At the molecular level, under conditions of high proteinase pre-treatment and detection using two antibodies reacting with either an epitope in the N terminal octapeptide repeat region or the core of PrP, BASE and cBSE were clearly distinguishable in primate. BASE was detectable only by the core antibody, whereas cBSE was detectable by both antibodies. We estimated that the proportion of octapeptide-resistant PrPres molecules in the BASE brain homogenate was only a small fraction (≤1/10) of that of the cBSE brain homogenate. The difference in octapeptide sensitivity to PK between cBSE and vCJD in macaques on the one hand, and Type 1 sporadic CJD in humans on the other hand, is similar to what was observed between cBSE and classical scrapie in sheep. This method can now be used to test both ruminant and human samples to identify similarities and differences in their molecular protein signatures, and to implement the classification of ruminant and possibly human strains.

Although classical epidemiological studies have not found any link between scrapie in sheep and goats and human CJD, newer molecular biological studies now indicate that about half of all cases of scrapie are due to previously undetected atypical strains [19] that are experimentally transmissible to sheep and mice [20]. Their risk for humans is unknown and is the subject of current studies in experimental models, including primates. cBSE has been shown to be responsible for human cases of vCJD, but the comparative risk for humans of BASE and other atypical strains of BSE is still unknown, and its clarification will require many years of epidemiological surveillance and molecular biological testing of both bovine and human populations.

The first cases of BASE in cattle had PrPres electrophoretic profiles similar to the MV2 subtype of sporadic CJD patients [2] that, together with the presence of amyloid plaques in both the cattle and the patients, suggested a possible link between BASE and this subtype of sCJD. However, our PrPres typing technique has shown that, in the primate, PrPres of other MV2 sCJD patients exhibited a resistance to proteolysis different from the BASE-infected primate, whereas PrPres from vCJD-infected patients and primates behave similarly. This observation, together with the absence of amyloid plaques in the BASE-infected primate, weakens the likelihood of a direct link between BASE and MV2 subtype sCJD patients.

In contrast, the specific signature of PrPres in the BASE-infected primate was similar to that seen in three of four patients with the MM2 cortical subtype of sporadic CJD [7]. It is interesting that an

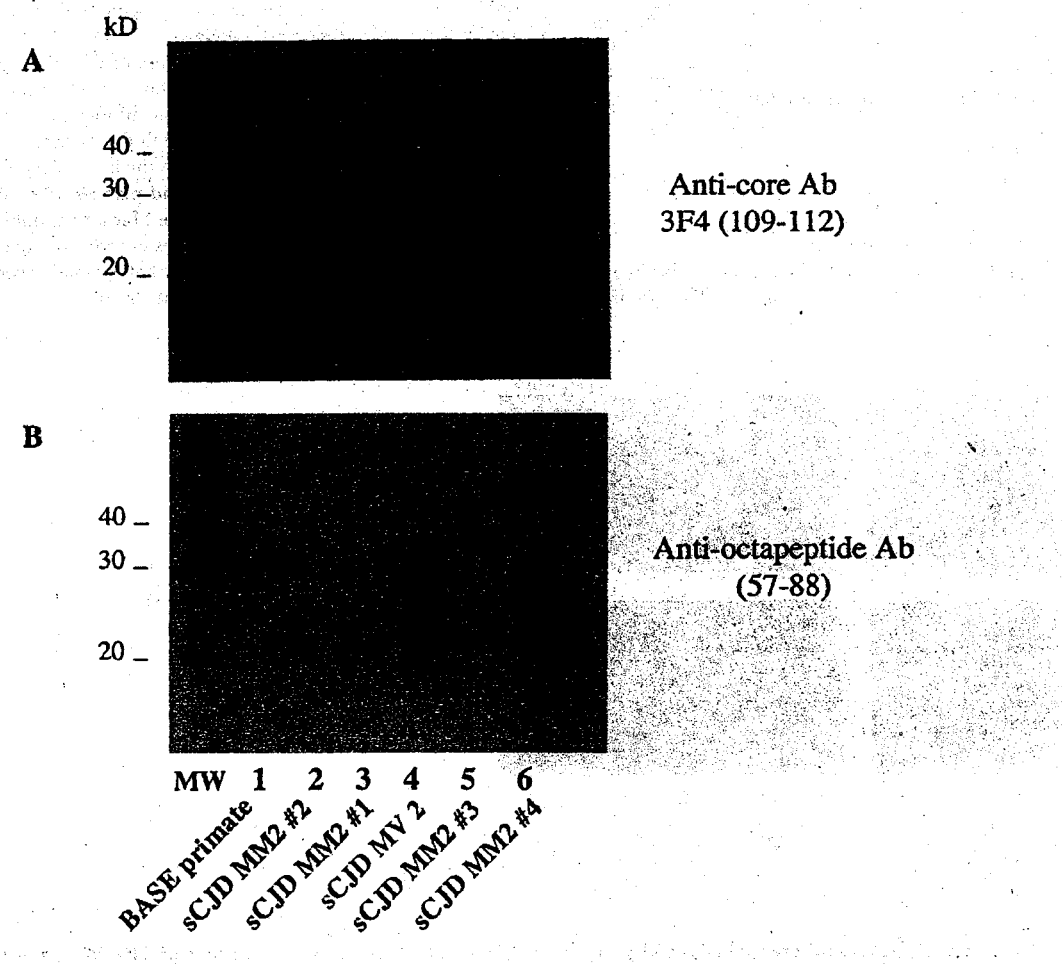

Figure 5. Electrophoretic analysis and differential sensitivity to proteolysis of PrPres in different MM2 CJD patients. PrPres from human brain homogenates (MM2 or MV2 subtypes of sCJD) and from primate experimentally infected with BASE were purified under high concentrations of proteinase K, and detected with monoclonal antibodies that recognize either the core (3F4, Panel A) or the octapeptide region (Panel B) of the protein.
doi:10.1371/journal.pone.0003017.g005

Table 2. Summary of MM2 subtype sporadic CJD patients.

Case #	1	2	3	4
[redacted]				
Age at onset	60 years	59 years	59 years	69 years
[redacted]				
Onset	Progressive memory impairment beginning with episodic memory disturbance, then attention loss, spatial and temporal orientation	Depression, insomnia, headache. Episodic memory impairment and poor language	Memory disturbance	Depression, memory impairment
[redacted]				
Terminal stage	Diffuse spastic rigidity; Pyramidal signs; dystonic movements and sporadic myoclonic jerks	Akinetic mutism	No information available	Akinetic mutism
[redacted]				
SPECT 99mTc-ECD	Not done	Hypo-perfusion of frontal, parietal and temporal cortices, bilaterally. Normal perfusion of subcortical ganglia and cerebellum	Hypo-perfusion of frontal, parietal and to a lesser extent temporal cortices	Not Done
[redacted]				
CSF 14-3-3 protein	Positive	Positive	Not done	Negative
[redacted]				
Neuropathology	Cortical spongiosis, Cerebellum relatively spared	Cortical spongiosis, Cerebellum relatively spared	Cortical spongiosis, normal cerebellum	Cortical spongiosis, cerebellum relatively spared
[redacted]				
Resistance of N-terminal part to proteolysis	No (BASE infected primate-like)	No (BASE infected primate-like)	No (BASE infected primate-like)	Yes

doi:10.1371/journal.pone.0003017.t002

important feature of the clinical-pathological syndrome in this BASE-infected macaque – the absence of cerebellar involvement – is also a common element in patients with the MM2 subtype of human sporadic CJD (Supplementary Figure S2). However, as illustrated by the clinical details of our four tested MM2 cases, there is considerable patient-to-patient variation, just as there can be variation among individual animals experimentally inoculated with a given strain of TSE. [21,22].

It is not known whether atypical strains of BSE have been circulating for years, or represent new forms of disease, and continuing research is clearly needed to answer both this and the equally important question about a possible relationship to at least certain forms of what are presently regarded as sporadic cases of human disease (sCJD) [4,23]. Moreover, the BASE strain has been described to evolve naturally towards BSE after successive transmissions in inbred mice [6]. The stability and pathogenicity of this strain in humans remains to be determined, and it is worth recalling that the stability of the cBSE/vCJD strain, which retains its specific molecular signature in different infected hosts, is the exception rather than the rule. As has been previously observed [24–26], one patient (Case No. 4, cf. figure 5 sample MM2#4) exhibited both types of PrP, i.e. type 2 typical of the MM2 subtype and type 1 observed in the MM1 subtype. On the one hand, this demonstrates the interest of such a simple biochemical test to refine PrP analysis, and on the other hand it raises a question about the existence of different PrPres signatures in the same patient, i.e., different prion strains linked to multiple infections or to variants selected by the host.

In summary, we have transmitted one atypical form of BSE (BASE) to a cynomolgus macaque monkey that had a shorter incubation period than monkeys infected with classical BSE, with distinctive clinical, neuropathological, and biochemical features; and have shown that the molecular biological signature resembled that seen in a comparatively uncommon subtype of sporadic CJD. We cannot yet say whether BASE is more pathogenic for primates (including humans) than cBSE, nor can we predict whether its molecular biological features represent a clue to one cause of apparently sporadic human CJD. However, the evidence presented here and by others justifies concern about a potential human health hazard from undetected atypical forms of BSE, and despite the waning epizoonosis of classical BSE, it would be premature to abandon the precautionary measures that have been so successful in reversing the impact of cBSE. We would instead urge a gradual, staged reduction that takes into account the evolving knowledge about atypical ruminant diseases, and both a permanent ban on the use of bovine central nervous system tissue for either animal or human use, and its destruction so as to eliminate any risk of environmental contamination.

Supporting Information

Figure S1 Resistance to proteolysis of different prion strains in sheep. PrPres from brain homogenates of sheep infected with classical scrapie, experimental cBSE, or atypical Nor-98 scrapie, and of an uninfected control sheep. Samples were purified using low (odd lanes) or high (even lanes) concentrations of proteinase K, and visualized with monoclonal antibodies that recognize either the core region (Panel A) or the octapeptide region (Panel B) of the protein. With the lower concentration of PK used in the purification step (in order to maximize test sensitivity) of one widely utilized BSE screening test [13], all three strains gave a positive result with both the anti-core and anti-octapeptide antibodies (odd lanes). Using a higher concentration of PK (even lanes) did not alter the positivity with either antibody for classical scrapie, but the cBSE strain no longer reacted with the anti-octapeptide antibody while Nor98 did not react with either antibody. Thus, by using the higher concentration of PK and two different antibodies, it is possible to discriminate between all three strains.
Found at: doi:10.1371/journal.pone.0003017.s001 (2.51 MB TIF)

Figure S2 Lesion profiles in cBSE- and BASE-infected macaque, and in MM2 sporadic CJD patients. The lesions were scored from 0 to 4 (negative, light, mild, moderate, and severe) for the different following gray matter regions: frontal (FC), temporal (TC), parietal (PC) and occipital (OC) neocortices, hippocampus (HI), parasubiculum and entorhinal cortex (EC), neostriatum (ST) (nuclei caudatus and putamen), thalamus (TH), substantia nigra (SN), midbrain periventricular gray (PG), locus ceruleus (LC), medulla (ME) (periventricular gray and inferior olive), and cerebellum (CE). Scoring for MME sCJD patient was issued from Parchi et al. [7].
Found at: doi:10.1371/journal.pone.0003017.s002 (1.52 MB TIF)

Acknowledgments

We thank Sebastien Jacquin for careful maintenance of primates, and Aurore Jolit for her precious help for histological studies; we thank Richard Kascsak (New York State Institute for Basic Research in Developmental Disabilities), Jacques Grassi, Eric Quemeneur and their colleagues (CEA/DSV) who provided the anti-PrP antibodies. We thank Sylvie Benestad (National Veterinary Institute, Oslo, Norway) for Nor-98 sheep samples, and Danny Matthews (VLA, Weybridge, UK) for the sheep sample inoculated with experimental cBSE.

Author Contributions

Conceived and designed the experiments: CC CIL. Performed the experiments: NLE SF DM FA. Analyzed the data: EEC NLE MMR SF JPD. Contributed reagents/materials/analysis tools: CC GZ MMR MC. Wrote the paper: EEC GZ PB JPD. Participated to the final reviewing of the manuscript: CC SM NS MC PL CIL.

References

1. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, et al. (1996) A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 347: 921–925.
2. Casalone C, Zanusso G, Acutis P, Ferrari S, Capucci L, et al. (2004) Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. Proc Natl Acad Sci USA 101: 3065–3070.
3. Biacabe AG, Laplanche JL, Ryder S, Baron T (2004) Distinct molecular phenotypes in bovine prion diseases. EMBO 5: 110–114.
4. Brown P, McShane L, Zanusso G, Detwiler L (2006) On the question of sporadic or atypical bovine spongiform encephalopathy and Creutzfeldt-Jakob disease. Emerg Infect Dis 12: 1816–1821.
5. Buschmann A, Gretzschel A, Biacabe AG, Schiebel K, Corona C, et al. (2006) Atypical BSE in Germany – Proof of transmissibility and biochemical characterization. Vet Microbiol 117: 103–116.
6. Capobianco R, Casalone C, Suardi S, Mangieri M, Miccolo C, et al. (2007) Conversion of the BASE prion strain into the BSE strain: the origin of BSE? PLoS Pathog; 3: e31. Doi:10.1371/journal.ppat.0030031.
7. Parchi P, Giese A, Capellari S, Brown P, Schulz-Schaeffer W, et al. (1999) Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. Ann Neurol 46: 224–233.
8. Collinge J, Whitfield J, McKintosh E, Beck J, Mead S, et al. (2006) Kuru in the 21st century – an acquired human prion disease with very long incubation periods. Lancet 367: 2068–2073.
9. Lasmézas CI, Deslys JP, Demaimay R, Adjou KT, Lamoury F, et al. (1996) BSE transmission to macaques. Nature 381: 743–744.
10. Lasmézas CI, Fournier JG, Nouvel V, Boe H, Marce D, et al. (2001) Adaptation of the bovine spongiform encephalopathy agent to primates and comparison with Creutzfeldt-Jakob disease: implications from human health. Proc Natl Acad Sci USA 98: 4142–4147.
11. Herzog C, Riviere J, Lescoutra-Etchegaray N, Charbonnier A, Leblanc V, et al. (2005) PrP^{TSE} distribution in a primate model of variant, sporadic, and iatrogenic Creutzfeldt-Jakob disease. J Virol 79: 14339–14345.
12. Herzog C, Salès N, Etchegaray N, Charbonnier A, Freire S, et al. (2004) Tissue distribution of bovine spongiform encephalopathy agent in primates after intravenous or oral infection. Lancet 363: 422–428.
13. Deslys JP, Comoy E, Hawkins S, Simon S, Schimmel H, et al. (2001) Screening slaughtered cattle for BSE. Nature 409: 476–477.
14. Deslys JP, Comoy E, Grassi J. Method for diagnosing a transmissible spongiform subacute encephalopathy caused by an unconventional transmissible agent strain in a biological sample. Patent N°99/14242, 12 November 1999.
15. Eloit M, Adjou K, Coulpier M, Fontaine JJ, Hamel R, et al. (2005) BSE agent signatures in a goat. Vet Rec 156: 523–524.
16. Gambetti P, Kong, Zou W, Parchi P, Chen SG (2003) Sporadic and familial CJD: classification and characterization. Brit Med Bull 66: 213–239.
17. Yutzy B, Holznagel E, Goulibaly C, Stuke A, Hahmann U, et al. (2007) Time-course studies of 14-3-3 protein isoforms in cerebrospinal fluid and brain of primates after oral or intracerebral infection with bovine spongiform encephalopathy agent. J Gen Virol 88: 3469–3478.
18. Kong Q, Zheng M, Casalone C, Qing L, Huang S, et al. (2008) Evaluation of the human transmission risk of an atypical bovine spongiform encephalopathy prion strain. J Virol 82: 3697–3701.
19. Benestad SL, Sarradin P, Thu B, Schonheit J, Tranulis MA, et al. (2003) Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. Vet Rec 153: 202–208.
20. Le Dur A, Beringue V, Andreoletti O, Reine F, Lai TL, et al. (2005) A newly identified type of scrapie agent can naturally infect sheep with resistant PrP genotypes. Proc Natl Acad Sci USA 102: 16031–16036.
21. Brown P, Gibbs CJ Jr, Rodgers-Johnson P, Asher DM, Sulima MP, et al. (1994) Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease. Ann Neurol 35: 513–529.
22. Williams L, Brown P, Ironside J, Gibson S, Will R, et al. (2007) Clinical, neuropathological and immunohistochemical features of sporadic and variant forms of Creutzfeldt-Jakob disease in the squirrel monkey (Saimiri sciureus). J Gen Virol 88: 688–695.
23. Krasnianski A, Meissner B, Schulz-Schaeffer W, Kallenberg K, Bartl M, et al. (2006) Clinical features and diagnosis of the MM2 cortical subtype of sporadic Creutzfeldt-Jakob disease. Arch Neurol 63: 876–880.
24. Puoti G, Giaccone G, Rossi G, Canciani B, Bugiani O, et al. (1999) Sporadic Creutzfeldt-Jakob disease: co-occurrence of different types of PrPSc in the same brain. Neurology 53: 2173–2176.
25. Polymenidou M, Stoeck K, Glatzel J, Vey M, Bellon A, et al. (2005) Coexistence of multiple PrP^{Sc} types in individuals with Creutzfeldt-Jakob disease. Lancet Neurol 4: 805–814.
26. Yull HM, Ritchie DL, Langeveld JPM, van Zijderveld FG, Bruce ME, et al. (2006) Detection of type 1 prion protein in variant Creutzfeldt-Jakob disease. Amer J Pathol 168: 151–157.

医薬品
医薬部外品　研究報告　調査報告書
化粧品

別紙 3-4

識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2008 年 5 月 8 日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称			研究報告の公表状況	Estimating the pathogen safety of manufactured human plasma products: application to fibrin sealants and to thrombin. Horowitz, B. and Busch, M. Transfusion, 48, 1739-53 (2008)	公表国 米国	
販売名（企業名）						

研究報告の概要	使用上の注意記載状況・その他参考事項等
本稿では，米国で最近上市された 2 種類の血液製剤（トロンビン及びフィブリノゲン）を用いて，パルボウイルス B19（B19V），A 型，B 型及び C 型肝炎，HIV ならびに変異型クロイツフェルト・ヤコブ病（vCJD）関連プリオンの病原体感染リスクを評価した。特に，A 型肝炎ウイルス（HAV）及び B19V は，エンベロープを持たないため不活化がより困難であることから本試験の対象とした。これら血液製剤の製造過程では 2 つの異なるウイルス除去工程が使われている。各病原体の安全域は，出発原料内の最大ウイルス量と製造工程の除去能との比較によって決定した。フィブリノゲン及びトロンビンは，ともに 1 バイアル当たりの病原菌が伝播する残遺リスクが極めて低く，A 型，B 型及び C 型肝炎及び HIV については 10^{15} 分の 1 未満と算出された。B19V については，トロンビンが 1000 万分の 1 未満，フィブリノゲンが 50 万分の 1 未満と算出された。同様に，vCJD の病原体伝播リスクも非常に低いと推定された。新型ウイルス（西ナイルウイルス，H5N1 型インフルエンザウイルス，重症急性呼吸器症候群（SARS）ウイルス又はチクングニヤウイルス－いずれもエンベロープを持つ）の脅威に関しては，現在の製造工程が完全な不活化をもたらすことがわかっている。したがって，血漿製造業界は，血液製剤の安全性を増強するために過去 10～20 年に実施された多くの改善点により，優良な安全性プロファイルをもつ製品の製造し，提供していると著者らは結論付けた。	BYL-2008-0323

報告企業の意見	今後の対応
著名な 2 研究機関が発表した本研究では，新たな脅威として現れたエンベロープウイルスに対する完全不活化法や非エンベロープウイルスやプリオンに対する実質的除去法など，病原体不活化法が常に改良され続けてきたことを確認している。したがって，実質的リスクが存在する血液製剤と違い，血漿分画製剤における病原体伝播リスクは極めて低いと考えられる。	現時点で新たな安全対策上の措置を講じる必要はないと考える。

(37)

Estimating the pathogen safety of manufactured human plasma products: application to fibrin sealants and to thrombin

Bernard Horowitz and Michael Busch

BACKGROUND: Plasma fractionators have implemented many improvements over the past decade directed toward reducing the likelihood of pathogen transmission by purified blood products, yet little has been published attempting to assess the overall impact of these improvements on the probability of safety of the final product.

STUDY DESIGN AND METHODS: Safety margins for human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), hepatitis A virus (HAV), parvovirus B19, and variant form of Creutzfeldt-Jakob disease (vCJD) were calculated for the two fibrin sealants licensed in the United States and for thrombin. These products were selected because their use in a clinical setting is, in most cases, optional, and both were relatively recently approved for marketing by the US Food and Drug Administration (FDA). Moreover, thrombin and fibrinogen both undergo two dedicated virus inactivation steps and/or removal steps in accord with the recommendations of regulatory agencies worldwide. Safety margins were determined by comparing the potential maximum viral loads in contaminated units to viral clearance factors, ultimately leading to the calculation of the residual risk per vial.

RESULTS: The residual risk of pathogen transmission per vial was calculated to be less than 1 in 10^{-15} for HIV, HCV, HBV, and HAV for both fibrinogen and thrombin. Owing to the greater quantities that can be present and its greater thermal stability, the calculated risk for parvovirus transmission was 1 in 500,000 vials for fibrinogen and less than 1 in 10^{7} per vial for thrombin. Assuming that vCJD is found to be present in plasma donations, its risk of transmission by these purified and processed plasma derivatives would appear to be very low.

CONCLUSIONS: The pathogen safety initiatives implemented by plasma fractionators over the past 10 to 20 years have resulted in products with excellent pathogen safety profiles. Of the agents examined, parvovirus continues to have the lowest calculated margin of safety. Despite this, parvovirus transmissions should be rare. Manufacturers are encouraged to continue exploring processes to further enlarge parvovirus safety margins and to continue exploring ways of eliminating prions.

Products derived from human plasma have important therapeutic uses, including substitution therapy for hemophilia and primary immune deficiency disorders, plasma expanders after trauma and surgery, and as hemostatic agents.[1-3] Plasma proteins and their functions are so diverse that new applications for currently licensed plasma protein products continue to be investigated[4] and novel plasma protein products continue to be developed.[5-7] Consequently, there has been an increase in the quantity of plasma processed worldwide, and significant improvements have been made in manufacturing procedures and in plant design and operation. Many of these improvements were implemented with the goal of assuring safety of plasma derivatives from transfusion-transmissible pathogens. These include 1) improved selection of donors, 2) use of plasma only from "qualified" donors who repeatedly pass viral screening procedures, 3) use of nucleic acid amplification testing (NAT) methods to detect and eliminate virus before the pooling of donor units, 4) inventory hold policies that allow interdiction of "window-phase"

ABBREVIATIONS: BVDV = bovine viral diarrhea virus; HAV = hepatitis A virus; ID(s) = infectious dose(s); PRV = pseudorabies virus; SARS = severe acute respiratory syndrome; S/D = solvent/detergent (method of virus inactivation); TNBP = tri-(*n*-butyl)phosphate (the solvent in S/D treatment); vCJD = variant form of Creutzfeldt-Jakob disease that infects man and presumptively has arisen from the epidemic of bovine spongiform encephalopathy in cattle; WNV = West Nile virus.

From Horowitz Consultants, LLC, Key Biscayne, Florida; and Blood Systems Research Institute and University of California at San Francisco, San Francisco, California.

Address reprint requests to: Bernard Horowitz, PhD, 785 Crandon Boulevard, Unit 1204, Key Biscayne, FL 33149; e-mail: horowitzconsult@aol.com.

Supported in part by Johnson and Johnson, Inc.

Received for publication November 1, 2007; revision received January 24, 2008, and accepted January 27, 2008.

doi: 10.1111/j.1537-2995.2008.01717.x

TRANSFUSION **;**:**-**.

units before pooling, 5) employment of purification procedures shown to remove virus or prions should they be present, 6) the use of two complementary or "orthogonal" methods of virus inactivation, and 7) the engineering and design of facilities so as to prevent contamination of downstream process streams with upstream fractions.

Products that promote hemostasis and tissue sealing following trauma and surgery are among the more recently licensed human plasma products in the United States. Two fibrin sealants, one from Omrix (New York, NY) and one from Baxter (Deerfield, IL), are licensed in the United States by the Food and Drug Administration (FDA), and Omrix also recently received approval of a topically applied thrombin. While they cannot be used in all surgical settings, such as to control high-pressure (arterial) bleeds, these products have been shown to improve surgical outcomes, reduce the time to hemostasis, reduce blood loss, and reduce surgical complications.[8] Substitutes for these human plasma-derived hemostatic agents have also been developed, including bovine thrombin and recombinant-derived human thrombin. Bovine thrombin is antigenically distinct from human thrombin and has been shown to elicit antibodies when used in man.[9] These antibodies, as well as antibodies elicited to bovine impurities in the product, especially antibodies to coagulation factor (F)V, have resulted in severe bleeding complications due to cross-reaction with their human counterparts.[10-13] Higher purification has reduced this complication, although a recent report[14] indicates that antibody formation still occurs. Products made by recombinant technology have their own, somewhat unique, issues. Depending on the gene construct used and the cell line chosen, the amino acid sequence may differ from that which occurs naturally, and differences in posttranscriptional processing often result in altered patterns of glycosylation or other molecular changes.[15-19] Consequently, immunogenicity is a potential problem that needs to be continually assessed. Also, depending on specific production details, manufacturing procedures must employ steps designed to inactivate and/or remove viral contaminants (and other potential pathogens) known to be present in the cell line and/or in the culture medium employed.[20] Additionally, in many circumstances, the higher cost associated with recombinant proteins limits their use.

In the past decade, many estimates of the viral safety of transfused whole blood and its components (i.e., red blood cells, platelet concentrates, and fresh-frozen plasma) have been published, with each passing year showing improved viral and bacterial safety.[21,22] In the same time frame, aside from monitoring clinical outcomes and despite the aforementioned improvements, little has been published to assess the parallel increase in safety of manufactured plasma products. A recent publication by Janssen and colleagues[23] used a probabilistic, Monte Carlo model to estimate the risk of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) in a hypothetical plasma derivative subjected to what appears to be a single method of viral inactivation. Based on their assumptions, they calculated that the risk per vial approximated 1 in 1 million. Given these improvements, the recent licensure of human plasma-derived topical thrombin, and the frequent surgical use of fibrin sealants, consisting of fibrinogen in addition to thrombin, it is timely to estimate their pathogen safety. These estimates are especially useful since the fibrinogen component of fibrin sealants is among the least processed blood derivatives, while the manufacturing procedures for thrombin, whether part of a fibrin sealant kit or used by itself, are typical of those employed with most newer plasma derivatives. Thrombin and fibrinogen both undergo two dedicated virus inactivation steps and/or removal steps. Fibrinogen and thrombin from each company are each treated by solvent/detergent (S/D). Additionally, Omrix pasteurizes its fibrinogen and nanofilters its thrombin, while Baxter vapor heats both components following lyophilization. The fibrinogen preparation cannot be nanofiltered without suffering large losses in fibrinogen and fibronectin due to their large size. The presence of fibronectin may be important since it contributes to cell adhesion.[24] For HIV, hepatitis C virus (HCV), and HBV, this report updates estimates made by one of us (BH) in 1990[25] using better information on viral loads than was available then and enlarges the pathogen list to include hepatitis A virus (HAV) and parvovirus, both of which are nonenveloped viruses, and the prion that causes variant form of Creutzfeldt-Jakob disease (vCJD). It is anticipated that the method of approach reported here can be applied to other existing or experimental blood protein products.

MATERIALS AND METHODS

Two distinct methods can be used to calculate pathogen safety. The first is to calculate safety margins by comparing the number of infectious units or doses of pathogen in the starting material to the clearance capacity of the manufacturing process. The second is to measure clinical outcomes, comparing the incidence of transmission to the quantity of product infused. Transmissions in a clinical setting should be considered the gold standard since they involve actually measuring what we want to know, and with this information, one can back-calculate clearance capacities of processes for known pathogen burdens. On the other hand, clinical studies of the type required can be extremely lengthy and expensive and the results possibly misleading. The former method has the advantage that estimates of safety can be made in advance of clinical testing. Moreover, the safety margins calculated for a wide range of viruses likely will also be applicable to unstudied and newly emerging viruses.

Product safety margins can be calculated by comparing potential viral loads with the viral clearance capacity by the formulas

$$VL = N \times C,$$

where VL is viral load, N is the number of units in a plasma pool containing infectious virus, and C is the concentration of virus in those units, and

$$\text{Safety Margin} = CC/VL,$$

where CC is the clearance capacity or the ability of the process to remove or inactivate the infectious agent being studied.

Viral load

The pathogens of interest for manufactured plasma products are largely viruses that are present in blood predominantly as cell-free virions (e.g., HBV, HCV, HIV, HAV, and parvovirus B19). Other examples include West Nile virus (WNV) and dengue viruses. The newly described vCJD agent, presumably a prion, is also a potential concern despite the absence of evidence that it is transmitted by purified plasma protein products.[26,27] Cell-associated viruses like cytomegalovirus, Epstein-Barr virus, and human herpes virus 8 are not a concern since infected cells are removed by the apheresis and filtration procedures in common use. Bacteria and fungi are also effectively removed by the terminal sterile filtrations applied to all biologic products, including plasma products, recombinant products, monoclonal antibodies, and so forth, and therefore will not be addressed here.

For the major transfusion-transmitted viral pathogens, the viral loads are typically measured as genome-equivalents (geq) per mL of plasma based on results of quantitative NAT. These loads vary dramatically during the progressive stages of infection with the highest viral loads seen transiently during the acute preseroconversion (i.e., so-called window period) stage of infection; moreover, infectiousness is also highest during this same period.[28-30] Subsequent to antibody seroconversion (and coincident with innate and adaptive cellular immune responses to infection), the agents are 1) eliminated from the body (e.g., eradication of infection, as occurs with WNV, HAV, and dengue); 2) cleared from plasma but with persistence of cell-associated virus in tissues (e.g., latent infections such as herpes viruses, parvovirus B19, and "occult" HBV infections); or 3) persistent at reduced concentrations in plasma (i.e., so-called set-point viremia after establishment of chronic HBV, HCV, and HIV infections). In addition to variations in viral load measured by NAT, as infections evolve the infectivity of viruses change profoundly.[31-39] For HIV (and its model agent simian immunodeficiency virus), HBV, HCV, and WNV, it is now well established that during the acute preseroconversion phase of infection (pre–ramp-up and ramp-up stages), virion particles in plasma are highly infectious, with 10 or fewer geq in the entire volume of plasma sufficient to transmit infection following parenteral injection. In contrast, viral particles present in plasma from the same infected individuals have significantly (10- to 1000-fold) reduced infectious potential weeks to years after seroconversion.[29-35,40-43] The reduced infectivity of plasma virus from postseroconversion phases of chronic infection is attributable to a combination of factors, including presence of endogenous neutralizing antibodies, generation of defective virions (i.e., lacking full genomes or other required infectivity factors), and immune selection of virions with reduced fitness. Hence, viral load distributions observed during acute versus chronic stages of infection need to be adjusted by a factor to account for the relative infectivity of virion particles to derive estimates for the functional viral load during each stage.

All donated blood in the United States, whether for the preparation of components or for use in manufactured plasma products, is screened by serologic assays for HIV-1 and -2, HBV, HCV, and human lymphotropic virus-1 and -2 and by NAT for HIV and HCV. Donors are also excluded if they have certain risk factors that make their exposure to viruses or prions more likely. Additionally, plasma manufacturers screen donated plasma in a minipool format for HBV, HAV, and parvovirus by NAT. The use of NAT greatly reduces viral loads since positive units missed by serologic screening procedures typically have the highest concentrations of virus, which is also highly infectious. Consequently, with very rare exceptions of concordant testing errors in serology and NAT screening, only units that test both serologically negative (i.e., window-phase units) and that have relatively low titers of infectious virus (<500-5000 infectious doses [IDs]/mL) are pooled. Furthermore, manufacturing pools are retested by NAT before fractionation to assure that high-titer viremic units were not missed as a result of erroneous testing. As a result, the probability that a fractionation pool contains a significant level of virus is extraordinarily remote.

Pathogen infectious load estimates are given in Table 1. To estimate the number of positive units missed by the screening procedures employed, currently observed NAT yields, expressed as number of positive samples per million donations, were adjusted to account for the amount the window period is believed to be closed through the use of NAT. From this, we conclude that few plasma pools will contain HIV, HCV, or HAV while contamination by HBV and parvovirus B19 will be considerably more frequent. Based on the analytical sensitivity of the NAT assays, the dilution factors during assay, and the volume of an individual donor unit, we calculated the

TABLE 1. Viral load estimates

Virus	NAT yield (number/million donations)* (A)	Percent NAT closes window period† (B)	Number of positive units missed by NAT/million donations (C) = (A/B – A)	NAT analytic sensitivity‡ (geq/ml)	NAT operational sensitivity‡ (geq/mL in the donor unit)	Maximum genomic load (log geq/ fractionation pool)§	Ratio of IDs to geq¶	Maximum viral load (log ID/pool)
HIV	0.58	48% (11/23)	0.63	1.40	717	5.7	1:1	5.7
HCV	4.08	88% (50/57)	0.56	3.10	1,587	6.0	1:1	6.0
HBV	13	23% (10/43)	44	0.66	338	5.4	1:10	4.4
HAV	0.30	71% (5/7)	0.12	2.0	1,024	5.9	1:1,000	2.9
Parvovirus B19	50.5	71% (5/7)	20.6	22.6	5,120,000	9.6	1:1,000	6.6
vCJD						4.3	1:1	4.3

* For HIV, HCV, and HBV, a NAT yield unit is defined as an antibody- or hepatitis B surface antigen (HBsAg)-negative donation detected by RNA and/or DNA screening using pooled NAT systems. Rates presented are published rates from United States and European whole-blood donor screening programs.[78-78] Although NAT yield rates for these viruses among source plasma donors are higher, this is offset by source plasma policies that stipulate that only plasma from "qualified donors" be released for fractionation and that frozen units be held in inventory enabling interdiction of quarantined potential window-phase units when donors later test reactive for infectious disease markers or are deferred for other reasons.[79] For HAV and parvovirus B19, we use the rate of detection of high titer viremic donations by low sensitivity NAT screening of whole-blood and plasma donors, irrespective of serostatus of viremic units.[80,81]

† The percentage is determined by dividing the number of days NAT detects positive samples by the number of days from when a donor becomes infectious until there is sufficient antibody to be detected serologically (HIV, HCV, HBV) or there is sufficient antibody to render the donation noninfectious (HAV, parvovirus; see Busch et al.[82] for conceptual basis for this approach and Kleinman and Busch[83] for application of this approach to HBV). The residual infectious window periods are defined as the number of days from viremia reaching the minimal infectious threshold (set as 1 copy per 20 mL of plasma; Busch et al.[84]) to the level of viremia detected by pooled-sample NAT, using the viral doubling-times during the acute ramp-up phases established for each agent (20.5 hr for HIV, 10.8 hr for HCV, 2.6 days for HBV, and approx. 1 day for HAV and parvovirus B19). This yielded pre-NAT infectious window periods of 12 days for HIV, 7 days for HCV, 33 days for HBV, and 2 days for HAV and parvovirus B19. The NAT detection windows are based on time from reaching the 50 percent sensitivity of the NAT screening assays to the point of seroconversion for HIV (11 days), HCV (50 days), and HBV (10 days) or the duration of the estimated NAT yield window period for HAV (5 days) and parvovirus B19 (5 days).

‡ We assumed that the 50 percent sensitivity levels for assays used by source plasma donors are in the same range as those reported by the National Genetics Institute (NGI). NGI NAT assays are used by approximately 60 percent of the source plasma sector for all five viruses, as well as by the American Red Cross for HAV and parvovirus B19. For HIV-1, HBV, and HCV, the analytic sensitivity quoted is that of the assay itself without taking sample dilutions or pooling into account (Schreiber et al.[79]). Operational sensitivity takes these dilutions into account and refers to the maximum quantity that could be present in the contaminated donor unit. For parvovirus, Omrix's acceptance requirement for a pool of 512 units is less than 10,000 geq per mL, and thus for operational sensitivity we used $10{,}000 \times 512$ (the number of units in the minipool).

§ NAT operational sensitivity was multiplied by 700, the assumed volume of the donation. Based on the number of units missed by NAT per million donations and a pool size of 6000 L, we assume that only one positive unit will enter a fractionation pool. For vCJD, we assumed 30 ID per mL in a contaminated unit.

¶ For HIV and HCV, the infectious load is considered to be equivalent to the viral load expressed in geq or copies, given that we are restricting consideration to the acute preseroconversion viremic phase, which is known to be highly infectious (see text), and that seropositive units from other donors, which might contain neutralizing antibodies, have been detected by serologic screening and excluded from the manufacturing pools. For HBV, we similarly assume high-level infectivity of window-phase donations[35,85] but reduce this to a ratio of 1 in 10, in part, because of the likely presence of anti-HBsAg in the plasma pool. We used a ratio of 1:1000 for HAV and parvovirus. We believe this to be justified since the neutralization capacity of anti-HAV is well established. While the ratio of infectious units to geq for parvovirus is unknown, results from tissue culture infectivity studies indicate that the ratio is 1:5000 for genotype 1 and 1:260,000 for genotype 2[86] for products devoid of parvovirus antibody, the lowest ID that has been reported on infusion into a seronegative recipient is 2×10^4 geq,[70] and the infectivity of products containing parvovirus antibody has been shown to be reduced considerably.[83,87,88]

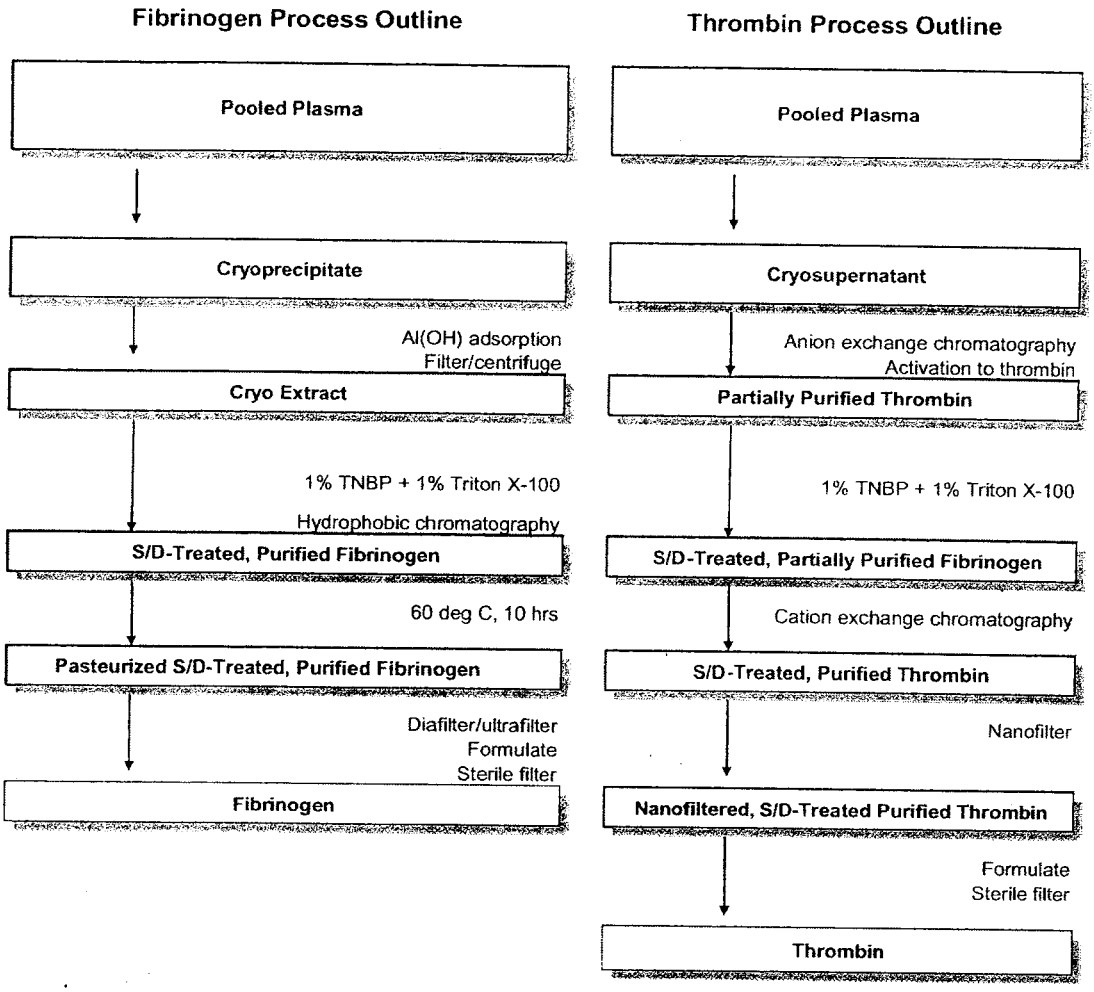

Fig. 1. Process outlines for fibrinogen and thrombin.

maximum genomic load likely to be present in a fractionation pool (Table 1, Column 7). This was adjusted downward for HBV, HAV, and parvovirus B19 to account for the reduced infectivity of virus that occurs as a result of neutralizing antibodies derived from other donors in the pool, since antibody screening is not performed for these prevalent agents. No adjustment was applied to HIV or HCV since later-stage infections with potential neutralizing antibodies are interdicted by the currently deployed serologic tests. Although attempts to transmit vCJD by human plasma have failed,[44] epidemiologic evidence supports its transmission by whole blood and blood components.[45-47] Based on animal models, its concentration is likely to be quite low, estimated at 20 to 30 IDs per mL.[48] With the use of this estimate, the maximum concentration in the plasma pool approximates 0.003 IDs per mL (3 IDs/L), and the total maximum load in a 6000-L plasma pool will approximate $10^{4.3}$ IDs (Table 1).

Clearance capacity

The clearance capacity for pathogens is a function of the extent to which pathogen is removed during steps designed to purify the protein of interest, the inclusion within the manufacturing process of dedicated viral inactivation and removal steps, the presence of neutralizing antibody in final product, and serendipitous inactivation that occurs. The process steps for fibrinogen and thrombin used by Omrix are outlined in Fig. 1. As is typical of modern plasma protein products, each process includes two dedicated, viral elimination steps: fibrinogen is treated with S/D and is pasteurized, and thrombin is treated with S/D and is passed through a purposefully designed, virus removal filter (so-called nanofiltration). Additionally, each chromatographic step and filtration in the presence of filter aid can contribute to pathogen removal. Baxter's Tisseel is processed similarly except, when first introduced in 1998, it utilized vapor heating as

TABLE 2. Validated viral elimination when processing fibrinogen: Omrix process

		Log kill or removal				
	Virus:	HIV-1	BVDV	PRV	HAV	CPV
	Model for:	HIV	HCV	HBV	HAV	Parvovirus B19
Step	Enveloped virus?:	Yes	Yes	Yes	No	No
Cryoprecipitation + Al(OH) treatment		ND	ND	ND	1.5	1.5
S/D treatment		>4.4*	>4.4†	>4.4†	ND	0
Pasteurization		>4.4‡	>5.5‡	ND	>5.8‡	1.3
	Sum:	>8.8	>9.9	>4.0	>7.3	2.8

* No infectivity after 5 minutes. Treatment is for 4 hours.
† No infectivity after 10 minutes, the first time point taken.
‡ 9 to 10 hours were required to achieve reported kills. Treatment is for 10 hours.
Al(OH) = aluminum hydroxide; CPV = canine parvovirus; ND = not done.

TABLE 3. Validated viral elimination when processing thrombin: Omrix process

		Log kill or removal						
	Virus:	HIV-1	BVDV	Sindbis	PRV	EMCV	CPV	MMV
	Model for:	HIV	HCV	HCV	HBV	HAV	Parvovirus B19	Parvovirus B19
Step	Enveloped virus?:	Yes	Yes	Yes	Yes	No	No	No
Cryo removal		ND	ND	ND	ND	ND	ND	ND
Anion-exchange chromatography		ND	ND	ND	ND	ND	ND	ND
S/D treatment		>5.8*	>4.7†	>5.3‡	>4.3†	ND	0	ND
Cation-exchange chromatography		ND	ND	ND	ND	ND	ND	ND
Nanofiltration		>4.4	ND	>5.3	>5.5	7.0	5.9	5.8
	Sum:	>10.2	>4.7	>10.6	>9.8	7.0	5.9	5.8

* No infectivity after 5 minutes. Treatment is for 6 hours for thrombin.
† No infectivity after 10 minutes, the first time point taken.
‡ No infectivity after 15 minutes, the first time point taken.
EMCV = encephalomyocarditis virus; MMV = mouse minute virus; ND = not done.

its sole, dedicated virus inactivation step; S/D treatment has been added recently. Another difference is that its thrombin component is isolated starting with Baxter's activated prothrombin factor complex.

The FDA and other applicable regulatory authorities demand that formal viral inactivation and/or removal studies be performed and that these adhere to international standards as they relate to the selection of viruses to be used, the conduct of these studies under Good Laboratory Practice guidelines and the calculations provided. We need not reiterate those guidelines here, except to say that the model viruses selected were chosen to represent multiple viral types and, in particular, the viruses of concern for products derived from human blood. Thus, viral elimination studies typically use HIV, bovine viral diarrhea virus (BVDV; model for HCV), pseudorabies virus (PRV; model for HBV), HAV or another picornovirus such as encephalomyocarditis virus, and canine parvovirus (or another model for human parvovirus B19).

The results from these formal studies for the fibrinogen and thrombin components of Omrix's and Baxter's fibrin sealant products are given in Tables 2 and 3 and Table 4, respectively (see product package inserts, with updates from manufacturers; see Acknowledgments). The clearance factors for enveloped viruses and the models for HIV, HCV, and HBV exceed the challenge dose for each of the dedicated viral elimination steps (i.e., S/D, pasteurization, nanofiltration, and vapor heating). Consequently, when the same virus has been studied in each of the two dedicated steps, the validated clearance factors exceed 9 log, and where higher doses of virus have been used or more steps validated, clearance factors as large as 18 log have been reported. The validated clearance of nonenveloped viruses is significantly less than for enveloped viruses since only one of the two dedicated viral elimination methods is effective against these viruses. Parvoviruses are a special case since they are especially heat-stable, and only 1 to 2 log of animal parvoviruses are inactivated by either pasteurization or vapor heating. It should be noted, however, that human parvovirus B19 may be more heat-sensitive than the models used here.[49] Nanofiltration is significantly more effective, and Omrix has shown for its thrombin preparation that nanofiltration removes approximately 6 log of parvoviruses.

A more complete estimate of safety margin needs to take into account the contribution of the other steps in the process that contribute to safety despite not being formally validated. It is commonly accepted that immune neutralization contributes to HAV and parvovirus B19 safety and that the neutralization capacity of antibodies to