Table 2 Distribution of bovine spongiform encephalopathy (BSE) 301V infectivity across different fractions collected during the purification of fibrinogen and factor VIII by ion-exchange chromatography

Stage/fraction	BSE titre ID_{50}/ml (log_{10})	Volume of fraction (ml)	Total BSE infectivity in fraction, ID_{50} (log_{10})	% BSE infectivity in fraction	Reduction factor (log_{10})
1. Microsomal inoculum	7·3[a]	10·0	8·3		
Factor VIII process					
2. Factor VIII solution (spiked)	6·7	93·2	8·7		
3. Factor VIII solution after S/D[b]	6·8	98·6	8·8	100·00	
4. Fibrinogen fraction (120 mM NaCl)	< 3·8	139·8	5·9[c]	0·13	≥ 2·9
5. Low-NaCl wash (145 mM NaCl)	< 3·4[d]	41·0	5·0[c]	0·02	≥ 3·8
6. Factor VIII fraction (250 mM NaCl)	4·8	20·0	6·1	0·20	2·7
Column cleaning					
7. First high-NaCl wash (2 M NaCl)	6·4[d]	15·2	7·6	5·75	1·2
8. NaOH wash (0·1 M NaOH)	< 3·2	39·0	< 4·8	< 0·009	> 4·0
9. Second high-NaCl wash (2 M NaCl)	< 3·2	8·1	< 4·1	< 0·002	> 4·7

[a]Transmissible spongiform encephalopathy (TSE) titre obtained previously [24].
[b]S/D, after treatment with solvent and detergent.
[c]Maximum value on the assumption that 100% of animals would have been positive if the sample had been tested at a 10^{-1} dilution.
[d]Approximate TSE titre, estimated from bioassay at one dilution using the dose–response curve obtained with sample 2.
ID_{50}, infectious doses 50%.

consistent with the original level of infectivity, suggesting that aggregates may have formed during the frozen storage of the microsomal fraction and that full dispersion was only achieved after the microsomal fraction had been added to the solution of intermediate-purity factor VIII. Although there was a small apparent increase in 301V titre following solvent/detergent treatment (Table 2), this was well within the margin of error for TSE bioassay titrations. Nevertheless, a small increase in TSE titre is often detected after mild detergent treatment or other disaggregating treatment (e.g. sonication) and is probably a result of disaggregation, but may also occur as an effect of the efficiency of titration [29].

The three fractions recovered from the ion-exchange process, including the factor VIII fraction, all contained 301V infectivity. However, the quantity of infectivity present in each of these fractions was much less than that of the starting material. From these data it was calculated that with respect to the feedstock to ion-exchange chromatography, 301V infectivity was reduced by 2·9 log_{10} in the fibrinogen fraction and by 2·7 log_{10} in the factor VIII fraction (Table 2). It was also estimated that less than 0·4% of the 301V infectivity present in the feed to the ion-exchange process (sample 3) was recovered in the fractions collected up to and including the factor VIII fraction (Table 2), indicating that 99·6% of the added infectivity remained bound to the ion-exchange matrix following the recovery of factor VIII.

In the procedure used to clean the ion-exchange gel between uses, we found that a significant degree of infectivity desorbed into the first 2 M NaCl wash (Table 2). Subsequently, no 301V infectivity was detected in either the fraction recovered following treatment with 0·1 M NaOH nor in the second 2 M NaCl wash, despite further protein being desorbed at this point, according to the OD_{280} profile (Fig. 2, peak f).

Discussion

Despite considerable concern that haemophilia patients might be infected with vCJD by treatment with plasma products [15,30–33], few studies have been undertaken to determine how TSE agents distribute over processes used to manufacture factor VIII concentrates [7,34]. In modern factor VIII concentrates, a high degree of purification is obtained by chromatographic processing [35], yet information is available from only two studies concerning chromatographic purification of factor VIII, both of which employed scrapie 263K as the TSE model. Foster *et al.* [16] reported a 3·1 log_{10} reduction of PrP^{Sc} using a DEAE ion-exchanger, and Drohan has observed removal of a total of 3·5 log_{10} ID_{50} over QAE ion-exchange and 4·6 log_{10} ID_{50} over immunoaffinity chromatography [34]. Similar studies concerning ion-exchange purification of albumin and other plasma proteins have reported log_{10} reduction factors ranging from 2·2 to 5·2 [16,36,37].

If the agents responsible for BSE and vCJD share distinct properties, which are preserved on transmission to humans and mice, then 301V in mice may be a more suitable model than scrapie in experiments of this type. Our study with 301V was designed to allow a comparison to be made with scrapie 263K and we found that the reduction factors for fibrinogen

agent. We used a microsomal fraction for this purpose, for two reasons: first, by removing whole cells and large fragments, the method of preparation was similar to the separation of plasma from whole blood; and, second, to permit comparison of the results from this study with those from our earlier experiments with the scrapie agent in which a microsomal fraction was also used [16,24]. No specific measurements were performed to characterize the microsomal fraction, other than to titrate it for TSE infectivity. However, no significant TSE reduction has been observed over leucofiltration, using either endogenously infected murine plasma [41] or blood spiked with the microsomal fraction [42], indicating that, with respect to leucofiltration, the microsomal fraction contains PrP^{Sc} of a comparable state to that derived from an endogenous source. Nevertheless, the extent to which 301V infectivity from the micosomal fraction represents the vCJD agent as it would exist naturally at the intermediate stage of the factor VIII manufacturing process, has still to be established. Finally, our measurements on the procedure used to clean the ion-exchange matrix, and our inability to achieve an exact mass balance, were limited by the sensitivity of the murine bioassay (Table 2). This was constrained by dilution of the samples to make them suitable for intracerebral inoculation, the small volume of sample tested and the number of animals employed, which was minimized for ethical reasons.

Conclusions

This experiment has resulted in a number of important observations. First we have confirmed that ion-exchange chromatography can substantially remove a BSE-derived agent from preparations of fibrinogen and factor VIII concentrate. Second, most of the added TSE agent remained bound to the ion-exchange matrix after elution of factor VIII. Third, the cleaning procedure used to sanitize the ion-exchange matrix between uses was effective in eliminating a significant proportion, and possibly all, of the BSE-derived agent that remained bound after the elution of factor VIII. Finally, our results were similar to those obtained previously using hamster-adapted scrapie, suggesting that scrapie 263K may be a suitable TSE model for using to estimate the partitioning behaviour of the vCJD agent over ion-exchange chromatography.

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Removal of TSE agents from blood products

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Introduction

Transmissible Spongiform Encephalopathies (TSEs) are fatal neuro-degenerative disorders. Creutzfeldt-Jakob disease (CJD) in humans is divided into classical CJD (cCJD), of which there are a number of forms (sporadic, familial, Gerstmann-Straussler-Scheinker (GSS) syndrome), and variant CJD (vCJD), the latter probably transmitted by food contaminated with bovine spongiform encephalopathy (BSE).

cCJD has been transmitted by medical procedures in which tissues with a high level of infectivity were involved [1] but transmission by blood products has not been observed [2] possibly because infectivity in blood is very low. By contrast, vCJD has probably been transmitted by transfusion of whole blood [3] consistent with experimental transmissions of BSE between sheep [4].

The prevalence of cCJD is 0·5–1·0 per million inhabitants per annum world-wide [5]. About 150 cases of vCJD have been recorded, but the subclinical prevalence of infection in the human population is not known. BSE has been discovered in over 20 countries and it is conceivable that large numbers of people have been exposed to infection. Without a suitable diagnostic test, the extent to which CJD agents may be present in blood donations is not known. It is therefore important to establish the extent to which TSE agents can be eliminated during the preparation of blood products.

TSE diseases are associated with conversion of prion protein (PrP) to a pathogenic conformation (PrPSc) that accumulates in the brain causing degeneration. TSE agents have been found to be highly resistant to physical and chemical treatments and methods for their inactivation [6] are too severe to be applied to blood products. Attention has therefore concentrated on removal using separations technologies. PrPSc has a number of properties which could be exploited to separate it from other biological substances; including a low solubility in aqueous solution, the ready formation of aggregates and a tendency to adhere to surfaces [7].

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Experimental approaches

Studies on the removal of TSE agents are not straightforward. Infectivity in blood is very low and the characteristics of the agent as it exists naturally in blood are not defined. Different experimental approaches have been adopted and it is necessary to appreciate their limitations.

Process scale-down

Experiments with infective material must be performed in containment facilities as TSE agents represent a bio-hazard. This, together with the difficulty of obtaining suitable infected tissue means that process studies are normally undertaken at small volume, typically 10–100 ml, whereas manufacturing processes operate at 100 s to 1000 s of litres. For results to be meaningful it is necessary to simulate the manufacturing operation reasonably accurately.

Form of TSE agent

Two basic forms of TSE material have been used: blood obtained from experimentally infected animals [8] and preparations derived from infectious brain tissue, such as brain homogenate (BH) [8], microsomal fraction (MF) [9], caveolae-like domains (CLD's) [10] and semipurified PrPSc [10]. Studies with infected blood are referred to as '*endogenous*' whilst those using brain-derived material are described as '*exogenous*'. In endogenous studies, the very low level of infectivity means that only a small degree of removal at the beginning of multistep processes can be observed. The higher titre of infectivity available in exogenous experiments enables greater capacities for TSE removal to be determined and more steps considered. However, there is uncertainty over the extent to which materials derived from brain represent TSE agents present naturally in blood.

Strain of TSE agent

Partitioning studies have been undertaken with a number of TSE strains. Endogenous studies have been performed with murine-adapted GSS, Fukuoka-1 strain [8], hamster-adapted scrapie, strain 263K (R. G. Rohwer, unpublished) and murine-adapted BSE, strain 301 V (H. E. Reichl, unpublished). Exogenous

experiments have employed high titre preparations infected with hamster-adapted scrapie (strains 263K [9], Sc237 [10] and ME7 [11]), murine-adapted BSE, 301 V [12] and three strains of human CJD, vCJD, sporadic CJD (sCJD) and GSS [13].

Determination of TSE agents

Two approaches have been used to determine the degree of removal of TSE agents: measurement of infectivity by rodent bioassay [8,12,14,15] and immuno-chemical determination of PrPSc using either Western blotting [9,16] or conformation-dependent immunoassay (CDI) [10]. Immunoassays are performed after PrP has been removed by digestion with proteinase-K (PK), PrPSc being resistant. Immunoassays are therefore dependent on the effectiveness of PK-digestion and the assumption that PrPSc is the infective agent, or that it partitions precisely with infectivity.

Studies on individual process steps

Leucocyte-filtration

Universal leucocyte-depleting-filtration of blood components was introduced as a precaution against vCJD transmission [17] following a report that B-lymphocytes were crucial to the pathogenesis of TSE disease [18], despite earlier findings [19]. In a small-scale study Brown *et al.* [14] filtered fresh plasma from symptomatic mice infected with GSS (Fukuoka-1 strain) using a white cell-reduction filter (Pall PLF1); no significant reduction in TSE infectivity was observed. Filtration has been studied at full-scale using a whole blood leucocyte-depleting filter (Pall WBF2) to filter 450 ml of blood from hamsters infected with scrapie-263K. Although infectivity was reduced by 45% (R. G. Rohwer, unpublished), this was within the error of the bioassay. Scrapie-263K was also employed in an exogenous experiment in which human blood spiked with MF was filtered using four different whole blood filters. Abnormal fragmentation of red cells occurred suggesting interference by the MF spike; nevertheless, no significant removal of PrPSc was observed over any of the filters [20]. Consequently, the ability of leucocyte-depleting filters to remove TSE agents from blood components has still to be established.

Protein precipitation

Separation of proteins according to differences in solubility is central to the manufacture of many plasma products. TSE partitioning has been studied over cryoprecipitation and a number of cold-ethanol precipitation steps (Table 1). Fraction III and Fraction IV, which are discarded from immunoglobulin and from albumin, respectively, gave a high degree of TSE removal. Separation is only achieved when the precipitate phase is removed from the solution phase. In routine manufacture, centrifuge supernatants are clarified by depth filtration to ensure that the resultant solutions are of uniform quality. Such filtration procedures are therefore an important adjunct to precipitation processes.

Depth filtration

In immunoglobulin manufacture, the supernatant remaining after removal of Fraction III (Supernatant III) and the solution obtained when Fraction II precipitate is re-dissolved are both subjected to depth filtration. Similarly in the preparation of albumin, both Supernatant IV and the solution obtained when Fraction V is re-dissolved are both treated by depth filtration. In these applications, added infectivity or PrPSc was removed to the limit of detection by Seitz filters, whereas filters from other manufacturers have given variable results (Table 2). PrPSc was not removed from Supernatant I by Seitz filtration [10], suggesting that the much broader spectrum of proteins present at this earlier stage of fractionation saturated the relevant binding sites on the filter. There are many types and grade of depth filter available and more comprehensive data are required to better define those suitable for removal of TSE agents.

	Foster	Lee	Lee	Stenland	Vey	Reichl
Ref	[9]	[15,16]	[15]	[13]	[10]	[12]
TSE agent	263K	263K	263K	vCJD	Sc237	301 V
spike	MF	BH	BH	BH	BH/MF/CLD/PrPSc	MF
assay	W blot	W blot	bioassay	W blot	CDI	bioassay
Log_{10} Redn						
cryopptn	1·0	1·0	1·0	0·9	0·3/0·2/0·4/2·4	
fraction I		1·1			0·9/0·9/0·7/3·1	
fraction II + III	1·3	≥ 4·7	6·0		3·6/3·1/3·1/4·0	
fraction (I) + III	≥ 3·7	≥ 4·3	5·3			2·1
fraction IV_1/IV_4		≥ 4·2/ ≥ 4·1	3·7/4·6			
fraction IV	≥ 3·0				3·2/3·4/3·2/2·2	

Table 1 Removal of TSE agents by precipitation, with each process studied individually

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Studies on process steps in sequence

As well as characterizing process steps individually, it is important to examine steps operated in sequence to determine if removal by successive steps is additive. The initial precipitation steps in plasma fractionation have been studied in endogenous [8,14, R. G. Rohwer, unpublished; H. E. Reichl, unpublished] and in exogenous [8,25] experiments. The results (Table 3) demonstrate a progressive reduction of the TSE agent over successive steps, indicating that different precipitation processes can complement one another. When precipitation was combined with depth filtration [12], or where two different filtration procedures were combined [12,22], the overall degree of TSE removal exceeded that of the first step but was less than the sum obtained from individual steps. These findings indicate that care must be taken in interpreting data obtained only from individual steps.

Conclusions

There is a body of data suggesting that processes by which plasma products are manufactured are capable of removing TSE agents. Nevertheless, there is uncertainty over the relevance of the spiking materials used in exogenous experiments and the range of steps studied in endogenous experiments has been restricted. Methods of detection are limited in sensitivity, and possibly in specificity. Additional studies are required, with advances in detection, to better determine the safety of plasma products.

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REVIEW ARTICLE

Factor VIII and transmissible spongiform encephalopathy: the case for safety

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Summary. Haemophilia A is the most common inherited bleeding disorder, caused by a deficiency in coagulation factor VIII (FVIII). Current treatment of haemophilia A is based on repeated infusions of plasma-derived FVIII concentrate or of recombinant FVIII, which may be exposed to plasma-derived material of human or animal origin used in its tissue culture production process. We review epidemiological and experimental studies relevant to blood infectivity in the transmissible spongiform encephalopathies (TSEs, or 'prion' diseases), and evaluate the hypothetical risk of TSE transmission through treatment with plasma-derived or recombinant FVIII.

Keywords: blood, factor VIII, prion disease, safety, transmissible spongiform encephalopathy, variant Creutzfeldt–Jakob disease

Haemophilia and replacement therapy

According to a survey of the World Federation of Haemophilia, approximately 400 000 individuals worldwide are affected with hereditary bleeding disorders that require lifetime therapeutic care. Haemophilia A is the most common bleeding disorder, which affects 1 : 5000 males and is caused by a deficiency or functional defects in coagulation factor VIII (FVIII) [1]. Haemophilia B or Christmas disease affects 1 : 30 000 males [2] and is caused by a hereditary defect in coagulation factor IX (FIX). Both conditions are X-linked recessive disorders caused by mutations in the corresponding genes, and are passed to the next generation through the female line. von Willebrand disease is a rare haemorragic condition, inherited in autosomal dominant fashion, caused by a deficiency or defect of von Willebrand factor (vWF), which leads to a secondary deficiency of FVIII [3].

FVIII is an essential component of the intrinsic pathway of the blood coagulation cascade. It serves as a cofactor for a serine protease factor IXa (FIXa), which, in its membrane-bound complex (Xase), activates factor X [4,5]. Activated factor X (FXa) then participates in the conversion of a zymogen prothrombin into thrombin, a key enzyme of the coagulation cascade. Subsequently, thrombin cleaves fibrinogen to fibrin and activates FXIII, which leads to formation of a stable clot. Immediately after release into circulation, FVIII binds to vWF to form a tight noncovalent complex. Association with vWF is required for maintaining the normal FVIII level in circulation and for preventing the interaction of FVIII with other components of the intrinsic Xase complex. In addition, vWF protects FVIII from inactivation by activated protein C, and activated FIX and FX. Upon activation of the FVIII/vWF complex by thrombin, FVIII is rapidly released from the complex with vWF [4,6].

While initiation of blood coagulation is ascribed to the extrinsic, tissue factor-dependent pathway in which small amounts of activated factors IX and X are generated, the intrinsic pathway catalyses activation of factor X approximately 50-fold more

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efficiently, dramatically amplifying the coagulation events triggered by the tissue factor-dependent pathway [7]. The requirement of a powerful amplification of the coagulation burst via the FVIII-dependent intrinsic pathway for maintaining normal haemostasis explains why the absence of FVIII disturbs the coagulation process and results in haemophilia A.

Based on the residual activity of FVIII in plasma, haemophilia A is categorized as severe (< 1 IU dL^{-1} of normal activity), moderate (1–5 IU dL^{-1} of activity) and mild (5–30 IU dL^{-1}). Clinically, the severe form of the disease is characterized by spontaneous recurrent painful bleedings into joints, muscles and soft tissues, and may result in a chronic and debilitating arthropathy. Haemophilic pseudotumours may occur in bones as a result of repeated subperiosteal haemorrhages with bony destruction and new bone formation. More serious complications and death can result from bleedings into the intracranial and retroperitonial space.

Current treatment of haemophilia A is based on correcting functional FVIII deficiency by intravenous infusions of plasma-derived, affinity-purified and, more recently, recombinant FVIII products [8]. Plasma-derived concentrates of FVIII became available for the treatment of haemophilia A in the early 1960s and provided a dramatic improvement in the life expectancy of haemophilic patients [9]. Due to a relatively short half-life of FVIII in circulation (12–14 h) [10], treatment of haemophilia A requires repeated (up to three per week) infusions of expensive FVIII products and in cases of severe disease, the cost of treatment may be as high as US$100 000 per year. The major disadvantage of plasma-derived FVIII therapy was the risk of transmission of blood-borne viruses, such as hepatitis B and C and human immunodeficiency virus [9, 11]. Recombinant gene technologies offer new therapeutic products that are considered safer in certain aspects than plasma-derived concentrates [12–14]. The safety of plasma-derived concentrates has greatly improved in the last decade because of careful donor selection, screening of donations for infectious viruses, and enhanced efficacy of specific antiviral steps in the manufacturing process [15]. Concerns remain about the transmission of thermo-resistant nonlipid-enveloped viruses, such as parvovirus [16], which may be addressed, in part, by introduction of testing using polymerase chain reaction, and the hypothetical risk of transmission associated with variant Creutzfeldt–Jakob disease (vCJD) [13].

Transmissible spongiform encephalopathies or prion diseases

Transmissible spongiform encephalopathies (TSEs or prion diseases) are a group of rare and always fatal neurodegenerative disorders that affect both humans and animals. The animal diseases are sheep scrapie, bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy, chronic wasting disease of deer and elk, feline spongiform encephalopathy and encephalopathy of exotic ungulates. In humans, the TSE manifests itself as a sporadic, hereditary or acquired disorder. Sporadic Creutzfeldt–Jakob disease (sCJD) is the most common form, occurring without known cause at a frequency of approximately 1 case per million per year (200–250 cases each year in the US) and represents 85–90% of all human TSE cases [17]. Usually, sCJD occurs in late middle age (average age 60 years) but occasionally affects younger people. Approximately 10% of TSE cases have an hereditary cause linked to pathogenic mutations in the *PRNP* gene located on chromosome 20, which encodes the prion protein [18]. This form includes familial CJD, Gerstmann–Sträussler–Scheinker disease (GSS) and fatal familial insomnia (FFI) [17]. Acquired TSE diseases are conditions in which evidence of human-to-human or animal-to-human transmission has been documented or suspected. Kuru, now almost extinct, but an epidemic disease during the 1950–60s in the endocannibalistic Fore population of Papua New Guinea, was the first recognized instance of human-to-human transmission of a TSE [19]. Interest in kuru was rekindled after emergence of vCJD, because both diseases represent infections by the oral route and predominantly affect younger individuals; the youngest cases were in a 4-year-old with kuru [20] and a 12-year-old with vCJD [21]. Iatrogenic CJD occurs in modern society as a result of human-to-human transmission due to various medical procedures such as injection of contaminated pituitary-derived hormones, transplantation of dura mater and corneal tissue, and contact with ineffectively decontaminated surgical instruments or implanted brain electrodes [17, 22, 23]. The variant form of CJD was originally identified in the UK in 1996 [24], where there are presently 113 cases [25], and has also occurred in France (five cases) [26,27] and Sicily (one case). Two additional affected individuals, one in Ireland [28], and one in Hong Kong [29] had spent time in the UK during the BSE epidemic, and it is therefore probable that they were infected during their residence in the

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