UK. Scientific evidence strongly supports the causal link between BSE and vCJD [30–33].

Prions as transmissible agents of TSEs

Today it is widely believed that TSEs develop when a host-encoded normal cell-surface glycoprotein, the prion protein (PrP^{C}, normal PrP) changes its conformation to a pathological isoform (PrP^{Sc}, abnormal PrP) that accumulates in the brain tissue of afflicted individuals [34]. Brain tissue of such individuals is highly infectious when introduced into susceptible species, especially by the intracerebral route of inoculation. The infectious agents responsible for the transmission of TSE disease are called prions. They are apparently devoid of nucleic acid and seem to be composed exclusively of a conformationally modified abnormal PrP [34], in which the α-helical content diminishes and the amount of β-sheet increases [35]. It is not understood how this conversion occurs, but studies using transgenic mice have suggested that another unknown factor is required [36], and the quest for the discovery of the nature of this unknown factor continues.

The physiological function of normal PrP has not been yet elucidated but several important observations imply a possible role in copper metabolism [37]. Normal PrP is widely expressed in most tissues throughout the body, including organs of the lymphoreticular system and blood cells [38–45]. In human blood, the highest level of normal PrP expression has been found in mononuclear cells and platelets [41–46], but a significant amount of cell-free PrP has also been detected in plasma [47].

TSEs and blood safety

During the past decade, CJD has been the object of considerable attention from the blood, plasma and fractionation industries. Initially, concern about the safety of blood products arose when it became apparent that donor pools contained plasma from patients who later developed CJD. However, several observations mitigated the possible risk associated with the use of such plasma pools. These included: (1) absence of epidemiological evidence for blood-related TSE transmission; (2) absence of definite evidence of transmission from experiments when human blood or blood components were inoculated into experimental animals (including chimpanzees); (3) very low levels of TSE infectivity in blood, compared to the brain, of rodents experimentally infected with various strains of prions; and (4) efficient reduction of TSE infectivity during validation studies of various steps used in the manufacture of plasma-derived products. However, new concerns about the safety of blood and plasma-derived products emerged when vCJD was identified in the UK [48], based upon the fact that the abnormal PrP was detected in lymphoreticular tissues, including tonsils, spleen and lymph nodes in vCJD patients [49–52], but not in sCJD patients, and in the appendix of a preclinical patient who eight months later developed vCJD [53]; in addition, spleens and tonsils of vCJD patients are infectious [54]. It has been argued that blood of vCJD patients interacting with lymphoreticular organs might contain the abnormal PrP and/or infectious prions. Concern is further heightened by the following observations: (1) BSE, causally linked to vCJD, has spread through many European countries; (2) the extent of exposure to BSE, the source and route of transmission, and transmissibility of different bovine tissues to humans have not been definitely established, and few epidemiological data are available to date; (3) the number of vCJD cases is increasing, and it is impossible to predict accurately the number of people who may have been infected with BSE and might develop vCJD in the future, because the incubation period may vary from 4 to 20 or even 40 years, as found with kuru; (4) epidemiological data are scarce concerning the risk of blood-related transmission of vCJD; (5) disease transmission by transfusion of blood from experimentally BSE-infected sheep has been reported [55]; (6) information is incomplete about the distinctive physico-chemical and biological properties of the vCJD agent in comparison to the other well-studied laboratory strains of TSEs; (7) there is no test available for early diagnosis of infected individuals; and (8) validation studies on the removal of TSE agents (including vCJD) during the manufacturing of plasma-derived products have not been completed and verified by different laboratories.

Experimental blood-related transmission studies

Animal-to-animal transmission

TSE infectivity has never been found in blood from animals with naturally occurring infections (scrapie in sheep, BSE in cattle) when inoculated into mice [56–58]. However, early TSE blood-related transmission studies were not extensive, and employed only a small number of donor and recipient animals (Table 1). The low susceptibility of conventional mice in these studies can be explained by the existence of an interspecies barrier. In addition, it is

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Table 1. Transmission studies to detect infectivity [illegible]

Donor species	Recipient species	[illegible]
Scrapie (natural)		
Goat	Mouse	[illegible]/serum
Sheep	Mouse	[illegible]/serum
BSE (natural)		
Cow	Mouse	[illegible]/serum
Cow	Mouse	[illegible]
Scrapie (experimental)		
Goat	Goat	[illegible]
Mouse	Mouse	[illegible]
Goat	Mouse	[illegible]
Sheep	Mouse	[illegible]
Rat	Rat	[illegible]
Mouse	Mouse	[illegible]
Mouse	Mouse	[illegible]
Hamster	Hamster	[illegible]
Hamster	Hamster	[illegible]
Hamster	Hamster	[illegible]
Hamster	Hamster[2]	[illegible] components
		[illegible]
		[illegible]
Mink encephalopathy (experimental)		
Mink	Mink	[illegible]
Mink	Mink	[illegible]
BSE (experimental)		
Cow	Mouse	[illegible]
	Cow[2]	[illegible]
Mouse	Mouse	[illegible]
Sheep	Sheep[2]	[illegible]
CJD (experimental)		
Guinea pig	Guinea pig	[illegible]
GSS (experimental)		
Mouse	Mouse	[illegible]
Mouse	Mouse	[illegible]plasma
		[illegible] plasma
		[illegible]

[1]In several of the studies, assays were conducted on serial [illegible] obtained [illegible]
[2]Ongoing experiments. Citations for the original studies can be found in [76] [illegible] intracerebral; i.m., intramuscular; i.p., intraperitoneal; [illegible]

possible that animals with natural disease [illegible] extremely low levels of TSE infectivity in [illegible] are not detectable in inbred mice, and [illegible] transgenic mice should therefore be used [illegible] studies.

In contrast to the negative results [illegible] most transmission studies using human [illegible] the blood of animals with natural disease, [illegible] sions have been consistently achieved when [illegible] blood components from experimentally TSE-[illegible] ted animals, primarily rodents, were used [illegible]

studies [illegible] blood [illegible] inoculati[illegible] animals [illegible] large [illegible] low le[illegible] ponents [illegible] times [illegible] adapted [illegible] hamsters [illegible] of scrapie [illegible]

Table 2. Transmission studies to detect infectivity in the blood of humans with CJD.

Diagnosis	Pos./total subjects	Animal assay	Inoculum	Route of Inoculation	Pos./total animals	Reference
Sporadic CJD	1/1	Guinea pig	Buffy coat	i.c.	2/2	73
Sporadic CJD	1/1	Guinea pig	Buffy coat	i.c.	0/5	
		Hamster	Buffy coat	i.c.	2/2	
Sporadic CJD	1/3	Mouse	Whole blood	i.c.	2/13	71
Sporadic CJD	1/1	Mouse	Leukocytes	i.c.	0/10	74
		Mouse	Plasma conc. ×3	i.c.	3/8	
Sporadic CJD	0/3	Chimpanzee	Whole blood units	i.v.	0/3	75
Sporadic CJD	0/1	Guinea pig	Whole blood	i.c., i.p.	0/2	
Sporadic CJD	0/1	Spider monkey	Whole blood	i.c., i.v., i.p.	0/3	
Sporadic CJD	0/1	Squirrel monkey	Whole blood	i.c., i.p., i.m.	0/1	
Sporadic CJD	0/4	Squirrel monkey	Buffy coat	i.c., i.v.	0/4	
hGH iatro. CJD	1/1	Hamster	Whole blood	i.c.	1/4	72
Sporadic CJD	0/13	Transgenic mouse	Buffy coat	i.c.	0/106	Safar *et al.* 2000[1]
			Plasma	i.c.	0/56	
Variant CJD	0/7	RIII mouse	Buffy coat	i.c.	0/34	54
			Plasma	i.c.	0/47	

[1]The transgenic mouse data has not been published [76]. Pos., positive; conc., concentrate.

inoculation was shown to be less efficient than the intracerebral route of the disease transmission for both buffy coat and plasma [67], and very low transmission rates were achieved by transfusion of whole blood [70; P. Brown and L. Cervenakova, unpublished data].

Taken together, these observations permit a confident statement that TSE infectivity occurs in the blood of experimentally infected animals, however, the relevance of these data to humans remains the subject of ongoing scientific debate.

Human-to-animal transmission

Attempts to transmit disease from human blood to animals are summarized in Table 2. Transmission of human CJD to rodents by intracerebral inoculation of whole blood [71,72], buffy coat [73] and plasma [74] has been reported. However, all these studies have been questioned on scientific grounds. In contrast, a number of attempts to transmit the disease have been made at the National Institutes of Health (NIH) Laboratory of Central Nervous System Studies [75] with negative results. Blood from 13 CJD patients, inoculated into either primates or rodents, including transfusion of units of blood from three sporadic CJD patients into three chimpanzees, did not transmit the disease. Another large study conducted recently using transgenic mice highly susceptible to human disease failed to record any positive transmissions from buffy coat and plasma collected from 12 sporadic patients and one patient with familial CJD [76]. In addition, no transmissions resulted from intracerebral inoculation of mice with buffy coat and plasma from four vCJD patients [54]. More experimental studies using transgenic mice and nonhuman primates have been initiated to explore the transmissibility of the vCJD through blood transfusion and the use of plasma-derived products. The results of these ongoing studies will help us better evaluate the risk of transmitting vCJD through blood and blood components.

Epidemiological blood-related Creutzfeldt–Jakob disease transmission studies

A number of epidemiological studies have evaluated the risk of TSE transmission by blood or plasma-derived products. None of these studies has provided evidence that classical sporadic, familial or iatrogenic TSE are transmitted via blood transfusion or via plasma-derived products. Two systematic reviews of case-control studies [77,78] have analysed data from Japan [79], the UK [80–82], Europe [83], and Australia [84] and found no association with risk of developing sporadic CJD from blood transfusion.

Three studies investigating the possibility of human-to-human CJD blood-related transmission among the most frequently exposed individuals with genetic bleeding disorders were performed in the US

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[85,86] and UK [87]. In the US study [85], neuropathological examinations of brain tissue from the few available autopsied patients with haemophilia A (22 cases), haemophilia B (one case) and von Willebrand disease (one case) revealed no features of CJD. All examined individuals, except one, received clotting factor concentrates for more than 10 years; one patient received cryoprecipitate. Most of the patients (21 cases) were HIV positive and the majority (15 cases) had clinical evidence of CNS involvement. Brain tissue from two cases was also evaluated for the presence of abnormal PrP; neither was positive by immunohistochemistry. Analysis of national mortality data in the US from 1979 to 1994 showed no evidence of CJD in patients with increased exposure to blood or blood products, specifically, patients with haemophilia A, haemophilia B, thalassemia and sickle cell disease [86]. In response to the emerging concern over vCJD, a retrospective neuropathological examination was conducted on 35 HIV positive UK haemophilic cases who were treated with clotting factor concentrates derived from predominantly UK donors during the years 1962–95 [87]. No evidence of spongiform encephalopathy was found and immunohistochemical analysis was negative in all cases. It was concluded that, at present, there is no evidence of the transmission of vCJD via clotting factor concentrates to patients with haemophilia.

An investigational retrospective study has been conducted by the US National Blood Data Resource Center since 1995 [88; personal communication from M. Sullivan]. Only the classical form of CJD has been under investigation because no cases of vCJD have occurred in the US. The study found no evidence of CJD transmission in 332 transfusion recipients of blood components from 23 CJD-implicated donors. None of the 212 (66%) deceased recipients for whom the cause of death was known died from CJD, and a subgroup of 120 surviving recipients (34%) continue to be followed. In addition, a subgroup of 42 long-term survivors have lived a minimum of 5 years after transfusion with no signs of neurological disease; some recipients were transfused as many as 28 years ago, and 17 of these survivors received components prepared from blood donated less than 1 year prior to the onset of disease in the donor. A report from Germany [89] identified one CJD patient who donated 55 units of blood during a 20-year period to 27 individuals. None of 18 deceased individuals died from dementia or neurological causes; nine patients were still alive 4–20 years after receiving transfusions from this patient, without any sign of mental deterioration.

Six years after [illegible] CJD Surveillance [illegible] several vCJD [illegible] sions b[illegible] developed [illegible] eight were [illegible] and [illegible] blood [illegible] recipients. [illegible] whole blood [illegible] recipients), [illegible] red [illegible] cells [illegible] ients), fresh-frozen plasma [illegible] cryo-depleted plasma a[illegible] recipient for each component. Plasma [illegible] from eight donors had entered plasma p[illegible] for the manufacturing of therapeutic proteins that were distributed to thousands of [illegible] [personal communication from R. Will]. [illegible] blood donors who have developed vCJD [illegible] have contributed to pools of plasma [illegible] treat patients with haemophilia A [illegible] B. A[illegible] there [illegible] recalls of products in 199[illegible] 2000 [illegible] unpublished data].

Developments in diagno[illegible] for Creutzfeldt–Jakob di[illegible]

Most of the [illegible] the risk [illegible] transmission of CJD through [illegible] plasma-[illegible] products [illegible] resolve[illegible] the early [illegible] infect[illegible] individuals [illegible] fication o[illegible] abnormal [illegible] of presymptomatic [illegible] the level of [illegible] PrP [illegible] CJD [illegible] is no greater than [illegible] patients [illegible] disease [illegible] control [illegible] recently, other [illegible] fluid) have not [illegible] used [illegible] a promising discovery has [illegible] been [illegible] by Shaked *et al.* [illegible] abnormal PrP [illegible] the urine of experimental [illegible] infected with prions long [illegible] the appearance of clinical signs. Abnormal PrP [illegible] also detected in the urine of cattle [illegible] BSE and in symptomatic [illegible] afflicted with a genetic [illegible] of CJD. The value of this technique as a diagnostic tool will need to be validated by other independent laboratories. Another encouraging observation was recently made [92] by the discovery that levels of an erythroid differentiation related factor (EDRF) transcript was decreased in the spleens [illegible] infected mice in both the preclinical and clinical phase of the disease and in the [illegible] of [illegible] ill [illegible] A significant decrease in the expression level was also observed in the [illegible] of cattle [illegible]

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signs of BSE and in whole blood of sheep with scrapie. Further analysis of mouse bone marrow revealed that EDRF is expressed in maturing erythroid cells. Preliminary analysis of normal human blood revealed EDRF expression in the nonlymphocyte fraction, and future studies will be required to determine whether EDRF might be used as a diagnostic marker of human TSEs.

Extensive reviews have recently been published on progress in the development of diagnostic screening tests for CJD by different laboratories [76,93]. All assays were aimed at detecting the presence of abnormal PrP as an indicator of TSE infection, and all except one were based on an immunological approach using appropriate PrP-specific antibodies. The sensitivity of classical immunoblotting assays has been significantly improved [52,93,94], variations of dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) have been introduced [95, 96], and new advanced technologies such as UV-fluorescence spectroscopy [97], capillary electrophoresis [98,99] and confocal laser spectroscopy [100] have been applied. None of these assays has yet achieved the required sensitivity to detect picogram levels of abnormal PrP equivalent to approximately 10–20 IU ml^{-1}, the estimated maximum concentration of infectivity in buffy coat during the preclinical phase of disease in experimental transmission studies [76]. One group reported the detection of abnormal PrP in blood from scrapie-infected sheep [99], but we have not been able to identify the presence of abnormal PrP in the blood of CJD-infected chimpanzees or humans afflicted with TSEs using this approach [Cervenakova *et al.*, unpublished data]. A potentially important discovery has been made by Saborio *et al.* [101] who reported that pulse sonication could convert *in vitro* normal PrP into a protease-resistant, abnormal PrP-like isoform in the presence of tiny quantities of the abnormal PrP template. Conceptually this procedure is analogous to polymerase chain reaction amplification; the initial templates of abnormal PrP aggregate with normal PrP to form new abnormal aggregates that are then disrupted by sonication to form smaller abnormal PrP units for continued formation of new abnormal molecules. This method yielded approximately 30 times more abnormal PrP (250 pg or 8.3 × 10^{-15} mol) compared to the input amount (6–12 pg or 0.2–0.4 × 10^{-15} mol). It may be possible by this novel approach to amplify a subthreshold amount of abnormal PrP from blood to detectable levels.

One problem that the field faces today is the absence of a high-affinity reagent that would specifically recognize only abnormal PrP. Recently, plasminogen, a protein of the fibrinolytic system present in blood, which has also been implicated in neuronal excitotoxicity, has been identified as the first naturally occurring protein that may specifically bind full-length native abnormal PrP from brain tissue of multiple species [102, 103]. Earlier, a protocadherin-2 was identified as a cellular receptor of high affinity (Kd < 25 nmol) for both normal and abnormal forms of PrP [104; personal communication from N. Cashman]. Ideally, the use of these or other reagents with similar properties, in combination with various approaches such as *in vitro* amplification, may achieve a concentration of abnormal PrP to levels that could be detected by presently available methods, and also find use in the removal of infectious TSE agents from blood and plasma-derived products.

Removal of TSE agents/prions during the manufacturing process of plasma-derived Products

To define the risk of vCJD being transmitted by plasma-derived therapeutic products, it is first necessary to define the partition of infectivity through the various separation steps used in the manufacture of plasma products. Two approaches are possible for validation studies: (1) use of plasma of experimentally infected animals (endogenous infected plasma) containing low levels of infectivity that can be detected only in bioassays; and (2) use of brain tissue (or tissue extract) from infected animals or humans as an infectivity 'spike' to evaluate the clearance of TSE infectivity in bioassays, or of abnormal PrP by an immunological method, for example Western blot [94,105,106] or conformation-dependent immunoassay (CDI) [95].

Two experiments have evaluated partitioning of endogenous TSE infectivity in plasma collected from clinically ill mice infected with mouse-adapted human TSE during Cohn fractionation, modified for small volumes [66,67]. The TSE infectivity was partitioned into various fractions using cold precipitation and different ethanol concentrations and pH. Even though some of the infectivity partitioned into cryoprecipitate, used by most manufacturers to produce FVIII, the level of infectivity was more than 10-fold lower than in plasma, and several log orders lower than levels in the brains of clinically ill animals. These very low levels of infectivity did not allow an evaluation of the removal capability of various steps. Therefore, most validation studies have been performed using the 'spiking' approach, which has documented a significant degree of

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abnormal PrP removal during precipitation, adsorption/desorption steps, including anion and cation exchange chromatography, hydrophobic interaction chromatography, nonspecific adsorption, and multiple ion-exchange procedures [106, 107].

Brown *et al.* [66] studied the partitioning of TSE infectivity during the modified Cohn fractionation of plasma separated from human blood 'spiked' with hamster-adapted 263K scrapie strain. Only a small proportion of the infectivity (3%) was recovered in plasma and only 0.71% and 0.[illegible]% in cryoprecipitate and fraction I + II + III. Table 3 shows the efficiency of TSE infectivity and prion removal by various steps used for manufacture of FVIII. Lee *et al.* [94,105] performed validation studies of certain plasma-purification steps used by Bayer for the manufacture of plasma-derived therapeutic proteins. Their principle purification steps for manufacture of FVIII (Koate DVI) employ multiple precipitations and size exclusion chromatography [106]. Two validated manufacture steps, cryoprecipitation and PEG precipitation, together [illegible] 2.2 log_{10} ID_{50} from FVIII [105]. Foster [illegible] calculated the cumulative removal efficiency [illegible] multiple steps employed by SNBT Protein Fractionation Centre (Edinburgh, UK) during the manufacture of plasma products, by analysing published data on the removal capacity of various steps. A [illegible] log_{10} ID_{50} reduction of TSE infectivity during the manufacturing of the FVIII concentrate, Liberate[illegible] was shown. In a subsequent large experimental study Foster *et al.* [108] showed a 6.[illegible] log_{10}

[illegible]

Table 3. Efficiency of prion protein and/or TSE infectivity removal by various [illegible]

Validated manufacturing steps	Spiking material: hamster scrapie[1]	Detection method	[illegible]
Cryoprecipitation, precipitation and adsorption, SD treatment and ion-exchange chromatography, membrane filtration	263K: microsomal fraction	Western [illegible]	[illegible]
Cryoprecipitation and cryoprecipitate/PEG separation	263K: brain [illegible]	[illegible]	[illegible]
Cryoprecipitation	Sc237: brain [illegible], microsomal [illegible] (CLD) Sc237: Purified [illegible]	[illegible]	[illegible]
Ethanol precipitation 8%	Sc237: brain [illegible], microsomal [illegible] (CLD) Sc237: Purified [illegible]	[illegible]	[illegible]
FVIII immunoaffinity column ion-exchange chromatography [109]	263K: Brain [illegible]	[illegible]	[illegible]

[1]Hamster-adapted scrapie (263K or Sc237); [2]CLD, cave[illegible]

MONARC-M™ and Baxter's Hemofil M). For validation of the efficiency of two main manufacturing steps (monoclonal antibody affinity chromatography and ion exchange chromatography), cryoprecipitate suspension and fresh eluate from the immunoaffinity column were spiked with 263 K scrapie strain [106]. The results showed removal of 4.57 $\log_{10}$ ID_{50} by the anti-FVIII immunoaffinity chromatography, and of 3.47 $\log_{10}$ ID_{50} by Q-Sepharose chromatography, for a total removal of 8.04 $\log_{10}$ ID_{50} by the complete process [110].

Taken together, accumulated data provide strong evidence that a substantial amount of TSE infectivity could be removed by the steps used during the manufacturing of coagulation factors. These data agree with the failure of epidemiological studies to identify iatrogenic blood-related transmission of TSEs. More studies are under way to address the safety issues associated with vCJD, not only for coagulation factors, but also for other plasma-derived therapeutics. Hopefully, the combination of different approaches and new developments in detection and/or removal methodologies for TSE infectivity will lead to even greater safety in regard to the still theoretical risk of iatrogenic transmission of vCJD through blood transfusion and plasma-derived products.

Conclusion

Treatment of haemophilic patients with high-quality therapeutics, and the elimination of risks associated with blood-transmitted diseases, deserves our highest priority. The emergence of vCJD in the UK has produced a new, albeit hypothetical, risk of infection for haemophiliac patients treated with coagulation factors, and some evidence suggests that the TSE agent causing vCJD might be more invasive to lymphoreticular tissue than classical CJD. Without a reliable diagnostic test for selection of donors and testing of blood products, a donor deferral policy will remain the main preventive measure. Producers of plasma-derived therapeutics, including FVIII, are working toward the development of appropriate methods to assure the removal of the vCJD agent/infectivity, if present in human blood. Treatment of haemophiliac patients with recombinant FVIII may further decrease the possible risk of human-to-human vCJD transmission. However, in the absence of available recombinant products, the hypothetical risk of vCJD from plasma products is surely outweighed by the real risk of inadequate medical treatment.

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Vox Sanguinis

Transfusion, [illegible]

Vox Sang [illegible]78:86–9[illegible]

Studies on the Removal of Abn[illegible] Protein by Processes Used in [illegible] of Human Plasma Products

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Abstract

Background and Objectives: To identify if any process steps used in plasma fractionation may have a capability of removing agents of human transmissible spongiform encephalopathy (TSE). ***Materials and Methods:*** Sixteen fractionation steps were investigated separately by adding a preparation of hamster adapted scrapie 263K to the starting material at each process step and determining the distribution into resultant fractions of protease-K-resistant (abnormal) prion protein by Western blot analysis. ***Results:*** A number of process operations were found to remove abnormal prion protein to the limit of detection of the assay. These were cold ethanol precipitation of fraction IV (log reduction, LR, ≥ 3.0) and a depth filtration (LR ≥ 4.9) in the albumin process; cold ethanol fraction I+III precipitation (LR ≥ 3.7) and a depth filtration (LR ≥ 2.8) in the immunoglobulin processes and adsorption with DEAE-Toyopearl 650M ion exchanger (LR ≥ 3.5) in the fibrinogen process. In addition, a substantial degree of removal of abnormal prion protein was observed across DEAE-Toyopearl 650M ion exchange (LR = 3.1) used in the preparation of factor-VIII concentrate; DEAE-cellulose ion exchange (LR = 3.0) and DEAE-sepharose ion exchange (LR = 3.0) used in the preparation of factor-IX concentrates and S-sepharose ion exchange (LR = 2.9) used in the preparation of thrombin. ***Conclusions:*** Plasma fractionation processes used in the manufacture of

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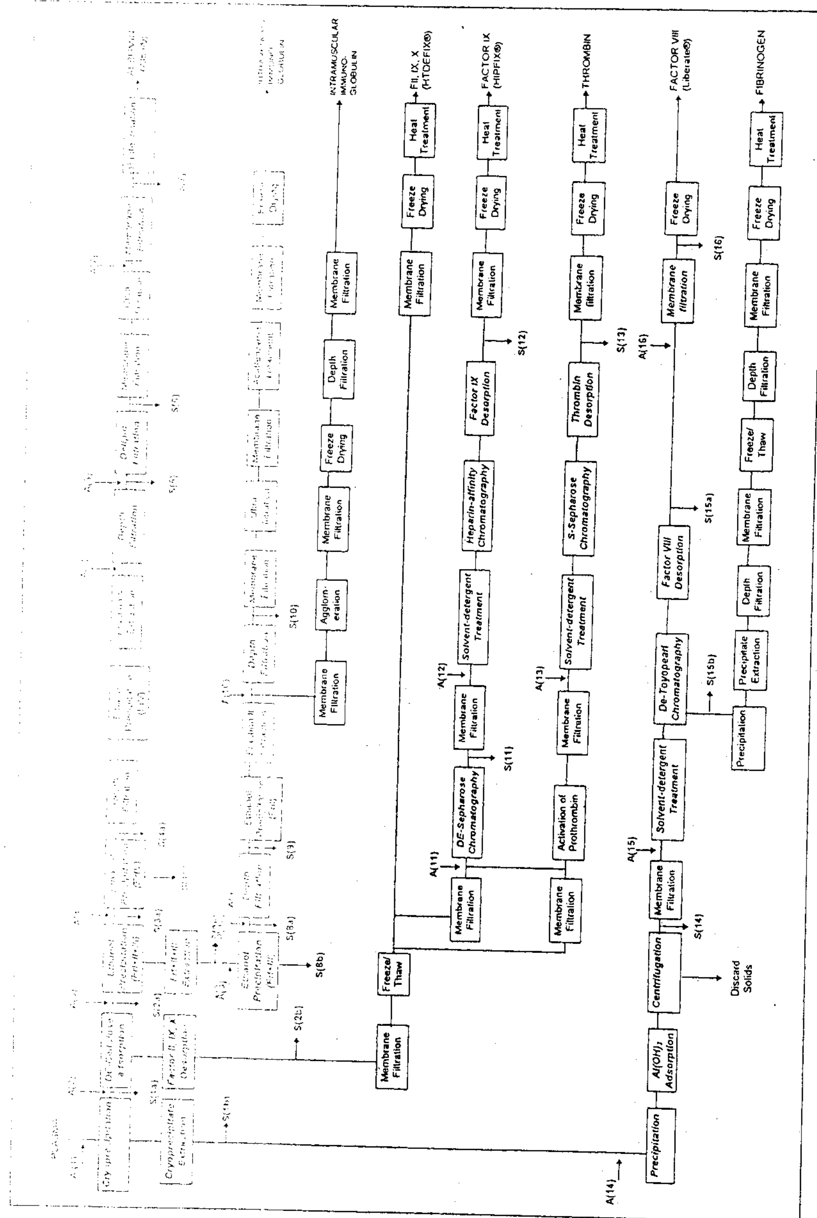

phosphate (5 mM) at pH 6.2. Factor II, IX and X solution [illegible] was applied to the column, which was then treated with [illegible] wash buffer, all at a flow rate of 8.4 ml/min, followed by 10 [illegible] wash buffer + 280 mM sodium chloride at 1.9 ml/min. Factor IX was eluted using 100 ml wash buffer +360 mM sodium chloride, pH [illegible] at [illegible] ml/min.

Solvent-Detergent Treatment and Affinity Chromatography of Factor IX (Step 12)

Microsomal inoculum (10 ml) was added to a solution of factor IX (108 ml) which had been prepared by diluting 36 ml of factor IX eluate (step 11) with 72 ml of a solution of citrate (20 mM) + arginine (4.5 g/l), at pH 7.55. Tri(n-butyl)phosphate and Tween-80 were added to 108 ml of 'spiked' factor IX solution to achieve a final concentrations of 0.3 and 1%, respectively [24], the mixture stirred at [illegible] for 19 h, then purified by affinity chromatography based on the method of Burnouf et al. [25]. 30 ml heparin-sepharose FF (Pharmacia) was packed into a 26-mm diameter chromatography column [illegible], Pharmacia) using 20 mM citrate. The solvent-detergent [illegible] factor IX mixture was applied to the column, the bed washed with [illegible] ml of 20 mM citrate, treated with 100 ml of 20 mM citrate + [illegible] sodium chloride and factor IX then eluted with 100 ml of a solution of citrate (20 mM) + arginine (4.5 g/l) + sodium chloride (50[illegible]) at a flow rate of 3.1 ml/min.

SD Treatment and Ion Exchange Chromatography of Thrombin (Step 13)

Microsomal inoculum (9.5 ml) was added to an unpurified solution of thrombin (197 ml), which had been prepared by calcium activation of the factor II, IX and X eluate (fig. 1: step 2) according to the method of MacGregor et al. [26]. Tri(n-butyl)phosphate and Tween-80 were added to achieve final concentrations of 0.3 and [illegible], respectively, and the mixture stirred at 25 °C for 19 h prior to purification of thrombin by ion exchange chromatography. 20 ml S-sepharose (Pharmacia) was packed into a 26-mm diameter chromatography column (XK 26/10, Pharmacia) and washed with 20 mM trisodium citrate (80 ml) at pH 6.5. The SD-treated thrombin mixture was applied to the column at a flow rate of 8.5 ml/min; the column was washed with 200 ml trisodium citrate (20 mM) and thrombin was then eluted with 80 ml of trisodium citrate (20 mM) + sodium chloride [illegible] at a flow rate of 4.2 ml/min.

Precipitation and Adsorption of Cryoprecipitate Extract (Step 14)

Microsomal inoculum (9.5 ml) was added to cryoprecipitate extract (215 ml) which had been prepared by resuspending [illegible] frozen washed cryoprecipitate in 20 mM Tris ([illegible]68 ml) at [illegible]. The pH of the extract was adjusted to 6.7 and zinc precipitant (zi[illegible] + sodium chloride + trisodium citrate + heparin) added to [illegible] concentrations of 0.5 mM zinc, 1 mM citrate and 2.5 IU/ml [illegible]. The mixture was stirred for 5 min at 20 °C, aluminium hydroxide ([illegible] hydrogel, Superfos, Copenhagen, Denmark) was added to a final concentration of 5%; after stirring for a further [illegible] min, the suspension was centrifuged at 5,500 g for 15 min at 20 °C to recover the supernatant, which was then formulated to 20 mM trisodium citrate and 2.5 mM calcium chloride.

Table 2. Removal of PrP^Sc in the preparation of high purity factor VIII (Liberate®) and fibrinogen

Process step[a]	Factor VIII CF[b]	Factor VIII RF[c]	Fibrinogen CF	Fibrinogen RF
1 Cryoprecipitation	1.7	1.0	1.7	1.[illegible]
14 Zinc precipitation + Al $(OH)_3$ adsorption	2.0	1.7	2.0	1.[illegible]
15 SD[d] + DEAE Tyopearl 650 M chromatography	3.8	3.1	≥4.1	≥[illegible]
16 Membrane filtration (0.45 μm/0.22 μm)	1.6	1.0	n/d[e]	n/[illegible]

[a] Number of process step in flowsheet (fig. 1).
[b] PrP^Sc clearance factor (log_{10}).
[c] PrP^Sc reduction factor (log_{10}).
[d] Solvent-detergent treatment.
[e] Not determined.

Table 3. Removal of PrP^Sc in the preparation of high purity factor IX concentrate (HIPFIX®), factor IX complex (HTDEFIX®) and thrombin

Process step[a]	Factor IX CF[b]	Factor IX RF[c]	FII, IX and X CF	FII, IX and X RF	Thrombin CF	Thrombin RF
1 Cryoprecipitation	<1.0	<1.0	<1.0	<1.0	<1.0	<1.[illegible]
2 DEAE-cellulose adsorption	2.8	3.0	2.8	3.0	2.8	3.0
11 DEAE-sepharose chromatography	4.4	3.0	n/a[d]	n/a	n/a	n/a
12 SD[e] + heparin-sepharose chromatography	2.7	1.4	n/a	n/a	n/a	n/a
16 SD + S-sepharose chromatography	n/a	n/a	n/a	n/a	3.3	2.9

[a] Number of process step in flowsheet (fig. 1).
[b] PrP^Sc clearance factor (log_{10}).
[c] PrP^Sc reduction factor (log_{10}).
[d] Not applicable.
[e] Solvent-detergent treatment.

Table 4. Distribution of PrP^Sc by precipitation

Process step[a]	Precipitation conditions: ethanol %	pH	temperature °C	time h	% distribution[b] of PrP^Sc: precipitate	supernatant
1 Cryoprecipitation	–	–	–	–	10	96
3 FrI+II+III precipitation	21	6.70	–5.0	15	84.4	4.7
4 FrIV precipitation	35	5.55	–5.0	17	>100	<0.1
8[c] FrI+III precipitation	8	5.10	–2.5	16	>100	<0.02
8[d] FrI+III precipitation	12	5.10	–2.5	16	>100	<0.02

[a] Number of process step in flowsheet (fig. 1).
[b] 100% = Total PrP^Sc measured in feedstock prior to precipitation.
[c] Process step used in the preparation of immunoglobulins for intramuscular administration.
[d] Process step used in the preparation of immunoglobulins for intravenous administration.

ly a small proportion of PrP^Sc could be accounted for in samples taken over chromatographic procedures, e.g., about 0.1% at steps 2, 11 and 13 (table 3). It is possible that PrP^Sc may have partitioned into wash fractions which were not sampled; however, it seems more probable, given its adherent nature [16], that most PrP^Sc remained adsorbed to chromatographic matrices following product elution.

The contribution made by each step in an overall process will be dependent on whether or not different steps are complementary to one another. As each process step was exam-

Partitioning over cryoprecipitation is less clear. On processing plasma from mice experimentally infected with a human TSE, the infectivity appeared to partition primarily into the cryoprecipitate, whilst in the comparative exogenous experiment using human blood 'spiked' with scrapie 263K, 8.1 log_{10} LD_{50} remained in plasma, but only 0.7% of this infectivity was detected in the cryoprecipitate [30]. In a subsequent larger-volume endogenous experiment, using blood from scrapie-infected hamsters, Rohwer [38] has estimated that about 20% of the plasma infectivity partitioned into cryoprecipitate. By contrast Petteway et al. [31], using human plasma to which scrapie 263K brain homogenate was added, reported that 90% of PrP^Sc partitioned into the cryoprecipitate. Our finding that about 10% of the scrapie 263K PrP^Sc added to human plasma partitioned into cryoprecipitate (table 4) is reasonably comparable with Rohwer's [38] figure of 20% from his larger-volume endogenous model, suggesting that the microsomal inoculum used in our study behaved similarly to a TSE agent present naturally in plasma. However, it is also possible that some of the different results reported may simply reflect variations between different manufacturer's procedures for the preparation of cryoprecipitate, rather than differences in the nature of the infective materials used.

Little information is available on the behaviour of TSE agents in chromatographic separations currently used in plasma fractionation. Drohan [34], in a study of factor VIII processing, has reported log_{10} RFs of 4.4 and 6.3 for immunoaffinity and ion exchange chromatography, respectively, using a 10% brain homogenate of hamster-adapted scrapie as the inoculum and with infectivity determined by bioassay. Additional chromatographic data are available from a variety of different bio-process industries [13, 14, 16, 39, 40] with log_{10} RFs ranging from 2.2 to 5.5. Our results on ion exchange are within this range, with essentially no difference being observed between anion exchange and cation exchange or between different ion exchange matrices (table 3). The somewhat smaller degree of PrP^Sc reduction observed over heparin-affinity chromatography (table 3; step 12) may have been due to a smaller charge difference,

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Assessment of the potential of plasma fractionation processes to remove causative agents of transmissible spongiform encephalopathy

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SUMMARY. Although there is no evidence that classical CJD (cCJD) can be transmitted by human blood or blood products in clinical practice, uncertainties surrounding new variant CJD (nvCJD) have led to the safety of plasma products derived from UK donors being questioned. To better define whether or not there is a risk of nvCJD being transmitted it is necessary to determine how the causative agent would partition across the separations processes used in the preparation of plasma products.

The abnormal prion protein which is associated with transmissible spongiform encephalopathies (TSEs), such as CJD, has a low solubility, a high tendency to form aggregates and adheres to surfaces readily. If the physico-chemical properties of the agent of nvCJD are similar to those of abnormal prion protein then nvCJD may be

Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative disorders including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease (CJD) in humans (Baker & Ridley, 1996). CJD is a rare disease which occurs uniformly world-wide, with an incidence of about 1 per 10^6 persons per annum. A new form of TSE in humans, termed new variant CJD (nvCJD), was first identified in 1996 in the UK and is believed to have resulted from the consumption of central nervous tissue from BSE-infected animals which entered the human food chain (Will *et al.*, 1996). The current clinical incidence of nvCJD in the UK is about 0·2 per 10^6 persons per annum (Scottish Centre for Infection & Environmental Health, 1998) but, in the absence of a suitable diagnostic procedure, the subclinical prevalence of the infection is not known.

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products in this regard, the FDA decided that batches of plasma products must be recalled where a donor had been diagnosed with CJD or was at increased risk of CJD (FDA, 1995). In the 12 months to 30 March 1998, the FDA recalled 175 batches of albumin products, 83 batches of immunoglobulins and 11 batches of coagulation factor concentrates on this basis. This extent of plasma product recall in North America resulted in shortages of critical therapeutic products (FDA, 1998a). Subsequently, the FDA position was revised to recommend the recall of products only where a donor had developed nvCJD (FDA, 1998b). In Europe, plasma products do not require to be recalled on the basis of classical CJD (cCJD), but a decision was taken to recall batches where nvCJD has been diagnosed in a contributing donor (CPMP, 1998). Three such UK donors were identified in 1997 and the subsequent product recalls, the lack of knowledge of the prevalence of subclinical nvCJD in the UK population together with some evidence that the distribution of nvCJD in human tissues may differ from that of cCJD (Hill *et al.*, 1997) resulted in the safety of plasma products derived from UK donors being questioned (Ludlam, 1997) and ultimately to a decision by the UK Government to ban the manufacture of plasma derivatives from plasma collected in the UK, as a precautionary measure (Warden, 1998).

In order to define the risk of either cCJD or nvCJD being transmitted by plasma products it is necessary to determine how the causative agents would partition across the separations processes that are employed in the manufacture of plasma products. The effect of pharmaceutical manufacturing procedures on TSE agents is normally assessed by challenging a scaled-down version of the process with a high titre of a defined strain of a rodent adapted scrapie agent and measuring the infectivity of samples, taken before and after processing, by intracerebral injection in animals. Such studies take a long time to complete and, because of the high costs involved, tend to be restricted to a small number of key process steps rather than a comprehensive examination of the complete manufacturing process. For example, in a study of the process used to manufacture Trasylol®, the examination of four individual process steps consumed 1600 mice and took 3 years to complete (Kozak *et al.*, 1996).

TSE agents are highly resistant to inactivation (Taylor, 1996) and therefore, for protein pharmaceuticals, it is their physical removal that is of particular interest. Preliminary data on TSE agent partitioning have been reported for some selected process steps used in the fractionation of human plasma using a rodent adapted strain of a human TSE agent (Brown *et al.*, 1998) and a rodent adapted strain of the scrapie agent (Brown *et al.*, 1998; Petteway *et al.*, 1998), but the outcomes expected over a complete plasma fractionation process have n yet been described. In the absence of comprehensi measurements of TSE agent partitioning across plasn fractionation processes, the behaviour of nvCJD can l estimated only by extrapolation of data obtained fro similar biopharmaceutical process operations. A prov sional assessment of how TSE agents might be expect to partition during plasma fractionation has been mac on this basis.

PLASMA FRACTIONATION

The Scottish National Blood Transfusion Servic (SNBTS) manufactures over 250 000 unit doses of range of different plasma products from ≈100 000 kg plasma. The preparation of each product involves exte sive processing via a carefully designed, closely co trolled series of operations (Fig. 1) (Foster, 1994). Eac process includes a number of steps in which macr molecular constituents are preferentially removed; the steps are summarized below on a product-by-produ basis.

Albumin (Alba®)

The SNBTS process for the manufacture of albumi involves removal by centrifugation of the precipita which forms when the frozen donations of plasma ar thawed (cryoprecipitate), removal by centrifugation c the precipitates which form at 21% ethanol, pH 6·7 – 5 °C (fraction I + II + III) and at 35% ethanol, pH 5·5 – 5 °C (fraction IV), depth filtration through a mixe bed of cellulose, kieselguhr and perlite at two stage depth filtration through a mixed bed filter incorporatin a cation exchange resin and membrane filtration a three different stages of the process, two of whic employ a cellulose acetate membrane. The final produc is pasteurized at 60 °C for 10 h to inactivate potentia viral contaminants.

Immunoglobulins

Similar purification procedures are used in the manu facture of immunoglobulin products. Following th removal of cryoprecipitate and the recovery of fractio I + II + III, the resuspended fraction I + II + III i adjusted (8 or 12% ethanol, pH 5·1, – 3 °C) to precipitat fraction I + III, which is removed by centrifugation, th supernatant being clarified by borosilicate glass dept filtration. The IgG solution is subsequently subjected to mixed bed depth filtration (cellulose, kieselguhr an perlite) and to membrane filtration at three differen stages of manufacture, two of which employ a cellulos acetate or similar membrane.