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Please cite this article as: J.D.F. Wadsworth, J. Collinge, Update on human prion disease, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbadis.2007.02.010

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Please cite this article as: J.D.F. Wadsworth, J. Collinge, Update on human prion disease, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbadis.2007.02.010

医薬品
医薬部外品　　研究報告　調査報告書
化粧品

識別番号・報告回数			報告日	第一報入手日 2007 年 9 月 28 日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	トロンビン		研究報告の公表状況	Biologicals 2007; AVAILABLE ONLINE 24 SEPTEMBER:1-10	公表国 日本	
販売名（企業名）	トロンビン-ヨシトミ（ベネシス）					

研究報告の概要

本研究で我々は、異なるポアサイズのウイルス除去膜を使用し、異なる処理を行ったスクレーピープリオン蛋白（PrP^{Sc}）の除去能力を評価した。たん白質を含まない系で超音波処理を行わなかった 263K 由来のミクロソーム画分（263K MF）をろ過すると、75nm 以下のポアサイズのウイルス除去膜ろ過により PrP^{Sc} がウエスタンブロット（WB）の検出限界以下まで除去された。しかし、超音波処理により粒子径分布を至適化するように調製した 263K MF をスパイク物質として使用したときは、75nm のろ液中に PrP^{Sc} が検出された。
血漿由来調製品存在下で超音波処理により粒子径分布を至適化するように調製した 263K MF をろ過すると、15nm のろ過のみが全ての条件で WB の検出限界以下まで PrP^{Sc} が除去されることが示された。しかし、1 条件下の 15nm ろ液のバイオアッセイの結果では、感染性 PrP^{Sc} が確認された。
この結果は、スパイク物質の性質が、プリオン除去のためのフィルター能力の評価において重要な要素であること、および超音波処理又は界面活性剤処理のような、スパイクするプリオン粒子サイズの分布が最小になるように設計された方法をスパイク物質の調製に使用しなければならないことを示している。

使用上の注意記載状況・その他参考事項等

2. 重要な基本的注意
(1) 略
1) 略
2) 略
3) 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
プリオンソースの処理の違いにより、ウイルス除去膜による PrP^{Sc} の除去効果が異なるとの報告である。 これまで血漿分画製剤によってvCJD、スクレイピー及びCWDを含むプリオン病が伝播したとの報告はない。しかしながら、万一vCJD感染者の血漿が本剤の原料に混入した場合には、製造工程においてプリオンを低減し得るとの報告があるものの、製剤から伝播する可能性を完全には否定し得ない。そのため、弊社の血漿分画製剤の製造工程におけるTSE感染性低減に関する検証実験を加速し、自社データを早期に取得し、工程評価を行い、必要に応じて工程改善を実施する予定である。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

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Prion removal by nanofiltration under different experimental conditions

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Received 21 December 2006; revised 11 April 2007; accepted 27 April 2007

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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doi:10.1016/j.biologicals.2007.04.005

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 waterbath. 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.