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研究報告の概要

血漿分画製剤は、原材料がヒト由来であるため、変異型クロイツフェルト・ヤコブ病(vCJD)を引き起こすプリオンを伝播する可能性があるが、血漿分画製剤によってプリオンが伝播したという報告はない。
様々な国で実施されている対策(BSE発生国での滞在歴や輸血または組織移植を受けたことがある献血者の保留、白血球除去など)や、いくつかの製造工程が、血漿分画製剤によるプリオン伝播防止に貢献している可能性がある。
脳由来の感染因子をスパイクする多数の実験的感染性試験により、いくつかの分画ステップ、特にエタノール分画、デプス・フィルトレーション(深層濾過)、イオン交換クロマトグラフィーは、プリオンを数log除去できることが実証された。更に、凝固因子や免疫グロブリン製剤からウイルスを除去するために使用されている75nm以下の多層膜を使ったナノろ過は、サイズ的排除とトラッピングメカニズムによって、スパイクしたプリオンを3〜5log以上除去することができる。
これらのことから、血漿分画製剤によるvCJDの伝染はほとんどないと思われるが、ヒト血液中における感染因子の生化学的性質についてはまだ分からないところがあるため、注意を怠るべきではない。

使用上の注意記載状況・その他参考事項等

重要な基本的注意
現在までに本剤の投与により変異型クロイツフェルト・ヤコブ病（vCJD）等が伝播したとの報告はない。しかしながら、製造工程において異常プリオンを低減し得るとの報告があるものの、理論的なvCJD等の伝播のリスクを完全には排除できないので、投与の際には患者への説明を十分行い、治療上の必要性を十分検討の上投与すること。

報告企業の意見	今後の対応
血漿分画製剤からのvCJD感染リスクに関する情報で、現時点まで血漿分画製剤からのvCJD伝播の報告はないこと、血漿分画製剤の製造工程でプリオンが除去できるとの情報である。	今後ともvCJDに関する安全性情報等に留意していく。

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Original article

Current strategies to prevent transmission of prions by human plasma derivatives

Mesures actuelles de prévention du risque de transmission de prions par les médicaments dérivés du plasma humain

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Abstract

Protein products prepared from pooled human plasma are an essential class of therapeutics used mostly to control bleeding and/or immunological disorders. Because of the human origin of the starting material, there is a risk that these products may possibly transmit prions causing variant Creutzfeldt–Jakob disease (vCJD). No case of transmission of prions by plasma products has been observed. Case-by-case measures implemented in various countries, and several technical factors may contribute, to various degrees, to the prevention of the risk of transmission of prions by plasma products. Those measures include (a) the epidemiological surveillance of population in countries with cases of vCJD and/or bovine spongiform encephalopathies (BSE), (b) the deferral of blood donors who traveled or resided, for specific periods of time, to countries with BSE, or who received transfusion or tissue transplant, (c) the removal of leucocytes in plasma used for fractionation, and, last but not least, (d) the removal of the prion agents during the complex industrial fractionation process used to prepare plasma products. Numerous experimental infectivity studies, involving the spiking of brain-derived infectious materials, have demonstrated that several fractionation steps, in particular ethanol fractionation, depth filtration, and chromatography, can remove several logs of prions. Removal is explained by the distinct hydrophobic and aggregative properties of the prion proteins. In addition, nanofiltration using multi-layer membranes of 75 nm or smaller, which is commonly used for removing viruses from coagulation factors and immunoglobulins products, can remove more than 3–5 logs of spiked prions, presumably by size-exclusion and trapping mechanisms. Therefore, the risk of transmission of vCJD by human plasma products appears remote, but caution should prevail since the biochemical nature of the infectious agent in human blood is still unknown.

Résumé

Les médicaments dérivés du plasma humain occupent une place thérapeutique essentielle en particulier dans le traitement de troubles hémorragiques ou immunologiques. Par l'origine humaine du plasma, ces produits sont une source possible d'infection par les agents transmissibles non conventionnels (ATNC ou prions), dont celui induisant la variante de la maladie de Creutzfeldt–Jakob (vMCJ). On n'a recensé toutefois à ce jour aucun cas de transmission de vMCJ par les produits plasmatiques industriels. Diverses mesures de précaution, mises en place au cas par cas dans différents pays, et des facteurs techniques paraissent prévenir les possibilités de transmission de ces agents infectieux par les médicaments dérivés du sang. Ils comprennent : (a) le contrôle épidémiologique de la population dans les pays où des cas de vMCJ et/ou d'encéphalopathie bovine spongiforme (EBS) ont été identifiés ; (b) l'exclusion des candidats donneurs de sang ayant voyagé ou séjourné pour une certaine période de temps dans des pays touchés par l'EBS, ou ayant été transfusés ou transplantés ; (c) la limitation du contenu du plasma en leucocytes ; et (d) l'élimination de la protéine prion pathologique au décours des étapes de fractionnement. De nombreuses études expérimentales, reposant le plus souvent sur des épreuves de surcharge par extraits de cerveaux d'animaux infectés par une souche d'ATNC, concourent à établir que diverses étapes de fractionnement, dont les précipitations en présence d'éthanol, les filtrations en profondeur, et les chromatographies, contribuent à une élimination importante des prions. Celle-ci paraît s'expliquer par les caractéristiques d'hydrophobicité et de tendance de l'agent infectieux à

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s'agréger. Par ailleurs, les étapes de nanofiltration sur des membranes multicouches d'une porosité de 75 nm ou moins, utilisées pour la sécurisation virale des facteurs de coagulation ou des immunoglobulines, retiennent, vraisemblablement par des mécanismes d'exclusion stérique et de piégeage, plus de trois à cinq logs de prions. Au regard de ces données expérimentales, le risque de transmission de vMCJ par les produits plasmatiques parait très mince, mais la portée réelle de ces études reste incomplète tant que la nature de l'agent infectieux présent dans le sang ne sera pas pleinement élucidée.

Keywords: Prions; Plasma products; Fractionation; PrP^{TSE}; Safety; vCJD

Mots clés : Prions ; Agents transmissibles non conventionnels ; Produits plasmatiques ; Fractionnement ; PrP^{TSE} ; Sécurité ; vCJD

1. Introduction

Human plasma is used for the manufacture of fractionated products called plasma products, plasma derivatives, or plasma-derived medicinal products. These products have no substitutes to treat various life-threatening congenital or acquired bleeding, thrombotic disorders, immunological deficiencies, tissues enzymatic degradations, and/or trauma. Plasma products are purified from pools of thousand liters of plasma. Production methods involve sophisticated purification procedures based on a series of precipitation, filtration, and chromatographic steps that constitute the plasma fractionation process [1]. As historical perspectives show, various transmissible infectious agents, most notably viruses, can contaminate human blood and be transmitted by industrial plasma products. It is now believed that the variant form of Creutzfeldt–Jakob disease (vCJD), a human disease thought to be primarily associated to the ingestion of food contaminated by the bovine spongiform encephalopathy (BSE) agent, may also be transmitted by transfusion of blood components [2]. Three human cases of transfusion transmitted prion infections (only two of which were symptomatic of vCJD) have been identified and ascribed to the infusion of non-leucoreduced red blood cell concentrates [3]. The third, pre- or sub-clinical, case was in an individual who was Met/Val heterozygous at codon 129 of the prion protein gene, suggesting that individuals with a genotypes other than Met–Met homozygous at residue 129 may also be infected [3,4].

As it is now apparent that vCJD can be transmitted by transfusion, concerns about the safety of pooled plasma derivatives have grow. A UK retrospective study on tissue samples suggests that more people than initially thought may be incubating the disease [5,6]. There is, so far, no evidence that vCJD has been transmitted by plasma derivatives. However, the possibility of long incubation period of the disease does not allow to draw definite conclusion on the absence of risks, and therefore preventative measures have been put in place in several countries. The purpose of this paper is to present the current strategies that are believed to restrict the risk of transmission of prions by industrial plasma products.

2. Preventative measures for the collection of plasma for fractionation

The occurrence of human-to-human transmission of vCJD by blood transfusion has alerted health regulatory authorities on the importance of implementing a set of precautionary measures. Table 1 summarizes the current preventative measures that are in place against the risk of transmission of prions by plasma products. The approach largely follows, when possible, the one that has been developed successfully over the last 20 years to avoid the transmission of viruses. The strategy needed to prevent transmission of viruses requires (a) an accurate information on the structure of the infectious agents and their mode of transmission, (b) the epidemiological surveillance of the population, (c) the accurate screening of the donors, (d) the testing of the donations by sensitive and relevant assays, (e) the implementation of validated inactivation or removal procedures during product manufacturing, and (f) the application of good manufacturing practices (GMP) at all stages of the production chain [7,8]. As indicated in this publication, not all of these measures can be used to date to reduce the risks of transmission of prions by industrial plasma products.

2.1. Biophysical characteristics and resistance of PrP^{TSE}

The physico-chemical properties of human blood-associated transmissible spongiform encephalopathy (TSE) agent are still unknown. The infectious agent is thought to be a misfolded, abnormal, prion protein, globally now referred to as PrP^{TSE} because of increasing complexity in the terminology for various forms of the prion protein [4]. Pathological PrP^{TSE} represents aberrantly folded isoforms of a normal cellular prion protein (PrP^c) whose physiological function is still largely unknown. PrP^c is a glycosyl phosphatidyl inositol-linked glycoprotein composed of approximately 256 amino acids, that undergoes facultative N-linked glycosylation at two sites [9, 10]. PrP^{TSE} present β-sheet structure that tends to aggregate and which, in vitro, makes it insoluble in detergent solutions, resistant to enzymatic degradation, and prone to adhere to surfaces [11]. Recent experimental studies in scrapie-infected (263K strain) hamster brain suggest that prion particles have a size and molecular weight range of 5–90 nm, and $155–15{,}220 \times 10^3$ KDa, respectively. Infectivity of this prion strain is highest in oligomers with apparent radii of 17–27 nm and a molecular weight of 300–600 kDa, and is apparently absent, or less, in large fibrils and in oligomers of ≤ 5 PrP molecules [12]. Non-fibrillar particles, with a mass equivalent to 14–28 PrP molecules, may be the most efficient initiators of TSE disease [12].

Table 1
Points to consider and measures to prevent the transmission of viruses and prions by plasma-derived medicinal products

Points to consider/measures	HIV, HBV, HCV	HAV, B19	Prion agent
Structural characteristics of the infectious agent	Well characterized (enveloped, size, shape, genome, resistance)	Well characterized (non-enveloped, size, shape, genome, resistance)	Unknown for the infectious agent in plasma
Infectious dose in human plasma	Known (can reach several logs)	Known (can reach several logs)	Limited information (estimated to be low; 2–30 infectious doses per ml)
Epidemiological information on prevalence, risk factors, and transmission modes	Well established	Well established	Unknown
Specific donor exclusion criteria	Yes	No	Yes[a]
Testing of individual donations for markers of the pathogenic agent	Anti-HIV 1 and 2 Ab HCV Ab HBsAg	No	No (tests in development)
Leucoreduction	No	No	About 50% reduction in infectivity [27]
Plasma pool testing	Yes (e.g. nucleic acid test)	Yes (e.g. nucleic acid test)	No
Dedicated inactivation steps	Yes (e.g. SD treatment, pasteurization, low pH, caprylic acid, dry heat)	Yes/No	No
Unspecific removal steps	Yes (e.g precipitation, chromatography)[b]	Yes (e.g precipitation, chromatography)[b]	Yes? (e.g. precipitation, depth filtration, chromatography)[c]
*Dedicated **robust** removal steps*	Yes (nanofiltration)	Yes (nanofiltration)	Yes (nanofiltration)?[c]
Final product testing to control markers of infectious risks	No[d]	No[d]	No

[a] In some countries exclusion criteria includes travel to countries with BSE, and previous transfusion or transplantation (see text for details).

[b] Often not regarded as a robust removal step by most regulatory agencies due to the difficulty in proving consistent viral removal.

[c] This assumes that PrP^{TSE} in plasma would behave the same as PrP^{TSE} from brain homogenates, which is still unknown.

[d] Tests to detect the presence of viral markers in final products have not been validated and do not guarantee product safety.

Brain-associated forms of PrP^{TSE} are resistant to the viral inactivation procedures used during the manufacture of human plasma products, such as solvent-detergent (SD), heat treatments, and low pH [1,13]. The methods known to inactivate PrP^{TSE} (such as oxidation, treatment with strong base, chaotropic agents, extreme heat, strong sodium hypochlorite solutions or hot solutions of sodium hydroxide [14]) would denature plasma proteins and therefore cannot be used in plasma fractionation.

2.2. Prion infectious dose in plasma

There is only limited information on the infectious dose of prion protein in human blood. Estimates based on animal models suggest that prion protein infectivity in blood is low, possibly comprised between 2 and 30 infectious dose per ml during both the incubation and symptomatic stages of disease [15, 16]. Half of the infectivity appears associated with plasma [17]. Possibility of abnormal prion protein transmission by transfusion in humans corroborated earlier experimental evidence that the blood of infected rodents, cows, and sheep may transmit infectivity [18] in both the incubation period and clinical phase [4]. Experimental evidence of blood transfusion transmission in deers with chronic wasting disease has also been reported recently [19]. At the early stages of the incubation period, blood-associated PrP(Sc) may originate from peripheral replication of prions, whereas during the symptomatic phase, it may leak from the brain [20]. Therefore, it can not be excluded that the risk of infectivity varies during the various phases of the disease.

2.3. Epidemiology surveillance

Human exposures to the BSE agent appear primarily linked to (a) the prevalence of BSE in native and imported cattle population and (b) the risk of contamination of local and imported food by the BSE agent. vCJD appears limited geographically to countries where BSE has been identified but not much is known on BSE agent infectivity and on the minimal oral doses able to transmit vCJD to humans [4]. In addition, prion strains distinct from that causing BSE have recently been isolated from cattle, and found to induce lethal neurological disorders in a transgenic mice model, raising potential additional public health concerns [21]. Proportion of asymptomatic carrier may be higher than initially thought [5,6] but further data must be obtained. Countries are therefore encouraged to establish epidemiological surveillance systems and conduct systematic assessment of possible cases of BSE and vCJD to follow the prevalence and trends of the disease so that appropriate deferral measures can be taken on a timely manner, if needed [4].

2.4. Deferral of "at-risk" donors

Deferral criteria of donors presumed at risk of vCJD have been implemented in some countries following a careful assessment of the risk/benefit ratio, the consideration on the long incubation period of vCJD after oral exposure to BSE contaminated beef, and the impact on the supply of blood and plasma products. The rationale for some deferral measures is based on the mathematical probability of "recycling" vCJD infection via blood transfusion and plasma products, consider-

ing the assumed number of infected individuals in a given country. Several countries defer donors who visited or resided in the UK and other European countries where BSE cases have been found, for a cumulative period of 3 months or more between 1980 and 1996. To prevent secondary spread, previously transfused donors are also deferred in countries like France (since 1998), UK (since 2005), Ireland, the Netherlands, and Switzerland. Canada, Australia, Italy and the US are currently deferring donors previously transfused in a country where BSE or vCJD cases have been identified [4].

2.5. *Testing*

There is as yet no assay available to detect misfolded prion proteins in human blood, one technical difficulty being linked to the low level of prion protein in blood and the fact that the disease does not induce conventional immune response and has no nucleic acid associated markers. Several assays using different principles are under development [4,22] but so far data have not been reproduced by independent groups. It is not known when validated screening tests will be available for routine use in blood establishments. Possible implementation would raise a number of concerns including how to deal with the potential high number of false-positive results. It is also expected that two validated tests, one used for primary determination, the other as a confirmatory assay, would be required [22].

2.6. *Leucoreduction*

Limiting the leucocytes number in plasma for fractionation to less than 10^6 per l is currently the practice in countries like France, as part of a set of precautionary measures to reduce the risks of vCJD transmission by plasma products [23]. Such limit in leucocyte content can be met by using leucoreduction filters during blood or plasma preparation [24] or by collecting plasma using specific apheresis procedures that reduce leucocytes [25]. The rationale for limiting leukocyte contamination was based on the belief that lymphocytes play a crucial role in TSE pathogenicity [26]. However, leucofiltration of whole blood from hamsters infected with scrapie 263K (endogenous TSE model) was recently found to remove "only" 42–72% infectivity from plasma [17,22,27]. These findings appear consistent with the low reduction of infectivity found by leucofiltration of blood from mice infected with a mouse-adapted strain of human TSE [28]. In an exogenous model where scrapie 263K was used to spike human blood, filtration with four different whole blood leucoreduction filters did not remove significant PrP^{sc} [29]. However, it is still not known how much prion infectivity is removed when using apheresis procedures that reduce the leucocyte content to a range similar to that of whole blood passed through dedicated leucoreduction filters [25].

2.7. *Prion removal filters*

Currently, two CE-marked filters are commercially available for the capture of prions from red blood cell concentrates [10,15], and a third one, that combines leucoreduction and prion reduction, is in development [30]. Coagulation factors have been found to bind on some of these filters. It is not known whether prion removal filters dedicated for the filtration of plasma for fractionation (or transfusion) will be developed and licensed. Use of prion reduction filters during the plasma fractionation process, rather than at the stage of plasma preparation, may be a more economical and rational approach.

3. Removal of prions during plasma fractionation

3.1. *Plasma fractionation technology*

Most plasma products are manufactured by an integrated technology encompassing cryoprecipitation, cold ethanol precipitation, filtration and chromatographic steps to achieve protein separation and polishing. Cryoprecipitation, the first step in the fractionation process, is a thawing of plasma at 2–4 °C that isolates a cold insoluble fraction (cryoprecipitate), used as a source of factor VIII (FVIII), von Willebrand factor (VWF), and fibrinogen, and a supernatant (cryo-poor plasma, or cryosupernatant), which is the starting material of other proteins. The ethanol fractionation process—used for instance in albumin, IgG, alpha 1-antitrypsin separation—comprises precipitation steps at 8–40% ethanol concentrations, under defined conditions of pH, temperature, and osmolality [1]. Precipitates and supernatants are separated by centrifugation or filtration using filter aids and depth filters. Chromatography is used for protein separation and purification from the various intermediates, as well as for removal of the solvents and detergents used in viral inactivation procedures [1,31]. Common chromatographic methods include anion-exchange, cation-exchange, immobilized heparin affinity, and immunoaffinity. To date most albumin and IgG preparations are produced by a process largely based on the ethanol fractionation method, while the manufacture of most coagulation factors, protease inhibitors, and anticoagulants preparations involves chromatography [31,32].

3.2. *Experimental prion clearance studies*

Studies have been carried out to evaluate the clearance of prions taking place during plasma fractionation. These studies are difficult, time-consuming, and expensive. Important factors in their design include the choice of the TSE strains and of the tissue used for spiking, the type of infectivity assay, and—as for viral validation studies [7,33]—the validity of the scale-down process to mimic the large-scale manufacture conditions. Since spiking agents may have different partitioning properties, process clearance is often evaluated using spikes exhibiting different biophysical properties [34]. TSE strains used in exogenous spiking experiments include (a) hamster-adapted scrapie (strains 263K [35], Sc237 [34] or ME7 [36], (b) murine-adapted BSE, 301 V [37] and (c) strains of human CJD, vCJD, sporadic CJD (sCJD) and GSS [38]. Endogeneous studies have used murine-adapted GSS, Fukuoka-1 strain [39], hamster-adapted scrapie, strain 263K and murine-adapted

BSE strain 301 V [40]. Two approaches are available to assess infectivity. Bioassays, which detect infectivity in rodent models [28,37,39,41], are the current "gold standard", but they have limitations linked to the limited availability of specialized animal facilities, their time-consumption, or the inadaptability for evaluation of process robustness [42]. Immuno-chemical determination of PrP^{TSE} is done either by Western blotting [35,43] or, less frequently, conformation-dependent immunoassay (CDI) [34], after digestion with proteinase-K. Immunochemical assays are rapid and relatively cheap, and are useful for an evaluation of clearance. Good correlation between both types of assays have been found [41,44]. Tissue culture infectivity assays (TCIA) may be new promising alternatives to animal assays. Sensitivity is equivalent to animal assay and almost 100 times more than the WB. Protein misfolding cyclic amplification (PMCA) which allows autocatalytic replication of minute quantities of infectious prions, is claimed to provide over 4000-times more sensitivity than the animal bioassay [45] and, if validated, may be of interest for clearance studies in the future.

3.3. Results of experimental clearance studies

3.3.1. Coagulation factors

Coagulation factor concentrates are prepared from plasma intermediates generated early in the fractionation process and could be, in principle, at higher risks of contamination by prions. Significant efforts have, understandably, been made to study TSE infectivity removal capacity of chromatographic purification and nanofiltration steps, which are commonly used to manufacture these products.

3.3.1.1. Chromatography. Table 2 summarizes clearance data obtained during chromatography. Various spikes have been used; assays included in vitro immunochemical methods and animal bioassays. Anion-exchange chromatography on DEAE-Toyopearl 650M, as used in the purification of FVIII and fibrinogen [46], and on DEAE-Sepharose, as used for FIX [47], contributes to a significant removal (typically in the 2 to > 3 logs range) of spiked TSE agents [35,42,48,49]. Upstream SD treatment of the cryoprecipitate extract did not impact prion removal [50]. Immobilized heparin affinity chromatography of FIX [47] removed 1.4 $\log_{10}$ of PrP^{TSE} [35], and S-Sepharose cation-exchanger during thrombin purification removed 2.9 $\log_{10}$ [35], and similar removal has been reported by monoclonal antibody chromatography [42]. The fact that consistent prion clearance factors are found in processes using chromatographic resins of different chemical structures and substitutions, and under different buffer systems, supports the occurrence of non-specific binding of the infectious agent onto the chromatographic support surface. Although prion removal appears reproducible, incomplete understanding of the removal mechanism raises questions, such as how to (a) determine the maximum capacity of chromatographic support to bind TSE agents, (b) ensure efficient sanitizing procedures of recycled gels, and (c) guarantee consistent prion removal over production cycles.

3.3.1.2. Nanofiltration. Nanofiltration is a proven, dedicated method using nm-membranes that are permeable to proteins but retain infectious agents (viruses) by size-exclusion partitioning [51,52]. Accumulating experimental evidence (Table 3) shows consistent removal of substantial doses of TSE agents spiked to plasma fractions through multi-layer filters with porosity of 75 nm or less [42]. The removal capacity of the larger pore-size nanofilters (75 and 35 nm) appears, as expected, somewhat influenced by the physico-chemical characteristics of the plasma fraction [53]. Removal of spiked scrapie agent ME7 by 15 nm nanofilters appears more robust and was not, in experimental studies, influenced by 0.9% sarkosyl [36].

3.3.2. Albumin and IgG

Significant work has evaluated the extent of TSE infectivity removal during the backbone plasma fractionation process steps that generate the various plasma intermediates used to manufacture several products (Table 3).

3.3.2.1. Cryoprecipitation. In initial endogenous experiments that studied the fractionation of murine plasma from animals infected with a human TSE, the infectivity was found to precipitate predominantly into cryoprecipitate (and precipitate I+II+III) [39]. However, in exogenous studies where human blood was spiked with hamster-adapted scrapie 263K, only 0.7% of the initial infectivity was recovered in the cryoprecipi-

Table 2
Extent of TSE agent removal during chromatography of plasma-derived coagulation factors. Adapted from Refs. [41,43]

Processing step evaluated	End-product	Agent	Spike	Assays	Reduction factor
IEC (DEAE-Toyopearl 650M)	FVIII	263K	MF	WB	*1.7* [42]
SD + IEC (DEAE-Toyopearl 650M)	FVIII	263K	MF	WB	*3.1* [35]
SD + IEC (DEAE-Toyopearl 650M)	FVIII	BSE (strain 301V)	MF	Bioassay	*2.7* [48]
IEC (DEAE-Toyopearl 650M)	vWF	263K	MF	WB	*3.9* [42]
IEC (DEAE-Toyopearl 650M)	Fibrinogen	263K	MF	WB	*3.8* [42]
SD+ IEC (DEAE-Toyopearl 650M)	Fibrinogen	263K	MF	WB	≥ *3.5* [35]
SD+ IEC (DEAE-Toyopearl 650M)	Fibrinogen	BSE (strain 301V)	MF	Bioassay	*2.9* [48]
DEAE-cellulose	Prothrombin complex/FIX	263K	MF	WB	*3.0* [35]
IEC (DEAE-Sepharose)	PCC	263K		WB	*3.3* [61]
IEC (DEAE-Sepharose)	FIX	263K	MF	WB	*3.0* [35]
Heparin-Sepharose	FIX	263K	MF	WB	*1.4* [35]
S-Sepharose	Thrombin (fibrin glue)	263K	MF	WB	*2.9* [35]

MF: microsomal fraction; WB: Western-blot.

Table 3
Extent of TSE agent removal during nanofiltration of plasma-derived coagulation factor concentrates. Adapted from Refs. [43,62]

Starting plasma fraction	Nanofilter	End-product	Agent	Spike	Assays	Reduction factor
DEAE-Toyopearl 650M eluate	Planova 35N	vWF	263K	MF	WB	≥ *3.1* [61]
DEAE-Toyopearl 650M eluate	Planova 35N + 15N	FVIII	263K	MF	Bioassay	≥ *3.3* [61]
SD + DEAE-Toyopearl 650M eluate	Planova 35N + 15N	FVIII	263K	MF	WB	≥ *5.1* [61]
Monoclonal antibody chromatography eluate	Planova 75N + 35N + 35 + 15N	FVIII	263K	BH + SD	Bioassay	*4 + 0.3 + 1.3 +* ≥ *0.5* [62]
Monoclonal antibody chromatography eluate	Planova 75N + 35N + 35N + 15N	FVIII	263K	PrPsc + SD	Bioassay	*3.1 + 0 + 0.8 +* ≥ *2* [62]
Ion-exchange chromatography + heparin-Sepharose eluate	Planova 15N	FIX	263K	MF	Bioassay	*4.8* [61]
Ion-exchange chromatography + heparin-Sepharose eluate	Planova 15N	FIX	BSE 6PB1	BH + sonication	Bioassay	*5.3* [42]

MF: microsomal fraction; BH: brain homogeneate; WB: Western-blot; SD: solvent-detergent.

tate [39]. Using blood from scrapie-infected hamsters, and in spiking studies where scrapie 263K PrP^{sc} was added to human plasma, 20% and 10% of the infectivity partitioned in the cryoprecipitate, respectively [35,54]. By contrast, when human plasma was spiked with scrapie 263K brain homogeneate (BH), 90% of PrP^{sc} was found in the cryoprecipitate. These contradictory results may highlight the influence of the nature of the experimental model used, in particular the physicochemical nature of the spike [34], or of the variations in the down-scaling of the cryoprecipitation procedure, or they actually illustrate the fact that cryoprecipitation does not ensure robust partitioning of TSE infectivity.

3.3.2.2. Ethanol precipitation. The capacity of precipitation steps to remove prions efficiently has first been shown by partitioning of endogenous infectivity using a rodent model. It is particularly well documented for the ethanol fractionation process isolating albumin and immunoglobulins. In the albumin fractionation procedure using either the Cohn-Oncley or the Kistler and Nitchman processes, major and consistent reduction factors (typically 3–5 logs) of TSE agent have been found by various groups, most specifically during the precipitation of fraction II + III, fraction III, and fraction IV, or their equivalents using slightly modified fractionation conditions [35,42] (Table 4). Similar experiments revealed 3–5 logs removal during the precipitation III or I+III used in the IgG process [35,42]. These data suggest that, in spite of variations in the conditions (such as ethanol concentration and pH) used, reproducible clearance of PrP^{TSE} takes place. Removal is achieved when the precipitate is separated. It has been speculated that prion removal in precipitates is possibly due to aggregation and is dependent upon pH and presence of alcohol [35,55]. Other precipitation steps, using caprylic acid during immunoglobulins production [56] or polyethylene glycol also contribute to prion removal [42].

3.3.2.3. Depth filtration. Depth filters are made of a combination of a matrix (generally based on cellulose), filter aids (diatomeous earth, resins, or other adsorbents), and a drainage system. They are used to clarify crude protein solutions and

Table 4
Extent of TSE agent removal during the ethanol plasma fractionation process in the manufacture of albumin and immunoglobulin G. Adapted from Refs. [41,43,62]

Step	Fraction evaluated	Agent	Spike	Assays	Reduction factor (log_{10})
Precipitation of fraction I	Supernatant	263K	BH	WB	1.1 [41,43]
Precipitation of I + II + III	–	263K	MF	WB	1.3 [34]
Precipitation of I + II + III	–	263K	MF	WB	≥ 2.8[a] [42]
Precipitation of fraction II + III	–	263K	BH	WB	≥ 4.7 [41,43]
–	–	263K	BH	Bioassay	6.0 [41]
–	–	Sc237	BH/MF/CLD/PrPSc	CDI	3.6/3.1/3.1/4.0 [34]
–	–	263K	BH	WB	≥ 4.2/ ≥ 4.1 [41,43]
–	–	263K	BH	Bioassay	3.7/4.6 [41]
Precipitation of fraction I + III[b]	–	263K	MF	WB	≥ 3.5[a] [42]
Precipitation of fraction IV[c]	–	263K	MF	WB	≥ 3.0 [35]
–	–	Sc237	BH/MF/CLD/PrPSc	CDI	3.2/3.4/3.2/2.2 [34]
–	–	263K	MF	WB	≥ 3.7 [35]
–	–	263K	MF	WB	≥ 4.3[a] [42]
–	–	263K	BH	WB	≥ 4.3 [41,43]
–	–	263K	BH	Bioassay	5.3 [41]

BH: brain homogeneate; MF: microsomal fraction; CLD: caveolae-like domain; WB: Western-blot; CDI: conformation-dependent immunoassay.

[a] Evaluated together with filter aids to remove precipitates.

[b] Precipitate discarded during the manufacture of IgG.

[c] Precipitate discarded during the manufacture of albumin.

Table 5
Extent of TSE agent removal during depth filtration of albumin and immunoglobulin G fractions. Adapted from Refs. [41,43,62]

Fraction	Filter	Agent	Spike	Assays	Reduction factor ($\log_{10}$)
Supernatant I	Seitz Supra P80	Sc237	BH/MF	CDI	–0.1/0.1 [34]
Supernatant III	Millipore AP20	BSE 301V	MF	Bioassay	2.4 [37]
–	Seitz KS80P	BSE 301V	MF	Bioassay	≥ 3.1 [37]
–	Cuno Zetaplus	263K	BH	WB	≥ 3.3 [55]
Supernatant IV	Seitz Supra P80	Sc237	CLD/PrPsc	CDI	≥ 0.9/ ≥ 2.4 [34]
–	Seitz AKS5 (carbon)	263K	MF	WB	2.7 [42]
Fraction V (albumin)	Cuno Delipid-1	263K	MF	WB	2.3 [35]
–	Seitz KS80P	263K	MF	WB	≥ 4.9 [35]
Fraction II (IgG)	Seitz K200P	263K	MF	WB	≥ 2.8 [35]
–	Ca_3PO_4 + filter aid + Cuno	263K	MF	Bioassay	2.5 [42]
–	Cuno	263K	BH	Bioassay	≥ 4.9 [57]

BH: brain homogeneate; MF: microsomal fraction; CLD: caveolae-like domains; CDI: conformation-dependent immunoassay; WB: Western-blot.

Table 6
Extent of TSE agent removal during nanofiltration of plasma-derived albumin, and IgG. Adapted from Refs. [43,62]

End-product	Nanofilter	Agent	Spike	Assays	Reduction factor
Albumin[a]	Planova 35N	CJD	BH	Bioassay	≥ 5.9 [53]
Albumin[a]	Planova 35N	ME7	BH	Bioassay	4.93 [36]
Albumin + detergent[a]	Planova 35N	ME7	BH	Bioassay	1.61 [36]
Albumin[a]	Planova 15N	ME7	BH	Bioassay	≥ 5.87 [36]
Albumin + detergent[a]	Planova 15N	ME7	BH	Bioassay	≥ 4.21 [36]
RhO (D) IgG	VIRESOLVE 180	263K	Detergent treated, sonicated and filtered BH	WB	≥ 2.5 [63]
IgG	DV50	263K	BH	Bioassay	4.4 [64]
IgG	Planova 75N + 35N	263K	MF	Bioassay	3.2 [61]

BH: brain homogeneate; MF: microsomal fraction; WB: Western-blot.

[a] Nanofiltration is not used during production of albumin preparations; a detergent was added for experimental purposes only.

remove precipitates. As such they are an important adjunct to the ethanol precipitation process. Principle of action encompasses both removal of particulates larger than the pore-size by size-exclusion, and of smaller elements by adsorption. During immunoglobulin manufacture, the supernatant of Fraction III (Supernatant III) and the re-dissolved Fraction II precipitate, and during production of albumin, the Supernatant IV and the re-dissolved Fraction V, generally undergo depth filtration steps. Experimental spiking experiments (Table 5) have shown that several types and grades of depth filters can remove prions [35,37,42]. Depending upon the type of depth filter or the physico-chemical parameters of the suspension, PrP^{TSE} removal may be due to an aggregation in the presence of alcohol [55] or to hydrophobic adsorption on the filter aids [42]. The impact of protein composition and content remains to be investigated and understood to demonstrate and guarantee the robustness of this non-specific removal.

3.3.2.4. Nanofiltration. Table 6 shows experimental data on the removal of TSE agents during nanofiltration of albumin and IgG. As for coagulation factors, high reduction factors have been found. Prion partitioning is presumed to result from a size-exclusion mechanism.

3.4. Cleaning and sanitization

Most equipment, including stainless steel reactors, chromatographic gels and columns, ultrafilters are re-used and, therefore, must undergo steam (SIP) or chemical (CIP) sanitizing procedures between production batches. These processes should be validated to ensure proper bacterial, pyrogenic, viral, and protein decontaminations. Experience gained with the sterilization of surgical instruments has shown that autoclaving at 134 °C for 18 min or 121 °C for 30 min reduced the transmission of prion infectivity by a factor > 5 $\log_{10}$ [57, 58] but autoclaving without immersion is less effective (4–4.5 log reduction) [58]. Standard chemical decontamination methods (NaOH 1 N, NaOCl 20,000 ppm) and hydrogen peroxide alone achieved a reduction of > 6.5 and 4.5 $\log_{10}$, respectively [58]. By experiments involving a hamster scrapie strain 263K BH model, it was shown that 0.1 M NaOH for 15 min, in the absence of detergent, at 4 and 18 °C caused a reduction of 3.5 and 4.0 $\log_{10}$ of the prion protein, respectively. In the presence of sarkosyl, a 60-min incubation in NaOH further enhanced PrPRES reduction to > or = 4.5 $\log_{10}$, with no residual infectivity. Therefore 0.1 N NaOH could also effectively inactivate prions, and its efficacy can be enhanced by the addition of sarkosyl [59]. A separate study shows that 0.1 M NaOH at 60 °C for 2 min and 0.25 M NaOH at 30 °C for 60 min inactivate 3.96 and 3.93 $\log_{10}$ of mouse-adapted scrapie strain ME7, respectively, and 0.5 M NaOH at 30 °C for 60 or 75 min inactivates ≥ 4.23 and 4.15 $\log_{10}$ [60].

4. Conclusion

There is so far no evidence of transmission of prions by plasma derivatives. The prevalence of the disease in the population is considered to be very low, although possibly not quite as low as initially considered [5,6]. Based on experimental models, it is believed that the infectivity in the plasma does

not exceed a few infectious doses per ml. By lack of knowledge of the nature of the agent associated to the infectivity in plasma, and in the absence of validated screening tests, alternative precautionary measures have been introduced to prevent the possibility of transmission of vCJD by plasma derivatives. Epidemiological surveillance of the population is in place in countries where BSE and vCJD cases have been identified. In some countries, blood donation deferral criteria include travel or residence of donors in BSE countries, and history of previous transfusion or tissue transplantation. Based on filtration experiments of blood collected from scrapie-infected hamsters, leucoreduction decreases by about 50% the prion infectivity in plasma [22,27]. Extent of removal of TSE agents during the plasma fractionation process appears to be substantial. Data from various laboratories and using different experimental models show several logs removal of TSE infectivity during the fractionation process. The most effective, but non-specific, removal steps are ethanol precipitation, depth filtration, and ion-exchange chromatography. Nanofiltration was also demonstrated to remove several logs of TSE infectivity, possibly based on a specific prion removal mechanism by size-exclusion. Uncertainty on the validity of these experimental studies remains, and additional studies are needed, since the biochemical features of the infective agent in blood and plasma is not known, nor the extent to which it may be present in blood donations. Research should continue, aiming at identifying the features of TSE agents in human plasma and at ensuring the robustness of prion removal steps and sanitizing procedures during plasma product manufacture.

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