資料4－3

CJD関連各種論文等について

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CJDに関する各種論文等(要約)一覧表

資料番号			概要
A. 血漿分画製剤とvCJDについて			
A-1	血液凝固第Ⅷ因子製剤投与歴のある患者脾臓に異常プリオンタンパクが集積していた事例について		
①	英国保健省(2009.6.5)	vCJD Risk Assessment Calculations for a Patient with Multiple Routes of Exposure	後にvCJDを発症した供血者からの血漿が入った原料血漿から製造された第Ⅷ因子を投与された血友病患者がvCJD以外の疾患で死亡し、剖検によって脾臓から異常プリオンタンパクが検出された。脳を含めた他臓器からは検出されなかった。なお、当該患者の脾臓検体24切片の異常プリオンタンパクが陽性であったものは1切片であり、他の23切片は陰性であった。 問題の第Ⅷ因子製剤のロットは、 FHB4547: 26303人のプールから製造され、DNVモデルから60 ID50の感染性があると推定され、このロット全体で18.38 ID50が含まれていることになる。患者は8025単位の投与を受けたので0.16 ID50に相当する異常プリオンタンパクを投与されたと推定されている。 FHC4237: 21330人のプールから製造され、0.05 ID50に相当する異常プリオンタンパクを投与されたと推定されている。 **一方、この患者は他に、39万単位の英国で採血された血漿由来の第Ⅷ因子製剤を投与されている。虫垂と扁桃の摘出検体の調査から、英国での献血者の中に未発症の感染者が1万人に1人存在していると推定されている。その推定に基づくと、2万人プールの原料血漿に2人の未発症の血漿が入っていることになり、ロット毎に感染価が異なるものの、平均すると第Ⅷ因子1単位あたり6X10^-5 ID50の異常プリオンタンパクが混入していたと推定される。その結果、患者に投与された総異常プリオンタンパク量は24 ID50に相当する達し、上記の2ロット以外のロットからの方が多くの異常プリオンタンパクに暴露されていたことになる。この患者は内視鏡、輸血等も受けていたが上記の計算から第Ⅷ因子製剤からの感染が疑われている。** **問題点:** **該当する製剤の製法が不明。また、linear dose-response modelを用いて個々のロットの感染リスクを加算することによってこの症例が、第Ⅷ因子製剤から感染したと推定しているが、化学物質と同様なことが異常プリオンタンパクの感染にも当てはまるのかについては不明である。さらに献血者1万人あたり1人未発症の感染者がいるという推定についても、献血後少なくとも10年が経つにもかかわらず発症者が問題となっているロットの1人のみということから考えても感染率を過大評価している可能性もある。また、現在も多くの感染者が潜伏期の状態にあるとした場合、10年から20年以上前に献血した血液中に感染性があるのか、という疑問が残る。しかし、該当する2ロットについては発症前の感染者の血漿が混入していたこと、及び他の文献等から当時の製造工程によっては最終製品にプリオンが混入する可能性があると考えられる。**

資料番号			概要
A-2	血漿分画製剤における異常プリオンの不活化・除去法について		
②	Vox Sanguinis 2004;86(2):92-99	Distribution of a bovine spongiform encephalopathy-derived agent over ion-exchange chromatography used in the preparation of concentrates of fibrinogen and factor VIII	Scottish National Blood Transfusion Serviceにより第Ⅷ因子製剤「Liberate」について。S/D処理と陰イオン交換クロマトを用いた製造法により、異常プリオンタンパクの感染性はBSE由来の異常プリオン株BSE301Vで2.7Log(フィブリノゲンは2.9Log)除去される。なお、イムノクロマトを用いた方法では4.57Log除去されると報告。
③	Vox Sanguinis 2004;87 supply2:7-10	Removal of TSE agents from blood products	クリオプリシピテートの精製工程では、1log程度の異常プリオンタンパク除去効果があると報告。
④	Haemophilia 2003;8:53-75	Factor VIII and transmissible spongiform encephalopathy: the case for safety	第Ⅷ因子製剤「Liberate」について、異常プリオンの感染性は異常プリオンタンパク株263Kに対して6.8Log除去されると報告。
	Vox Sanguinis	Studies on the Removal of [illegible] Process Used in the Manufacture of Human Plasma Products	[illegible] 試験されなかった。
⑤	Transfusion Medicine 1999;9:3-14	Assesment of the potential of plasma fractionation processes to remove causative agents of transmissible spongiform encephalopathy	各種クロマトグラフィーや各種フィルトレーション等の製造工程における異常プリオンタンパクの除去効果について報告。
		The distribution of infectivity in [illegible]	
		[illegible]	
		[illegible]	
		[illegible] under different experimental [illegible]	

資料番号			概要
⑩	Biologicals 2006;34:227-231	CJD PrPsc removal by nanofiltration process: Application to a therapeutic immunoglobulin solution (Lymphoglobuline)	製造工程で人由来の赤血球や胎盤を使用するウマ抗人胸腺細胞免疫グロブリンにプリオン病に感染した人の脳乳剤を添加し、ナノフィルトレーションを行なったところ、1.6～3.3 Logのプリオン除去が可能であった。
A-3	白血球除去による異常プリオンの除去について		
⑪	Vox Sanguinis 2006;91:221-230	Creutzfeldt-Jakob disease and blood transfusion: results of the UK Transfusion Medicine Epidemiological Review study	白血球除去フィルターが導入された1999年以降、献血後にvCJDを発症した供血者から輸血を受けた27例の受血者については、今までのところ感染発症したとの報告はない。
⑰	Lancet 2004;364:529-531	Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood	白血球除去工程によって異常プリオンタンパクの感染性を60%減少させることができるが、すなわち血漿には感染性が40%残存する。(WBCは1X10^6/unit未満になっており赤血球製剤や血小板製剤の感染リスクはバッグに残存する血漿に依存している。文献②)
⑬	Lancet Neurology 2006;5:393-398	Predicting susceptibility and incubation time of human-to-human transmission of vCJD	ヒト型のプリオンタンパクを発現するトランスジェニック(Tg)マウスを作製し、BSE感染牛及びvCJD由来の脳乳剤をそれぞれ脳内接種により感染実験を行った。ヒト型TgにBSE由来異常プリオンタンパクは感染しなかったが、遺伝子型がMM(メチオニン・メチオニン)型やMV(メチオニン・バリン)型のTgはvCJD由来の異常プリオンタンパクに感受性を示し、VV型のTgに対しては他の遺伝子型よりも抵抗性を示したが感染が成立した。vCJDの感染効率はヒトからヒトの方が、牛からヒトよりも高い。
⑭	Blood 2008;112;4739-4745	Prion diseases are efficiently transmitted by blood transfusion in sheep	プリオン病が輸血で感染することをヒツジの系で詳細に解析した報告である。TSE発症前の状態を含めた異常プリオンに感染したヒツジの血液を輸血することによって、BSE由来のプリオン病では36%、scrapie由来のプリオン病では43%の輸血を受けたヒツジがプリオン病を発症した。これまでのマウスなどの小動物を用いた実験と異なり、ヒトの輸血に使用する量を投与できること及び長期間の観察が可能(マウスでは2年以下)な点がヒトに近い。
B. 英国、フランス、アメリカ、カナダにおける対応			
⑮	Transfusion 2009;49:797-812	From mad cows to sensible blood transfusion: the risk of prion transmission by labile blood components in the United Kingdom and in France	英国においてはBSE感染牛のピークから12年後にvCJD発生のピークを迎えている。現在、英国でのvCJD新規発症者数が減少しているが、今後70例(10～190例)くらい発症すると推定されている。一方、扁桃と虫垂における異常プリオン陽性率(3/12500例)から更に3000例発生すると推定されている。(現在、さらに10万検体を目標に追跡プロジェクトは進行している(0/45000、2008年))。この違いは感染者の93%が長い潜伏期の状態にいるとのことである。その結果からすると供血者の1/4000人が感染しており、血液や組織、医療機器を介して2次感染が起こる可能性がある。なお、虫垂から検出された2人の遺伝子型はVV型であった。 フランスでは1996～2007年に計23症例のvCJDが報告されている。この中には、供血後にvCJDを発症した3例が含まれている。これまでにこの3例の供血については、42人に投与され、うち16人が生存していることが判明している。
⑯	British journal of haematology 2008;144:14-23	An update on the assessment and management of the risk of transmission of variant Creutzfeldt-Jakob disease by blood and plasma products	
⑰	British journal of haematology 2005;132:13-24	Managing the risk of transmission of variant Creutzfeldt Jakob disease by blood products	

資料番号			概要
⑱	FDA(2009.6.18)	Questions and Answers on "Guidance for Industry: Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease (vCJD) by Blood and Blood Products	アメリカにおいては、英国滞在歴通算3ヵ月以上、フランス滞在歴通算5年以上の者については献血制限を行う施策を続行する(2009.6.18現在)。
⑲	Health Canada	Donor Exclusion to Adress Theoretical Risk of Transmission of variant Creutzfeldt-Jakob Disease (vCJD) through the Blood Supply	カナダにおいては、1980～1996年に英国滞在歴通算3ヵ月以上、フランス滞在歴通算3ヵ月以上の者の献血制限を行っている。
⑳	Health Canada	Additional Donor Exclusion Measures to Address the Potential Risk of Transmission of variant Creutzfeldt-Jakob Disease (vCJD) through the Blood Supply	
㉑	オーストラリア		1980～1996年に英国滞在歴通算6ヶ月以上の者からの献血制限を行なっている。

vCJD Risk Assessment Calculations for a Patient with Multiple Routes of Exposure

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Preface

This paper was developed in response to a request from the CJD Incidents Panel following the finding of abnormal prion protein in the spleen of a patient with haemophilia. Assuming that the abnormal protein represents a marker of vCJD infection, the paper sets the various possible routes through which such infection could have occurred, and considers their relative likelihood in various scenarios. As well as dealing with this specific "incident", the paper sets out a more general methodology for assessing multiple possible infection routes. The analysis was considered by the Panel at its meeting on 28th May 2009, and informed the advice subsequently issued. This version of the paper repeats the analysis presented to the Panel, while giving slightly more background information for other readers, and is placed here for public record.

Introduction

1. This paper offers an analysis of the recent finding of the spleen of a haemophiliac. This involves a patient of potential vCJD infection routes (including multiple transfusions, repeated receipt of UK-sourced fractionated including some units linked to a donor who later went vCJD, and several invasive biopsies) who was found abnormal prion protein in a spleen sample.

2. If this finding is interpreted as an instance of vCJD raises questions as to the operational meaning of the The discovery of abnormal protein in a single sole result after exhaustive investigation of tissues from haemophilia patient who died of other causes and other neurological conditions. All other tissues the presence of abnormal prion protein – fixed samples vessel, appendix, spleen and lymph node and fresh occipital lobe, cerebellum, lymph node and were negative. This information would not vCJD prevalence tests conducted so far, and spleen survey (depending on the size of the whether someone with this limited distribution be infective - and if so, by what routes of

3. For present purposes, however, these issues simply assume that the abnormal prion protein for asymptomatic vCJD infection: the likelihood of the infection having come from done in order to inform discussion by the CJD to the implications of the finding, and in particular warrants any change to the "at risk" status of

4. The ideal would be to quantify these likelihoods is not possible due to the multiple uncertainties rehearsed. We do not know the prevalence instance, some of the potential routes of are not, so the relativities change. The potential component transmitting infection is uncertain approach adopted by the Panel, it is possible implicated plasma derivatives transmitting vCJD However, they can be estimated using methods assessment by independent consultants CJD used in drawing up Panel recommendations have been regarded as "precautionary", i.e. giving infectivity likely to be present.

5. Given these unknowns, we make no attempt though illustrative examples are provided. limited task of determining whether different contacts associated with the index patient can

the additional 1% (over the UK population risk derived from consumption of beef and beef products) "risk threshold" used by the CJD Incidents Panel to trigger decisions on notification of increased risk status. We also consider the wider implications for groups that are or might be classed as "at risk". Although the analysis does throw some light on these questions, it also highlights some conundrums for our understanding of vCJD prevalence and transmissibility.

Summary of findings

6. Specifically, we conclude that on the evidence available:

(i) The chance of **the patient having been infected via an endoscopic procedure is very small**, probably comparable to that of having been infected via primary (dietary) exposure. The potential risk associated with the endoscopies can be disregarded in assessing the risks associated with the possible blood-borne transmission routes, and no specific action is called for with regard to other patients on whom those endoscopes may have been used.

(ii) Comparing the blood-borne routes, **the patient is much more likely to have been infected through receipt of plasma products, rather than any of the 14 units of red cells known to have been received.** The implied risk of each of these 14 donors being infected appears to lie below the 1% threshold that would trigger "at risk" status.

(iii) Given the large pool sizes involved (of the order of 20,000 donations per pool), the risk **differential between "implicated" and "non-implicated" batches of blood product is not marked.** Unless the prevalence of infection is very low, there is a strong possibility of *any* given batch of blood products prepared from large pools sourced from UK donors in the period 1980-2001 containing at least one infected donation. This reinforces the logic of the CJD Incidents Panel's 2004 decision to consider all haemophilia and blood disorder patents exposed to such UK-sourced plasma products as an "at risk" group. There is no strong case for differentiating between sub-groups.

(iv) Given the precautionary assumptions in the DNV risk assessment, any patient exposed to substantial quantities of UK plasma product (as this haemophilia patient was) would almost certainly have received a substantial infective dose, *whether or not* any of the batches were "implicated" (i.e. traceable to a donor who later went on to develop clinical vCJD). In fact, this patient may have been **more likely to have** been infected **by receipt of large quantities of "non-implicated"** plasma, than by **the smaller quantities of "implicated"**.

(v) The lack of any clinical vCJD cases to date amongst patients with haemophilia may suggest that the DNV infectivity scenario is overly-pessimistic. Risk assessments carried out elsewhere assume that a greater proportion of the infectivity would be removed during the manufacturing processes. This raises issues beyond the scope of this paper. Nevertheless, we have re-run the analysis using a markedly lower infectivity assumption with regard to plasma products, and the conclusions listed in (ii) – (iv) above still hold.

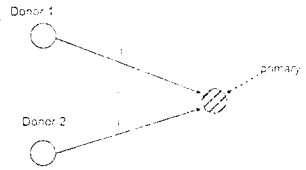
Method

7. The following analysis starts from the "reverse risk assessment" by the Panel to assess the implied risks of donors to vCJD infected (DH, 2005a; Bennett, Debra and Gronlund, [illegible] with this much more complex incident. We start with a simple [illegible] build up the analysis step-by-step. This is both to demonstrate conclusions are reached in this case, and to show how the [illegible] used to handle other complex incidents that may arise.

Example 1

8. We therefore start with a simple incident as shown in Figure 1 [illegible] has received two single-unit Red Cell transfusions, one from [illegible] The recipient goes on to develop vCJD, and the timing of the [illegible] not rule either of the donors out as the route of infection. What [illegible] each of these donors carrying vCJD infection?

Figure 1 (a) Two component donors, neither known to be infected



9. The answer to this depends primarily on the chance [illegible] one of the donors were to be infected – i.e. the [illegible] on definition, this lies between 0 and 1: if r = 1, transmission [illegible] that case, and all else being equal[1], the patient's disease would have come from primary infection, or from either of the two [illegible] infected. So by implication, each donor would have a [illegible] 5

[1] "All else being equal" essentially means that there is no prior reason [illegible] recipient were particularly likely or unlikely to have been infected [illegible] risk" surgery, or conversely not having lived in the UK during years [illegible]

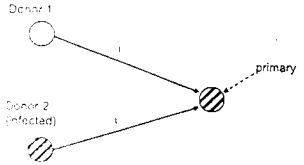
infected.[2] More generally, if there are n donors, the chance of each being infective would be 1/(n+1).

10. The implied risks to the donors clearly diminish if t <1. However, the CJD Incidents Panel has used a precautionary approach, concentrating on scenarios in which t is at least 0.5. With t in this range, the implied risk to donors remains high unless the number of donors to the vCJD case is large. For example, if t = 0.5, then with two donors the chance of either being infected would be roughly 0.25. Note that none of these calculations depend on the underlying prevalence of infection, provided this is the same for donors and recipients.

Example 2

11. The situation would clearly be very different if one of the donors was later diagnosed with vCJD, as in Figure 1(b).

Figure 1 (b) Two component donors, one known to be infected



This creates a marked asymmetry between the infection routes, dependent on the prevalence of infection in the donor population. Whilst Donor 2 is now known to be infected, Donor 1's prior probability of infection is simply the prevalence of infection (p), unknown but assumed to be small. This situation provides an exemplar for analyses in which some routes are prevalence-dependent and others are not.

Let:

P(D1) be the probability of the recipient's infection having come via Donor 1

P(D2) be that ... in having ...

and P(prim) be ... bility of the ...

- For simplicity, suppose that the chance of the ... than one route is negligible. Then (given this ...) P(D2) and P(prim) must add up to 1.

- Furthermore, the "balance" between the three ... by t and p. Specifically:
  - P(D1) will be proportional to ... t (transmission probability)
  - P(D2) will also be proportional to ...
  - and P(prim) will only be proportional to ...

12. Provided p is small (e.g. 1/1,000 or 1/10,000) ... larger than either of the other two probabilities. ... P(D2) = 1 and P(D1) and P(prim) are zero. ... infection came from Donor 2. In practical terms, ... Donor 2 means that ... should not be ... CJD Incidents Panel ...

Example 3

13. In the last two examples, the two secondary ... probability, t. But suppose now that there are ... e.g. transfusion of blood ... and recipients and ... Figure 2 below shows a ... in which the ... contrasting secondary routes:

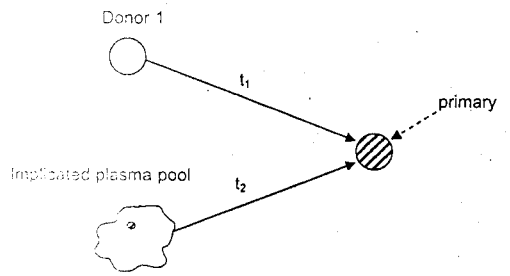
- a blood component transfusion, associated with a ... probability (t1) if the donor (D1) is infected, but with ... that this is the case, and
- a plasma product ... with a contribution from D2 ... infected , but with a ... transmission probability (t2)

As before, the three probabilities P(D1), P(D2) and P(prim) ... and now:

- P(D1) will be proportional to p and t1
- P(D2) will be proportional to t2
- and P(prim) will be proportional to p

[2] The arguments expressed here can be expressed more formally using Bayes' Theorem to update probabilities in the light of new information. However, this is presentationally more clumsy, especially in the more complex examples considered below.

Figure 2: One component donor, **not known to be infected: plasma pool, containing** an implicated donation



14. To illustrate numerically, suppose p is 10^{-4} i.e. prevalence of infection is 1 in 10,000, that $t_1 = 1$ and $t_2 = 10^{-3}$ (that is, transmission via the product pool is less efficient than via the transfused component by a factor of 1,000).

In that case, it can be shown that:

$$P(D1) = 1/12 \qquad P(D2) = 10/12 \qquad \text{and} \qquad P(\text{prim}) = 1/12$$

The infected plasma pool is thus clearly the most likely transmission route, by a factor of 10 over each of the other two possibilities.

15. The principles used to analyse these simple cases are now extended to consider the case of the haemophilic patient with a finding of abnormal prion protein in the spleen.

Analysis

16. Potential secondary transmission routes in this instance consisted of the following (where an "implicated" donor means one for which there is now evidence of having been infected with vCJD):

- 5 invasive endoscopic procedures (biopsies) and a larger number of endoscopies without biopsy.
- exposure to 14 units of Red Cells, each from different ("non-implicated") donors
- exposure to just over 9,000 units of Factor VIII made from two plasma pools with an "implicated" contributing donor (8,025 units from one batch and 1,000 from the other)

- exposure to many other units of UK-sourced plasma products, including nearly 400,000 units of Factor VIII, with no known link to "implicated" donors

To simplify the subsequent discussion, we consider the risk arising from each of these routes in turn.

Transmission risks from the endoscopic

17. vCJD transmission risks from endoscopy have been examined by the CDP TSE WG subgroup, informed by an outline risk assessment. It is important to appreciate that these procedures involve a very small instrument [illegible] being passed down a very long, thin, channel. The possible transmission of infection therefore differs from other surgical procedures. The principal concern is that a significant risk of onward transfer of infective material to a recipient site would require the procedure to be invasive, as distinct from standard procedures involving the instrument sliding against the wall of the gut. On this assumption, the relative risk from endoscopic procedures not involving biopsy is [illegible].

18. So concentrating on procedures involving biopsy, the question arises of whether the heads used would have been single-use. This would [illegible] transmission risks considerably, but not eliminate them (due to the possibility of a new head being contaminated on its way down the endoscope channel). [illegible] do not know whether the heads involved in these procedures were single-use, let us suppose they were not.

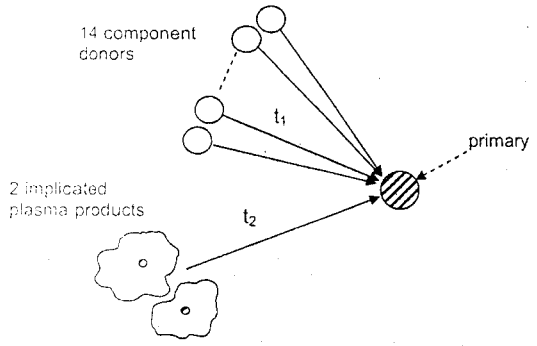
19. For endoscopy with re-used heads, the best existing [illegible] the current surgical risk assessment as applied to procedures encountering [illegible] tissue. Depending on assumptions on the efficacy of decontamination [illegible] standard" model suggests that indefinite re-use of a set of instruments might [illegible] 1 – 10 secondary infections per operation on an infective patient. The [illegible] risk to a random patient resulting from all previous re-uses of the instruments would be in the same range multiplied by the prevalence of infection [illegible]. However, the surgical model considers the transmission risks from a set of 20 [illegible] instruments, rather than just one (very small) biopsy head. For the latter, it [illegible] seems reasonable to reduce the estimated risk by a factor of at least 20. [illegible] on pessimistic assumptions, therefore, the risk of infection from a "standard" biopsy would be in the range (0.1 – 1)p. In other words, the chance of a patient being infected via any of 5 such biopsies would be similar to the chance of having been infected through the "primary" route of dietary exposure.

20. As will be seen below, the chance of this particular patient having been infected by the primary route are very small (in all scenarios as examined). The risk of infection through a blood-borne route. On the above assumptions, the same applies to the endoscopic route. For simplicity, this route will therefore be disregarded in the following calculations. It should be noted that even if the risk of transmission via endoscopy were much greater than assumed, the only effect on subsequent calculations would be to reduce the probabilities associated with all the blood-borne routes slightly.

Blood components and "implicated" plasma products

21. We now consider the relative probability of the patient's infection having come from the implicated plasma products, versus the 14 Red Cell transfusions. As discussed in the "methods" section, we need to balance the greater transmission probability for blood components (Red Cells in this instance) against the existence of an implicated donor contributing to the pooled plasma products. The situation is shown schematically in Figure 3, omitting for now the other "non implicated" plasma products.

Figure 3: 14 component donors, **none known to be infected; 2 plasma products,** each from a pool containing **an implicated donation**



22. The key additional variable here is t_2 – the chance of transmission from an implicated pool. This can be quantified using the infectivity assumptions originally generated in DNV's risk assessment (DNV, 2003). As discussed further below, the calculations initially use the more pessimistic of alternative infectivity scenarios considered by DNV.

23. For the present, we also suppose that the *only* infected donation in the plasma pools came from the identified infected donor – though this is reconsidered below. As detailed in the first part of Annex A, calculations then suggest that this one infected donor would have resulted in the Factor VIII received by the patient containing a total infective dose of about 0.2 ID_{50} (0.16 via one pool and 0.05 via the other). Using the simple linear dose-response model that has informed Panel recommendations to date, this implies a transmission probability t_2 of approximately 0.1.

24. We can then use the approach set out before to assign probabilities to the possible infection routes in different scenarios. Table 1 below shows the results, using this value for t_2 and alternatives of 1 and 0.5 for t_1 and 1 in 4,000 and 1 in

10,000 for the prevale[illegible]. The [illegible]sive [illegible] infection having come [illegible] the implicated plasma [illegible] 14 component (Red Cell) [illegible], and from the [illegible] that in all scenarios, the [illegible] illustrative figures, using [illegible] they do suggest that the [illegible] plasma products, with [illegible] clearly below 1%.

Table 1: Relative probabilities of potential infe[illegible] into "pooled plasma" pro[illegible]

Prevalence, p	
Transmission probability, t1	
Probability implicated plasm[illegible]	
Probability of each of the 14 [illegible]	
Probability primary	

Note: these are illustrative [illegible] probabilities) indicate an up per [illegible]

Implicated and "Non-implicated" p[illegible] products

25. Although the above analysis provides some rob[illegible] infection routes consid[illegible] far, the calculati[illegible] chance of the infection [illegible] some routes the [illegible] – i.e. those manufactur[illegible] pools [illegible] contributing donor. The [illegible] (of the order of 20,00[illegible] them did, in fact, contain [illegible] Crudely, if the prevale[illegible] contain about 2 infecte[illegible]

26. This argument does [illegible] non-implicated p[illegible] donor (and nothing [illegible] have the same probabi[illegible] 10,000 and typical poo[illegible] implicated" pools to [illegible] contain 3. Nevertheless, [illegible] that unless the prevale[illegible] here, there is only a [illegible] and non-implicated pl[illegible] considering recipients [illegible]

[3] More strictly, the expected number of infective donations in each [illegible] distribution. However, the distri[illegible] not essential to this argu[illegible] receiving high volumes of produc[illegible] from many different p[illegible] fluctuations will tend to even out

applying additional measures to those with known exposure to implicated batches.

27. This specific haemophilia patient had received such large quantities of Factor VIII – almost 400,000 units, the majority since 1980)] - that on these calculations, the cumulative risk from the "non-implicated" batches may well have exceeded that from the smaller number of "implicated" ones. This can be illustrated by considering the expected number of ID_{50} received via each route. This is illustrated in the second part of Annex A. In summary:

- If the two "implicated" pools contained 3 infected donations, this route would have exposed the patient to a total dose of 0.6 ID_{50}.
- If the other "non-implicated" pools each contained 2 infected donations, this route would have exposed the patient to an expected total of 24 ID_{50}.

28. Simple application of the linear dose-response model would then suggest that whereas Factor VIII from the two "implicated" pools would have contained a dose liable to transmit infection with a probability of 0.3, the large number of units sourced from "non-implicated" pools would have contained more than enough infectivity to transmit. Crudely, this suggests that the "non-implicated" pools represent the more probable source of infection, by a factor of just over 3.[4]

29. This last calculation is reflected in Table 2 below, for prevalence scenarios of both 1 in 10,000 and 1 in 4,000. However, we stress that this is very simplistic. It rests on accepting the linear model uncritically, and assuming that doses received on successive occasions can simply be added together in calculating an overall risk of infection. Nevertheless, the comparison between "implicated" and "non-implicated" routes is instructive, in showing how the sheer number of exposures may come to dominate the presence of a known infection.

Table 2: Relative probabilities of potential infection routes (including "non implicated plasma" products)

Prevalence, p	1 in 4,000		1 in 10,000	
Transmission probability, t1	0.5	1	0.5	1
Probability implicated plasma products	38%	38%	24%	24%
Probability of each of the 14 component donors	<0.03%	<0.03%	<0.02%	<0.02%
Probability primary	<0.03%	<0.03%	<0.02%	<0.02%
Probability non-implicated plasma products	61%	61%	76%	76%

Note: these are illustrative calculations only. All figures are rounded to the nearest %, or (for small probabilities) indicate an upper bound.

[4] Note that the differential between *infectious doses* is much greater, but the practical effect is limited by infection being regarded as certain once the dose reaches 2 ID_{50}. As seen in following paragraphs, the risk differential between routes is therefore more pronounced in lower-infectivity scenarios.

30. As can be seen, the previous conclusion about the low implied [illegible] 14 component (red cell) donors still applies, with even greater [illegible] However, these results also highlight something of a paradox. Combining [illegible] infectivity scenario taken from the DNV assessment, the pool size / prevalence calculations suggest that many recipients of plasma products would have received very high infectious doses, *whether or not* they had received any "implicated" units with known linkage to an infected donor. This opens the question of why no clinical vCJD cases have been seen in the population of haemophilia / blood disorder patients designated as "at risk" because of their exposure to UK sourced blood products.[5] It might therefore be argued that the infectivity assumptions applied to plasma products are overly pessimistic.

31. Although this question is impossible to answer definitively, and [illegible] issues beyond the scope of this paper, it is appropriate [illegible] conclusions we have already suggested about relative [illegible] be overturned were we to assume lower levels of infectivity [illegible] The DNV report itself suggests two possible methods [illegible] infectivity present in each plasma derivative, using [illegible] effect of the various manufacturing processes. In line with the [illegible] precautionary approach adopted by CJD Incidents Panel [illegible] use figures based on the more pessimistic of these. The [illegible] alternative suggested by DNV (using the "highest single [illegible] manufacturing process) leads to an infectivity estimate for [illegible] lower by a factor of 4. However, it should also be noted that [illegible] carried out elsewhere take the clearance factors achieved [illegible] at least partly additive, which would lead to much smaller [illegible]

32. In fact, reducing the assumed infectivity *increases* the relative [illegible] via "non-implicated" as compared to "implicated" plasma. [illegible] the presumed infectivity in all the Factor VIII received was reduced [illegible] 100 (2 logs). Modifying the calculations in paragraph 27, [illegible] have received an expected:

- 0.006 ID_{50} from the two "implicated" pools (representing a [illegible] of 0.003)
- 0.24 ID_{50} from all the other "non-implicated" pools (a [illegible] infection risk of 0.12).

33. Albeit with the same caveats as before about using the [illegible] the cumulative risks from successive doses, this suggests [illegible] outweigh the former by a factor of 10. Table 3 above [illegible] for this patient would change under this revised [illegible]

[5] *Possible* explanations include the following: prevalence of infectivity [illegible] lower than in the scenarios considered here, but [illegible] more infectivity [illegible] processing of plasma products than suggested by the DNV analysis, [illegible] dose-response effect and most recipients fall below this. Genotypic [illegible] providing resistance to infection or extending the time to clinical disease [illegible] substantial proportion of this group to be MM homozygotes - the most [illegible]

even, the previous conclusions still hold, in particular regarding the small implied risk to each of the 14 red cell donors.

Table 5: Relative probabilities of potential infection routes (including "non implicated plasma" products and using lower infectivity estimates for plasma products)

Prevalence, p	1 in 4,000		1 in 10,000	
Transmission probability, t1	0.5	1	0.5	1
Probability implicated plasma products	2%	2%	3%	3%
Probability of each of the 14 component donors	<0.05%	<0.09%	<0.05%	<0.09%
Probability, summary	<0.09%	<0.09%	<0.09%	<0.09%
Probability non-implicated plasma products	97%	97%	97%	96%

Note: for illustrative calculations only. All figures are rounded to the nearest %, or (for small probabilities) indicate an upper bound.

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Annex A: Application of D[illegible] Risk Calculation [illegible]

(a) Implicated Donations

Key points: FHB4547

- There was one implicated [illegible] donations (pool size supplie[illegible]
- Factor VIII is derived from [illegible] ID_{50}s / donation of infected [illegible]
- 70.45kg of cryoprecipitate [illegible] in the FHB4547 batch.
- This implies that (21.53kg [illegible] batch (18.38 ID_{50}s)
- 1,844 vials each of 50[illegible] estimate of 0.00997 ID_{50}s [illegible]

Professor Frank Hill's report indicates that the index case [illegible] batch, giving an estimated 0.16 ID_{50} from the implicated donation.

Key points: FHC4237

- There was one implicated [illegible] donations (pool size [illegible]
- Factor VIII is derived from [illegible] ID_{50} / donation of whole [illegible]
- 67.6kg of cryoprecipitate [illegible] FHC4237 batch
- This implies that the full [illegible]
- 5,074 vials each of 250 [illegible] 0.0118 ID_{50} per vial or [illegible]

Professor Frank Hill's [illegible] batch, giving an estimated [illegible]

Conclusion

In total, these calculations [illegible] ID_{50} from the "implicated" [illegible] translates into a transmission [illegible] transmission probability of [illegible] 10.4%.

(b) Non-implicated Donations

In addition to the implicated donations, we have also to consider the possibility of other donors contributing to a pool being infective. With pool sizes of the order of 20,000 donations, each pool will be likely to contain contributions from one or more infected donors by chance, unless p is very small. For implicated pools, these will be *in addition to* the "known" implicated donor.

With a prevalence of 1 in 10,000, one might therefore expect the two implicated pools to contain two *further* infected donations, taking the total from 1 to 3 per pool.

This would make the infective dose received via the implicated units three times that calculated above, i.e. a total of roughly 0.6 ID_{50}, yielding a transmission probability of 0.3.

This patient also received approximately 391,000 iu of UK-sourced Factor VIII plasma treatment *not* known to be associated with any infected donor. In round figures, this can be visualised in terms of 20 exposures to pools of 20,000 donors, each typically containing 2 donations from infected donors. The exact infective dose passed on to the patient will vary from batch to batch. However, the two examples given in part (a) suggest an eventual dose of 2-5 x 10^{-5} ID_{50} per unit, per infected donor. For illustration, therefore, suppose that each unit exposed the recipient to 6 x 10^{-5} ID_{50}, 400,000 such units would therefore have exposed the recipient to 24 ID_{50}.

ORIGINAL PAPER

Distribution of a bovine spongiform [illegible]-derive agent over ion-exchange chromato[illegible] the preparation of concentrates of [illegible] VIII

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Background and Objectives. [illegible] Jakob disease (vCJD) via treatment [illegible] fore, in order to determine the risk [illegible] the preparation of factor VIII [illegible] encephalopathy (BSE)-derived agent [illegible] used to prepare the Scottish Nation[illegible] VIII concentrate (Liberate®).

Materials and Methods. Murine-passaged BSE [illegible] somal fraction prepared from infected brain, [illegible] VIII of intermediate purity. The 'spiked' material [illegible] detergent treatment and then to anion-exchange [illegible] DEAE-650M. All fractions were tested for BSE [illegible] including the procedures used to clean the ion-exchange [illegible] the case.

Results. BSE 301V infectivity was [illegible] by 2·7 $\log_{10}$ to the factor VIII fraction. [illegible] remained bound to the ion-exchange [illegible] quantity of infectivity was subsequently [illegible] media with 0·4 M NaCl. No further BSE [illegible] after treatment with 0·1 M NaOH or [illegible]

Conclusions. Results using [illegible] be substantially removed by the ion-exchange [illegible] fibrinogen and factor VIII concentrates [illegible] bound to the ion-exchange matrix [illegible] be eluted away by the processing [illegible] each use.

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Key words: Creutzfeldt-Jakob [illegible]

Introduction

Variant Creutzfeldt–Jakob disease (vCJD) [illegible] a fatal, neurodegenerative disorder of transmissible spongiform [illegible] [illegible] been [illegible] [illegible] formed [illegible] [illegible] first [illegible] [illegible] the UK [illegible] [illegible] TSE agent [illegible] [illegible] pathy (BSE) [illegible] [illegible] being the [illegible] originated [illegible] [illegible] detected in [illegible] in 25 different countries [illegible] of all cases [illegible]

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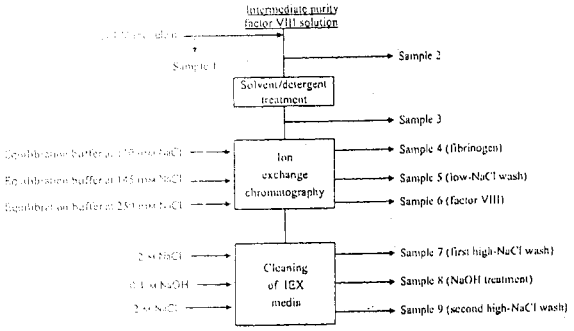

Fig. 1 Flow diagram of the processes over which partitioning of bovine spongiform encephalopathy (BSE) 301V infectivity was measured. IEX, ion-exchange chromatography.

of equilibration buffer, with the breakthrough (unadsorbed) fraction (139·8 ml) being collected (fibrinogen fraction). Forty-one millilitres of equilibration buffer, containing 145 mM NaCl, was then applied and the resultant wash fraction collected (low-NaCl wash). This was followed by 26 ml of equilibration buffer containing 250 mM NaCl, at a flow-rate of 4·8 ml/h, to elute factor VIII (factor VIII fraction).

Cleaning of the ion-exchange gel

Following collection of the factor VIII eluate, the chromatography bed was cleaned *in situ* by washing with 2 M NaCl, followed by 0·1 M NaOH and then again with 2 M NaCl. First, 25 ml of 2 M NaCl was applied to the column and the eluate (15·2 ml) was collected from the beginning of the 'salt front' (first high-NaCl wash). Subsequently, 0·1 M NaOH (70 ml) was applied to the column and an eluate (39 ml) was collected when the pH increased from 6·3 to > 12 (NaOH wash). When the application of 0·1 M NaOH was complete, the column was allowed to soak in NaOH for 1 h and then subjected to a second wash with 2 M NaCl (42 ml). An eluate volume of 8·1 ml was collected to capture the protein-containing fraction observed at this stage (second high-NaCl wash).

Determination of protein elution during the ion-exchange process

Throughout the ion-exchange procedure, the output from the column was monitored continuously by inline measurement of the solution optical density at a wavelength of 280 nm (OD_{280}) to detect total protein being eluted (Fig. 2).

Scale-down of the ion-exchange process

The small-scale ion-exchange procedure used in this study was designed to give yields and purification for factor VIII and fibrinogen equivalent to the full-scale process. Although the degree of scale-down was ≈ 1300-fold, all materials and surfaces were the same as in routine manufacture, except that chromatography eluates were collected into polypropylene containers rather than stainless steel vessels. The OD_{280} profile obtained in the presence of added 301V (Fig. 2) was the same as that obtained in the absence of 301V, both in the small-scale model and in the routine full-scale chromatography process, demonstrating the accuracy of down-scaling achieved.

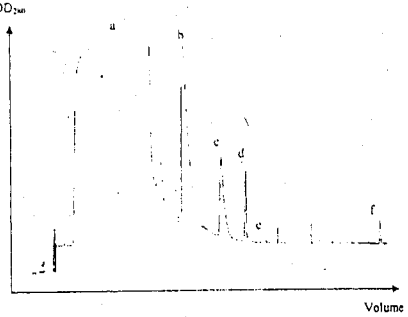

Fig. 2 Optical density of fractions eluted during ion-exchange chromatography of intermediate-purity factor VIII to which the bovine spongiform encephalopathy (BSE) 301V microsomal inoculum had been added. (a) Fibrinogen fraction (110 mM NaCl); (b) low-NaCl wash (145 mM NaCl); (c) factor VIII fraction (250 mM NaCl); (d) first high-NaCl wash (2 M NaCl); (e) NaOH wash (0·1 M NaOH); (f) second high-NaCl wash (2 M NaCl).

Determination of BSE 301V infectivity

The BSE 301V infectivity of samples from the ion-exchange process was determined by bioassay. Samples for assay were diluted in saline and injected intracerebrally (20 µl) into

Table 2 Distribution of bovine spongiform encephalopathy (BSE) 301V infectivity in factor VIII by ion-exchange chromatography

Stage/fraction	BSE titre (ID_{50}/ml [log$_{10}$])	Volume of fraction (ml)	Total infectivity (ID_{50} [log$_{10}$])	Infectivity removed (log$_{10}$)
1. Microsomal inoculum	7·3[a]			
Factor VIII process				
2. Factor VIII solution (spiked)	6·7			
3. Factor VIII solution after S/D[b]	6·8			
4. Fibrinogen fraction (110 mM NaCl)	< 3·8			
5. Low-NaCl wash (145 mM NaCl)	< 3·4[c]			
6. Factor VIII fraction (250 mM NaCl)	4·6			
Column cleaning				
7. First high-NaCl wash (2 M NaCl)	5·4[d]			
8. NaOH wash (0·1 M NaOH)	< 3·2			
9. Second high-NaCl wash (2 M NaCl)	< 3·2			

[a]Transmissible spongiform encephalopathy (TSE) titre of microsomal inoculum (24).
[b]S/D, after treatment with solvent and detergent.
[c]Maximum value on the assumption that 100% of animals would have been positive if the starting material had contained infectivity.
[d]Approximate TSE titre, estimated from bioassay at one dilution using the dose-response curve of the starting material (24).
ID_{50}, infectious doses 50%.

consistent with the original level of infectivity, suggesting that aggregates may have formed during the frozen storage of the microsomal fraction and that full dispersion was only achieved after the microsomal fraction had been added to the solution of intermediate-purity factor VIII. Although there was a small apparent increase in 301V titre following solvent/detergent treatment (Table 2), this was well within the margin of error for TSE bioassay titrations. However, such a small increase in TSE titre is often detected after solvent/detergent treatment or other disaggregating treatments (sonication) and is probably a result of disaggregation, but may also occur as an effect of the efficiency of titration [25].

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

In the procedure used to clean the ion-exchange gel between uses, we found that a significant degree of infectivity desorbed into the first 2 M NaCl wash (Table 2). Subsequently,

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

Conclusions

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

Acknowledgements

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Removal of TSE agents from blood products

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Introduction

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

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

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

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

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Experimental approaches

Studies on the removal of TSE [illegible] infectivity in blood is very [illegible] agent as it exists naturally [illegible] experimental approaches [illegible] sary to appreciate their [illegible]

Process scale-down

Experiments with infective material [illegible] containment facilities as TSE agents represent a [illegible] This, together with the difficulty of [illegible] infected tissue means that process studies are usually undertaken at small volume, typically [illegible] manufacturing processes operate at [illegible] For results to be meaningful it is necessary to simulate the manufacturing operation reasonably accurately.

Form of TSE agent

Two basic forms of TSE material have been used [illegible] obtained from experimentally infected animals [illegible] preparations derived from [illegible] brain homogenate (BH) [illegible] caveolae-like domains (CLDs) [illegible] [10]. Studies with infected [illegible] *genous* whilst those using [illegible] as 'exogenous'. In [illegible] infectivity means that [illegible] beginning of multistep [illegible] titre of infectivity available [illegible] enables greater capacities [illegible] and more steps considered. However, [illegible] over the extent to which materials [illegible] represent TSE agents present naturally [illegible]

Strain of TSE agent

Partitioning studies have been undertaken [illegible] TSE strains. Endogenous studies have [illegible] murine-adapted GSS, Fukuoka-1 strain [illegible] scrapie, strain 263K (R. G. Rohwer, unpublished) and mouse-adapted BSE, strain 301 V (R. G. Rohwer, unpublished). Exogenous

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

Determination of TSE agents

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

Studies on individual process steps

Leucocyte-filtration

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

Protein precipitation

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

Depth filtration

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

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

	Foster	Lee	Lee	Stenland	Vey	Reichl
Ref	[8]	[15,16]	[15]	[13]	[10]	[12]
TSE agent	263K	263K	263K	vCJD	Sc237	301 V
spike	MF	BH	BH	BH	BH/MF/CLD/PrPSc	MF
assay	W blot	W blot	bioassay	W blot	CDI	bioassay
$\log_{10}$ Redn						
cryoppt	1·0	1·0	1·0	0·9	0·3/0·2/0·4/2·4	
fraction I		1·3			0·9/0·9/0·7/3·1	
fraction II + III	1·0	≥ 4·7	6·0		3·6/3·1/3·1/4·0	
fraction II + III	≥ 2·7	≥ 4·0	5·3			2·1
fraction IV/V		≥ 4·2/≥ 4·1	3·7/4·6			
fraction IV	≥ 3·0				3·2/3·4/3·2/2·2	

Studies on process steps in sequence

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

Conclusions

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

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REVIEW ARTICLE

Factor VIII and transmissible spongiform encephalopathy: the case for safety

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

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

Haemophilia and replacement therapy

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

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

While initiation of blood coagulation is ascribed to the extrinsic, tissue factor-dependent pathway in which small amounts of activated factors IX and X are generated, the intrinsic pathway catalyses activation of factor X approximately 50-fold more efficiently, dramatically amplifying the coagulation events triggered by the tissue factor-dependent pathway [7]. The requirement of a powerful amplification of the coagulation burst via the FVIII-dependent intrinsic pathway for maintaining normal haemostasis explains why the absence of FVIII disturbs the coagulation process and results in haemophilia A.

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

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

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 of the transgenic mice have suggested that another unknown factor is required [36], in 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 from [illegible]

Donor species	Recipient species	[illegible]
Scrapie (natural)		
Goat	Mouse	[illegible]
Sheep	Mouse	[illegible]
BSE (natural)		
Cow	Mouse	[illegible]
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]
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]

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

possible that animals with natural disease might have extremely low levels of TSE infectivity in blood, that are not detectable in inbred mice, and more sensitive transgenic mice should therefore be used for such studies.

In contrast to the negative results obtained in most transmission studies using human blood or the blood of animals with natural disease, transmissions have been consistently achieved when blood or blood components from experimentally TSE-infected animals, primarily rodents, were used [illegible] studies [illegible] blood and [illegible] incubation [illegible] animals [illegible] large numbers [illegible] low levels [illegible] ponents [illegible] times less [illegible] adapted [illegible] hamster–mouse [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	1/1	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 intro. 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	KIII 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 the first [illegible] CJD Surveillance Unit [illegible] several vCJD [illegible] sions [illegible] developed vCJD [illegible] eight were [illegible] recipients. [illegible] whole blood [illegible] recipients), [illegible] recipients), fresh-frozen plasma [illegible] cryo-depleted plasma and cryoprecipitate [illegible] recipient for each component. Plasma [illegible] from eight donors [illegible] manufacturing of therapeutic proteins [illegible] distributed to thousands [illegible] (personal communication from B. Will). [illegible] who have developed vCJD [illegible] transfused to pools of plasma [illegible] patients with haemophilia A and B. [illegible] recalls of product in 1997 [illegible] unpublished data).

Developments in diagnostic screening tests for Creutzfeldt–Jakob disease

Most of the [illegible] the risk of transmission of CJD through [illegible] products and [illegible] the early [illegible] infection [illegible] fication of [illegible] abnormal [illegible] of presymptomatic or [illegible] the level of [illegible] no greater than [illegible] disease [illegible] recently, other [illegible] the cerebrospinal fluid) have not [illegible] promising discovery has [illegible] Shaked *et al.* [illegible] abnormal PrP in the urine [illegible] infected with prions long [illegible] clinical signs. Abnormal PrP [illegible] urine of cattle with BSE [illegible] afflicted with a genetic [illegible] of this technique as a diagnostic tool will need to be validated [illegible] Another encouraging observation was recently made [92] by the discovery that levels of an erythroid differentiation [illegible] decreased in the spleens [illegible] both the preclinical and clinical phase of the disease and in the [illegible] of [illegible] observed in the same manner [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 $0.2–0.4 \times 10^{-15}$ 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 [illegible] 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}

cumulative [illegible] steps [illegible] tion of [illegible] matography [illegible] production of [illegible]

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Removal [illegible] ted in a v[illegible] Baxter [illegible] coagulation [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
Cryoprecipitation, precipitation and adsorption, SD treatment and ion-exchange chromatography, membrane filtration	263K: microsomal fraction	Western [illegible]
Cryoprecipitation and cryoprecipitate/PEG separation	263K: brain homogenate	[illegible]
Cryoprecipitation	Sc237: brain homogenate, microsomal fraction/CLD[illegible]; Sc237: Purified [illegible]	[illegible]
Ethanol precipitation 8%	Sc237: brain homogenate, microsomal fraction/CLD[illegible]; Sc237: Purified [illegible]	
FVIII immunoaffinity column ion-exchange chromatography [109]	263K: Brain [illegible]	[illegible]

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

Vox Sang

Studies on the Removal of Abnormal P… Protein by Processes Used in the Manu… 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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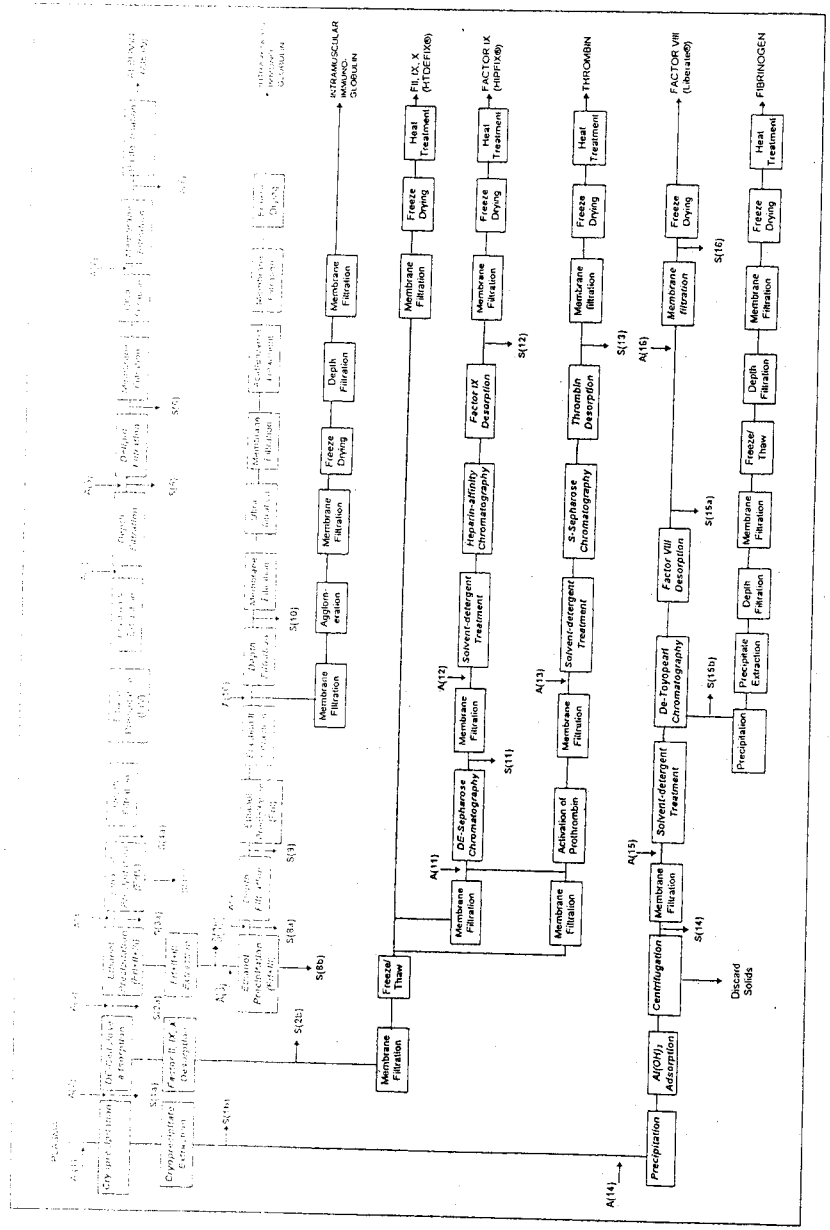

phosphate (5 m*M*) at pH 6.2. Factor II, IX and X solution (215 ml) was applied to the column, which was then treated with 1.0 column wash buffer, all at a flow rate of 8.4 ml/min, followed by 10 column wash buffer + 280 m*M* sodium chloride at 1.9 ml/min. Factor IX was eluted using 100 ml wash buffer +360 m*M* sodium chloride, pH 6.8 at 1.9 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 m*M*) + 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 25 °C 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 (XK 26/10, Pharmacia) using 20 m*M* citrate. The solvent-detergent (S/D)-treated factor IX mixture was applied to the column, the bed washed with 100 ml of 20 m*M* citrate, treated with 100 ml of 20 m*M* citrate + 1 *M* sodium chloride and factor IX then eluted with 100 ml of a solution of citrate (20 m*M*) + arginine (4.5 g/l) + sodium chloride (500 m*M*) 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 1.0%, 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 m*M* 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 m*M*) and thrombin was then eluted with 80 ml of trisodium citrate (20 m*M*) + sodium chloride (1.0 *M*) 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 45 g of frozen washed cryoprecipitate in 20 m*M* Tris (168 ml) at 2 °C. The pH of the extract was adjusted to 6.7 and zinc precipitant (zinc acetate + sodium chloride + trisodium citrate + heparin), added to obtain concentrations of 0.5 m*M* zinc, 1 m*M* citrate and 2.5 IU/ml heparin. The mixture was stirred for 5 min at 20 °C, aluminium hydroxide (Al(OH)3 hydrogel, Superfos, Copenhagen, Denmark) was added to a final concentration of 5%; after stirring for a further 15 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 m*M* trisodium citrate and 2.5 m*M* calcium chloride.

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

Process step[a]	Factor VIII		Fibrinogen	
	CF[b]	RF[c]	CF	RF
1 Cryoprecipitation	1.7	1.0	1.7	1.0
14 Zinc precipitation + Al $(OH)_3$ adsorption	2.0	1.7	2.0	1.7
15 SD[d] + DEAE Tyopearl 650 M chromatography	3.8	3.1	≥4.1	≥3
16 Membrane filtration (0.45 µm/0.22 µm)	1.6	1.0	n/d[e]	n/d

[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 (HIPRIX®), factor IX complex (HYPERFIX®) and thrombin

Process step[a]	Factor IX		FII, IX and X		Thrombin	
	CF[b]	RF[c]	CF	RF	CF	RF
1 Cryoprecipitation	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
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				% distribution[b] of PrP^{Sc}	
	ethanol %	pH	temperature °C	time h	precipitate	supernatan
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 differen[illegible]

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Assessment of the potential of plasma fract[illegible] to remove causative agents of trans[illegible] 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 [illegible]

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 expecte to partition during plasma fractionation has been mac on this basis.

PLASMA FRACTIONATION

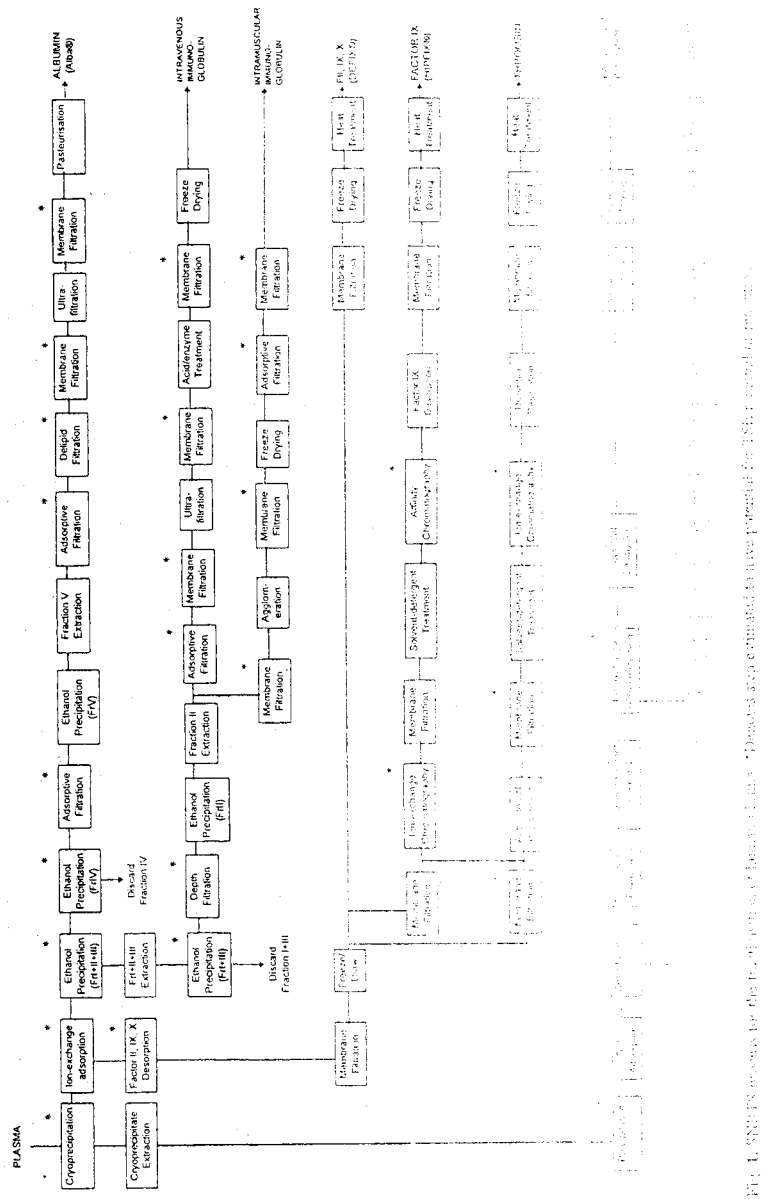
The Scottish National Blood Transfusion Servic (SNBTS) manufactures over 250 000 unit doses of range of different plasma products from ≈100 000 kg plasma. The preparation of each product involves exte sive processing via a carefully designed, closely co trolled series of operations (Fig. 1) (Foster,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



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 eluate, in this instance by cation exchange chromatography, with a total of six separate membrane filtration steps, [illegible] of 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 (Alba®) 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	
Precipitation	
Cryoprecipitation	
Cohn fraction I	
Other Cohn fractions	
Other precipitation methods	
Adsorption chromatography	
Packed bed	
Packed bed	
Suspension	
Adsorptive filtration	
Depth filter (mixed bed)	
Depth filter (single bed)	
Membrane filter (cellulose acetate)	

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 [illegible] tion (Budnik *et al.* [illegible] *et al.*, 1996; [illegible] & Spaltro, 1996 [illegible] fractionation [illegible] from hepatitis [illegible] & Barker, 1979 [illegible] These technologies [illegible] tially remove [illegible] 1998). Whether [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 [illegible] tious agent, additional [illegible] mine whether or not [illegible] material may remain in [illegible] question.

In precipitation [illegible] solubility of the infectious agent [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

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.

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.

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. $ID_{50}\,mL^{-1}$.

TSE infectivity [illegible] white blood cells [illegible] the separation of [illegible] would be expected [illegible] a TSE agent [illegible] assumed in the [illegible] from whole blood [illegible] concentration of nvCJD [illegible] would be 250 i.v. [illegible] single infective 300 mL [illegible] a total nvCJD infectivity of up [illegible] Further reduction of [illegible] leucofiltration (Rider *et al.*, 1998 [illegible] nvCJD content, but as the [illegible] any contribution that [illegible] has been discounted.

To examine the [illegible] of nvCJD in the UK [illegible] of product contamination [illegible] from processing a [illegible] been estimated that [illegible] have been infected [illegible] represents a [illegible] UK population and [illegible] to support such a [illegible] taken as a [illegible] theoretical [illegible] contaminated to [illegible] nvCJD in a plasma [illegible] $ID_{50}\,mL^{-1}$. Therefore [illegible] tive in this scenario [illegible] must be [illegible] question.

From the information [illegible] TSE agents, the [illegible] plasma fractionation [illegible] manufactured using [illegible] to remove TSE agents [illegible] the impact of a 0·15 [illegible] case scenario) has been [illegible] infectivity that would be [illegible] purified product pool prior to dispensing [illegible] of dispensed product [illegible] by the SNBTS and [illegible] which are included [illegible] have been present [illegible] tion since the early [illegible]

If it is assumed that [illegible] genous and that nvCJD [illegible] amongst all of the [illegible] calculated that [illegible] contain an infectious [illegible] these the [illegible] incidence of nvCJD [illegible]

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^{6}), 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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TRANSFUSION [illegible]

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

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

BACKGROUND: The administration of blood components from donors who subsequently develop Creutzfeldt-Jakob disease has raised the issue of blo[illegible] as a possible vehicle for iatrogenic disease.
STUDY DESIGN AND METHODS: We examined infectivity in blood components and Cohn plasma fractions [illegible] normal human blood that had been "spiked" with trypsinized cells from a scrapie-infected hamster bra[illegible] and in blood of clinically ill mice that had been inoculated with a mouse-adapted strain of human transmissible spongiform encephalopathy. Infectivity was assayed by intracerebral inoculation of the bl[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 fac[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 detectable 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, suggest 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 600 × *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 6900 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 0.5 $\log_{10}$ 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 P102L 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 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 all 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	27.0 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	0.30 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	1.46 g	2.5	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 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 buffy coat (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 10^{-5} 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 found its way to the cryoprecipitate and fraction I+II+III, and virtually none (<0.01%) in 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 assayed 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 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 $10^{[illegible]}$ LD_{50} per g and $10^{[illegible]}$ 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 successes 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] primates inoculated with blood from humans with CJD [illegible]. None of these studies examined the distribution of infectivity in different blood components of a single specimen, 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 the much lower levels of endogenous infectivity that would be 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 in successive processing steps. Scrapie-infected hamster brain satisfies this condition of high-input infectivity. The choice of trypsinized and washed intact brain cells was based on evidence that blood infectivity is most likely cell-associated,[1] and thus, insofar as could be predicted, infected cells represent a more appropriate infectious vehicle than either infectious tissue homogenate or purified cell-free PrP. We could not know in advance whether trypsiniz[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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ORIGINAL PAPER

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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 1 (SN1) 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.

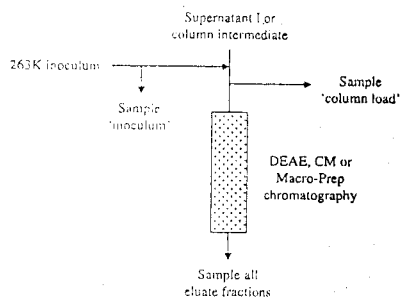


Fig. 1 Flow diagram showing spiking points and sampling points for each column in 263K PrP^{Sc} studies. The diagram applies to each of the chromatography columns, as they were each spiked separately.

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

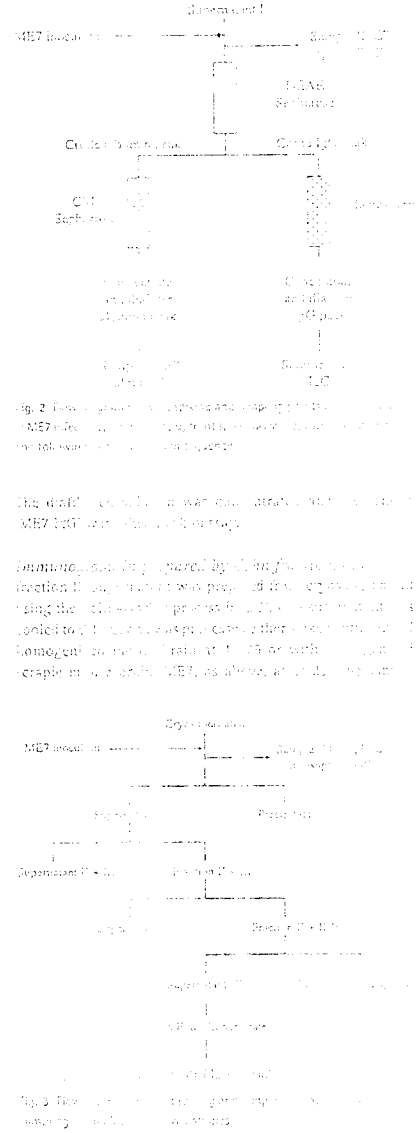
Scrapie ME7 was incubated in C57 black mice, and brains were harvested from mice in the late clinical stage of infection. The brains were homogenized in PBS at 10% wt/v using a Duall tissue grinder (Kontes, Vineland, NJ), and the homogenate was centrifuged at 10 000 *g* for 30 min to remove cellular 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 SN1 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 SN1' 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),



'ME7 spiked supernatant' was taken for bioassay, leaving a volume of 315 ml for further processing.

Cold ethanol at ≤ −5 °C was added to achieve a final ethanol concentration of 7·5–8·5% v/v, and Fraction I (fibrinogen) precipitate was separated by centrifugation at 20 000 *g* for 10 min. Cold ethanol was added to the SNI to give a final ethanol concentration of 18·5–22·5% v/v. The mixture was centrifuged at 20 000 *g* for 10 min at −5 ± 1 °C and the Fraction II + III precipitate (immunoglobulin plus lipoprotein) was collected. Sufficient ethanol at ≤ −5 °C was then added to achieve an ethanol concentration of 20·0% v/v, to precipitate immunoglobulins while leaving albumin in solution. Fraction II + III(W) precipitate was separated by centrifugation at 20 000 *g* for 10 min at −5 ± 1 °C. The fraction III precipitate (lipoprotein and IgM) was separated by centrifugation at 20 000 *g* for 10 min at −5 ± 1 °C. Filter aid Diacel 150 (CFF, Gehren, Germany) was added to the fraction III supernatant and filtered through Seitz EK1 disks (Pall, East Hills, NY). The filtrate was adjusted to pH 4·0 and diafiltered at this pH using 10 kDa ultrafiltration membranes. The sample 'ME7 IgG Cohn' was taken for bioassay.

Bioassay

Samples collected from one control run and the TSE partitioning run were used for intracerebral (IC) inoculation of mice. The test materials were subjected to tenfold dilutions in PBS, and weanling C57 black mice (Animal Resources Center, Perth, WA) were IC inoculated with 30 µl of test dilution in sets of five mice per cage. As shown in results in the tables, some dilutions were inoculated into more than one cage, to improve sensitivity when low prion infectivity was expected (given Western blot study results). The study period for the bioassay was 18 months. Mice showing clinical symptoms of scrapie [22] throughout the study or that died within incubation periods consistent with TSE were harvested for TSE evaluation by haematoxylin and eosin staining to detect spongiform change. Further testing using MAb 6H4 (Prionics, Schlieren, Switzerland) for immunohistology and MAb SAF83 (Cayman, Ann Arbor, MI) for Western blot was performed if required. Mice were scored as scrapie positive when clinical signs were confirmed by two or more methods. At the end of 18 months, histology was performed on all surviving mice in dilutions from which scrapie mice had been culled. Histology was also performed on all mice in the lowest dilution for which there were no scrapie cases recorded.

Negative mouse controls within the bioassay component were deemed to be satisfactory when they showed no signs of toxicity over the period of the study or did not contract scrapie over the full study. The 50% end point for infectious dose (ID_{50}) for the bioassay titration was calculated using the Spearman Kärber method [23]. When no infectivity was present in a sample, a 95% probability formula was used to estimate residual infectivity in the sample [24]. The log reduction factor (LRF) of infectious scrapie over the processes was determined by subtracting the scrapie log load of the final concentrated eluates from the log load of the spiked starting material [24].

Results

Scale-down validity

Protein intermediates from control runs showed that the processes were scaled down accurately and were representative of production processes with regard to protein purity, concentration and chemical composition. Chromatographic profiles as shown for the scrapie ME7 spiked scale-down runs accurately represented those obtained from the industrial-scale production process [25]. All buffers and column eluates achieved the same HETP, pH, and conductivity limits as production processes.

Experiments using microsomal scrapie 263K with Western blot detection

Log reduction factors and recovery of PrP^{sc} are shown for the ion exchange columns used for the production of albumin and IVIG (Table 1). All eluate streams from the columns were assayed for PrP^{sc} using Western blot. Substantial partitioning of PrP^{sc} away from the target proteins was achieved in all ion exchange steps examined. The log reductions across the DEAE Sepharose and CM Sepharose for albumin were ≥ 4·0 and ≥ 3·0, respectively. The log reductions across the DEAE Sepharose and Macro-Prep for immunoglobulin were 3·3 and ≥ 4·1, respectively.

Summation of all the PrP^{sc} recovered from all eluates of each column shows that the overall percentage recovery of PrP^{sc} for the DEAE Sepharose, CM Sepharose and Macro-Prep columns are ≤ 0·34, ≤ 1·84 and ≤ 0·03%, respectively. Mass balance was therefore not achieved in all three ion exchange columns up to the final wash with 1 M NaCl. The 1 M NaOH sanitation washes were not studied as NaOH renders PrP^{sc} sensitive to digestion by proteinase K [26], and could lead to aberrant results. The results indicate that some PrP^{sc} was eluted from the DEAE Sepharose and CM Sepharose, but most of the PrP^{sc} was either not recovered or bound to the chromatography gel prior to the NaOH sanitation step.

Scrapie ME7 spike with bioassay detection

Limiting dilution bioassay was used to determine the titre of the spiked supernatant I starting material and the final concentrated eluates from the CM Sepharose and Macro-Prep columns (Table 2). The control mice for all studies remained normal throughout the observation period, indicating that the inocula were non-toxic and that there was no cross-contamination from cages housing TSE-positive mice.

Table 1 Partitioning of PrP^{sc} microsomal fraction during albumin and immunoglobulin purification across ion exchange columns as determined by Western blot

Step/Fraction	Total PrP^{sc} (log_{10})	% PrP^{sc} in fraction	Reduction (log_{10})[b]
DEAE Sepharose™ FF			
Inoculum	3·80		
Column load	4·30	100·00	
Unbound IgG[a]	0·98	0·05	3·3
Transferrin peak	≤ 0·84	≤ 0·03	≥ 3·5
Wash – 10 mM NaAc	≤ 0·92	≤ 0·04	≥ 3·4
Eluted albumin[a]	≤ 0·32	≤ 0·01	≥ 4·0
Wash – 150 mM NaAc	1·63	0·20	2·7
0·5 M NaCl	≤ 0·11	≤ 0·01	≥ 4·2
CM Sepharose™ FF			
Inoculum	3·11		
Column load	3·64	100·00	
Unbound protein	≤ −0·34	≤ 0·03	≥ 4·0
Wash 110 mM NaAc	≤ 0·41	≤ 0·06	≥ 3·2
Eluted albumin[a]	≤ 0·68	≤ 0·13	≥ 3·0
Wash 400 mM NaAc	≤ 0·23	≤ 0·04	≥ 3·4
0·5 M NaCl	1·83	1·56	1·8
Macro-Prep High Q			
Inoculum	3·80		
Column load	4·18	100·00	
Purified IgG (unbound)[a]	≤ 0·08	≤ 0·01	≥ 4·1
Wash 10 mM NaAc	≤ −0·19	≤ 0·01	≥ 4·4
1 M NaCl	≤ −0·07	≤ 0·01	≥ 4·2

[a]Eluates shown in bold are main column eluents used for ongoing processing of albumin or immunoglobulin. All other eluates are waste streams.
[b]If PrP^{sc} (proteinase K-resistant scrapie prion protein) could not be detected in the neat sample, the PrP^{sc} log reduction was recorded as '≥'.

Table 2 Bioassay of test materials from albumin and immunoglobulin chroma[a]

Sample	Parameter	Sample dilution 10^{-2}	10^{-3}
Control	Mice infected/inoculated	0/5	
SN1	incubation period (days)[a]		
Control	Mice infected/inoculated	0/10	
Albumin	incubation period (days)		
Control	Mice infected/inoculated	0/5	
IgG	incubation period (days)		
ME7	Mice infected/inoculated	5/5	5/5
spiked SN1	incubation period (days)	194 ± 0	193 ± 7
ME7	Mice infected/inoculated	5/5	5/5
Albumin	incubation period (days)		
ME7	Mice infected/inoculated		
IgG	incubation period (days)	0/20	0/5

[a]Mean ± standard deviation.

Table 3 Bioassay of test materials from Normal Immunoglobulin 2VI (Cohn) process

Sample	Parameter	Sample dilution							
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Control cryosupernatant	Mice infected/inoculated incubation period (days)[a]	0/8							
Control IgG (Cohn)	Mice infected/inoculated incubation period (days)	0/9							
ME7 spiked cryosupernatant	Mice infected/inoculated incubation period (days)	5/5 186 ± 6	5/5 235 ± 26	5/5 223 ± 8	4/5 279 ± 67	1/5 279	1/5 347	1/5 230	0/4
ME7 IgG (Cohn)	Mice infected/inoculated incubation period (days)	0/20	0/5	0/5	0/5				

[a]Mean ± standard deviation.

Step/Fraction	Infectivity (log_{10} ID_{50}/ml)	Volume (log_{10})	Total infectivity (log_{10})	Reduction (log_{10})
Chromatography				
ME7 spiked SN1	5·4 (4·5–6·3)[a]	2·1	7·5	
ME7 albumin	≤ 0·7	1·2	≤ 1·8	≥ 5·6
ME7 IgG	≤ 0·7	1·4	≤ 2·1	≥ 5·4
Cohn				
ME7 spiked cyrosupernatant	5·4 (4·4–6·5)[a]	2·5	7·9	
ME7 IgG (Cohn)	≤ 0·7	1·6	≤ 2·3	≥ 5·6

Table 4 Calculation of infectivity in spiked process starting materials and final materials of chromatography and Cohn process

[a]95% confidence interval of Spearman Kärber estimate of ID_{50}.

Discussion

The potential risk of vCJD transmission has led producers of plasma products to examine the prion clearance capacity of their fractionation processes. Whereas it is an accepted principle to add viral log reduction factors attained by mechanistically complementary steps [27], different approaches are needed to establish overall prion removal. The European Agency for the Evaluation of Medicinal Products (EMEA) guidance [28] advises that validation studies of removal/inactivation procedures for TSEs are difficult to interpret due to the necessity to take into consideration the nature of the spiked material and its relevance to the natural situation, the design of the study (including scaling-down of processes) and the method of detection of the agent.

This study programme looked initially at PrP^{sc} removal capacity of individual process steps then examined the potential of two or more combined process steps to remove prion infectivity. The study programme used scrapie 263K and ME7 as models for human prions, an approach that is supported by the finding that partitioning of human prions is similar to that observed in the hamster scrapie model [29]. The studies used different spiking materials (microsomal 263K and 10 000 g supernatant ME7) because the ME7 study sought to use an infectious spike which would consist not only of microsomal infectious units, but of smaller units of infectivity as soluble PrP^{sc} [17]. The study programme found substantial partitioning of prions away from the product streams of chromatographic albumin and immunoglobulin, and for immunoglobulin produced by Cohn fractionation. Importantly, this investigation shows that removal of infectivity from immunoglobulin preparations is similar whether chromatographic or Cohn purification processes are used.

The study with microsomal scrapie 263K showed substantial partitioning of PrP^{sc} away from the target proteins in all ion exchange steps examined. The log reductions across the anion exchange DEAE Sepharose and cation exchange CM Sepharose for the albumin process were ≥ 4·0 and ≥ 3·0, respectively. The log reductions across the DEAE Sepharose and anion exchange Macro-Prep for the immunoglobulin process were 3·3 and ≥ 4·1, respectively.

At the loading pH buffer ranges used for this experiment (pH 5·2 for DEAE Sepharose, pH 4·5 for CM Sepharose, and pH 6·2 for Macro-Prep), scrapie should be below its isoelectric point (pI) of pH 5·4–9·3 [30] on the DEAE and CM Sepharose columns, and hence would be positively charged. While pH 6·2 is within the pI range for scrapie, it is likely that scrapie is predominantly positively charged when loaded onto Macro-Prep. If scrapie bound to chromatography columns purely based on charge, it would be predicted that more binding should occur with the cation exchanger CM Sepharose, and less to the anion exchangers DEAE Sepharose and Macro-Prep. The substantial

removal of PrP^{sc} by the anion and the cation exchange gels, and lack of substantial amounts of PrP^{sc} in the wash fractions indicated that PrP^{sc} removal was more dependent on adsorption to the gel matrix than to the exchange group. There was a partitioning of 0·05% of loaded PrP^{sc} in the unbound IgG eluted from DEAE Sepharose. However, as the Macro-Prep column removed ≥ 4·1 logs, there is a level of confidence that this remaining PrP^{sc} would be removed from the product stream.

Similar results were reported using murine bioassay of BSE 301 V over Toyopearl DEAE-650 M [31], in which LRFs of 2·9 and 2·7 were found in eluted fibrinogen and factor VIII, leading to the conclusion that over 99% of BSE infectivity remained bound to the ion-exchange column. A 2 M NaCl wash removed 5·75% of this infectivity, and infectivity could not be detected in eluates following a 0·1 M NaOH wash.

In our study, new chromatography gels were used, as opposed to production gels that had been exposed to previous cycles. The possibility of infectivity binding to chromatography gels has led to further experimental work examining prion removal and or inactivation of infectivity from chromatography gels, in which it was ascertained that infectious prions did bind to DEAE Sepharose, and the cleaning cycle was able to remove or inactivate this infectivity [32].

A LRF of ≥ 5·6 across the DEAE and CM Sepharose ion exchange columns in the albumin process and a LRF of ≥ 5·4 across the DEAE Sepharose and Macro-Prep ion exchange columns in the immunoglobulin process was achieved. Both processes include a final concentration/diafiltration step using 30 kDa ultrafiltration, with the retentate containing either albumin or immunoglobulin. It is unlikely that substantial prion infectivity would be lost in the permeate stream, as infectious units are believed to have a minimum molecular weight of approximately 55 kDa [33]. Conversely, it is possible that some infectivity is adsorbed to the ultrafilter membrane surface; however, the membrane types used (polyethersulphone for albumin and regenerated cellulose for immunoglobulin) are both specified as low protein binding by the respective manufacturers.

If the starting titre for the ME7 bioassay study had been higher it may have been possible to show removal capacity equal to the addition of removals attained for each column in the Western blot study. Previous studies using scrapie 263K for validation of prion removal in bovine serum albumin production with sequential columns [34] have shown a 5·2 log removal of scrapie 263K over the first ion exchange column, and ≥ 6·2 when the second ion exchange column is included. This implies that the result is limited by the starting titre, and the question of additivity cannot be resolved without a higher infectivity spike. In our study programme, the spike material preparations and the method of detection (Western blot vs. bioassay) were different between the two studies. Higher titre spiking material would be needed to further elucidate the additive vs. non-additive nature of prion removal over sequential columns.

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Conclusion

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Acknowled

We wish to [illegible] Elaine Bem[illegible] the studies [illegible] performed b[illegible] Peter Gomm[illegible] Hartel helpe[illegible]

Referenc

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Prion removal by nanofiltration under [illegible]

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Abstract

Manufacturing processes used in the production of biopharmaceuticals [illegible] potential contaminants, including TSE agents. In the present study, we have evaluated [illegible] different starting materials, using virus removal filters of different pore sizes. [illegible] Western blot (WB) analysis when a "super-sonicated" microsomal fraction derived [illegible] used as the spike material. In contrast, no PrP^{Sc} was detected when an untreated [illegible] ner designed to optimize the particle size distribution within the preparation [illegible] of detection of the WB assays used under all the experimental conditions. However, [illegible] under one experimental condition. The results obtained suggest that the [illegible] ability of filters to remove prions, and that procedures designed to minimize [illegible] sonication" or detergent treatments described herein, should be used for the prep[illegible]

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Keywords: Prion; Removal; Filter; Clearance study; Spike material

1. Introduction

The transmission of variant Creutzfeldt–Jakob disease (vCJD) through blood transfusion has been of increasing concern, since a fourth possible transmission case was reported [1]. In addition, prions have been detected in the buffy coat separated from the blood of hamsters infected with scrapie, using a biochemical assay (protein misfolding cyclic amplification, or PMCA) [2]. Infectious prions are [illegible]

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consider the effect of the prion spike material when evaluating process steps for prion clearance. A rationale for the choice of the spike preparation used for such evaluation studies should be provided [4].

Several prion strains have been used to evaluate manufacturing processes for their ability to remove TSE agents, including hamster scrapie prion protein (PrP^{Sc}, 263K or Sc237), and mouse PrP^{BSE} (301V). In a polyethylene glycol (PEG) fractionation process, hamster PrP^{Sc} and human PrP^{CJD}, prepared using the same methodology, were reported to behave in a very similar manner [6]. Different prion spike preparations have been used to investigate prion removal, including crude brain homogenate (BH), microsomal fraction (MF), caveolae-like domains (CLDs), and purified PrP^{Sc}. Of these materials, purified PrP^{Sc} was reported to behave differently from the other preparations in an 8% ethanol fractionation step [7]. This result suggests that the methods used to prepare the prion spike material may be a critical factor in prion clearance studies. Furthermore, these reports are useful in providing a rationale for the choice of the prion source and spike preparation used for such evaluation studies [8].

Tateishi et al. reported that sarkosyl influenced the ability of BMM40 filters to remove prions, using BH derived from CJD-infected mice [9]. The presence of sarkosyl was also shown to significantly reduce the capacity of Planova (P)-35N to remove the scrapie agent ME7, while filtration with P-15N resulted in the complete removal of infectivity, to below the limit of detection of the bioassay used, in both the presence and absence of sarkosyl [10]. Van Holten et al. evaluated the capacity of Viresolve 180 membranes (designed for virus removal from proteins of <180 kDa) to remove prions by using BH which was lysolecithin-treated, sonicated, and subsequently passed through a 100 nm filter (SBH), and demonstrated removal of PrP^{Sc} down to the limit of detection of the Western blot assay used. They argued that by using a better defined spike material, where the size of the scrapie particles was limited, the results may be more relevant with respect to the removal of potential TSE infectivity in plasma than previous studies that used a less well-defined BH [11].

Aggregation of the prion protein is a critical parameter when evaluating nanofiltration steps. The actual form of the infectious agent present in plasma in natural infection is not known. In addition, nanofiltration is typically performed late in the downstream processing, after protein purification steps, which may result in removal of larger or aggregated prion forms. Therefore, use of a spike preparation containing large aggregates may result in an over-estimate of the prion removal capacity of a filter. Although the reports described above, and others, have shown excellent prion removal ability for a number of filters, most reports have not described the particle size distribution of the prion protein in the spike preparations used. Therefore, in this study we have investigated the prion removal capacity of P-35N, P-20N and P-15N filters under diverse conditions, considering the particle size distribution of the MF preparations used.

2. Materials and methods

2.1. Preparation of microsomal fraction (MF)

Brains removed from hamsters infected with scrapie strain 263K [12] (originally obtained from the Institute for Animal Health, Edinburgh, UK), were homogenized in phosphate buffered saline (PBS) until homogeneous, to a final concentration of 10% (w/v). The homogenate was clarified by low speed centrifugation, to remove larger cell debris and nuclei, and the supernatant material was then further clarified by centrifugation at 8,000 × g for 10 min at 4 °C, before being ultracentrifuged at 141,000 × g for 60 min at 4 °C, to concentrate the scrapie fibrils, and small membrane vesicles and fragments. The pelleted material was resuspended in PBS, aliquoted, and stored at −80 °C. This material was designated 263K MF. Prior to use, stocks were thawed at 37 °C, and sonicated 2 × 4 min on ice water (Ultrawave ultrasonic bath model #U100, 130 W 30 kHz, Ultrawave Ltd., Cardiff, UK). Six independent batches of 263K MF were used in this study. These batches are designated 263K MF preparation lots A–F (Tables 1–3). Normal MF, derived from normal (i.e. uninfected) hamster brain material, was also prepared as described above.

Since we were unable to measure the particle size distribution of contaminated materials in our facility, we used normal MF, and investigated changes in the particle size distribution following strong sonication or treatment with detergent. Various concentrations of sarkosyl (*N*-lauroylsarcosine sodium salt, Nacalai Tesque, Inc., Kyoto, Japan), lysolecithin (L-α-lysophosphatidylcholine, Sigma-Aldrich Corp., St. Louis, USA), Triton X-100 (polyethylene glycol mono-*p*-isooctylphenyl ether, Nacalai Tesque, Inc.), TNBP (tri-n-butyl phosphate, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and/or 1% Tween 80 (Nacalai Tesque, Inc.) were added to normal MF. Changes in the particle size distribution were then monitored by dynamic light scattering method using volume-weighted gaussian analysis using a submicrometer particle sizer (NICOMP Type 370, Particle Sizing Systems, Inc., Santa Barbara, USA). To evaluate the effect of strong sonication, normal MF was sonicated using a closed system ultrasonic cell disruptor (Bioruptor UCD-200T, CosmoBio Co. Ltd., Tokyo, Japan) with a resonance chip set in the tube. Sonication was performed for 1 min at 20 kHz, 200 W in a cold waterbath. Ten cycles of sonication were performed, with a 1 min interval between each sonication treatment. During the treatment cycle, the particle size distribution was monitored. We named this treatment cycle "super-sonication".

Table 1
Scrapie infectivity in different 263K MF preparations[a]

	Log_{10} LD_{50}/ml	SE at 95% probability
Non-super-sonicated 263K MF lot C	5.7	0.44
Super-sonicated 263K MF lot C	6.0	0.53
Super-sonicated 263K MF lot D	5.3	0.69
SD-treated, ultracentrifuged, super-sonicated and 220 nm-filtered 263K MF lot C	6.9	0.69

[a] This bioassay study was performed in accordance with GLP regulations.

Table 2
Removal of PrP^{Sc} from PrP^{Sc}-inoculated PBS

	PVDF filter				Planova filter					
	220 nm		100 nm		P-75N (72 ± 2 nm)		[illegible] (35 ± 2 nm)		[illegible]	
Super-sonicated	+	−	+	−	+	−	[illegible]	[illegible]	[illegible]	[illegible]
Before filtration	4.2/3.5[a]	3.5/4.2	4.2/3.5	3.5/4.2	4.2/4.2	3.5/4.2	[illegible]	[illegible]	[illegible]	[illegible]
Filtered	3.8/3.8	3.1/3.8	3.8/3.1	2.4/3.1	2.4/2.4	<1.0/<1.0	[illegible]	[illegible]	[illegible]	[illegible]
LRF[b]	0.4/−0.3	0.4/0.4	0.4/0.4	1.1/1.1	1.8/1.8	≥2.5/≥3.2	[illegible]	[illegible]	[illegible]	[illegible]

Data represents total PrP^{Sc} present in samples, expressed as log_{10} arbitrary units, following Western [illegible] in accordance with GLP regulations.
[a] Two independent batches of 263K MF were used: lot C (left) and lot D (right), respectively.
[b] LRF, log reduction factor = total PrP^{Sc} in input/total PrP^{Sc} in filtrate, expressed as a log_{10} [illegible]

Different preparations of 263K MF, treated with various combinations of detergent, ultracentrifugation and/or "super-sonication", were used as the spiking agent in the process evaluation studies, and are described in the relevant methods sections below.

2.2. Detection of PrP^{Sc} by Western blotting (WB)

To determine the relative levels of PrP^{Sc} present in different samples, WB assays were performed. Three slightly different WB methodologies were applied over the course of the studies, all of which are based on detection of the disease-associated, protease-resistant form of the prion protein (PrP^{Sc}), using the monoclonal antibody 3F4 (Signet Laboratories, Inc., Dedham, USA) [13]. WB methods 1 and 2 were developed independently, and use different approaches to calculate the titer of PrP^{Sc}. As these assays were performed as part of GLP studies intended for regulatory [illegible] sion, the [illegible] in these st[illegible]

2.2.1. [illegible]

Samples [illegible] trols were [illegible] ultracentrifuged at 141,000 × g for [illegible] pelleted material was resuspended in [illegible] was performed to concentrate [illegible] ume samples, and to remove soluble [illegible] nents that might interfere with the [illegible] digested with proteinase K (Roche [illegible] berg, Germany) for 60 min at 37 °C. [illegible] tion of proteinase K, to [illegible] interfere with the detection of PrP^{Sc} [illegible] covery of the [illegible] protein [illegible] each sample [illegible] sample buffer [illegible] mM Tris-HCl [illegible] erol, 2% [illegible] and [illegible] BioRad Laboratories, Inc., Hercules, [illegible] β-mercaptoethanol. After [illegible]

Table 3
Removal of PrP^{Sc} from PrP^{Sc}-inoculated plasma preparations[a]

Filter	P-35N (35 ± 2 nm)		P-20N (19 ± 2 nm)		[illegible] (35 ± 2 [illegible])			
Preparation	IVIG	Haptoglobin	IVIG	Haptoglobin	[illegible]rombin			
Spike material	263K sMF[c]	263K sMF[c]	263K sMF[d]	263K dsMF[e]	[illegible]MF[f]	[illegible]	[illegible]	[illegible]MF
MF preparation lot.	C/D	B	E/F	E/F	[illegible]	[illegible]		
Spike ratio	1/100	1/200	1/20	1/200	[illegible]	[illegible]		
Detection method[b]	WB1	WB3	WB2	WB2	[illegible]	[illegible]	[illegible]	[illegible]
Before filtration	3.2/2.5	2.4	6.8/6.8	6.7/6.1	[illegible]	[illegible]	[illegible]	[illegible]
Filtered	0.8/0.8	<1.0	4.8/4.3	4.8/4.7	[illegible]	[illegible]	[illegible]	[illegible]
Log reduction factor	2.4/1.7	≥1.4	2.0/2.5	1.9/1.4	≥3.1	[illegible]	[illegible]	[illegible]

Abbreviations used: 263K MF, microsomal fraction derived from hamster adapted scrapie strain [illegible]; IVIG, intravenous [illegible] "super-sonicated" 263K MF; WB, Western blotting; 263K dsMF, detergent treated and "super-sonicated" [illegible] 263K [illegible] assay; +ve, scrapie positive.
[a] Scaled down conditions were designed according to current guidelines. However, in a study using [illegible] and the filtration was subsequently terminated.
[b] WB1, WB2, and WB3 mean Western blotting methods 1, 2 and 3, respectively. The studies involving the use of WB1 and WB2 [illegible] with GLP regulations; the studies involving the use of WB3 and the qualitative BA shown in this [illegible] performed [illegible]
[c] 263K MF was "super-sonicated" then 220 nm-filtered prior to spiking.
[d] 263K MF was ultracentrifuged at 141,000 × g for 60 min at 4 °C, resuspended in buffer equivalent to the starting material [illegible]" and 220 nm-filtered prior to spiking.
[e] 263K MF was "SD-treated", ultracentrifuged at 141,000 × g for 60 min at 4 °C, resuspended [illegible] starting material [illegible] "super-sonicated". These materials were 220 nm-filtered prior to spiking.
[f] 263K MF was treated with 0.1% sarkosyl for 30 min at room temperature.

the sample were then prepared and subjected to electrophoresis using 12% (w/v) SDS-polyacrylamide gels. Proteins were transferred from the gels to 0.45 μm PVDF membranes (Immobilon-P, Millipore Corp., Billerica, USA), and non-specific binding sites on the membranes were then blocked by overnight incubation in buffer containing dried milk and Tween 20. The blocked membranes were incubated with monoclonal antibody 3F4, washed extensively, and then incubated with a secondary horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Sigma-Aldrich Corp.). After further extensive washing, bound antibody was detected using an ECL-Plus detection system (GE Healthcare UK Ltd, Buckinghamshire, UK) and exposure to blue-light sensitive film.

The level of PrP^{Sc} present in each sample was calculated based on the end-point dilution after analysis by WB. The end-point dilution for each titration was taken as the first dilution at which the 28 kDa PrP^{Sc} protein could not be detected. The reciprocal of this dilution was then taken as the titer of the agent, and expressed in arbitrary units/ml.

2.2.2. Method 2 (WB2)

WB was performed essentially as described by Lee et al. [14]. Briefly, samples were digested with proteinase K at approximately 6 U/ml for 60 min at 37 °C and centrifuged at approximately 20,000 × *g* for 60 min at 4 °C. The pellet was then resuspended and denatured in a 1:1 mix of supernatant and sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS and 0.025% (w/v) bromophenol blue, Invitrogen Corp., Carlsbad, USA), by heating at approximately 100 °C. Serial 3.2-fold (0.5 $\log_{10}$) dilutions of the sample were prepared, and loaded onto 12% (w/v) SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Invitrogen Corp.), and the membranes blocked using buffer containing dried milk and Tween 20 for 1–2 h at room temperature. The blocked membranes were then incubated with monoclonal antibody 3F4, washed extensively, and incubated with a secondary alkaline phosphatase (AP)-conjugated anti-mouse antibody (Cambridge Biosciences Ltd., Cambridge, UK). After further extensive washing, bound antibody was detected using a CDP Star/Nitroblock II detection system (Applied Biosciences, Bedford, USA) and exposure to blue-light sensitive film.

The titer of PrP^{Sc} present in each sample was calculated slightly differently from WB1 and WB3. The end-point dilution for each titration was taken as the last dilution at which the 28 kDa PrP^{Sc} protein could be detected. The reciprocal of this dilution was then taken as the amount of agent in the sample volume tested, and was adjusted for the volume tested and any concentration factors, to give a titer/ml for the original process sample.

2.2.3. Method 3 (WB3)

Samples were ultracentrifuged twice at 150,000 × *g* for 1 h. The samples in the precipitates were then resuspended in PBS at 1/1 or 1/10th volume of the original. Resuspended samples were treated with proteinase K at a final concentration of 10–100 μg/ml. After incubation at 37 °C for 60 min, samples were treated with 10 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride (AEBSF) at room temperature for 10 min, then mixed with 5× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (300 mM Tris–HCl, 12% (w/v) SDS, 25% (v/v) glycerol, and 0.025% (w/v) bromophenol blue, pH 6.8, with 25% (v/v) β-mercaptoethanol) and heated at 100 °C for 5 min. Samples were serially 5-fold diluted with 1× PAGE dilution buffer (60 mM Tris–HCl, 2.4% (w/v) SDS, 5% (v/v) glycerol, and 0.005% (w/v) bromophenol blue, pH 6.8). SDS-PAGE was performed at 30 mA per gel for approximately 42 min. The proteins in the gel were transferred to 0.45 μm PVDF membranes. After treating with blocking buffer (5.0% (w/v) skimmed milk in PBS, 0.05% (v/v) Tween 20), the membrane was incubated with monoclonal antibody 3F4 at 4 °C overnight, then incubated with HRP-conjugated sheep anti-mouse IgG (Sigma-Aldrich Corp.). Bound antibody was visualized by chemiluminescence (ECL-Plus) on X-ray film. The titer of PrP^{Sc} present in the samples was calculated as described for method 1 in Section 2.2.1.

2.3. Evaluation of PrP^{Sc} removal by filtration

A 10% (v/v) concentration of "super-sonicated" 263K MF was prepared in PBS, and 10 ml aliquots were then filtered through a 220 nm or a 100 nm 4 cm^2 PVDF filter (Millex-GV or -VV, Millipore Corp.). In addition, 25 ml aliquots of "super-sonicated" 263K MF in PBS were filtered through a 0.01 m^2 P-75N (72 ± 2 nm), P-35N (35 ± 2 nm), or P-15N (15 ± 2 nm) filter (Asahi Kasei Medical Co., Ltd. Tokyo, Japan). Two independent batches of 263K MF were used. WB1 analysis of samples before and after filtration was performed to determine the removal of PrP^{Sc} under the different conditions. Non-sonicated 263K MF (from the same batch of 263K MF) was also filtered as a control.

2.4. Hamster bioassay to determine the infectious titer of 263K scrapie stocks

Three- to four-week-old female specific pathogen-free (SPF) Syrian hamsters were used in these experiments. Serial 10-fold dilutions of each sample or positive control were prepared in PBS. Six hamsters per sample dilution were inoculated intra-cerebrally with 0.02 ml per animal. The inoculated animals were monitored daily for general health, and weekly for clinical evidence of scrapie. Animals were euthanized once advanced signs of scrapie were evident, or at the end of the assay period (200 days). The brain was removed from each hamster following euthanasia: one half was fixed for histopathology and the other half was stored frozen at −70 °C for further analysis if required. For histopathological analysis, sections taken at four standard coronal levels, to cover the nine areas of the brain which are recognized to be mostly infected by the scrapie agent, were stained with hematoxylin and eosin, and scored for the presence or absence of scrapie lesions [15]. Histopathological analysis was performed on samples from around the clinical end-point of the titration assays, to confirm the clinical results. Hamsters that died during the course of the study for reasons other than scrapie infection were not included in the final calculation of infectious titers. Infectious titers were expressed as a 50% lethal dose (LD_{50}) according to the method of Kärber [16].

Samples taken before and after filtration during the P-15N/antithrombin (AT; previously named antithrombin-III) study were tested for the presence of scrapie infectivity using a qualitative hamster bioassay. Syrian hamsters were inoculated with undiluted samples only, as described above, except that only three animals were used per sample.

2.5. Evaluation of PrP^{Sc} removal in the presence of plasma preparations

To investigate whether differences in how the scrapie spike material was prepared influenced our evaluation of prion removal, two different spiked preparations were compared using the manufacturing process for preparing AT (Neuart®, Benesis Corp., Osaka, Japan). Samples taken during the actual manufacturing process, immediately before the Planova step, were spiked with 263K MF treated with 0.1% (w/v) sarkosyl for 30 min at room temperature, or with 220 nm-filtered "super-sonicated" 263K MF. The spiked AT materials were then passed through a P-15N filter. The influence of different filtration conditions on the removal of PrP^{Sc} was compared for the same spike preparations, and for different spike preparations, using heat/PEG-treated intravenous immunoglobulin (IVIG) (Venoglobulin-IH, Benesis Corp.) and haptoglobin (Haptoglobin Injection-Yoshitomi, Benesis Corp.). Samples taken during the actual manufacturing process, immediately before the Planova step, were spiked with: 220 nm-filtered "super-sonicated" 263K MF (IVIG/P-35N and haptoglobin/P-35N); 263K MF ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended in buffer equivalent to the starting material without protein, "super-sonicated" and 220 nm-filtered (IVIG/P-20N); or 263K MF treated with 0.3% (v/v) TNBP/1% (v/v) Tween 80 for 6 h at 30 °C ("SD treatment"), ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended in saline, "super-sonicated", and 220 nm-filtered (i.e., haptoglobin/P-20N). The spiked material was then passed through either a P-35N filter or a P-20N filter (19 ± 2 nm). Although not part of the manufacturing process for haptoglobin, the SD treatment was included for the spiked preparation in an effort to reduce the clogging of the filter that occurs following the addition of a prion spike. Filtration processes for the thrombin preparation (Thrombin-Yoshitomi, Benesis Corp.) were also investigated. For thrombin, a sample taken during the actual manufacturing process immediately before the Planova step was spiked with 263K MF subjected to "SD treatment" followed by ultracentrifugation at 141,000 × *g* for 60 min at 4 °C, resuspended in the starting material, "super-sonicated" and 220 nm-filtered, and the spiked material then passed through a P-15N filter.

The experimental conditions used in the prion removal studies were designed to mimic the conditions used during the actual manufacturing process for the relevant product. For all processes, samples were analyzed by WB. The $\log_{10}$ reduction factor (LRF) for PrP^{Sc} was calculated for each

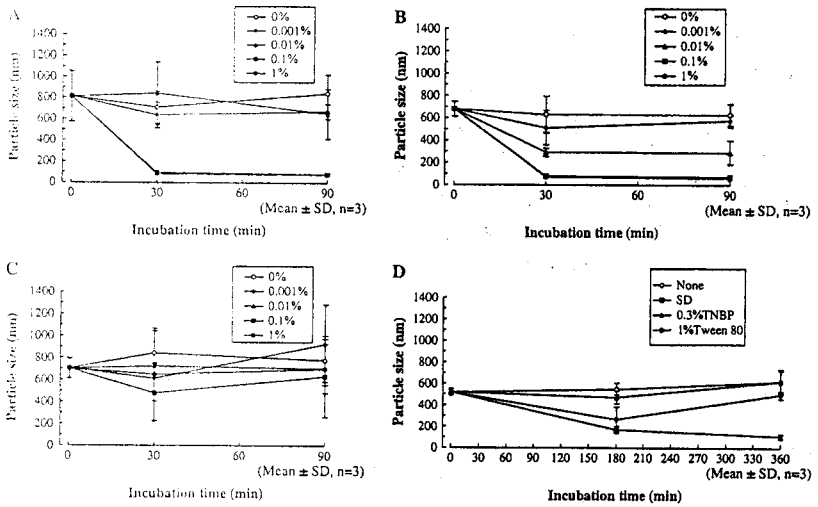

Fig. 1. Change of particle size in normal MF following treatment with various detergents. To normal MF, sarkosyl (A), lysolecithin (B), or Triton X-100 (C) was added to a final concentration of 1%, 0.1%, 0.01%, and 0.001%, respectively. The change in the average particle size was then monitored at room temperature for 90 min. In addition, TNBP or Tween 80 was added to normal MF to a final concentration of 0.3% and 1%, respectively, either alone, or in combination ("SD treatment"). The change in the average particle size was then monitored at 37 °C for 6 h (D).

with 0.1% sarkosyl, 0.1% lysolecithin, "SD treatment", or "super-sonication". The use of detergent or "SD treatment", in combination with "super-sonication", was also shown to effectively reduce the average particle size in normal MF preparations, to comparable levels to the individual treatments alone (data not shown). "Super-sonication" has an advantage over the other treatments in that it can minimize the change of composition of samples taken from the manufacturing process, as it does not require the addition of reagent(s) to the normal MF. For this reason, "super-sonication" is considered to be a useful approach for the treatment of 263K MF for process evaluation. "SD treatment", although slightly less effective, is used in many manufacturing processes, and may therefore be useful alone, or in combination with "super-sonication", for the process evaluation of products whose manufacturing process includes an "SD treatment" step. These approaches, alone or in combination, may also be useful to prevent the clogging of filters that can occur during spiking studies.

3.2. Infectivity of PrP^{Sc} in 263K MF and influence of 263K MF preparation methods on infectivity

The effect of "super-sonication" and "SD treatment" on the infectivity of 263K MF was studied. Infectious titers of

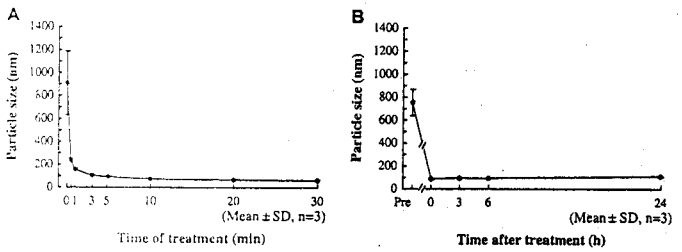

Fig. 2. Change of particle size in normal MF following intense sonication ("super-sonication"). Normal MF in a test tube equipped with a resonance chip (20 kHz, 200 W) was sonicated for 1 min in an ice bath. After 1 min, the sonication step was repeated. The change in average particle size was monitored during 30 cycles of sonication (A). After 10 cycles of sonication ("super-sonication"), normal MF was held at room temperature for 24 h, and the change in particle size was monitored (B).

263K MF, "super-sonicated" 263K MF, and 263K MF subjected to "SD treatment", ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended with thrombin starting material, "super-sonicated", and 220 nm-filtered, were determined using a hamster bioassay. The results are summarized in Table 1.

The titers of two independent batches of 263K MF treated by "super-sonication" were 6.0 and 5.3 $\log_{10}$ LD_{50}/ml, respectively. The titer of the "non-super-sonicated"263K MF used to generate one of these stocks was 5.7 $\log_{10}$ LD_{50}/ml. These results suggest that "super-sonication" does not influence the infectivity of 263K MF. The titer of the 263K MF subjected to "SD treatment", ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended with the thrombin starting material, "super-sonicated", and 220 nm-filtered, was 6.9 $\log_{10}$ LD_{50}/ml, which was approximately 1 log higher than that of the corresponding stock treated by "super-sonication" alone. Whether this difference is significant is unclear. The process to generate the "SD-treated" spike materials included an ultracentrifugation step. We were therefore concerned about recovery of infectivity following centrifugation, as the particle size of 263K MF was highly reduced by the "SD treatment" step. However, these results suggested that the recovery of infectious particles following ultracentrifugation was satisfactory.

Although it is possible that use of a 200 day bioassay may under-estimate the infectious titer of the 263K MF stocks, the use of a relatively short duration bioassay is considered unlikely to affect the main conclusions drawn. At least the last two dilution groups tested showed no animals with evidence of scrapie infection in all four titrations, and only three animals in the study (one in each of three separate titrations) developed clinical symptoms necessitating euthanasia later than day 131 (euthanized on days 160, 183 and 183, respectively), suggesting the titers obtained for all the stocks are close to end-point (data not shown). In addition, as others have demonstrated that treatment with detergent, and exposure to treatments that result in inactivation of the scrapie agent, such as heat or NaOH, may result in extended incubation periods for clinical scrapie, if anything the results may under-estimate the relative titers of the treated stocks [17,18]. Therefore, the bioassay results support the conclusion that "super-sonication" of 263K MF stocks, with or without "SD treatment", does not appear to significantly reduce the infectious titer of the stock, and that these preparations are therefore suitable for use in prion clearance studies.

3.3. Removal of PrP^{Sc} by various filters

To determine whether "super-sonication" influenced the $\log_{10}$ reduction observed for PrP^{Sc} following filtration under defined conditions, "super-sonicated" or "non-super-sonicated" stocks of 263K MF were diluted in PBS, and then filtered through 220 nm, 100 nm, P-75N, P-35N and P-15N filters. Samples were analyzed by WB. The results are summarized in Table 2. The use of "super-sonicated" 263K MF appeared to result in lower $\log_{10}$ reduction values, supporting the idea that "super-sonication" of 263K MF produces a

Table 4
Scrapie [illegible] samples generated using the P-15N/AT study

	Before filtration			Filtrate		
	Animal number			Animal number		
	1	2	3	1	2	3
Appearance of clinical signs (day euthanized)	87	87	87	94	143	105
PrP^{Sc} in brain by WB	Detected	Detected	Detected	Detected	Detected	Detected
Lesions by [illegible]	+ve	+ve	+ve	+ve	+ve	+ve
Medulla [illegible]	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P
Cerebellum [illegible]	D	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P
Midbrain	D,P	D,V,P	V,P	D,P	D,P	D,V,P
Hypothalamus	D,P	D,V,P	D,P	D,V,P	D,P	D,P
Thalamus	D,P	D,V,P	D,P	D,P	D,P	D,P
Hippocampus	NR	D,V	D	D	D,V,P	D,V
Parahippocampal gyrus	D,P	D,P	D,P	NR	D,V,P	P
Cerebral cortex (at lateral midline)	D,P	D,P	D,P	D,P	D,V,P	D,V,P
Cerebral cortex (at dorsal midline)	D,P	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P

Abbreviations: +ve, scrapie positive; NR, no remarkable change; D, degeneration of nerve cell; V, vacuolation; P, proliferation of glial cell.

corresponding hamster brain material on histopathological observation (Table 4). Typical nerve lesions are shown in Fig. 3. Thus, P-15N filtration did not result in the complete removal of infectivity for this process step.

4. Discussion

In this study we have investigated the capacity of P-35N, P-20N and P-15N filters to remove the 263K scrapie prion protein, PrP^{Sc}, under the conditions used for the manufacture of four different plasma-derived products, using spike preparations designed to present a serious challenge to the filters.

Validation studies to evaluate the capacity of manufacturing processes to remove potential contaminants, including prions, are required for biological or biopharmaceutical products intended for human use. When designing these studies, a worst-case challenge should be used wherever possible, to minimize the risk of over-estimating the capacity of the process to remove such contaminants. Virus removal filters (or nanofilters) are designed to remove contaminants predominantly on the basis of size. The worst-case challenge for such steps should therefore be a preparation containing the smallest possible form of the contaminating agent.

TSE clearance studies provide a particular challenge in that the nature of the infectious agent is still uncertain, and the forms of infectious agent present in plasma, and/or during the different stages of a manufacturing process, are not clearly understood. The causative agent of TSE diseases is believed to be strongly associated with, if not solely composed of, the disease-associated prion protein, PrP^{Sc}. Normal cellular PrP is a membrane-bound glycoprotein, which associates with membranes through a glycosylphosphatidylinositol (GPI) anchor. Prion infectivity is associated with heterogeneous particles, including membranes, liposomes and protein aggregates, so called prion rods. Therefore, methods which result in solubilization of membrane proteins, or dispersal of membrane fragments, vesicles and/or protein aggregates, may be expected to reduce the size of particles associated with prion infectivity.

Treatment of MF preparations derived from brains of uninfected (normal) hamsters with either detergent (0.1% lysolecithin or 0.1% sarkosyl) or extensive sonication ("super-sonication") resulted in a rapid reduction in the average particle size, to approximately 100 nm. SD treatment (1% Tween 80 and 0.3% TNBP for 6 h) also resulted in a reduction in particle size, although this was slower and less effective, reducing the average particle size to the order of 200 nm.

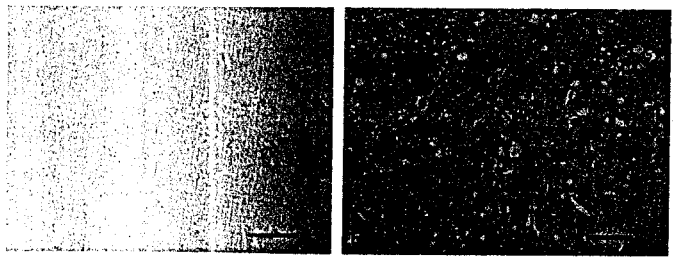

Fig. 3. Typical [illegible] lesions in the hippocampus of a hamster brain, taken from an animal inoculated with a P-15N-filtered sample (B), in comparison with the corresponding region from an uninfected normal (A). Arrows, vacuolation; Arrowheads, degeneration of nerve cells; scale bar = 50 μm; HE staining used.

"Super-sonication" has the advantage that it is a physical disruption process, and does not alter the chemical composition of the spike material, thus minimizing changes to the start material used for nanofiltration. SD treatment is included in many manufacturing processes for plasma-derived products, and therefore, although not as effective as "super-sonication", use of this treatment might be expected to result in a spike material more closely mimicking the form of infectious prion present in the relevant start material during the manufacturing process. Use of these treatments alone or in combination may therefore be useful in reducing the size of infectious particles present in TSE spike materials for prion clearance studies.

The effect of the above treatments was studied using normal MF, as the facility was unable to handle infectious TSE materials. Although some care should be taken in extrapolating these results to TSE-infected brain material, "super-sonication" of 263K MF preparations appeared to reduce the removal of PrP^{Sc} following filtration, while detergent-treated spike preparations have previously been shown to present a more significant challenge to nanofiltration steps than untreated preparations ([9,10] and own unpublished observations). Furthermore, "super-sonication", with or without SD treatment, does not appear to reduce the level of infectivity present within the 263K MF, supporting the use of such preparations for prion clearance studies.

Using 263K MF treated with 0.1% sarkosyl, "super-sonication" or SD plus "super-sonication", we investigated the prion removal capacity of P-15N, P-20N and P-35N filters in the manufacturing processes used for four different plasma products. The results obtained suggest that both the composition of the materials to be filtered and the prion load influences the removal of prions. PrP^{Sc} was recovered in the filtrate fraction from three out of the four processing steps performed for P-20N and P-35N. In contrast, under all conditions tested, P-15N filtration resulted in removal of PrP^{Sc} to below the limit of detection of the Western blot assays used. Thus, P-15N would appear to be a more robust method for the removal of prions, reproducibly giving LRF in the order of 3 logs, under the conditions tested. In practice, however, it is not feasible to incorporate P-15N filtration into the manufacturing process for all plasma derivatives. From the results shown in Table 2, it may also be possible to optimize processing conditions to allow effective removal of PrP^{Sc} using P-20N or P-35N filters.

WB assays were used to monitor the partitioning of PrP^{Sc} during the nanofiltration processes. WB assays are semi-quantitative and serve to provide an indication of the relative levels of PrP^{Sc} present in different samples. However, there are limitations to the sensitivity of available WB assays, and these assays provide only an indirect measure of infectivity. Therefore, to confirm that removal of PrP^{Sc} does reflect removal of infectivity, bioassays need to be performed.

Although PrP^{Sc} was not detected in any of the P-15N filtered samples by WB assay, infectivity was recovered in a filtrate fraction tested by bioassay for one process run. Foster also noted that infectivity was detected in a filtrate fraction after P-15N filtration ([8] reported as personal communication; data not shown). Thus, even with P-15N, depending on the

Planova filters. Some of the data presented in this study has been summarized in a recent review [22].

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CJD PrP^{sc} removal by nanofiltration [illegible] therapeutic immunoglobulin solution [illegible]

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Abstract

The characteristic of transmissible spongiform encephalopathies (TSE) is an acc[illegible] prion protein (PrP^{sc}). This pathological prion protein is very resistant to c[illegible] such as Creutzfeldt–Jakob disease (CJD), by biopharmaceutical products [illegible] tration process has been proved to be effective in removing viruses and scrapie agent. [illegible] efficacy in removing infectious particles without altering biopharmaceutical charact[illegible]

This study focused on the removal of human PrP^{sc} by means of a nanofiltration [illegible] a CJD brain homogenate. Lymphoglobuline® equine anti-human thymocyte immun[illegible] mainly on human T lymphocytes. The therapeutic indications are:

- immunosuppression for transplantation: prevention and treatment of graft [illegible]
- treatment of aplastic anemia.

In our study, CJD homogenate was spiked at three different dilutions [illegible] filtration process was performed on each sample. Using the western blot te[illegible] that obtained with a reference scale (dilution series of CJD brain homogenate in [illegible] 3.3 log). After nanofiltration, the PrP^{res} western blot signal was detected with a si[illegible] was undetectable in the two other samples.

These are the first data in CJD demonstrating a clearance between 1.6 and [illegible] nanofiltration process confirms its relative efficacy in removing human CJD PrP^{sc}.

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Keywords: TSE; CJD; Prion protein; Nanofiltration

1. Introduction

The safety of biopharmaceutical products used for human therapy has taken on the same importance as the therapeutic effects; this point was highlighted these last years by the contamination of children developing CJD after extractive growth hormone therapy using unsafe lots with respect to prion disease. More than [illegible] children died [illegible] are reported [illegible] clinical progres[illegible]

Products of human origin have [illegible] from therapeutic [illegible] but some [illegible] blood cells [illegible] as reagents [illegible] cation step [illegible] pharmaceutical [illegible] ity has never been [illegible] in human [illegible] process able [illegible] prion infe[illegible] same extent [illegible] transferr[illegible] (viruses, [illegible] could be of [illegible]

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