Factor II, IX and X concentrate (DEFIX®)

The supernatant which remains following the removal of cryoprecipitate is subjected to a batch anion exchange adsorption with coagulation factors II, IX and X being recovered by chromatographic desorption. The solution containing factors II, IX and X then undergoes two separate membrane filtration operations prior to being freeze dried and heat treated at 80 °C for 72 h for virus inactivation.

Factor IX concentrate (HIPFIX®)

Factor IX concentrate is purified from the desorbed factor II, IX and X eluate (above) using anion exchange chromatography and heparin affinity chromatography. A total of five separate membrane filtration steps are employed, as well as a solvent-detergent treatment to inactivate lipid-enveloped viruses, prior to the product being freeze dried and heat treated at 80 °C for 72 h.

Thrombin

Thrombin is also purified from the desorbed factor II, IX and X elution, in this instance by cation exchange chromatography, with a total of six separate membrane filtration steps, [illegible] which employ a cellulose acetate membrane, and a solvent-detergent treatment prior to the product being freeze dried and heat treated at 80 °C for 72 h. Albumin (Alb) is added as a stabilizer and must also be considered in the assessment of risk.

Factor VIII concentrate (Liberate®)

In the preparation of factor VIII concentrate, the extract obtained from cryoprecipitate is partially purified by precipitation and by adsorption with aluminium hydroxide gel. Following removal of the solids by centrifugation, the supernatant is treated with tri(n)-butyl phosphate + polysorbate 80 for the inactivation of lipid-enveloped viruses and by anion exchange chromatography for further purification of factor VIII. Membrane filtration is employed at two different stages of processing.

Fibrinogen

The preparation of fibrinogen is similar to that of factor VIII except that the unadsorbed fraction from anion exchange chromatography is processed rather than the desorbed fraction. The fibrinogen-rich solution is then subjected to three precipitation operations followed by two depth filtration and three membrane filtration procedures prior to freeze drying and heat treatment at 80 °C for 72 h.

THE PARTITIONING OF TSE AGENTS IN BIO-SEPARATION PROCESSES

Background

Although a number of different TSE diseases are known the causative agents are generally believed to posses similar physicochemical properties (Groschup *et al.* 1997) and to consist of a conformationally altered forn of cellular prion protein (PrP^c), referred to as abnorma prion protein (e.g. PrP^{Sc}). Whether or not PrP^{Sc} is itsel the causative agent of disease is not known; however removal of PrP^{Sc} is generally associated with removal o infectivity (Farquhar *et al.*, 1998).

PrP^{Sc} has still to be fully characterized (Donne *et al.* 1997; Edenhofer *et al.*, 1997), but the molecule i: believed to be based on a 27–30-kDa glycoproteir subunit (Meyer *et al.*, 1986) and, with both hydrophobic and hydrophilic domains (Bolton *et al.*, 1987), tends tc form large amorphous or rod-shaped aggregates *in vitrc* (McKinley *et al.*, 1991). PrP^{Sc} has a low aqueous solubility below pH 9 (Gasset *et al.*, 1993) and is readily precipitatec by ethanol (Prusiner *et al.*, 1980), ammonium sulphate and polyethylene glycol (PEG) (Turk *et al.*, 1988).

Therefore, it can be postulated that certain bioseparation technologies that are used in the preparation of plasma products, such as precipitation, adsorption and filtration, may well be capable of removing significant quantities of the abnormal prion protein associated with nvCJD. Indeed, the potential of these technologies for the removal of TSE agents has been identified previously in guidelines concerning the preparation of medicinal products (CPMP, 1992).

Measurement of TSE agent partitioning

Most information on the partitioning of TSE agents has been obtained from studies in which the behaviour a rodent adapted scrapie agent (PrP^{Sc}) was measured. PrP^{Sc} has similar biochemical properties to cCJD (Bendheim *et al.*, 1985) and has been accepted by Regulatory Authorities as a suitable model for studies of the inactivation and removal of BSE (Bader *et al.*, 1998). nvCJD is believed to be the human form of BSE (Almond & Pattison, 1997) and therefore PrP^{Sc} is also likely to be regarded as a suitable marker for determining the partitioning behaviour of the agent of nvCJD. Nevertheless, it is by no means sure that data from animal model systems are predictive for the human situation.

The transmissibility or infectivity of a TSE agent may be influenced by the strain of agent used, the dose of the agent, the route of administration and the presence or absence of a species barrier. Most studies of the infectivity of TSEs measure the dose that causes infection in 50% of the animals tested (ID_{50}), following inoculation

by the intracerebral route (i.c.). Intravenous (i.v.) administration is believed to result in a 10-fold reduction in infectivity compared to the i.c. route, whilst a species barrier may result in up to a 10^3-fold reduction in infectivity (Bader *et al.*, 1998).

To determine the partitioning behaviour of a TSE agent across a preparative process or an individual process step, measurements of the concentrate of infective agent ($ID_{50}\,mL^{-1}$) and the respective process volumes can be used to calculate a TSE agent reduction factor (RF) where

$$RF = \text{total } ID_{50} \text{ before processing} \div \text{total } ID_{50} \text{ after processing.}$$

The same units of measurement are used in the numerator and the denominator and therefore the RF is a dimensionless number which, as values can be high, is often expressed in the logarithmic ($\log_{10}$) form.

Protein precipitation technology

The very low aqueous solubility of PrP^{Sc} suggests that abnormal prion proteins will generally tend to partition into the solids phase in a precipitation process and be separable from proteins which remain in solution and to copurify with proteins which partition into the solids phase.

Cryoprecipitation. The solids phase which forms when plasma is thawed is known as cryoprecipitate; it is where the least soluble proteins tend to precipitate (i.e. fibrinogen, fibronectin, factor VIII, von Willebrand factor) and is the first stage in the overall fractionation process (Fig. 1).

Some information concerning the partitioning behaviour of TSE agents during cryoprecipitation is available from the work of Brown *et al.* (1998) who reported that infectivity from a mouse adapted strain of a human TSE, Gerstmann–Sträussler–Scheinker syndrome (GSS), was found to concentrate in the precipitate phase with an infectivity about one order of magnitude greater in cryoprecipitate than in the plasma from which it was prepared. A similar observation has been reported by Petteway *et al.* (1998), using an immunochemical method of analysis, who found that 90% of hampster adapted PrP^{Sc} (strain 263K) added to plasma partitioned into cryoprecipitate.

Ethanol precipitation. The iso-electric precipitation of proteins in the presence of ethanol forms the basis of cold-ethanol (Cohn) fractionation which is used in the preparation of albumin and immunoglobulins. A number of successive precipitation steps are employed, in which the least soluble proteins are precipitated first and the more soluble proteins being concentrated into later fractions (Cohn *et al.*, 1946). Brown *et al.* (1998) have reported that GSS infectivity partitioned preferentially

Table 1. Reduction of scrapie infectivity (ID_{50}) by chromatographic separations

Method	Product	Scrapie strain	Scrapie reduction factor: unadsorbed fraction	Scrapie reduction factor: desorbed fraction	References
Ion-exchange chromatography					
DEAE-cellulose (anion)	n/a*		$2{\cdot}5\times10^{1}$†	1×10^{2}†	Hunter & Millson, 1964
Q-sepharose (anion)‡	plasma protein	ME7	n/d*	$>2{\cdot}5\times10^{2}$	
SP-sepharose (cation)‡	plasma protein	ME7	n/d	$1{\cdot}6\times10^{2}$	
Resin I (undisclosed)	aprotinin	ME7	n/d	$1{\cdot}6\times10^{5}$	Kozak *et al.*, 1996; Golker *et al.*, 1996
Resin II (undisclosed)	aprotinin	ME7	n/d	1×10^{4}	Kozak *et al.*, 1996; Golker *et al.*, 1996
Ion exchange (undisclosed)	aprotinin	263K	n/d	$1{\cdot}2\times10^{5}$	Blum *et al.*, 1998
Ion exchange (undisclosed)	bovine albumin	263K	n/d	$1{\cdot}6\times10^{5}$	Blum *et al.*, 1998
Hydrophobic chromatography					
Phenyl sepharose‡	plasma protein	ME7	n/d	$>1{\cdot}6\times10^{3}$	
Ion exchange + hydrophobic chrom.					
DEAE-spherodex/LS® + DEA-spherosil/LS®	human albumin	C506/M3	n/d	$3{\cdot}1\times10^{5}$	Grandgeorge *et al.*, 1997
Nonspecific adsorption					
Calcium phosphate	n/a		$>1{\cdot}5\times10^{4}$†	$1{\cdot}4\times10^{2}$†	Hunter & Millson, 1964

* = n/a, not applicable; n/d, not done. ‡ M. McNaughton & A. Shepherd, personal communication, April 1997. † = approximation.

proteins has been studied using anion exchange chromatography, cation exchange chromatography, hydrophobic interaction chromatography, nonspecific adsorption and a number of ion exchange procedures for which the details were not disclosed. The results, summarized in Table 1, demonstrate removal of PrP^{Sc} infectivity by all of these procedures ranging from 10^2-fold to 10^5-fold reduction.

In their study of the Lowry process used to prepare human growth hormone, Taylor *et al.* (1985) observed a 10-fold reduction in PrP^{Sc} (ME7) infectivity after filtration through a 0·45-μm cellulose acetate membrane, even though the membranes were pretreated to prevent adsorption. Taylor *et al.* (1985) also noted that 'substantial amounts of scrapie infectivity can be lost by adsorption to membrane filters', and therefore a similar degree of removal of abnormal prion protein might also be expected to occur in comparable membrane filtration operations used in plasma fractionation.

Extrapolation of existing knowledge to plasma fractionation processes

From data available on the behaviour of PrP^{Sc} in a variety of bioprocess operations, it is possible to estimate how a TSE agent might be expected to partition across similar unit operations used in the preparation of pharmaceutical protein products from human blood plasma. Where removal of a TSE agent by a particular plasma fractionation procedure is anticipated, a value for the reduction factor has been assigned (Table 2) using conservative values from a relevant study. For process operations not listed in Table 2, it is assumed that abnormal prion protein will copurify with the plasma product being prepared.

Precipitation. From the information available the causative agents of TSEs would be expected to partition into the solids phase during protein precipitation operations. Where the solubility of a TSE agent is zero and the product protein remains in solution, separation of the product from the TSE agent will be possible. The degree of separation achieved will be influenced by the effectiveness of the technology used to separate the solid phase from the liquid, with a greater assurance of TSE agent removal where two solid–liquid separation operations are carried out in series (e.g. centrifugation followed by filtration).

Adsorption/desorption. Studies concerning a number of biopharmaceutical products have demonstrated that

Table 2. Estimated ability of bioprocess technologies to remove TSE agents

Process technology	[illegible]
Precipitation	
Cryoprecipitation	[illegible]
Cohn fraction I	[illegible]
Other Cohn fractions	[illegible]
Other precipitation methods	[illegible]
Adsorption chromatography	
Packed bed	[illegible]
Packed bed	[illegible]
Suspension	[illegible]
Adsorptive filtration	
Depth filter (mixed bed)	[illegible]
Depth filter (single bed)	[illegible]
Membrane filter (cellulose acetate)	[illegible]

PrP^{Sc} infectivity binds to a range of adsorbents, resulting in its partial or complete removal from the manufacturing process (Table 1). These data suggest that similar procedures in plasma fractionation processes should also be capable of removing a TSE agent from the product stream to a comparable extent.

In these circumstances the TSE agent reduction factor will be determined not only by the relative binding characteristics of the macromolecules, but also by the unit capacity of the adsorbent and by the technology employed for contacting the process solution with the adsorptive media, with flow through a packed bed (column) being expected to afford the highest degree of separation.

Separation of PrP^{Sc} occurred with all of the adsorbents examined (Table 1), despite the use of different ligands, matrices and principles of adsorption. Therefore, the outcome was not determined by a single well-defined property of PrP^{Sc} (e.g. charge), but must have involved either a number of different properties which caused PrP^{Sc} to be adsorbed in all of these different circumstances, or some form of binding which was common to all of these different methods.

If it is assumed that the reduction in PrP^{Sc} (ME7) infectivity by membrane filtration observed by Taylor *et al.* (1985) was a result of adsorption of the TSE agent to the membrane, rather than removal by a sieving mechanism, then TSE agent removal would be expected to be influenced by the chemical nature of the membrane. Therefore, a TSE agent reduction factor (Table 2) has been assigned only to SNBTS membrane filtration steps (Fig. 1) where the chemical composition of the filter is comparable to that used by Taylor *et al.* (i.e. cellulose acetate).

DISCUSSION

The development [illegible] viruses such as [illegible] (HIV) and the virus [illegible] C (HCV) from [illegible] 1991; Foster *et al.* [illegible] ment. Procedures [illegible] important; however, [illegible] technology has [illegible] contaminants can [illegible] tion (Budnik *et al.*, [illegible] *et al.*, 1996, and [illegible] & Spaltro, 1996 [illegible] fractionation [illegible] from hepatitis [illegible] & Barker, 1976 [illegible] These technologies [illegible] tially remove [illegible] 1998). Whether or not [illegible] is achievable is less [illegible] or less processed [illegible] to appreciate that a [illegible] necessarily indicate [illegible] removed completely by [illegible] the reduction factor [illegible] the capacity of process operations [illegible] tious agent, additional information [illegible] mine whether or not [illegible] material may remain in [illegible] question.

In precipitation [illegible] it is necessary to define the solubility of the [illegible]

conditions being employed. Unless the solubility is zero, then a quantity of the agent will remain in solution. Brown *et al.* (1998) were able to detect PrP^{Sc} infectivity in a fraction V precipitate prepared from normal human blood which had been 'spiked' with hamster adapted scrapie (263K), but with a 10^6-fold reduction from the original titre in the whole blood. Whether this small degree of infectivity resulted from a small proportion of PrP^{Sc} remaining soluble prior to the fraction V precipitation or if there was incomplete removal of earlier solids fractions is unclear. Taylor *et al.* (1985) were unable to detect PrP^{Sc} (ME7) in the supernatant following precipitation of human growth hormone with 10% ethanol at pH 4·8. However, the limit of detection quoted was 0·5 $\log_{10} ID_{50}\,mL^{-1}$ (i.e. 3 $ID_{50}\,mL^{-1}$) so it is possible that this concentration of PrP^{Sc} (ME7) could have been soluble and remained undetected in solution.

Different considerations apply to methods involving adsorption (and desorption) as the reduction factor should largely be indicative of whether or not a separation can be achieved and what the capacity of a process operation would be. The potential for interference by the TSE agent inoculum being added to challenge a process step must also be considered as constituents of a brain homogenate used as a source of PrP^{Sc} might either occupy adsorption sites which would otherwise be available for the binding of the TSE agent or, alternatively, might provide specific binding sites for PrP^{Sc} that would not otherwise exist. Where adsorption technology is employed for TSE agent removal then to avoid cross-contamination of subsequent batches it will be necessary either to use new adsorption media on each occasion or to sanitize media and equipment effectively before re-use.

Most TSE agent clearance studies have involved the addition of a brain homogenate to the process solution to be studied. How accurately this model represents the behaviour of endogenous TSE agents in human plasma is an important question. Brown *et al.* (1998) have reported two partitioning studies, one using human blood spiked with scrapie (263K) infected hampster brain and the other using murine blood obtained from mice infected with a strain of a human TSE (GSS). Comparable results were obtained in the fractionation of plasma from each experiment, indicating that the use of brain homogenate reasonably represented the behaviour of an endogenous TSE agent. Whether or not this finding will apply equally to processes or experimental procedures other than those employed by Brown *et al.* (1998) remains to be determined.

To appreciate the significance of the magnitude value of a reduction factor over an individual stage, it is necessary to relate its value to the potential quantity of the infectious agent that requires to be removed or inactivated. For example, where there is a high concentration of a virus in a plasma donation (e.g. HIV, HBV, HCV, B19 parvovirus) then a relatively high degree of reduction (e.g. 10^4-fold) may be required over individual process steps to assure product safety (Darling & Spaltro, 1996). However, where the concentration of the infective agent is relatively low (e.g. TSE agents in plasma) then a small degree of reduction may be significant (Brown, 1998).

Whether or not the individual reduction factors for each step in a process (Fig. 1) can be added together to provide a notional overall reduction factor across a complete process (Table 3) is dependent on the properties and state (e.g. degree of aggregation) of the infectious agent, the principles of the separation technologies concerned, the conditions at each step, the relative positions of different technologies within a process and other factors which might limit the effectiveness or capacity of a particular step or technology (Hageman, 1991). For TSE agents, where different operating conditions are employed in a series of successive steps, then each removal step is generally, but not always, regarded as additive (Rohwer, 1996). Where the same or similar step is used more than once, reduction factors may be additive if TSE agent removal is limited by the capacity of the step, but not where an equilibrium relationship (e.g. solubility of the TSE agent) is limiting.

Much remains to be learned concerning the physico-chemical properties of TSE agents in general (Edenhofer *et al.*, 1997) and nvCJD in particular. In the absence of such data it is inevitable that uncertainty will exist over the ability of particular process steps, either individually or in combination, to fully remove any nvCJD agent which may be present. In these circumstances the availability of a number of process steps which would be expected to remove a TSE agent by different mechanisms will provide a greater assurance of product safety than reliance on either a single step or a single mechanism of removal. The fact that plasma products are manufactured via a number of process steps which would be expected to operate in a complementary manner may be of particular importance in this regard.

Table 3. Estimated TSE agent reduction for each SNBTS plasma product

Product	No. process steps contributing to TSE agent reduction			Sum of estimated TSE agent reduction factors
	Precipitation	Adsorption (gel)	Adsorption (filter)	
Albumin (Alba®)	3	1*	5	10^{13}
Immunoglobulins	2	1*	5	10^9
Factor IX (HIPFIX®)	1	3	–	10^7
Thrombin	1	2	2	10^7
Fibrinogen	1	2	2	10^5
Factor VIII (Liberate®)	1	2	–	10^4
Factor II, IX, X (DEFIX®)	1	1	–	10^3

* Step applied only to 1/3rd of plasma pools and discounted in summation of reduction factors.

POSSIBLE nvCJD CONTENT OF PLASMA PRODUCTS

In order to estimate the possible nvCJD content of a plasma product it is necessary to first estimate the nvCJD content of the starting plasma pool, secondly to calculate the quantity of nvCJD infectivity remaining after processing and thirdly to consider how this material may be distributed in the vials or bottles of the dispensed product.

To determine the quantity of nvCJD infectivity that could potentially be present in a plasma pool, it is necessary to know the dose of nvCJD needed to transmit infection from human to human by intravenous or intramuscular administration, the number of infectious doses present in the plasma of an infected blood donor and the number of infected donations present in the plasma pool.

There are as yet no data available on the nvCJD content ($ID_{50}\,mL^{-1}$) of human blood or plasma. However, as nvCJD is believed to be human BSE (Almond & Pattison, 1997), then bovine data probably represent the best information currently available for the purpose of estimating the infectivity of blood from a person infected with nvCJD. BSE was not detected in the blood or serum of infected cattle, by i.c. injection into mice (Kimberlin, 1996). However, the limit of detection in these studies was 25 $ID_{50}\,mL^{-1}$ and, given the species barrier involved, the within-species infectivity could have been as high as 25 000 i.c. $ID_{50}\,mL^{-1}$. Correction for the route of infusion (from i.c. to i.v.) could give a within-species infectivity of blood of up to 2500 i.v. $ID_{50}\,mL^{-1}$.

Table 4. Theoretical estimates of the quantity of nvCJD in products prepared from pooled plasma where 0·15% of donations contain nvCJD

SNBTS product	Volume plasma per batch (L)	Total nvCJD (i/v. ID_{50}) In plasma pool*	In product pool pre-dispensing	In final vial†
Albumin, 4·5% (Alba®)	2000	$7{\cdot}5\times10^{5}$	$7{\cdot}5\times10^{-8}$	$3{\cdot}0\times10^{-11}$
Albumin, 20% (Alba®)	2500	$9{\cdot}7\times10^{5}$	$9{\cdot}7\times10^{-8}$	$3{\cdot}4\times10^{-11}$
IgG i/m	1500	$6{\cdot}0\times10^{5}$	$6{\cdot}0\times10^{-4}$	$3{\cdot}8\times10^{-9}$
IgG i/v	2000	$7{\cdot}5\times10^{5}$	$7{\cdot}5\times10^{-4}$	$3{\cdot}7\times10^{-7}$
Thrombin	3000	$1{\cdot}1\times10^{6}$	$2{\cdot}2\times10^{-3}$‡	$1{\cdot}9\times10^{-6}$
Factor IX HIPFIX®	2700	$1{\cdot}0\times10^{6}$	$1{\cdot}0\times10^{-1}$	$1{\cdot}2\times10^{-4}$
Fibrinogen	2000	$7{\cdot}5\times10^{5}$	$7{\cdot}5\times10^{0}$	$6{\cdot}2\times10^{-3}$
Factor VIII Liberate®	4000	$1{\cdot}5\times10^{6}$	$1{\cdot}5\times10^{2}$	$9{\cdot}2\times10^{-2}$
FII, IX, X DEFIX®	3000	$1{\cdot}1\times10^{6}$	$1{\cdot}1\times10^{3}$	$6{\cdot}2\times10^{-1}$
Factor VIII (Z8)§	1000	$3{\cdot}7\times10^{5}$	$3{\cdot}7\times10^{3}$	$3{\cdot}7\times10^{0}$
Factor VIII (NY)§	1000	$3{\cdot}7\times10^{5}$	$3{\cdot}7\times10^{4}$	$2{\cdot}7\times10^{1}$

* Based on nvCJD infectivity of 250 i.v. $ID_{50}\,mL^{-1}$ in plasma from each infected donation (300 mL). † Assumes an even distribution of nvCJD amongst all vials in a batch of product. ‡ Only about 2% of plasma pool processed to thrombin. § Products discontinued in 1992 (Z8) and 1986 (NY).

10^{5}), a relatively high infectivity of nvCJD in plasma (i.e. 250 i.v. $ID_{50}\,mL^{-1}$) and generally low values for the TSE agent process reduction factors (Table 2).

However, these calculations also involved a number of assumptions concerning process reduction factors that were extrapolated from a small number of studies that were themselves based on animal model systems not necessarily predictive for the human situation. Therefore, it is inevitable that uncertainty remains over whether or not there may be a risk of nvCJD being transmitted by any of the plasma products assessed. To obtain a more certain estimate of risk it will be necessary to determine the infectivity of the causative agent of nvCJD, its prevalence in the UK blood donor population and the effectiveness of plasma fractionation processes in removing TSE agents using appropriate measurements.

CONCLUSIONS

All of the available evidence concerning the properties and behaviour of the causative agents of TSEs suggests that a number of the bioseparations technologies used in the manufacture of human plasma products should have a potential to remove the causative agent of nvCJD. For each SNBTS product, the estimated potential for nvCJD removal involves processing by multiple unit operations and different principles of separation, both of which provide a greater degree of assurance than would be obtained with reliance on either a single step or a single mechanism of separation.

This assessment suggests that should there be a major epidemic of nvCJD in the UK, then most SNBTS plasma products prepared from plasma collected in the UK should have a very low risk of being contaminated. Nevertheless, many uncertainties remain and it will be necessary to establish the accuracy of these estimates in appropriate validation studies. Such studies should also indicate whether or not adsorption or precipitation technologies used in plasma fractionation could be exploited further to provide an increased capacity for the removal of human agents of TSE.

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TRANS[illegible]

The distribution [illegible] plasma derivatives [illegible] transmissible spong[illegible]

P. Brown, R.G. Rohwer, B.C. Dunstan [illegible]

BACKGROUND: The administration of blood compo- nents from donors who subsequently develop Creutzfeldt-Jakob disease has raised the issue of [illegible] as a possible vehicle for iatrogenic disease.
STUDY DESIGN AND METHODS: We examined infec- tivity in blood components and Cohn plasma fractions [illegible] normal human blood that had been "spiked" with trypsinized cells from a scrapie-infected hamster brain and in blood of clinically ill mice that had been inocu- lated with a mouse-adapted strain of human transmis- sible spongiform encephalopathy. Infectivity was as- sayed by intracerebral inoculation of the [illegible] specimens into healthy animals.
RESULTS: Most of the infectivity in spiked human [illegible] was associated with cellular blood components; the smaller amount present in plasma, when fractionated, was found mainly in cryoprecipitate (the source of [illegible] VIII) and fraction I+II+III (the source of fibrinogen and immunoglobulin); almost none was recovered in fraction IV (the source of vitamin-K-dependent proteins) and fraction V (the source of albumin). Mice infected with the human strain of spongiform encephalopathy had very low levels of endogenous infectivity in buffy coat, plasma, cryoprecipitate, and fraction I+II+III, and no de- tectable infectivity in fractions IV or V.
CONCLUSION: Convergent results from exogenous spiking and endogenous infectivity experiments, in which decreasing levels of infectivity occurred in cellular blood components, plasma, and plasma fractions, sug- gest a potential but minimal risk of acquiring Creutzfeldt- Jakob disease from the administration of human plasma protein concentrates.

MATERIALS AND METHODS

High input infectivity ("spiking") experiment

Preparation of material used in spiking experiment. One half of each brain from two terminally ill golden Syrian hamsters that had been infected with the 263K strain of scrapie agent were combined (total 1.0 g wet tissue) and minced into very fine fragments. The fragments were then suspended in 9 mL of phosphate-buffered saline (PBS) at pH 7.0 containing 0.025-percent trypsin and 0.05-percent EDTA, and incubated with constant stirring at 37°C for 30 minutes to disperse cells. Residual fragments were resuspended and similarly incubated in fresh trypsin-EDTA solution. No fragments remained after the second trypsinization, and the pooled pellets from each specimen (following centrifugation at 500 × *g* for 15 min) were washed two times in 50 mL of PBS. The final washed pellet contained 1.6×10^9 neuronal and glial cells, of which 99 percent were viably intact as evidenced by failure to stain with trypan blue, and contained 9.1 mean lethal dose ($\log_{10}/LD_{50}$) infectious units as determined by endpoint dilution assay in hamsters. The pellet was resuspended in 46.8 mL of normal whole human blood containing CPD (United States Pharmacopoeia) at an anticoagulant-to-blood ratio of 1:9.

Separation of blood into its components. A scaled-down version of the "three-bag" protocol used by the American Red Cross was used for component separation. Anticoagulated whole blood was centrifuged (Sorvall SS-34 rotor, DuPont Medical Products Clinical Diagnostics, Wilmington, DE) at 4300 rpm (2280 × *g*) for 4 minutes at ambient temperature. The supernatant plasma was carefully withdrawn by pipette down to the edge of the buffy coat overlying the red cell sediment, transferred to a new 50 mL tube, and centrifuged at 5800 rpm (4200 × *g*) for 8 minutes at ambient temperature. The supernatant plasma was pipetted into a new tube, leaving behind a very small sedimented pellet. Without disturbing their contents, all specimens were frozen intact at –70°C. While frozen, the buffy coat layer overlying the red cell sediment was sliced apart and combined with the pellet from the plasma centrifugation step to yield a single white cell and platelet specimen for assay.

Cohn fractionation of plasma component. Fractionation was carried out in a scaled-down version of a protocol in wide commercial use,[3] and yielded a protein profile similar to that of the production-scale process. Approximately 10 mL of plasma was transferred from –70°C to –20°C for overnight "tempering," then exposed to a final 30-minute thaw inside a 50-mL jacketed reaction beaker connected to a refrigerated circulating bath set at 1 to 2°C. The thawed plasma was transferred to a weighed, cold, 15-mL centrifuge tube and centrifuged at 6800 rpm (5600 × *g*) for 15 minutes at 1 to 2°C. The pellet was weighed and then frozen at –70°C (cryoprecipitate).

The supernatant was again placed into the reaction beaker-circulating bath apparatus set at 1 to 2°C, and the pH was adjusted to 6.65 to 6.70 with acetate buffer, pH 4.0. (10.9 g sodium Acetate, 24 g glacial acetic acid, 71 mL water). Slowly, over a period of 1 hour, repeated small amounts of cold 95-percent ethanol were added to achieve a final ethanol concentration of 20 percent. After addition of one half of the ethanol, the pH was verified to be in range of 6.80 to 7.00, and the circulating bath temperature was lowered from 1 to 2°C to -5°C. The plasma-ethanol mixture was transferred to a weighed, cold centrifuge tube and centrifuged at 6800 rpm (5600 × *g*) for 15 minutes at -5°C. The pellet was weighed and frozen at –70°C (fraction I+II+III).

The supernatant was again placed into the reaction beaker-circulating bath apparatus set at -5°C. The pH was adjusted to 5.16 to 5.22 with acetate buffer in 20-percent ethanol, pH 4.0, and then further adjusted to a final pH of 5.75 with 1 M $NaHCO_3$. Slowly, over a period of 1 hour, small quantities of cold 95-percent ethanol were added to achieve a final ethanol concentration of 40 percent and a final pH of 5.92 to 5.98. The plasma-ethanol mixture was transferred to a weighed, cold centrifuge tube and centrifuged at 6800 rpm (5600 × *g*) for 15 minutes at -5°C. The pellet was weighed and frozen at –70°C (fraction IV_1/IV_4).

The supernatant was placed into a tube containing 2 mg of filter aid per mL of supernatant, mixed, and filtered through a 20-mL syringe containing a filter (CPX70, Cuno, Meriden, CT). The filtrate was placed into the reaction beaker-circulating bath apparatus set at -5°C. The pH was adjusted to 4.78 to 4.82 by slowly adding acetate buffer in 40-percent ethanol, pH 4.0. The plasma mixture was placed into a weighed, cold centrifuge tube and centrifuged at 6800 rpm for 15 minutes at –5°C. The pellet was weighed and frozen at –70°C (fraction V). The supernatant was also frozen at –70°C (fraction V supernatant).

Infectivity bioassays. On the day of the test, specimens (inoculum, whole blood, blood components, and Cohn fractions) were thawed, serial 1-in-10 dilutions were made in PBS (pH 7.4), and specimens were inoculated intracerebrally in volumes of either 30 µL (for components) or 50 µL (for fractions) to groups of 4 to 8 female weanling hamsters per dilution. Two cages of uninoculated hamsters served as "sentinels" to monitor laboratory cross-contamination. Animals were observed for 8 months, and the brains from a random sampling of clinically positive animals in all higher dilution groups were examined to verify the presence of spongiform neuropathology. None of the uninoculated sentinel animals showed clinical or neuropathological signs of scrapie.

Using the method of Reed and Muench,[4] $\log_{10} LD_{50}$ infectivity titers were calculated except for the plasma specimen, for which infectivity was estimated comparing its incubation period curve to that of whole blood at dilutions 10^{-1} through 10^{-4} (the highest dilution of plasma that was

inoculated). This estimate makes use of the inverse relationship between the amount of infectivity and the length of the incubation period (the greater the infectivity, the shorter the interval between inoculation and disease)—a type of "dose-response" curve. Although not as precise as an endpoint dilution titration, it is reassuring that the whole blood, red cell, and buffy coat specimens, which had nearly identical endpoint dilution titers, also had nearly superimposable incubation period curves, and that the plasma curve was parallel to the whole blood curve at a [illegible] $\log_{10}$ unit lower level.

Endogenous infectivity experiment

Experimental model. Weanling Swiss-Webster mice (Charles River Laboratories, Wilmington, MA) were inoculated intracerebrally with a 10-percent clarified homogenate of a mouse-adapted Fukuoka-1 strain of human [illegible] Gerstmann-Sträussler-Scheinker disease (GSD).[5,6] When mice began to show symptoms of disease (approx. 4 months after inoculation), they were lightly anesthetized and bled by open chest direct cardiac puncture into CPD containing 5 units of heparin per mL blood to counteract the unusually strong clotting tendency of mouse blood. At the time of exsanguination, brains and spleens were also removed from each animal; tissue pools of each organ were made into separate 10-percent tissue suspensions in PBS for infectivity titrations performed at the same time as those for the blood specimens.

Collection and processing of blood specimens. A total of 75 mice yielded a pooled sample volume of 52 mL ([illegible] mL of blood and 7 mL of citrate containing 225 units of heparin). The blood was immediately separated into red cell, white cell-platelet, and plasma components, frozen at –70°C. A portion of the plasma was later thawed and processed into Cohn fractions, as described in the spiking experiment. The only difference was that, in this experiment, we did not combine the buffy coat layer of the red cell sediment with the centrifuged plasma pellet, choosing instead to assay the two specimens separately.

Infectivity bioassays. All specimens were inoculated intracerebrally in 30-µL volumes into groups of weanling Swiss-Webster mice, and two cages of uninoculated sentinel animals were included as cross-contamination controls. Because of anticipated low or undetectable infectivity levels in most specimens, this experiment was conducted in a facility that had never been used for TSE experiments, and specimens were inoculated into groups of up to 130 mice. Undiluted inocula proved to be highly toxic, causing nearly instantaneous death that was probably due to a combination of high osmolarity, anticoagulant, and (in the case of Cohn fractions) residual alcohol; dilutions of 1-in-4 to 1-in-5 were well tolerated and were therefore used for most inoculations. Serial 1-in-10 dilutions were inoculated for specimens expected to have higher infectivity titers, such as brain, spleen, and the white cell-platelet component of blood.

TABLE 1. Distribution of infectivity among blood components and Cohn plasma fractions in normal human blood "spiked" with $10^{9.4}$ LD_{50} of scrapie infectivity contained in a trypsinized suspension of viable brain cells from hamsters infected with the 263K strain of scrapie agent*

Specimen	Specimen vol (or wt)	Infectivity concentration ($\log_{10}LD_{50}$/mL or g)	Total infectivity ($\log_{10}LD_{50}$)†	Fractional recovery of infectivity(%)†
Whole blood	46.8 mL	8.3	9.3 × 10^9	100
Red cells	[illegible] mL	8.0	2.0 × 10^9	22
White cells/platelets‡	2.0 mL	8.5	6.3 × 10^8	7
Plasma§	24.0 mL	7.1	3.0 × 10^8	3
Fractionated plasma (11 mL)				
Plasma§	11.0 mL	7.1	1.4 × 10^8	100
Cryoprecipitate	0.20 g	6.6	1.0 × 10^6	0.71
Fraction I+II+III	[illegible] g	6.1	1.2 × 10^6	0.86
Fraction IV_1+IV_4	0.57 g	4.0	8.7 × 10^3	0.006
Fraction V	[illegible] g	3.5	0.5 × 10^3	0.0004
Fraction V supernatant	11.5 mL	ND¶		

* Specimens were assayed by intracerebral inoculation of healthy weanling hamsters.
† For components, the amount of infectivity in the component compared to the amount of infectivity in the whole blood; for fractions, the amount of infectivity in the fraction compared to amount of infectivity in the plasma sample used for fractionation. Note that because differences of less than ±0.5 log in infectivity concentration between any two specimens are not necessarily significant, fractional recovery percentages could be correspondingly higher or lower in a repeat experiment.
‡ Recovered from centrifuged plasma (4200 × g for 8 min).
§ Infectivity estimated from comparison of incubation period time curve to that of whole blood (see Methods section).
¶ ND = none detected (no disease transmissions in groups of four hamsters inoculated with undiluted through [illegible] dilutions).

considered to be significant in single-assay comparisons); somewhat lower levels in plasma and the first two plasma fractions, and substantially lower levels (4-6 $\log_{10}$ reduction) in the last two fractions (Table 1). The absence of transmissions in the small group of animals inoculated with the final fraction V supernatant is consistent with a range of infectivity (using a Poisson distribution calculation) from zero to 1.4 $\log_{10}$, that is, less than the demonstrated infectivity in fraction V.

Considering the total amount of infectivity (rather than its concentration) in these blood components and fractions, about equal amounts (3-7%) of infectivity were recovered in buffy coat and plasma, of which a very small amount of plasma infectivity [illegible] was to the cryoprecipitate and fraction I+II+III, and virtually none [illegible] the last two fractions.

It may be remarked that a significant proportion of input spike infectivity was not recovered, either in the blood components or in the plasma fractions. Some of this apparent "disappearance" could have been due to the imprecision of the bioassay (±0.5 $\log_{10}$ variability of LD_{50} titers), and some could have resulted from adherence of infective particles to containers used for experimental manipulations. It is also possible that some infectivity was lost as a result of Cohn fractionation, although low pH and ethyl alcohol by themselves have previously been shown not to inactivate the agents of TSE.[7,8]

Endogenous blood infectivity in TSE mouse model

From clinically ill mice that had 4 months earlier been inoculated intracerebrally with a mouse-adapted strain of human TSE, specimens of buffy coat, plasma, cryoprecipitate, and Cohn fraction I+II+III transmitted disease to a few animals, but no transmissions occurred from whole blood, red cells, or Cohn fractions IV and V (Table 2).

TABLE 2. Infectivity in blood components and plasma fractions processed from the pooled blood of 75 mice experimentally infected 4 months earlier with a mouse-adapted strain (Fukuoka-1) of Gerstmann-Sträussler-Scheinker disease*

Specimen	Specimen vol (or wt)	Proportion of specimen inoculated (%)†	Specimen dilution	Positive animals‡	Negative animals‡
Whole blood	45.0 mL	0.15	1-in-5	0	11
Red cells	18.0 mL	0.22	1-in-5	0	7
Buffy coat§	3.5 mL	2.3	1-in-5	2	10
			1-in-50	0	6
Plasma pellet¶	0.2 mL	60	1-in-6	4	19
			1-in-60	0	10
Plasma‖	22.6 mL	3.5	1-in-5	8	124
			1-in-50	0	10
Fractionated plasma (11.3 mL)					
Cryoprecipitate	0.15 g	29	1-in-4	5	6
			1-in-40	1	3
Fraction I+II+III	0.40 g	37	1-in-4.5	6	37
Fraction IV_1+IV_4	0.86 g	38	1-in-4	0	86
Fraction V	1.22 g	30	1-in-4	0	94

* Specimens were assayed by intracerebral inoculation of healthy weanling mice.
† Amount of inoculated specimen divided by the amount contained in the 45-mL volume of whole blood (taking into account the volume and dilution of each inoculated specimen; dilution of anticoagulant; and for fractions, the fractionated plasma volume).
‡ Confirmed by Western blot tests for PrP in brain extracts. Sixteen animals inoculated with higher dilutions of the plasma pellet, fraction IV, and fraction V, tested negative.
§ Sliced from top 5 mm of red cell sediment frozen after centrifugation of whole blood. The amount of infectivity may be greater than shown, as several more animals that died at about the same time as the positive animals were not tested for PrP and were thus excluded from the table.
¶ Pellet after plasma centrifugation for 8 minutes at 4200 × g (see Methods).
‖ Supernatant after plasma centrifugation for 8 minutes at 4200 × g (see Methods).

The presence of infectivity in the separately [illegible] specimens of buffy coat and the centrifuged plasma pellet probably reflects the presence of white cells in both specimens, but raises the possibility that platelets as well as white cells might contain the infectious agent. It should also be noted that the absence of transmissions from the whole blood and red cell specimens does not imply the absence of infectivity (which would be unreasonable in view of its presence in buffy coat and plasma), because only very small proportions of these specimens were assayed, due to the necessity of using diluted inocula. The separate pools of brains and spleens collected from the same 75 animals had infectivity titers of approximately [illegible] LD_{50} per g and [illegible] LD_{50} per g, respectively, similar to titers observed in an earlier experiment using the same mouse model.[illegible]

DISCUSSION

Several earlier studies of TSE have tested one or another component of whole blood specimens for the presence of infectivity, with conflicting results: most of the [illegible] were from buffy coat, but in a few studies, whole or extracted blood, and serum or concentrated serum [illegible] found to be infectious; and no infectivity was detected in nearly half of such studies (including [illegible] of sheep naturally infected with scrapie, and [illegible] mates inoculated with blood from humans with CJD [illegible]). None of these studies examined the distribution of infectivity in different blood components of a single [illegible] and none examined infectivity in the Cohn fractions that represent an intermediate stage between crude plasma and therapeutic plasma protein concentrates.

Experimental design considerations

The primary goals of these experiments were to determine the effect of a standard protocol for blood separation and plasma fractionation in blood containing a high enough level of infectivity to permit an estimate of the degree to which processing caused a reduction in infectivity (agent clearance) and provide an idea of the distribution of [illegible] much lower levels of endogenous infectivity that would [illegible] expected to occur in the blood of experimentally infected animals.

No single experimental design can answer both questions. For clearance studies, a much higher level of infectivity is needed than occurs in the blood of experimentally infected animals to measure serial infectivity reductions [illegible] successive processing steps. Scrapie-infected hamster brain satisfies this condition of high-input infectivity. [illegible] choice of trypsinized and washed intact brain cells [illegible] based on evidence that blood infectivity is most likely [illegible] associated,[1] and thus, insofar as could [illegible] fected cells represent a more appropriate infectious [illegible] than either infectious tissue homogenate [illegible] free PrP. We could not know in advance [illegible]

cell-associated rather than cell-free origin, but further work needs to be done to resolve the issue.

Infectivity estimates and risk assessment

What might be the likely limits of infectivity in the plasma of a patient with CJD? For this speculative calculation, we can reason as follows: if each of the assay mouse transmissions resulted from a single infectious unit, which seems likely in view of the small proportion of positive to inoculated animals in the 1-in-5 dilution and the absence of transmissions in the 1-in-50 dilution, then the number of observed transmissions (8) multiplied by the reciprocal of the percentage of plasma inoculated (100/3.5) predicts the number of infectious units (230) that would have been observed if all 22.6 mL of plasma had been inoculated. Thus, the mouse plasma contained approximately 10 infectious units per mL. Similar calculations yield infectivity estimates per mL of processed plasma of about 5 infectious units in cryoprecipitate, and one infectious unit in fraction I+II+III.

If the 10 infectious units per mL of plasma are considered as a concentration of infectivity applicable to both humans and mice, then a standard 450-mL blood donation (containing approx. 250 mL of plasma) would contain about 2500 infectious units. Even if an intravenously inoculated plasma specimen were only 1-in-100th as likely to produce infection as the intracerebral inoculation assay used in this experiment,[13] the consequent estimate of 25 infectious units still seems far too high in view of the fact that no case of CJD has yet been linked to the administration of blood or blood products.[14-17] It is possible that peripheral routes of infection are even less efficient than supposed, or that dilution of this comparatively low number of infectious units in large donor pools comes into play in further reducing the risk of disease transmission.

A question of immediate practical importance is the issue of which plasma products deserve the most attention as possible vehicles for the transmission of CJD. Our results suggest that the potential for transmission would be comparatively higher for cryoprecipitate and fraction I+II+III than for fractions IV and V. Albumin, made from fraction V, is an especially important product because it is used as an excipient and stabilizer in other plasma protein concentrates, as well as in various non-plasma-derived biologicals, including products as varied as vaccines, injectable diagnostic radiology dyes, and embryonic cultures for in-vitro fertilization procedures. Judging from the nearly 5 $\log_{10}$ reduction in infectivity in fraction V as compared to plasma in the spiking experiment, and the absence of fraction V infectivity in the TSE mouse model, the risk of contracting CJD from exposure to albumin must be extremely low.

CONCLUSIONS

The distribution of blood infectivity in two different experimental models of TSE—one using an infectious cellular spike of normal blood and the other using blood from experimentally infected mice—confirmed the previously demonstrated association of infectivity with buffy coat. An unexpected finding was the presence of infectivity in plasma, which may have resulted from the imperfect separation of cells and plasma in the course of a standard centrifugation separation protocol. Cohn fractionation of the infectious plasma further reduced its infectivity to very low or undetectable levels.

The levels of infectivity demonstrated in these model studies may not be fully representative of the actual risk of disease transmission from human blood components because: 1) blood from a CJD patient included in a donor pool will contribute only a minute proportion of plasma to the pool, which is usually made up from as few as 6000 to more than 100,000 donors[18]; 2) many therapeutic protein concentrates are derived from plasma fractions processed through chromatography columns that are known to adsorb (although not inactivate) TSE infectivity[19,20]; and 3) plasma products are administered via intravenous and parenteral injections, which have been shown to be comparatively inefficient routes of TSE disease transmission.[13]

Our results represent only the beginning of a rational approach to an assessment of the risk, if any, of acquiring CJD from the administration of blood components or plasma products. Among urgently needed additional pieces of information are answers to the following questions: 1) is there a similar amount and distribution of blood infectivity in the preclinical stage of disease (when humans would usually be donating blood)?; 2) is the infectivity present in plasma the result of contamination by white cells or white cell debris (special interest in white cells comes from the demonstration that B cells are important for neuroinvasion and clinical infection[21])?; 3) can the low levels of endogenous blood infectivity detected by intracerebral inoculation of assay animals also be detected by intravenous or intramuscular inoculation (the routes by which most therapeutic blood products are administered)?; 4) will such infectivity, if present in Cohn fractions, be carried through the additional processing steps used to produce therapeutic end products?; and finally, 5) does "new variant" CJD have the same biological characteristics with respect to blood infectivity as other types of TSE?

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Prion-removal capacity of chromatographic and ethanol precipitation steps used in the production of albumin and immunoglobulins

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Background and Objectives Although there is no epidemiological evidence to suggest that classical Creutzfeldt-Jakob disease (CJD) is transmitted through blood or blood products, the variant form (vCJD) has been implicated in transmission via packed red blood cells. The potential threat of the infectious agent contaminating plasma pools has led to manufacturing processes being examined for capacity to remove prions. The objective of these studies was to examine the prion-removal potential of the chromatographic purification and ethanol precipitation steps used to fractionate immunoglobulins and albumin from human plasma.

Materials and Methods Western blot assay was used to examine the partitioning of proteinase K-resistant scrapie prion protein (PrP^{sc}) over DEAE Sepharose, CM Sepharose and Macro-Prep High Q chromatographic columns, utilizing microsomal scrapie 263K spiked into each scaled down feedstream and assayed after each chromatographic step. In further studies, bioassay in C57 black mice was used and spikes of 10 000 g clarified brain homogenate of scrapie ME7 were added to feedstreams before sequences of scaled down chromatographic or Cohn fractionation process steps.

Results The microsomal spiking study with Western blot detection demonstrated substantial partitioning of PrP^{sc} away from the target proteins in all ion exchange chromatographic steps examined. The $\log_{10}$ reduction factors (LRF) across DEAE Sepharose and CM Sepharose columns for albumin were ≥ 4.0 and ≥ 3.0 respectively. The reductions across DEAE Sepharose and Macro-Prep High Q for intravenous immunoglobulin were 3.3 and ≥ 4.1 respectively. Bioassay demonstrated LRFs of ≥ 5.6 across the combination of DEAE Sepharose and CM Sepharose columns in the albumin process and ≥ 5.4 across the combination of DEAE Sepharose and Macro-Prep High Q columns in the intravenous immunoglobulin process. Bioassay studies also demonstrated a LRF of ≥ 5.6 for immunoglobulin produced by Cohn fractionation.

Conclusions Using rodent-adapted scrapie as a model, the studies indicated that ion exchange chromatography, as well as Cohn immunoglobulin fractionation have the potential to effectively reduce the load of TSE agents should they be present in plasma pools.

Table of Contents Ion exchange columns used for production of human albumin and immunoglobulins, as well as Cohn immunoglobulin fractionation, effectively reduce the load of TSE agents should they be present in plasma pools.

Key words: bioassay, chromatography, prion, scrapie, transmissible spongiform encephalopathy, Western blot.

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Introduction

The outbreak of the variant form of Creutzfeldt-Jakob disease (vCJD), linked to a bovine spongiform encephalopathy (BSE) in the UK, and the propensity of this form to accumulate in peripheral lymphoid tissues, has raised the theoretical possibility of blood-borne transfusion of the vCJD agent. Experimental studies in a sheep model in which BSE was transmitted via blood transfusions [1] demonstrate proof of principle for this possibility. It is probable that transmission has occurred in humans with the report of vCJD in a blood transfusion recipient 6·5 years after receiving red blood cells from a presymptomatic vCJD donor [2]. This report led to the identification of 20 U of plasma from individuals who later developed vCJD that were pooled to produce fractionated products used to treat thousands of recipients. To date, no cases of vCJD have been identified in recipients of these fractionated plasma products.

Evidence that vCJD may be transmitted by red blood cell transfusion followed the post-mortem detection of proteinase K-resistant scrapie prion protein (PrP^{sc}) in the spleen and lymph node of a patient who died of other causes, having previously receiving a red blood cell transfusion from a donor that subsequently developed vCJD [3]. More recently, the UK National CJD Surveillance Unit has announced a 'probable' third case of transfusion-related vCJD, in which the patient (who is still living) developed symptoms of vCJD about 8 years after receiving a blood transfusion from a donor who developed symptoms of vCJD about 30 months after donating this blood [4]. In contrast to vCJD, classical CJD transmission by blood transfusion has never been reported in humans [5].

The potential risk of vCJD transmission led producers of plasma products to examine the prion-removal capacity of their fractionation processes [6–10]. A difficulty with accurately modelling the removal of blood-borne infectious prions from plasma processes is identifying the form of 'spiking' material that best represents what might be present in blood. The best representation of blood-borne infectivity is the use of blood ex-sanguinated from test animals with clinical TSE [11,12]; however, the low infectivity level found in blood does not enable a high infectivity challenge of plasma fractionation processes. TSE-infected brain material offers much higher levels of infectivity and a variety of preparations have been reported. Ideally, a range of different spiking materials would be tested on each process step [9]; however, in practice, investigators have selected one or two preparations for their experiments because of practical limitations including the many test animals required for bioassays.

Rodent-adapted scrapie has been used extensively as a model for the study of prion partitioning during plasma processing steps [6,10,13,14]. The incubation period of

Experiments using microsomal 263K spiking and Western Blot assay

Preparation of microsomal inoculum

Brain homogenate from hamsters without disease, or in the late clinical stage of infection with hamster adapted scrapie (strain: 263K), was used to prepare a microsomal fraction as described [21]. Briefly, crude brain homogenate (10% wt/v) was prepared by Dounce homogenization of brains in phosphate-buffered saline (PBS). This was pelleted at 10 000 *g* for 7 min to remove nuclei, unbroken cells and mitochondria. The microsomes remaining in the supernatant were then pelleted by centrifugation at 100 000 *g* for 90 min, followed by resuspension in PBS.

DEAE Sepharose chromatography

De-lipidated and euglobulin (non-IgG globulins)-depleted Supernatant I (SNI) was obtained from the production plant, and 135 ml was 'spiked' at 10% v/v with microsomal control or scrapie 263K and sampled (Fig. 1). DEAE Sepharose™ Fast Flow (DEAE Sepharose) was obtained from GE Healthsciences, Uppsala, Sweden. A 17·5 cm bed height column was equilibrated with 10 mM sodium acetate (NaAc) at pH 5·2, and one-third of the spiked material was loaded. Following loading, the column was washed with 10 mM NaAc buffer and protein elution was monitored by ultraviolet (UV) absorption at 280 nm. The non-retained crude immunoglobulin was collected until the onset of the second peak, in which transferrin was eluted.

The 10 mM NaAc wash was continued until the elution of the transferrin peak was complete. Albumin was then eluted with approximately 2·5 column volumes (CV) of 25 mM NaAc buffer. The column was regenerated with 2 CV of 150 mM NaAc, pH 4·0. The loading and elution cycle was repeated a further two times to load the entire starting volume as per the production process, before regeneration and sanitization in reverse flow with 1 CV of 0·5 M NaCl, 1 CV of 1 M NaOH and 2·5 CV of 150 mM NaAc. All corresponding peak fractions from each cycle (other than the 1 M NaOH eluate) were pooled and assayed by Western blot.

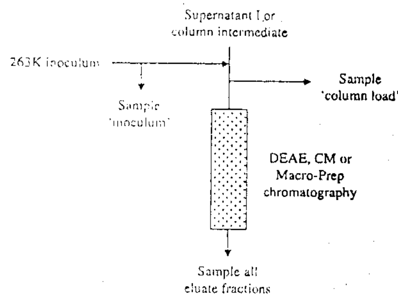


Fig. 1 Flow diagram showing spiking points and sampling points for each column in 263K PrP^{sc} studies. The diagram applies to each of the chromatography columns, as they were each spiked separately.

CM Sepharose chromatography

CM Sepharose™ Fast Flow (CM Sepharose) was obtained from GE Healthsciences, Uppsala, Sweden. A 17·5 cm bed height column was equilibrated with 25 mM NaAc (pH 4·5). Pooled crude albumin from the DEAE Sepharose column was obtained from the production plant, and 150 ml was spiked at 10% v/v with microsomal control or scrapie 263K. After sampling, one-third of the volume was loaded onto the column, and then flushed with 1·8 CV of 25 mM NaAc to elute the unbound proteins. Albumin was then eluted with approximately 3 CV of 110 mM NaAc buffer. The column was regenerated with 1·5 CV of 400 mM NaAc pH 8·0. The loading and elution cycle was repeated a further two times to load the entire starting volume as per the production process, before the column was regenerated and sanitized in reverse flow with 1 CV of 0·5 M NaCl, 1 CV of 1 M NaOH and 2·5 CV of 150 mM NaAc. All corresponding peak fractions from each cycle (other than the 1 M NaOH eluate) were pooled and assayed by Western blot.

Macro-Prep chromatography

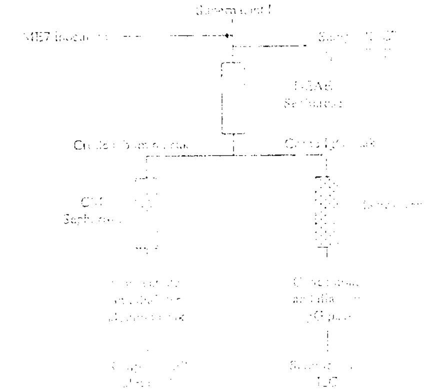
Macro-Prep High Q (Macro-Prep) gel was obtained from Bio-Rad, Hercules, CA. A sample of non-retained crude IgG solution from DEAE Sepharose was obtained from an actual production process and 100 ml was spiked at 10% v/v with microsomal control or scrapie 263K. The pH adjusted crude IgG solution was loaded onto a 17·5 cm bed height column that had been equilibrated with 6 CV of 10 mM NaAc, pH 6·2. Two CV of 10 mM NaAc pH 6·2 were used to elute the non-retained immunoglobulins from the column. The column was regenerated with 2 CV of 1·0 M NaCl and 2 CV of 1·0 M NaOH. All column eluates (other than the 1 M NaOH eluate) were assayed by Western blot.

Western blot

Samples were ultracentrifuged at 150 000 *g* for 1 h and the pellet was resuspended in a minimal volume of PBS prior to digestion with proteinase K (Roche, Mannheim, Germany) at 250 µg/ml for 1 h at 37 °C. Digestion was terminated by 1 : 1 addition of sample buffer (125 mM Tris-hydrochloric acid, 20% v/v glycerol pH 6·8, containing 4% w/v sodium dodecylsulphate, 5% v/v 2-mercaptoethanol), then boiled for 3 min. Samples were run on 12% polyacrylamide gels (Bio-Rad, Hercules), and transferred onto Immobilon P (Millipore, Billerica, MA). Membranes were blocked with PBS/Tween 20 (0·05%) containing 5% skim milk and were probed with monoclonal antibody (MAb) 3F4 (Signet

Laboratories, Dedham, MA) at 1/10 000 for 1 h. Rabbit antimouse secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich, St Louis, MO) was used at 1/1000 for 1 h. Blots were developed with ECL reagents (GE Healthcare, Uppsala) and were visualized on Hyperfilm M (GE Healthcare, Uppsala).

After Western blot, the dilution was recorded at which PrP^{sc} could no longer be detected. If PrP^{sc} could not be detected in the neat sample, the total PrP^{sc} ($\log_{10}$) reduction was recorded as '≤'. The formula used to calculate the number of units of PrP^{sc} was: reciprocal of the end point dilution of the sample × the total fraction volume in ml × correction factor applied to control for concentration of the sample following ultracentrifugation. Scrapie reduction was calculated by dividing the total scrapie in the spiked starting material by the total recovered scrapie. Variability of the data could not be assessed, as one Western blot was run per sample.



Experiments using bioassay with ME7 spike

Scrapie inoculum

Scrapie ME7 was incubated in C57 black mice, and brains were harvested from mice in the late clinical stage of infection. The brains were homogenized in PBS at 10% wt/v using a Duall tissue grinder (Kontes, Vineland, NJ), and the homogenate was centrifuged at 10 000 *g* for 30 min to remove cellular debris [17].

Chromatography

All chromatographic conditions described for the Western blot study were replicated for the bioassay study; however, columns were run sequentially without intermediate spiking (Fig. 2). De-lipidated and euglobulin-depleted SNI was obtained from a production batch and was 'spiked' with clarified brain homogenate from control mice or ME7-infected mice to give a final spike concentration of 3·3% v/v. For the TSE spiked run, sample 'ME7 spiked SNI' was taken, and 133 ml of the material was separated on DEAE Sepharose. The albumin and immunoglobulin-containing peaks from each cycle were pooled with the corresponding peaks from each of the three cycles and were further processed on CM Sepharose or Macro-Prep.

The pooled crude albumin was loaded onto a CM Sepharose column. The purified albumin peak eluted from each cycle was pooled with the corresponding peak from the other cycles and was concentrated 10-fold with a Pellicon XL 30 kDa polyethersulphone membrane (Millipore, Billerica), and the sample 'ME7 Albumin' was taken for bioassay.

Crude IgG eluate from the DEAE Sepharose column was loaded onto the Macro-Prep column, and the eluted pure IgG concentrated and diafiltered using a 30 kDa regenerated cellulose YM30 ultrafiltration membrane (Millipore, Billerica).