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 2002;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。
⑤	Vox Sanguinis 2000;78:86-95	Studies on the Removal of Abnormal Prion Protein by Processes Used in the Manufacture of Human Plasma Products	263Kに感染したハムスター脳の細胞内小器官の一つであるマイクロゾームのフラクションをパルスし、除去効果をウエスタンブロット法にて評価。グロブリンよりも下流の分画から感染性が証明されなかった。
⑥	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検討。
⑦	Transfusion Medicine 1998;38:810-816	The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy	プリオン感染マウス血液の各分画ごとに異常プリオンタンパクの感染性を評価。クリオプリシピテートが一番感染性が高い。免疫グロブリン分画でも感染性がある。
⑧	Vox Sanguinis 2006;91:292-300	Prion-removal capacity of chromatographic and ethanol precipitation steps used in the production of albumin and immunoglobulins	種々のクロマトグラフィーとコーンの分画法によるアルブミン製剤と免疫グロブリン製剤の製造工程における異常プリオンタンパク除去効率を検討し、それぞれ、3-5Log程度reductionするとされている。
⑨	Biologicals 2008;36:27-36	Prion removal by nanofiltration under different experimental conditions	異常プリオン株263Kを感染させたハムスター脳の細胞内小器官の一つであるマイクロゾームのフラクションを血液製剤にパルスしてプラノバによるプリオン除去能を評価した。35nm、20nm、15nmの各ポア（小孔）サイズのプラノバでプリオンの除去が可能であったが、15nmのものが除去効率が最も高く、WB法では3 Log前後の除去が可能であった。また、評価に用いるプリオンの性状は重要な要素であってプリオンの大きさを小さくするように調整することが必要である。

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。
⑫	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	Brithish 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	Brithish 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	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 20th 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 abnormal prion protein in the spleen of a haemophilic. This involves a patient exposed to a large number of potential vCJD infection routes (including multiple blood component transfusions, repeated receipt of UK-sourced fractionated plasma products including some units linked to a donor who later went on to develop clinical vCJD, and several invasive biopsies) who was found post mortem to have abnormal prion protein in a spleen sample.

2. If this finding is interpreted as an instance of asymptomatic vCJD infection, this raises questions as to the operational meaning of the "prevalence" of infection. The discovery of abnormal protein in a single spleen sample was the only positive result after exhaustive investigation of tissues taken at autopsy of an elderly haemophilia patient who died of other causes with no symptoms of vCJD or other neurological condition. All other tissues from this patient tested for the presence of abnormal prion protein – fixed samples of brain, heart, liver, blood vessel, appendix, spleen and lymph node and frozen samples of frontal lobe, occipital lobe, cerebellum, lymph node and 23 other samples from the spleen – were negative. This individual would not have tested "positive" on any of the vCJD prevalence tests conducted so far, and possibly not even in a post mortem spleen survey (depending on the size of spleen sample used). Nor do we know whether someone with this limited distribution of abnormal prion protein would be infective - and if so, by what routes of transmission.

3. For present purposes, however, these issues of interpretation are ignored. We simply assume that the abnormal prion protein found in this patient is a marker for asymptomatic vCJD infection: the task is then to investigate the relative likelihood of the infection having come from the various possible routes. This is done in order to inform discussion by the CJD Incidents Panel ("the Panel") as to the implications of the finding, and in particular whether the new evidence warrants any change to the "at risk" status of any individuals or groups.

4. The ideal would be to quantify these likelihoods in a robust way. However, this is not possible due to the multiple uncertainties involved. These are well-rehearsed. We do not know the prevalence of infectious donors – and in this instance, some of the potential routes are dependent on prevalence while others are not, so the relativities change. The probability of an infected blood component transmitting infection is uncertain - though on the precautionary approach adopted by the Panel, it is presumed to be substantial. The risks of an implicated plasma derivatives transmitting infection are even more uncertain. However, they can be estimated using methods suggested in an existing assessment by independent consultants DNV (DNV, 2003), which have been used in drawing up Panel recommendations to date. These calculations have also been regarded as "precautionary", i.e. giving a pessimistic view of the levels of infectivity likely to be present.

5. Given these unknowns, we make no attempt at definitive probability calculations, though illustrative examples are provided. Instead, we concentrate on the more limited task of determining whether different groups in the complex chain of contacts associated with the index patient can be robustly placed under or above

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.

3

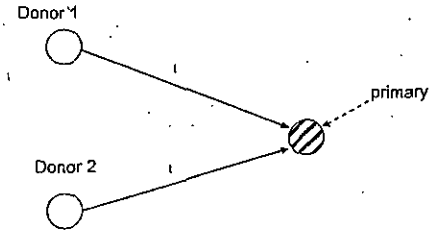
Method

7. The following analysis starts from the "reverse risk assessment" previously used by the Panel to assess the implied risks of donors to vCJD clinical cases being infected (DH, 2005a; Bennett, Dobra and Gronlund, 2006), and extends it to deal with this much more complex incident. We start with a simple example and then build up the analysis step-by-step. This is both to demonstrate how the conclusions are reached in this case, and to show how the same approach can be used to handle other complex incidents that may arise.

Example 1

8. We therefore start with a simple incident as shown in Figure 1(a). Here, a patient has received two single-unit Red Cell transfusions, one from each of two donors. The recipient goes on to develop vCJD, and the timing of the transfusions does not rule either of the donors out as the route of infection. What is the chance of 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 of transmission occurring *if* one of the donors were to be infected – i.e. the transmission probability, t. By definition, this lies between 0 and 1: if t = 1, transmission would be certain. In that case, and all else being equal[1], the patient's disease would be equally likely to have come from primary infection, or from either of the two donors having been infected. So by implication, each donor would have a 1 in 3 chance of being

[1] "All else being equal" essentially means that there is no prior reason to suppose that donors or recipient were particularly likely or unlikely to have been infected with vCJD, e.g. through "high risk" surgery, or conversely not having lived in the UK during years of high BSE exposure.

4

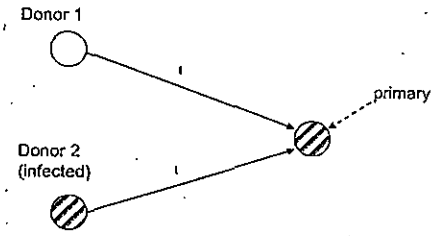
infective.[2] More generally, if there are n donors, the chance of each being infective would be 1/(n+1).

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

[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.

P(D2) be that of the infection having come via Donor 2

and P(prim) be the probability of the recipient having a primary infection

- For simplicity, suppose that the chance of the patient being infected by more than one route is negligible. Then (given that infection has occurred) P(D1), P(D2) and P(prim) must add up to 1.

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

12. Provided p is small (e.g. 1/4,000 or 1/10,000) and t is not, P(D2) will be *much* larger than either of the other two probabilities. To a very close approximation, P(D2) = 1 and P(D1) and P(prim) are zero. We can be virtually certain that the infection came from Donor 2. In practical terms, this new information about Donor 2 means that Donor 1 need not be considered as "at risk" according to CJD Incidents Panel criteria.

Example 3

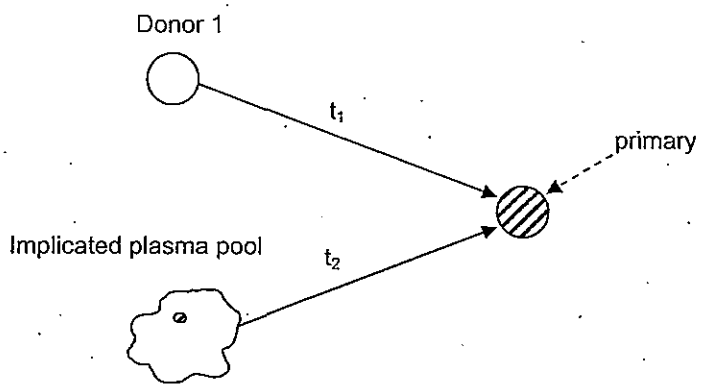
13. In the last two examples, the two secondary routes had the same transmission probability, t. But suppose now that there are routes with different values of t – e.g. transfusion of blood components and receipt of fractionated blood products. Figure 2 below shows a situation in which the calculations need to balance two contrasting secondary routes:

    - a blood component transfusion, associated with a high transmission probability (t_1) *if* the donor (D1) is infected, but with no reason to believe that this is the case, and
    - a plasma product pool with a contributing donor (D2) now known to be infected , but with a low transmission probability (t_2)

    As before, the three probabilities P(D1), P(D2) and P(prim) must add up to 1, and now:

    - P(D1) will be proportional to p and t_1
    - P(D2) will be proportional to t_2
    - and P(prim) will be proportional to p

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:

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

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)

7

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

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

Transmission risks from the endoscopies

17. vCJD transmission risks from endoscopy have been examined by an ACDP TSE WG subgroup, informed by an outline risk assessment. It is important to appreciate that these procedures involve a very small instrument (head) being passed down a very long, thin, channel. The possible "mechanics" of infection therefore differs from other surgical procedures. The group considered that any significant risk of onward transfer of infective material to a receptive site would require the procedure to be invasive, as distinct from examinations that involve the instrument sliding against the wall of the gut. On that argument, the relative risk from endoscopic procedures *not* involving biopsy would be negligible.

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

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

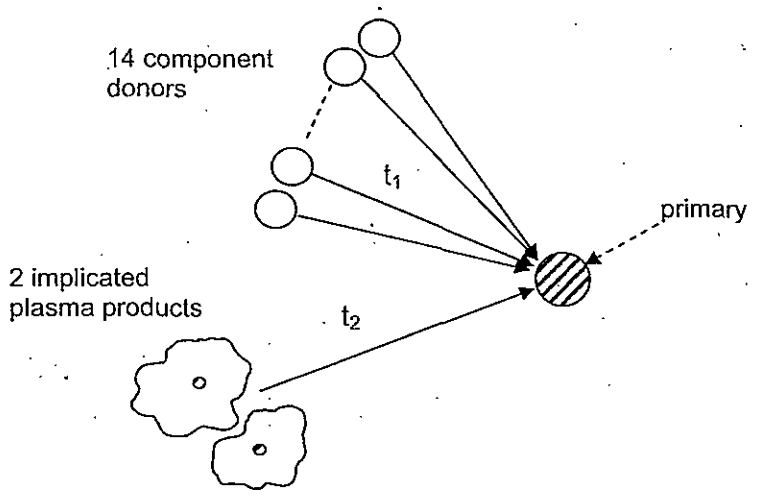
20. As will be seen below, the chance of this particular patient having been infected by the primary route are very small (in all scenarios) as compared to that of infection through a blood-borne route. On the above argument, the same applies to the endoscopic route. For simplicity, this route will therefore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

8

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

9

10,000 for the prevalence, p. The successive rows show the probability of infection having come from the implicated plasma products, from any *one* of the 14 component (Red Cell) donors, and from the primary outbreak. It can be seen that in all scenarios, the first route strongly dominates. Note that these are illustrative figures, using assumptions subject to much uncertainty. Nevertheless, they do suggest that the infection is much more likely to have come from the plasma products, with the implied risk to the component donors remaining clearly below 1%.

Table 1: Relative probabilities of potential infection routes (omitting "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	98%	97%	99%	99%
Probability of each of the 14 component donors	<0.3%	<0.3%	<0.1%	<0.1%
Probability primary	<0.3%	<0.3%	<0.1%	<0.1%

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

Implicated and "Non-implicated" plasma products

25. Although the above analysis provides some robust conclusions about the infection routes considered so far, the calculations ignore one further factor: the chance of the infection having come from the "non-implicated" plasma products – i.e. those manufactured from plasma pools not *known to have* an infected contributing donor. The problem here is that because the pool sizes are so large (of the order of 20,000 donations each), there is a high probability that many of them did, in fact, contain infective donors even if one has not been identified. Crudely, if the prevalence were 1 in 10,000, one would expect each pool to contain about 2 infected donations.[3]

26. This argument does not entirely remove the distinction between implicated and non-implicated pools. Where there is known to be an infected contributing donor (and nothing is known about the rest), the other donors to that pool also have the same probability p of being infected. So with a prevalence of 1 in 10,000 and typical pool sizes of 20,000, one would reasonably expect a "non-implicated" pool to contain 2 infected donations and an "implicated" pool to contain 3. Nevertheless, this is not a great differential. The calculation suggests that unless the prevalence of infection is very low - much lower than considered here, there is only a modest difference in the risks posed by receipt of implicated and non-implicated plasma. This observation supports the existing policy of considering recipients of UK-sourced plasma products as a group, rather than

[3] More strictly, the expected number of infected donations in each pool will be subject to a binomial distribution. However, the distribution is not essential to the argument, especially for patients receiving high volumes of product sourced from many different pools, when these statistical fluctuations will tend to even out.

10

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 risk to each of the 14 component (red cell) donors still applies, with even greater force. However, these results also highlight something of a paradox. Combined with the 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 definitely, and in any case raises issues beyond the scope of this paper, it is appropriate to check that the conclusions we have already suggested about relative likelihoods would not be overturned were we to assume lower levels of infectivity in plasma derivatives. The DNV report itself suggests two possible methods for calculating the infectivity present in each plasma derivative, using different assumption about the effect of the various manufacturing steps. In line with the generally precautionary approach adopted by CJD Incidents Panel, the calculations so far use figures based on the more pessimistic of these. The less pessimistic alternative suggested by DNV (using the "highest single clearance factor" in the manufacturing process) leads to an infectivity estimate for Factor VIII that is lower by a factor of 4. However, it should also be noted that risk assessments carried out elsewhere take the clearance factors achieved at different stages to be at least partly additive, which would lead to much smaller infective loads.

32. In fact, reducing the assumed infectivity *increases* the relative chance of infection via "non-implicated" as compared to "implicated" plasma. For example, suppose the presumed infectivity in all the Factor VIII received was reduced by a factor of 100 (2 logs). Modifying the calculations in paragraph 27, this patient would then have received an expected:

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

33. Albeit with the same caveats as before about using the linear model to quantify the cumulative risks from successive doses, this suggests that the latter risk would outweigh the former by a factor of 40. Table 3 shows how the previous results for this patient would change, under this revised infectivity scenario. As can be

[5] *Possible* explanations include the following: that prevalence of infection amongst donors is much lower than in the scenarios considered here; that much more infectivity is removed during processing of plasma products than suggested by the DNV analysis; and/or there is a threshold dose-response effect and most recipients fall below this. Genotype effects may also be relevant (in providing resistance to infection or extending the time to clinical disease), but one would expect a substantial proportion of this group to be MM homozygotes – the most susceptible genotype.

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

Table 3: 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 primary	<0.09%	<0.09%	<0.09%	<0.09%
Probability non-implicated plasma products	97%	97%	97%	96%

Note: these are 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 DNV Risk Calculation to Factor VIII Units

(a) Implicated Donations

Key points: FHB4547

- There was one implicated (presumed infective) donation in a start pool of 26,303 donations (pool size supplied by Professor Frank Hill via email)
- Factor VIII is derived from cryoprecipitate, which has an estimated infectivity of 60 ID_{50}s / donation of infected whole blood according to the DNV model
- 70.45kg of cryoprecipitate was made from the start pool, of which 21.58kg was used in the FHB4547 batch
- This implies that (21.58kg / 70.45kg) of the 60 ID_{50}s made its way into the FHB4547 batch (18.38 ID_{50}s)
- 1,844 vials each of 500 units (iu) were made from the batch, which results in an estimate of 0.00997 ID_{50}s per vial or 1.99×10^{-5} ID50s per iu

Professor Frank Hill's report indicates that the index case received 8,025 units from this batch, giving an estimated 0.16 ID_{50} from the implicated donation.

Key points: FHC4237

- There was one implicated (presumed infective) donation in a pool of 21,330 donations (pool size again supplied by Professor Frank Hill)
- Factor VIII is derived from cryoprecipitate, which has an estimated infectivity of 60 ID_{50} / donation of whole blood
- 67.6kg of cryoprecipitate was made from the start pool, of which all was used in the FHC4237 batch
- This implies that the full dose of 60 ID_{50} made its way into the FHC4237 batch
- 5,074 vials each of 250 iu were made from the batch, resulting in an estimate of 0.0118 ID_{50} per vial or 4.73×10^{-5} ID_{50} per iu

Professor Frank Hill's report indicates that the index case received 1,000 units from this batch, giving an estimated dose of 0.05 ID_{50}.

Conclusion

In total, these calculations suggest that index case would have received an estimated 0.21 ID_{50} from the "implicated" donor. Using a linear dose-response model (where 1 ID_{50} translates into a transmission probability of 0.5 and 2 ID_{50} or more translates into transmission probability of 1) this represents a transmission probability of 0.104 or 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}.

15

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ORIGINAL PAPER

Distribution of a bovine spongiform encephalopathy-derived agent over ion-exchange chromatography used in the preparation of concentrates of fibrinogen and factor VIII

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Background and Objectives The risk of haemophiliacs contracting variant Creutzfeldt-Jakob disease (vCJD) via treatment with factor VIII concentrates is not known. Therefore, in order to determine the extent to which the vCJD agent might be removed during the preparation of factor VIII concentrate, the partitioning of a bovine spongiform encephalopathy (BSE)-derived agent was measured over the main purification step used to prepare the Scottish National Blood Transfusion Service high-purity factor VIII concentrate (Liberate®).

Materials and Methods Murine-passaged BSE (strain 301V), in the form of a microsomal fraction prepared from infected brain, was used to 'spike' a solution of factor VIII of intermediate purity. The 'spiked' starting material was subjected to solvent-detergent treatment and then to anion-exchange chromatography with Toyopearl DEAE-650M. All fractions were tested for 301V infectivity using a murine bioassay, including the procedures used to clean the ion-exchange media after use.

Results BSE 301V infectivity was reduced by 2·9 $\log_{10}$ in the fibrinogen fraction and by 2·7 $\log_{10}$ in the factor VIII fraction. Over 99% of the added 301V infectivity remained bound to the ion-exchange column after elution of factor VIII. A large quantity of infectivity was subsequently removed by washing the ion-exchange media with 2 M NaCl. No further BSE 301V infectivity was detected in column eluates after treatment with 0·1 M NaOH or a second wash with 2 M NaCl.

Conclusions Results using a BSE-derived agent suggest that vCJD infectivity would be substantially removed by the ion-exchange process used in the preparation of fibrinogen and factor VIII concentrate. Although 301V infectivity remained bound to the ion-exchange matrix following elution of factor VIII, this appeared to be eliminated by the procedure used for cleaning the ion-exchange media after each use.

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Key words: Creutzfeldt–Jakob disease, chromatography, factor VIII, fibrinogen.

Introduction

Variant Creutzfeldt–Jakob disease (vCJD) is an incurable, fatal, neurodegenerative disorder of transmissible spongiform encephalopathy (TSE), of which there have been 150 confirmed or probable cases diagnosed since the condition was first reported [1]; 143 of these 150 subjects were resident in the UK at some time. Evidence that vCJD is caused by the TSE agent responsible for bovine spongiform encephalopathy (BSE) in cattle is convincing [2], with dietary exposure being the most probable route of transmission [3]. BSE originated in the UK [4] and has now been detected in cattle in 25 different countries, although 98% of all cases found

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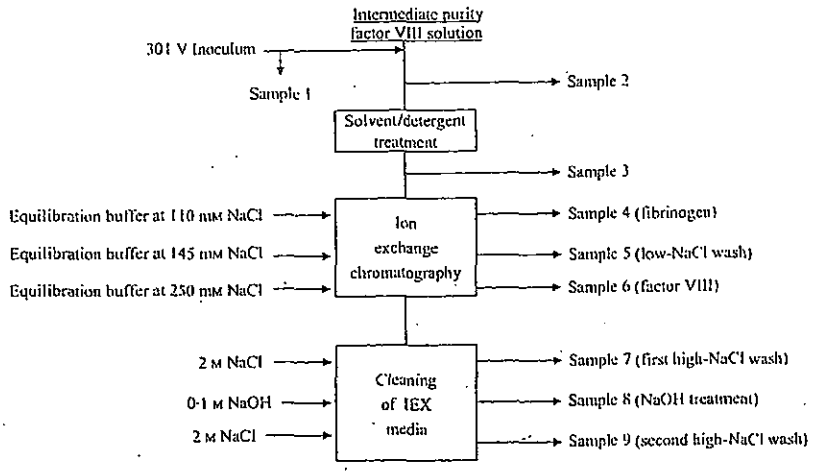

Fig. 1 Flow diagram of the processes over which partitioning of bovine spongiform encephalopathy (BSE) 301V infectivity was measured. IEX, ion-exchange chromatography.

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 48 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

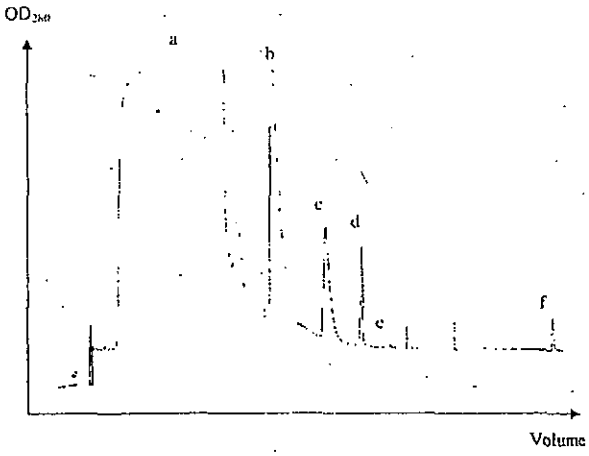

Fig. 2 Optical density of fractions eluted during ion-exchange chromatography of intermediate-purity factor VIII to which the bovine spongiform encephalopathy (BSE) 301V microsomal inoculum had been added. (a) Fibrinogen fraction (110 mM NaCl); (b) low-NaCl wash (145 mM NaCl); (c) factor VIII fraction (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).

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.

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 across different fractions collected during the purification of fibrinogen and factor VIII by ion-exchange chromatography

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

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

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

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

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

Discussion

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

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

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

Conclusions

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

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Removal of TSE agents from blood products

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Introduction

Transmissible Spongiform Encephalopathies (TSEs) are fatal neuro-degenerative disorders. Creutzfeldt-Jakob disease (CJD) in humans is divided into classical CJD (cCJD), of which there are a number of forms (sporadic, familial, Gerstmann-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 agents are not straightforward. Infectivity in blood is very low and the characteristics of the agent as it exists naturally in blood are not defined. Different experimental approaches have been adopted and it is necessary to appreciate their limitations.

Process scale-down

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

Form of TSE agent

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

Strain of TSE agent

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

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

Determination of TSE agents

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

Studies on individual process steps

Leucocyte-filtration

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

Protein precipitation

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

Depth filtration

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

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

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

Studies on process steps in sequence

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

Conclusions

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

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REVIEW ARTICLE

Factor VIII and transmissible spongiform encephalopathy: the case for safety

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

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

Haemophilia and replacement therapy

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

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

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

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

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

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

Transmissible spongiform encephalopathies or prion diseases

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

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

Prions as transmissible agents of TSEs

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

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

TSEs and blood safety

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

Experimental blood-related transmission studies

Animal-to-animal transmission

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

Table 1. Transmission studies to detect infectivity in the blood of animals with TSE.

Donor species	Recipient species	Inoculum[1]	Route of inoculation	Positive/total donors
Scrapie (natural)				
Goat	Mouse	Blood clot/serum	i.c.	0/3
Sheep	Mouse	Blood clot/serum	i.c.	0/18
BSE (natural)				
Cow	Mouse	Blood clot/serum	i.c. + i.p.	0/2
Cow	Mouse	Buffy coat	i.c. + i.p.	0/2
Scrapie (experimental)				
Goat	Goat	Whole blood	i.c.	0/14
Mouse	Mouse	Whole blood	i.c.	0/39
Goat	Mouse	Blood clot	i.c. or s.c.	0/20
Sheep	Mouse	Serum	i.c.	1/1
Rat	Rat	Serum	i.c.	1/1 (pool)
Mouse	Mouse	Serum	i.c.	1/1 (pool)
Mouse	Mouse	Whole blood	i.c.	3/13
Hamster	Hamster	Whole blood	i.c.	0/9
Hamster	Hamster	Blood extract	i.c.	5/5 (pools)
Hamster	Hamster	Blood extract	i.c.	10/11 (pools)
Hamster	Hamster[2]	All blood components	i.c.	1/1 (large pool)
		Whole blood	i.c.	25–50%
		Whole blood	i.v.	< 1%
Mink encephalopathy (experimental)				
Mink	Mink	Serum	i.c.	0/1
Mink	Mink	Whole blood, plasma, red cells, white cells, platelets	i.c.	0/8 (pools)
BSE (experimental)				
Cow	Mouse	Buffy coat	i.c. + i.p.	0/11 (pools)
	Cow[2]	Buffy coat	i.c.	0/4 (pools)
Mouse	Mouse	Plasma	i.c.	4/48
Sheep	Sheep[2]	Whole blood	i.v.	1/19
CJD (experimental)				
Guinea pig	Guinea pig	Buffy coat	i.c., s.c., i.m., i.p.	10/28
GSS (experimental)				
Mouse	Mouse	Buffy coat	ip	4/7 (pools)
Mouse	Mouse	Buffy coat/plasma	i.c.	5/5 (pools)
		Buffy coat/plasma	i.v.	2/2 (pools)
		Whole blood	i.v.	1/20[3]

[1]In several of the studies, assays were conducted on serial specimens obtained during both the incubation and clinical phases of disease. [2]Ongoing experiments. Citations for the original studies can be found in [76]. [3]Brown P & Cervenakova L, unpublished data. i.c., intracerebral; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous.

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 the studies.

In contrast to the negative results observed in most transmission studies using human blood and 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 for the studies (Table 1). Infectivity has been found in blood and blood components during both the incubation phase of the disease and in clinically ill animals [59–71]. More recent studies employing large numbers of animals have reported extremely low levels of infectivity in blood, and blood components of mice < 100 infectious U mL^{-1}, that is 10^4 times less than in the brain) infected with mouse-adapted GSS strain of human TSEs [66,67] and hamsters infected with hamster-adapted 263K strain of scrapie [68–70]. The intravenous route of

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

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

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

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

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

Human-to-animal transmission

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

Epidemiological blood-related Creutzfeldt–Jakob disease transmission studies

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

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

[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 identified case of vCJD, the CJD Surveillance Unit in the UK has identified several vCJD patients who received blood transfusions but none has been linked to a donor who later developed CJD or vCJD. Of 14 vCJD blood donors, eight were traced and had donated blood to 22 recipients. Transfused blood components included whole blood (two recipients), red blood cells (13 recipients), buffy-coat reduced red cells (two recipients), fresh-frozen plasma (three recipients), and cryo-depleted plasma and cryoprecipitate (one recipient for each component). Plasma donations from eight donors had entered plasma pools for the manufacturing of therapeutic proteins that were distributed to thousands of donors [personal communication from R. Will]. At least four blood donors who have developed vCJD in the UK have contributed to pools of plasma used to treat patients with haemophilia A and B. As a result there were two recalls of product in 1997 and 2000 [C. A. Lee, unpublished data].

Developments in diagnostic screening tests for Creutzfeldt–Jakob disease

Most of the uncertainties about the risk of transmission of CJD through blood or plasma-derived products could be resolved by an accurate test for the early detection of infected individuals or identification of contaminated blood or plasma pools, but abnormal prions have never been detected in blood of presymptomatic or symptomatic individuals, and the level of normal PrP in plasma of CJD patients is no greater than in patients with other neurological diseases or healthy control individuals [90]. Until recently, other body fluids (except the cerebrospinal fluid) have not been used for detection of PrP, but a promising discovery has recently been reported by Shaked *et al.* [91] who detected the presence of abnormal PrP in the urine of experimental hamsters infected with prions long before the appearance of clinical signs. Abnormal PrP was also detected in the urine of cattle with BSE and in symptomatic humans afflicted with a genetic form of CJD. The value of this technique as a diagnostic tool will need to be validated by other independent laboratories. Another encouraging observation was recently made [92] by the discovery that levels of an erythroid differentiation-related factor (EDRF) transcript was decreased in the spleens of scrapie-infected mice in both the preclinical and clinical phase of the disease and in the blood of terminally ill animals. A significant decrease in the expression level was also observed in the bone marrow of cattle with early

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

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 0.86% in cryoprecipitate and fraction I + II + III. Table 3 shows the efficiency of TSE infectivity and prion removal by various steps used for manufacture of FVIII. Lee *et al.* [94,105] performed validation studies of certain plasma-purification steps used by Bayer for the manufacture of plasma-derived therapeutic proteins. Their principle purification steps for manufacture of FVIII (Koate DVI) employ multiple precipitations and size exclusion chromatography [106]. Two validated manufacture steps, cryo-precipitation and PEG precipitation, together removed 2.2 $\log_{10}$ ID_{50} from FVIII [105]. Foster [107] calculated the cumulative removal efficiency of 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 4 $\log_{10}$ ID_{50} reduction of TSE infectivity during the manufacturing of the FVIII concentrate, Liberate® was shown. In a subsequent large experimental study Foster *et al.* [108] showed a 6.8 $\log_{10}$ cumulative reduction of abnormal PrP by four main steps (cryoprecipitation, precipitation and adsorption of cryoprecipitate extract, ion exchange chromatography and membrane filtration) used in the production of FVIII.

Some of these same issues have been addressed in an extensive study conducted recently by Aventis Behring [109]. The company uses multiple precipitation steps during the manufacture of Haemate P (equivalent to Haemate HS), and ion-exchange chromatography for the manufacture of Beriate P (equivalent to Beriate HS) [106]. The study evaluated the efficiency of TSE removal by various precipitation steps, using four different spikes prepared from the brain tissue of hamsters infected with Sc237 strain of scrapie, and compared the partitioning of various prion strains, including vCJD. The question of proper spiking material for inactivation/removal experiments is important because the physico-chemical state of the agent, should one exist in human blood, is not known. Removal of less than 1 $\log_{10}$ of PrP was achieved by cryoprecipitation and a precipitation step utilizing 8% ethanol when plasma was spiked with any of three different materials, brain homogenate, microsomal membranes and caveolae-like domains. However, significantly more removal was achieved for cryoprecipitate (2.4 $\log_{10}$) and 8% ethanol precipitate (3.1 $\log_{10}$) when purified abnormal PrP was used as a 'spike' [109].

Removal of TSE infectivity has also been documented in a validation study of the American Red Cross/Baxter manufacturing process used for preparation of coagulation factor VIII concentrates (Red Cross'

Table 3. Efficiency of prion protein and/or TSE infectivity removal by various steps used in manufacture of plasma-derived factor VIII.

Validated manufacturing steps	Spiking material: hamster scrapie[1]	Detection method	PrP^{Sc} or infectivity reduction factor	Company
Cryoprecipitation, precipitation and adsorption, SD treatment and ion-exchange chromatography, membrane filtration	263K: microsomal fraction	Western blot	6.8[3] $\log_{10}$	SNBTS Protein Fractionation Centre [108]
Cryoprecipitation and cryoprecipitate/PEG separation	263K: brain homogenate	Western blot Bioassay	2.2[3] $\log_{10}$ mL^{-1} 3.0 log LD_{50} mL^{-1}	Bayer [105]
Cryoprecipitation	Sc237: brain homogenate, microsomal fraction, CLDs[2] Sc237: Purified PrP^{Sc}	Conformation-dependent immunoassay	< 1 $\log_{10}$ 2.4 $\log_{10}$	Aventis-Behring [109]
Ethanol precipitation 8%	Sc237: Brain homogenate, microsomal fraction, CLDs Sc237: Purified PrP^{Sc}		< 1 $\log_{10}$ 3.1 $\log_{10}$	
FVIII immunoaffinity column ion-exchange chromatography [109]	263K: Brain homogenate	Bioassay	4.57 log LD_{50} mL^{-1} 3.47 log LD_{50} mL^{-1}	American Red Cross/Baxter [110]

[1]Hamster-adapted scrapie (263K or Sc237); [2]CLD, caveola-like domains; [3]sum of all steps tested.

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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Studies on the Removal of Abnormal Prion Protein by Processes Used in the Manufacture 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 albumin, immunoglobulins, factor-VIII concentrate, f tor-IX concentrates, fibrinogen and thrombin all cont steps which may be capable of removing causal agents of human TSEs.

Introduction

The fatal neurodegenerative disorder Creutzfeldt-Ja disease (CJD) has been transmitted iatrogenically vi number of routes [1] suggesting the possibility that causative agent might also be transmissible by blood pr ucts [2]. The identification of a new form of human tra missible spongiform encephalopathy (TSE), 'new varia CJD (vCJD) [3, 4], confirmation of an association with agent of bovine spongiform encephalopathy (BSE) [5 and evidence that the distribution of the agent of vCJ human tissues may differ from that of classical CJD [7 led to increased concern that vCJD may be transmissi by plasma products [9]. Consequently, as a precaution measure, the UK government decided to ban the fractio tion of plasma donated in the UK [10] and replaced with plasma purchased outside the UK. Despite this portation of plasma, the risk of transmission of vCJD mains to be defined for those patients who previously been treated with plasma products derived from donors.

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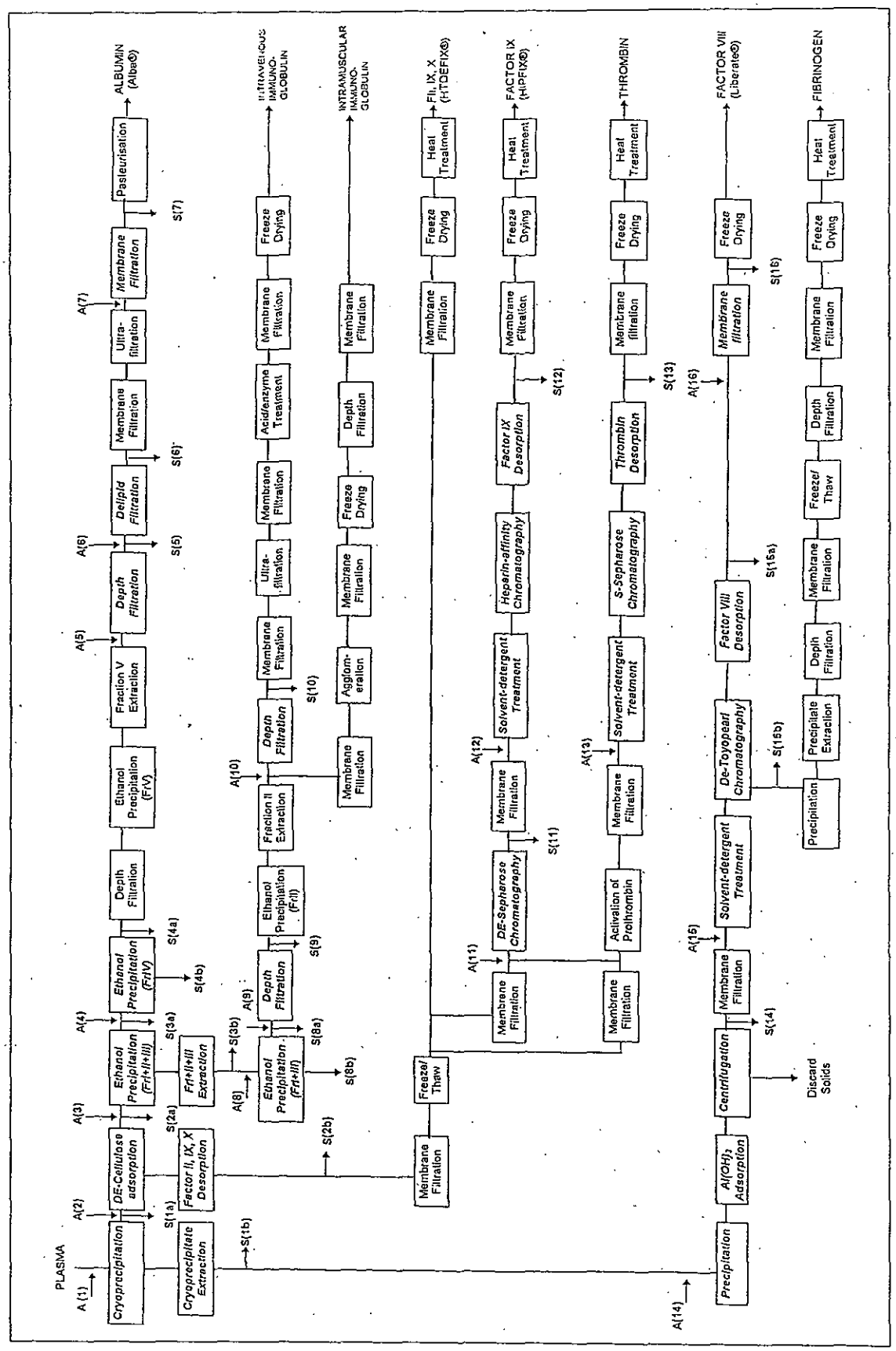
PLASMA
A(1)
Cryoprecipitation
A(2)
DE-Cellulose adsorption
A(3)
Ethanol Precipitation (Frl+II+III)
A(4)
Ethanol Precipitation (FrIV)
Depth Filtration
Ethanol Precipitation (FrV)
Fraction V Extraction
A(5)
Depth Filtration
A(6)
Delipid Filtration
Membrane Filtration
Ultra-filtration
A(7)
Membrane Filtration
Pasteurisation
ALBUMIN (Albaⓡ)
S(7)
S(6)
S(5)
S(4a)
S(4b)
S(1a)
Cryoprecipitate Extraction
S(1b)
Factor II, IX, X Desorption
S(2a)
S(2b)
Frl+II+III Extraction
S(3a)
S(3b)
A(8)
Ethanol Precipitation (Frl+II)
S(8a)
S(8b)
A(9)
Depth Filtration
S(9)
Ethanol Precipitation (FrI)
Fraction II Extraction
A(10)
Depth Filtration
Membrane Filtration
Ultra-filtration
Membrane Filtration
Acidenzyme Treatment
Membrane Filtration
Freeze Drying
S(10)
Agglom-eration
Membrane Filtration
Freeze Drying
Depth Filtration
Membrane Filtration
INTRAMUSCULAR IMMUNO-GLOBULIN
Membrane Filtration
Freeze Drying
Heat Treatment
Fll, IX, X (HTDEFIX®)
Membrane Filtration
Freeze Drying
Heat Treatment
FACTOR IX (HPFIX®)
S(12)
A(12)
Solvent-detergent Treatment
Heparin-affinity Chromatography
Factor IX Desorption
Membrane Filtration
S(11)
A(11)
DE-Sepharose Chromatography
Membrane Filtration
A(13)
Solvent-detergent Treatment
S-Sepharose Chromatography
Thrombin Desorption
Membrane filtration
Freeze Drying
Heat Treatment
THROMBIN
S(13)
Membrane Filtration
Activation of Prothrombin
Membrane Filtration
A(16)
Membrane filtration
Freeze Drying
FACTOR VIII (Liberateⓡ)
S(16)
A(15)
Solvent-detergent Treatment
Factor VIII Desorption
S(15a)
De-Toyopearl Chromatography
S(15b)
Membrane Filtration
Centrifugation
S(14)
Discard Solids
Precipitation
Depth Filtration
Precipitate Extraction
Precipitation
Membrane Filtration
Freeze/ Thaw
Depth Filtration
Membrane Filtration
Freeze Drying
Heat Treatment
FIBRINOGEN
A(14)
Al(OH)3 Adsorption
Membrane Filtration
Freeze/ Thaw

phosphate (5 m*M*) at pH 6.2. Factor II, IX and X solution (210 ml) was applied to the column, which was then treated with 150 ml wash buffer, all at a flow rate of 8.4 ml/min, followed by 10 ml of 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 7.8 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 26/20, Pharmacia) using 20 m*M* citrate. The solvent-detergent (SD)-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 + 200 m*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*), all 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 (500 m*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 48 g of frozen washed cryoprecipitate in 20 m*M* Tris (168 ml) at 20 °C. The pH of the extract was adjusted to 6.7 and zinc precipitant (zinc acetate + sodium chloride + trisodium citrate + heparin) added to obtain final 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-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.

SD Treatment and Ion Exchange Chromatography for Factor VIII and Fibrinogen (Step 15)

Microsomal inoculum (10 ml) was added to a solution of fact VIII (104 ml) of intermediate purity, containing 20 m*M* trisodium c rate, 2.5 m*M* calcium chloride, 109 m*M* sodium chloride, 4.5% w sucrose, 0.3% tri(n-butyl)phosphate and 1% Tween-80. The mixtu was incubated with stirring at 25 °C for 17 h prior to purification factor VIII by ion exchange chromatography [27]. 14 ml DEA Toyopearl 650M (TosoHaas GmbH, Stuttgart, Germany) was pack into a 10-mm diameter column (C10/20, Pharmacia) using 2 *M* soc um chloride and equilibrated with 110 ml of buffer containing 1 m*M* glycine, 16 m*M* lysine, 10 m*M* trisodium citrate, 1 m*M* calciu chloride and 110 m*M* sodium chloride at pH 7.0. The SD-treated fa tor VIII solution was applied to the column followed by 30 ml equi bration buffer and 45 ml equilibration buffer raised to 145 m*M* soc um chloride, all at a flow rate of 1.3 ml/min. Factor VIII was th eluted using 26 ml of equilibration buffer raised to 250 m*M* sodiu chloride, at a flow rate of 0.8 ml/min. A sample was also taken of t flowthrough (unadsorbed fraction) from the starting material, as th fraction is used in the manufacture of fibrinogen (fig. 1).

Membrane Filtration of Factor VIII (Step 16)

Microsomal inoculum (9.7 ml) was added to factor VIII solutic (105.4 ml) with a total protein content of 0.43 g/l, which had been fo mulated in elution buffer (step 15) plus 0.1% w/v sucrose. Two mer brane filters (47-mm discs, Durapore 0.45 µm and 0.22 µm, Mill pore) were assembled in series and primed with 12 ml formulatic buffer, prior to filtration of factor VIII solution at a rate of 6.4 ml/mi Filtration was halted, due to blockage of the filters, after 40 ml of t 'spiked' factor VIII solution had been processed. The analysis of th step was therefore based on the 40 ml of starting material that was a tually filtered.

All samples taken were adjusted to approximately pH 7.0, if ne essary, and store at ≤ –70 °C pending analysis. Negative control san ples consisted of samples of each starting material taken prior to t addition of microsomal inoculum and samples of fractions obtaine from equivalent processing carried out without addition of micros mal inoculum.

Western Blot Determination of PrP^{Sc}

In order to reduce the background signals in the Western blots, a process samples were ultracentrifuged to pellet the PrP^{Sc} fibrils, fo lowed by re-suspension in PBS. For Western blot analysis, two 50-µ aliquots of each sample were incubated at 37 °C for 1 h, one in t presence of protease-K (1 µl ; 50 µg/ml) and the other serving as a undigested control. After the addition of an equal volume of SD boiling mix (10% SDS; 50 m*M* Tris/HCl, pH 6.5; containing β-me captoethanol), antigens were denatured by incubation in boiling wat for 3 min. 30 µl of each sample was then loaded onto a Tris-base 12% polyacrylamide gel (Bio-Rad), and the gels electrophoresed 150 V until the blue marker was ~1 cm from the bottom of the ge Pre-stained molecular weight standards were run on the gel to facil tate accurate identification of the size of the immunolabelled band Proteins were transferred to Imobilon-P using a semi-dry blotting pr cedure, and the membranes washed in TTBS (25 m*M* Tris-HCl, pl 7.6, 0.05% Tween 20 and 0.5 m*M* NaCl) for approximately 10 mi followed by blocking with TTBS containing 5% skimmed milk pow der (Marvel) for approximately 1 h. PrP protein was detected usin the monoclonal antibody 3F4 specific for hamster PrP [28]. This anti body (supplied by Senetek PLC) reacts with residues 109–112 Pr

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	R
1	Cryoprecipitation	1.7	1.0	1.7	1.
14	Zinc precipitation + Al $(OH)_3$ adsorption	2.0	1.7	2.0	1.
15	SD[d] + DEAE Tyopearl 650 M chromatography	3.8	3.1	≥4.1	≥
16	Membrane filtration (0.45 µm/0.22 µm)	1.6	1.0	n/d[e]	n/

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

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

	Process step[a]	Factor IX		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	<
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	supernata
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 chro matographic matrices following product elution.

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

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

Little information is available on the behaviour of TSE agents in chromatographic separations currently used in plasma fractionation. Drohan [34], in a study of factor VIII processing, has reported $\log_{10}$ RFs of 4.4 and 6.3 for immunoaffinity and ion exchange chromatography, respectively, using a 10% brain homogenate of hamster-adapted scrapie as the inoculum and with infectivity determined by bioassay. Additional chromatographic data are available from a variety of different bio-process industries [13, 14, 16, 39, 40] with $\log_{10}$ RFs ranging from 2.2 to 5.5. Our results on ion exchange are within this range, with essentially no difference being observed between anion exchange and cation exchange or between different ion exchange matrices (table 3). The somewhat smaller degree of PrP^{Sc} reduction observed over heparin-affinity chromatography (table 3; step 12) may have been due to a smaller charge difference, together with the relatively high concentration of sodi chloride (i.e. 500 m*M*) at product elution. The probabi that most PrP^{Sc} remained bound to chromatographic ma ces emphasises the importance of either limiting the re- of adsorbents or in developing suitable cleaning procedu

The observation that a high degree of PrP^{Sc} removal be obtained by depth filtration (table 1) may be the most i portant finding from our study, as depth filters are stand items used in most albumin and immunoglobulin process In addition, the low cost of filter media means that fil pads are normally disposed of after each use, thereby avo ing the possibility that subsequent product batches could exposed to any TSE infectivity which might have been sorbed.

Conclusions

The data obtained from our study suggest that if vC infectivity was present in plasma pools obtained from U donors, then the quantity of abnormal prion present wo have been reduced substantially during the preparation each of the plasma products manufactured by SNB Whether or not these processes would have been capable completely removing all vCJD infectivity is not known, this may have been dependent on the quantity of vCJD fectivity actually present as well as on the accuracy of experimental models from which our data were obtain Further studies are required to address these points:

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

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

The abnormal prion protein which is associated with transmissible spongiform encephalopathies (TSEs), such as CJD, has a low solubility, a high tendency to form aggregates and adheres to surfaces readily. If the physicochemical properties of the agent of nvCJD are similar to those of abnormal prion protein then nvCJD may be removed by precipitation and adsorption technologies used in plasma fractionation.

Available data on the removal of TSE agents by such bioprocess technologies have been used to estimate the potential degree of reduction expected from each step in the plasma fractionation processes used by the SNBTS. The overall process reduction factors estimated are: 10^{13} (albumin), 10^{9} (immunoglobulins), 10^{7} (factor IX, thronibin), 10^{5} (fibrinogen), 10^{4} (factor VIII) and 10^{3} (factor II, IX and X); however, it will be necessary to establish the accuracy of these estimates by practical validation studies.

Key words: Creutzfeldt–Jakob disease, new variant CJD, partitioning of CJD/scrapie, plasma fractionation, plasma products.

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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Transmission of CJD by human growth hormone derived from human pituitary glands and by other medical procedures (Brown *et al.*, 1992) has led to concerns that BSE could be transmitted by medicinal products manufactured using bovine substances (CPMP, 1992) and that CJD may be transmissible by products derived from infected blood donors (Esmonde *et al.*, 1993).

A number of studies have been undertaken to determine whether or not CJD has been transmitted by blood or plasma products, with no evidence of transmission being found to date (Brown, 1995; Ricketts *et al.*, 1997; Evatt *et al.*, 1998; Evatt, 1998). This apparent absence of CJD transmission could be due to the disease not being transmissible by blood or blood products in clinical practice, the low prevalence of the disease in the blood donor population, the absence or low concentration of the CJD agent in plasma for fractionation, the removal of the causative agent of CJD by the processes used to manufacture human plasma products or to a long incubation period for the disease in recipients.

Because of uncertainty over the safety of plasma

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 yet been described. In the absence of comprehens measurements of TSE agent partitioning across plas fractionation processes, the behaviour of nvCJD can estimated only by extrapolation of data obtained fr similar biopharmaceutical process operations. A pro sional assessment of how TSE agents might be expec to partition during plasma fractionation has been ma on this basis.

PLASMA FRACTIONATION

The Scottish National Blood Transfusion Servi (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). Ea process includes a number of steps in which maci molecular constituents are preferentially removed; the steps are summarized below on a product-by-produ basis.

Albumin (Alba®)

The SNBTS process for the manufacture of albun involves removal by centrifugation of the precipita which forms when the frozen donations of plasma a thawed (cryoprecipitate), removal by centrifugation 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 mix bed of cellulose, kieselguhr and perlite at two stage depth filtration through a mixed bed filter incorporati a cation exchange resin and membrane filtration three different stages of the process, two of whi employ a cellulose acetate membrane. The final produ is pasteurized at 60 °C for 10 h to inactivate potenti viral contaminants.

Immunoglobulins

Similar purification procedures are used in the man facture of immunoglobulin products. Following t removal of cryoprecipitate and the recovery of fracti I + II + III, the resuspended fraction I + II + III adjusted (8 or 12% ethanol, pH 5·1, −3 °C) to precipita fraction I + III, which is removed by centrifugation, t supernatant being clarified by borosilicate glass dep filtration. The IgG solution is subsequently subjected to mixed bed depth filtration (cellulose, kieselguhr ar perlite) and to membrane filtration at three differe stages of manufacture, two of which employ a cellulo acetate or similar membrane.

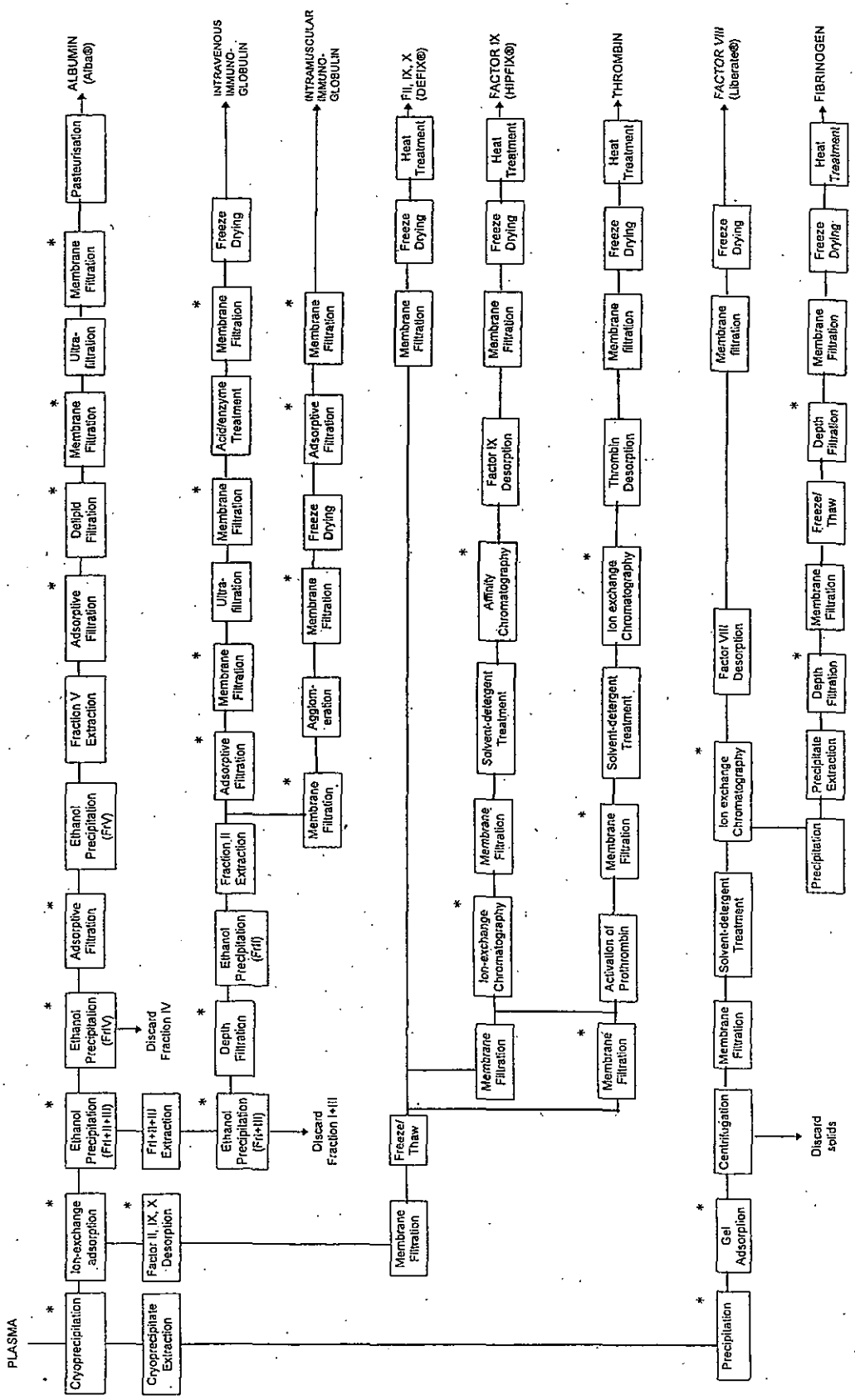

Fig. 1. SNBTS process for the fractionation of human plasma. *Denotes step estimated to have potential for TSE removal or reduction.

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 solution, in this instance by cation exchange chromatography, with a total of six separate membrane filtration steps (two 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 know the causative agents are generally believed to posse similar physicochemical properties (Groschup *et a* 1997) and to consist of a conformationally altered for of cellular prion protein (PrP^{c}), referred to as abnorm prion protein (e.g. PrP^{Sc}). Whether or not PrP^{Sc} is itse the causative agent of disease is not known; howeve removal of PrP^{Sc} is generally associated with removal infectivity (Farquhar *et al.*, 1998).

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

Therefore, it can be postulated that certain biosepar tion technologies that are used in the preparation plasma products, such as precipitation, adsorption an filtration, may well be capable of removing significa quantities of the abnormal prion protein associated wi nvCJD. Indeed, the potential of these technologies fo the removal of TSE agents has been identified previousl in guidelines concerning the preparation of medicin products (CPMP, 1992).

Measurement of TSE agent partitioning

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

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

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 into the earlier fractions in a cold-ethanol (Cohn) fractionation scheme. Similar observations were reported by Petteway *et al.* (1998) who observed a 10-fold reduction of PrP^{Sc} (263K) in the supernatant following a Cohn fraction I precipitation, a $\geq 3\times10^2$-fold reduction over a Cohn fraction III precipitation and $\geq 3\times10^2$-fold reduction across a Cohn fraction IV precipitation.

Iso-electric precipitation in the presence of ethanol was used as an early purification step in the manufacture of human growth hormone where, in a scrapie clearance study, mouse adapted PrP^{Sc} (strain ME7) in the supernatant was reduced from $3\times10^7\,ID_{50}\,mL^{-1}$ to $\leq 3\,ID_{50}\,mL^{-1}$ after a clarifying precipitation at pH 6 followed by ethanol precipitation (10% ethanol, pH 4·8) of the growth hormone (Taylor *et al.*, 1985). As the growth hormone was precipitated, any CJD infectivity present would have been expected to copurify with the product at this stage of manufacture.

Other solubility methods. Petteway *et al.* (1998), using an immunochemical method of analysis, have reported a 3×10^2-fold reduction of PrP^{Sc} (263K) in a precipitation step used in the preparation of a factor VIII concentrate from cryoprecipitate.

PEG precipitation has also been the subject of a scrapie clearance study, with an overall $\geq 4\times10^5$-fold reduction of PrP^{Sc} (ME7) infectivity being measured following filtration of the supernatant which remained after a light surface phase and two PEG precipitates had been discarded (M. Macnaughton and A. Shepherd, personal communication, April 1997).

In the preparation of aprotinin from bovine lung, Golker *et al.* (1996) studied the distribution of PrP^{Sc} (ME7) infectivity during a water/salt/methanol extraction of lung tissue. The concentration of PrP^{Sc} in the extract was reduced from $10^7\,ID_{50}\,mL^{-1}$ to $\leq 50\,ID_{50}\,mL^{-1}$ (mean of two runs), giving a mean reduction factor across the methanol extraction process of $\geq 2{\cdot}5\times10^4$.

Adsorption/desorption technology

As abnormal prion proteins are strongly membrane bound (Stahl *et al.*, 1990), possess hydrophobic and hydrophilic domains (Bolton *et al.*, 1987) and tend to adhere to surfaces, it is probable that they will interact with chromatographic (Foster, 1994; Burnouf, 1995) and filtration (Meltzer, 1987) media used in plasma fractionation. In these circumstances, a high degree of separation from abnormal prion protein may be possible where a plasma protein does not adsorb to a given matrix. Where a plasma protein is adsorbed, separation from abnormal prion protein will be dependent on the relative strength of binding of each of the macromolecular components.

The separation of PrP^{Sc} infectivity from a variety of

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	Recovered fraction	Estimated TSE agent reduction factor
Precipitation		
Cryoprecipitation	Supernatant	10^1
Cohn fraction I	Supernatant	10^1
Other Cohn fractions	Supernatant	10^2
Other precipitation methods	Supernatant	10^1–10^2
Adsorption chromatography		
Packed bed	Desorbed	10^2
Packed bed	Nonadsorbed	10^1
Suspension	Nonadsorbed	10^1
Adsorptive filtration		
Depth filter (mixed bed)	Non-adsorbed	10^2
Depth filter (single bed)	Non-adsorbed	10^1
Membrane filter (cellulose acetate)	Non-adsorbed	10^1

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 of methods for the elimination of viruses such as the human immunodeficiency virus (HIV) and the viruses of hepatitis B (HBV) and hepatitis C (HCV) from labile plasma products (Cuthbertson *et al.*, 1991; Foster *et al.*, 1997) has been a significant achievement. Procedures that can inactivate viruses are especially important; however, the removal of viruses by separations technology has also contributed to product safety. Viral contaminants can be preferentially removed by precipitation (Budnick *et al.*, 1994), depth filtration (Bhattacharya *et al.*, 1996) and chromatography (Burnouf, 1993; Darling & Spaltro, 1996), with removal by cold ethanol (Cohn) fractionation contributing significantly to the freedom from hepatitis transmission of human albumin (Hoofnagle & Barker, 1976) and immunoglobulin (Pennell, 1957). These technologies would also be expected to preferentially remove causative agents of TSEs (Blum *et al.*, 1998). Whether or not complete removal of a TSE agent is achievable is less certain, especially with less purified or less processed products. It is important in this context to appreciate that a very high reduction factor would not necessarily indicate that an infectious agent had been removed completely by the process concerned. Although the reduction factor provides a useful indication of the capacity of process operations to remove an infectious agent, additional information is required to determine whether or not some residual (resistant) infectious material may remain in a process stream after a step in question.

In precipitation processes it is necessary to define the solubility of the infectious agent under the precipitation

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 pH4·8. 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 aggregration) 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 physicochemical 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 has been found to be associated with white blood cells (Kuroda *et al.*, 1983) and consequently the separation of plasma from the cellular components would be expected to result in a significant proportion of a TSE agent being removed. If a 10-fold reduction is assumed in the routine centrifugal separation of plasma from whole blood (Brown *et al.*, 1998), then the estimated concentration of nvCJD in an infective plasma donation would be 250 i.v. $ID_{50}\,mL^{-1}$. With these assumptions, a single infective 300-mL donation of plasma could contain a total nvCJD infectivity of up to $7{\cdot}5\times10^4$ i.v. ID_{50}. Further reduction of the white cell content of plasma by leucofiltration (Rider *et al.*, 1998) may also reduce the nvCJD content, but as the degree of reduction is uncertain, any contribution that may be afforded by this technology has been discounted.

To examine the possible implications of an epidemic of nvCJD in the UK it is necessary to calculate the degree of product contamination that could result, in theory, from processing a contaminated pool of plasma. It has been estimated that up to 80 000 people in the UK could have been infected by BSE (Cousens *et al.*, 1997). This represents a cumulative incidence of 1500 per 10^6 of the UK population and, although there is as yet no evidence to support such a figure, this particular prediction may be taken as a 'worst case' scenario for the purposes of theoretical calculations. If plasma from UK donors is contaminated to this extent then the concentration of nvCJD in a plasma pool could be $3{\cdot}7\times10^{-1}$ i.v. $ID_{50}\,mL^{-1}$. Therefore, for a plasma product to be infective in this scenario, the nvCJD agent in the plasma pool must be concentrated into one or more of the products in question.

From the information available on the behaviour of TSE agents, the opposite might be expected during plasma fractionation, in that all plasma products are manufactured using procedures which would be expected to remove TSE agents to some extent. To illustrate this, the impact of a 0·15% incidence of infection (i.e. a worst case scenario) has been calculated in terms of the total infectivity that would be present in the plasma pool, the purified product pool prior to dispensing and in each vial of dispensed product for current products manufactured by the SNBTS and for some earlier products (Table 4), which are included as it is conceivable that nvCJD may have been present subclinically in the blood donor population since the early to mid-1980s (Cousens *et al.*, 1997).

If it is assumed that the final product pool is homogenous and that nvCJD infectivity is distributed equally amongst all of the vials of product dispensed, it can be calculated that no vial of any current product would contain an infectious dose (ID_{50}). It should be noted that these theoretical values were determined assuming a high incidence of nvCJD in the human population (1500 per

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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16

The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy

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BACKGROUND: The administration of blood components from donors who subsequently develop Creutzfeldt-Jakob disease has raised the issue of blood as a possible vehicle for iatrogenic disease.

STUDY DESIGN AND METHODS: We examined infectivity in blood components and Cohn plasma fractions in normal human blood that had been "spiked" with trypsinized cells from a scrapie-infected hamster brain, and in blood of clinically ill mice that had been inoculated with a mouse-adapted strain of human transmissible spongiform encephalopathy. Infectivity was assayed by intracerebral inoculation of the blood specimens into healthy animals.

RESULTS: Most of the infectivity in spiked human blood was associated with cellular blood components; the smaller amount present in plasma, when fractionated, was found mainly in cryoprecipitate (the source of factor 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.*

Concern has mounted in recent years about the possibility of transmitting Creutzfeldt-Jakob disease (CJD) through blood or blood components because a proportion of patients dying of CJD have been regular blood donors, and because the blood of experimentally infected animals, and humans with CJD, may sometimes contain low levels of the infectious agent.[1]

See also: Dodd RY, Sullivan MT. Creutzfeldt-Jakob disease and transfusion safety: tilting at icebergs? (editorial). Transfusion 1998;38:221-3.

The purposes of this study were, first, to determine the distribution of infectivity among the various components and plasma fractions of normal human blood that had been "spiked" with a high concentration of infectious agent, to obtain information about agent clearance during the process of blood separation and fractionation; and, second, to determine the distribution of infectivity (if present) in the same components and fractions in an experimental model of transmissible spongiform encephalopathy (TSE) characterized by a low blood level of endogenous circulating pathogen, analogous to the probable situationin humans with CJD.[2]

ABBREVIATIONS: CJD = Creutzfeldt-Jakob disease; GSSD = Gerstmann-Straüssler-Scheinker disease; LD_{50} = mean lethal dose; PBS = prosphate-buffered saline; PrP = protein-resistant protein; TSE = transmissible spongiform encephalopathy.

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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 6800 rpm (5600 × *g*) for 15 minutes at 1 to 2°C. The pellet was weighed and then frozen at –70°C (cryoprecipitate).

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

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

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

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

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

inoculated). This estimate makes use of the inverse relationship between the amount of infectivity and the length of the incubation period (the greater the infectivity, the shorter the interval between inoculation and disease)—a type of "dose-response" curve. Although not as precise as an endpoint dilution titration, it is reassuring that the whole blood, red cell, and buffy coat specimens, which had nearly identical endpoint dilution titers, also had nearly superimposable incubation period curves, and that the plasma curve was parallel to the whole blood curve at a 1.2 $\log_{10}$ 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 (45 mL of blood and 7 mL of citrate containing 225 units of heparin). The blood was immediately separated into its red cell, white cell-platelet, and plasma components, frozen at –70°C. A portion of the plasma was later thawed and processed into Cohn fractions, as described in the spiking experiment. The only difference was that, in this experiment, we did not combine the buffy coat layer of the red cell sediment with the centrifuged plasma pellet, choosing instead to assay the two specimens separately.

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

Animals were observed for clinical signs of disease for a period of 13 months, at which time all surviving mice were sacrificed. The brains from most animals that died during this observation period, as well as the brains of all animals sacrificed at the conclusion of the experiment, were removed and stored at –70°C for Western blot detection of proteinase-resistant protein (PrP).

Of a total of 537 mice that survived the immediate postinoculation period, 21 mice inoculated with various blood specimens died during the next 6 months, when the earliest verified deaths from spongiform encephalopathy began to occur in animals used for parallel titration of brain infectivity. Brains from 10 of these 21 mice were examined and found by Western blot to have been PrP-negative. We therefore presumed that all 21 deaths were due to intercurrent non-CJD-related illness and excluded them from our analysis.

Of the remaining 516 animals, 264 later died while under observation, and 252 remained well until the conclusion of the experiment. Only one of these 252 mice was PrP-positive, so that the "lethal" and "infectious" doses were essentially identical. Our final analysis was based upon 461 animals in these two groups whose brains were examined for the presence of PrP (brains from 55 mice in various groups that died during the course of the experiment, and that were found dead more than 24 hours postmortem, were considered unsatisfactory for Western blot examination). None of the uninoculated sentinel animals became ill or had PrP in their brains.

For Western blots, 100 µL of a 10-percent brain homogenate of each brain was digested at 37°C in the presence of 100 µg per mL of Proteinase K (Boehringer-Mannheim, Indianapolis, IN) and 2-percent sodium dodecyl sulfate (Gibco, Gaithersburg, MD). A second 100-µL aliquot was incubated with a 0.0125 M solution of the protease inhibitor phenylmethylsulfonylfluoride (PMSF, Gibco). After 20 minutes, PMSF was added to the sample containing Proteinase K, and both samples were autoclaved at 121°C for 30 minutes to denature the proteins and inactivate infectivity. The samples were electrophoresed in adjacent lanes on precast 14-percent tris-glycine acrylamide gels (Novex, San Diego, CA), and electroblotted to membranes (Immobilon, PVDF, Millipore, Burlington, MA). PrP was detected using chemiluminescence (ECL + Western Blot Detection System, Amersham, Arlington Heights, IL) with a mouse PrP antibody (#78295) kindly provided by Dr. Richard Rubenstein (Institute for Basic Research, Staten Island, NY). Control specimens of scrapie-infected and uninfected mouse brain were included with every digestion and gel.

RESULTS

Scrapie-infected hamster brain cell spike of normal human blood

Infectivity was found at comparable concentrations in whole blood, red cells, and buffy coat (values of ±0.5 $\log_{10}$ are not

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	20.0 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	0.26 g	6.6	1.0×10^6	0.71
Fraction I+II+III	0.93 g	6.1	1.2×10^6	0.86
Fraction IV_1+IV_4	0.87 g	4.0	8.7×10^3	0.006
Fraction V	1.66 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 infectivity concentration between any two specimens are not necessarily significant, fractional recovery percentages could be correspondingly higher or lower in a repeat experiment.
‡ Recovered from centrifuged plasma (4200 × g for 8 min).
§ Infectivity estimated from comparison of incubation period time curve to that of whole blood (see Methods section).
¶ ND = none detected (no disease transmissions in groups of four hamsters inoculated with undiluted through 10^{-6} 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 same 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%) to 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 also be noted that the absence of transmissions from the whole blood and red cell specimens does not imply the absence of infectivity (which would be unreasonable in view of its presence in buffy coat and plasma), because only very small proportions of these specimens were assayed, due to the necessity of using diluted inocula. The separate pools of brains and spleens collected from the same 75 animals had infectivity titers of approximately 10^5 LD_{50} per g and 10^2 LD_{50} per g, respectively, similar to titers observed in an earlier experiment using the same mouse model.[2]

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 were found to be infectious; and no infectivity was detectable in nearly half of such studies (including assays on the blood of sheep naturally infected with scrapie, and assays in primates inoculated with blood from humans with CJD).[1,9-11] 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 homogenates or purified, cell-free PrP. We could not know in advance whether trypsinized infected brain cells would partition during separation in exactly the same manner as blood cells; in the event, they turned out to be a more accurate predictor of endogenous infectivity distribution than we imagined possible.

Relevance of experiments to human disease

The choice of mice inoculated with the Fukuoka-1 strain of human GSSD to investigate endogenous infectivity was based on its standing as a well-studied model in which, beginning about half-way through the incubation period, low but rising levels of infectivity were detected in the buffy coat.[2] It may be objected that this GSSD strain might not be an appropriate choice to investigate the behavior of CJD. However, most human TSE strains produce similar clinical signs and neuropathology when inoculated into rodents, and induce the same molecular species of host PrP in the infected animal; thus, there is every reason to suppose that the biology of blood infectivity can be considered as a generic phenomenon of TSE (the only potential exception would be "new variant" CJD, which has shown some degree of biologic distinctiveness).

Similarly, although experimental modeling of medical problems may or may not yield results that can be transposed to the human condition, rodents have provided a vast amount of information about the pathogenesis of TSE that appears to be widely applicable across many agent strains and host species, and there is thus reason to suppose that rodent models can also provide useful (even if not definitive) information on the question of blood infectivity and disease transmission. The ideal experiment, in which chimpanzees would be inoculated with human strains of CJD, would be almost forbiddingly expensive, and require many years to obtain meaningful results (nearly 2 years for the inoculated animals to become sick, and another several years to bioassay the collected blood specimens in healthy squirrel monkeys).[12]

Both the exogenous spike and endogenous infectivity experiments gave consistent results: infectivity was present in plasma as well as white cells, and when the plasma was subjected to Cohn fractionation, infectivity distributed almost entirely into the first two precipitates. Thus, although infectivity in the brain cell spike was added in a necessarily unnatural cellular element, the similarity of its distribution to that observed in the infected mouse model indicates that it accurately reflected the endogenous situation (the only major difference was the predictably lower level of infectivity in the endogenous model). The presence of infectivity in plasma raises a question as to its origin from either white cell (or white cell fragment) contamination of adjacent components during the comparatively low speed centrifugation separation, or as distinct cell-associated and cell-free forms of infectivity. The finding of infectivity in the plasma component of normal human blood that had been spiked with intact infected brain cells argues the case of a

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

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

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Vox Sanguinis

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.

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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 20 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 murine-adapted scrapie strain ME7 has been characterized as 171 ± 2 days in C57 black mice [15] which carry the Sinc s7 gene for short scrapie incubation time. The hamster-adapted 263K strain has also been widely used.

Most published studies investigating the prion-removal potential of processes used to purify albumin and immunoglobulins (IgG) from plasma have focused on studies of Cohn fractionation, and comparatively little information has been published on prion-removal potential of chromatography-based processes. The prion-removal potential of the chromatographic portion of processes used to fractionate intravenous immunoglobulins and albumin [16] was examined using two methodologies. The first study utilized a microsomal preparation of hamster-adapted scrapie 263K, detected by Western blot. This study focused on clearance over individual columns in the processes.

The second study used mouse-adapted scrapie ME7, with detection by bioassay in C57 black mice. This study used a 10 000 g clarified homogenate spiking material, which is characterized as containing both microsomal and 'soluble PrP^{sc}' [17], to assess overall removal capacity of the ion exchange chromatography steps in the production process. The TSE infectivity reduction potential of the Cohn–Oncley [18,19] process employed in manufacturing hyperimmune immunoglobulins was also examined.

Materials and methods

Experimental design

All parameters of the industrial processes were scaled down to give accurate representations of chromatography and other process conditions. New chromatography gels were used for all experiments. Columns sizes were held at the same height as production columns, however, with smaller diameter. The scale-down factor for the DEAE and CM Sepharose columns was 1 : 5625, and for the Macro-Prep High Q column was 1 : 14 667. The number of column volumes of buffers was exactly the same as the production processes; protein loadings and linear flow rates were set at the maximum allowable in the production processes. It was required that all buffers and column conditions achieved the same pH, conductivity and height equivalent of a theoretical plate (HETP) limits as are applied to the production process. Control runs were performed with feedstreams spiked with uninfected brain preparations as described below. Extensive testing of all buffers and protein eluates determined whether the control runs accurately represented the industrial processes, as previously described [20]. Due to the limited scope of assays that could be performed in containment conditions for the TSE spiked runs, the validity of the TSE spiked run was assured by following exactly the scale-down conditions used in the control runs.

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.

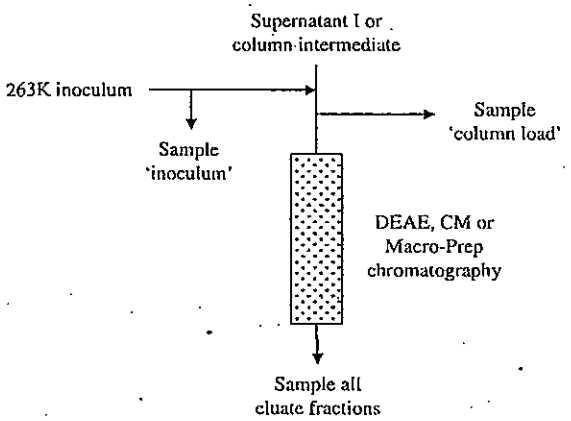

Fig. 1 Flow diagram showing spiking points and sampling points for each column in 263K PrP^{sc} studies. The diagram applies to each of the chromatography columns, as they were each spiked separately.

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 SNI was obtained from a production batch and was 'spiked' with clarified brain homogenate from control mice or ME7-infected mice to give a final spike concentration of 3·3% v/v. For the TSE spiked run, sample 'ME7 spiked SNI' was taken, and 133 ml of the material was separated on DEAE Sepharose. The albumin and immunoglobulin-containing peaks from each cycle were pooled with the corresponding peaks from each of the three cycles and were further processed on CM Sepharose or Macro-Prep.

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

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

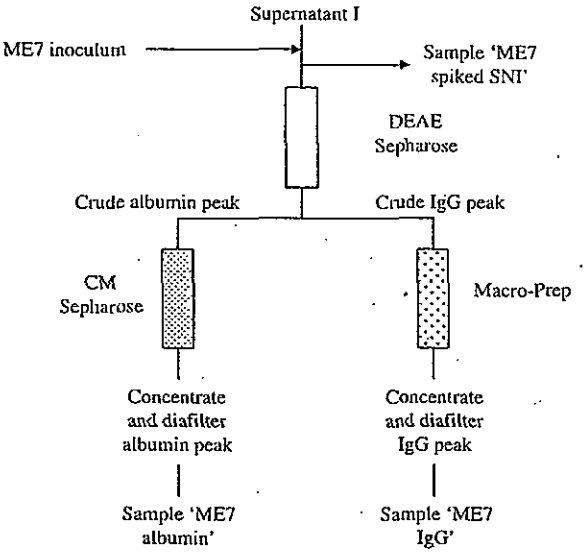

Fig. 2 Flow diagram showing spiking and sampling points for each column in ME7 infectivity studies. One upfront spike was made, with bioassay before and following each purification sequence.

The diafiltered solution was concentrated and the sample 'ME7 IgG' was taken for bioassay.

Immunoglobulin prepared by Cohn fractionation

fraction III supernatant was prepared from cryosupernatant using the Cohn–Oncley process (Fig. 3). Cryosupernatant was cooled to ≤ 1 °C, and was processed either spiked with control homogenized mouse brain at 1 : 29 or with homogenized scrapie mouse brain (ME7, as above) at 1: 29. The sample

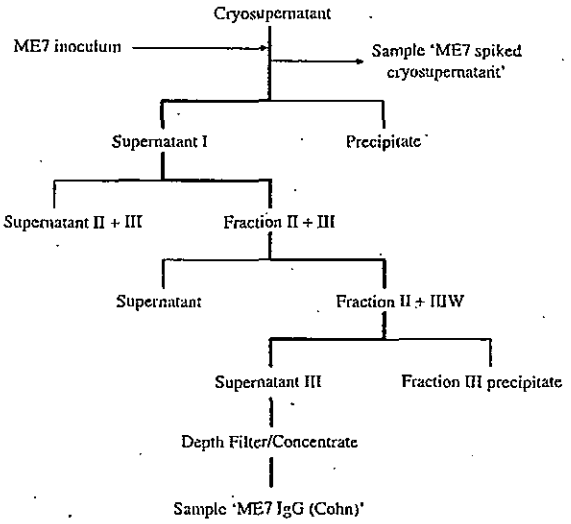

Fig. 3 Flow diagram showing spiking and sampling points for Cohn immunoglobulin ME7 infectivity studies.

'ME7 spiked cryosupernatant' was taken for bioassay, leaving a volume of 316 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 + IIIW 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}) of 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	1·58	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 '≥'.

The period between inoculation and days of onset of clinical symptoms for the spiked starting material ranged from an average of 184 days for the neat material through to 252 days in the 10^{-3} dilution. The trend of increasing incubation time with higher dilutions of inoculum was not observed at the higher 10^{-4} and 10^{-5} dilutions, with the single positive mice showing disease onset at 474 and 260 days, respectively. This variability could be due to factors such as IC injection placement or due to the specific nature of the infectivity associated with a single infectious prion unit. TSE was not found in any mice inoculated with the concentrated post-CM Sepharose (albumin) nor in the concentrated post-Macro-Prep eluate (immunoglobulin).

For the study of removal capacity of the Cohn immunoglobulin process, the period between inoculation and days of onset of clinical symptoms for the spiked starting material ranged from an average of 186 days for the neat material through to 347 days in the 10^{-5} dilution (Table 3). The trend of increasing incubation time with higher dilutions of inoculum was not observed at the 10^{-6} dilution, with the single positive mouse showing disease onset at 230 days.

The titre of the ME7 spiked starting material for the chromatography process experiment was found to be 5·4 ID_{50}/ml, with a 95% confidence interval of 4·5–6·3 (Table 4). The LRFs for the combined chromatographic processes were calculated as ≥ 5·6 for the albumin process, and ≥ 5·4 for the immunoglobulin process.

Titration of the ME7 spiked starting material for Cohn fractionation also found the titre to be 5·4 ID_{50}/ml, with a 95% confidence interval of 4·4–6·5. No TSE was found in mice inoculated with the Cohn-purified immunoglobulin, with the LRF for the process calculated as ≥ 5·6.

Table 2 Bioassay of test materials from albumin and immunoglobulin chromatographic processes

Sample	Parameter	Sample dilution 10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Control SN1	Mice infected/inoculated incubation period (days)[a]	0/8							
Control Albumin	Mice infected/inoculated incubation period (days)	0/10							
Control IgG	Mice infected/inoculated incubation period (days)	0/9							
ME7 spiked SNI	Mice infected/inoculated incubation period (days)	5/5 184 ± 0	5/5 193 ± 7	5/5 228 ± 14	5/5 252 ± 18	1/5 474	1/5 260	0/5	0/5
ME7 Albumin	Mice infected/inoculated incubation period (days)	0/15	0/5	0/5	0/5				
ME7 IgG	Mice infected/inoculated incubation period (days)	0/20	0/5	0/5	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	5/5	5/5	4/5	1/5	1/5	1/5	0/4
		186 ± 6	235 ± 26	223 ± 8	279 ± 67	279	347	230	
ME7 IgG (Cohn)	Mice infected/inoculated incubation period (days)	0/20	0/5	0/5	0/5				

[a]Mean ± standard deviation.

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

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	≤ 1·9	≥ 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

[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.

The LRF calculated for the Cohn immunoglobulin process was ≥ 5·6, which is similar to that shown previously, where a clearance of ≥ 4 logs of PrP^{sc} in fraction III effluent was detected by Western blot [7]. Removal over Fraction I + III precipitation with microfiber glass depth filtration and Seitz depth filtration has previously been reported [8] to give an overall removal of BSE 301 V infectivity of ≥ 2·9. The non-additive nature of ethanol precipitation and depth filtration was established by these authors, and further work [35] suggests that the likely cause of prion removal is the precipitation of PrP^{sc} by ethanol, and removal in centrifugation, followed by the 'polishing' effect of depth filtration.

Conclusion

Using both scrapie 263K and ME7 as spiking agents, these experiments have shown that the ion exchange chromatographic steps used in the production of albumin and immunoglobulin have the capacity to remove at least 5·6 and 5·4 logs, respectively, of infectious prions. Production of immunoglobulin by ethanol precipitation was shown to have the capacity to remove at least 5·6 logs of prion infectivity. Importantly, this investigation shows that removal of infectivity from immunoglobulin preparations is similar whether chromatographic or Cohn purification processes are used. In all the bioassay studies, the process steps removed scrapie ME7 to below the limit of detection, and further studies with a higher titred challenge, or more concentrated final product would be needed to finitely measure the removal capacity of individual process steps. The Western blot study indicated that prion removal by the columns was more dependent on adsorption to the gel matrix than the exchange group, and a ≥ 3·0 log removal of PrP^{sc} was achieved for all individually spiked columns.

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Prion removal by nanofiltration under different experimental conditions

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Abstract

Manufacturing processes used in the production of biopharmaceutical or biological products should be evaluated for their ability to remove potential contaminants, including TSE agents. In the present study, we have evaluated scrapie prion protein (PrP^{Sc}) removal in the presence of different starting materials, using virus removal filters of different pore sizes. Following 75 nm filtration, PrP^{Sc} was detected in the filtrate by Western blot (WB) analysis when a "super-sonicated" microsomal fraction derived from hamster adapted scrapie strain 263K (263K MF) was used as the spike material. In contrast, no PrP^{Sc} was detected when an untreated 263K MF was used. By using spike materials prepared in a manner designed to optimize the particle size distribution within the preparation, only 15 nm filtration was shown to remove PrP^{Sc} to below the limits of detection of the WB assays used under all the experimental conditions. However, infectious PrP^{Sc} was recovered following 15 nm filtration under one experimental condition. The results obtained suggest that the nature of the spike preparation is an important factor in evaluating the ability of filters to remove prions, and that procedures designed to minimize the particle size distribution of the prion spike, such as the "super-sonication" or detergent treatments described herein, should be used for the preparation of the spike materials.

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 thought to be the causative agent of the transmissible spongiform encephalopathy (TSE) diseases, which include Creutzfeldt–Jakob disease (CJD), vCJD, and bovine spongiform encephalopathy (BSE). Therefore, to reduce the risk of transmission when raw materials for protein products (such as plasma) are contaminated with infectious prions, measures should be introduced to decrease the prion load, to evaluate the risk to the product, and to introduce prion removal/inactivation step(s) in the manufacturing process, if feasible [3–5]. Unlike viruses, the minimum infectious prion unit does not exist as a single particle. The infectious prion unit is believed to be composed of protein polymers/aggregates, rather than a prion particle. The unusual nature of the prion agent makes it particularly important to

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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^{vCJD}, 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 water-bath. Ten cycles of sonication were performed, with a 1 min

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)		P-35N (35 ± 2 nm)		P-15N (15 ± 2 nm)	
Super-sonicated	+	−	+	−	+	−	+	−	+	−
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	4.2/4.2	3.5/4.2	4.2/4.2	3.5/4.2
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	<1.0/<1.0	<1.0/<1.0	<1.0/<1.0	<1.0/<1.0
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	≥3.2/≥3.2	≥2.5/≥3.2	≥3.2/≥3.2	≥2.5/≥3.2

Data represents total PrP^{Sc} present in samples, expressed as $\log_{10}$ arbitrary units, following Western blot analysis as described for WB1. This study was performed 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 $\log_{10}$ value.

interval between each sonication treatment. During the treatment cycle, the particle size distribution was monitored. We named this treatment cycle "super-sonication".

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 submission, the results are presented as reported in these studies.

2.2.1. Method 1 (WB1)

Samples and controls were either tested directly, or first ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, and the pelleted material then resuspended in PBS. Ultracentrifugation was performed to concentrate the PrP^{Sc} present in large volume samples, and to remove soluble proteins or buffer components that might interfere with the WB assay. Samples were digested with proteinase K (Roche Diagnostics, GmbH, Penzberg, Germany) for 60 min at 37 °C. The optimal concentration of proteinase K, to remove any background that could interfere with the detection of PrP^{Sc} and to allow effective recovery of the PrP^{Sc} protein, was previously established for each sample. Digested samples were mixed 1:1 with Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, and 0.01% (w/v) bromophenol blue, BioRad Laboratories Inc., Hercules, USA) containing 5% (v/v) β-mercaptoethanol. After boiling, serial 5-fold dilutions of

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

Filter	P-35N (35 ± 2 nm)		P-20N (19 ± 2 nm)		P-15N (15 ± 2 nm)			
Preparation	IVIG	Haptoglobin	IVIG	Haptoglobin	Antithrombin			Thrombin
Spike material	263K sMF[c]	263K sMF[c]	263K sMF[d]	263K dsMF[e]	263K dMF[f]	263K sMF[c]		263K dsMF[e]
MF preparation lot.	C/D	B	E/F	E/F	A/A	B		C/D
Spike ratio	1/100	1/200	1/20	1/200	1/50	1/21		1/20
Detection method[b]	WB1	WB3	WB2	WB2	WB1	WB3	BA	WB1
Before filtration	3.2/2.5	2.4	6.8/6.8	6.7/6.1	3.1/3.1	3.6	+ve	3.7/3.7
Filtered	0.8/0.8	<1.0	4.8/4.3	4.8/4.7	0.0/0.0	<0.8	+ve	<0.2/<0.2
Log reduction factor	2.4/1.7	≥1.4	2.0/2.5	1.9/1.4	≥3.1/≥3.1	≥2.8	NA	≥3.5/≥3.5

Abbreviations used: 263K MF, microsomal fraction derived from hamster adapted scrapie strain 263K; IVIG, intravenous immunoglobulin; 263K sMF, "super-sonicated" 263K MF; WB, Western blotting; 263K dsMF, detergent treated and "super-sonicated" 263K MF; 263K dMF, detergent treated 263K MF; BA, bioassay; +ve, scrapie positive.

[a] Scaled down conditions were designed according to current guidelines. However, in a study using P-35N filter and haptoglobin, clogging of the filter occurred, 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 were performed in accordance with GLP regulations; the studies involving the use of WB3 and the qualitative BA shown in this table, were performed as non-GLP studies.

[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 without protein, "super-sonicated", 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 in the starting material (thrombin) or saline (haptoglobin), and "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 0.0025% (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 (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 filtration run, by comparing the total amount of PrP^{Sc} present in samples before and after filtration. All studies involving the use of WB1 and 2, and the quantitative bioassays, were performed in facilities in compliance with current GLP regulations. Studies involving the determination of average particle size in normal MF preparations, the use of WB3, and the qualitative bioassay, were performed as non-GLP studies.

3. Results

3.1. Influence of MF preparation method on particle size distribution

Ideally, to represent a "worst case" challenge for a filter, the smallest form of prion protein, or infectious agent, should be used. Studies to investigate the optimum method for preparing the prion spike material were therefore performed. In these studies, changes in the average particle size in normal MF were investigated, as 263K-infected brain material could not be handled within our facility. Although prion particles in MF derived from 263K-infected brain material were not investigated directly, we tried to optimize the design of our experiments by minimizing the size of particles in normal MF, as particle size may influence filtration performance (both with respect to filter blockage, and removal of PrP^{Sc}). The results are shown in Figs. 1 and 2.

Treatment of normal MF with sarkosyl or lysolecithin reduced the average size of particles to approximately 100 nm, when 0.1% or higher concentrations of the detergents were used. However, below that concentration, the particle size did not change significantly, with the exception of 0.01% lysolecithin which reduced the average particle size to approximately 300 nm (Fig. 1A,B). Treatment with Triton X-100 did not result in a significant change in particle size, even at 1% (Fig. 1C). Treatment with 0.3% TNBP or 1% Tween 80 alone was not able to reduce the particle size below 200 nm. However, when combined, one of the conditions generally used for viral inactivation ("SD treatment"), 0.3% TNBP and 1% Tween 80 reduced the average particle size to below 200 nm (Fig. 1D). These results suggest that the reduction in average particle size in normal MF depends on the choice of detergent(s), and the concentration and combination of detergent(s) used.

We also studied the effect of "super-sonication" on the particle size in normal MF. The results showed that "super-sonication" could reduce the average particle size to a very fine level in a short time, without the need to change the composition of the normal MF material (Fig. 2A). Since "super-sonication" is a temporary physical procedure, reversal of the particle size reduction may possibly occur. To exclude this possibility during the experiments, we conducted a stability study on the particle size in normal MF after "super-sonication". There was no significant change in the particle size up to 24 h after "super-sonication", with the size remaining at approximately 100 nm (Fig. 2B).

The results showed that the particle size of normal MF preparations could be reduced significantly by treatment

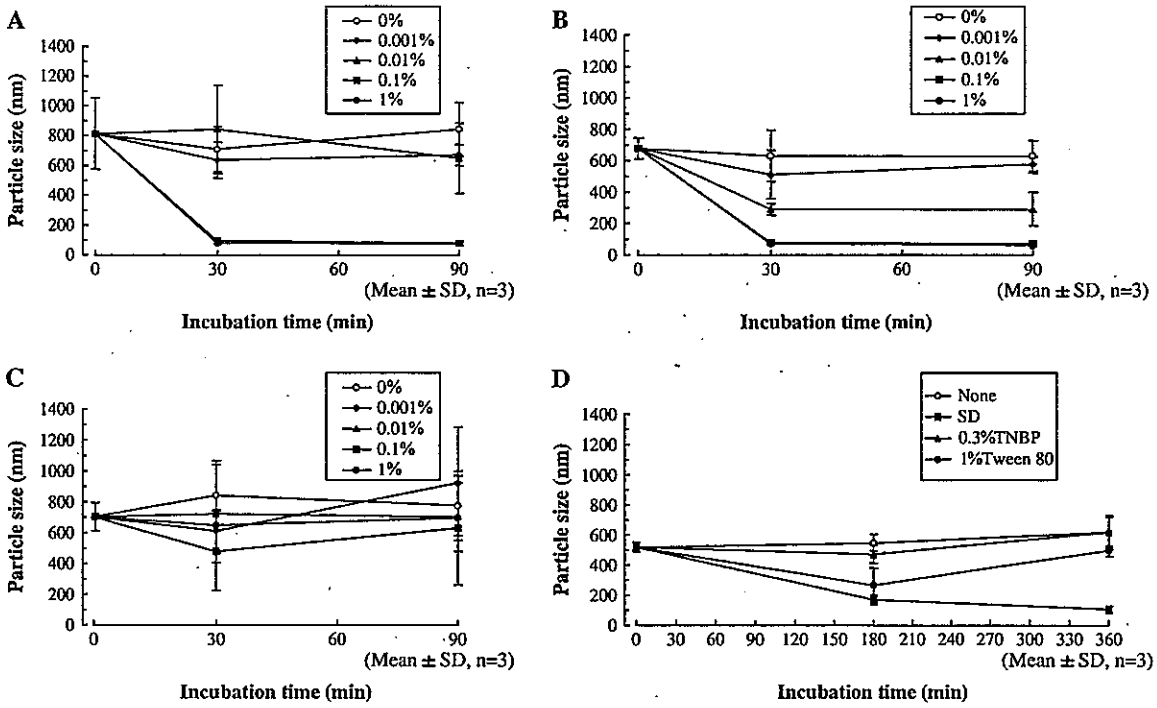

Fig. 1. Change of particle size in normal MF following treatment with various detergents. To normal MF, sarkosyl (A), lysolecithin (B), or Triton X-100 (C) was added to a final concentration of 1%, 0.1%, 0.01%, and 0.001%, respectively. The change in the average particle size was then monitored at room 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

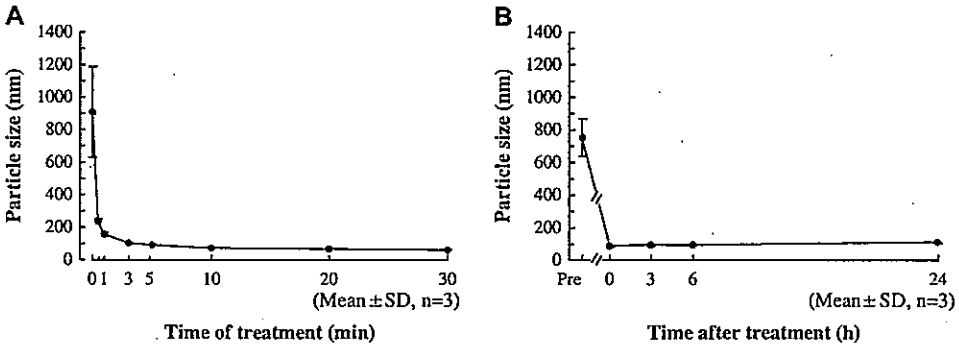

Fig. 2. Change of particle size in normal MF following intense sonication ("super-sonication"). Normal MF in a test tube equipped with a resonance chip (20 kHz, 200 W) was sonicated for 1 min in an ice bath. After 1 min, the sonication step was repeated. The change in average particle size was monitored 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 more severe challenge for a filter step. An approximately 5-fold higher $\log_{10}$ reduction factor was observed for "non-super-sonicated" stocks, for the 100 nm and P-75N filters, for both stocks tested. No significant loss of PrP^{Sc} was observed with either spiking material with 220 nm filtration, and no PrP^{Sc} was detected in the filtrates following P-35N and P-15N filtration.

Previously, we have observed some removal of PrP^{Sc} in some lots of "non-super-sonicated" 263K MF by 220 nm filtration. Strict control of the methodology used to generate the 263K MF stocks appeared to prevent this, suggesting that the method of preparing the 263K MF itself may influence the particle size distribution (data not shown).

3.4. Removal of PrP^{Sc} by Planova filters in the presence of plasma preparations

Removal of PrP^{Sc} by P-15N, P-20N, and P-35N filters was evaluated in the presence of a number of different plasma preparations, under conditions designed to mimic the relevant manufacturing process. The design of the experiments was similar to that of virus clearance studies. Samples were analyzed by WB, and the $\log_{10}$ reduction factor (LRF) was calculated for each filter step. The results are shown in Table 3.

Under all the experimental conditions tested, PrP^{Sc} was not detected by WB after filtration through P-15N. The LRF values were $\geq$2.8. In contrast, PrP^{Sc} was detected by WB in samples following filtration through P-20N and P-35N filters, in three out of the four processes tested, giving LRF values in the order of 2 logs. In one study, P-35N/haptoglobin, using "super-sonicated" 263K MF, PrP^{Sc} was not detected in the filtrate. However, the sensitivity of this study was low, giving a LRF of $\geq$1.4, and therefore the robustness of this filtration process was not evaluated. In the initial studies (Table 2), PrP^{Sc} was not detected in the fractions after P-35N filtration of either "super-sonicated" or "non-super-sonicated" 263K MF in PBS, resulting in log reduction factors in the order of 3 logs. The variance in the results obtained for these filters could be due to a combination of factors, including how the scrapie spike material was prepared, the composition of the starting material, and the precise filtration conditions used.

3.5. Removal of prion infectivity by Planova filters in the presence of plasma preparations

P-15N filtration was shown in these studies to be able to remove PrP^{Sc} to levels below the detection limit of the WB assays used, regardless of the method used to prepare the spike material, the composition of the start material, or the filtration conditions. However, a bioassay study for samples generated in a P-15N/AT study using 220 nm-filtered "super-sonicated" 263K MF, demonstrated that infectivity was recovered following filtration, as clinical signs appeared in all hamsters inoculated with the filtrate, and analysis of hamster brain material confirmed the clinical results. PrP^{Sc} was detected in the brain homogenates from all clinically positive hamsters by WB, and scrapie-associated lesions were observed in all the

Table 4
Scrapie infectivity in samples generated during 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 WB3	Detected	Detected	Detected	Detected	Detected	Detected
Lesions by histopathology	+ve	+ve	+ve	+ve	+ve	+ve
Medulla (oblongata)	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P
Cerebellum (cortex)	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
Paraterminal body	D,P	D,P	D,P	NR	D,V,P	P
Cerebral cortex (posterior midline)	D,P	D,P	D,P	D,P	D,V,P	D,V,P
Cerebral cortex (anterior midline)	D,P	D,V,P	D,V,P	D,V,P	D,V,P	D,V,P

Abbreviations used: +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 infectious 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.

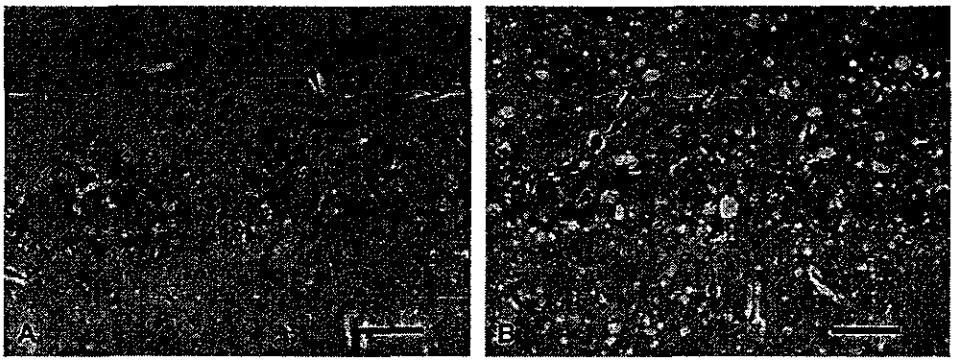

Fig. 3. Typical nerve lesions in the hippocampus of a hamster brain, taken from an animal inoculated with a P-15N-filtered sample (B), in comparison with the corresponding region from an uninfected animal (A). Arrows, vacuolation; Arrowheads, degeneration of nerve cells; scale bar = 50 μm; HE staining 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 processing conditions, there may be incomplete removal of prion contaminants.

Although infectivity was detected in the filtrate fraction from the one process step studied, longer and more variable incubation periods were observed in the animals inoculated with the filtrate sample (Table 4), suggesting a lower prion titer following filtration. However, it was not possible to estimate the relative levels of prion infectivity present in the input and filtrate samples, as no data was available to correlate incubation periods and prion titers for this study. Based on the titers typically observed for 263K MF stocks, the bioassay used could theoretically detect reductions in prion infectivity in the order of 4 logs for this process step. Detection of infectivity in the filtrate fraction by bioassay is therefore not necessarily incompatible with the WB results obtained (LRF $\geq$2.8 logs), and may simply reflect a difference in sensitivity between the two assays used.

As discussed above, uncertainties about the nature of the infectious agent in plasma, and during the manufacturing process, raise concerns about the design and interpretation of prion clearance studies. No single spike preparation is likely to contain all potential forms of the infectious agent. Infectivity is associated with membranes and protein aggregates. In addition, it has recently been shown that the GPI anchor is not required for infectivity, suggesting that endogenous proteolytic release of PrP^{Sc} from cell surfaces may also contribute to the spread of the infectious agent *in vivo* [19,20]. Whether significant levels of infectivity in human plasma are associated with GPI-anchorless prion protein is not yet clear. These different forms of infectivity, with different biophysical properties, could show different partitioning properties through the same manufacturing process [7]. Furthermore, different forms of the agent may differ in their level of infectivity. For example, it was recently reported that particles in the order of 17–27 nm appeared to have the highest relative level of infectivity, in comparison to levels of PrP^{Sc} [21]. Therefore, a better understanding of the nature and forms of the infectious agent is essential to allow the design of more accurate models for prion clearance studies, and a more confident evaluation of the safety of manufacturing processes with respect to potential TSE contamination.

In summary, we used methods intended to reduce the size of particles present within MF preparations in an effort to present a worst-case (smallest) prion challenge during nanofiltration. Using such preparations, P-15N filtration consistently reduced the level of PrP^{Sc} to below the limits of detection of the Western blot assays used, suggesting that this process step is effective for the removal of prions. However, data from a single process step studied suggested that infectivity could be recovered following P-15N filtration, and thus even P-15N filtration may not result in complete removal of prions, at least when used under some conditions.

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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 process: Application to a therapeutic immunoglobulin solution (Lymphoglobuline®)

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Abstract

The characteristic of transmissible spongiform encephalopathies (TSE) is an accumulation of partially protease resistant (PrP^{res}) abnormal prion protein (PrP^{sc}). This pathological prion protein is very resistant to conventional inactivation methods. The risk of transmission of TSE, such as Creutzfeldt–Jakob disease (CJD), by biopharmaceutical products prepared from human cells must be taken into account. The nanofiltration process has been proved to be effective in removing viruses and scrapie agent. The major advantages of this technique are flexibility and efficacy in removing infectious particles without altering biopharmaceutical characteristics and properties.

This study focused on the removal of human PrP^{sc} by means of a nanofiltration method after spiking a Lymphoglobuline® solution with a CJD brain homogenate. Lymphoglobuline® equine anti-human thymocyte immunoglobulin is a selective immunosuppressive agent acting mainly on human T lymphocytes. The therapeutic indications are:

- immunosuppression for transplantation: prevention and treatment of graft rejection;
- treatment of aplastic anemia.

In our study, CJD homogenate was spiked at three different dilutions (low, moderate and high) in the Lymphoglobuline® product. The nanofiltration process was performed on each sample. Using the western blot technique, the PrP^{res} signal detected in nanofiltrates was compared to that obtained with a reference scale (dilution series of CJD brain homogenate in Lymphoglobuline® detected by western blot and elaborated on 3.3 log). After nanofiltration, the PrP^{res} western blot signal was detected with a significant reduction in the less dilute sample, whereas the signal was undetectable in the two other samples.

These are the first data in CJD demonstrating a clearance between 1.6 and 3.3 log with a Lymphoglobuline® recovery of over 93%. The nanofiltration process confirms its relative efficacy in removing human CJD PrP^{sc}.

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 90 children died in France and young adults are reported to show clinical progression of iatrogenic CJD.

Products of human origin have generally been withdrawn from therapeutic protocols but some human products such as blood cells can be used as reagents needed in different purification steps for biopharmaceutical products. Though infectivity has never been detected in human red blood cells, a safety process able to decrease prion infectivity significantly to the same extent as infectivity transferred by conventional agents (viruses, bacteria, etc.) could be of great interest.

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Prion infectivity is closely related to neurological disorders called TSE which include human diseases such as Creutzfeldt–Jakob disease (CJD), Fatal Familial Insomnia and Gerstmann Straüssler Scheinker syndrome.

CJD is classified as sporadic, genetic, iatrogenic including the CJD variant associated with bovine spongiform encephalopathy. The infectious agent responsible for this disease was called a "prion" by Prusiner [1]. It is a glycoprotein which is normally present in the physiological form (PrP^c) which becomes pathological in CJD (PrP^{sc}). The transition process from the physiological to the pathological form is complex [2]. Scientists have demonstrated a trans-conformational change between both protein forms [3]. PrP^c primarily contains α helix, though PrP^{sc} contains more β sheets in its three-dimensional structure [4]. CJD is characterized by intra-cerebral accumulation of abnormal prion protein which is partially protease resistant (PrP^{res}). Cleavage of PrP^{sc} by proteinase K results in two types of PrP^{res} after western blot, type 1 and type 2, according to Parchi's classification [5].

Studies on the elimination of viral contamination from biopharmaceutical products (which are prepared from human cells) have been conducted using nanofiltration [6–8]. This nanofiltration method has been effective on many viruses. Albumin solution safety nanofiltration has been conducted on scrapie prion protein [9].

The purpose of this research is to study the efficacy of nanofiltration on CJD PrP^{sc} in order to introduce this method into the manufacturing process of a therapeutic immunoglobulin solution Lymphoglobuline®.

Lymphoglobuline® equine anti-human thymocyte immunoglobulin is a selective immunosuppressive agent acting mainly on human T lymphocytes. It recognizes most of the molecules involved in the cascade of T-cell activation during graft rejection, such as T-cell receptor and CD3, HLA class 1 molecules, CD4 and CD8 co-receptors, co-activation molecules or adhesion molecules CD2, CD5, and CD18. The therapeutic indications are the followings:

- immunosuppression for transplantation: prevention and treatment of graft rejection;
- treatment of aplastic anemia.

During the process of purification of the equine anti-human thymocytes a step of nanofiltration was added for the viral security.

2. Materials and methods

2.1. Biopharmaceutical product

Lymphoglobuline® is an anti-thymocyte equine immunoglobulin that induces immunosuppression as a result of T-cell depletion and immune modulation. It is approved for the prevention and treatment of rejection episodes in kidney, pancreas or liver transplantation. In hematology, Lymphoglobuline® is approved for treatment of aplastic anemia and in the treatment of steroid resistant graft versus host disease.

In the Lymphoglobuline® manufacturing process, human thymocytes, membrane red blood cells and placenta are used. These human elements represent a virtual potential source of contamination of Lymphoglobuline®.

2.2. Human source of pathological prion protein

After the histological, immunohistochemical and biochemical analyses of post-mortem human brains, one case of definite CJD, and one non-CJD were chosen. The anatomic site chosen was the frontal cortex. The CJD case selected was characterized by the presence of PrP^{res} type 1 in western blot analysis according to Parchi's classification and by synaptic deposits of PrP^{sc} with an immunohistochemical technique. The same human cortex was used as source of PrP^{sc} for the reference scale and for the nanofiltration samples.

2.3. Sample preparation

2.3.1. Human brain homogenate

Frontal cortex of CJD and non-CJD cases was spiked in PBS buffer, 1:10 at final dilution. These homogenates were filtered successively with needles of 0.6 mm and 0.5 mm diameter in order to obtain homogenous preparation. After centrifugation at 1000g for 5 min, supernatants were applied to nanofiltration process.

2.3.2. Reference scale

This reference scale was prepared with series of dilutions of CJD brain homogenate in Lymphoglobuline® from 1:10 to 1:20,000. This reference scale was based on the technique used by Lee et al. [10,11].

2.3.3. Nanofiltration samples

These samples were prepared using CJD brain homogenate dilutions in Lymphoglobuline®. Three different samples were produced; samples at a high PrP^{sc} dilution (1:500), samples at a moderate PrP^{sc} dilution (1:100) and samples at a low PrP^{sc} dilution (1:10). Each dilution was prepared for three samples, one non-nanofiltrated (control) and two nanofiltrated. These samples were prepared as a reference scale with an adaptation of the method used by Lee et al. [11].

2.4. Filtration

Small-sized (membrane diameter: 47 mm) Pall® filters (hydrophilic Polyvinylidene fluoride microporous membrane) with mean pore sizes for Pall® DVD of about 0.1 μm, Pall® DV50 of about 50 nm and Pall® DV20 of about 20 nm were used successively in the nanofiltration process. The filtration mode was conducted at a constant membrane pressure of 3 bars. The samples underwent nanofiltration in the following order: negative control, CJD samples at a high PrP^{sc} dilution (1:500), CJD samples at a moderate PrP^{sc} dilution (1:100), CJD samples at a low PrP^{sc} dilution (1:10) and negative control (Fig. 1).

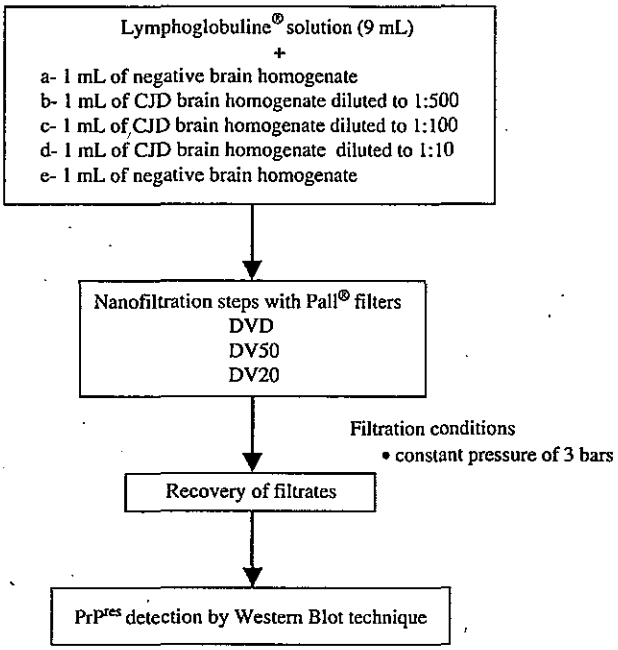

Fig. 1. Nanofiltration process. (a) Negative control sample at 1:10 in Lymphoglobuline®; (b) CJD sample at a high PrP^{sc} dilution (1:500) in Lymphoglobuline®; (c) CJD sample at a moderate PrP^{sc} dilution (1:100) in Lymphoglobuline®; (d) CJD sample at a low PrP^{sc} dilution (1:10) in Lymphoglobuline®; and (e) negative control sample at 1:10 in Lymphoglobuline®, ×2, produced in duplicate.

The nanofiltration material was treated with sodium hydroxide (2 M) for 1 h between each nanofiltration of different PrP^{sc} dilution samples.

2.5. PrP^{res} detection

The western blot technique was used to detect PrP^{res} after proteinase K treatment [12]. The anti-prion protein antibody revealed three strips of a molecular weight between 30 and 22 kDa (Fig. 2) corresponding to the biglycosylated, monoglycosylated and unglycosylated forms. Then, PrP^{res} was revealed by chemiluminescence. This technique was used to detect PrP^{res} in reference scale samples and in samples before and after nanofiltration.

The reference scale samples and samples for nanofiltration were produced and developed by the western blot technique under the same conditions and in the same time.

2.6. Determination of reduction factors

The reduction factors defined as the reduced titer versus the real titer present in the spiked sample were determined by comparing the PrP^{res} signal of samples before and after nanofiltration with the PrP^{res} signal of reference scale. After this comparison, we determined a reduction factor (log) for each sample.

3. Results

The reference scale ranges from 1:10 to 1:20,000 dilutions of CJD brain homogenates. From the 1:10 to 1:2000 dilutions,

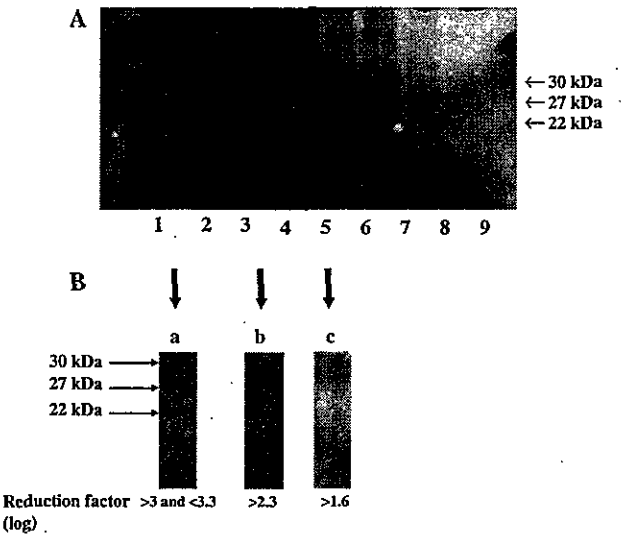

Fig. 2. (A) Reference scale. (1) PrP^{sc} sample dilution at 1:10 in Lymphoglobuline®; (2) PrP^{sc} sample dilution at 1:20 in Lymphoglobuline®; (3) PrP^{sc} sample dilution at 1:100 in Lymphoglobuline®; (4) PrP^{sc} sample dilution at 1:200 in Lymphoglobuline®; (5) PrP^{sc} sample dilution at 1:1000 in Lymphoglobuline®; (6) PrP^{sc} sample dilution at 1:2000 in Lymphoglobuline®; (7) PrP^{sc} sample dilution at 1:10,000 in Lymphoglobuline®; (8) PrP^{sc} sample dilution at 1:20,000 in Lymphoglobuline®; and (9) negative sample dilution at 1:10 in Lymphoglobuline®. (B) Western blot signal after nanofiltration steps. a. Signal of PrP^{sc} sample dilution at 1:10 after nanofiltration, three strips of PrP^{res} were shown, the reduction factor was between 3 and 3.3 log to the reference scale. b. Signal of PrP^{sc} sample dilution at 1:100 after nanofiltration, strips of PrP^{res} were not detected, the reduction factor was strictly greater than 2.3 log in comparison to the reference scale. c. Signal of PrP^{sc} sample dilution at 1:500 after nanofiltration, strips of PrP^{res} were not detected, the reduction factor was strictly greater than 1.6 log in comparison to the reference scale.

the three strips of PrP^{res} (biglycosylated, monoglycosylated and unglycosylated) were visualized, and for the 1:10,000 and 1:20,000 dilutions, only the monoglycosylated strip was detected. The reference scale was elaborated over a 3.3 log range by the western blot technique (1:10–1:20,000). (Fig. 2A).

The PrP^{res} strips of samples at a low PrP^{sc} dilution (1:10) were detected after nanofiltration with a western blot signal corresponding to the reference scale dilution between 1:10,000 and 1:20,000 (Fig. 2B). The nanofiltration process removed PrP^{sc} by a reduction factor of 3–3.3 log.

The PrP^{res} strips of samples at a moderate PrP^{sc} dilution (1:100) and of samples at a high PrP^{sc} dilution (1:500) were not detected after nanofiltration, these samples after nanofiltration corresponding to reference scale dilutions were greater than 1:20,000 (Fig. 2B). PrP^{sc} was removed by the nanofiltration process with a reduction factor greater than 2.3 log and 1.6 log: the real reduction factors are not available due to limited detection of the western blot assay.

The western blot signal of the negative control sample was negative, no strips of PrP^{res} were detected.

4. Discussion

Pathological prion protein (PrP^{sc}) is highly resistant to inactivation [13] and is not destroyed by known viral inactivation

processes [14]. Effective methods include for example exposure to 1 M sodium hydroxide during autoclaving at 121 °C. This kind of method using chemical agents (sodium hydroxide, chlorine at high concentrations) and physical treatment by autoclaving is very drastic and it is a real problem to inactivate PrP^{sc} in biopharmaceutical products without modifying their therapeutic properties. The reduction of any risk associated with a pharmaceutical product will be dependent on the physical removal of infective material during product manufacture. Many techniques for plasma-derived products, such as ethanol fractionation, depth filtration and chromatographic processes, may contribute to a significant partitioning of prion protein [10,15–18]. Although early applications of nanofiltration targeted viral removal [7,8,19], new data suggest that it may be a specific removal system for prion proteins as well. Human TSE pathogens in diluted brain homogenate were reported to be removed by a Millipore screen-type 0.025 μm membrane filter employed during production of growth hormone [20]. However, only a small quantity of diluted brain homogenate could pass through the membrane. Planova® cartridges with mean pore sizes from 75 to 10 nm were used to filter brain homogenate from mice infected with human TSE [21]. No infectivity was detected in the 35 nm filtrate. The pathogenic agent was estimated to be approximately 40 nm in size. However, some residual infectivity was found in the 10 nm filtrate when 1% Sarkosyl was added to the homogenate [22]. Recently, removal of scrapie agent ME7, a mouse adapted strain of scrapie used as a model for the BSE or vCJD agents by using nanofiltration of a 2% albumin solution spiked with a brain homogenate [23]. The albumin recovery was over 90%. Extent of removal was influenced by the filter type and by the addition of an anionic detergent (Sarkosyl) to the protein solution. An infectivity of 4.93 and 1.61 log was removed using a 35-nm filter without and with detergent, respectively. Moreover, a reduction of infectivity of >5.87 and 4.21 log was obtained using a 15-nm filter in the absence and presence of detergent, respectively. No residual infectivity was detected in any filtrate when using 15 nm or smaller porosity filters. Studies have shown an efficacy of 35–15 nm filters in achieving some removal of prions from biological solutions with the best removal with a 15-nm filter. The data, although encouraging, should be analyzed more accurately due to the tendency of prion spikes to aggregate under the experimental conditions used and with human prion protein because this removal could be dependent on the "strain" of prion protein.

In our study, we wanted to study the efficacy of nanofiltration on human PrP^{sc} in a biopharmaceutical product (Lymphoglobuline®). We used human PrP^{sc} from CJD patients as the contaminant. This contamination condition was important to study the Lymphoglobuline® nanofiltration technique under conditions as close as possible to a possible contamination by human cells used for the preparation of this product. The extent of removal may be influenced by the aggregation, type [24] and conformation of prion proteins and the physicochemical nature of the solution filtered. These parameters were important to choose the PrP^{res} type for the study. Amyloid plaques or focal deposits of PrP^{sc} still remain after homogenizing the cerebral cortex and the hypothesis was made that this kind of PrP^{sc} aggregation could be the result of a bias in the methodology. For this reason, PrP^{res} type 1 associated with synaptic deposits with an immunohistochemical technique was chosen in order to test the Lymphoglobuline® nanofiltration process under worst conditions to test the filters. In this study, Lymphoglobuline® was spiked with brain homogenate at different dilutions (1:10, 1:100, and 1:500). These PrP^{sc} dilutions can be correlated with World Health Organization (WHO) classification of organ infectivity: the low PrP^{sc} dilution corresponding to 1:10 (brain and spinal cord), moderate PrP^{sc} dilution corresponding to 1:100 (spleen, tonsil, lymph node, intestine, placenta...) and high PrP^{sc} dilution corresponding to 1:500 (brain stem, thymus, liver, pancreas, lungs...).

The comparison of the samples before and after nanofiltration showed a reduction factor between 3.3 and 1.6 log in comparison with the reference scale. The reduction factor of samples at a low PrP^{sc} dilution (1:10) was between 3 and 3.3 log. This dilution could correspond to a brain or a spinal cord PrP^{sc} concentration (WHO). The reduction factors for a very high PrP^{sc} concentration obtained illustrate a very good efficacy of the nanofiltration process.

In samples at a moderate PrP^{sc} dilution (1:100) and samples at a high PrP^{sc} dilution (1:500), the PrP^{res} strips were not detected after nanofiltration, the reduction factor was strictly greater than 2.3 and 1.6 log, respectively. The 1:100 dilution could correspond at a spleen or tonsil or lymph node or intestine or placenta PrP^{sc} concentration (WHO) and the 1:500 dilution could correspond to a brain stem or thymus or liver or pancreas or lungs PrP^{sc} concentration (WHO). In conclusion, the data obtained on both these PrP^{sc} dilutions are encouraging because, after nanofiltration, the PrP^{res} signal was not detected, although they are only indicative with probably underestimated reduction factors. Finally, the reduction factor obtained is 3.3 log and seem to demonstrate the efficacy of the nanofiltration process on human CJD PrP^{sc} with a good protein recovery.

Removal may be based on a sieving mechanism or due to adsorption on the membrane. The potential to use nanofiltration as a dedicated step for prion removal may have a significant impact on the safety of biopharmaceutical products and recombinant proteins, when production involves the use of human or animal derived materials, or medicinal products derived from bovine sources [25,26]. This technique has the ability to extend the concept of sterility of biological products from bacteria to, at least, some viruses. Our results suggest that nanofiltration could be also of interest for the removal of human pathological prion proteins.

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ORIGINAL PAPER

Creutzfeldt–Jakob disease and blood transfusion: results of the UK Transfusion Medicine Epidemiological Review study

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Vox Sanguinis

Background and Objectives This paper reports the results to 1 March 2006 of an ongoing UK study, the Transfusion Medicine Epidemiological Review (TMER), by the National CJD Surveillance Unit (NCJDSU) and the UK Blood Services (UKBS) to determine whether there is any evidence that Creutzfeldt–Jakob disease (CJD), including sporadic CJD (sCJD), familial CJD (fCJD), and variant CJD (vCJD) is transmissible via blood transfusion.

Materials and Methods Sporadic CJD and fCJD cases with a history of blood donation or transfusion are notified to UKBS. All vCJD cases aged > 17 years are notified to UKBS on diagnosis. A search for donation records is instigated and the fate of all donations is identified by lookback. For cases with a history of blood transfusion, hospital and UKBS records are searched to identify blood donors. Details of identified recipients and donors are checked against the NCJDSU register to establish if there are any matches.

Results *CJD cases with donation history:* 18/31 vCJD, 3/93 sCJD, and 3/5 fCJD cases reported as blood donors were confirmed to have donated labile components transfused to 66, 20, and 11 recipients respectively. Two vCJD recipients have appeared on the NCJDSU register as confirmed and probable vCJD cases. The latter developed symptoms of vCJD 6.5 years and 7.8 years respectively after receiving non-leucodepleted red blood cells (RBCs) from two different donors who developed clinical symptoms approximately 40 and 21 months after donating. A third recipient, given RBC donated by a further vCJD case approximately 18 months before onset of clinical symptoms, had abnormal prion protein in lymphoid tissue at post-mortem (5-years post-transfusion) but had no clinical symptoms of vCJD. CJD cases with history of transfusion: Hospital records for 7/11 vCJD and 7/52 sCJD cases included a history of transfusion of labile blood components donated by 125 and 24 donors respectively. Two recipients who developed vCJD were linked to donors who had already appeared on the NCJDSU register as vCJD cases (see above). No further links were established.

Conclusion This study has identified three instances of probable transfusion transmission of vCJD infection, including two confirmed clinical cases and one pre- or sub-clinical infection. This study has not provided evidence, to date, of transmission of sCJD or fCJD by blood transfusion, but data on these forms of diseases are limited.

Key words: blood, CJD, familial, sporadic, transfusion variant.

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Introduction

Until 2004, it was generally accepted that Creutzfeldt–Jakob disease (CJD) had not been transmitted by blood transfusion.

Preliminary findings from sheep studies indicate that bovine spongiform encephalopathy (BSE) and scrapie can be transmitted by blood transfusion [1,2]. It is vital to find out whether this also applies to human transmissible spongiform encephalopathies (TSEs) and, in particular, variant CJD (vCJD). The UK is the only country where a significant outbreak of vCJD has occurred and is in a unique position to study this question which has important implications for public health policy. The results reported in this paper are from a study which is being carried out, with ethical approval, to investigate whether or not there is any evidence for the transmission of any type of CJD (sporadic, familial and variant) by blood transfusion.

Materials and methods

CJD surveillance

A surveillance system for CJD, the National CJD Surveillance Unit (NCJDSU), was established in the UK in 1990 with the aim of identifying all cases of CJD in the UK. The methodology of this study has been described previously [3], but in brief involves referral of suspected cases to the Unit from targeted professional groups, including neurologists and neuropathologists; review of suspects by a neurologist from the Unit and review of investigation results and neuropathological material when available. Cases are classified according to standard diagnostic criteria [4,5]. Onset of clinical symptoms for vCJD cases are estimated to the nearest month by NCJDSU on the basis of available clinical information. Details of past medical history, including blood donation or transfusion, are obtained from the family of suspected cases. Following the identification of vCJD in 1996 a collaborative study, the Transfusion Medicine Epidemiological Review (TMER), was established between the NCJDSU and UK Blood Services (UKBS) to search for evidence of transfusion transmission of CJD. The study was granted ethical approval by the local Research Ethics Committee.

Notification of CJD cases with a history of donation

Sporadic CJD (sCJD) and familial CJD (fCJD) cases with a history of blood donation are notified to UKBS retrospectively. For vCJD, all patients who are old enough to have donated blood (> 17 years of age) are notified to UKBS at diagnosis, whether or not there is a known history of blood donation. Upon receipt of notification from the NCJDSU, a search is made for donor records. Current computer databases and archived records (computerized and paper-based records where appropriate) based at individual blood centres are searched using name, date of birth, and previous addresses as identifiers. For CJD cases reported as blood donors, information on dates and places of donation is also used to help locate past donor records. Where donor records are found, all components produced and issued to hospitals are identified and their fate determined, as recorded in hospital blood transfusion laboratory records. Recipient details are then checked against the NCJDSU register to establish if there is a match between these individuals and patients who have developed CJD. Recipients details are also flagged with the Office for National Statistics (ONS) to establish date and certified cause of death.

Plasma used for UK fractionation

Independent of this study, and for regulatory reasons, all plasma derived from donations made prior to a diagnosis of vCJD is notified to UK fractionators so that appropriate actions can be taken.

Notification of CJD cases with a history of transfusion

Information provided by relatives of CJD cases with a previous transfusion history is also passed to UKBS, who then liaise with appropriate hospital blood transfusion laboratories. The laboratories identify whether transfusions took place at the time and place indicated, and, if so, identify the components transfused to the case. Details of donation numbers, component type and date transfused are passed back to the local blood centre and this information is used to identify the donors. Donor details are checked against the NCJDSU register and with ONS. For both donors and recipients, searches before 1980 are now impractical as most hospital records are no longer extant.

Related public health measures

The Department of Health (England) set up a committee (the CJD Incidents Panel) in 2000 to advise health authorities on the management of incidents where patients may have been put at risk of CJD through medical procedures. Cases where a blood donor or blood recipient has later developed vCJD have been referred to the Incidents Panel for consideration and further actions have been recommended and implemented. These further actions are outside the scope of this study and are not reported in this paper.

Results

vCJD cases with history of blood donation

Identification of donors

A total of 150 vCJD cases (out of a total of 160 cases on the NCJDSU register) who were old enough to have been potential blood donors have been notified to UKBS as of 1 March

Table 1 Recipients of blood donated by variant Creutzfeldt–Jakob disease cases by year and blood component transfused ($n = 66$)

Year of transfusion	Blood component transfused	Number of recipients
1980–1984	Whole blood	1
	Red blood cells	1
1985–1989	Red blood cells	2
1990–1994	Red blood cells	9
1995–1999	Whole blood	1
	Red blood cells	15
	Red blood cells – buffy coat depleted[a]	2
	Red blood cells – leucodepleted[b]	2
	Fresh frozen plasma	3
	Cryo-depleted plasma	1
	Cryoprecipitate	1
	Platelets (pooled)	1
2000–2004	Red blood cells – leucodepleted	23
	Fresh frozen plasma – leucodepleted	2
	Platelets (pooled, leucodepleted)	2

[a]Red cells with buffy-coat (containing most of the platelets and white cells) removed by centrifugation and physical separation.
[b]Red cells leucocyte-depleted by pre-storage filtration to $< 5 \times 10^6$/unit according to UK guidelines [6].

2006. Of these, 31 of 150 (21%) were reported to have been blood donors at various times in the past, although there is variation in the details of available information and the confidence of families in donation history.

Donor records were found for 24 vCJD cases, comprising 20 reported by relatives as blood donors and four additional cases with no reported donation history. Of these, 18 vCJD cases (12% of the total eligible to donate blood) were confirmed to have donated labile blood components, with the number of components made and issued for use in UK hospitals ranging from 1 to 14 per donor. Six vCJD cases were registered as donors, but had not donated labile blood components. Two of these had never attended sessions, three were deferred (due to past medical history, low haemoglobin value and illness, respectively) and one case had donated plasma for fractionation only (made from a single donation from which the red cells were discarded).

The search for donor records was negative in 11 of 31 (35%) vCJD cases reported as putative donors (three of whom allegedly donated well before the onset of the BSE epidemic in the 1980s). The information provided in these negative cases was minimal, except in one case where relatives were confident that regular donations (up to 50) had been made in the years leading up to 1993. Despite extensive searches no records were found; moreover, blood collection sessions had never been made at the purported venue. No explanation has been found for the lack of records, although discrepancies in some of the details given suggest that the history was not as certain as initially thought.

Labile components issued to hospitals

Sixty-six labile components originating from 18 donors were issued to UK hospitals over the period 1981–2004 and transfused to patients according to blood transfusion laboratory records. A further nine components issued between 1982 and 1996 could not be traced by the relevant hospital. Table 1 gives the number of recipients transfused by year and the type of blood component transfused. Fifty-six recipients (85%) received red cells or whole blood, seven (11%) were transfused with labile plasma components or derivatives and three (4%) received pooled platelets made according to UK specifications in which the buffy-coat preparation containing platelets from the implicated vCJD donor was pooled with buffy coats from three other donors and resuspended in plasma from one of the four donations. Nearly half of the red cell recipients received red cells that had been leucocyte-depleted by pre-storage filtration to $< 5 \times 10^6$ leucocytes per unit (in 99% of units with 95% statistical confidence according to UK guidelines [6]) after the introduction of universal leucocyte depletion of the UK blood supply in 1999.

Recipients of blood components

Patient identifiers are available for 66 recipients who received blood from 18 different donors who went on to develop vCJD. None of the 66 recipients had themselves donated blood between receiving their transfusion and early 2004 when the UKBS implemented a policy of excluding all donors transfused in the UK since 1 January 1980. It is of note that 41 (62%) recipients were aged over 60 years at the time of transfusion and were not eligible to donate. All living recipients ($n = 26$) have been informed of their risk and advised not to donate blood, tissues or organs. Three instances of probable transfusion transmitted vCJD infection have occurred, including two confirmed clinical cases and one pre- or subclinical infection. Of these, two cases have died, and one is still alive (see succeeding discussion). Figures 1 and 2 show the survival period for dead (transfusion to death) and live recipients (transfusion to 1 March 2006) of vCJD components, respectively, according to the interval between transfusion and onset of clinical symptoms in the donor.

Dead recipients

Forty recipients (61%) are known to be dead, with mean age at death 66 ± 19 years. Table 2 gives the time and cause of death as stated on death certificates for the recipients known to have died. Around half ($n = 21$) of the dead recipients died within a year of receiving their transfusion, with only seven surviving for more than 5 years. Two recipients, who died 4 months and 14 months, respectively, after transfusion had

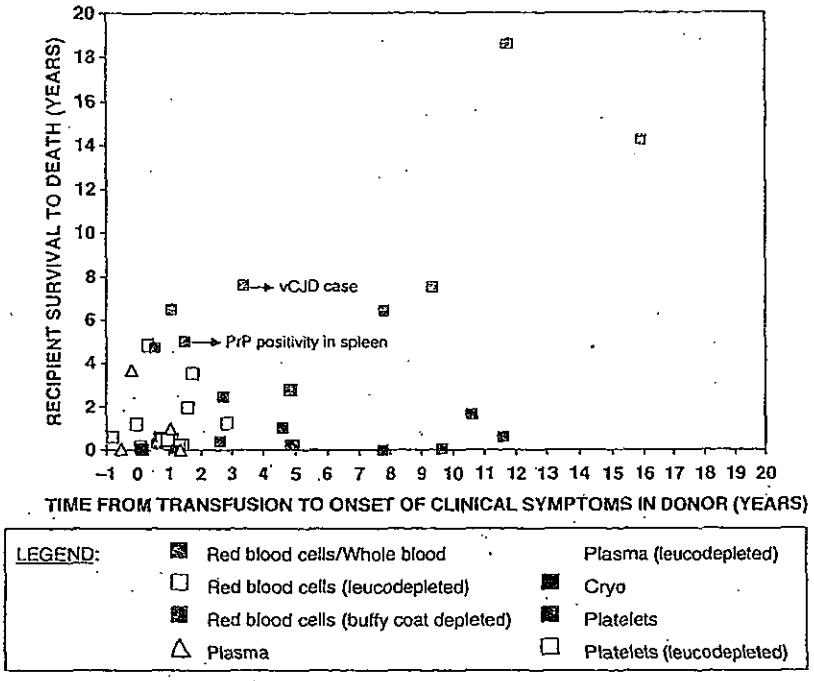

Fig. 1 Survival period (transfusion to death) for recipients of variant Creutzfeldt–Jakob disease components according to interval between transfusion and onset of clinical symptoms in the donor.

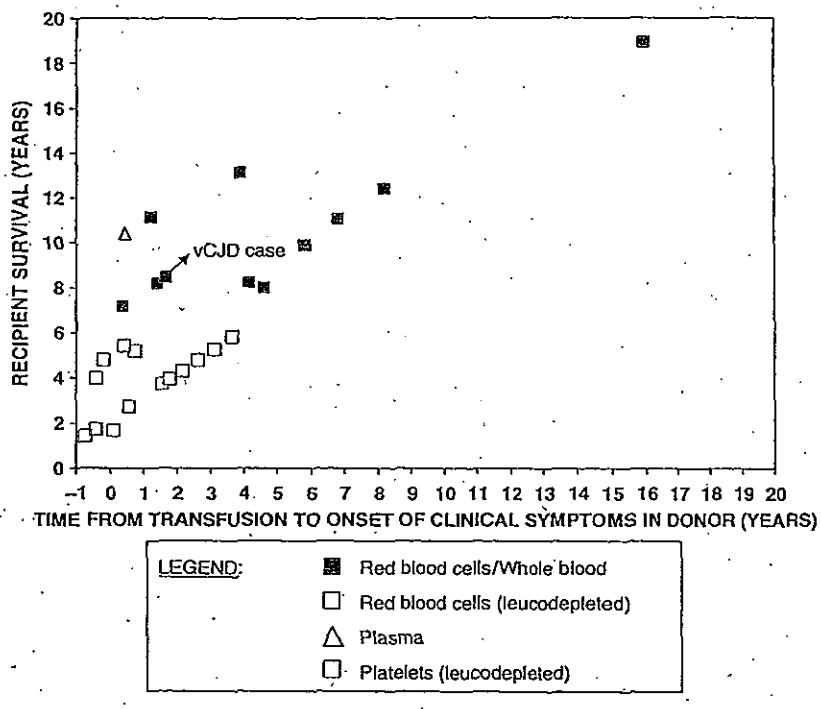

Fig. 2 Current survival period (since transfusion to 1 March 2006) for recipients of variant Creutzfeldt–Jakob disease components according to interval between transfusion and onset of clinical symptoms in the donor.

'dementia' recorded on the death certificate, but examination of case notes indicated that neither case had features to suggest vCJD. All the other recipients were certified as dying of causes unrelated to vCJD, except for a recipient whose cause of death on the death certificate was recorded as '1A dementia and II. prostate cancer' and was later confirmed neuropathologically as suffering from vCJD [7]. This patient, who had received a transfusion of red cells 6.5 years before onset of clinical symptoms, was a methionine homozygote at codon 129 of the human prion protein gene (*PRNP*). The case that donated to this individual also had a neuropathological diagnosis of vCJD, with clinical onset approximately 40 months after donating. In a second red cell recipient (of a different donor who developed clinical symptoms approximately

Table 2 Cause of death of variant Creutzfeldt–Jakob disease recipients known to have died (n = 40)

Interval from transfusion to death	Number of recipients	Cause of death
< 1 month	7	Acute renal cortical necrosis
		Cancer (2)
		Myocardial infarction
		Septicaemia (2)
		Sepsis (pancreatitis)
1– < 6 months	11	Aspiration pneumonia (sigmoid resection)
		Cancer (4)
		Myelodysplasia (2)
		Myelofibrosis
		Peritonitis (2)
		Stroke/diabetes mellitus/dementia
6– < 12 months	3	Cancer (3)
1– < 5 years	12	Acute myeloid leukaemia (2)
		Bronchopneumonia/senile dementia
		Cancer
		Ischaemic heart disease (3)
		Chronic obstructive airways disease (COAD)
		Hypertensive heart disease, chronic renal failure
		Myelodysplasia
		Disseminated sepsis
		Spinal haemangioblastoma
5– < 10 years	5	Cerebrovascular accident
		Ischaemic heart disease
		Acute lymphoblastic leukaemia
		Dementia[a], prostatic cancer
		Ruptured aortic abdominal aneurysm/severe atheroma/COAD[b]
≥ 10 years	2	Bronchopneumonia
		Ischaemic heart disease

[a]Confirmed variant Creutzfeldt–Jakob disease case [7].
[b]PrP positivity in lymphoid tissue, pre- or subclinical vCJD infection [8].

18 months after donating and was later diagnosed with neuropathologically confirmed vCJD), protease-resistant prion protein (PrP^{res}) was detected in the spleen and one lymph node (but not in the brain) at post-mortem [8]. This recipient, who died 5 years after transfusion without any clinical symptoms of vCJD, was a codon 129 *PRNP* heterozygote and is thought to represent pre- or subclinical infection.

Live recipients

Twenty-six recipients (39%) are alive as of 1 March 2006 with a mean age of 63 ± 19 years. Table 3 shows the number of live recipients according to the time elapsed since transfusion, along with their current age, component transfused and the interval between donation and onset of clinical symptoms of vCJD in the donor. Fifty per cent of live recipients were transfused with components from vCJD donors whose donations were made within 20 months of clinical onset, in seven cases around the time of development (n = 3) or shortly after (n = 4) the first signs of clinical illness. These cases would have appeared healthy when attending donor sessions and passed the normal medical checks as being fit to donate. Sixteen recipients have survived longer than 5 years, with six surviving > 10 years (one for over 18 years). These patients, mean age currently 61 ± 19 years, were given blood from donors who developed vCJD symptoms at intervals ranging from around 5 months to 191 months after making the donation (see Table 3). Recently, a diagnosis of probable vCJD has been made in one of these surviving recipients who had received a transfusion of red cells 7 years and 10 months before onset of clinical symptoms [9]. The donor of this third probable transfusion-transmitted vCJD infection developed vCJD approximately 21 months after the donation, and the recipient is a codon 129 *PRNP* methonine homozygote.

Plasma for UK fractionation

Twenty-five units of plasma originating from 11 different donors, bled between 6 months and 17 years, 11 months before onset of clinical vCJD symptoms, were supplied for UK fractionation during the period 1986–1998. Product batches manufactured from 23 plasma units derived from nine donors have been traced. The fate of batches of product derived from the two remaining plasma donations, from two different donors, has not yet been traced, and this search is still ongoing. Table 4 lists the plasma products derived from the 23 traced donations and the number of batches implicated, divided into risk categories as used in the plasma product notification exercise (www.hpa.org.uk/infections/topics_az/cjd/Recommendations.pdf). The fate of batches of products has not been traced to individual recipients as part of this study. It is known, however, that haemophilia centres have traced the ultimate fate of the batches of factor VIII. It is also known that no case of vCJD has been identified in a patient with haemophilia in the UK.

sCJD cases with history of blood donation

Ninety-three cases of sCJD identified between 1980 and 2000 were reported to have been blood donors, with only 38 reported to have donated from 1980 onwards. Donation records for most sCJD cases were untraceable since most dated back many years before 1980, in some cases to the 1940s. Donation records were found for eight sCJD cases; but only three had actually donated labile blood components for hospital use (one with 18 recipients, and one each with one recipient) which could be traced to recipients. A total of 20 recipients were transfused between 1995 and 1999 with components from these three donors who went on to develop

Table 3 Live recipients of labile blood components donated by variant Creutzfeldt–Jakob disease cases (n = 26)

Time elapsed since transfusion[a]	Current age of recipient (years)[a]	Blood component transfused	Interval between blood donation and onset of clinical symptoms in donor (months)[b]
1 – < 2 years	48	Platelets (leucodepleted)	–9 months
	49	Red cells (leucodepleted)	–5 months
	83	Red cells (leucodepleted)	2 months
2 – < 3 years	38	Red cells (leucodepleted)	7 months
3 – < 4 years	58	Red cells (leucodepleted)	19 months
	83	Red cells (leucodepleted)	22 months
	90	Red cells (leucodepleted)	–5 months
4 – < 5 years	59	Red cells (leucodepleted)	26 months
	67	Red cells (leucodepleted)	–2 months
	89	Red cells (leucodepleted)	32 months
5 – < 6 years	30	Red cells (leucodepleted)	37 months
	52	Red cells (leucodepleted)	9 months
	64	Red cells (leucodepleted)	44 months
	71	Red cells (leucodepleted)	5 months
6 – < 7 years	–	–	–
7 – < 8 years	42	Red cells	5 months
8 – < 9 years	31[c]	Red cells	21 months
	74	Red cells	17 months
	76	Red cells	49 months
	87	Red cells	55 months
9 – < 10 years	75	Red cells	70 months
> 10 years	33	Cryo-depleted plasma	7 months
	49	Red cells	15 months
	67	Red cells	46 months
	70	Red cells	191 months
	75	Red cells	98 months
	87	Red cells	82 months

[a]As at 1 March 2006.

[b]A negative interval denotes that donation was made by individual while (retrospectively recognized) clinical symptoms were present.

[c]Probable variant Creutzfeldt–Jakob disease case [9].

sCJD between 1 and 5 years after donation. Of these, 11 (55%) received red cell components, eight recipients (40%) received platelets and one (5%) received fresh frozen plasma.

As of 1 March 2006, 12 recipients are confirmed dead with a mean age at death of 74 ± 15 years. Of these, five died soon after transfusion (four within a week, and one 2 months later) and seven survived for between 1 and 8 years after receiving their transfusion before dying of a variety of non-CJD-related causes (cerebrovascular accident/stroke, n = 3; acute myeloid leukaemia, n = 3, general debility/old age, n = 1). Seven recipients are not known to be dead from ONS flagging to date, and are therefore presumed to be alive. The mean age of these seven recipients is 58 ± 19 years. The time elapsed since their transfusion ranges from 7 to 9 years. The fate of a further recipient is unknown. None of the sCJD recipients identified as having received blood from donors who went on to develop sCJD have appeared on the NCJDSU register to date.

fCJD cases with history of blood donation

Donation records were found for three out of five cases of fCJD identified between 1992 and 2000, all reported to have donated blood after 1980. These three cases had all donated labile blood components (one with five recipients, one with four recipients and one with two recipients) for hospital use which could be traced to individual recipients. A total of 11 recipients were transfused between 1977 and 1992 with labile components from these three donors who went on to develop fCJD between 1 and 15 years later. Nine of the 11 (82%) recipients received red cell components (whole blood, n = 6, red cells n = 3) while two received platelets.

Five of 11 recipients identified have since died with a mean age at death of 75 ± 6 years. Three of these survived for 3, 10 and 17 years after transfusion before dying of non-CJD-related causes (cancer, n = 2; bronchopneumonia, n = 1); and two died of cancer shortly after receiving their transfusion.

Table 4 Product batches made by UK fractionators derived from plasma donated by individuals who later developed variant Creutzfeldt–Jakob disease[a,b]

Infectivity Classification[c]	Plasma product	Number of implicated batches
Low	Factor VIII (excipient[d])	77
	Albumin 20%	21
	i.m. immunoglobulin	12
Medium	*Albumin 4·5%*	28
	i.v. immunoglobulin	11
High	Factor VIII	16
	Factor IX	8
	Anti-thrombin	1
	TOTAL	174

[a]Twenty-three plasma donations from nine variant Creutzfeldt–Jakob disease donors, data courtesy of Health Protection Agency.
[b]Excludes fate of two plasma units from two further vCJD cases (see text for explanation).
[c]Risk categories as used in plasma product notification exercise.
[d]Albumin from implicated plasma donation used as excipient (inert substance added to provide bulk) in preparation of batch of Factor VIII.

Three recipients are not known to be dead from ONS flagging to date, and are therefore presumed to be alive. The mean age of these three recipients is 44 ± 20 years. The time elapsed since their transfusion ranges from 13 to 21 years. The fate of a further three recipients is not known. None of the fCJD recipients identified as having received blood from donors who went on to develop fCJD have appeared on the NCJDSU register to date.

vCJD cases with history of transfusion

Eleven vCJD cases were reported to have received past blood transfusions between 1962 and 1999. A further case received a blood transfusion after onset of illness. This case is excluded from further analysis. For two cases, hospital records showed that they had not been transfused. No hospital records could be found for another two cases reported to have been transfused in 1962 and 1971, respectively. Hospital transfusion records were found for seven vCJD cases (64% of those reported as transfused) who had been transfused with components donated by 125 donors (121 identified), with one vCJD case, who also received a solid organ transplant, receiving components from 103 donors. The identity of four donors who donated red cell/whole blood components to two cases (case 2 and case 7, see Table 5) is unknown. Table 5 shows the transfusion date, number of donors and blood components donated, and the interval from transfusion to onset of clinical symptoms of vCJD in these seven recipients. These cases had been exposed to between two and 103 donors, respectively (NB search for donors to case 6 is incomplete). To date, one donor who gave red cells to case 5 and another donor who gave red cells to case 6 are also registered on the NCJDSU database as vCJD cases. These are the donors of the two clinical cases of transfusion-transmitted vCJD referred to previously (see vCJD cases with history of donation).

sCJD cases with history of transfusion

Fifty-two cases of sCJD identified between 1980 and 2000 were reported to have received a blood transfusion, of which 28 received a transfusion after 1980. Transfusion records were found for seven sCJD cases transfused between 1984 and 1997. Donor details were found for 24 donors who donated components transfused to these seven sCJD cases. One of these donors is known to have died, with a cause of death not related to CJD. Twenty donors are not known to have died from ONS flagging to date, and are therefore presumed to be alive. The fate of a further three donors is not known. The mean age of the donors presumed still alive is 51 ± 9 years. None of the traced donors who gave blood to patients who were subsequently diagnosed with sCJD have appeared on the NCJDSU register to date.

fCJD cases with history of transfusion

One case of fCJD identified in 1992 was reported to have received three blood transfusions in 1965, 1970, and 1987 none of which could be traced.

Discussion

This study has identified three instances in which a recipient of a transfusion derived from a 'vCJD' donor has developed infection with vCJD, including two clinical cases and one pre- or subclinical infection [7–9]. These are three different donor/recipient pairs. In view of the small size of the total at-risk recipient population ($n = 66$) and the background mortality rate for vCJD in the general UK population (0·24/million/annum), these observations provide strong evidence that vCJD can be transmitted from person to person through blood transfusion. This finding has had important implications for public health policy nationally and internationally.

The risk of developing vCJD infection in the surviving recipient population is significant but cannot be precisely estimated because of variables including the timing of blood donation in relation to clinical onset in the donor, the influence of the codon 129 genotype of donor and recipient and the effect of the introduction of leucodepletion in 1999. Furthermore, the currently observed number of infections in the recipient population may be an underestimate as some surviving recipients may yet develop vCJD and there is limited available information on the outcome in the cohort of

Table 5 Donors (n = 125) of labile blood components given to variant Creutzfeldt–Jakob disease cases[a] (n = 7) with identifiable past hospital transfusion records

Case	Transfusion date	Number of donors of labile blood components transfused	Blood component donated to vCJD recipient	Interval from transfusion to onset of illness
1	1993	38	Cryoprecipitate (4) Fresh frozen plasma (11) Platelets (8) Red cells (14) Whole blood (1)	4 years, 9 months
1	1993	65	Cryoprecipitate (12) Fresh frozen plasma (25) Platelets (17) Red cells (11)	4 years, 6 months
2	1983	2[b]	Red cells	15 years, 11 months
2	1993	3	Fresh frozen plasma	6 years, 3 months
3	1994	4	Red blood cells	5 years, 4 months
4[c]	1999	5	Red blood cells (2) Red blood cells (Leucocyte-depleted) (3)	8 months
5[a]	1996	5[d]	Red blood cells	6 years, 6 months
6[a]	1997	1+[e]	Red blood cells	7 years, 10 months
7	1982	2[b]	Whole blood	13 years, 11 months

[a]Two of these cases linked to donors already on the National CJD Surveillance Unit (NCJDSU) register as vCJD cases [7,9].
[b]Component details traced, but donors not identifiable.
[c]Timing of clinical illness excludes blood transfusion as the source of infection in this case.
[d]One of the donors already on NCJDSU register as vCJD case, others presumed not to be source of infection.
[e]One donor already on NCJDSU register as vCJD case. Search for 40+ donors to Case 6 not complete, as of 1 March 2006.

deceased recipients; a significant proportion of these individuals may not have survived long enough to express clinical disease even if infected. The minimum incubation period in CJD transmitted from person to person by a peripheral route is 4·5 years in kuru and growth-hormone-related CJD [10,11] and only nine deceased recipients survived for longer than this period. An investigation of the hospital records of the deceased recipients is underway, and to date, none had clinical features of vCJD pre-mortem. However, the identification of the individual with 'preclinical' vCJD infection was dependent on post-mortem examination of peripheral lymphoreticular tissues, and, to date, no equivalent tissues have been available in the deceased transfusion recipients. Extrapolating from the three observed infections in the total recipient population is likely to lead to an underestimate of the overall risk of transfusion transmission of vCJD, although the introduction of leucodepletion in 1999 may have reduced the risk to recipients transfused after this date.

A further important variable in estimating individual risk is the time from blood donation to clinical onset in the donor and, although evidence from animal studies in relation to this issue is conflicting [12–14], it is likely that an extended gap between blood donation and clinical onset in the donor will reduce the risk of transfusion transmission. All tested clinical cases of vCJD have been methionine homozygotes at codon 129 of *PRNP*, but the individual with 'pre-clinical' transfusion transmitted infection was heterozygous at this locus [8], indicating that individuals with this genotype are susceptible to secondary infection with vCJD. Except for the three cases infected through blood transfusion, the codon 129 genotypes of the recipient population are not known. Although the relative risk of secondary infection in relation to the codon 129 genotype is uncertain, a recent study in a transgenic mouse model suggests that individuals with all human codon 129 genotypes may be susceptible to secondary infection with vCJD, with a hierarchy of risk from methionine homozygotes to heterozygotes to valine homozygotes [15]. Risk may vary according to genetic background, but it cannot be assumed that some recipients will possess an absolute genetic barrier to infection.

The analysis of vCJD cases with a history of blood transfusion has identified over 100 donors to these cases, although the great majority were linked to one vCJD case who had undergone an organ transplant. A risk assessment has suggested that these donors are themselves at significant risk of developing vCJD and these individuals have been informed of this risk and have been advized not to act as blood or organ donors. To date, none of these individuals have developed vCJD, with the exception of the two donors linked to the two clinical cases of vCJD described above.

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Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood

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In 1999, the UK implemented universal leucoreduction as a precaution against transmission of variant Creutzfeldt-Jakob disease by transfusion of domestic blood or red blood cells. We aimed to assess how effectively leucoreduction reduced infectivity of transmissible spongiform encephalopathies (TSEs) in blood. 450 mL of whole blood collected and pooled from scrapie-infected hamsters was leucoreduced with a commercial filter. Blood cell concentrations were quantified, and infectivity titres measured. Blood cell recovery and white blood cell removal complied with American Association of Blood Banks standards. Leucofiltration removed 42% (SD 12) of the total TSE infectivity in endogenously infected blood. Leucoreduction is necessary for the removal of white-cell-associated TSE infectivity from blood; however, it is not, by itself, sufficient to remove all blood-borne TSE infectivity.

Transmissible spongiform encephalopathies (TSEs) are fatal CNS infections that can incubate asymptomatically for a decade or more in human beings before the appearance of clinical disease. People in the asymptomatic phase of variant Creutzfeldt-Jakob disease (vCJD) appear healthy and donate blood with the same frequency as any healthy person. Transmission of vCJD by transfusion was recently recognised in Great Britain.[1] To reduce the risk of transfusion transmission of such diseases in human beings, the UK implemented universal leucoreduction of donated blood in 1999. This measure was based on the expectation that infectivity would be associated with white blood cells.[2] However, findings in blood from infected mice and hamsters suggested otherwise; at least 40% of the infectivity was plasma-associated, suggesting that leucoreduction would not eliminate infectivity (Rohwer laboratory, unpublished).[3] Other investigations showed no loss of infectivity when small amounts of TSE-infected plasma were passed through scaled-down filters.[4] Similarly, no significant removal of abnormal prion protein was detected when units of human whole blood, spiked with a microsomal fraction from TSE-infected brain, were passed through leucoreduction filters from any of the four major suppliers.[5] Because of reservations about the relevance of these experiments, none of these findings aroused concern.

We investigated the effectiveness of leucoreduction in removal of TSE infectivity from a human-sized unit of pooled hamster blood. To ensure that the 150 hamsters needed for a 450 mL blood pool were at the same symptomatic stage of disease (wobbling gait and head bobbing) for each of two separate experiments, 400 weanling golden Syrian hamsters (Harlan, Madison, WI, USA) were inoculated intracranially with 50 µL of brain homogenate containing about 250 infectious dose$_{50}$ (ID$_{50}$) of hamster-adapted scrapie-strain 263K. A low dose of infectivity was used to preclude re-isolation of the inoculum in the blood. This animal protocol was approved by the University of Maryland Institutional Animal Care and Use Committee.

We obtained two pools of blood from the hamsters, one at 106 days and one at 111 days after inoculation. Under carbon dioxide anaesthesia, 3·5 mL of blood was drawn from the right ventricle into 0·5 mL of CP2D anticoagulant. Care was taken not to touch any other tissue. Only perfect bleeds containing 12·5% CP2D with no visible clots were pooled.

Two in-line leucofiltration systems from Pall Corporation (Port Washington, NY, USA) were evaluated. We selected the Leukotrap WB collection set for the infectivity study because filtration and component separation of hamster blood was fully compliant with American Association of Blood Banks (AABB)[6] specifications, and required only two titrations for interpretation. The Leukotrap RC-PL system

	Volume (mL)*	White blood cells†		Red blood cells, total (% of total)	Platelets, total (% of total)
		Total (% of total)	Log$_{10}$ reduction		
Whole blood	448·5	$2·1\times10^{9}$ (100%)	0	$3·7\times10^{13}$ (100%)	$1·4\times10^{12}$ (100%)
Leucoreduced blood	424·2	$3·0\times10^{6}$ (0·15%)	2·9	$3·6\times10^{13}$ (100%)	$1·5\times10^{12}$ (100%)
Plasma	179	$3·0\times10^{5}$ (0·02%)	3·8	0 (0%)	$1·1\times10^{12}$ (8%)
Red blood cells + AS3	305·9	$2·0\times10^{6}$ (0·15%)	3	$3·1\times10^{13}$ (86%)	1×10^{11} (71%)

*Volume measurements were obtained by weight using experimentally determined densities of whole hamster blood, 1·04 g/mL. †Values are average of at least three separate microscopic determinations using a haemocytometer and by flow cytometric measurements with white cells stained with propidium iodide. AS3 is a preservative and stabiliser.

Table 1: Blood component cell numbers and volumes before and after leucoreduction

	Volume inoculated (mL)	Total animals inoculated	Total animals infected	Titre in ID/mL (SD)	Fractional distribution of infectivity
Whole blood	5·2	104	50	13·1 (1·6)	1
Leucoreduced blood	5·4	108	34	7·6 (1·2)	0·58

Titre and SD calculated from the Poisson distribution as described in the text.

Table 2: Concentration of TSE infectivity in whole and leucoreduced blood

approached, but did not fully achieve all specifications; furthermore, because more than one filter is involved, more titrations would have been required to evaluate the removal of infectivity.

For the infectivity study, 448·5 mL of CP2D-anticoagulated whole hamster blood was pooled into the whole-blood receiving bag of a Leukotrap WB collection set and processed within the 8-h time limit specified by the AABB. Filtration was done at room temperature under gravity with a 60-inch pressure head on the in-line WBF2 filter, and was completed in 30 min. After removal of a 19 mL sample of the leucoreduced whole blood for subsequent testing, the remainder was centrifuged at 4150 rpm (about 5000 g) for 8 min at room temperature in a Sorvall RC-3C centrifuge. The plasma fraction was expressed into a satellite in-line bag. A preservative and stabiliser, AS3, was added to the red blood cells. Samples of the pre-filtration whole blood, post-filtration whole blood, red blood cells, and plasma were removed for analysis of cell composition and for titration in animals.

Cellular composition of the blood was assessed with a HemaVet five-part differential cell counter calibrated for hamster blood cells (Drew Scientific, Oxford, CT, USA). The residual white blood cell concentrations in the leucoreduced samples were measured by manual count and flow cytometry.

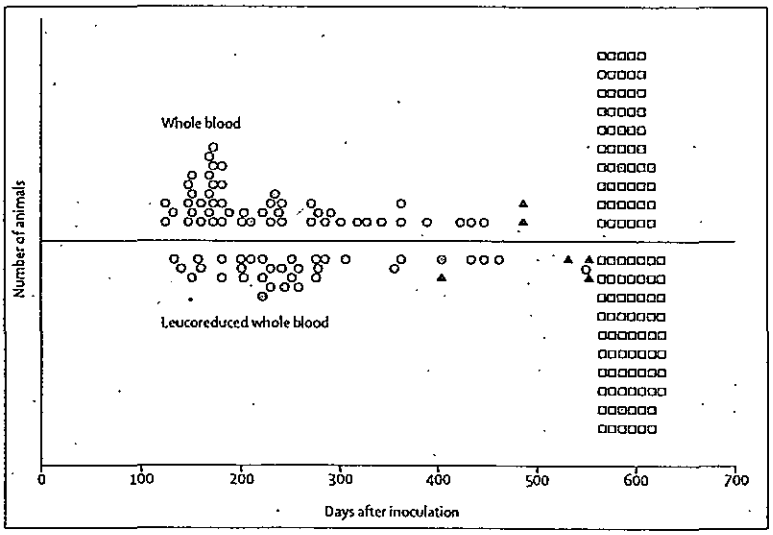

Figure: Incubation times of infections from whole and leucoreduced blood
Results of inoculations of whole blood are represented by data above the horizontal line; those from inoculations of leucoreduced blood are shown below the line. Circles represent infected animals. Squares represent uninfected animals that survived to the end of the experiment. Triangles represent animals that died intercurrently of causes other than the inoculum.

Infectivity of whole and leucoreduced blood was quantified by limiting dilution titration, a method developed in the Rohwer laboratory. The two samples were processed and inoculated separately and sequentially. Each sample of blood was sonicated with a separate sterile probe to lyse cells and disperse infectivity. It was then immediately inoculated intracranially, 50 µl at a time, into about 100 weanling golden Syrian hamsters that were deeply anaesthetised with pentobarbital. Animals were maintained for 566 days; those that contracted scrapie were killed when the clinical diagnosis was conclusive, and animals still alive at the end of the study were killed. All brains were tested for the presence of the proteinase K-resistant form of prion protein by western blot using 3F4 antibody.

The limiting dilution of an endpoint dilution titration is that at which not all of the inoculated animals become infected. At limiting dilution, the distribution of infectivity into individual inoculations is described by the Poisson distribution, where P(0)=probability of no infections at that dilution and inoculation volume, or (1–probability of infection). From the Poisson distribution $P(0)=e^{-(titre)}$ and titre=–ln(P[0]) expressed as ID/(inoculation volume). SD of the limiting dilution titre is the square root of the titre in ID/mL divided by the total volume inoculated in mL.

Table 1 shows the distribution of cells in each component of the scrapie-infected blood. Leucofiltration reduced the number of white blood cells by 2·9 log, thereby meeting the AABB standard. White cell contamination of the red blood cell fraction and red blood cell recovery were within AABB specifications of less than 5×10^6 and greater than 85%, respectively. Hamster platelets are not removed by the WBF2 filter, and partition with the red cells during centrifugation.

The incubation times of infections in each measurement are shown in the figure. At limiting dilution, incubation times begin at the end of the predictable dose-response seen in endpoint dilution titrations (about 140 days) and rarely extend beyond 500 days. All clinical and western blot results were consistent.

The limiting dilution titre of the whole blood pool (table 2) was close to the values from titrations of similar pools of whole blood by this method (unpublished data). Leucofiltration of whole blood removed only 42% (SD 12) of the initial TSE infectivity (table 2); of the 5900 ID present in the original unit of blood, 3400 ID were recovered in the leucofiltered blood.

Ideally, leucoreduction would be validated by measuring infectivity concentrations before and after leucoreduction of full units of vCJD-infected human blood. However, it is not currently possible to assay

either infectivity or the infection-specific form of the prion protein in human blood. By contrast, limiting dilution titration of rodent blood can detect less than 1 ID/mL of TSE infectivity and can readily show a difference of less 20% between samples. With this technique we did a study that: avoided the issue of spikes by using endogenously infected blood; avoided the question of scale by using a human-sized unit of fresh hamster blood obtained within the time limits specified for human blood; minimised the possibility of artefact by using a commercial blood collection set with integral filtration unit and a blood centre centrifuge and expressor; and achieved precision in the infectivity measurements by limiting dilution inoculation of 5 mL of each fraction. We assessed the performance of the filter by measuring the level of white blood cell reduction obtained and the cell recoveries of each component. The leucoreduction met or exceeded AABB specifications for all relevant variables.

Leucoreduction removed only 42% of the initial TSE infectivity from whole blood. This distribution is consistent with that obtained in a centrifugal separation of TSE-infected hamster whole blood, in which the buffy coat contained 70% of the total white cells but only 45% of the total whole blood infectivity (unpublished data). Both methods showed that a substantial proportion of the TSE infectivity was not associated with white cells. We have shown previously[7] that TSE infectivity is not associated with highly purified platelets, and we are currently testing purified red blood cells. We presume that the majority of blood-borne infectivity is plasma-associated.

Although leucoreduction is a necessary step for removing white-cell-associated TSE infectivity from blood, this process is insufficient to remove the risk from an infected transfusion unit. Due to the low concentration of TSE infectivity in blood and the absence of screening or inactivation alternatives, removal is an attractive strategy. However, the feasibility of removal depends upon the actual associations and distributions of TSE infectivity in blood itself, which can only be ascertained by assessment of endogenous blood-borne infectivity.

Contributors

The overall design and execution of the experiment, including management of the logistics and all the infectivity work, was by L Gregori and R G Rohwer with the assistance of the staff of the Molecular Neurovirology Laboratory. A Giulivi, N McCombie, D Palmer, and P Birch supplied expertise on blood centre operations, blood collection, component separation, leucoreduction, and quantitation of white blood cells. D Palmer and P Birch undertook and interpreted flow cytometry. S Coker supplied expertise on the use of the collection set and leucofilter.

Conflict of interest statement

R G Rohwer is a cofounder and part owner of Pathogen Removal and Diagnostics Technologies, which is developing technologies for the removal of TSE infectivity from blood and other materials. L Gregori receives contract support from Pathogen Removal and Diagnostics Technologies for studies on TSE removal. S Coker is an employee of Pall Corporation, which produces leucofilters and is developing TSE removal strategies for blood. The remaining authors declare that they have no competing financial interests.

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Predicting susceptibility and incubation time of human-to-human transmission of vCJD

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Summary

Background Identification of possible transmission of variant Creutzfeldt-Jakob disease (vCJD) via blood transfusion has caused concern over spread of the disease within the human population. We aimed to model iatrogenic spread to enable a comparison of transmission efficiencies of vCJD and bovine spongiform encephalopathy (BSE) and an assessment of the effect of the codon-129 polymorphism on human susceptibility.

Methods Mice were produced to express human or bovine prion protein (PrP) by direct replacement of the mouse *PrP* gene. Since the human *PrP* gene has variation at codon 129, with MM, VV, and MV genotypes, three inbred lines with an identical genetic background were produced to express human PrP with the codon-129 MM, MV, and VV genotypes. Mice were inoculated with BSE or vCJD and assessed for clinical and pathological signs of disease.

Findings BSE was transmitted to the bovine line but did not transmit to the human lines. By contrast, vCJD was transmitted to all three human lines with different pathological characteristics for each genotype and a gradation of transmission efficiency from MM to MV to VV.

Interpretation Transmission of BSE to human beings is probably restricted by the presence of a significant species barrier. However, there seems to be a substantially reduced barrier for human-to-human transmission of vCJD. Moreover, all individuals, irrespective of codon-129 genotype, could be susceptible to secondary transmission of vCJD through routes such as blood transfusion. A lengthy preclinical disease is predicted by these models, which may represent a risk for further disease transmission and thus a significant public-health issue.

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See Reflection and Reaction page 374

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Introduction

After the identification of variant Creutzfeldt-Jakob disease (vCJD) in 1996,[1] there have been many attempts to estimate the extent of the UK epidemic. Many individuals are likely to have been exposed to bovine spongiform encephalopathy (BSE) material through their diet; however, there have been only 161 cases of the disease in the UK. The predicted total number of future cases has ranged from the low hundreds[2] to hundreds of thousands.[3] However, findings from a retrospective immunocytochemical study that aimed to detect prion protein (PrP) in appendix and tonsil specimens suggested a prevalence of BSE infection of 237 per million people in the UK.[4] DNA sequence analysis of the PrP gene (*PRNP*) in vCJD has shown that 100% of tested cases are homozygous for methionine at the codon-129 polymorphism compared with about 40% of the general white population and about 70% of sporadic CJD cases. The methionine homozygous genotype (MM) has been included as a limiting variable in most mathematical predictions of the size of the epidemic.[2,3] Identification at autopsy of preclinical vCJD infection in a methionine/valine (MV) heterozygous individual who had received a transfusion of red cells from a donor who later died of vCJD, was the first indication that MM might not be the only susceptible genotype.[5]

Polymorphisms and mutations in *PRNP* in various species can affect disease susceptibility, although the precise mechanisms by which these effects are mediated have not been established.[6,7] Codon 129 of the human *PRNP* gene has been shown to affect the clinicopathological phenotype of disease in CJD and fatal familial insomnia.[8–13] Heterozygosity at *PRNP* codon 129, when compared with homozygous individuals, has been reported to lengthen incubation times in iatrogenic CJD cases associated with growth hormone treatment, and in kuru,[9,14] whereas valine homozygosity (VV) has been proposed to be protective for both BSE and vCJD transmission in studies that used murine models overexpressing human PrP.[15] At a molecular level, the biophysical properties of PrP refolding into the disease associated form (PrP^{Sc}) have been shown to be affected by the codon-129 genotype, with the methionine variant having an increased propensity to form PrP^{Sc}-like structures.[16]

We sought to analyse the transmission characteristics of BSE and vCJD to four inbred lines of transgenic mice after intracerebral inoculation with brain homogenate from cases of vCJD and BSE. We then aimed to use these models to address the apparent low level of vCJD in the human population resulting from exposure to BSE and to predict the potential for human-to-human spread of vCJD and the susceptibility of different genotypes in the human population.

Methods

Transgenic mice

Details of how the gene-targeted transgenic lines were created are supplied as supplementary information

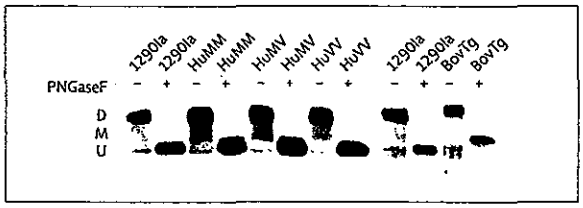

Figure 1: **Western blot of brain extract from uninoculated mice showing that PrP^{C} is detected with equivalent electrophoretic mobility and glycoform ratio in all three human transgenic lines**
D=diglycosylated PrP^{C} band; M=monoglycosylated PrP^{C} band; U=unglycosylated PrP^{C} band. In the BovTg line, a deglycosylated band is detected of increased molecular weight due to the additional N-terminal octapeptide repeat motif. Protein levels are similar to the wildtype line used in generating the transgenics (129Ola). Glycosylation is confirmed by the reduction to a single band after deglycosylation with the enzyme PNGaseF. The anti-PrP antibody 7A12 was used for the HumTg blot as it will react with both murine and human PrP, and 8H4 was used for the BovTg blot.

See Online for webappendix

(webappendix). Transgenic mice were anaesthetised with halothane and then injected with 0·02 mL of brain homogenate into the right cerebral hemisphere. The vCJD tissue homogenate (at 10^{-2} dilution) was supplied by the UK National Institute for Biological Standards and Control (Code NHBY0/0003). BSE-infected cattle brain (Veterinary Laboratories Agency, reference BBP 12/92) was prepared by maceration of the tissue in sterile saline to a dilution of 10^{-1}. From 100 days they were scored each week for signs of disease.[17] Mice were killed by cervical dislocation whether they had clinical signs of transmissible spongiform encephalopathy (TSE) or another non-specific disorder. The brain was recovered at post mortem. Half the brain was snap-frozen in liquid nitrogen for biochemical analysis and the remaining half was fixed for histology.

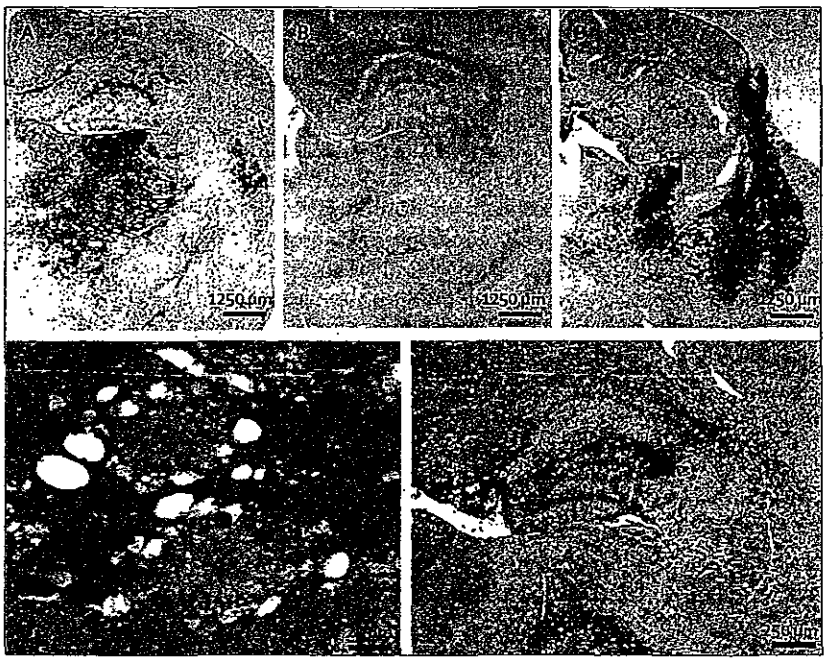

Figure 2: **Immunocytochemistry of histological sections with anti-PrP antibody 6H4 showing the cortex, hippocampal, and thalamic regions of the mouse brain with PrP detection (brown)**
A–D: Human transgenic mice with vCJD inoculum. A: HuMM mouse 693 days post inoculation. B: HuMV mouse 707 days post inoculation. C: HuVV mouse 693 days post inoculation. D: Florid plaques found in the hippocampus of the HuMM mouse in panel A. Each plaque has an eosinophilic core with a paler halo and is surrounded by a ring of vacuolation (haematoxylin and eosin stain). E: Hippocampal region of a BovTg mouse inoculated with BSE. PrP is deposited in a more diffuse/granular form with occasional plaques.

Procedures

Immunocytochemical detection of disease-associated PrP (PrP^{Sc}) deposits in the brain is a key pathological marker of TSE transmission, and variation in location and morphology of PrP^{Sc} deposits can be affected by both the strain of TSE agent and by the host PrP.[7,18] After fixation in 10% formal saline, brains were treated for 1·5 h in 98% formic acid (to reduce the titre of infectivity for safety reasons), cut transversely into four sections, and embedded in paraffin. We used the Vectastain Elite ABC Kit (Vector Labs, UK) with overnight primary antibody incubation (6H4 at 1:2000; Prionics, Switzerland) for PrP detection. Identification of antibody binding was through deposition of 3,3′-diaminobenzidine chromogen via a horseradish peroxidase reaction. The BSE-inoculated human transgenics were also studied using the Catalysed Signal Amplification kit (DAKO K1500). This kit uses the same principles as the Vector Labs kit, but has an additional step, which amplifies the final detected signal and therefore improves sensitivity.

Scoring of the abundance and location of TSE-associated vacuolation in grey and white matter of the brain is routinely used for diagnosis and strain classification in non-transgenic mice[17,19] and was used to assess all the mice in this study. TSE-related vacuolation was assessed at nine grey-matter regions and three white-matter regions to produce a lesion profile, as previously described.[20,21]

Analysis

Frozen brain samples from the human transgenic mice were homogenised in 0·9% saline to give a 10% suspension. This material was cleared by centrifugation and the supernatant treated with 0·05 g/L proteinase K for 1 h at 37°C, as previously described in detail.[22] The digested product was denatured then loaded onto a 10% Bis/Tris NuPAGE Novex gel (Invitrogen, UK). After electrophoresis the gel was blotted onto polyvinylidine difluoride (PVDF) membrane. We used the ECL+ technique (Amersham Biosciences, UK) with primary antibody 6H4 (Prionics, Switzerland) at 1:40000 and an anti-mouse IgG peroxidase-linked secondary (Amersham Biosciences, UK) at 1:40000 for the detection of PrP. Chemiluminescence was captured on radiographic film. Samples prepared for figure 1 were digested overnight at 37°C with 500 units of PNGaseF (New England Biolabs, UK) and not with proteinase K; the primary antibody was 7A12.[23]

Frozen brain samples from the bovine transgenic mice were homogenised in an NP40 buffer (0·5% v/v NP40,

98

0·5% w/v sodium deoxycholate, 0·9% w/v sodium chloride, 50mM Tris-HCl pH 7·5) to give a 10% suspension. This material was cleared by centrifugation and the supernatant digested with PNGaseF. The products were denatured then loaded onto a 12% Novex Tris/Glycine gel (Invitrogen, UK). After electrophoresis the gel was blotted onto PVDF membrane. PrP was identified with the SuperSignal West Dura chemiluminescence detection kit (Pierce, UK) with primary antibody 8H4[24] at 1:20000 and an anti-mouse IgG peroxidase-linked secondary (Jackson Immuno Research Laboratories, UK) at 1:10000. Images were captured on radiographic film and with a Kodak 440CF digital imager (figure 1).

Role of the funding source

The sponsors of this study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We first investigated the potential effects of the species barrier between BSE and human beings and any alteration in that barrier once BSE had passed through people in the form of vCJD. We then investigated the effect of the codon-129 polymorphism on human-to-human transmission of vCJD using gene-targeted inbred mice developed by direct replacement of the murine *PrP* gene for the human gene. These mice produce PrP under the control of the normal regulatory elements for PrP and thus express physiological concentrations of PrP with the correct tissue distribution (figure 1). Three inbred lines with an identical genetic background were produced to express human PrP with the codon-129 MM, MV, and VV genotypes (designated HuMM, HuMV, and HuVV, respectively). Each line differs by only a single codon in *PRNP* and in all other respects the mice were genetically identical. Additionally, in an identical manner, we produced mice that express bovine PrP to enable direct comparisons to be made not only between transgenic and wild-type mice, but also between each of the transgenic lines.

Typical clinical signs of TSE disease were seen in more than half (15/22) the BovTg mice inoculated with BSE material with a mean incubation period of 551 days (SD 47). These clinical cases were confirmed by a positive test for the presence of TSE vacuolation or PrP^{Sc} deposition by immunocytochemistry. The lesion profiles generated for targeting and degree of vacuolation showed similar patterns for all positive mice. Immunocytochemical data showed PrP^{Sc} deposition mainly in a diffuse and synaptic form, and also as plaque-like structures, frequently associated with areas of spongiform change (figure 2). Deposition was most abundant in the thalamus and hippocampus, but was recorded throughout other regions of the brain. The cerebral cortex showed only occasional plaque-like structures and the cerebellum had only a few areas of PrP^{Sc} deposition limited to the granule cell layer. Further pathological analysis was undertaken on mice that were culled for reasons other than clinical TSE (intercurrent deaths). This analysis showed that all the brains had pathological signs of TSE disease in terms of vacuolation or PrP deposition. Thus, all the bovine transgenic mice (22/22) seemed to be susceptible to BSE infection, although not all developed clinical signs of infection (tables 1 and 2).

HuMM, HuMV, and HuVV mice were inoculated with BSE material and after extensive pathological analysis all were confirmed as negative for TSE transmission (table 1). Mice of each genotype line were inoculated with vCJD material. Two pathologically confirmed clinically positive mice were seen in the HuMM line (at 497 and 630 days post inoculation), one in the HuMV line (at

	Clinically positive	Vacuolation positive	PrP positive*	Negative†
BovTg (n=22)				
0–400	0	3	6	0
401–500	1	1	0	0
501–600	10	11	5	0
>600	4	4	2	0
HuMM (n=18)				
0–400	0	0	0	4
401–500	0	0	0	5
501–600	0	0	0	2
>600	0	0	0	7
HuMV (n=23)				
0–400	0	0	0	3
401–500	0	0	0	6
501–600	0	0	0	4
>600	0	0	0	10
HuVV (n=22)				
0–400	0	0	0	9
401–500	0	0	0	4
501–600	0	0	0	7
>600	0	0	0	2

*Because most mice were positive by both clinical and vacuolation scoring not all mice were tested by immunocytochemistry for PrP deposition. †Negative by clinical or pathological analysis, or positive by clinical scoring but not confirmed by pathology.

Table 1: Clinical and pathological scoring of BovTg and human transgenic mice, by number of days after BSE inoculation

	BSE				vCJD		
	BovTg	HuMM	HuMV	HuVV	HuMM	HuMV	HuVV
Susceptibility	22/22	0/18	0/23	0/22	11/17	11/16	1/16

Positives confirmed by immunocytochemistry or lesion profile.

Table 2: Susceptibility to TSE disease comparison of BovTg and human transgenic mice inoculated with BSE or vCJD

	Clinically positive	Vacuolation positive	PrP positive	Negative*
HuMM (n=17)				
0–400	0	0	2	2
401–500	1	1	1	2
501–600	0	1	3	2
>600	1	4	5	0
HuMV (n=16)				
0–400	0	0	0	0
401–500	0	0	0	0
501–600	0	0	4	3
>600	1	1	7	2
HuVV (n=16)				
0–400	0	0	0	0
401–500	0	0	0	1
501–600	0	0	0	5
>600	0	1	1	9

*Negative by clinical or pathological analysis, or positive by clinical scoring but not confirmed by pathology.

Table 3: Clinical and pathological scoring of human transgenic mice, by number of days after vCJD inoculation

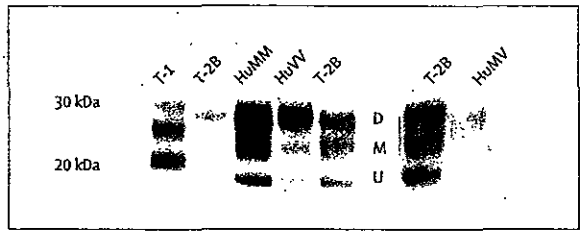

Figure 3: Western blots of brain extract from three transgenic lines inoculated with vCJD

D=diglycosylated PrP^{Sc} band; M=monoglycosylated PrP^{Sc} band; U=unglycosylated PrP^{Sc} band. T-2B corresponds to human vCJD brain homogenate showing the typical PrP^{Sc} type 2B and T-1 corresponds to human sCJD brain homogenate showing the typical PrP^{Sc} type 1 signature. Type 2B and 1 differ in mobility of the unglycosylated band (~19 kDa and ~20 kDa respectively) and the degree of glycosylation (diglycosylated dominant and mono/unglycosylated dominant respectively). All samples were treated with proteinase K. The anti-PrP detection antibody was 6H4. The HuMV and T2-B control blot had to be overexposed as the signal from the HuMV was weak, due to the low levels of PrP^{Sc} seen by immunocytochemistry.

665 days post inoculation), and none in the HuVV line (table 3). HuMM mice were more likely to show disease-associated vacuolation, beginning at around 500 days post inoculation. Six were scored positive and showed similar distribution of vacuolation in the brain, with the highest levels found in the dorsal medulla, thalamus, and cerebellar white matter. By contrast, only a single mouse in each of the HuMV and HuVV groups scored positive for vacuolation at approximately 700 days post inoculation.

Most of the HuMM mice (11/15) showed PrP^{Sc} deposition in most areas of the brain at a relatively early stage (from around 370 days post inoculation), before the vacuolar pathology became evident. From 500 days post inoculation the appearance of vacuolation was accompanied by a significant increase in PrP^{Sc} deposition. By contrast, although PrP^{Sc} deposition was identified in many HuMV mice (11/13), they had little deposition restricted to only a few areas (including the ventrolateral and ventromedial thalamic nuclei and the red nucleus of the mid-brain), even after 700 days post inoculation (figure 2, table 4). Although PrP^{Sc} deposition was clearly present at 581 days, the timing of initial onset of deposition in this line was not established.

Significant levels of PrP^{Sc} deposition were noted in the brain of the subclinical HuVV case. Indeed, these were similar in intensity to those observed in the clinical HuMM cases. Patterns of PrP deposition and plaque formation show differences among the three genotypes, including the presence of florid plaques only in the HuMM mice (table 4).

PrP^{Sc} found in vCJD brain is characterised by a 19 kDa non-glycosylated fragment and the predominance of the diglycosylated form (type 2B).[22] Both biochemical properties of PrP^{Sc} are maintained when vCJD is transmitted to the human transgenic mice, irrespective of their codon-129 genotype (figure 3). Preliminary densitometric analysis suggested that there was an increase in the diglycosylated form in the HuVV mouse compared with the HuMM mouse. Additionally, comparison of PrP^{Sc} from the BSE inoculum and brain material from BovTg mice also confirmed propagation of the predominantly diglycosylated glycoform signature of PrP^{Sc} associated with the BSE/vCJD agent strain (data not shown).

	HuMM	HuMV	HuVV
Vacuolation*	Thalamus (severe); cerebral cortex and hippocampus (mild); cerebellar cortex (minimal)	Thalamus, cerebral cortex, hippocampus, and cerebellar cortex (minimal)	Thalamus and cerebral cortex (severe); hippocampus (mild); cerebellar cortex (minimal)
Plaque formation*	Fibrillary amyloid plaques; florid and non-florid plaques in cerebral cortex and hippocampus; no evidence of plaques in cerebellum	No evidence of amyloid plaques	Amorphous non-fibrillary structures often forming into clusters in cerebral cortex and thalamus
PrP deposition†	Intense staining of plaques in hippocampus and cerebral cortex; plaque-like, pericellular, and amorphous deposits in the hippocampus; synaptic, peri-neuronal, and diffuse perivascular deposits in the thalamus	Occasional small plaque-like deposits and pericellular deposits in the thalamus	Strongly positive large amorphous deposits and clusters of plaques, small plaque-like structures, perivascular aggregates, and sub-pial deposits in the cerebral cortex and thalamus

*Analysed with haematoxilin and eosin staining. †Analysed with immunocytochemical techniques.

Table 4: Comparison of TSE-associated neuropathology in human transgenic mice inoculated with vCJD

Discussion

Although the cattle BSE epidemic in the UK has amounted to more than 180 000 cases since the 1980s, the extent of the human vCJD epidemic has so far remained limited with the total number of cases worldwide currently at 190. One explanation for this apparent discrepancy is that there exists a significant species barrier between cattle and human beings, which limits the susceptibility of the human population to BSE. The data shown here suggest that this could indeed be the case since BSE was readily transmissible to the bovine transgenic mice but not to the human transgenic mice. However, once BSE has passed through human beings in the form of vCJD, the transmissibility of this TSE strain is altered for the human population.

All the human transgenic lines inoculated with BSE were negative for TSE transmission, which suggests that either the human transgenic lines are relatively resistant to transmission of BSE or the incubation time is longer than the length of the experiment (approximately 700 days). BSE transmission previously observed by others, in human transgenic lines overexpressing the human prion protein, could be due to overexpression of the *PrP* gene and may not therefore give a true reflection of the species barrier between BSE and human beings.[15,25,26] This apparent resistance of human transgenic mice to BSE could be explained by a large species barrier and this in turn could explain the low number of vCJD cases in the human population.

vCJD was transmitted to all three human lines with different pathological characteristics for each genotype, and a gradation of transmission efficiency from MM to MV to VV. The greater transmission efficiency in HuMM mice suggests that homozygosity for methionine at codon 129 leads to earlier onset of TSE-related pathological features and clinical disease than for the other two genotypes. The differences in PrP^{Sc} deposition in the HuMM and HuMV lines suggest that the codon-129 polymorphism in human beings is likely to affect the distribution of PrP^{Sc} deposition in the brain. Moreover, the similar numbers that scored positive for PrP deposition in each of the MM and MV groups (11/15 and 11/13 respectively) suggest that the two genotypes might be equally susceptible to vCJD, but with different incubation periods. Titration experiments are needed to fully compare the susceptibility of each line. The single HuVV mouse positive for PrP^{Sc} shows that VV individuals may be susceptible to vCJD with very long incubation times, including a lengthy subclinical phase. Transmission studies from all three genotype mice are now underway to examine the infectious nature of the disease and determine any alterations in the strain characteristics on passage through human transgenic mice. By contrast with published data suggesting that VV individuals cannot propagate the vCJD biochemical phenotype,[15] the data presented here suggest that the PrP^{Sc} type will remain a useful diagnostic feature of secondary vCJD infection irrespective of codon-129 genotype, as has been observed for the two extant cases of transfusion-associated vCJD infection.[5,27]

Transmission of vCJD to the three lines of human transgenic mice indicates that the human population could be at significantly heightened risk of developing disease after iatrogenic exposure to vCJD. Secondary transmission of vCJD has partly removed the cattle-to-human species barrier and has resulted in an agent that can be transmitted from human to human with relative efficiency. Transmission studies in cynomolgus macaques provide further evidence for this agent adaptation as they show reduction in incubation times after serial passage of BSE.[28] Our BSE inoculation at 10^{-1} dilution was compared with vCJD inoculation at 10^{-2} because the latter inoculum was found to be toxic to the mice at 10^{-1}. Use of a higher dose of vCJD inoculum would have maintained or increased the transmission efficiency of vCJD and enhanced the current findings.

Our findings raise concerns relevant to the possibility of secondary transmission of vCJD through blood transfusion, fractionated blood products, or contaminated surgical instruments. For this study mice were injected intracerebrally, whereas the probable human exposure to these agents is by peripheral routes (eg, oral or intravenous), and thus human-to-human exposures might be significantly less efficient. However, it is difficult to know for sure what the practical implications might be in human beings. Peripheral route challenge is in progress; however, BSE transmission studies in primates have shown the intravenous route to be as efficient as the intracerebral route, with an extension of the incubation time.[28]

Although all cases of vCJD up to now have been observed in the MM genotype, this model of human-to-human vCJD transmission suggests that other genotypes are also susceptible. In our experimental setting, all *PRNP* codon-129 genotypes are susceptible to vCJD infection; however, progressive development of pathological TSE features (vacuolation and PrP deposition) is more rapid in the MM-genotype mice. An explanation for this finding might be provided by in-vitro conversion of recombinant human PrP by BSE and vCJD agents, which has shown that PrP with methionine at position 129 is more efficiently converted than PrP with valine, and that conversion by vCJD is significantly more efficient than by BSE.[29] Long incubation periods during which PrP^{Sc} is deposited predicts that, in human beings, infection could be present in all genotypes for a significant period before clinical onset. Incubation periods of more than 30 years have been reported in the human TSE disease kuru.[30]

The possibility that an MV or VV genotype could result in a phenotype distinct from that recognised in vCJD draws attention to the importance of systematic assessment of the clinical, genetic, pathological, and

biochemical features of all human prion diseases. Our findings indicate that for human-to-human vCJD infection it should be assumed that all codon-129 genotype individuals (not just MM) can be infected, that long incubation times can occur, and that a significant level of subclinical disease might be present in the population.

Contributors

MTB, PH, and CP did immunocytochemical and western blot analysis; JCM, NT, HNB, and LA produced the transgenic mouse lines; JWI supplied vCJD case material and reviewed the neuropathology; VT did the mouse inoculations; and MTB, PH, MWH, RGW, JWI, and JCM prepared the manuscript.

Conflicts of interest

We have no conflicts of interest.

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Prion diseases are efficiently transmitted by blood transfusion in sheep

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The emergence of variant Creutzfeld-Jakob disease, following on from the bovine spongiform encephalopathy (BSE) epidemic, led to concerns about the potential risk of iatrogenic transmission of disease by blood transfusion and the introduction of costly control measures to protect blood supplies. We previously reported preliminary data demonstrating the transmission of BSE and natural scrapie by blood transfusion in sheep. The final results of this experiment, reported here, give unexpectedly high transmission rates by transfusion of 36% for BSE and 43% for scrapie. A proportion of BSE-infected tranfusion recipients (3 of 8) survived for up to 7 years without showing clinical signs of disease. The majority of transmissions resulted from blood collected from donors at more than 50% of the estimated incubation period. The high transmission rates and relatively short and consistent incubation periods in clinically positive recipients suggest that infectivity titers in blood were substantial and/or that blood transfusion is an efficient method of transmission. This experiment has established the value of using sheep as a model for studying transmission of variant Creutzfeld-Jakob disease by blood products in humans. (Blood. 2008;112: 4739-4745)

Introduction

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases, which include Creutzfeld-Jakob disease (CJD) in man, scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle. A new variant of CJD (termed vCJD) was recognized in the United Kingdom in the mid-1990s, apparently as a result of transmission of BSE to humans.[1] To date, there have been 166 cases of vCJD recorded in the United Kingdom, as well as several cases in other countries. Human TSEs are characterized by long asymptomatic incubation periods (usually several years), and there is no reliable test for detecting infection before the onset of clinical disease. It is not known how many people in the United Kingdom harbor vCJD, although estimates based on screening of tonsil and appendix samples suggest there could be up to 4000.[2] These infected persons pose a risk of human-to-human transmission via blood transfusion or contaminated surgical instruments.

In patients with vCJD, there is widespread replication of the infectious agent and deposition of PrP^{Sc} (disease-associated form of prion protein) in lymphoreticular tissues, such as the tonsil, spleen, and lymph nodes, in contrast to sCJD, where lymphoreticular involvement is minimal.[3] The fact that lymphocytes continuously recirculate between blood and lymphoreticular tissues strongly suggests that the blood of vCJD patients is probably infectious. Data from rodent TSE models had shown that the highest levels of infectivity in blood were associated with leukocytes and, to a lesser extent, plasma.[4] As a result, costly control measures such as leukodepletion (filtration of blood and blood products to remove leukocytes) and importation of plasma were introduced to protect United Kingdom blood supplies, despite the limited data that were then available to judge the size of the risk and the efficacy of the control measures.

The potential for using sheep as a model for studying the risks of vCJD transmission by blood transfusion was highlighted by the similarity between the distribution of infectivity and PrP^{Sc} in sheep infected with TSEs and humans infected with vCJD.[5-7] One factor limiting the successful transmission of TSEs by blood in rodent models was the small volumes of blood that could be injected. In contrast, the relative similarity in size of sheep and humans means that volumes of blood comparable with those used in human transfusion practice can be collected from and transfused into sheep. Using this model, we previously reported preliminary results showing that both BSE and natural scrapie could be transmitted between sheep by blood transfusion.[8,9] Although scrapie is not thought to be transmissible to humans, it was included as a representative of infection acquired under field conditions, which may give different results to those obtained from experimentally infected animals. Our blood transfusion experiment in sheep is complete after 9 years, and this paper presents the full data from the study. The overall transmission rates for both scrapie and BSE are surprisingly high when factors such as the stage of infection and genetic background are taken into account, suggesting that blood transfusion represents an efficient route of transmission.

Methods

Donor and recipient sheep

The animal work was reviewed and approved by internal ethical review procedures at the Institute for Animal Health, United Kingdom, and carried out under the authority of Home Office Project Licences.

PrP genotypes of all sheep were confirmed by sequencing the coding region of the *PrP* gene[10] and are represented by single letter amino acid

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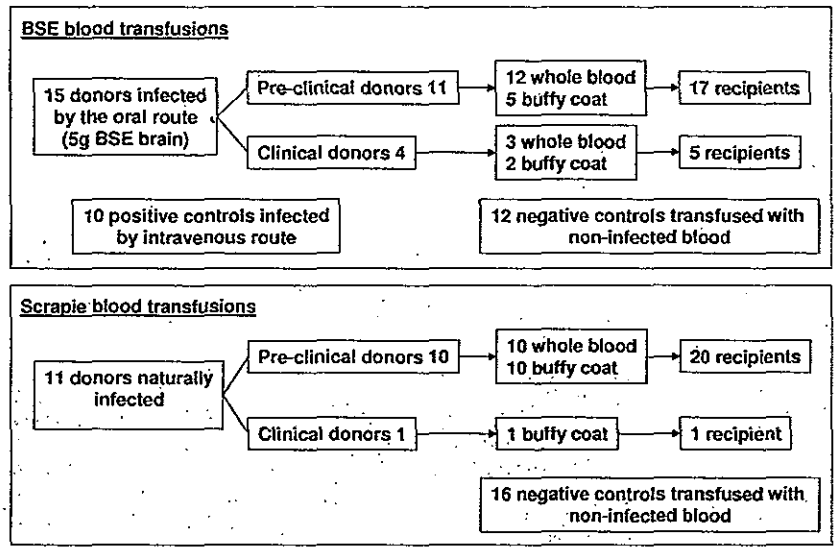

Figure 1. Overview of experimental design.

code for codons 136, 154, and 171, which have been linked to scrapie susceptibility (eg, ARQ represents alanine, arginine, and glutamine, respectively, at codons 136, 154, and 171).

All donor sheep were from the Edinburgh NPU Cheviot flock, which has endemic natural scrapie. The recipient sheep (including scrapie negative control donors) were Cheviots derived from the DEFRA scrapie-free (DEFRA/SF) flock of New Zealand origin. Transfusion recipients and positive and negative controls were housed in a purpose-built isolation unit on a different site to the donors, with strict procedures in place to minimize the risk of cross-contamination between groups, as described.[9] The sheep were scored at weekly intervals for clinical signs of TSEs and killed when they reached humane endpoints agreed with the Home Office. For experimentally inoculated animals (BSE donors, positive controls, and transfusion recipients), the incubation period (IP) in clinically positive sheep was defined as the period between the date of inoculation and the date of death. For scrapie-exposed donors, the IP in clinically positive sheep was defined as the age at death (ie, they were assumed to have become infected immediately after birth).

Blood collection and transfusion

Procedures for blood collection/transfusion were as previously described.[9] Briefly, venous blood (450-500 mL = 1 unit) was collected into sterile collection bags (NBPI-Fresenius, Emmer-Compascuum, The Netherlands) containing citrate phosphate dextrose adenine solution as anticoagulant. From donors that were about to be killed, 2 units was collected just before postmortem, whereas from donors that were to be left alive, separate collections of 1 unit were made at least 28 days apart. However, for practical reasons, it was not always possible to collect 2 units of blood from every donor sheep. In most cases where 2 units of blood was obtained, one was transfused as whole blood (without leukodepletion) and the other was used to prepare a buffy coat fraction.

BSE blood transfusions

Fifteen sheep experimentally inoculated either orally (n = 14) or intracerebrally (n = 1) with 5 g or 0.05 g, respectively, of BSE-infected cattle brain homogenate were used as blood donors. The donor *PrP* genotypes were ARQ/ARQ (n = 3), ARQ/AHQ (n = 5), or AHQ/AHQ (n = 7), which are resistant to natural scrapie in the NPU flock but produce the shortest IPs after inoculation with BSE. Two sheep previously reported as donors[9] were excluded from the study (along with their recipients) when regenotyping showed them to be ARQ/ARR and VRQ/AHQ, respectively, genotypes that result in relative resistance to oral infection with BSE.

Eleven donor sheep provided blood for transfusion at the preclinical stage of infection. Eight of these were culled at the time of donation as part of a separate time course pathogenesis experiment. The remaining 3 preclinical donors went on to develop clinical signs of BSE, with respective IPs of 629, 761, and 2131 days after infection. Four sheep were used as blood donors once they had developed clinical signs of BSE at 561 to 671 days after infection. PrP^{Sc} deposits in brain and/or in peripheral tissues were confirmed in all clinically affected donors by immunohistochemistry (IHC). In 2 donors culled at the preclinical stage, sparse PrP^{Sc} deposits were found in only one tissue in each sheep: Peyer patch (58 × 81) and dorsal root ganglion (60 × 49). However, a negative result was obtained when the same tissues were immunostained in another laboratory. There were 15 ARQ/ARQ recipients of whole blood and 7 ARQ/ARQ recipients of buffy coat from BSE-infected donors. Figure 1 gives a summary of the experimental design, whereas details of the donor and recipient sheep are in Table 1.

Scrapie blood transfusions

The donors for this experiment were 10 VRQ/VRQ and one VRQ/ARQ Cheviot sheep from the Edinburgh NPU flock, where sheep of these genotypes show a disease incidence approaching 100%. Epidemiologic and pathologic evidence suggests that infection occurs around the time of birth. Blood collections were made from animals in 3 different age groups (200-250 days, 450-500 days, 700-850 days) to represent donors at different preclinical stages of disease, as well as from one clinical case. Seven donors were culled after developing clinical signs of scrapie at ages ranging from 1081 to 1556 days, and were confirmed positive by histopathology and IHC. Two donors were culled before the onset of clinical signs at 1197 and 1350 days of age, respectively, but PrP^{Sc} was detected in their tissues by IHC. Two donors died prematurely at 349 and 974 days of age: one was IHC negative, in the other the tissues were too decomposed to allow analysis. There were 21 recipients (all VRQ/VRQ PrP genotype) of blood from scrapie-exposed donors: 11 were transfused with buffy coat and 10 with whole blood. See Figure 1 for a summary of the experimental design and Table 2 for details of donor and recipient sheep.

Positive and negative controls

Seven ARQ/AHQ and 3 ARQ/ARQ sheep were infected intravenously with 0.2 g of the same BSE-infected cattle brain homogenate as given orally to the blood donors and served as positive controls. No positive controls were used in the scrapie transfusion experiment. As negative controls for the BSE transfusion experiment, 12 ARQ/ARQ recipients were given transfusions of whole blood (n = 6) or buffy coat (n = 6) from 7 uninfected donors (6 ARQ/AHQ, 1 ARQ/ARR). Two recipients died at 633 days and 1181 days after transfusion, respectively, and the remaining 10 recipients were culled between 2462 and 2586 days after transfusion. As negative controls for the scrapie experiment, 16 VRQ/VRQ sheep received either whole blood (n = 8) or

Table 1. Outcome of transfusions from BSE-exposed donor sheep

Donor sheep details								Recipient sheep details				
Donor sheep ID	Donor genotype	Clinical status at donation	Percentage of actual or average incubation period at donation*	Clinical outcome	IHC result	Incubation period, d	Component transfused	Recipient sheep ID	Recipient PrP 168 codon genotype	Clinical outcome	IHC result	Incubation period, d
58x51	ARQ/ARQ	Preclinical	12	+	+	2131	WB	D529	PP	+†	–	–
60x49	ARQ/ARQ	Preclinical	22	–	+/–	–	WB	D433	PL	–	–	–
			44		(DRG)‡		WB	F14	PL	–	–	–
J2747	ARQ/AHQ	Preclinical	42	–	–	–	BC	F182	PP	–	–	–
			44				WB	F181	PP	–	–	–
61x24	ARQ/AHQ	Preclinical	42	–	–	–	BC	F238	PP	+†	–	–
			43				WB	F234	PP	–	–	–
J2746	AHQ/AHQ	Preclinical	45	–	–	–	WB	F19	PP	+	+	536
J2559	AHQ/AHQ	Preclinical	51	+	+	629	WB	D505	PP	+	+	610
58x81	ARQ/AHQ	Preclinical	61	–	+/–(IPP)‡	–	BC	D358	PP	–	–	–
58x28	ARQ/AHQ	Preclinical	61	–	–	–	WB	D421	PP	–	–	–
			61				BC	D384	PP	–	–	–
58x27	AHQ/AHQ	Preclinical	61	–	–	–	WB	D452	PP	–	+§	–
			61				BC	D318	PP	–	–	–
58x39	ARQ/AHQ	Preclinical	62	–	–	–	WB	D337	PP	–	+‖	–
			62				WB	D386	PP	–	–	–
J2499	AHQ/AHQ	Preclinical	86	+	+	761	WB	D341	PP	–	–	–
J2771	AHQ/AHQ	Clinical	100	+	+	561	BC	G61	PL	–	+	–
J2770	AHQ/AHQ	Clinical	100	+	+	589	WB	G74	PP	+	+	594
60x69	AHQ/AHQ	Clinical	100	+	+	660	WB	G78	PP	+	+	556
							BC	G49	PP	+	+	531
D383	ARQ/ARQ	Clinical	100	+	+	671	WB	G92	PL	–	+	–

WB indicates whole blood; BC, buffy coat; DRG, dorsal root ganglion; IPP, ileal Peyer patch; +, positive; and –, negative.

*Calculated from the days after infection at the time of donation, as a percentage either of the final incubation period (in sheep kept alive until the development of clinical signs) or of the average incubation period in orally infected donors (640 days), excluding the outlying incubation period of 2131 days (58x51).

†No evidence of infection was found on postmortem examination of tissues from these clinical suspects; therefore, it is most likely they were clinically misdiagnosed.

‡These tissues were initially scored weakly positive by IHC, but the results were not reproducible in two laboratories and can therefore be considered as inconclusive.

§This sheep died of unrelated causes (ie, without showing clinical signs of BSE) at 1139 days after transfusion but was positive by IHC.

‖This apparently healthy sheep was culled 3018 days after transfusion and found to be positive by IHC; however, further analysis suggested this was a case of "atypical" scrapie and therefore unlikely to be transfusion related.

buffy coat (n = 8) collected from 8 uninfected VRQ/VRQ donors. There were 2 intercurrent deaths at 397 days and 464 days after transfusion, and the other 14 animals were culled between 2052 and 2409 days after transfusion. None of the negative controls for the BSE or scrapie experiments showed clinical signs of TSEs, and all were IHC negative for PrP^{Sc}.

PrP^{Sc} detection by immunohistochemistry

Tissue samples from the brain, spleen, mesenteric lymph node, and palatine tonsil of the sheep under study were fixed in formaldehyde and processed according to standard procedures. Sections were immunolabeled for PrP^{Sc} detection by IHC with primary antibody R145, which recognizes the 222-226 amino acid sequence of ovine PrP,[11] as described previously.[12,13]

Results

BSE transfusion experiment

A total of 5 transfusion recipients showed clinical signs of TSEs and were confirmed positive by IHC and/or Western blot (Table 1; Figure 2). These included 2 (F19 and D505) of 12 sheep transfused with whole blood from donors in the preclinical phase of infection (at 45% and 50% of estimated IP, respectively), as reported previously.[8,9] Two of 3 recipients of whole blood and one of 2 recipients of buffy coat from donors clinically affected by BSE developed clinical BSE. The IPs in the 5 clinically positive recipient sheep ranged from 531 to 610 days after transfusion (mean ± standard deviation [SD] = 565 ± 35 days), and there was no obvious difference in the IPs of those that received blood from preclinical or clinical donors.

One recipient (D452) of whole blood from a preclinical donor died of unrelated causes at 1139 days after transfusion but had PrP^{Sc}-positive IHC labeling in brain and other tissues. One of 3 recipients of whole blood (G92) and one of 2 recipients of buffy coat (G61) from clinical donors showed weak PrP^{Sc} deposition in the brain and lymphoid tissues after being culled at 2003 and 2497 days after transfusion, respectively, in the absence of clinical signs. Full sequencing of the *PrP* gene of these sheep revealed that they carried an additional proline (P) to leucine (L) substitution at codon 168,[14,15] which appears to be associated with the prolonged survival of these infected sheep. The polymorphism was also identified in 2 recipients of blood from a preclinical BSE-challenged donor, neither of which showed evidence of infection.

Taking the results for all 22 recipients of blood from BSE-exposed donors, 5 clinical cases and 3 sheep showing evidence of infection in the absence of clinical signs were identified, giving an overall transmission rate of 36%.

One recipient was culled for health reasons at 1444 days after transfusion, 2 were culled with suspected TSE clinical signs at 2480 and 2160 days after transfusion, respectively, and the remaining clinically negative sheep were culled between 2239 and 3068 days after transfusion. With one exception, examination of the tissues by IHC did not find evidence of infection. The exception (D337) was culled at 3018 days after transfusion and showed

Table 2. Outcome of transfusions from scrapie-exposed donor sheep

Donor sheep details								Recipient sheep details			
Donor sheep ID	Donor genotype	Clinical status at donation	Percentage of actual or average incubation period at donation*	Clinical outcome	IHC result	Incubation period, d	Component transfused	Recipient sheep ID	Clinical outcome	IHC result	Incubation period, d
67x42	VRQ/VRQ	Preclinical	17	+	+	1274	BC	G247	–	–	–
			19				WB	G230	–	–	–
66x45	VRQ/VRQ	Preclinical	17	–	–	–	WB	G267	–	–	–
			19				BC	G265	–	–	–
67x23	VRQ/VRQ	Preclinical	18	+	+	1207	BC	G241	–	–	–
			20				WB	G228	–	–	–
65x13	VRQ/VRQ	Preclinical	28	+	+	1556	WB	F275	–	–	–
			30				BC	F273	–	–	–
65x02	VRQ/VRQ	Preclinical	34	–	+	–	WB	F310	–	–	–
			37				BC	F309	+	+	1101
65x03	VRQ/VRQ	Preclinical	34	–	+	–	WB	F277	+	+	1138
			37				BC	F276	+†	–	–
61x75	VRQ/ARQ	Preclinical	53	+	+	1324	BC	F149	+	+	782
			57				WB	F144	+	+	672
61x68	VRQ/VRQ	Preclinical	64	+	+	1113	BC	F152	+	+	853
			69				WB	F153	+	+	660
61x66	VRQ/VRQ	Preclinical	62	–	ND	–	WB	F286	–	–	–
			64				BC	F284	–	–	–
59x27	VRQ/VRQ	Preclinical	73	+	+	1137	BC	F126	+	+	826
			77				WB	F141	+	+	575
59x28	VRQ/VRQ	Clinical	100	+	+	1081	BC	F143	+	+	737

+ indicates positive; and –, negative.

*Calculated from the age at the time of donation, as a percentage either of the final incubation period (for sheep that survived until the development of clinical signs) or of the average incubation period (1296 days) for sheep that died or were culled before developing clinical signs.

†No evidence of infection was found on postmortem examination of tissues from this clinical suspect; therefore, it is most likely it was clinically misdiagnosed.

positive PrP^{Sc} labeling in the brain, but with a pattern distinct from that observed in other BSE-infected sheep. The brain PrP^{Sc} distribution involving major white matter tracts and sparing the dorsal motor nucleus of the vagus was similar to that of Nor98 (or "atypical" sheep scrapie) and therefore doubtful to be transfusion-related. No other sheep in the present study showed evidence of being infected with atypical scrapie.

Of the 10 sheep that were infected intravenously with BSE as positive controls, 8 developed clinical signs confirmed by IHC, with an average IP of 702 days (± 61 days, SD). The remaining 2 animals were culled at 2591 days after infection and, although not demonstrably clinically affected, IHC showed PrP^{Sc} deposition in the brains and lymphoid tissues of both animals. These 2 sheep were heterozygous (PL_{168}) for the PrP polymorphism P168L, whereas the other 8 were homozygous (PP_{168}).

The PrP^{Sc} profile obtained by IHC from BSE-positive recipients was the same as that found in the orally inoculated donors and in the positive controls.[16] In addition, characteristic BSE glycoform patterns were obtained by Western blot analysis of PrP^{Sc}-positive donor and recipient sheep (data not shown),[9] and inoculation of brain homogenates from infected donors and recipients into a panel of inbred mouse strains produced IPs and lesion profiles characteristic of BSE (data not shown). Taken together, these results confirm that the strain characteristics were not altered after transmission via blood.

Scrapie transfusion experiment

Four of 10 recipients of whole blood and 4 of 10 recipients of buffy coat from donors in the preclinical phase of scrapie infection developed clinical signs of scrapie, which were confirmed by positive IHC results. One sheep transfused with buffy coat from the single clinical donor was also clinically affected and IHC positive (Table 2; Figure 2). Four of these cases (F144, F153, F141, and F143) were reported previously.[9] There were 4 intercurrent deaths at 354, 753, 1237, and 1615 days after transfusion, respectively, and the 8 remaining recipients were culled between 2329 and 2484 days after transfusion. These 12 animals were clinically negative at the time of death and showed no detectable PrP^{Sc} by IHC. Thus, 9 of 21 recipients of blood from scrapie-exposed sheep developed clinical scrapie, giving an overall transmission rate of 43%.

The majority of confirmed scrapie cases in recipients (n = 7) occurred in the groups that received transfusions from donors in the late preclinical (> 50% of estimated IP) or clinical phase of infection. Only 2 of 9 recipients in these groups remained free of infection. The other 2 positive recipients were in the group of 6 sheep that received transfusions from donors at 28% to 37% of estimated IP, and their IPs were much longer than the rest (1101 and 1138 days after transfusion compared with a range of 575-853 days in recipients of blood from donors at > 50% of estimated IP). No disease was confirmed in the 6 recipients that received blood from donors at less than or equal to 20% of estimated IP.

The PrP^{Sc} profile obtained from brains of donors and recipients highlighted some differences in terms of presence of vascular plaques or glia-associated PrP^{Sc} in donors but not in recipients, or vice versa (S.S., unpublished data, December 16, 2005). Such discrepancies were interpreted as presence of more than one natural scrapie strain in the flock of origin.

Discussion

The outcome of the blood transfusion experiments showed that 2 different TSE agents, scrapie and BSE, could be efficiently

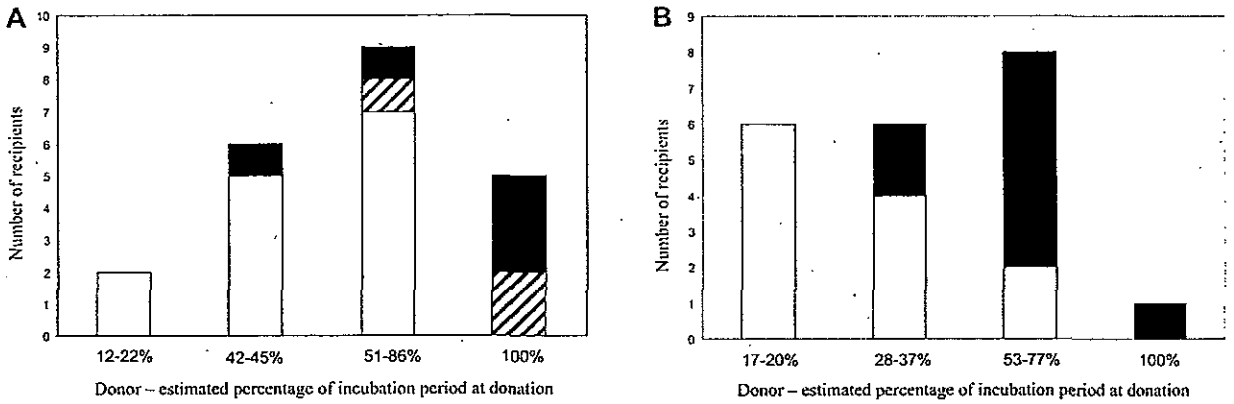

Figure 2. Outcome of transfusions as a function of the stage of disease incubation in the donor. (A) BSE-infected donors. (B) Scrapie-infected donors. For each stage of infection in the donor sheep, the number of uninfected (□), clinically positive/ IHC-positive (■), and clinically negative/IHC-positive (▨) recipients are shown.

transmitted between sheep by blood transfusion, using volumes similar to those used in human transfusions. The overall transmission rates (percentage of all recipients that became infected) were 36% for BSE and 43% for scrapie. For BSE, the figure was much higher than anticipated because 3 of the 8 BSE-infected recipients survived for long periods without showing clinical signs, whereas all the scrapie-infected recipients identified by IHC were also clinically positive. The greater probability of subclinical infection in recipients of blood from BSE-exposed donors is largely the result of variability in the genetic susceptibility to infection among sheep used in the BSE experiment, which will be discussed in "Effect of genetic variation in susceptibility." The results are consistent with the known facts about transmission of vCJD by blood transfusion in humans.[17] Sixty-six patients known to have received labile blood products from 18 donors who subsequently developed vCJD were followed up in an ongoing study. Three of these recipients have been confirmed clinically and pathologically as vCJD cases, with intervals between transfusion and the development of clinical signs ranging from approximately 6.5 years to 8.5 years.[18-20] Another patient, who died of unrelated causes 5 years after transfusion, showed PrP^{Sc} deposits in lymphoid tissues but not brain postmortem, and is thought to represent preclinical or subclinical infection.[21] These 4 patients represent 6% of the total recipients, or 12.5% of recipients surviving longer than 5 years.

Various factors influence the transmission rate by transfusion in both sheep and humans, including: (1) the interval between blood donation and the onset of clinical signs in the donors, (2) genetic variation in susceptibility of donors and recipients, and (3) the blood component transfused.

Stage of incubation period of the donors at the time of blood donation

The effect of the stage of incubation can best be deduced from the results of the scrapie transfusion experiment because the *PrP* genotype of the sheep used (VRQ/VRQ) renders them almost 100% susceptible to natural and experimental infection.[22] The stage of incubation of the donor has a strong influence on the probability of transmission to the recipient (Figure 2). When donations were made at less than or equal to 20% of the estimated IP, there was no disease transmission, whereas donations made at more than 50% of the estimated IP produced an 80% transmission rate, with a mean IP of 729 days (± 99, SD) in the recipients. Blood collected at 28% to 37% of the estimated IP transmitted infection at a lower rate of approximately 33%, and with longer IPs in the recipients of more than 1000 days. The data are consistent with a gradual increase in infectivity in the blood, from approximately 30% to 50% of IP until the clinical phase.

In the BSE transfusion experiment, the correlation between stage of infection and transmission is not clear-cut but shows the same general trend of increasing probability of transmission to recipients as infection progresses in the donors (Figure 2). Possible explanations for the lower transmission rates from preclinical BSE-infected blood donors compared with preclinical scrapie-infected donors include the following:

(a) Variation in susceptibility to infection of both donor and recipient sheep.

(b) Differences in the pathogenesis of natural scrapie and experimental BSE. VRQ/VRQ sheep naturally infected with scrapie have detectable PrP^{Sc} deposits in lymphoid tissues early after infection (ie, < 50% estimated IP).[23,24] Time course studies of ARQ/ARQ sheep orally infected with BSE showed that PrP^{Sc} was not consistently detected in lymphoid tissues before at least 65% of the average IP.[7] If infectivity in blood correlates with its presence in lymphoid tissues, this could explain the differences observed in the 2 transfusion experiments.

The probability of transmission from preclinical donors is of greatest relevance to the human situation. In the case of the 4 transfusion-related transmissions of vCJD, the donors developed clinical signs between 17 and 42 months after donation. The mean IP for vCJD has been estimated to be 16.7 years, with a lower 95% confidence interval of approximately 12.4 years.[25] Therefore, it is probable that the transfusion-related vCJD cases resulted from donations made at least halfway through the IP, which is in agreement with the data from the sheep experiments. In vCJD cases, the timing of detectable lymphoid replication in the preclinical stages of disease is unknown; therefore, it is not clear whether the peripheral pathogenesis more closely resembles BSE or natural scrapie in sheep.

Effect of genetic variation in susceptibility

A small proportion of sheep with $A_{136}Q_{171}/A_{136}Q_{171}$ PrP genotypes do not die of infection after natural or experimental exposure to scrapie and BSE, or have very prolonged incubation periods.[26-28] The reasons for this variability in response are not clearly understood, but it can be predicted to reduce infection rates in both donor and recipient sheep in the BSE transfusion experiment. The majority of preclinical donor sheep (8 of 11) in the BSE transfusion experiment were killed at, or shortly after, the time of donation, and

none showed conclusive evidence of infection, although 2 transmitted infection to their respective transfusion recipients. It is potentially significant that donors that failed to transmit infection were heterozygous at *PrP* codon 154, whereas those that did transmit infection were homozygous. Thus, variable susceptibility to infection among the donor sheep may be the result of a protective effect of codon 154 heterozygosity to oral challenge with BSE, although more data are required to confirm this association.

A novel polymorphism, resulting in a proline to leucine substitution at codon 168 of the *PrP* gene, was identified in 4 BSE transfusion recipients and 2 positive control sheep inoculated intravenously with BSE.[14] All 6 survived more than 2000 days without developing clinical signs of BSE, but on postmortem examination 4 showed PrP^{Sc} deposition in brain and lymphoid tissues. This suggests that the P168L polymorphism can protect against clinical disease but does not prevent infection by the intravenous route. This polymorphism has not been identified in the Edinburgh NPU Cheviots used as donors in the BSE experiment or in sheep with the VRQ/VRQ genotype.

Although the genetic basis of susceptibility to BSE infection in sheep and humans is not directly comparable, the variability in response to BSE found in ARQ/ARQ sheep provides a more realistic reflection of the situation with vCJD in the human population than the very uniform susceptibility of VRQ/VRQ sheep to scrapie infection. In addition, the survival of BSE-infected transfusion recipients for up to 7 years without clinical signs demonstrates that prolonged secondary incubation periods and/or a subclinical/"carrier" state are possible after transfusion in sheep. The existence of such subclinical or prolonged preclinical infection states in humans is recognized as one of the important factors influencing the probability of onward transmission, and thus the potential size of the vCJD epidemic.[29] Susceptibility to human TSEs has been linked to codon 129 of the *PrP* gene, which can encode either methionine (M) or valine (V). Until recently, all clinical cases of vCJD (including the 3 transfusion-related cases) that have been tested have been homozygous for methionine at 129 (129MM). Interestingly, the "preclinical" patient thought to have been infected by transfusion was heterozygous (129MV).[21] There is accumulating evidence to suggest that all human 129 genotypes may be susceptible to vCJD infection, with apparently greater likelihood of subclinical infection in 129MV and 129VV persons.[30-32]

Effect of blood component

The 4 transfusion-related vCJD infections occurred in patients who received transfusions of red cells that had not been leukodepleted. Leukodepletion was introduced in the United Kingdom in 1999 to control the risk of transmission of vCJD by blood transfusion because previous studies in rodents had shown that infectivity appeared to be concentrated in the buffy coat, which contains most of the blood leukocytes.[4] Subsequently, leukodepletion of blood from scrapie-infected hamsters was shown to remove up to 72% of infectivity.[33,34] In the sheep experiments, only whole blood and buffy coat were transfused because we were seeking to establish proof of principle of transmission of TSEs by blood transfusion, and assessing whether infectivity appeared to be concentrated in the buffy coat. The effect of leukodepletion was not investigated but is being addressed in a follow-up study, along with estimates of the distribution of infectivity among other blood components, including plasma, platelets, and red cells.

In our experiments, transmission rates did not appear to be significantly different in recipients receiving whole blood compared with recipients transfused with buffy coat. The number of sheep transfused with buffy coat in the BSE experiment was too small to allow statistical analysis. In the scrapie experiment, 5 of the positive recipients were transfused with buffy coat, and 4 with whole blood. The similarity in transmission rates for both components suggests that they contain approximately equivalent amounts of infectivity.

We have shown that, for sheep infected with scrapie and BSE, high transmission rates can be achieved using blood transfusion, particularly when donors are at more than 50% of incubation period. The results also revealed the possibility of prolonged incubation periods and/or subclinical infections in some recipients of BSE-infected blood, which is at least partly because of genetic variation in the sheep PrP gene. The suggestion of relatively high titers of infectivity in blood is perhaps surprising in view of the need for ultrasensitive methods of detection for PrP^{Sc} in blood.[35,36] It may be that, in blood, infectivity is not closely correlated with levels of protease-resistant PrP, but comparative titrations of brain and blood-borne infectivity in sheep will be required to further define the relationship. The results of our sheep transfusion experiments are consistent with what is known about transfusion-associated vCJD transmission in humans, and support the use of sheep as an experimental model in which to study the risks associated with different blood products, the effectiveness of control measures, and the development of diagnostic and screening tests.

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Authorship

Contribution: F.H. designed the study, performed transfusions and postmortems on recipient sheep, analyzed data, and wrote the paper; A.C. performed Western blots; S.M. performed Western blots and reviewed the report; J.F. coordinated collection of blood and postmortems on donor sheep; W.G. analyzed and interpreted PrP genotype data and reviewed the report; S.S. and L.G. examined tissues, interpreted IHC results, analyzed data, and reviewed the report; M.J. contibuted to the interpretation of IHC results and reviewed the report; and N.H. designed the study, analyzed data, and reviewed the report.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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TRANSFUSION

Transfusion

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REVIEWS

From mad cows to sensible blood transfusion: the risk of prion transmission by labile blood components in the United Kingdom and in France

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ABSTRACT

Transfusion transmission of the prion, the agent of variant Creutzfeldt-Jakob disease (vCJD), is now established. Subjects infected through food may transmit the disease through blood donations. The two nations most affected to date by this threat are the United Kingdom (UK) and France. The first transfusion cases have been observed in the UK over the past 5 years. In France, a few individuals who developed vCJD had a history of blood donation, leading to a risk of transmission to recipients, some of whom could be incubating the disease. In the absence of a large-scale screening test, it is impossible to establish the prevalence of infection in the blood donor population and transfused patients. This lack of a test also prevents specific screening of blood donations. Thus, prevention of transfusion transmission essentially relies at present on deferral of "at-risk" individuals. Because prions are present in both white blood cells and plasma, leukoreduction is probably insufficient to totally eliminate the transfusion risk. In the absence of a screening test for blood donations, recently developed prion-specific filters could be a solution. Furthermore, while the dietary spread of vCJD seems efficiently controlled, uncertainty remains as to the extent of the spread of prions through blood transfusion and other secondary routes.

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ARTICLE TEXT

The first case known in the history of medicine as "mad-cow disease" dates back to 1985. It was observed in the United Kingdom (UK),[1] where the epidemic spread widely: by 2001, close to 180,000 animals had been affected by bovine spongiform encephalopathy (BSE). Most likely, the epidemic appeared as a result of feeding of bovine livestock with animal food prepared from residues from slaughtering and quartering, including carcasses of sheep which died from scrapie and cattle affected by a sporadic form of BSE. The use of such animal feed was forbidden in 1988 in the UK and in 1994 in France. On March 20, 1996, the UK Department of Health announced that the BSE agent was transmissible to man. A new human pathology appeared that year: a previously unknown form of Creutzfeldt-Jakob Disease (CJD), transmitted by a variant of the prion, the agent of sporadic CJD.

Unlike other transfusion-transmissible agents, the prion (**P**roteinaceous **I**nfectivity **O**nly) contains no nucleic acids

and is composed purely of protein.[2,3] The "normal" prion or PrP^{c} ("proteinaceous particle") is a protein expressed on the cellular membrane by a number of tissues, but the greatest amount is found on the neurons in the brain. Sensitive to the action of proteolytic enzymes, PrP^{c} has a half-life of a few hours. Despite having an identical amino acid sequence to that of the normal form, the BSE agent is a prion of different conformation, designated by the abbreviation PrP^{sc} (**sc** for **sc**rapie) and is derived from the isoform of the normal protein by a posttranslational structural modification and conversion to a richly beta-pleated sheet.[4] The abnormal prion has a tendency to aggregate and above all a resistance (from which is derived its other abbreviation, "PrP^{res}") to proteolytic enzymes (notably proteinase K), resistance lying in the majority conformation in beta-pleated sheets.[5] The prion itself plays a role of cofactor in this conformational change. In infected subjects, PrP^{sc} induces, on native PrP molecules, a conformation that confers on them their pathologic character, and the phenomenon of amplification is self-propagated.[6] Because it affects the accumulation of a protein present in its natural state in the body, there is no immunologic response: neither the production of antibodies nor a specific cellular response. Accumulation of abnormal prions generates vacuoles in the cerebral tissue, giving eventually a spongiform appearance and hence the title "spongiform encephalopathy."

HUMAN PRION DISORDERS

The human transmissible spongiform encephalopathies (TSEs) generally follow a long incubation period, but with subsequent rapid evolution and death. Various forms are described:

- The idiopathic disorders, principally the sporadic form of CJD (sCJD), which continues to be the commonest form. It appears predominantly in the seventh decade, with an annual incidence of 1 to 1.5 cases per million population.[7] Death follows within 6 months. This form, which is associated with a pathologic prion, has unknown etiology. It probably results from a spontaneous conformational modification of PrP to PrP^{sc}.
- The genetic forms: familial CJD (fCJD), Gerstmann-Strausler-Scheinker syndrome, and fatal familial insomnia.
- The acquired forms were, until 1966, of human origin: kuru, found in New Guinea and linked to funeral practices, and the iatrogenic form of CJD, seen after use of contaminated neurosurgical instruments, corneal grafts, dura mater, and intramuscular injection of pituitary hormones, obtained from cadavers. Acquired disease of bovine origin, that is, variant CJD (vCJD) is seen in subjects infected with the BSE agent.[8,9] This is the only human prion disorder that has crossed the species barrier. The first series of 10 patients was described in 1996 by the UK National CJD Surveillance Unit (NCJDSU) based in Edinburgh. The disease was found chiefly in adults under 40 years of age, contrasting with the mean age for sCJD. The disease is fatal in a mean of 14 months, which is slower than the sporadic form.[10] Nuclear magnetic resonance imaging scanning shows hypersignals situated in the posterior thalamus ("pulvinar sign"). Unlike other human prion disorders, notably sCJD, in which the accumulation of abnormal prion protein affects the central nervous system with a minimal peripheral involvement, vCJD progresses with invasion, by the abnormal protein, of the central nervous system, the peripheral nervous system, and other tissues, notably lymphoid: tonsils, appendix, Peyer's patches, and thymus.[11] Tonsillar biopsy may reveal the presence of PrP^{sc}, but a negative result does not categorically exclude the diagnosis.

GENETIC INFLUENCES

The prion protein gene is situated on the short arm of chromosome 20 and codes for 254 amino acids, with either valine (V) or methionine (M) at position 129. With two copies of the gene, an individual can be MM (39% of the normal population), MV (50%), or VV (11%). This polymorphism is fundamentally important in the development of the variant type of the disease, since there is a host susceptibility linked to the genetic type.[12,13] Homozygosity for M (MM) appears to confer susceptibility to clinical expression and to influence the incubation period of the disease: all vCJD cases, to date, in whom codon 129 typing has been performed, are MM homozygotes.[14] In one case of infection, where the individual died 5 years after an implicated blood transfusion but did not have any clinical symptoms of vCJD at the time of death, the genotype was MV. Furthermore, PrP^{sc} has been detected in the appendix of two VV cases.[15] The MV genotype and perhaps more the VV genotype could confer a protective effect, but this remains true only until a symptomatic case of vCJD is described in a MV or VV subject. In fact, we know that individuals with all three genotypes can accumulate PrP^{sc} in vCJD-specific tissues, but we do not know whether symptomatic cases will develop in all genotypes.

EPIDEMIOLOGY OF vCJD

To December 2007, a cumulative total of 166 cases of vCJD have been recorded in the UK, and this number accounts for the majority of cases worldwide. Mean age of affected individuals was 28 years (range, 12-74 years), with a slight male predominance. The mean duration of the symptomatic period was 14 months (range, 6-40 months). All tested patients were homozygous MM. The most probable origin for the majority of cases was dietary: no risk factor of other kinds of CJD was observed.[16,17] A small number of cases had a history of blood transfusion, and some of these have been linked with a known infected donor.

In France, vCJD incidence was, as in the UK, proportional to dietary exposure to contaminated beef. Importation of contaminated beef into France increased regularly from 1985 to 1995, while the ingestion of these meats decreased in the UK over the same period.[18] However, the level of exposure in France is estimated[19-21] to have been 10 to 20 times lower than that of the UK, with moreover a difference between the two countries in terms of dates of occurrence: the comparison between the number of French cases and that of UK cases (taking into account the year of the beginning of the symptomatic phase) indicates that a maximal incidence of French cases occurred 5 years after the peak of the epidemic in the UK, where the number of recorded cases has regularly decreased since 1999.[17] This temporal gap in the epidemic in the UK and in France is attributable to the period of maximal exposure of the general population in the two countries.

Between 1996 and 2007, 23 vCJD cases have been registered in France, with a mean age of 36 years (range, 19-58 years) and an equal sex ratio (12 males, 11 females). The clinical and genetic characteristics were comparable to those of the British vCJD patients. The mean duration of the symptomatic period was 15 months (range, 8-24 months). All the analyzed cases were homozygous MM, without any risk factor of other kinds of CJD.[17] Short stays in the UK (less than 10-day periods) were mentioned in 3 patients; a fourth one had stayed several times in the UK, for long periods, between 1987 and 1996.

In other countries, vCJD cases remain exceptional. A small number of these cases are attributable to residence in the UK, but there remain a number which appear to have been acquired outside the UK and France: two cases each in Ireland, the Netherlands, Saudi Arabia, Spain, and Portugal and one in each of Italy and Japan.

Several studies have been conducted to estimate the extent of the vCJD epidemic in the UK and in France. A British retrospective study revealed the presence of the abnormal prion in surgically removed appendix in 3 of 12,674 individuals without clinical vCJD:[22] this rate appears higher than suggested by the number of vCJD cases recorded in the general population. From these results, a prevalence of 237 cases per million of British citizens was proposed (95% confidence interval, 49-792). In the most pessimistic hypothesis (i.e., taking into account the highest range of this interval), 41,250 of 60 million individuals would be infected by the abnormal prion. However, the latest count of vCJD cases in the UK is more in agreement with the lower prediction of epidemiologists.

In France, where the level of exposure was lower than in the UK, estimates of 6 to 300 vCJD cases during the next 60 years have been suggested in one study,[19] and 205 cases in another.[23] In 2003, a model of incidence prediction suggested a total number of 33 cases (0-100), with 14 cases (2-30) over the 2004-2005 period, and 11 (1-20) over the 2006-2010 period.[18] These data are compatible with the most recent epidemiologic reports. A recent study predicted 39 (6-99) subsequent cases.[21] The worst case scenario of 300 cases over the next 60 years is, however, maintained in the epidemiologic estimations, in particular to estimate the prevalence of prion infection in the blood donor population.

Measures taken against the vCJD epidemic, with screening for the BSE agent in cattle and elimination of positive animals from the food chain, and the latest epidemiologic observations suggest that a vCJD pandemic of dietary origin is unlikely in the coming years. The unknowns now reside in other sources of contamination, through blood or cellular vectors: blood transfusion, grafts of tissues or organs, or use of medical or surgical instruments contaminated with the abnormal prion. Transfusion transmission is especially feared due to its potential extent. Nowadays, since food contamination, which was the main source of infection, seems fully controlled, has transmission by blood components taken its place? In the UK, as in France, while prions have probably been eliminated from beef, they are likely to be present in the blood of asymptomatic human carriers and transmissible to the recipients of their blood donations. The fear of human-to-human transmission has thus replaced the fear of interspecies contamination.

EXPERIMENTAL BASIS OF vCJD TRANSMISSION BY TRANSFUSION

Until about 1996 it was acknowledged that the CJD agent was not transmitted by transfusion: several studies failed to show any association between the occurrence of sCJD and a past transfusion.[24-26] The prion load being

higher in vCJD than in sCJD,[27] the number of infectious particles in blood and/or their distribution in individuals affected by sCJD are presumably too low to cause transmission through a blood transfusion. Thus, before the first vCJD cases, the two main circumstances of prion transmission between humans had been kuru and iatrogenic contamination by injection of growth hormones of pituitary origin. It should be noted that transmission of the kuru agent belongs to the past since the prohibition of certain rituals in New Guinea and that the exclusive use of growth hormones of recombinant origin put an end to iatrogenic transmissions through this route. Though no cases of human transmission of vCJD had yet been described, the possibility of transmission by blood transfusion remained a theoretical risk.[28-31] Unlike major transfusion-transmitted viruses observed in the past decades (hepatitis B virus, human immunodeficiency virus [HIV], hepatitis C virus), the vCJD agent did not immediately enter into the family of blood-borne agents.

In experimental models, invasion of lymphoid tissue by abnormal prion has been observed rapidly after infection, with persistence throughout the whole incubation period. It has been suggested (but not demonstrated) that the lymphoid infiltration is brought about by circulating cells, which led to the hypothesis that infected lymphocytes could transmit the prion to recipients of blood components containing lymphocytes.[32] Intracerebral injection in mice of buffy coats and plasma collected from patients with vCJD has not shown such transmissibility,[33] but these experiments only involved a small number of cases and the sensitivity of the technique may have been insufficient to detect low level of infectivity. Subsequently, transfusion transmission of prions was shown in rodents,[34] in particular in mice made susceptible to vCJD.[35,36] However, the turning point was the result of experiments aiming to show transmissibility through blood from orally infected sheep to healthy sheep:[37] it was then found that the abnormal prion was present in circulating blood and that blood could be a vector of transmission. Blood infectivity being thus demonstrated, at least in certain circumstances, French and British Health Authorities, as a precaution, considered the possibility of transmission of the vCJD agent by transfusion.

In another experiment, transfusion of healthy sheep with blood from infected sheep led to transmission rates of 17 percent for BSE and 19 percent for scrapie.[38] A more recent animal experiment was based on detection of PrP^{sc} in blood of hamsters experimentally contaminated by the scrapie agent through intraperitoneal inoculation of infected brain tissue.[39] In both cases, the infectious agent was present in circulating blood during a part of the incubation phase of the disease, and the transmission rate was shown to be quite high. However, it is important to distinguish the studies conducted with a Western blot assay detecting the amplified amyloid protein and those involving a titration of endogenous infectivity.

Finally, even before the description of the first human transfusion cases, these animal experiments had shown blood transmissibility of prions and the possibility of a short incubation period of the disease through this transmission route.

SURVEILLANCE OF TRANSFUSION RISK OF CJD IN THE UK

The first UK epidemiologic studies did not suggest transfusion as a mode of transmission of the vCJD agent, and the first descriptions of recovery of abnormal prions within the body had indicated that the blood route would be an improbable source of contamination. Subsequently, experiments into blood-borne transmission of the BSE agent in sheep and the observation of a wider distribution of PrP^{sc} in the body of subjects infected by the variant agent compared with subjects infected with sCJD led to reconsideration of this view.

In 1990, the UK, the country most exposed to the BSE risk, put in place a national surveillance system named "The National Creutzfeldt-Jakob Disease Surveillance Unit" or NCJDSU, charged with identifying and monitoring all cases of CJD.[40,41] All suspected cases were to be reported by health professionals (principally neurologists and neuropathologists) and then confirmed and categorized according to the defined diagnostic criteria. As far as transfusion is concerned, the medical history of each patient was examined and family members were interviewed, looking for history of blood donation or of receipt of transfusion. A collaborative study between the NCJDSU and the UK Blood Transfusion Services, called "TMER" (Transfusion Medicine Epidemiology Review), was set up in 1997 to examine all cases of CJD, including sCJD, fCJD, and vCJD, who had either donated or received blood in the past. On December 1, 2007, among the 166 UK cases of vCJD, 150 were old enough to have been blood donors and, among these, 31 (21%) had, at least according to their families, donated their blood at least once.[42] Records were checked and the dates and places of the donations were established. The fate of the donations was traced, including whether they were used for blood component preparation and/or for fractionated plasma products, and the fate of recipients of blood components was established. These enquiries identified donor records relating to 24 individuals who later developed vCJD: 18 of whom had donated blood that had been used to prepare components issued for hospital use. A total of 66 recipients were identified from these 18 donors: 23 of these are still alive. Blood donor records were identified for only 3 of 93 individuals who later developed sCJD and were reported to have been donors in the past, with 20 recipients identified, of whom 12 are known to be dead: 5 died within 1

year of the blood transfusion and 7 between 1 and 7 years after transfusion. Seven recipients are not known to be dead and have survived 7 to 9 years after transfusion. Three cases who developed fCJD (of 5 reported as blood donors) were associated with 11 identified recipients, of whom 2 died within 1 year of blood transfusion and 3 died 3, 10, and 17 years after transfusion. Three recipients not known to be dead have survived 13 to 21 years after transfusion. Among the 97 recipients thus identified, 4 have developed evidence of infection and 3 of these have died of the disease: these all belong to the first group, those exposed to risk of vCJD. Because there is no evidence that either sCJD or fCJD has been transmitted by blood transfusion, none of those recipients have been informed and none were tested for evidence of infection.

THE FIRST UK CASES OF vCJD IN RECIPIENTS OF BLOOD COMPONENTS

All four transfusion-associated vCJD infections occurred in patients transfused in the UK with nonleukoreduced red blood cells (RBCs). There have been no transfusion-associated cases of sCJD or of fCJD: no cases have ever been described in these two latter groups, even in retrospective lookbacks or in case control studies,[43-47] and no such infections have been detected in the blood in experimental animal studies, even in transgenic mice rendered susceptible to the disease, although it is not possible to formally exclude transfusion cases of sCJD, which might have passed unnoticed if they possessed exceptional features and/or a particularly long incubation period.

The first of the four patients infected with vCJD through blood transfusion was a male in the 60- to 70-year age group, who developed the illness in 2002 and died the next year. During surgery in 1996, he received 5 units of nonleukoreduced RBCs, one of which was donated by a young donor who developed vCJD in 1999 and died the following year. Both donor and recipient were MM homozygous. Infection of dietary origin could not be completely excluded in this case (as in the others), but the transfusion was the most plausible explanation, given the age of the recipient which was greater than the median for cases believed exposed through the dietary route, and by the association of this rare disease in both donor and recipient: statistical analysis demonstrated that the possibility these two observations of vCJD would have happened independently, if transfusion was not the source of infection, was in the order of 1:15,000 (and rose to 1:30,000 taking into account the age of the recipient). This first transfusion-associated case in world literature was reported in December 2003.[48]

The second case was an elderly recipient, who died of cardiovascular causes without developing any clinical signs of vCJD. Asymptomatic infection with PrP^{Sc} was established by postmortem examination, which demonstrated the presence of abnormal prion protein in lymphoid tissue (the spleen and one cervical lymph node, but not in the tonsils or appendix), but not in the brain. This patient had been identified as "at risk" since, 5 years previously, in 1999, a nonleukoreduced RBC component had been provided from a donor who died of vCJD 18 months after the donation. The PrP^{Sc} isolated from the spleen had an isoform identical with that observed in cases of vCJD. The donor was an MM homozygote, but the recipient was a heterozygote (MV), which may explain the asymptomatic nature of this case, assigned as "preclinical" or "subclinical" vCJD. Alternatively, the recipient may have developed vCJD at a later date, if survival had been longer. This second case of possible transfusion transmission of vCJD infection was reported in July 2004.[49]

The third case, reported in 2006,[42,50,51] was also one of the cohort of blood recipients who had received nonleukoreduced RBCs and had been notified of their risk. This recipient was in the 20- to 30-year age group and had received transfusion support during a surgical intervention complicated by serious bleeding. The recipient developed vCJD in 2005, 7 years after the transfusion episode, and died 2½ years later. One of the donors presented with vCJD in 1999 (18 months after the donation) and died the following year (21 months after the blood donation). Both donor and recipient were MM homozygotes.

The fourth and last case to date was a recipient who developed vCJD 8½ years after transfusion with RBCs from a donor who presented with vCJD 17 months after donation. This donor was the same as that to case number three. The recipient, genotype MM, died 1 year after presentation.[52]

These cases reported in professional journals (and subsequently in general reports) have led to the risk of transfusion-associated vCJD moving progressively from "theoretical risk" to "possible," then to "probable" and finally "demonstrated." There are a number of unknowns in the variables of risk of blood contamination by vCJD, but the combination of the low prevalence of vCJD in the general population (the estimation of the risk from an individual unit varies between 1:15,000 and 1:30,000 in the UK)[48] and of the high prevalence of vCJD cases in the small group of recipients who have been rendered at risk (and it should be noted that a sizeable proportion of these at-risk recipients have died without surviving long enough to develop an eventual vCJD and were never tested for the presence of infection) makes highly probable a transfusion origin rather than diet. The occurrence of these cases reinforces the theory that the blood of a donor in the asymptomatic phase of the disease could be infective for recipients. This evidence of the transfusion transmissibility of vCJD thus largely justifies the preventative measures previously applied in the UK and in France.

In fact, despite the small number of reported transfusion cases, many observations have been proposed or are already known:

1. The possibility of a relatively short incubation period with a transfusion source: 6½ years between the transfusion and the first clinical signs in Case 1, 6 years in Case 3, 8½ years in Case 4. This short incubation period demonstrates the efficacy of the transfusion route. It might suggest a particular pathogenic character of the abnormal prion circulating in the blood and transmitted by this route, even if it is established that intraspecies transmission is usually accompanied by a shorter incubation than interspecies transmission. Indeed, the shortest incubation period has been observed in kuru, in the iatrogenic form after injection of growth hormone,[53] and in transfusion-associated vCJD.[54]
2. The rate of transmission in the population of at-risk recipients is high, even though it is not inevitable in the relatively short follow-up period.[55] A review of the UK's TMER study published in 2006 gave an indication of the transfusion risk of vCJD and of the incubation period of the first observed cases:[42] among the 66 blood component recipients transfused from donors who later developed vCJD, 37 died within the first 5 years posttransfusion with a cause of death linked to the existing illness. Apart from the one case shown to have evidence of infection, none of the other deceased recipients were tested for evidence of infection because their deaths predated the information that their donors had developed vCJD. Furthermore, no postmortem tissue was available for retrospective testing.

Among the 29 who survived over 5 years, 20 are still alive and have no signs of vCJD, and 9 are now deceased. Among these 9, 6 died of pathology not linked to vCJD (although only 1 of these had a postmortem to look specifically for infection, which was demonstrated) and 3 developed (and died from) vCJD.

3. The influence of codon 129 genotype is not refuted in the context of the transfusion route: the sole recipient known to be infected but asymptomatic was a heterozygote (MV), although it should be noted that the observation period was the shortest of the series of infected recipients, since this recipient died 5 years after transfusion.
4. All the infected recipients had received nonleukoreduced RBCs between 1996 and 1999. Routine leukoreduction was introduced in the UK by October 1999.
5. The four recipients who developed evidence of infection had been transfused respectively with components from 5, approximately 8-10 (figure uncertain), 56, and 23 blood donors. [Correction added after online publication 2-Jan-2009: Number of donors has been updated.]
6. In the UK and France, no case of vCJD has been reported in recipients of fractionated plasma products. As indicated in the title of this article, we have limited our review to labile blood components, aware of the additional procedures that contribute to the safety of plasma products with respect to prions.

SURVEILLANCE OF TRANSFUSION RISK IN FRANCE: FIRST CASES OF vCJD WITH PREVIOUS BLOOD DONATIONS AND FIRST MEASURES TAKEN WITH REGARD TO THE RECIPIENTS

Although epidemiologic investigations conducted in France have not revealed previous blood transfusions during the "risk period" for vCJD (one case had received a blood transfusion, but in 1971, before the epidemic), some patients had been blood donors, as would be predicted, in the same period. In 1992, a national surveillance network for cases of CJD was set up in France, coordinated by Inserm Unit U708 and including representatives of various medical specialities and the health services: neurologists, neuropathologists, reference laboratories, and the "Institut de Veille Sanitaire" (InVS). The aim of this network was to collect and investigate reports of suspected cases of CJD, follow their progress, classify the type (sporadic, familial, iatrogenic) and the degree of probability (distinguishing confirmed cases from probable cases), and establish epidemiologic characteristics. In cases with previous history of blood donation, InVS was charged with informing the French Blood Service ("Etablissement Français du Sang") so that a transfusion investigation could be started. It appeared that three of the French vCJD cases, who had developed the disease in 2004, had a history of blood donation.

The first case (eighth in the series, reported in February 2004) was a 32-year-old female who donated blood between 1993 and 2003. The components prepared from these donations were 13 concentrated RBCs (of which 10 were leukoreduced) and one platelet (PLT) concentrate. Fourteen recipients, of whom 10 were still alive, were traced. Ten plasma donations were used for fractionated plasma products.

The second case (ninth in the series, reported in April 2004) was a 52-year-old man who had donated blood since 1984, chiefly between 1996 and 2002. No investigations were carried out into donations which preceded the vCJD

epidemic. The blood components were 5 concentrated RBC units (all leukoreduced) and 3 PLT concentrates (2 leukoreduced). For donations made after 1994, 7 recipients were traced, of whom 2 were still alive. Twelve plasma donations were used for fractionation.

The third case (13th in the series, reported in October 2004) was a 48-year-old man who donated blood between 1991 and 2004. The components were one fresh-frozen plasma (FFP) and 15 concentrated RBC units (half of them leukoreduced). All 16 recipients were identified, of which 6 were alive.

In total, these 3 donors account for 42 recipients of RBCs or PLTs, of whom 16 were alive at the time of the investigation: 2 of these, transfused before 1984, were not informed, but 14 were notified because they received transfusions between 1991 and 2004. To date, none has presented with symptoms of vCJD. None of the deceased recipients were tested for evidence of infection, because all died several years before the diagnosis of vCJD in the donor. There were clearly more recipients of fractionated plasma products prepared from plasma from the affected donors. Two of the donors had given plasma destined for fractionation in the period 1991 to 2004 (10 donations in one case, 12 in the other). These 2 donors accounted for around 50,000 recipients: 2000 for treatment of chronic disorders (hemophilia, immunodeficiency), the rest for occasional treatment (albumin, immunoglobulins).

In response to the first three cases of blood donors who later developed vCJD, the following measures were put into place in France:

- Immediate recall of in-date fractionated plasma products and labile blood components prepared from these donors. When the illness was discovered in the donor, blood products had almost always already been transfused, but this strategy allowed the following actions.
- Information to the prescribers of the labile blood components implicated in the investigation.
- Direct and personal information to the recipients of blood components (except those transfused before the epidemic); exclusion of all recipients as donors of organs, tissues, and cells (they were already excluded from blood donation because of their history of transfusion); and finally, putting in place long term clinical follow-up.
- A decision to not inform individual recipients of fractionated plasma products, except hemophiliacs who had received Factor (F)VIII or F IX produced from the affected donations.
- Information aimed at the general population and at health professionals about the possibility of transfusion transmission of vCJD.

The information given to the blood transfusion recipients by their doctor proved more difficult than that given, more than 20 years previously, to the first blood donors to be found "LAV positive," who were already aware of the large number of uncertainties at the time about the prognosis of infection by the agent responsible for AIDS. Those who supported not informing recipients of the risk of prion transmission through blood transfusion use the following arguments: it is not possible to quantify the absolute risk, because of a number of unknowns and the absence of a diagnostic test; the existence of preventive measures applied in recent years against the risk linked to labile blood components; the major psychological harm resulting for such information, which can only generate major anxiety; absence of any diagnostic or prognostic tests (except for codon 129 status); and finally, lack of any prophylaxis or treatment. On November 4, 2004, the National Ethical Consultative Committee of France (CCNE) confirmed its position expressed in 1997: to not worry without benefit, notably where no preventive action is available, and to take into account the risk of excluding a patient from health care in the name of the precautionary principle. Finally, the CCNE insisted on the need for complete traceability of donations of blood from donors who had subsequently developed vCJD.

Those in favor of informing recipients of the risk pointed out the need to inform them that they could no longer donate (in principle, because they had been transfused, the subjects were already excluded permanently from blood donation), but also that the patients had "the right to know," imposed by French law on March 4, 2002, which puts an obligation on the doctor to alert the patient to all "newly identified risks," even if the degree of the individual risk is not quantifiable and there is no available diagnostic procedure and no means of prevention. Another factor favoring informing recipients is to reduce the risk of secondary spread to health care workers, dentists, and other patients. The French circular number 138 of March 14, 2001, defined the management principles of the risks of transmission of "nonconventional transmissible agents" during medical and surgical procedures and had classified the recipients of labile blood components in the category of patients at individual risk of contamination by the vCJD agent. For all these reasons, in France, it was ultimately decided to inform patients at high risk of prion infection.

PRECAUTIONARY MEASURES FOR DONORS AND LABILE BLOOD COMPONENTS IN THE UK AND FRANCE

Since the removal of infected beef products from the food chain, a public health measure taken to protect the general population, precautionary measures to reduce the risk of transfusion transmission of prions were implemented in the UK and France in line with advances in epidemiologic knowledge. Some were put in place before the emergence of the first case of transfusion-associated vCJD, primarily to reduce the risk of transmission of other forms of CJD and in particular the iatrogenic forms. The first case of transfusion transmission of vCJD provoked the health authorities in the UK and France to take new and complementary risk reduction measures. Along with the exclusion of at-risk donors, the introduction of leukoreduction has contributed to the reduction of the infectious load in prion transmission by blood[56] (it has been shown that this could reduce the infectivity of whole blood by almost 50%[57]). Despite this, as the cases of vCJD transmission by blood transfusion observed in the UK were all due to nonleukoreduced blood components, it could be concluded that the decrease is by definition accounted for by leukoreduced blood components, and above all that it has been established that the white blood cell (WBC) layer does not contain all the infectivity: an equal amount of infectivity exists, we now know, in plasma. Leukoreduction therefore appears a necessary measure, but certainly not sufficient.

Table 1 lists, in chronological order, the precautionary measures, specific or nonspecific, put in place in the UK[58,59] against the risk of transfusion transmission of vCJD. In France, the precautionary measures followed the same pattern in a number of complementary actions. The circular of September 23, 2005,[60] concerning the reports of the first probable British cases of transfusion transmission of vCJD and the first case of a French donor who developed the illness, raised the issue of secondary transmission by transfusion of labile blood components or by use of surgical instruments or endoscopes on patients who had received transfusions of blood components originating from donors who later developed vCJD. The successive measures instituted in France and including those taken for the other forms of CJD before the emergence of vCJD, are shown in Table 2.

TABLE 1. Preventive measures in the UK against the transfusion risk of vCJD

1997	Recall and discard of labile blood components and of plasma derivatives obtained from donors who later developed vCJD.
1998	Importation of plasma destined for fractionation from non-UK sources.
1999	Leukoreduction of all labile blood products.
2002	Importation of FFP for recipients born after January 1, 1996.
2004	Permanent donor deferral in case of transfusion after January 1, 1980.
2005	Importation of FFP for recipients age less than 16 years.
	Permanent donor deferral in case of transfusion anywhere in the world after January 1, 1980.
	Permanent deferral and notification of donors whose donations have been transfused to recipients who later developed vCJD.
	Progressive replacement of PLT pools with apheresis (single-donor) PLTs. Apheresis PLTs recommended for children age less than 16 years.

TABLE 2. Preventive measures in France against the transfusion risk of vCJD

1992-1995	Permanent donor deferral in case of treatment by injection of growth hormones of pituitary origin.
	Permanent donor deferral in case of history of neurodegenerative disease.
	Recall and discard of labile blood components and batches of plasma products containing plasma from donors who later developed sCJD, fCJD, or iatrogenic CJD; having a history of fCJD; or having been treated with hormones of pituitary origin.
	Permanent donor deferral in case of history of neurosurgery.
1997	Tracing of recipients of labile blood components collected from donors who later developed CJD.
	Permanent donor deferral in case of transfusion of graft.
	Recall and discard of labile blood components and plasma products obtained from donors who later developed vCJD.
1998	Leukoreduction of cellular blood products for a residual level $<1 \times 10^6$/unit.
2000	Permanent deferral of donors who lived in UK for 1 year or more between 1980 and 1996.
2001	Leukoreduction of all plasma (FFP or plasma destined for fractionation) to a residual level $<1 \times 10^6$/unit.
	Residual WBC level $<1 \times 10^4$/unit for plasma not destined for fractionation.
	Reduction of volume of plasma in PLT components through use of PLT additive solution, potentially reducing an infectious load.

Information for doctors, recipients, and donors.

One difficulty with the current situation is that individuals incubating vCJD but still asymptomatic will not know they are at risk and may be donating their blood. This is relevant as those affected to date with vCJD have been relatively young and could donate their blood several times per year, for many years. In these circumstances, the only specific preventive measure against prion contamination of blood transfusions would be to rely on a diagnostic blood test for qualification of donors, or a general measure which could prove absolute efficacy (while one waits for prion filters), or both.

In the prevention of any transfusion risk, an equilibrium between the deferral of at-risk donors and the need for blood components is necessary. Being the most exposed country, the UK has taken major measures against prion transmission by blood transfusion, such as the importation of all plasma for fractionation.[61] Although leading to the exclusion of numerous blood donors,[62] these measures were imposed by the "precautionary principle."[63,64]

Transfusion measures taken in other countries are essentially based on the exclusion of potential blood donors who have stayed in an "endemic" area. For example, the Canadian authorities took successively more stringent measures to exclude from donation individuals who became at risk through a stay in a country affected by the vCJD epidemic: in 1999, all people who had spent a cumulative period of 6 months in the UK since 1980 were excluded from donation; in 2000, it was the same criterion for a stay in France; in 2001, the duration of a stay in France or the UK was reduced to 3 months; and that of a stay elsewhere in Europe must not exceed a cumulative period of 5 years.[65,66]

After a case of vCJD in an individual who visited the UK for less than 1 month in the 1980s and who developed in 2001 an illness that led to his death in 2004, Japan also took precautionary measures for blood transfusion, considering that the patient had become infected in the UK, even though approximately 15 cows born in Japan have been identified with BSE. Having already excluded donors who had stayed more than 1 month in the UK, the Japanese health authorities took the decision to exclude all individuals who had stayed for even 1 day in the country between 1980 and 1996. One can see that prion infection and the precautionary principle have at least two common points: they cross all frontiers and spread in an unforeseen manner.

THE VARIABLES OF RISK OF TRANSFUSION TRANSMISSION OF PRIONS BY LABILE BLOOD COMPONENTS

At this stage of medical knowledge, it is clear that all the elements of risk for transfusion transmission of prions are not clarified. Certain elements are however identifiable:

1. The number of labile blood components received by the patient and the date of their production in relation to the dates of the epidemic and to the application of precautionary measures (exclusion of at-risk donors, leukoreduction, etc.).
2. The prevalence of infection in blood donors: a great uncertainty exists about this prevalence that will depend upon that in the general population who were exposed through diet in the UK and in France. A calculation taking into account the higher end of the estimate of between 6 and 300 persons infected in the general French population and imagining these 300 cases among the 36 million subjects who are in the age range to be blood donors (18-65 years) and eligible to be donors, and assuming that they could be infective during the whole incubation period, results in a prevalence of 1 in 120,000: that is approximately 8 infected individuals per 1 million donors, which is close to 11 infected donations per year—in a *worst case scenario*.[67] In a recent reevaluation, the number of expected vCJD cases in France was revised downward (maximum of 100 cases instead of 300), leading to an estimate of 1 donor infected in 1/360,000. The equivalent calculation in the UK gives a prevalence of 1 in 10,000 donors, although better estimates may become available in the UK with results from the National Anonymous Tonsil Archive testing that is currently in progress.
3. Infectivity of a labile blood component with regard to prions is still ill understood. One measures and refers to an "infectious dose," defined as the minimal dose capable of transmitting the infection in an animal model for the mode of contamination given. At present, the infection of a unit of blood depends on two factors:
   - The stage of infection in the donor: the level of circulating prion and thus the infectivity certainly increases with the duration of the incubation period.[68] This ignores the delay before which the blood of

the infected subject becomes infective for the recipient of the blood: an infected donor, donating during the early part of the incubation period, may not be infectious to a recipient. According to animal studies, blood infectivity can be demonstrated at least at the start of the second half of the incubation period and perhaps also earlier (the infectivity of blood precedes the presence of pathological prion in the brain and the organs).[68] Even though experiments suggest that infectivity will be absent or minimal during the first third of the incubation period, caution dictates, in the current state of knowledge, that a labile blood component originating from a donor in the incubation period contains at least one infectious dose.[69] As many years have passed since the peak of the dietary epidemic, infected individuals are no longer in the initial stages of infection. The paradox could be that even though the number of infections is no longer increasing, the number of infectious subjects could still increase over time.

- Second, the efficacy of leukoreduction for cellular components and for plasma: leukoreduction of hamster blood contaminated with a scrapie prion removed only a little less than half (42%) of the infectivity present, because the infectivity divides almost equally between the WBCs and the plasma.[57,70,71] Leukoreduction may therefore be less effective than originally calculated. As demonstrated in studies based on experimentally infected rodent blood, total blood infectivity will be, during the asymptomatic phase, from 20-30 IU per mL,[35] and the distribution in the compartments of blood is in the order of 30 percent in the buffy coat and 50 percent in the plasma.[71] The presence of RBC and PLT infectivity has not been established in a formal manner: it seems at any rate to be little or none.[72,73] Thus, after the implementation of leukoreduction of labile blood components (which must have a residual WBC count of $<1 \times 10^6$/unit), the infectivity of RBC or PLT components is dependent on the amount of residual plasma. Use of optimal additive solutions for cellular components helps to reduce the quantity of plasma and therefore the infectious dose in the case of an infected blood component.

4. Recipient methionine homozygosity at codon 129 has an impact on the risk of developing illness, with perhaps a hierarchy of risk, moving in descending order from MM homozygotes to MV heterozygotes to VV homozygotes. Furthermore, nonhomozygosity for MM does not appear to confer absolute protection from infection, as indicated by the second UK recipient case (an MV heterozygote, nonetheless infected through the transfusion route) and in experimental animals.[13] What is certain is that the clinical outcome of transfusion transmission appears to be greatest for MM homozygotes, since they alone of the "exposed" population at risk have developed the disease.
5. Finally, the length of the incubation period, an essential factor and of which much is currently unknown and to which must be added two important parameters: the age of the recipient and the posttransfusion survival, which is heavily influenced by deaths due to the underlying illness in the initial years after the blood transfusion.

PRION FILTERS

Specific prion reduction filters applicable for certain labile blood components have been undergoing validation. The first donations processed with these prion filters demonstrated their capacity to reduce spiked infectivity of blood by three logs, which would without a doubt make a significant contribution to reducing transfusion risk.[74] These filters have been produced by two companies with a view to use for RBC preparations: application to PLT preparations and to plasma await further work. The validation work has been carried out on the Pall leukotrap affinity prion reduction filter, integrated in the filter CompoSafe Pr Fresenius,[75-77] and the TSE affinity ligand of the pathogen removal and diagnostic technologies, integrated in the P-Capt MC (MC for Macopharma) filter.[78] Changes were made to the Pall filter after the initial validation, which affected performance and led to its withdrawal. A new combined leukoreduction and prion removal filter from the same manufacturer is now under development. These affinity filters are assumed to remove all detectable traces of infection in a contaminated unit and to reduce infectivity by transfusion. This capacity has been demonstrated by a study based on inoculation, in hamsters, of leukoreduced whole blood taken from animals infected by a TSE. When the blood was treated with passage over a filter, no hamster became infected. When the blood was not filtered, some hamsters developed illness associated with the presence of prions in tissues.[79] Nevertheless, although the potential of these filters has been demonstrated by experimental infectivity transmissions in animal models, their efficacy in the prevention of

human transfusion transmission remains to be validated.[80] Indeed, the amount and the form of the pathological prion circulating in different human blood components may differ from that in an animal infected in an experimental manner, in particular from brain extracts. These artificial situations cannot reproduce exactly the qualitative and quantitative characteristics of the human prionemia. The most infectious aggregates of pathologic prion protein are those that are formed of 14 to 28 molecules,[81] but the size of circulating aggregates in the blood remains unknown. Furthermore, the consequences of using prion reduction filters on blood constituents (notably in the maintenance of PLT function) and on plasma proteins is totally unknown.[82] Chief of the possible consequences are the risk of neoantigenicity and induction of inhibitors.

A MUCH-AWAITED DIAGNOSTIC TEST

Lacking nucleic acid and not provoking any immune response by the infected host, the pathologic prion cannot be detected by molecular or serologic methods usually used in viral diagnosis. Furthermore, research for indirect markers has, up to now, reached a dead end.[83-85]

In asymptomatic or symptomatic infection, the most useful diagnostic test will be based on detection of the pathologic prion in the blood. However, the form that the prion takes in the blood is different from its form in the central nervous system. PrP^{sc} has an aggregated form in the brain and a much more soluble fraction in the blood: that difference could influence the effectiveness of diagnostic tests, the majority of which are based on the capacity to detect the cerebral form. Furthermore, the pathologic form only represents part of the circulating prions, but it is this pathologic form that the test must detect. Most of the diagnostic tools developed up to now depend on the physicochemical differences in the two forms of prion, the normal and the pathologic, in particular on the resistance of the pathological form to proteinase K.[11,86-90]

A large number of unknowns relating to the transmissibility, epidemiology, and natural history of prion infection will no doubt be resolved when one or several diagnostic tests, having the necessary characteristics of sensitivity, specificity, and reproducibility, become available and usable on a large scale.[59,91,92] Major efforts are presently being made to develop such tools, which could be used in the screening of blood donations and would help to reduce even further the risk of transfusion transmission of prions. These tests should, nevertheless, meet strict criteria:[93,94]

1. A very high sensitivity, to detect an infectious load that may be very low in asymptomatic subjects, because a low level of PrP^{sc} in circulating blood is likely to be infectious for recipients of blood components.
2. High specificity is essential, since the normal protein is present in the circulating blood.[95] False-positive results could have disastrous consequences, in terms of notifying individuals whose blood donation had been concluded "positive," not to mention the unjustified deferral of a large number of donors. For other infections, every reactive result obtained through blood donation screening tests must be verified by a confirmatory test to separate true-positive results from false-positive results. At present, it is not known if a true confirmatory test will be available, whether the solution for prions will be two screening tests performed simultaneously, or if one will be used for "confirmation" of a positive result by the other. On the problem of specificity, it has been calculated that, if a diagnostic test having a 99 percent sensitivity and an equivalent specificity was applied to the screening of blood donations in a population having a prevalence of vCJD of 1 in 10,000 (which is the estimate for donors in the UK), 99 individuals would be detected in the incubation phase of the infection and would correspond to "true positives" for 1 million tested donors, but 10,000 donors would give a false-positive result. On the other hand, there would only be one false-negative result in 1 million tests.[96] In France, where an estimate of prevalence is 1 donor infected in 360,000, fewer than 10 carriers of the variant would be detected, but the number of false-positive results would be just as high as the UK: 10,000 per 1 million donors, who would not be allowed to give their blood and who would need to be informed of their biologic status.
3. Finally, these tests will have to be reproducible, usable on a large scale, and able to be carried out within a time scale that is compatible with the shelf life of PLT components, criteria that have been required (and obtained) for nucleic acid testing in transfusion.

The lack of a test with the above-mentioned characteristics has major consequences: the impossibility of deferring from blood donation all those who are carriers of vCJD and the necessity of basing the screening of donors and of blood donations on nonspecific or partially effective measures such as existence of a risk factor in the donor, leukoreduction of blood donations, and so forth; the impossibility of detecting infected recipients and of testing at-risk recipients; the difficulty in collecting data about the mean duration of incubation of the illness; and in France

and in other countries, the impossibility of rehabilitating donors excluded because of a stay in the UK during the affected years (all the more because, among the cases of vCJD identified in France, such a history has been found only once and it becomes a paradox to exclude donors on the pretext of a visit to the UK when almost all the French patients who had the illness were infected in their own country). It would be necessary, furthermore, to take care that the positive effect of such a "rehabilitation" for some donors was not offset by a negative effect, by announcing, in the media, the use of a specific transfusion screening test. This could raise concern in the donor population, of learning, through giving blood, that they carry the infectious agent of an illness for which there is no preventive or curative treatment.

While awaiting validation, and preceding their potential use in detecting donors who are infected by vCJD, the first tests could be usefully applied in studies of sample repositories, to determine the spread of the epidemic in the overall population and in the transfused population. Assessment of the prevalence of vCJD in donors and recipients of blood, as well as its transmissibility through plasma products, could be carried out via anonymous plasma samples of matched donors and recipients. This is one of the possibilities provided by the repository presently undertaken on a European scale, called "BOTIA" (Blood and Organ Transmissible Infectious Agents).[97] Indeed, for obvious ethical reasons, one cannot use imperfectly validated tests on nonanonymous samples.

Meanwhile, the absence of a diagnostic test and strong uncertainties about a transfusion epidemic of vCJD requires maintenance of the preventive measures established by the UK, France, and other countries. If a specific test is used in transfusion in the future, there will be the opportunity to consider relaxation of these measures.

UNCERTAINTIES

An illness whose pathogenicity is not well known, with an uncertain prevalence of the infectious agent in at-risk groups and in the general population, the absence of a screening test, infectiousness and duration of incubation poorly defined, and the absence of any therapy, make up the elements that influence the transfusion risk of vCJD and handicap its prevention. Many questions have no answers, and the order in which we enumerate them probably does not correspond to the sequence in which solutions will be found:

1. After the end of the UK dietary epidemic and after the peak of the vCJD epidemic in 1999, will there be a second peak of transfusion origin? Up to now, the epidemic has remained relatively limited: approximately 200 cases worldwide, of which three-quarters have been in the UK. The initial pessimistic hypotheses on future number of cases have been revised downward. Furthermore, the peaks that followed the initial peak of CJD cases linked to injection of contaminated growth hormone were smaller and smaller, as if patients of other genotypes were less susceptible to infection and/or to the development of clinical illness. It is not known if the same will happen with vCJD, but the hypothesis of a secondary transfusion epidemic, with reamplification of the phenomenon through asymptomatic carriers of the prion, cannot be excluded. Nevertheless, it is now 14 years since the first cases of vCJD occurred, and no evidence of clinical cases in heterozygotes has appeared, in contrast to observations in the growth hormone epidemic. Finally, intraspecies transmission of prions induces, compared to interspecies transmission, a shorter incubation period and increased effectiveness of transmission. This could cause a larger outbreak of infection through transfusion than through contamination by food.
2. What is the prevalence of infection in the general population of the UK and in France, and how many potentially infected donors are there? The results of a retrospective British study on the prevalence of vCJD in surgical tissues from appendectomies and tonsillectomies pointed in the direction of a much higher prevalence of asymptomatic carriers than was implied by the known number of symptomatic cases. Furthermore, since, in the British MV transfused recipient carrying the variant, PrP^{res} was only detectable in the spleen and the cervical lymph nodes, and not in the appendix or the tonsils, this retrospective epidemiologic study based on detection of the pathologic prion in the appendix could have underestimated the size of the epidemic in the general population.
3. What are the kinetics of the appearance of circulating prion during the incubation phase? For estimation of the transfusion risk, the working hypothesis is that of blood infectivity and thus potential transmissibility throughout this phase, but the prion level in circulating blood may be too low, in the first months or first years of infection, to transmit infection by transfusion.
4. What is the effect of the current precautionary measures in transfusion, especially leukoreduction? The margin of safety that this measure gives is unknown. Has a reduction in infectivity prevented, or will it prevent, some transmissions by blood components? Up to now, the most feared contradiction would be the appearance of vCJD in a recipient transfused solely with leukoreduced components. Such a finding has not yet been reported.

5. Do non-MM subjects (that is, 60% of the general population) have absolute resistance to the disease, or might they develop it after a longer period of incubation? This latter instance would imply a second wave of the epidemic, which might, furthermore, be partially masked by other causes of death.[98] But in both situations, infected asymptomatic subjects would not be less infectious if they donated their blood, and recipients could become symptomatic if they have the MM status. Such a situation would be epidemiologically disastrous: an MV or VV infected donor could transmit vCJD to any number of recipients and not become ill him- or herself, but the recipients would develop illness if they are MM. Such circumstances have been observed in viral transfusion transmissions, notably in infection by HIV, where an infected recipient could develop symptomatic illness several years before the donor. In these conditions, donors who are carriers of vCJD but do not develop the illness because of a protective genotype at codon 129 would not be identifiable without a specific diagnostic test, except by transfusion investigations showing their common donor status in two (or more) recipients infected by vCJD who had developed the illness because of a nonprotecting genotype. Such studies would be critical as the only ones capable of identifying a regular, infected donor and of interrupting a chain of transmissions by transfusion. One can perceive to what extent the deferral of transfused patients from giving blood is an essential measure in breaking a "contamination cycle" between the donor population and that of recipients. Implemented several years ago, this precaution has probably avoided several transfusion transmissions of vCJD, even if a German study, based on a mathematical model, has concluded that the effect of this strategy is minimal: arguing that the majority of donors were infected from dietary sources and, having never been transfused, would not be excluded from blood donation.[99]
6. How many donors and recipients will develop vCJD during the next few years, each time leading to transfusion lookbacks and investigations? In the TMER study, among recipients of blood collected from vCJD-infected donors, the proportion of recipients who developed the illness has appeared high in a relatively short period of time (less than a decade), taking into account that the truly at-risk group was reduced by the genetic status of codon 129.
7. Will the threat of transfusion transmission of prions be limited solely to the UK and France? The description, in other countries such as Spain and Saudi Arabia, of cases of vCJD with a past of blood donations, suggest that the problem has now taken on an international dimension, including, and above all, in its aspect of transfusion safety.

CONCLUSIONS

The possibility of a blood component recipient developing vCJD 10, 20, or 30 years after transfusion, and that of a regular donor developing it after the same amount of time, are two situations that will not find any solution except with the help of a transfusion traceability almost as prolonged as the human life span. The basic danger of the past dietary epidemic is now a problem of chronic asymptomatic carriers who donate their blood or can transmit the infection via medical devices used in surgery or in endoscopies,[100] creating and thus enlarging a secondary wave of epidemic.

An essential notion is that of protection provided by leukoreduction of blood components. In a worst-case scenario, where cases of vCJD would show up in recipients who have been exclusively transfused with leukoreduced blood components and thus infected by the residual plasma, the only solution, in the absence of a biologic test for the screening of blood donations, would be to have recourse to prion filters, if the question of their effectiveness and harmlessness is resolved, or to only use washed RBCs, with all the accompanying logistical and financial concerns[101]—even if a partial reduction of prion level through leukoreduction would help to reduce the number of infected recipients and/or to induce a longer incubation period (in the hypothesis that this period would be proportional to the original contaminating infection).[102]

Many professionals in the field of transfusion infection are betting on the efficacy of prion filters, while acknowledging that demonstration of their clinical efficacy remains difficult for reasons we have already described and which are dominated by the absence of a diagnostic test usable on a large scale. At present, a decision to use these filters is problematic and leads to as many questions as not using them. In addition, perfecting a screening test that would be applicable for blood donations will raise a no less difficult question: filter or test? Filter and test?

Procedures for the inactivation of infectious agents in labile blood components will probably be implemented, but since they are aimed at the nucleic acids of these agents, they will not be effective against prions. For this reason,

the prion could become the ultimate transfusion-transmissible agent, while the risk connected to viruses, bacteria and parasites, known or emerging, would be controlled by pathogen inactivation.

If transfusion transmission of vCJD is a certainty from now on, benefits of transfusion obviously remain immeasurable compared to this risk. One must put in perspective the number of lives saved every day by transfusion and the number of cases of transfused vCJD counted on a worldwide scale. One also must compare this risk, which mainly concerns two European countries, with the infectious risks faced by transfused patients in parts of the globe where the means are so limited that safety is not always assured even for major blood-borne agents.

Never before have so many measures been taken in transfusion to counteract a risk that is numerically so low, some taken even before the first case of vCJD by blood transfusion had been reported. The precautionary principle has not just gone into the law: it has also penetrated the senses.

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bjh review

16

An update on the assessment and management of the risk of transmission of variant Creutzfeldt-Jakob disease by blood and plasma products

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Summary

There have been four highly probable instances of variant Creutzfeldt-Jakob disease (vCJD) transmission by non-leucocyte depleted red cell concentrates and it is now clear that the infectious agent is transmissible by blood components. To date there in no reported evidence that the infectious agent has been transmitted by fractionated plasma products, e.g. factor VIII concentrate. This review outlines current and potential risk management strategies including donor deferral criteria, the potential for donor screening, blood component processing and prion reduction filters, plasma product manufacture and the difficulties in identification and notification of those considered 'at risk of vCJD for public health purposes'.

Keywords: Creutzfeldt-Jakob disease, blood, plasma products.

This review offers an update on our recent assessment and management of the risk of transmission of variant Creutzfeldt-Jakob disease (vCJD) by blood components and plasma products (Ludlam & Turner, 2005). As that review surveyed perceptions on the nature of the prion agent, the spectrum of prion diseases in animals and man, and the range of animal studies relating to pathogenicity and infectivity (much of which still represents the current level of knowledge), these topics are not reviewed again here, other than where significant new relevant studies have been published. This current review focuses on the state of the art in relation to the safety of blood components and plasma products, which has also been reviewed elsewhere (Farrugia *et al*, 2005; Dolan, 2006; Ironside, 2006 and Clarke *et al*, 2007).

To date, a total of 203 probable, or definite, cases of vCJD have been reported worldwide, of which 166 have arisen in the UK, 23 in France, four in Eire and Spain, three in the USA, and one in each of Holland, Portugal, Italy, Saudi Arabia, Japan and Canada (http://www.cjd.ed.ac.uk/vcjdworld.htm). Of these, two of the Irish and US cases and those in Canada and Japan are thought to have been infected in the UK. The third US case is thought to have been infected in Saudi Arabia. The other cases are thought to have been infected in their countries of origin either through exported UK meat products or exported animals or animal foodstuffs. The UK outbreak of vCJD appears to have reached a peak around the year 2000 and has waned such that in 2007 there were only five new cases, though the frequency of new cases continues to increase in France and Spain. All clinically affected individuals thus far have been methionine homozygous at codon 129 of the prion protein gene (*PRNP*). Mathematical projections based on the current incidence of vCJD suggest a maximum likelihood estimate of 70 further cases (95% confidence interval 10–190) (Clarke & Ghani, 2005). This could prove to be an underestimate, however, if individuals of other codon 129 genotypes are also capable of being infected and/or secondary transmissions occur from asymptomatic individuals.

Two observations give pause for thought. The first is that the median age of onset of clinical disease (26 years) has not altered over the past 10 years as one might expect if a cohort of individuals were exposed to infection during a specific window of time. The best fit mathematical model suggests an age-related exposure/susceptibility during the teenage years. The second is the data from a retrospective study of tonsils and appendices (Hilton *et al*, 2004) in which 3/12 500 samples showed evidence of abnormal prion accumulation, giving a maximum likelihood estimate of 3000 future cases. The discrepancy between this estimate and that based on current clinical incidence is best explained by the proposition that around 93% of infected individuals may experience long-term pre- or sub-clinical infection (Clarke & Ghani, 2005). This is consistent with experimental animal studies and clinical studies in patients with iatrogenic CJD and kuru, which suggest that individuals who are heterozygous or valine homozygous at codon 129 have a longer incubation period and a lower incidence of development of clinical disease than those who are codon 129 methionine homozygous. These observations give rise to concern however that a significant cohort of individuals, maybe as many as 1/4000 of the general population in the UK, may have sub-clinical vCJD infection

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and be at risk of transmitting the disease through blood and tissue products or surgical and medical instrumentation, despite being asymptomatic themselves.

As there is no currently accepted blood test that reliably identify vCJD infected individuals (see below), further studies have been carried out to try to refine the estimate of the prevalence of sub-clinical disease. The National Anonymised Tonsil Archive aims to test 100 000 tonsil samples. Currently, there have been no confirmed positive samples out of 45 000 tested (http://www.hpa.org.uk/infections/topics_az/cjd/tonsil_archive.htm). However there are reservations around the interpretation of these data, given that the sensitivity of the assays in detecting subclinical vCJD is uncertain, the frequency of involvement of the tonsil as a site of preclinical infection is unknown, and a large proportion of the study population are too young to have been exposed to dietary bovine spongiform encephalopathy (BSE). The Spongiform Encephalopathy Advisory Committee (SEAC) has therefore not felt it appropriate to amend the current prevalence estimates within the UK at present (http://www.seac.gov.uk).

Infectivity in the peripheral blood

Infectivity remains undetectable in the peripheral blood of patients with vCJD despite the fact that clinical transmission has clearly occurred. This apparent contradiction is probably explained by the presence of a species barrier between man and mouse and the limited volumes of blood that can be inoculated into test animals.

Studies in hamsters infected with the 263K strain of scrapie showed similar results to those in the Fukuoka-1 GSS strain in mice (Brown *et al*, 1998; Ludlam & Turner, 2005), with a point estimate of 1–10 infectious doses (ID)/ml of whole blood of which around 40% was associated with the leucocytes and most of the remainder in the plasma (Gregori *et al*, 2004). Further studies in this model suggest that the majority of cell-associated infectivity is only loosely bound and can be washed off and therefore that the plasma form of infectivity probably predominates. Further studies in mice suggest that the level of infectivity is similar in vCJD-infected animals (Cervenakova *et al*, 2003a). Studies in sheep naturally infected with scrapie, or experimentally infected with BSE, suggest a transmission frequency of up to 50% from blood taken during the preclinical or clinical phase of disease and transfused into recipients from a scrapie-free flock (Hunter *et al*, 2002). BSE has also been transmitted through buffy coat to the primate *Microcebus* (Bons *et al*, 2002).

Variant CJD transmission by blood transfusion

Within the UK, the Transfusion Medicine Epidemiology Review (TMER) has proved an effective system for collating evidence of possible transmission of vCJD by blood components (Hewitt *et al*, 2006). The UK CJD Surveillance Unit in Edinburgh shares information about new cases of vCJD with the Blood Transfusion Services, which search their databases to ascertain whether these patients have been blood donors in the past. In this event attempts are made to identify the fate of the blood components (http://www.cjd.ed.ac.uk/TMER) and trace, notify and monitor living recipients. The 'reverse' arm of the TMER study attempts to identify which individuals who develop vCJD have received blood transfusions and to identify the donors.

Eighteen patients with vCJD have, or had previously, been blood donors, from whom a total of 66 recipients have been identified, 26 of whom are still alive. Of those who have died, four cases of transmission of vCJD prions have been identified (see below). Many of these patients however will have died of their underlying conditions within 5 years of the implicated transfusion and will not have had time to show clinical evidence of vCJD if infected.

The first symptomatic case of vCJD disease associated with blood transfusion was identified in December 2003. This individual developed vCJD 6·5 years after transfusion of red cells donated by an individual who developed symptoms of vCJD 3·5 years after donation (Llewelyn *et al*, 2004).

A second case of transmission was identified a few months later in a recipient of red cells from a donor who developed symptoms of vCJD 18 months after donation. This patient died from causes unrelated to vCJD 5 years after transfusion. Postmortem investigations found abnormal prion protein accumulation in the spleen and a cervical lymph node, but not in the brain, and no pathological features of vCJD were found (Peden *et al*, 2004).

A third patient developed symptoms of vCJD 6 years and died 8·7 years after receiving a transfusion of red blood cells from a donor who developed vCJD about 20 months after this blood was donated (Health Protection Agency 2006).

The fourth case of transmission developed symptoms of vCJD 8·5 years after receiving a transfusion of red blood cells from a donor who developed vCJD about 17 months after this blood was donated. The donor to this patient also donated the vCJD-implicated blood transfused to the third patient (Editorial Team, 2007).

All four patients received transfusions of non-leucodepleted red blood cells between 1996 and 1999. Since October 1999, leucocytes have been removed from all blood used for transfusion in the UK.

These data therefore demonstrate clearly that non-leucodepleted red cells from asymptomatic individuals incubating vCJD can transmit the infection by blood transfusion to other individuals and that the risk of them doing so is relatively high.

Donor deferral criteria

There has been little substantive change in blood donor criteria since our previous review (Ludlam & Turner, 2005). Whilst other countries continue to defer those who have spent more than a specified cumulative period of time in the UK, within

the UK only those considered by the CJD Incidents Panel to be 'at risk of vCJD for public health purposes' on account of exposure to implicated surgical instruments, blood components or plasma products, and those who themselves have received blood components, are deferred (http://www.hpa.org.uk/infections/topics_az/cjd). There is considerable complexity relating to the introduction of similar donor deferral criteria in the context of cell, tissue and organ donation. Broadly, whilst all forms of donation are excluded for patients with CJD or those considered potentially infected, donation of haematopoietic stem cells and solid organs is permitted from those considered 'at risk for public health purposes' and those previously transfused, subject to a risk assessment that weighs the risk of vCJD transmission against the potentially life-saving nature of an otherwise suitable transplant. Donation of other tissues is based on the same donor deferral criteria as blood. Donor deferral criteria remain, however, blunt risk management tools with potential deleterious effects on blood, tissue and organ supply.

Importation of blood components

Since our last report (Ludlam & Turner, 2005) the use of imported methylene-blue treated fresh frozen plasma (FFP) has been extended to all patients under the age of 16 years and to high users. Solvent detergent-treated FFP is recommended for patients undergoing plasma exchange for thrombotic thrombocytopenic purpura on the grounds that there is some evidence to suggest that methylene-blue treated FFP has a deleterious impact on outcome in this patient group (Alvarez-Larran *et al*, 2004). Consideration continues to be given around the possibility of importing FFP and cryoprecipitate for additional groups of patients. Importation of platelets is likely to be impractical given the short shelf-life of these products. However, it may be possible to import red cell concentrates for some groups of patients, for example for children up to 16 years of age. Consideration also has to be given to cost, quality and regulatory requirements and countervailing risks of transmission of other infectious diseases or of component shortages.

Advances in the development of a screening test

As previously noted (Ludlam & Turner, 2005), neither nucleic acid transmission nor immunological responses have been clearly identified in association with transmission of prion diseases, rendering standard molecular and serological screening assays unfeasible. Surrogate markers, such as 14-3-3, S100 and erythroid differentiation-related factor, have thus far proved insufficiently sensitive and specific to be of clinical value. Considerable progress has however been made in the development of assays for the abnormal conformer of prion protein, PrP^{TSE}.

Normal prion protein (PrP^{C}) is a widely expressed 35 kDa 230 amino acid glycosyl-phosphatidylinositol anchored membrane glycoprotein with two N-linked glycosylation sites and a secondary structure that includes three alpha helices and a single beta-pleated sheet. During the development of prion diseases there is a change in secondary and tertiary structure with a substantial increase in the proportion of beta-pleated sheet which leads to a change in the physico-chemical characteristics of the molecule, rendering it relatively resistant to breakdown by endogenous proteases and leading to deposition of amyloid-like plaques in affected tissues. Because PrP^{C} and PrP^{TSE} have the same primary structure and post-translational modifications, both forms tend to be recognised by most conventional monoclonal antibodies. PrP^{TSE}-based assays, therefore, have to utilise alternative ways of distinguishing the normal from the abnormal conformational form. PrP^{TSE} was originally defined by its resistance to digestion by proteinase K (PK). However this is a relative phenomenon; PrP^{TSE} can be digested by higher concentrations and longer exposure to proteolytic enzymes and, in addition, there is recent evidence for proteolysis-sensitive forms of PrP^{TSE} (Safar *et al*, 2005).

Five general approaches have been developed for the detection of PrP^{TSE}

Differential proteinase-K digestion. Immunohistochemistry distinguishes PrP^{TSE} from PrP^{C} by disrupting the latter through the use of proteolytic enzymes or chaotropic agents, coupled with the use of standard anti-PrP monoclonal antibodies and *in situ* visualisation for detection. This technique enables the demonstration of PrP^{TSE} deposition in the central nervous system and, in the case of vCJD, in follicular dendritic cells in lymphoid tissue of clinically affected patients. Western blot relies on the extraction of PrP^{TSE} from blood or tissues, proteolytic digestion and electrophoresis with visualisation by anti-PrP monoclonal antibodies. Proteolysis leads to complete digestion of PrP^{C}, but removes only the membrane-distal part of PrP^{TSE}, leading to three bands on gel electrophoresis which corresponds to the three different glycosylation states. The migration rates of these bands vary between different strains of prion disease, allowing clarification of strain type (Collinge *et al*, 1996). Partially selective precipitation of PrP^{TSE} from large sample volumes by sodium phosphotungstic acid coupled with enhanced detection of bound antibody by chemiluminescence have been used to enhance the sensitivity of Western blot and demonstrate PrP^{TSE} in a variety of peripheral tissues in both sporadic and vCJD (Wadsworth *et al*, 2001). The World Health Organisation Working Group on International Reference Materials for the Diagnosis and Study of Transmissible Spongiform Encephalopathies oversaw a collaborative study in which a number of different laboratories used their versions of the immunoblot (Minor *et al*, 2004). Generally it was concluded that, at present, immunoblot is not sufficiently sensitive to detect PrP^{TSE} in the peripheral blood of animals or humans with clinical prion disease.

Immunocapillary electrophoresis was amongst the first methods that claimed to be able to detect PrP^{TSE} in the peripheral blood. The test material is treated with proteinase and subject to a competitive antibody inhibition assay using a labelled peptide (as the competitor) and a monoclonal antibody that recognises both PrP^{TSE} and the peptide (Schmerr *et al*, 1999; Yang *et al*, 2005). The technique has however proved difficult to reproduce in other laboratories and failed to discriminate between infected and uninfected blood samples in a blinded study (Cervenakova *et al*, 2003b).

Epitope unmasking/masking. More success has been achieved with the conformation-dependent immunoassay (CDI), which is predicated on the observation that some PrP epitopes are masked within the PrP^{TSE} aggregate. An increase in signal intensity produced by a labelled monoclonal antibody by a sample denatured using guanidine hydrochloride when compared with the native (un-denatured) sample denotes the presence of PrP^{TSE} (PrP^{C} gives the same signal intensity under both conditions). The sensitivity of the technique is increased through the use of highly sensitive dissociation-enhanced lanthanide fluorescence immunoassay for antibody detection and, in some versions of the assay, the use of PK to reduce background signal (Safar *et al*, 1998, 2002). CDI appears to achieve greater sensitivity than immunoblot (Bellon *et al*, 2003) and, in the format including PK, may approximate the sensitivity of infectivity assays (Bruce *et al*, 2001). In the absence of PK it appears able to detect PK-sensitive forms of PrP^{TSE}, though it remains unclear as to whether these are infectious or not (Bellon *et al*, 2003).

The epitope-protection assay developed by Amorfix uses a chemical modification process which alters epitopes on normal PrP but not those buried within PrP^{TSE} aggregates. The latter are then disaggregated and the conserved epitopes detected using immunodetection methods (http://www.amorfix.com).

PeopleBio have developed an approach where a single antibody is used for both capture and detection steps leading to the blocking of available epitopes by the capture of PrP^{C} but not PrP^{TSE}.

PrP^{TSE}- specific monoclonal antibodies. Several antibodies have now been developed that appear to be specific for conformation-dependent epitopes present in PrP^{TSE} but not PrP^{C} (Korth *et al*, 1997; Paramithiotis *et al*, 2003; Curin Serbec *et al*, 2004; Zou *et al*, 2004). On these, the antibody 15B3, described by Korth *et al* (1997) and manufactured by Prionics, is the best characterised and has proved capable of detecting infectivity in the peripheral blood of scrapie-infected sheep and BSE-infected cattle in the absence of PK digestion (http://www.fda.gov/ohrms/dockets/AC/06/slides/2006-4240S1_9.ppt). Three other antibodies (Paramithiotis *et al*; 2003; Curin Serbec *et al*, 2004; Zou *et al*, 2004) also appear specific to PrP^{TSE} but have not yet been translated to routine assay format.

PrP^{TSE} – specific ligands. A variety of other ligands have been shown to bind selectively to the abnormally conformed molecule. Plasminogen has been proposed as a means of selective binding PrP^{TSE}, but as it can also bind to a variety of other proteins it is therefore unlikely to be sufficiently specific for assay development (Fischer *et al*, 2000).

Polyanionic compounds are known to selectively bind PrP^{TSE} and this property has been employed in the Seprion assay (Lane *et al*, 2003), which uses coated magnetic beads to capture the molecule. The assay is not dependent on PK treatment and is not species-specific provided a suitable detection antibody is used. It is licensed for postmortem diagnosis of BSE and Chronic Wasting Disease and is reported to be able to distinguish between infected and uninfected blood in scrapie-infected sheep and a small number of human samples.

The approach developed by BioMerieux involves PK digestion, precipitation and denaturation followed by reticulation by streptomycin, chemical capture by calyx-6-arene and detection of the macromolecular aggregates by labelled monoclonal antibody (http://www.fda.gov/ohrms/dockets/AC/06/slides/2006-4240S1_9.ppt). Detection of PrP^{TSE} in a small number of plasma samples from scrapie-infected sheep, BSE-infected cattle and CJD-infected humans has been reported.

Adlyfe have developed a third approach utilising a synthetic peptide based on the region of the PrP molecule involved in the PrP^{C}–PrP^{TSE} conformational transition. The peptide sequence is coupled to its mirror image as a palindromic molecule fluorescently labelled at each end. When incorporated into PrP^{TSE} the peptide folds into a hairpin with a beta-sheet conformation and the flurophores stack and change their fluorescence wavelength. Further, the folded ligand induces further molecules to adopt the folded conformation and thus amplifies the signal (Grosset *et al*, 2005). The assay is reported to have discriminated infected from uninfected plasma in natural and experimental scrapie, BSE and CJD.

Chiron have utilised (http://www.fda.gov/ohrms/dockets/AC/06/slides/2006-4240S1_9.ppt) a synthetic PrP polypeptide to capture PrP^{TSE} on magnetic beads with detection by monoclonal antibody in an ELISA format.

Amplification. Two methods have been used to amplify the detection signal. Screening for intensively fluorescent targets utilises double labelled antibodies, more of which bind to PrP^{TSE} aggregates than to PrP^{C} and giving rise to a stronger fluorescence signal (Bieschke *et al*, 2000). Immuno-polymerase chain reaction (PCR) also provides a method of amplifying the signal from an antibody or ligand conjugated to a nucleotide sequence utilising the PCR (Barletta *et al*, 2005).

Two further approaches have been developed that result in the amplification of PrP^{TSE} itself. The first of these, protein misfolding cyclic amplification (PMCA) has given rise to considerable excitement. PrP^{TSE} seeded into an excess of PrP^{C} leads to formation of new PrP^{TSE}. That PrP^{TSE} is then

fragmented through sonication or shaking and leads to a new round of PrP^{TSE} formation (Kocisko *et al*, 1994; Saborio *et al*, 2001). Recurrent cycles therefore of incubation and fragmentation lead to amplification of the original PrP^{TSE} (Castilla *et al*, 2005). Immunoblot and CDI have been used for detection of PrP^{TSE} and infectivity. Studies show that 140 sonication cycles produced an increase in signal intensity of around 6000-fold, whilst a second 'nested' set of 118 cycles with a fresh source of normal PrP led to an approximate 10^7-fold amplification. The technique has proved capable of discriminating infected from uninfected blood from hamsters experimentally infected with scrapie, however there are recent reports of detection of PrP^{TSE} in uninfected animal brain implying the possibility of low levels of abnormally conformed PrP in 'normal' individuals.

A number of cell-based amplification techniques have been described in which the rodent cell lines N2a (Nishida *et al*, 2000), PK-1 (Klohn *et al*, 2003), Rov9 (Birkett *et al*, 2001) and CAD-5 are infectable by natural or experimental strains of scrapie and demonstrate amplification of PrP^{TSE} detected by immunoblot. No cell-based amplification has yet been successfully reported for CJD.

Both these kinds of amplification take several days (PMCA) to weeks (cell-based assays) and would therefore be better positioned as confirmatory rather than screening assays.

Considerations with regard to assay assessment. Whilst the above is not a comprehensive list of all the assays under development, it does provide a flavour of the range and variety of approaches and their relative strengths and weaknesses. Some of these are now approaching the point at which they may be Council of Europe (CE) marked and marketed as potential clinical assays. There are, therefore, a series of further considerations relating to the potential assessment and utility of prion assays prior to clinical implementation.

The required sensitivity is difficult to gauge because the level, spatial distribution and temporal variation of infectivity in the blood of patients with vCJD or healthy individuals with subclinical infection is unknown. The generalizability of experimental data from mouse and hamster experiments to the human condition cannot be assumed (Castilla *et al*, 2006). Moreover, the relationship between infectivity and PrP^{TSE} is complex. Although many authorities believe PrP^{TSE} to be causal, there is experimental evidence both of infectivity with very low levels of PrP^{TSE} and of the presence of PrP^{TSE} in the absence of infection in human brain (Yuan *et al*, 2006). Recent studies have shown that following PK digestion, particles in the size range 300–600 kDa have the highest associated specific infectivity (Silveira *et al*, 2005), suggesting that a PrP^{TSE} oligomer of 14–28 might represent an infectious dose. The contribution of PK-sensitive PrP^{TSE} to infectivity is uncertain. It is reasonable, therefore, to regard PrP^{TSE} as a marker of infection, provided it is recognised that there is not a simple linear correlation with the level of infectivity or the development of disease. The National Institute of Biological Standards and Controls have established a reference panel of homogenised human CJD-infected brain and spleen spiked into plasma, peripheral blood from natural and experimentally infected animals and a small number of peripheral blood samples from patients with vCJD, to provide independent evaluation of the sensitivity of prion assays (http://www.nibsc.ac.uk).

Whereas the sensitivity of an assay is mostly related to its technical aspects, the specificity is also highly dependent on the population into which it is deployed. Thus an assay which may be highly specific (i.e. a high proportion of true to false positives) in a group of patients with suspect clinical disease, can show very poor specificity in a normal population where the number of false positives may significantly exceed the number of true positives. This point is demonstrated in Fig 1. The UK Blood Services have established a Test Assessment Facility containing samples from 5000 UK and 5000 US blood donors to evaluate the likely positivity rates amongst the general population.

Finally, there are a number of other considerations in weighing the likely impact of the introduction of a vCJD assay. Such an assay is unlikely to only be used amongst blood donors, it may also be used in patients with suspect CJD or other psychiatric or neurological conditions (to exclude CJD); in those considered 'at risk for public health purposes' on account of exposure to implicated surgical instruments, blood components or plasma products; or for population prevalence studies and the 'worried well'. In the absence of a true confirmatory assay (i.e. the ability to demonstrate infectivity) it will prove difficult to discriminate between false and true positive individuals and, of course, the likelihood of a truly positive individual developing clinical vCJD is unknown.

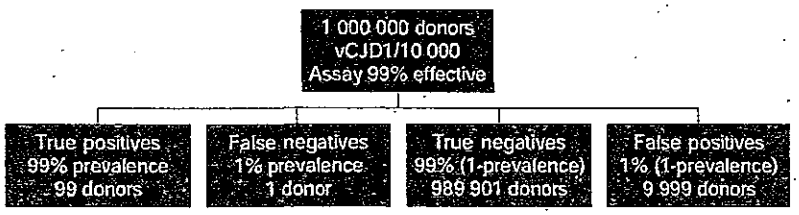

Fig 1. Outcome of screening of a 'normal' population of one million donors in whom there is a true positive prevalence of 1/10 000 for subclinical variant Creutzfeldt-Jakob disease using an assay with a sensitivity and specificity of 99%. As can be seen, a very poor specificity results where the number of false positives greatly exceeds the number of true positives.

Blood donors would not be able to continue to donate if they tested positive, it is illegal for example to take the donation with the intent of discarding it, even if the donor consents to such a strategy. Test positive individuals will therefore have to be told of this outcome and (presumably) managed as 'at risk for public health purposes'. Clearly this will cause significant distress and give rise to psychological and social problems for some people, act as a disincentive to blood donation and therefore a negative impact on the blood supply. Moreover, it is likely that previous recipients of blood components from these donors will also have to be traced and contacted (lookback), giving rise to a much larger group of individuals in the population considered 'at risk of public health purposes' and requiring specific precautionary measures to be taken in the event of surgery or medical investigation (see below). A comprehensive health and economic evaluation will therefore have to weigh the positive impact of reducing potential secondary transmission of vCJD against these potential negative consequences.

Table I. Residual infectivity distribution in a unit of leucodepleted red cell concentrate.

Log reduction in infectivity	Residual leucocytes	Residual plasma	Residual infectivity
Leucodepletion alone	0·2	130	130·2
1 Log	0·2	13	13·2
2 Log	0·2	1·3	1·5
3 Log	0·2	0·13	0·33
4 Log	0·2	0·013	0·213

The data represents the likely distribution of residual infectivity in a unit of leucodepleted red cell concentrate prepared by a bottom and top processing method (with a residual plasma volume of around 10 ml).
Assuming 10 ID/ml infectivity in whole blood with 40% (i.e.4 ID/ml) being removed by leucodepletion and the remainder residing in the plasma (i.e. for a haematocrit of 0·45 a plasma concentration of approximately 13 D/ml), around 130 ID remains in the unit's plasma. Hence up to approximately a 3 log further reduction is required to reduce the risk of transmission to <1 ID/unit.

Blood component processing

Universal leucodepletion was introduced in the UK in 1999 as a measure to reduce the risk of secondary transmission of vCJD. The experimental data from mice infected with the Fukuoka-1 strain of Gerstmann-Straussler-Scheinker disease (Brown *et al*, 1998, 1999) suggests that leucodepletion filters have little impact on plasma-borne infectivity. Studies in the 263K hamster model (Gregori *et al*, 2004) similarly suggest a 40–70% reduction in whole blood infectivity, consistent with the removal of leucocyte-associated infectivity, but not that present in the plasma. Table I illustrates the likely distribution of residual infectivity in a unit of leucodepleted red cell concentrate prepared by bottom and top processing method (with a residual plasma volume of around 10–15 ml). Assuming 10 ID/ml infectivity in whole blood, just over 130 ID would be left in the unit and that up to a 3 log further reduction is required to impact upon the risk of transmission (i.e. achieve <1 ID/unit). Red cell concentrates prepared by the more common top-top methodology contain greater amounts of residual plasma (around 20 ml) and would consequently require a 4-log reduction. The absence of data on the level of infection in human blood means an uncertainty of at least 1-log around these point estimates. It can be said in summary, however, that it is unlikely that current blood component processing will suffice to reduce the risk of transmission in most plausible infectivity scenarios.

Three companies are working on the development of prion reduction filters. One has a CE-marked dock-on filter which is used in series with a leucodepletion filter. Published studies using this filter material show >3 log reduction in infectivity on brain homogenate spikes and to the limit of detection (>1 log) in endogenous infectivity studies (Gregori *et al*, 2006). Two other companies are working on the development of combined leucodepletion/prion reduction filters. All prion reduction filters will have to undergo independent assessment of clinical safety and efficacy within a series of studies managed by the UK and Irish Blood Services and agreed with SEAC and the Advisory Committee on the Safety of Blood, Tissues and Organs (http://www.advisorybodies.doh.gov.uk/acsbto/index.htm). Part of the problem for both manufacturers and Blood Services is the absence of assays capable of detecting either PrP^{TSE} or infectivity in the peripheral blood of patients with vCJD. Assessment of the efficacy of the technology is therefore based on brain homogenate spikes (where baseline infectivity is sufficient to detect a 3–4 log reduction but the physico-chemical form of the spike is unlikely to be similar to that of plasma based infectivity), and endogenous infectivity studies (where the form of infectivity is likely to be more relevant, but the baseline infectivity is sufficiently low that little more than a 1-log reduction is detectable). There remain, therefore, fundamental questions relating to the clinical relevance of different forms of spike material and general applicability of these kinds of studies to the human situation. The potential for deleterious effects on the red cell concentrate itself are also a matter for concern, both in terms of the possibility of alterations to the rheological or antigenic profile of the red cells and the loss in the volume of the additional filter. The latter would have a particular impact if used in conjunction with bottom and top processing, the combined effect of which may reduce the red cell mass in a concentrate below current standards, necessitating additional transfusions for some individuals.

With regard to platelet concentrates, re-suspension in optimal additive solution rather than plasma would reduce the amount of residual plasma by around 65% to 80–90 ml. This would still contain more than enough infectivity to transmit infection to the recipient under even the most

optimistic of the current infectivity assumptions and is likely to be ineffectual. Prion reduction filters are not currently applicable to either platelet concentrates or FFP.

Plasma product manufacturing

It is reassuring that to date no recipient of a pooled plasma product has developed vCJD. However in 1997, shortly after the first description of vCJD as a new condition, there was concern that the UK plasma supply might have the potential to transmit the infectious agent and that plasma collected from countries where there were few or no cases of vCJD might pose a lower risk (Ludlam, 1997). Although this view gave rise to controversy, the regulatory authorities moved to a position of allowing, and subsequently mandating that pooled plasma products manufactured in the UK should only be made from plasma imported from parts of the world at low risk of vCJD.

In an attempt to help define the risk of PrP^{TSE} transmission by plasma-derived products, detailed studies have been undertaken to assess how prions are partitioned during the plasma fractionation process, mainly by spiking the starting plasma with 'exogenous' prion derived from brain homogenates of experimentally infected animals. The strengths and weaknesses of this approach are similar to those described above in the discussion around the assessment of prion filters. In general there was least clearance of prion in the manufacture of factor VIII, IX and antithrombin concentrates, greater clearance in the preparation of intravenous immunoglobulin, and greatest clearance in the manufacture of albumin (Foster, 1999).

The way in which different countries responded to the risk that plasma products might transmit the infectious agent varied and depended partly on the perceived relative number of donors who might be infectious as well as details of the plasma fractionation techniques used in each country.

In the UK, using data on partitioning of prion infectivity during manufacture of plasma products, along with the animal data on the likely range of infectivity in individuals with subclinical infection, a risk assessment was undertaken to quantify the risk of recipients of such products being infected. The CJD Incidents Panel have taken the view that an individual with a >1% additional risk of exposure to an infectious dose of vCJD should be notified and managed as 'at risk for public health purposes'.

To date a total of 174 'implicated' batches of plasma products have been identified as having been manufactured from a pool of plasma to which an individual contributed who subsequently developed vCJD (Hewitt *et al*, 2006). For each of these batches a detailed risk assessment was carried out that included the total number of donations included in the pool, the details of the plasma fractionation process used during manufacture and (conservative) estimates of the likely cumulative reduction in infectivity over the manufacturing process. The outcome was expressed as the likely mass of product to which an individual would have had to be exposed to increase their risk of exposure to infection by 1% over background. Although these estimates varied between manufacturers dependent on the details of the manufacturing process, broadly speaking plasma products could be classified into 'high risk of exposure' products, such as coagulation factor concentrates, where a single adult dose would suffice to place a patient beyond the 1% additional exposure threshold; 'medium risk of exposure' products, such as immunoglobulin, where only patients receiving repeated doses of the implicated batch would be likely to pass the 1% threshold; and 'low risk of exposure' products, such as albumin, where unfeasibly large exposure to the implicated batch would be required to move the patient beyond the 1% threshold. Categorisation as 'at risk for public health purposes' requires that a patient be notified and that precautions be taken in the use of surgical instruments and other invasive medical interventions (such as endoscopy with biopsy), to reduce the risk of onward transmission to other patients.

Consideration was given to whether to categorise as 'at risk for public health purposes' only those who had been exposed to specific implicated batches, or whether to categorise all individuals who had received UK manufactured products between 1980 and 2001 (the dates between which implicated plasma products pools could have used). After much debate it was agreed that for 'low and medium risk of exposure' products (immunoglobulins and albumin), the former approach would suffice, whereas for 'high risk of exposure' products (coagulation factors), an umbrella approach would be more appropriate because it was uncertain how many batches of plasma might be 'infectious' from donors with subclinical vCJD and because if additional donors developed vCJD, it would be necessary to inform further groups of recipients, who might have previously been told they had not received 'implicated' batches.

Other countries have responded differently to the UK. In France the assessment took into account the local plasma fractionation processes and concluded that the risk posed by 'implicated' batches to recipients was very small and it was not appropriate to take any special precautions to prevent further spread by surgical instruments. A similar view was formulated in Germany, though no cases of vCJD have yet arisen in that country (Seitz *et al*, 2007).

Within the UK advice on prevention of spread of PrP^{TSE} by surgical, medical and dental instruments is given by the Advisory Committee on Dangerous Pathogens and advice on individual clinical incidents by the CJD Clinical Incidents Panel. Instrument contamination was estimated to be of 'high risk' where there was contact with tissue from the nervous system and 'moderate' risk if there was exposure to lymphoid tissue (http://www.advisorybodies.doh.gov.uk/acdp/index.htm). Surgery involving these tissues should be undertaken with disposable instruments where possible, and if not they should be 'quarantined' thereafter and not reused because it would not be possible to ensure adequate decontamination prior to use on the next patient. This guidance has led to major

difficulties in the performance of biopsies with gastrointestinal endoscopes because the samples obtained would probably contain lymphoid tissue. The financial implications are significant because the endoscopes cannot be decontaminated and must effectively be discarded. Both upper and lower gastrointestinal endoscopies without biopsy do not result in the instrument being considered as potentially 'contaminated' and it can therefore be reused on other patients after standard cleaning procedure. The concern about possible contamination of instruments has also led to an increased use of capsule endoscopies, which give good images but cannot be used to biopsy or treat gut lesions.

Although no individuals with haemophilia have thus far developed vCJD and a retrospective study of autopsy samples from individuals with haemophilia in 1998 showed no evidence of sub-clinical infection, it has been important to try and gather more data (Lee *et al*, 1998). This has not been easy and depends upon procuring appropriate tissue samples prospectively from individuals undergoing clinically necessary surgery in addition to consent for autopsy. In addition it has been important to try and develop a record of the extent of exposure of individuals to 'implicated' batches of concentrate, as well as all recipients of UK clotting factor concentrates over the 22-year period of exposure. This is being co-ordinated by UK Haemophilia Centre Doctors' Organisation by accumulating the data for subsequent anonymised studies.

Communication with patients and the general public

Keeping recipients of blood and blood products informed about the current state of knowledge and in particular informing individuals about their individual risks has proved challenging because of the complexity and uncertainty inherent in our understanding of the field. It has been important for there to be close collaboration between those able to assess the risk of vCJD infection, physicians responsible for clinical services and patient organisations representing those potentially affected. For those who have received blood components from donors who subsequently developed vCJD, the risk of exposure to vCJD is judged to be high and these individuals have been contacted on an individual basis and offered counselling and specialist follow-up. Similarly, blood donors who have donated blood administered to a patient who later developed vCJD have been contacted and are managed as 'at risk for public health purposes'. In 2004, all patients with haemophilia were sent a letter stating whether or not they had or had not received UK plasma-derived clotting concentrates between 1980 and 2001, irrespective of whether or not they had received UK plasma products, because in an earlier mailing about this topic only those in the 'at risk' group were contacted and this left non-recipients of letters not knowing whether they had not been potentially exposed or whether their letter had got lost in the post. All were offered the opportunity for individual counselling. It is this attention to the detail of how patients are informed that is critical in trying to ensure that individuals feel confident in the arrangements.

For patients potentially exposed to other implicated plasma products, the issue of traceability and notification have proved more problematic. Whilst patients with primary immunodeficiency share a similar close long-term relationship with their physicians, those receiving immunoglobulin for other clinical indications or high doses of albumin (for example during plasma exchange), are often discharged following their acute care. The absence of a general system of traceability for plasma products and of searchable clinical notes has made the follow-up of the latter groups of potentially exposed patients highly problematic.

Concluding remarks

Three years after our last review (Ludlam & Turner, 2005), the management of the risk of transmission of vCJD by blood and plasma products remains highly challenging. Whilst the diminishing number of clinical cases is reassuring, there are continuing uncertainties surrounding the prevalence of sub-clinical disease, the level of infectivity in peripheral blood of such individuals, and the overall risk of transmission and development of clinical disease. Much progress has been made in the development of new technologies, such as prion filters and prion assays, but assessment of these is problematic and cost and countervailing risks need to be considered. Accurate and timely communication with the general public and with those who are considered to be at increased risk of exposure remains essential given the continuing complexity and uncertainty of the field.

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bjh review

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Managing the risk of transmission of variant Creutzfeldt Jakob disease by blood products

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Summary

Whereas plasma-derived clotting factor concentrates now have a very good safety record for not being infectious for lipid enveloped viruses, concern has arisen about the possibility that prion diseases might be transmitted by blood products. There is epidemiological evidence that classical sporadic Creutzfeld Jakob disease (CJD) is not transmitted by blood transfusion. There is now good evidence that the abnormal prion associated with variant CJD can be transmitted by transfusion of fresh blood components and infect recipients. To reduce the risk of the pathological prion in the UK infecting recipients of clotting factor concentrates, these are now only manufactured from imported plasma collected from countries where there has not been bovine spongiform encephalopathy (BSE) in cattle and the risk of variant CJD in the population is, therefore, considered negligible. The safety of these concentrates is also enhanced because prion protein is, to an appreciable extent, excluded by the manufacturing process from the final product. To help reduce the chance of prion transmission by fresh blood products, donations are leucodepleted, there is increasing use of imported fresh frozen plasma (especially for treating children) and potential donors, who have been recipients of blood since 1980 (the beginning of the BSE epidemic in cattle) are deferred.

Keywords: variant Creutzfeld Jakob disease, transfusion, epidemiology, safety, haemophilia.

Emerging pathogens will always challenge the safety of blood transfusion. Whilst the risk of hepatitis B virus (HBV), hepatitis C virus (HCV) or human immunodeficiency virus (HIV) transmission by blood components and plasma products is now small (http://www.eurosurveillance.org), new potentially transfusion-transmissible pathogens continue to emerge.

Many challenges were posed by the emergence of variant Creutzfeldt Jakob disease (CJD) in 1996 (Will *et al*, 1996). Whereas there has been little evidence of transmission of sporadic CJD by blood components or plasma products, it was recognised that variant CJD represented a different strain of prion disease, with a different distribution of peripheral disease (*vide infra*) and that absence of evidence of transmission could not be considered to equate with evidence of absence of risk (Ricketts, 1997; Ricketts *et al*, 1997). A number of precautionary measures were therefore taken to manage the uncertain risk. The description of two cases of transmission of variant CJD prions by red-cell concentrates over the last 18 months (Llewelyn *et al*, 2004; Peden *et al*, 2004), coupled with continuing concerns over the prevalence of subclinical disease in the UK population (Clarke & Ghani, 2005), has led to the introduction of further precautionary measures. The Departments of Health have considered it a high priority to prevent secondary spread of variant CJD by transfusion as this could lead to the infection becoming endemic in the UK population (Fig 1). However, these measures are likely to be of limited efficacy. The development of prion reduction filters and/or of peripheral blood screening assays pose significant challenges but hold the possibility of achieving better control over the risk of transmission of this disease by blood products. In the meantime, public health policy and medical practice will have to continue to balance the risks and benefits associated with human blood and tissue products (Flanagan & Barbara, 1996).

This review considers the unique aetiological and pathophysiological features of prion diseases, the measures that have been taken or might be taken in future to manage that risk, and the implications for those who prescribe and receive blood components and plasma products.

The biochemistry of prions

Prion protein (PrP^c) is a 30–35 kDa glycoprotein that is widely expressed by many cells and tissues in animals and man. It has two N-glycosylation sites and its secondary structure includes three alpha-helices and one beta-pleated sheet in the membrane-proximal carboxy-terminal of the protein. The membrane-distal amino-terminal of the molecule is largely unstructured. The protein is encoded by a single gene (*PRNP*) and with no spliced isoforms. In man, 20 different single nucleotide polymorphisms have been described that predispose to familial disease (de Silva, 1996a) (*vide infra*). In

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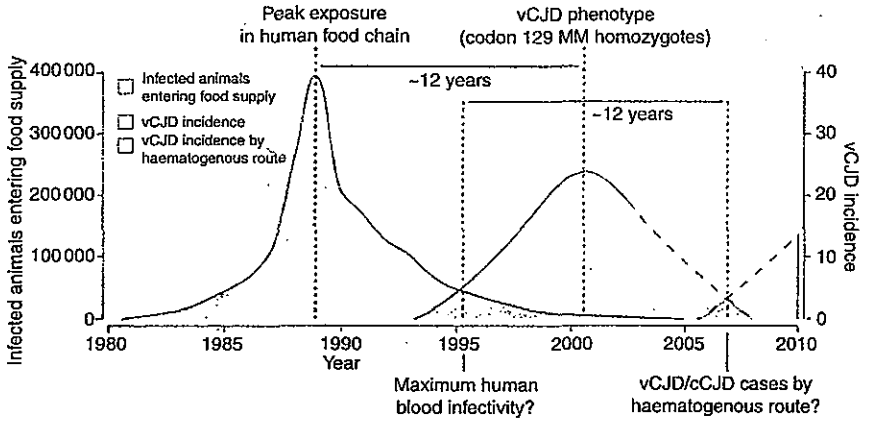

Fig 1. Incidence of bovine spongiform encephalopathy and variant Creutzfeldt Jakob disease in the UK (- - - - -, predicted cases). The right hand peak illustrates the potential for secondary spread by haematogenous spread. Reprinted from Collins *et al* (2004) with permission from Elsevier.

addition, a critical polymorphism at codon 129 coding for methionine or valine leads to significant variation in the susceptibility to, and incubation period of, human prion diseases. In the UK, 37% of the general population are homozygous for methionine at this locus, 11% are homozygous for valine and 52% heterozygous. Methionine homozygosity is much more common than expected amongst patients with CJD (*vide infra*). PrP is inserted into the cell membrane predominantly via a glycosylphosphatidyl inositol (GPI) anchor, although transmembrane and soluble forms have also been described. The glycoprotein is predominantly located in calveolar zones in the cell membrane and is estimated to have a half-life of around 6 h, being internalised into endosomes with a proportion recycling to the cell surface (Shyng *et al*, 1993). The function of the protein remains unclear, it has been shown to bind to a laminin receptor precursor protein (Martins *et al*, 1997; Rieger *et al*, 1997) and act as a copper metalloproteinase (Brown *et al*, 1997a). PrP null mice appear to develop normally although some strains show subtle neurological abnormalities (Tobler *et al*, 1996). Prion formation involves changes in the secondary and tertiary conformations of the PrP molecule: up to 40–50% of the molecule can be in the form of beta-pleated sheet, mainly at the expense of the membrane-distal unstructured region. This changes the physicochemical properties of the molecule and engenders relative resistance to proteinase digestion. Prion protein aggregates (PrP^{Sc}) are deposited in cells and tissues leading to the formation of amyloid-like plaques and in the nervous system to neuronal death, astrogliosis and spongiform change.

The mechanism by which PrP^{c} is converted to PrP^{Sc} remains unclear, as does its precise role in the aetiology of the disease. The prion hypothesis (Prusiner, 1998) proposes that the PrP^{Sc} molecule itself converts PrP^{c} to the abnormal conformation, either through a process of heterodimerisation or through nuclear polymerisation (Aguzzi & Weissmann, 1997). PrP^{Sc} is relatively resistant to proteinase-K digestion and different molecular strains of disease can be identified by the balance of di-glycosylated, mono-glycosylated and non-glycosylated species. Several molecular strains of PrP^{Sc} occur in sporadic CJD; however, only a single strain of PrP^{Sc} is found in variant CJD, which is similar to that seen in naturally occurring bovine spongiform encephalopathy (BSE) in cattle, and BSE transmitted naturally and experimentally to other animals (Collinge *et al*, 1996; Hill *et al*, 1997a). Evidence that variant CJD and BSE represent the same strain of prion disease also stems from infectivity studies in a prion disease strain typing panel of inbred experimental mice, where the patterns of incubation period and neuropathological targeting were similar and differed from those seen in sporadic CJD, scrapie and other prion diseases (Bruce *et al*, 1997).

Prion diseases in other species

A range of prion disorders have been described including those involving the SuP35p and Ure2p proteins in yeast, which appear to be non-pathogenic and convey a survival advantage under certain circumstances (Burwinkel *et al*, 2004).

Scrapie was first described as a disease of sheep and goats over 250 years ago and demonstrated to be experimentally transmissible 50 years ago (Aguzzi & Polymenidou, 2004). There is no evidence that scrapie has ever transmitted to man. The only other known self-sustaining animal prion disease is chronic wasting disease in mule deer and elk in several states of the USA. Again there is no current evidence that this disease has transmitted to man.

BSE was first described in UK cattle in 1985 (Wells *et al*, 1987) and is thought to have spread through oral consumption of ruminant-derived meat and bone meal (Wilesmith *et al*, 1988; Brown, 1998). The disease spread widely, peaking in 1992 with over 180 000 clinical cases in the UK, although mathematical estimates suggest that 1–2 million cattle could have been infected but slaughtered and entered the human food chain before they were old enough to demonstrate evidence of clinical disease (Fig 1) (Anderson *et al*, 1996). BSE has crossed into up to 20 other species, including domestic and exotic cats (Wyatt *et al*, 1991; Kirkwood & Cunningham,

1994) and exotic ungulates in British zoos. In July 1988, the spread of BSE led the UK Government to restrict the use of ruminant-derived meat and bone meal as an animal feed and in November 1989 specified that bovine offals were banned for human consumption.

Sporadic Creutzfeldt Jakob diseases

Sporadic CJD was the first described human prion disease, is of uncertain aetiology, has a worldwide distribution and an incidence of around one per million population per year (Will *et al*, 1998). The median age at onset is around 68 years and the disease is characterised by a rapidly progressive dementia leading to death in around 4–6 months. The incidence of the disease varies with the codon 129 genotype of the *PRNP* gene, with 83% of patients homozygous for the expression of methionine at this locus (Deslys *et al*, 1998). Molecular strain typing suggests that six forms of disease are dependent on codon 129 phenotype and strain of prion disease. One of the pathological hallmarks of sporadic CJD is the restriction of accumulation of plaques of prion protein to the central nervous system (CNS). However, with recently developed, more sensitive techniques, prion accumulation has also now been reported to be present in peripheral nerve (Favereaux *et al*, 2004) as well as in muscle, lymphoid tissue and olfactory epithelium (Glatzel *et al*, 2003) at an advanced stage of clinical disease.

Although there are a small number of reports claiming transmission of sporadic CJD by inoculation of blood from patients with clinical disease into experimental rodents (Manuelidis *et al*, 1985; Tateishi, 1985), these results have not been supported by further studies in primates (Brown *et al*, 1994). Similarly, although there are a handful of reports of sporadic CJD arising after blood or plasma product transfusion (Klein & Dumble, 1993; Creange *et al*, 1995, 1996; de Silva, 1996b; Patry *et al*, 1998), in none of these has a causal link to a donor with CJD been established. Moreover a series of epidemiological case control (Kondo & Kuroiwa, 1982; Davanipour *et al*, 1985; Harries-Jones *et al*, 1988; Will, 1991; Wientjens *et al*, 1996; Van Duijin *et al*, 1998; Collins *et al*, 1999), lookback (Esmonde *et al*, 1993; Heye *et al*, 1994; Operskalski & Mosley, 1995) and surveillance (Evatt, 1998; Evatt *et al*, 1998; Lee *et al*, 1998) studies carried out over almost 25 years have failed to demonstrate evidence of transmission of sporadic CJD by blood components or plasma products. It seems likely therefore that the preclinical incubation period in sporadic CJD is sufficiently short, or peripheral blood infectivity is sufficiently low, as to make transmission of the disease by blood components and/or plasma products at worst a very rare event (de Silva & Mathews, 1993; Brown, 1995; Ricketts *et al*, 1997; Will & Kimberlin, 1998).

Thus, although individuals suspected of having sporadic CJD are permanently deferred from blood donation, no other precautions, such as withdrawal of plasma products if the donor has contributed to the plasma pool, are undertaken. This is because although sporadic CJD is a rare disease, the large number of donations contributed to a plasma pool leads to frequent withdrawal and product shortages.

Familial human prion diseases

Familial human prion disorders are uncommon, which include Gerstmann–Straussler–Scheinker disease (GSS), fatal familial insomnia and familial CJD, and are associated with mutations in the prion gene (*vide supra*). Although there is no evidence of familial human prion disease transmission via blood products, individuals with two or more blood relatives with prion disease, or who have been advised that they are at risk of prion disease as a result of *PRNP* gene sequencing, are deferred from blood donation as a precautionary measure.

Acquired human prion diseases

The transmission of human prion disease (Kuru) was first reported in the Fore people of Papua New Guinea in the late 1950s (Gajdusek & Zigas, 1957) and is thought to have been transmitted during ritual cannibalistic or sacrificial funeral rites. The clinical features differ from those of sporadic CJD with more prominent ataxia and a longer clinical course. At one time Kuru was a leading cause of death amongst the Fore people and interestingly, despite abandoning these practices around 1960, there are still occasional people presenting with clinical disease – testimony to the fact that the incubation period in prion diseases can be very long.

Iatrogenic transmission of CJD has been well documented by direct inoculation of the CNS through contaminated neurosurgical instruments, stereotactic intracerebral electrodes, dura mater and corneal grafts. Iatrogenic transmission has also occurred via cadaveric human pituitary growth hormone and gonadotrophins administered by intramuscular injection (Buchanan *et al*, 1991; Brown *et al*, 1992). The clinical presentation varies depending on the route of infection; centrally transmitted cases tend to have a shorter incubation period of around 2 years and develop a rapidly progressive dementia reminiscent of sporadic CJD, whilst peripherally transmitted cases tend to have a much more prolonged incubation period of around 13–15 years and present with ataxia and sensory disturbance (Table I) (Brown *et al*, 2000).

Variant CJD

Variant CJD was first described almost 10 years ago (Will *et al*, 1996) as a result of systematic monitoring of the incidence and clinical phenotype of CJD in the UK by the National CJD Surveillance Unit in Edinburgh. Clinically, the disease is unusual in that it presents with neuropsychiatric symptoms, such as anxiety or depression, dysaesthesia and ataxia. Patients develop progressive dementia, myoclonus and choreoathetosis with an average clinical course to death of 6 months–2 years (Will, 2004; Will & Ward, 2004). Non-specific electroence-

Table I. Iatrogenic transmission of Creutzfeldt Jakob disease.

	Number	Incubation period (months)
Neurosurgical instruments	5	12–28
Intracerebral electrodes	2	16–20
Dura mater graft	120	18–216
Corneal graft	4	16–320
Human growth hormone	142	550–456
Human gonadotrophin	5	144–192

The incubation period for infections transmitted by peripheral inoculation is shorter than that when infection is directly in the brain (from Ironside and Head, 2003, with permission from Blackwell Publishing).

phalogram changes are observed, but magnetic resonance imaging (MRI) is more informative, with changes in the pulvinar (posterior thalamus) in the majority of cases.

Neuropathologically, the disease is characterised by neural cell loss, astrogliosis and spongiform change with particularly florid amyloid plaques as a pathognomic feature (Fig 2) (Ironside & Head, 2003; Peden & Ironside, 2004). To date all clinical cases of variant CJD have occurred in methionine 129 homozygous individuals; it seems likely that valine homozygous and methionine/valine heterozygous individuals are more resistant to infection or, if infected, to the development of clinical variant CJD. In this context it may be relevant that methionine 129 human prion protein oligomises more rapidly with beta-sheet formation whereas 129 valine tends to form alpha-helix rich monomers (Tahiri-Alaoui *et al*, 2004). Furthermore it is of interest that following inoculation with prions, mice homozygous for human methionine developed 'typical' variant CJD, whilst those that were homozygous for valine appeared more resistant to infection and when this occurred, the clinical and pathological features were more similar to sporadic CJD (Wadsworth *et al*, 2004). It is noteworthy, in this context that the second case of probable variant CJD prion transmission by blood transfusion was recorded in a methionine/valine heterozygous patient who did not develop clinical features of the disease despite surviving 5 years after transfusion (Peden *et al*, 2004). This patient had been identified as part of the variant CJD lookback process and postmortem examination was requested following death from unrelated causes (*vide infra*).

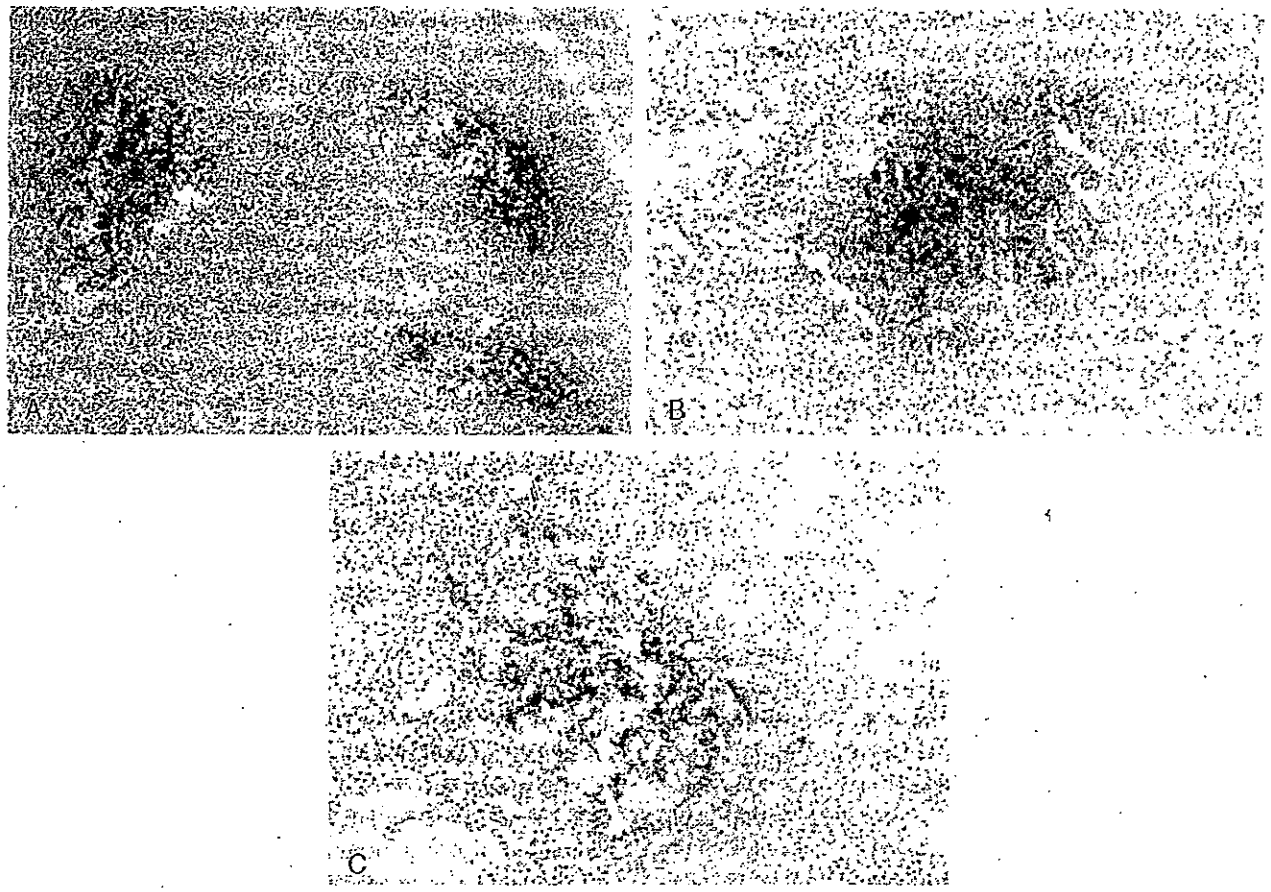

Fig 2. Immunocytochemistry for the prion protein (PrP) in lymphoid tissues in variant Creutzfeldt Jakob disease shows staining of follicular dendritic cells and macrophages in (A) the tonsil, (B) spleen and (C) lymph node. Anti-PrP antibody (KG9) with haematoxylin counterstain [from Ironside and Head (2003) with permission from Blackwell Publishing].

Unlike sporadic and familial forms of CJD, patients with variant CJD show evidence of abnormal prion accumulation in follicular dendritic cells in peripheral lymphoid tissue including tonsils (Hill *et al*, 1997b; Kawashima *et al*, 1997), appendices, spleen (Hilton *et al*, 1998) and lymph nodes (Hill *et al*, 1999). In two patients, appendices removed 8 months and 2 years prior to the onset of clinical disease have also shown evidence of prion accumulation, although a sample removed 10 years prior to onset of clinical disease did not (Glatzel *et al*, 2004).

The median age at death is 29 years (range 14–74 years) and has not altered over the first 10 years of the outbreak, suggesting an age-related susceptibility or exposure (Ghani *et al*, 1998a; Boelle *et al*, 2004). At the time of writing there have been 154 definite and probable cases of variant CJD in the UK, nine in France, two in Ireland and one in each of the USA, Canada, Italy, Saudi Arabia and Japan. In the UK, the incidence of clinical disease appears to have peaked around 2000 and has since fallen significantly (http://www.cjd.ed.ac.uk). However, although the outbreak thus far has been very much less than that which was initially feared (Cousens *et al*, 1997; Ghani *et al*, 1998b), with an upper boundary of around a further 70 new cases now predicted based on the pattern of clinical disease (Will, 2003; Smith *et al*, 2004; Sneath, 2004), a recent retrospective study of tonsil and appendix samples demonstrated three of 12 500 samples positive for abnormal prion accumulation, suggesting that up to 3500 people could be infected with a prevalence of pre- or subclinical disease amongst the 10 to 30-year-old UK population of one of 10 000 (Hilton *et al*, 2004). Ghani *et al* (1998a) have suggested that up to 90% of individuals infected may have prolonged preclinical or true subclinical disease and that this could be related to codon 129 genotypes encoding valine homozygosity or methionine/valine heterozygosity. If transmissible prion infectivity is present in the peripheral blood of such asymptomatic individuals, the concern is that blood-derived products could provide a route to long-term persistence of variant CJD within the population.

Animal studies of peripheral blood infectivity and transmissibility

The route by which the prions disseminate and replicate following peripheral inoculation is of importance in understanding the likely distribution of infectivity and has been recently reviewed (Mabbott & Turner, 2005). Studies in knockout mice with deficiencies in PrP expression, or lacking various cellular compartments of their immune systems, have led to the conclusion that initial accumulation or replication in follicular dendritic cells is essential to peripheral transmission (McBride *et al*, 1992; Bueler *et al*, 1993; Fraser *et al*, 1996; Brown *et al*, 1997b; Klein *et al*, 1997, 1998; Mabbott *et al*, 1998). Indeed, infection and abnormal prion accumulation can be demonstrated in the lymphatic tissues of scrapie-infected rodents and sheep prior to the stage at which it can be detected in the nervous system (Diringer, 1984; Casaccia *et al*, 1989; Farquhar *et al*, 1994; Mabbott *et al*, 1998). The mechanism by which prions are transmitted from a site of initial exposure, such as the intestinal lumen, to the lymphoid germinal centres where they replicate is uncertain. It is possible that follicular dendritic cells (FDCs) trap circulating cell-free prion or that agent is transported by lymphocytes, macrophages or migratory dendritic cells. Once prion replication has occurred within FDCs, retrograde neuroinvasion occurs via sympathetic and parasympathetic peripheral nerves to the CNS. Again it is unclear whether direct FDC–neuronal interaction or dendritic cell-mediated spread is responsible for this step. These observations suggest that it is quite plausible that the peripheral blood also harbours infectivity from an early stage in the preclinical phase of the disease.

As a generalisation, peripheral blood infectivity has been shown to be detectable in rodents experimentally infected with scrapie, BSE and variant CJD and in experimentally infected sheep and goats. Peripheral blood infectivity has not been demonstrated in the peripheral blood of sheep naturally infected with scrapie or BSE-infected cattle. The reason for these differences is unclear but may relate to the route and size of the primary infectious inoculum.

In mice infected with the Fukuoka 1 strain of GSS, 100 infectious units (i.u.)/ml have been demonstrated in the peripheral blood during the clinical phase of disease and 10 i.u./ml during the preclinical phase of disease. Around 70% of the infectivity is associated with the buffy coat (inclusive of the leucocyte and platelets) and the remainder with the plasma (Brown *et al*, 1998, 1999). Similar findings have been demonstrated in the 293K hamster model. The latter also suggested an efficiency of transmission via the intravenous route of approximately 5–10% of that of the intracerebral route of inoculation.

In sheep naturally infected with scrapie or experimentally infected by an oral dose of BSE, peripheral blood drawn during the clinical and preclinical phases of disease has been shown to transmit infection to 20–25% of secondary recipients. This study amounted to proof of principal that prion diseases are transmissible by transfusion (Hunter *et al*, 2002).

Transmission of variant CJD by blood transfusion

The UK has established surveillance to assess the transmissibility of CJD by blood components (http://www.cjd.ed.ac.uk/). For each individual who develops CJD, close relatives are questioned and Blood Transfusion Service records searched to try to establish whether he/she was a blood donor. If so, the recipients of blood components manufactured from those donations are traced and notified. They themselves are subject to public health restrictions around blood, tissue and organ donation and invasive medical and surgical procedures and are flagged to the UK Office of National Statistics.

The reverse arm of the surveillance scheme addresses the question as to whether any of the patients who have developed variant CJD could have become infected via a previous blood transfusion. The transfusion history of all patients developing variant CJD is assessed and the donors are traced and also flagged to the UK Office of National Statistics.

To date 17 variant CJD patients are known to have been blood donors (15 in the UK and two in France). Of the 50 recipients of blood components, 17 are still alive. Plasma from 23 donations was fractionated to produce albumin, immunoglobulin and clotting factor concentrates that were used in the UK, France, Belgium, Germany and Italy. In the UK it appears that the incidence of variant CJD peaked in about 2001 and is now declining (Fig 1).

To date there have been two cases of probable transmission of variant CJD prions via non-leucodepleted red cell concentrates. In the first episode, a 24-year-old individual gave a blood donation in 1996 (Llewelyn *et al*, 2004). Three years later he developed variant CJD and died the subsequent year. The recipient of this donation in 1996 was aged 62 years and also received four other units of red cell concentrate to cover a surgical operation. In 2002 he became depressed and developed blurred vision, motor difficulties including a shuffling gait and cognitive impairment. An MRI of his brain was reported as normal. In 2003 he died of dementia. At autopsy, histology of his brain revealed characteristic features of variant CJD, and this was confirmed by proteinase-K resistance and typical features on Western blotting. Analysis of his *PRNP* gene revealed him to be homozygous for methionine at codon 129. A statistical assessment concluded that there was only a 1:15 000–1:30 000 chance of this occurring by coincidence.

A second individual was reported in 2004 as a result of the national surveillance of recipients of transfusions from donors who later developed variant CJD. This patient very likely became infected with variant CJD prions by a unit of red cell concentrate in 1999 from a donor who developed variant CJD 18 months later (Peden *et al*, 2004). Although this patient died 5 years after the transfusion of unrelated causes with no clinical features of variant CJD, analysis of her lymphoid tissue at autopsy revealed that prion accumulation was present in the spleen and one cervical lymph node. There were no histological features or evidence of prion accumulation in her CNS. The other unusual feature as noted above, was that the *PRNP* gene was heterozygous at codon 129 for methionine/valine.

These two cases are therefore of great importance because they have demonstrated that variant CJD prions can be transmitted by blood transfusion from donors who are in a preclinical phase of disease at the time of donation and that methionine/valine heterozygous individuals can also be infected, although whether they are as susceptible to infection and/or the development of clinical disease as methionine homozygous individual remains uncertain (Aguzzi & Glatzel, 2004).

Blood donor selection

Many countries have instituted policies of donor deferral for those who have spent time in the UK, France or more broadly Europe, based on the likely comparative level of risk with their indigenous population, the extent or pattern with which their population visit affected areas and the likely impact on their blood donor base.

In the UK, there are few epidemiological criteria that would allow identification of a 'high-risk' donor population. In response to the blood transfusion related transmissions of variant CJD, in 2004, a policy of deferral of donors who themselves have been recipients of blood components since 1980 was instituted to reduce the risk of tertiary or higher-order transmissions leading to a self-sustaining outbreak. This policy also has the advantage of reducing the risk of other blood borne infectious agents being recycled in the community by transfusion. There was concern that this would lead to a significant reduction in the donor base and that a sometimes precarious blood supply would be further compromised. Whilst about 5–10% donors have been lost from the UK blood donor panels, the impact has been mitigated by proactive recruitment campaigns to enlist more new donors.

Importation of blood components

It is not likely to be feasible to import red cell or platelet concentrates due to the large volumes required, the short shelf life and lability of these components and concerns over the risk of other transmissible agents in some overseas donor populations. To reduce the risk of variant CJD transmission to children, in 2002 the decision was made to only use imported non-UK plasma to treat those born after 31 December 1995. This date was chosen because it was considered that BSE-infected foods had been largely eliminated from the diet by this date, and therefore, children born after this time were unlikely to be infected from food. In addition, with relatively small volumes of plasma, the product can be stored, transported frozen and be virus-inactivated.

Donor screening

No immunological response to prion infection has yet been identified nor has DNA been found associated with disease transmission. Therefore, traditional serological and molecular biological approaches to donor screening are not currently feasible.

Several groups have looked at the possibility of using surrogate markers. The proteins 14·3·3 (Zerr *et al*, 1998) and S100 (Otto *et al*, 1998) are non-specific markers of CNS damage and are therefore likely to be elevated only in the clinical stages of disease. It has been shown that transcription of erythroid differentiation associated factor (EDAF) is depressed in the peripheral blood of animals suffering from prion disease (Miele *et al*, 2001). The cause of this observation

is uncertain and it also currently remains unclear whether this could be translated into the setting of human clinical and preclinical disease and whether an appropriate differential exists between patients incubating variant CJD and normal individuals.

Infectivity has not thus far been detected in the peripheral blood of patients with clinical variant CJD by intracerebral inoculation into rodents despite the evidence of clinical transmission, reflecting the limitations of infectivity bioassays due to the species barrier and the small amounts of blood inoculated.

A central difficulty in the development of molecular assays is the differentiation of PrP^{Sc} from PrP^{c} (Minor, 2004). There are currently no monoclonal antibodies or other reagents of sufficient analytical specificity to differentiate between the normal and abnormal isoforms. Most assays therefore depend on differential physicochemical characteristics, such as resistance to proteinase-K digestion or display of additional or novel PrP epitopes following treatment with chaotropic agents, such as guanidine hydrochloride. The level of sensitivity required is challenging. Brown *et al* (1999) has estimated that in the order 1 pg of PrP^{Sc}/ml may be present in the peripheral blood of individuals in the pre- or subclinical phases of disease, in the context of around 100 ng/ml of PrP^{c}, i.e. a ratio of 1 PrP^{Sc} molecule:1 million PrP^{c} molecules. There are also significant challenges in validating such assays. This would normally be undertaken using samples from individuals with the disease in question. However, there are very few patients alive at any one time with variant CJD and large amounts of blood cannot be drawn for ethical reasons. As it is not currently possible to determine who may, or may not, be incubating the disease, the assays will therefore need be validated on brain homogenate-spiked human blood or animal endogenous infectivity samples posing questions around the extrapolation of the data to the human setting. Finally it should be borne in mind that it will not be possible to determine which of the donors with positive assays are actually incubating variant CJD and which of these are likely to go on to develop clinical disease. There is no treatment available at the present time to offer such individuals. There is concern, therefore, over the number of donors who may need to be deferred due to positive assay results and the potential impact of the introduction of such assays on the willingness of donors to donate (Blajchman *et al*, 2004; McCullough *et al*, 2004).

Blood component processing

In October 1997, the UK Spongiform Encephalopathy Advisory Committee advised that universal leucodepletion be considered. The UK Departments of Health commissioned an independent risk assessment by Det Norske Veritas Consulting (DNV) and asked the Blood Services to consider the feasibility (Comer & Spouge, 1999). Implementation was recommended in July 1998 and completed by the autumn of 1999 (Department of Health, 1998a,b). The measure was predicated on studies suggesting that B lymphocytes were likely to be involved in the initial phases of disease and that leucocytes were an important locus of infectivity in the peripheral blood. Subsequently, it has become apparent that in animal studies leucodepletion does not reduce infectivity in plasma and is likely to reduce the prion concentration in blood by only about 40% (Prowse & Bailey, 2000; Gregori *et al*, 2004; St Romaine *et al*, 2004). Universal leucodepletion is also considered to offer a number of additional benefits, e.g. reduction in transmission of cell-based viruses such as cytomegalovirus and human T-cell lymphotropic virus, rates of alloimmunisation, immunomodulatory effects and transfusion-mediated graft *versus* host disease (Roddie *et al*, 2000)

Other approaches under consideration in the UK to reduce infection risk include the greater use of apheresis platelets from a single donor (rather than a pool from four individual donors), extension of imported fresh frozen plasma and cryoprecipitate to all patients under the age of 16 years and further reduction in residual plasma in red cell and platelet concentrates.

Two companies are developing filtration devices, which may reduce the prion concentration in blood by several orders of magnitude. Such a reduction could significantly reduce the likelihood of transmission of variant CJD from a donor with sub- or preclinical disease. However, validation is likely to pose a significant challenge as studies cannot be carried out on naturally infected human peripheral blood and data will therefore have to be extrapolated from studies using homogenised infected brain spikes in human blood and endogenously infected rodent blood, raising issues around the relevance of these models.

Plasma product manufacture

In 1997, the Committee for Proprietary Medicinal Products (Committee for Propriety Medicinal Products, 1998), as the licensing authority, recommended recall of two batches of in date, factor VIII concentrate that had been manufactured from a plasma pool containing donations from two patients who had subsequently developed variant CJD. The UK Haemophilia Centre Doctors Organisation (UKHCDO) in November 1997 recommended that as 'variant CJD occurs almost exclusively in the UK, it is likely that any risk of transmission would be reduced by using concentrate prepared from blood donor plasma collected in other countries, e.g. USA, where there are no cases of variant CJD or BSE' (Ludlam, 1997). The UK government was keen to try to evaluate the risk of blood transmission of variant CJD so that other rational safety measures could be taken and, in 2004, the original DNV risk assessment was reviewed using further estimates of quantitative risk that had emerged from animal studies and fresh guidance offered.

Although prions are highly resistant to physical and chemical degradation and methods for their inactivation would be too severe to be used on plasma products, their physicochemical features suggest that they will partition selectively during the

plasma fractionation process (Foster, 1999). Studies with blood from endogenously infected animals (Brown *et al*, 1998; Foster, 2004) and blood spiked with high-titre brain homogenates (Foster *et al*, 2000, 2004; Tateishi *et al*, 2001; Reichl *et al*, 2002; Stenland *et al*, 2002; Vey *et al*, 2002), suggest that a number of steps in existing plasma fractionation processes should contribute individually to reduction in infectivity, including cryoprecipitation and cold ethanol fractionation, depth filtration, adsorption chromatography and nanofiltration. Some of these steps have also been studied in sequence, where it has been shown that, in general, the overall degree of prion removal exceeds that of any one individual step but is less than the sum of the individual steps (Foster, 2004).

Other measures

For the past 5 years the UK Transfusion Services have had an active policy of trying to optimise the use of all blood and blood products. An important component of this policy has been to ensure the appropriate use of red cell concentrates. The aim has been to prevent unnecessary red cell use as exemplified by Sirchia *et al* (1994). Such a policy not only reduces the risk of all transfusion-transmitted infections to each individual patient but it allows more patients to be treated with a scarce red cell resource.

Non-blood transfusion related strategies to prevent secondary spread of variant CJD horizontally in population

Between 1996 and 2004 several attempts were made to assess the risk of horizontal spread of variant CJD transmission by mechanisms other than blood products and make rational recommendations on appropriate safety measures (Bird, 2004). There has been concern about transmission in health care settings by invasive medical and surgical procedures. The second 2004 DNV risk assessment was informed by animal studies, which provided some measure of risk related to prion load in the inoculum. The aim was to try and identify the patients and procedures for which specific safety precautions should be instituted. Clearly some level of precaution was appropriate for patients who had clinical variant CJD, but for what other groups of individuals should precautions be taken? It was proposed that precautions should be taken for individuals who could be identified as having more than a 1% risk of exposure to an infectious dose of variant CJD prions (two ID_{50} extrapolated from experimental rodent studies).

The UK CJD Incidents Panel and Health Protection Agency offered advice based on the 2004 DNV risk assessment in relation to recipients of blood components and plasma products. Precautions were to be taken with all identified recipients of fresh blood components from donors who went on to develop variant CJD. For those who received fractionated plasma products, the risk from each was calculated on a product-by-product basis, dependent on the size of the donor pool, detail of the manufacturing process, and the dose of product that would give a 1% risk of exposure to an infectious dose (as defined above) was estimated. The products were divided into three groups based on the assessed risk. Those that were considered to pose a high risk were factors VIII/IX and antithrombin concentrates, where less than one injection of a therapeutic dose for an adult would exceed the risk threshold. Products in the medium risk group were those where the risk threshold would be exceeded if several or more treatments were given and included intravenous immunoglobulin and high doses of albumin. The low risk group consisted of products where very high doses, far in excess of those used in normal medical practice would be required to exceed the risk threshold, e.g. albumin used as an excipient in other products, intramuscular immunoglobulin.

Having defined the threshold dose of 'implicated' product it was necessary to identify which patients were likely to have received such a dose. For those with haemophilia and antithrombin deficiency, it would have been possible in principle to have identified all those patients known to have received implicated concentrates. But this was likely to represent a significant proportion of all UK haemophiliacs as, by September 2004, 16 batches of factor VIII and eight batches of factor IX were implicated and furthermore, it is likely that more batches used in treatment several years ago will become implicated as further former blood donors develop variant CJD in the future. It was therefore decided to use a 'population' approach and consider all haemophiliacs who had received clotting factor concentrate manufactured from UK plasma between 1980 (the beginning of the BSE epidemic) and 2001 (the expiry date of the last batch of product prepared from UK plasma) as being 'at risk of variant CJD for public health purposes'. Such a policy strongly advocated by UKHCDO, was seen as the simplest and least threatening way to categorise those for whom extra precautions would need to be taken for certain invasive procedures. For other groups, e.g. those with immunodeficiency, patients are being reviewed individually and a decision made as to whether they would fall into the 'at additional risk of exposure to variant CJD for public health purposes' category (Hewitt, 2004).

For those considered to in the 'at additional risk of exposure to variant CJD for public health measures' group, either on the basis of population or individual assessment, the arrangements to prevent horizontal transmission have been laid out by the Advisory Committee on Dangerous Pathogens (http://www.hpa.org.uk/infections/topics_az/cjd/blood_products.htm). In such individuals CNS tissue constitutes a high risk of tissue infectivity and therefore potential contamination of surgical instruments. Surgery on lymphoid tissue or olfactory epithelium and the anterior chamber of the eye, e.g. cataract surgery, involved tissue of medium risk infectivity. Instruments for all these procedures should either be disposable or 'quarantined' after surgery and not reused. It has been suggested that some of these could profitably be used for research studies into decon-

tamination techniques. All other surgeries, including dental and orthopaedic, were not considered to pose a significant risk of contaminating instruments with prions as the tissues were considered at low risk of infectivity and therefore no special precautions were advised.

With the publication of the primate study (Herzog *et al*, 2004), in which, following infection of Macaques with BSE prion both orally and intravenously, PrP^{Sc} was clearly demonstrated in the gut subepithelial neural plexuses as well as Payer's patches, it became clear that endoscopic biopsies of the gut mucosa could potentially contaminate the biopsy forceps and its channel in the instrument with PrP^{Sc}. Whilst the current recommendation is that endoscopes used for non-invasive procedures be cleaned and reused in the normal way, those used for invasive procedures, e.g. colonic biopsies, should be 'quarantined' and not reused. This has had major financial implications for hospitals.

Concluding remarks

Management of the risk of transmission of variant CJD and indeed, other prion diseases by blood and plasma products remains highly problematic (Wilson & Ricketts, 2004a,b). Although the relatively small and falling number of clinical cases in the UK is reassuring, data indicating that up to 90% of infected individuals may sustain long-term preclinical or subclinical disease and that most such individuals are likely to be currently in the 20–40 years age group suggests a significant pool of potentially infectious blood donors. Blood donor selection criteria are a blunt instrument for risk management and current measures, such as universal leucodepletion, seem likely to be only of limited efficacy. Blood donor screening assays and prion reduction filters offer a better chance of control, but much of the validation will need to be based on animal experimentation, the extrapolation of which to the human setting is problematic. Most new risk reduction measures are likely to be highly expensive and engender the possibility of alternative risks, including critical blood shortages. In this context, it is of increasing importance that health services work to ensure prescription of blood products only where they are required (Hart *et al*, 2004; McClelland & Contreras, 2005).

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(18)

Vaccines, Blood & Biologics

Questions and Answers on "Guidance for Industry: Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease (CJD) and Variant Creutzfeldt-Jakob Disease (vCJD) by Blood and Blood Products"

Why do we recommend new blood donor deferrals for possible exposure to BSE and vCJD?

FDA is taking this step as a prudent measure to assure the safety of the blood supply by further reducing the theoretical risk from vCJD. In 1999, we recommended the first donor deferral for people who may have been exposed to the vCJD agent, which is believed to be the same as the agent of bovine spongiform encephalopathy (BSE, or "mad cow" disease). We recommended deferral of donors who resided in the United Kingdom (U.K.) for 6 months or more between 1980 and 1996. At this time, we are recommending new blood donor deferrals for possible exposure to BSE and vCJD for the following reasons:

1. Since 1999, the rate of vCJD cases in the U.K. has been on the rise.
2. Significant exposures to potentially contaminated U.K. beef occurred in France and cases of vCJD have appeared in France.
3. Significant exposures to potentially contaminated U.K. beef occurred at U.S. military bases in Europe
4. In Europe, outside the U.K., the BSE epidemic has been increasing.
5. Particularly in the U.K., transfusion recipients may have been exposed to donors already infected with vCJD.

What are the new donor deferrals for possible exposure to vCJD?

1. Residence in the U.K. for 3 months or more, between 1980 and 1996.

Rationale: The U.K. has experienced the largest epidemic of BSE, and also has the largest number of cases of vCJD (over 100). However, in 1996, the U.K. instituted and enforced rules to prevent contaminated cattle from entering the human food chain (www.defra.gov.uk/animalh/bse/public-health/public-health-index.html). Due to these effective food chain protections, the risk of exposure to the BSE agent has been greatly reduced. For this reason, the donor deferral extends only through 1996.

2. Military personnel (current and former), and their dependents, who spent time in military bases in northern Europe 1980-1990, or southern Europe (1980-1996), for 6 months or more.

Rationale: British beef was eaten at military bases during these time periods. The maximum amount of U.K. beef eaten was about 35% of the total beef diet.

3. Donors who lived in France for 5 years or more, between 1980 and the present.

Rationale: The French imported at least 5% of their beef supply from the U.K. before 1996. There are also 5 cases of vCJD in France. This deferral will go into place before the European deferral (# 5., below).

4. Donors who received a transfusion in the U.K. between 1980 and the present.

Rationale: Although there are no known cases of transfusion of vCJD, it is too early to rule out this possibility. Since the U.K. has the highest number of vCJD cases, and is likely to also have the highest number of people incubating vCJD, we recommend deferral of people who have received blood products from U.K. donors.

5. Blood donors who lived in Europe for 5 years or more, between 1980 and the present.

Rationale: Most European countries now have reported BSE, although in fewer cattle than in the U.K. However, methods to prevent BSE from getting into human food are not completely in place in all European countries, so we recommend deferral up to the present time.

How effective are the new donor deferrals at reducing risk of vCJD from transfusion?

Combined with the effect of our previous recommendations, our new recommendations, added to the previous U.K. deferral, eliminate an estimated total 90% of overall risk (calculated by "risk-weighted" person-days of exposure to infected beef), and may decrease the number of donors an average of an additional 5% nationwide. The new deferrals reflect an attempt to minimize the theoretical risk of transmission of vCJD, while maintaining critical supplies of blood products.

Why can people who have lived in Europe for 5 years or more, give Source Plasma, but not blood?

Blood donors are deferred, but donors of "Source Plasma," who have lived in Europe (except France and the U.K. as above), may continue to donate. Unlike blood, Source Plasma undergoes manufacturing into highly processed products ("plasma derivatives"), several of which have been in short supply. Donors who have lived in Europe have a low likelihood of incubating vCJD, compared to people who lived in France or the U.K. Furthermore, published studies show that some of the steps used in plasma derivative manufacturing remove agents which are similar to the vCJD agent, thus adding a potential

margin of safety. Thus we consider the risks and benefits of deferring Source Plasma donors, as opposed to blood donors, for residence in Europe to be different.

How will the new deferrals affect the blood supply?

Based upon a 1999 survey, we estimate that about 5% of blood donors may be deferred. However, in some locations, such as in large coastal cities, where more people travel, up to 10% of donors may be deferred.

What measures are being taken to attenuate the impact of the new donor deferrals?

1. We have recommended two separate phases of donor deferrals, to spread out the potential impact on supplies over time. Phase I will start May 31, 2002, and includes deferral of people who lived in the U.K. (3 months or more, 1980-1996), in France (1980-present), or on military bases (as described above), or who had a transfusion in the U.K. Phase I will provide 82% of the additional risk reduction accomplished by the revised deferral policy and is estimated to eliminate approximately 59% of current potential vCJD risk.

For blood donors who lived in Europe for 5 years or more, deferrals will start on October 31, 2002. Phase II will provide the balance (18%) of the additional risk reduction accomplished by the revised deferral policy, and is estimated to eliminate an additional 13% of current potential risk.

2. We have asked blood banks that choose to have broader deferrals than those we recommend, to implement pilot studies, to see whether the loss of donors can be tolerated without causing local blood shortages.
3. The Department of Health and Human Services has instituted a system for monitoring the blood supply, nationwide, in an effort to detect blood supply shortages.
4. We continue to encourage more blood donations, as well as cooperation among blood banks to assist each other in cases of local shortages.

If I am deferred, will I ever be able to donate again?

Because it is still uncertain whether blood can transmit vCJD, and because it is possible that donor screening tests may be developed to exclude anyone carrying the disease, it is possible that you will be able to donate again in the future. Along with our expert Transmissible Spongiform Encephalopathy Advisory Committee (TSEAC), we are continuing to monitor the BSE epidemic, human exposure to BSE, possible testing methods for blood, and scientific advances which will help us understand whether or not blood or blood components are able to transmit vCJD. New advances in science and epidemiology may enable you to donate again in the future.

What will happen when new countries, not now on the blood donor deferral list, are discovered to have BSE?

Since the publication of our draft guidance in August 2001, BSE was diagnosed in Japan, which is not on the blood donor deferral list. The source of this outbreak is believed to be contaminated material from BSE cattle, which was imported and fed to Japanese cows. The news media has reported that other countries may also have received potential BSE-contaminated material which they could have fed to their own cows. We may consider additional deferrals based upon possible exposure to BSE in Asia or elsewhere, but only after additional information about the potential level of BSE exposure and food chain controls in these other countries is acquired and, preferably, would anticipate doing so after the currently recommended deferrals have been implemented and their impact is assessed.

How is FDA monitoring the risk of vCJD transmission by blood?

We monitor the risk by keeping up to date with new published, and unpublished scientific work from academia and industry. Much of this material is made publicly available at meetings of the TSEAC. We maintain close contacts, and consult with experts in other agencies that are also involved in BSE and vCJD, such as the U.S. Department of Agriculture and the Centers for Disease Control and Prevention, as well as with international government agencies. FDA also maintains its own pool of scientific experts in these diseases who perform active research to address questions of transmission of spongiform encephalopathies, such as BSE and vCJD by blood.

Where can I obtain more information?

1. Previous TSEAC transcripts, containing discussion and information about many of the issues and decisions, above:

- TSEAC Transcripts, December 18, 1998
- TSEAC Transcripts June 1-2, 2000
- TSEAC Transcripts, January 18-19, 2001
- TSEAC Transcripts June 28, 2001

Referenced Guidance

- Guidance for Industry: Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease (CJD) and Variant Creutzfeldt-Jakob Disease (vCJD) by Blood and Blood Products (PDF) (PDF - 93KB)

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(19)

Donor Exclusion to Address Theoretical Risk of Transmission of variant Creutzfeldt-Jakob Disease (vCJD) through the Blood Supply

UNITED KINGDOM, FRANCE & *WESTERN EUROPE*

1. PURPOSE

The purpose of this Directive is to advise all licenced Canadian blood establishments to take further measures to reduce the theoretical risks of transmission of vCJD through the blood supply. This is to be accomplished by excluding from donating blood, all persons who:

- have spent a cumulative period of time of 3 months or more in the United Kingdom(UK) consisting of England, Scotland, Wales, Northern Ireland, Isle of Man, the Channel Islands between the years 1980 to 1996; or
- have spent a cumulative period of time of 3 months or more in France between the years 1980 to 1996; or
- have spent a cumulative period of time of 5 years or more in countries of Western Europe(WE) consisting of Germany, Italy, Netherlands, Switzerland, Austria, Belgium, Spain, Republic of Ireland, Portugal, Denmark, Luxembourg, Liechtenstein between the years 1980 and ongoing; or
- have received a transfusion of whole blood or blood components in the UK between the years 1980 and ongoing.

The period of time of three months or more spent in the UK or France is not based on a combination of time in either country. The period spent in the above noted WE countries considers either the time spent individually in each country or any combination of time spent in the various countries so that cumulatively, the residence period requiring deferral amounts to 5 years or more .

2. BACKGROUND

Variant Creutzfeldt-Jakob disease (vCJD), first described in 1996, is a "new" disease, linked with the outbreak of Bovine Spongiform Encephalopathy (BSE) in cattle.

While there have been no cases of vCJD attributable to the use of human blood or plasma derivatives to date, lack of experience with this condition and the causative agent, together with limited knowledge available on certain biological effects associated with this infection (e.g. the lack of information on the concentration and infectivity of the vCJD prion in blood), do not allow for conclusion that it can not occur. In addition, a report that BSE in sheep can be transmitted within that species through blood transfusion, suggests that theoretically, vCJD may have the potential to spread through human blood or blood derivatives. Scientific knowledge of the Transmissible Spongiform Encephalopathies (TSEs) has been hampered by the long incubation period of the known TSE infectious agents (e.g. vCJD and BSE) and the lack of diagnostic procedures available for early detection. Consequently, Health Canada (HC) wishes to mitigate the risks of potential human to human transmission of vCJD with policies on blood donor deferral for persons who have spent time in the UK, or France or WE.

In considering this potential risk and measures to deal with it, the principle has been adopted that one must seek to apply measures which will reduce the targeted risk without jeopardizing the safety of the blood system in other ways. Using this rationale, Health Canada issued Directives on August 17, 1999 and August 20, 2000 requiring the exclusion from blood donation of all persons who had spent time amounting cumulatively, to a period of 6 months or more in

the UK or France between the years 1980 to 1996, inclusive. Based on recent scientific knowledge available since the issuance of the 1999 and 2000 Directives, Health Canada, in consultation with stakeholders including Canadian Blood Services(CBS) and Héma-Québec(HQ), is directing industry to tighten the blood donor deferral for the UK and France to 3 months or more and to add a deferral based on 5 years or more spent in the above-noted countries of WE.

This new Directive is based on recent scientific knowledge available since the issuance of the 1999 and 2000 Directives and the following new information:

- The total number of cases of vCJD is increasing, with a cumulative total that reached 110 in August, 2001, with 106 in the UK, France reporting 3 cases and one case in the Republic of Ireland;
- The number of observed BSE cases is increasing steadily in West European countries once thought to be free of the disease;
- Brain tissue from BSE-infected primates, injected intravenously into other primates, has been shown to transmit disease;
- Recent research has shown experimental sheep-to-sheep transmission of the BSE agent by blood transfusion.

Recent surveys conducted by CBS and HQ indicate that reducing the deferral period to three months or more for either France or the UK and the addition, of a deferral based on 5 years or more time spent in the above-noted countries of WE, will not jeopardize the blood supply. Health Canada's Population and Public Health Branch has carried out a number of modeling studies to estimate the theoretical risk of acquiring vCJD for those persons who have spent time in the UK. Similar modeling studies have been done to estimate vCJD risk for persons spending time in France and the above noted countries of WE. These risks are not identical and consequently, HC would not require a deferral based on a combination of time in the UK with time spent in France; or a combination of times spent between the above-noted WE countries and either the UK or France. However, WE deferral does allow for a combination of times spent among the above-noted WE countries.

A theoretical risk reduction of 72% is achieved under the 1999 and 2000 Directives. With the implementation of the current Directive, there is expected to be an additional 16-18% reduction of the theoretical risk for an estimated overall risk reduction value of 88-90%. A blood donor loss of around 3% or less is estimated under the current Directive.

3. SCOPE

This Directive applies to all Canadian blood establishments that are licensed to fabricate blood and blood components for transfusion or for further manufacture. Products affected by the Directive include all blood components for transfusion with the exception of: autologous donations, peripheral blood stem cells collected for autologous transplants, rare blood types and products derived from USA-sourced plasma.

4. REGULATORY REQUIREMENTS

Blood establishments are required to submit a Licence Amendment Submission to the Blood and Tissues Division of the Biologics and Genetic Therapies Directorate (BGTD) for review.

An attachment must be included which indicates both the impacts that this measure will have on the donor base and plans to mitigate any such effects. Operators are also encouraged to develop materials to be used in explaining these deferral actions to affected donors in order to foster an appropriate understanding of these precautionary actions.

Regarding the withdrawal of prior donations by deferred donors, Health Canada, will require that all available components collected from these deferred donors, that have not been transfused or pooled for further manufacture, be retrieved.

5. COMPLIANCE DATE

The exclusion is to be introduced as soon as operationally feasible, but not later than three months from the date of this Directive.

6. ADDITIONAL INFORMATION

Blood operators will be required to report semi-annually on the impact of this policy on their donor bases and the supply of blood.

On an ongoing basis, Health Canada may update its guidance in response to new scientific knowledge. If other cases of vCJD are confirmed in a specific country, a risk assessment will be carried out to determine specifically what deferral measures will be required.

The Directive, with a list of supporting references on the Background science, will be posted on an HC website.

Questions concerning the " Donor Exclusion to Address Theoretical Risk of Transmission of variant CJD through the Blood Supply" should be directed to:
Biologics and Genetic Therapies Directorate
Blood and Tissues Division
3rd Floor LCDC Building #6
Postal Locator 0603C
Tunney's Pasture
Ottawa, Ontario
KIA 0L2

7. REFERENCES

Scientific references used in the development of the Directive's "Background"Section:

1. Monthly statistics on the United Kingdom's CJD cases

http://www.doh.gov.uk/cjd/stats/aug01.htm

and EUROCJD and NEUROCJD: The European and Allied Countries Collaborative Study Group of CJD(EUROCJD) plus the Extended European Collaborative Study Group of CJD(NEUROCJD)

http://www.eurocjd.ed.ac.uk/

2. Monthly statistics on the cases of BSE determined through testing in the European countries. Monthly BSE testing - Cumulative table from January to May 2001 http://europa.eu.int/comm/food/fs/bse/testing/bse_test06_en.pdf - BSE testing - May 2001

and Office International des Epizooties - Number of reported cases of BSE worldwide

http://www.oie.int/eng/info/en_esbmonde.htm

3. Corinne Ida Lasmézas et al. PNAS, March 27, 2001, vol.98(7),4142-4147 "Adaptation of the bovine spongiform encephalopathy agent to primates and comparison with Creutzfeldt-Jakob disease: Implications for human health"

http://www.pnas.org/cgi/doi/10.1073/pnas.041490898

4. Houston F, Foster J.D., Chong A, et al. Transmission of BSE by blood transfusion in sheep. Lancet 2000; 356:999-1000

The modelling studies carried out by Health Canada's Population and Public Health Branch to estimate the theoretical risk of acquiring vCJD under the conditions of the Directive can be found on the Health Canada website with URL:
http://www.hc-sc.gc.ca/sab-ccs/sep2000_BSE_vCJD_slide11_e.html

(20)

Health Canada Santé Canada

April 22, 2005

Additional Donor Exclusion Measures to Address the Potential Risk of Transmission of variant Creutzfeldt-Jakob Disease (vCJD) through the Blood Supply

1. PURPOSE

The purpose of this new Directive is to advise all Canadian blood establishments licenced to fabricate blood and blood components for transfusion of the requirement to implement further measures to reduce the potential risk of transmission of vCJD through the blood supply. This is to be accomplished by screening and excluding from donating blood, all persons who have received a transfusion of whole blood or blood components in France or Western Europe (WE) between the years 1980 and ongoing. These new requirements are in addition to those detailed in Health Canada's Directive *Donor Exclusion to Address Theoretical Risk of Transmission of variant Creutzfeldt-Jakob Disease (vCJD) through the Blood Supply UNITED KINGDOM, FRANCE & WESTERN EUROPE* dated August 30, 2001[1].

To summarize the current requirements, risk reduction is to be achieved by excluding from donating blood, all persons who:

- have spent a cumulative period of time of 3 months or more in the United Kingdom(UK) consisting of England, Scotland, Wales, Northern Ireland, Isle of Man, the Channel Islands between the years 1980 to 1996; or
- have spent a cumulative period of time of 3 months or more in France between the years 1980 to 1996; or
- have spent a cumulative period of time of 5 years or more in countries of WE consisting of Germany, Italy, Netherlands, Switzerland, Austria, Belgium, Spain, Republic of Ireland, Portugal, Denmark, Luxembourg, and Liechtenstein between the years 1980 and ongoing; or
- have received a transfusion of whole blood or blood components in the UK, **France or** WE between the years 1980 and ongoing.

2. BACKGROUND

Variant Creutzfeldt-Jakob disease (vCJD), first described in 1996, is a fatal disease linked with the outbreak of Bovine Spongiform Encephalopathy (BSE) in cattle and the consumption of beef and beef products from cattle infected with BSE[2].

Scientific knowledge of the Transmissible Spongiform Encephalopathies (TSEs) has been hampered by the long incubation period of the known TSE infectious agents (e.g. vCJD and BSE) and the lack of diagnostic procedures available for early detection. Consequently, Health Canada (HC) wishes to mitigate the risks of potential human to human transmission of vCJD with policies on blood donor deferral for persons who have spent time or received transfusion of blood or blood components, in the UK, or France or WE.

In considering this potential risk and measures to deal with it, the principle has been adopted that one must seek to apply measures which will reduce the targeted risk without jeopardizing the availability or safety of blood in Canada. Using this rationale, Health Canada issued Directives based on the scientific knowledge available at the time, on August 17, 1999[3], August 20, 2000[4] and August 30, 2001[1]. The first two directives required the exclusion from blood donation of all persons who had spent time amounting cumulatively, to a period of 6 months or more in the UK or France between the years 1980 to 1996, inclusive, based on the BSE epidemic and the occurrences of vCJD in the UK and France. The August 30, 2001 Directive was issued to tighten the blood donor deferral for the UK and France to 3 months or more, to add a deferral based on 5 years or more spent in the above-noted countries of WE, and to add a deferral for donors who received a blood transfusion in the UK, between the years 1980 and ongoing.

The scientific knowledge related to vCJD since the issuance of the 2001 Directive has increased, including the following:

- A study in 2002 demonstrating that scrapie infected asymptomatic sheep could transmit the disease to other sheep by transfusion[5].
- Research indicates that the intravenous route of transmission of BSE is highly efficient[6]
- There have been two recent reports of potential human to human transmission of vCJD by blood transfusion[7,8]. The two blood donors involved did not develop symptoms of vCJD until 40 and 18 months after the donation. One of two recipients of the suspected blood component was a methionine-valine heterozygote (MV) at codon 129 of the prion protein gene (PRNP), contrary to previous data suggesting that susceptibility to vCJD was restricted to the methionine homozygous (MM) PRNP genotype[7].
- There has been an increase in BSE and vCJD cases reported worldwide[9,10,11]. The total number of definite and probable cases of vCJD has reached 168 as of February 7, 2005, with 154 cases in the UK, 9 in France, and one case each in the Republic of Ireland, Canada, Italy and United States[12,13].

3. REGULATORY REQUIREMENTS

Based on the current scientific knowledge, Health Canada is directing all Canadian blood establishments that are licenced to fabricate blood and blood components for transfusion to further reduce the risk of vCJD transmission through the blood supply by expanding the exclusion of donors who received a blood transfusion in the UK between the years 1980 and ongoing, to include France and WE. These blood establishments are required to submit a Licence Amendment Submission to the Biologics and Genetic Therapies Directorate (BGTD) for review.

An attachment must be included which indicates both the impacts that this measure will have on the donor base and plans to mitigate any such effects. Establishments are also encouraged to develop materials to be used in explaining these deferral actions to affected donors in order to foster an appropriate understanding of these precautionary actions.

Regarding the withdrawal of prior donations by deferred donors, Health Canada, will require that all available components collected from these deferred donors, that have not been transfused or pooled for further manufacture, be retrieved.

4. SCOPE

This Directive applies to all Canadian blood establishments that are licenced to fabricate blood and blood components for transfusion. Products affected by the Directive include all blood components for transfusion with the exception of: autologous donations, peripheral blood stem cells collected for transplants, and rare blood types.

It is recommended that Canadian and non-Canadian manufacturers of plasma-derived products follow the donor exclusion requirements outlined in this directive.

5. CONSULTATIONS

The scientific finding have been discussed and advised upon by Health Canada's Expert Advisory Committee on Blood Regulation as well as the Health Products and Food Branch Public Advisory Committee. Also, Canadian Blood Services, Cangene, and Héma-Québec have been consulted in the development of this Directive.

The blood donor loss as a result of this new exclusion criteria is estimated to be very low.

6. COMPLIANCE DATE

The exclusion is to be introduced as soon as operationally feasible, but not later than three

months from the date of this Directive.

7. ADDITIONAL INFORMATION

Blood operators will be required to report semi-annually on the impact of this policy on their donor bases and the supply of blood.

On an ongoing basis, Health Canada may update its guidance in response to new scientific knowledge.

Questions concerning the " Donor Exclusion to Address Theoretical Risk of Transmission of variant CJD through the Blood Supply" should be directed to:
Biologics and Genetic Therapies Directorate
Centre for Biologics Evaluation
Director's Office
3rd Floor LCDC Building #6
Postal Locator 0603D
Tunney's Pasture
Ottawa, Ontario
KIA 0L2

8. REFERENCES

1. Donor exclusion to address theoretical risk of transmission of variant Creutzfeldt-Jakob disease (vCJD) through the blood supply. Health Canada Directive, 2001 http://www.hc-sc.gc.ca/hpfb-dgpsa/bgtd-dpbtg/blooddeferral_uk_france_we_e.html

2. Corinne Ida Lasmézas et al. PNAS, March 27, 2001, vol.98(7),4142-4147 "Adaptation of the bovine spongiform encephalopathy agent to primates and comparison with Creutzfeldt-Jakob disease: Implications for human health" http://www.pnas.org/cgi/doi/10.1073/pnas.041490898

3. Directive 99-01: Donor Exclusion to Address Theoretical Risk of Transmission of Variant CJD through the Blood Supply. http://www.hc-sc.gc.ca/hpfb-dgpsa/bgtd-dpbtg/d99-01_e.html

4. Directive D2000-01: Donor Exclusion to Address Theorerical Risk of Transmission of Variant CJD Through the Blood Supply http://www.hc-sc.gc.ca/hpfb-dgpsa/bgtd-dpbtg/d2000-01_0830_e.html

5. Hunter, N., et al. Transmission of prion diseases by blood transfusion. J. Gen. Virol., 83: 2897- 2905, 2002.

6. Herzog, C., et al. Tissue distribution of bovine spongiform encephalopathy agent in primates after intravenous or oral infection. Lancet, 363: 422-428, 2004.

7. Llewelyn, C A, et al. "Possible Transmission of variant Creutzfeldt-Jakob disease by blood transfusion." The Lancet (2004) 363:417-421.

8. Peden, Alexander, et al. "Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient." The Lancet (2004) 364:527-528.

9. Monthly statistics on the cases of BSE determined through testing in the European countries.
http://europa.eu.int/comm/food/food/biosafety/bse/mthly_reps_bse2004_en.htm

10. Office International des Epizooties - Number of reported cases of BSE worldwide
http://www.oie.int/eng/info/en_esbmonde.htm

11. EUROCJD and NEUROCJD: The European and Allied Countries Collaborative Study Group of CJD(EUROCJD) plus the Extended European Collaborative Study Group of CJD(NEUROCJD)
http://www.eurocjd.ed.ac.uk/

12. Statistics on the United Kingdom's CJD cases
http://www.cjd.ed.ac.uk/figures.htm

13. Number of cases of vCJD in France
http://www.invs.sante.fr/publications/mcj/donnees_mcj.html