染性因子は十分に除去されていることを示唆している。ハムスターにおける感染力の研究は、ウェスタンブロットアッセイよりも、約1,000倍感度が高いことを示した。両研究データは、これらの血漿製剤が、TSE伝播の可能性に関して安全であるという更なる信頼感を与えるものである。

報告企業の意見	今後の対応
アルブミンや免疫グロブリン製剤の製造工程でのTSE因子除去の可能性のため、免疫グロブリンにナノ濾過を利用することに関して検討した報告である。	これまでの疫学研究等では、ヒトにおいて、血漿分画製剤を介してvCJDが伝播するという証拠はない。また異常プリオンがアルブミン製剤の製造工程で効果的に除去されるとの報告もあるが、理論的リスクを完全には排除できないため、今後も情報の収集に努める。

使用上の注意記載状況・その他参考事項等

クロスエイトM250
クロスエイトM500
クロスエイトM1000
血液を原料とすることに由来する感染症伝播等
理論的なvCJD等の伝播のリスク

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Partitioning of TSE infectivity during ethanol fractionation of human plasma

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Abstract

The practice of validating processes for their capacity to inactivate a range of non-enveloped and enveloped viruses also provides confidence that plasma products will be safe from emerging viral pathogens with known aetiology. Of greater concern are diseases of unknown or poorly defined actiology such as the group of neurological diseases collectively called the transmissible spongiform encephalopathies (TSEs), or prion diseases, for which the best known human disease is Creutzfeldt–Jakob Disease (CJD) and its variant form (vCJD). The goal of the current study was to investigate the potential for manufacturing steps used in the production of albumin and immunoglobulin products by Kistler–Nitschmann fractionation, and the utility of nanofiltration of immunoglobulin to remove TSE agents.

Two different scrapie model systems were used. In the first system infectious material used for spiking was scrapie sheep brain homogenate with infectivity titres being measured in hamsters. In the second system purified scrapie agent was used (PrP fibrils) with Western blot analysis measuring reduction in the proteinase K resistant form being used as a measure of removal.

The data demonstrated substantial removal of the infectious agent by the manufacturing process in both model systems although some differences were observed in partitioning of the two different infectious materials. The hamster infectivity studies were shown to be approximately 1000 fold more sensitive than the Western Blot assay.

The data from both studies provide added confidence that these plasma products are safe with respect to their potential to transmit TSE.

1. Introduction

The screening of donors, testing of donations for viral markers and the use of viral inactivation and elimination steps, result in the provision of plasma derived products having excellent safety records with respect to transmission of the major blood borne viral pathogens (HIV, HBV, HCV) [1,2]. The practice of validating processes for their capacity to inactivate a range of non-enveloped and enveloped viruses also provides confidence that plasma products will be safe from emerging viral pathogens with known aetiology. Of greater concern are diseases of unknown or poorly defined aetiology such as the group of neurological diseases collectively called TSEs, or prion diseases, for which the best known human disease is CJD.

The aetiologic agent of the TSE diseases is disputed. The prion hypothesis proposes that the infectious agent is the TSE prion protein PrP, which has an abnormal conformation and spreads infection by inducing conformational change in normal PrP proteins [3]. Other investigators, claiming the case for prions has been overstated, emphasize the virus-like properties of the TSE agents or the involvement of a nucleic acid component [4–6]. Human TSEs include sporadic and iatrogenic CJD and a variant form vCJD that is believed to have evolved through the consumption of cattle products infected with bovine spongiform encephalitis [7,8].

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Quantitation of risk to the blood supply from TSE diseases cannot be assessed because the infectivity titre in the human blood as well as the incubation time of the infection in humans is unknown. However, low levels of TSE infectivity can be detected and have been quantified in the blood of experimentally infected laboratory rodents [9], and infectivity has been transmitted from one rodent to another by transfusion in the laboratory [10] (MacAuley et al., personal communication). More recently, Houston et al. have demonstrated infectivity in blood of naturally infected sheep and in blood of sheep experimentally infected with bovine spongiform encephalopathy and scrapie [11,12]. Most importantly, the sheep experiments indicated that infectivity is present at a pre-clinical phase of the disease and it can be transmitted to other animals by transfusion. In addition, at least in one case, blood-borne TSE was detected in non-human primates intra-cerebrally challenged with a purified fraction of buffy coat [13]. From these studies, it appears that TSE blood infectivity is present and can be detected in blood of experimental animal models. On the other hand, there is still no evidence that CJD or vCJD has ever been transmitted from one human to another by the use of blood or blood products. Epidemiological studies of human blood transfusion show that transfused blood has not been associated with an increased risk for CJD [14] nor has there been a case of CJD among the haemophiliac population which is regularly and frequently exposed to plasma products [15,16]. Additionally, there have been no reports of vCJD transmission by blood or blood products [17].

In individuals with vCJD, the disease-specific form of the prion protein, PrP^{res}, has been found in both tonsils and appendix, organs involved in the processing of blood cells, whereas PrP^{res} is not found in these organs in individuals with classical CJD [18,19]. This, along with pathological and clinical manifestations that are distinct from classical CJD, suggests that transmissibility by blood and plasma products might be greater for vCJD. Thus, although no proof of human to human transmission has surfaced, it is not possible to conclude that blood and blood products cannot and will not transmit vCJD and similar diseases.

The theoretical risk of transmission of vCJD by blood and blood products has led to a range of precautionary initiatives being taken by regulatory agencies. The UK ceased fractionating plasma from British donors in 1998 due to the risk of vCJD exposure. However, whole blood and cellular components are still collected and transfused in Britain and there is still no evidence of transmission of vCJD by blood or blood products. As of 1999, the US FDA recommended deferring blood donors who had spent 6 months or more in the UK since 1986. Other countries also implemented this practice. Whether or not these deferrals increase the safety of plasma products remains unknown. Since people could harbour the infectious agent for well over 10 years without showing any clinical signs of the disease, the majority of all plasma pools that are fractionated anywhere in the world could theoretically contain at least one donation obtained from an "at risk" donor [20].

What then can be done to improve the safety of blood and blood products with respect to transmission of vCJD? The traditional tools are screening tests, inactivation, and removal. Several laboratories throughout the world are currently working on the development of a mass screening test for blood and other tissues. However, the assay development presents enormous challenges. Inactivation of the CJD agent requires harsh conditions [21,22] and is usually incompatible with blood cells and plasma proteins. Nanofiltration, increasingly used for the removal of viruses from plasma proteins, has been used for TSE removal with some success [23,24]. Since TSE infectivity depends on the aggregation state of the agent, additives such as detergents greatly influence the efficacy of nanofiltration. In addition, pre-treatment (sonication, filtration through 0.1 μm filter, etc) of the brain homogenate before nanofiltration is also critical to the removal of TSEs [24]. It is therefore accepted that TSE removal by physical methods such as filtration, precipitation, nanofiltration and others must be experimentally determined and it cannot be extrapolated by analogy to similar studies. In the absence of any test for the TSE agent in human blood, the best approach available to provide confidence that the plasma products are free from TSE agents is to examine the potential of the manufacturing process to remove or inactivate model TSE agents.

We know from past experience that viruses tend to accumulate in precipitates during cold ethanol fractionation and subsequent filtration in the presence of filter aids [25] and are thus removed in discarded precipitate fractions [26]. Since it is also known that TSE agents have a propensity for adhering to surfaces [27], we wanted to investigate whether TSE agents can be physically removed from plasma products prepared by ethanol fractionation.

In the study described here, fractionation steps used in the preparation of plasma have been tested for their potential to remove scrapie infectivity and purified PrP fibrils, respectively.

2. Materials and methods

2.1. Plasma fractionation-spiking with brain homogenate

Plasma was fractionated according to the method of Kistler and Nitschmann [28]. The production scale method had previously been down-scaled and the laboratory scale procedure validated against production scale (results not shown). The volumes used in the

laboratory were on the order of 40 to 180 ml. For each step indicated in Fig. 1, aliquots from production scale runs were spiked with 1% of their volume of scrapie-infected hamster brain homogenate (see below) and subjected to fractionation. The liquid phase at each step was titrated in hamsters, after phase separation. Titration of the solid phase (filter aid plus adsorbed proteins and mother liquor) was difficult, because recovery of infectivity in the presence of filter aid has not been investigated. Step 4 in Fig. 1 incorporates two filtrations: filtration a and filtration b. We decided to perform step 4 in two different ways. In one experiment, separate samples were obtained from production (step 4a and step 4b), spiked, filtered, and the filtrates assayed for infectivity. In the second experiment, we spiked another sample at step 4a, but carried out filtration a, the subsequent pH4/pepsin treatment, and filtration b in sequence and measured infectivity in the second filtrate.

2.2. Preparation of scrapie brain homogenate-spiked solutions

A suspension of 10% (w/v) brain homogenate from hamsters infected with the 263K strain of scrapie was prepared, sonicated, and used as the spike material for the experiments described. The titre of the scrapie brain homogenate was 9.00 $\log_{10}ID_{50}$/ml as calculated by the endpoint dilution method [29]. A 1:100 dilution of scrapie brain homogenate into each production scale run at steps 1, 2, 3, 4, 4a, 4b and 5 was prepared and the spiked solutions were fractionated according to the protocol outlined in Fig. 1.

2.3. Titration of infectivity

Infectivity in the solid material used for filtration (filter cakes) was not investigated. Aliquots from the challenge and the filtrate from each step of the protocol in Fig. 1 were titrated by bioassay. That is, serial 10-fold dilutions of each challenge and filtrate were prepared and 50 µl aliquots of each dilution were inoculated intra-cerebrally into hamsters. The clinical progression of the disease was closely monitored and recorded. Sick animals were sacrificed when they reached the clinical stage of inability to rear and to feed. All surviving animals were terminated at one year after inoculation. The brain of each animal was harvested and one hemisphere was used for biochemical confirmation of the disease by the presence of PrP^{res} on Western blot (see below). The results of the clinical assessment of the animal together with the Western blot results were evaluated and a final score (of scrapie-infected or normal) was assigned to each animal. At the completion of the study, all data were tabulated and the Reed and Muench method was used to estimate the ID_{50} endpoint [29]. The logarithmic removal factor (LRF) was calculated as the difference between the infectivity in the spike and the infectivity recovered in the filtrate. The final results after normalization to the spike are reported in Table 1 and in Fig. 3.

2.4. Western blot analysis and proteinase K digestion of hamster brain homogenate

Brain tissue was homogenized by sonication in 50 mM Tris, pH 7.4 and 10 mM NaCl, to give a final concentration of 10% brain homogenate. For each brain sample, two 100 µl aliquots were taken, and SDS was added each to give a final concentration of 2%. One aliquot was treated with proteinase K ("PK", Roche Diagnostics, Indianapolis, IN, USA) at a final concentration of 100 µg/ml and the other aliquot was incubated in the absence of PK. After incubation for 20 min at 37 °C, 5 µl of 0.25 M phenylmethylsulfonyl fluoride (a PK inhibitor) was added to both samples, followed by boiling the samples for 10 min. Samples with and without PK were mixed with SDS–PAGE loading buffer (50 mM Tris pH 6.8, 20% glycerol, 0.02% bromophenol blue, 2% SDS and 0.1 M 1,4-dithiothreitol) and boiled for 5 min. Samples were electrophoresed on a pre-cast 14% SDS–PAGE gel (Invitrogen, Carlsbad, CA, USA) and the proteins were transferred from the gel on to Immobilon-P membrane (Millipore Bedford, MA, USA) in the presence of Tris/glycine buffer, 20% methanol and 0.1% SDS. The membrane was blocked with blocking buffer: 5% non-fat milk in TBST (10 mM Tris, pH 8, 150 mM NaCl and 0.05% Tween 20) for one hour and then probed with 3F4 antibodies (a gift from Dr Kascsak, IBR, NY, USA) at a dilution of 1:10,000 in the blocking buffer for one hour [30]. After extensive washing of the blot with TBST, the membrane was incubated with the secondary antibody conjugated to horse radish peroxidase (Amersham-Pharmacia, Piscataway NJ, USA) diluted 1:3000 in blocking buffer for one hour. After extensive washing of the blot with TBST, the membrane-bound PrP was detected using a chemiluminescence reaction (ECL Plus, Amersham Pharmacia, Piscataway, NJ, USA). Western immunoblots were analyzed for the presence or absence of PK-resistant PrP (PrP^{res}) and scored scrapie-positive or normal, respectively.

2.5. Plasma fractionation-spiking with PrP fibrils

In a parallel experiment, samples from the production scale runs were spiked with 1% of their volume of PrP fibrils (see below) and plasma separation was then performed as outlined in Fig. 1. The nanofiltration or step 5 was not analyzed in the fibrils experiment.

2.6. Preparation of fibril-spiked solutions

Fibrils were prepared according to Beekes M. et al. [31] with no major modifications. The final titre of the

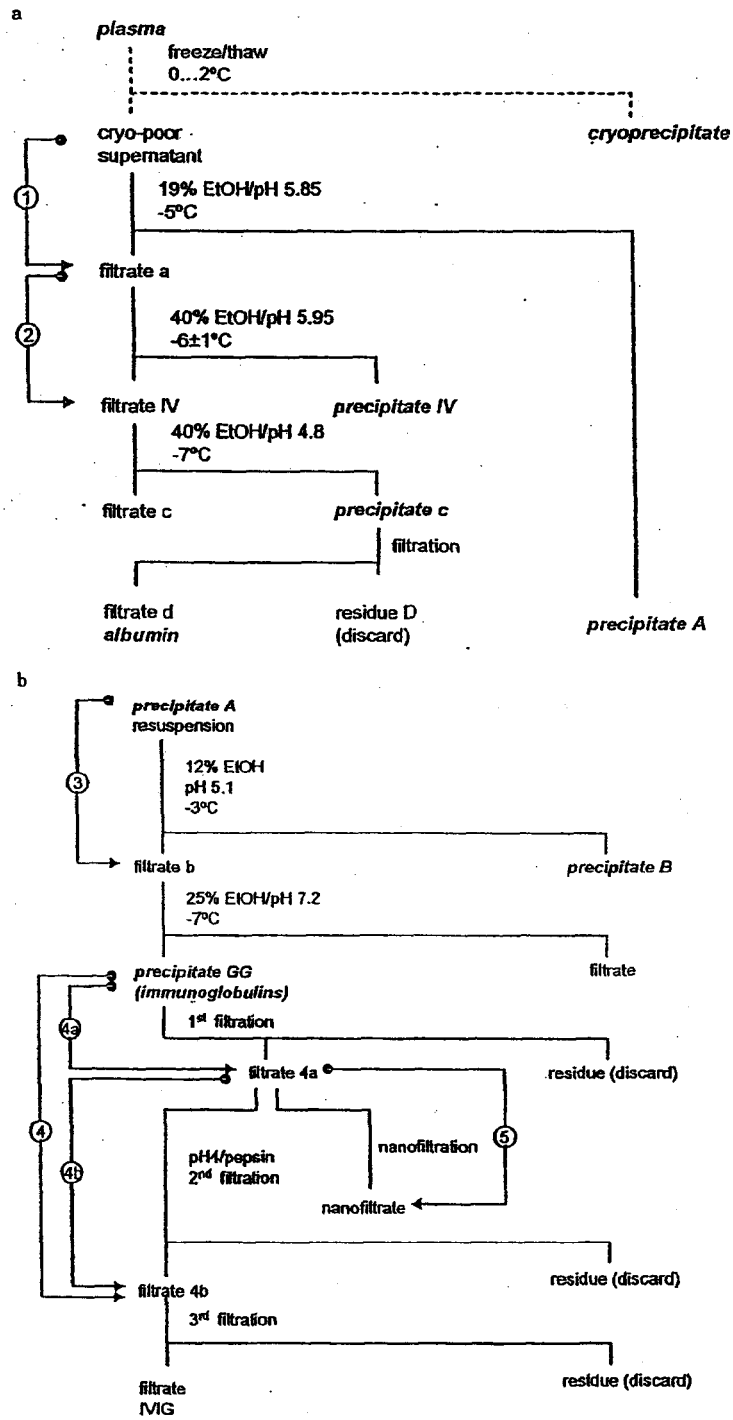

Fig. 1. a and b. Kistler–Nitschmann fractionation scheme. Flowsheet of ethanol fractionation of plasma according to the method of Kistler and Nitschmann illustrating the steps from production runs where clearance of scrapie-infected hamster brain homogenate was assessed. Filtrate a, IV and c are also referred to in the Kistler and Nitschman fractionation method as supernatant a, IV and c (shown in bold and italic). The steps tested for infectivity and fibril removal are numbered (steps 1–5). Each fractionation step was fulfilled with fresh feedstock and was subsequently spiked.

Table 1
Partitioning of hamster scrapie infectivity during cold ethanol plasma fractionation

Fractionantion step	Infectivity concentration		Fractional removal	Log_{10} reduction factor
	Expected with no removal	Measured after removal		
1	$9.2*10^6$	63,246	$6.9*10^{-3}$	−2.2
2	$9.6*10^6$	9283	$9.6*10^{-4}$	−3.0
3	$6.0*10^6$	2000	$3.3*10^{-4}$	−3.5
4a	$1.2*10^7$	431	$3.5*10^{-5}$	−4.5
4b	$1.4*10^7$	20,000	$1.4*10^{-3}$	−2.8
4	$9.0*10^6$	<0.63	$7.8*10^{-8}$	−7.2
5	$1.5*10^7$	632	$4.3*10^{-5}$	−4.4

purified fibrils was 10.7 $log_{10}ID_{50}$/ml as determined by the endpoint dilution method, with 38% recovery of infectivity. The purified fibrils were analyzed by Western blot to verify the complete digestion of PK-sensitive PrP. An aliquot of the purified fibrils was also analyzed by SDS–PAGE and the gel was stained with Coomassie Blue to determine the homogeneity of the preparation. No protein band other than PrP^{res} and a minor contaminant of proteinase K were detected. Fibrils were diluted 1:100 into each production scale run for step 1 to step 4b of Fig. 1a, and the fibril-spiked challenges were separated as described for brain-derived spike. Infectivity recovery for the experiment with fibrils was not measured. Instead, samples of the challenges, the filtrates and the filtercakes were collected and analyzed by Western blot for the presence of PrP^{res}. No PK digestion was necessary since the preparation of fibrils included PK digestion [31].

2.7. Treatment of the filter cake

The filtercake material was stored as a wet powder. An aliquot of the filtercake was removed, weighed and resuspended to a final concentration of 10% (w/v) in 2% SDS. The filtercake slurry was boiled for 10 min and microcentrifuged for 10 min. The supernatant containing solubilized and denatured PrP^{res} was analyzed as the filtercake.

2.8. Isolation and concentration of fibrils

Direct detection of PrP^{res} was complicated by the high plasma protein content in the spiked solutions and filter cakes, and by the extremely low concentration of PrP^{res} in the filtrates. Thus, a capture method which selectively captures PrP (Gregori et al., personal communication) was used for isolation and concentration of the fibrils to levels detectable by Western blot.

Due to different levels of PrP^{res} content, only 5 μl of challenge and 10 μl of filtercake were necessary for Western blot quantitation, while 200 μl of filtrate were used to detect PrP^{res}. Fibril-spiked samples were mixed with 10 μl of the affinity resin pre-equilibrated in 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100 and 0.5% Na deoxycholate. The unbound material was removed by centrifugation and the resin was washed three times with 1 ml of equilibration buffer. Proteins bound to the resin were eluted and analyzed by Western blot.

2.9. Semi-quantitation of fibril-derived PrPres signal on Western blot

Twelve microliters of the supernatant from the challenge, and two different volumes—0.3 μl and 15 μl—of the supernatant from the filtrates, corresponding to 1× and 50× volume equivalents respectively, were loaded on pre-cast 14% SDS–PAGE (Fig. 2A). Similarly, 12 μl of the supernatant from the filter cake were analyzed by Western blot (Fig. 2B). After immunostaining of the blots with 3F4 antibody, STORM fluorimager (Molecular Dynamics, Amersham, Sunnyvale, CA, USA) was used to quantitate the ECL Plus signal. In the quantitation experiments, the whole filtrate was run in one lane to maximize sensitivity of detection and three dilutions were run for the spike and the filter cake. In the case of the filtrate, the PrP^{res} signal in each lane, measured in arbitrary fluorescence units (AFU), was normalized for a volume unit and expressed as AFU/volume unit of that filtrate (relative concentration). The total relative amount of PrP in each filtrate was calculated as the relative concentration multiplied by the total volume of the filtrate. After normalization to the spike, the fractional recovery of fibrils for each filtrate was calculated and reported in Fig. 3. In the case of filter cake, the relative concentration was expressed in AFU per gram of filter cake. The total relative amount of PrP^{res} in the filter cake was calculated by adjusting the relative concentration to the final weight of the filter cake. Multiple dilutions were run for the spike and filter cake samples and the data regressed with the equation $y=1/[a+b/(x^c)]$ where a was fixed as $a=2.5\times10^{-8}$ and c was fixed as $c=1.18$. The parameter b was varied to optimize a family of curves with the same slope at low concentrations and converging on $y=1/a$ at high concentrations. The displacement of the filter cake curves from the spike curves was taken as the dilution of filter cake relative to spike. Using PrP^{res} concentration values from the regression

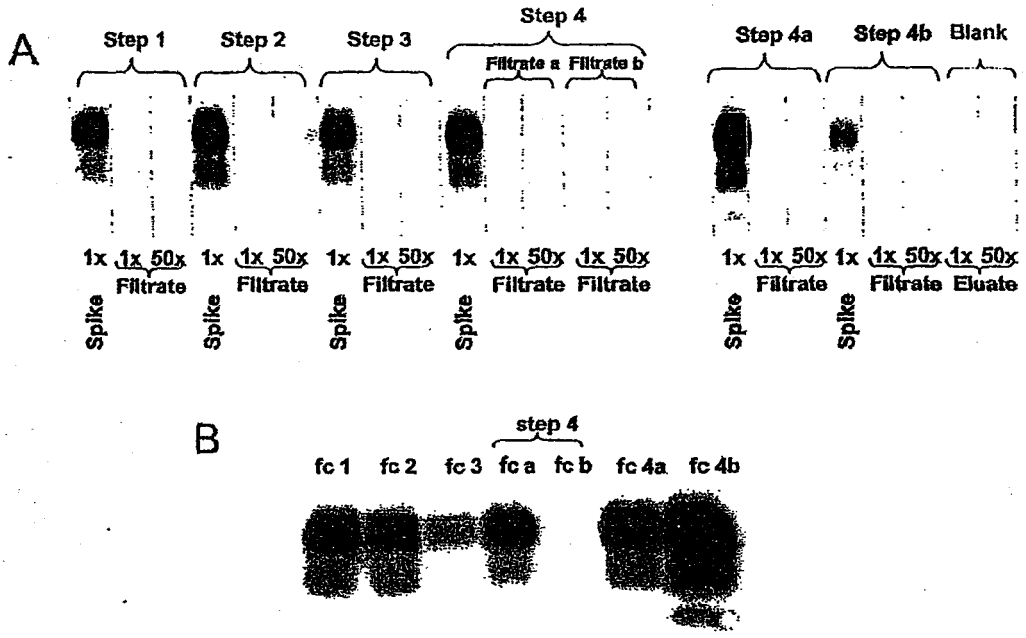

Fig. 2. Western blot analysis of fibrils immunoblots developed with 3F4 antibodies. Purified fibrils were spiked at each step of the Kistler–Nitschmann fractionation protocol (Fig. 1). At each step, a PrP concentration protocol was applied, and the challenge solution, the filtrate (panel A) and the filter cake (panel B) were tested for the presence of PrP^{res} by the Western blot method. In the lanes labelled (× 1), the same equivalents of challenge and filtrate were loaded. In the lanes labelled (× 50), a 50-fold greater filtrate volume equivalent was loaded. The two lanes labeled "blank" represent the negative control for the experiment showing no 3F4-immunoreactive protein was captured by the resin. Step 5 was omitted on this experiment.

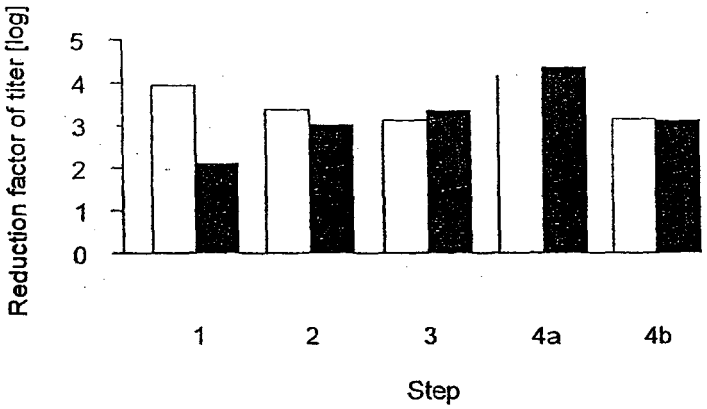

Fig. 3. Removal of PrP fibrils and infectivity-combined schematic representation of the calculated removal of infectivity (black bars) and PrPres (white bars). The results from Table 1 and quantitation of PrPres removal from Western blot analysis (Fig. 2) were combined in a single figure

curves for filter cakes normalized to spike, we were able to estimate the recovery of fibrils from the filter cake.

3. Results

3.1. Removal of infectivity

The fractionation scheme is shown in Fig. 1; the steps that were investigated in this study are numbered. The dotted lines at the beginning of the scheme indicate that removal of cryoprecipitate was not always performed; subsequent fractionation was not affected by the presence or absence of the proteins contained in cryoprecipitate, which only amount to about 1% of the total protein mass. Published work on the distribution of TSE infectivity in TSE-infected rodent blood has shown that cryoprecipitate contributes approximately 1% of the total blood infectivity [9].

Two titrations of the 10% scrapie brain homogenate used to spike all material obtained from production scale runs were performed. The two titrations indicated infectivity levels of 9.07 and 9.30 $\log_{10}ID_{50}$/ml for 10% brain homogenate. The average value of 9.19 $\log_{10}ID_{50}$/ml was used for calculation of the results shown in Table 1. Table 1 lists the expected and measured concentrations of the spike used in each of the various steps after phase separation. From these values the logarithmic reduction factors were calculated.

In step 1, we spiked cryo-poor supernatant with scrapie infectivity, performed the ethanol precipitation and measured the removal in Filtrate a. The conditions of the ethanol precipitation are described in Fig. 1. Infectivity recovery in Filtrate a was 4.8 $\log_{10}ID_{50}$/ml with more than 2 logs of removal of input infectivity. In the next step, we spiked a new solution of Filtrate a with scrapie brain homogenate and followed the ethanol precipitation conditions described in Fig. 1. The results indicated more than 3 logs removal of infectivity by this step. Steps 1 and 2 together are part of the albumin branch of the process. In step 3, we investigated the immunoglobulin branch of the purification procedure. Precipitate A from the step 1 obtained from ZLB's production plant was re-suspended and spiked with scrapie infectivity. Also in this step more than 3 logs of infectivity removal was demonstrated. Step 4 in the production scheme (Fig. 1) consisted of a tandem filtration on Filter cake a and Filter cake b. Step 4 was performed in two different ways. In one experiment, the same spike was carried through two consecutive steps: the solution of step 4 containing immunoglobulin was spiked, filtered on Filter cake a and the Filtrate a was filtered on Filter cake b. Infectivity was measured at the end of the second step. In the second method, a new spike was added to each step and infectivity was measured for individual steps, Filtrate 4a and Filtrate 4b. Step 4 was the only case in which the results from a sequential filtration (filtration a and filtration b) were compared with those of two individual spikes (step 4a and step 4b). The results showed a good agreement, i.e., 7.3 $\log_{10}ID_{50}$ for the combined removal and 7.2 $\log_{10}ID_{50}$ for the serial filtration (Table 1). In addition, in step 4, none of the animals inoculated with the supernatant Filtrate b showed any sign of scrapie. The titre of this solution was therefore calculated based on the worst case assumption that at the next higher concentration—had it been possible to assay it—all animals would have died of scrapie. The LRF of 7.2 therefore represents a lower limit.

3.2. Removal of PrP^{res}

In a parallel experiment, samples from the production scale runs were spiked with a purified fibril preparation. Although these samples were not tested for infectivity, we were interested in establishing whether the partitioning of fibril-derived PrP^{res} would follow that of brain-derived infectivity. The purified fibrils consisted, almost exclusively, of aggregated PrP^{res} as confirmed by SDS–PAGE.

The results of the Western blot analysis of the spiked samples and filtrates (panel A) and filter cakes (panel B) after the capture protocol are shown in Fig. 2. The last two lanes in Fig. 2A (blank) show the eluate of the resin which was incubated without fibril-containing samples. This control showed that no 3F4-immunoreactive protein band was eluted from the resin alone under the experimental conditions. When the same volume equivalents of spike and filtrate were loaded (×1) no signal was detected in the filtrate. These results were observed for all steps tested and indicated that every step of the protocol in Fig. 1 was effective in removing PrP^{res}. When 50 fold more volume equivalents were loaded for the filtrate samples (×50), a weak PrP^{res} signal was detected in steps 1, 2, 3 and 4 indicating that none of the filtration steps alone was able to capture and remove all PrP^{res}.

Assuming a correlation between PrP^{res} and infectivity, the presence of PrP^{res} in the filtrate indicates that none of the single steps was able to completely remove all input infectivity. This is consistent with the infectivity results for the brain homogenate experiment shown in Table 1 in which a reduction but not a total removal of infectivity was reported in the supernatant (filtrate) of each step with the exception of the tandem filtration in step 4. Fig. 2 shows that the two bands present in step 4 Filtrate a were still present in step 4 Filtrate b, suggesting that no detectable additional PrP^{res} removal was accomplished by the second filter cake. This observation was consistent with the results in panel B, in which step 4 Filter cake b shows no detectable PrP^{res}. However, this result is not in agreement with the infectivity data since step 4 Filtrate b solution showed no measurable infectivity in this fraction. This result could reflect subpopulations of PrP. When the experiment was repeated with two separate spike steps, step 4a and step 4b, the results showed PrP^{res} signal only in Filtrate b, with scarcely any signal in Filtrate a (Fig. 2A). Consistent with these observations, Filtrate 4b showed a partial infectivity removal equal to $10^{2.8}$ and Filtrate a showed the highest single step infectivity removal, equal to $10^{4.5}$. Lack of PrP^{res} signal in Filtrate 4a was attributed to the relatively limited sensitivity of the Western blot when compared to the animal bioassay.

In addition to the qualitative comparison between PrP^{res} recovery with a fibril spike and infectivity removal with a brain spike, we also attempted a semi-quantitative evaluation and comparison of the two experiments. Fig. 3 compares the quantitative results obtained from both the infectivity experiments obtained with brain homogenate and the Western blot analysis

obtained from the fibril experiments with the exception of nanofiltration which was not tested by fibril spike.

Infectivity reduction correlated well with the fractional fibril recovery for all steps except step 1 which represents the 19% ethanol precipitation. Differences in the removal of PrP^{res} between different spike preparations have been reported by Vey et al. [32].

Furthermore, infectivity results do not include filter cakes because infectivity was not determined in these fractions due to intolerability of i.c. injection of precipitates. The infectivity removed by each filtration step was presumed to be trapped in the filter cakes. Quantitation of the PrP^{res} signal trapped in the filter cakes was consistent with these conclusions. In fact, when the signals in Fig. 2B were converted into the total relative PrP^{res} concentration and normalized to the spike, all filter cakes, except step 4 Filter cake b, had a fibril fractional recovery >8%, thus, showing a relatively good recovery of the fibrils in the waste fractions.

4. Discussion

We used scrapie infected brain homogenate to spike the plasma fractions taken from our production plant. The results with brain spike demonstrated that the TSE agent was bound, to various levels, to all the filter aids used in the separation of protein precipitates by cold ethanol fractionation of human plasma. Because each solution was spiked anew at each step, the total infectivity removal for the whole plasma fraction scheme could not be determined experimentally. In virus validation studies it is common that the overall reduction of a process is estimated by adding the individual logarithmic reduction factors. However the correctness of this approach can be questioned. Step 4 is especially interesting in this context. We have investigated steps 4a (depth filtration after reconstitution of γ-globulin (GG) precipitate) and 4b (depth filtration after low pH treatment) separately and sequentially. The results showed that step 4a and step 4b, performed separately, had an LRF of 4.5 and 2.8, respectively, adding to a total LRF of 7.3. This was in excellent agreement with the value of 7.2 for the combined step 4, the latter being limited by the input titre. Although for some cases it is true that the overall removal of a multi-step process may not be equivalent to the sum of individual removal steps [33] this result clearly demonstrates that in this case the two process steps were additive.

Our results indicated a significant overall removal of scrapie infectivity in the purification of albumin and IVIG. Specifically, in the albumin branch of the process, we found 2.2 $\log_{10}$ removal of TSE infectivity in the preparation of Filtrate a (Fraction I+II+III) and 3 $\log_{10}$ of removal for Filtrate IV (Fraction IV) suggesting an overall reduction of 5.2 log. These results are in agreement with those published by other investigators [9,34–36]. Interestingly, Foster et al. and Lee et al. investigated TSE removal by Western blot and showed similar results to those presented here. In the immunoglobulin branch of the process, we found the ethanol precipitation and subsequent filtration in presence of filter aids resulting in Filtrate b and all other filtration steps investigated removed between 2.8 and 4.5 $\log_{10}$ of input infectivity, respectively, with an overall reduction >10 log.

It should be pointed out that these LRFs are minimum values, because some steps were not investigated for their partitioning potential, namely removal of residue D in the case of albumin and the third filtration in the case of immunoglobulin (Fig. 1).

Infected brain homogenate is by far the most common spike material used in TSE validation studies. One advantage is its high infectivity titre which allows the demonstration of a wide range of infectivity removal. However, brain homogenate is a crude material with a high level of protein and lipid content. We tested an alternative to brain homogenate, purified brain-derived fibrils. The advantage of the fibrils spike is that it retains a high concentration of infectivity but has a much more simplified protein content compared to that of brain homogenate, with no membrane-bound infectivity. Although fibrils were purified in the presence of detergents which, together with extensive sonication, improved the dispersion of TSE infectivity, fibrils remain in a highly aggregated state. It is unknown whether this may be very representative of blood infectivity. A more disperse and soluble infectivity could better represent blood-derived infectivity and endogenous infected blood is probably the most biologically relevant choice in spike material for the validation of blood and plasma products. However, such a material is seldom used in process validation studies of TSE removal due to the extremely low infectivity titre and high cost of the animal bioassay.

The choice of the spiking format for TSE infectivity removal studies is a critical parameter that must be carefully evaluated. In a recent study, several brain-derived TSE spike formats were tested in different plasma separation processes [32]. The results of that extensive analysis indicated that removal of TSE during the first two steps of the Cohn fractionation backbone, namely cryo-precipitation and 8% ethanol fractionation as determined by the conformation-dependent immunoassay was dependent on the type of spike used. In that context, the purified PrP^{sc} appeared to behave most differently from the other spike types tested. Further down the Cohn fractionation, reduction of the different spikes was comparable.

In the present studies, we analyzed the partitioning of fibril-derived PrP^{res} throughout the steps of the Kistler–Nitschmann plasma fractionation. Infectivity recovery for this experiment was not tested and thus, a direct

comparison to infectivity recovery in brain spike experiment was not possible. Lee et al. have demonstrated that the infectivity removal for several plasma purification steps measured by animal bioassay correlates positively with PrP^{res} removal as measured by Western blot signal [34,37]. Our results indicated that based on a qualitative analysis of the Western blot in Fig. 2, fibril-derived PrP^{res} was largely removed by each step in the plasma fractionation procedure but none of the steps alone completely removed infectivity. These results are consistent with brain-derived infectivity recovery.

When we combined the fibril-PrP^{res} quantitation results and the infectivity recovery results (Fig. 3) we found that all but one step showed a comparable removal irrespective of the spike's nature. These data are in good agreement with what is known from the literature, namely that at higher ethanol concentrations in plasma fractionation different spike types behave similarly [32].

Another question concerns the validity of using the hamster adapted scrapie strain as a model for human TSE's. A recent study by Stenland et al. addressed this issue. They investigated the partitioning of PrP^{sc} associated with the hamster adapted scrapie, human vCJD, human CJD, and Gerstmann–Sträussler–Scheinker syndrome (GSS), respectively. The outcome of this study clearly indicated that all PrP^{sc} partitioned equally [38].

All together, the data presented suggest that the Kistler–Nitschmann fractionation and subsequent polishing steps (filtration in presence of filter aids representing depth filtrations; nanofiltrtaion) have a high capacity in clearing TSE agents, thus rendering safer products.

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