Table IV-B. Comparison of results from FDA 2006 and 2009 Risk Assessments for vonWillebrand disease (vWD) patients with severe disease: Predicted potential annual exposures to vCJD agent in iv ID50 and vCJD risk assuming 4-6 LRF by manufacturing process

ADULT vWD (> 15 yrs of age)

				4-6 Log_{10} Reduction Factor (LRF)			
				Model Output for LOWER vCJD Case Prevalences based on Clark and Ghani (2005)		Model Output for HIGHER vCJD Infection Prevalences based on Hilton et al (2004)	
Prophylaxis	73	186,880 IU	2009	5.2×10^{-7}	1 in 3.9 million	4.1×10^{-5}	1 in 49,000
			2006	4.89×10^{-7}	1 in 4.1 million	4.32×10^{-5}	1 in 46,300
Episodic	78	86,923 IU	2009	2.2×10^{-7}	1 in 9.3 million	2.22×10^{-5}	1 in 75,000
			2006	1.99×10^{-7}	1 in 10 million	1.90×10^{-5}	1 in 53,000#

#The original risk estimate for this cell in the FDA Risk Assessment of 2006 (FDA 2006) was incorrect – the corrected estimate is provided in this table

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化粧品

識別番号・報告回数		回		報告日 年 月 日	第一報入手日 2009年7月3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						公表国 オーストリア	使用上の注意記載状況・その他参考事項等 BYL-2009-0387
販売名（企業名）				研究報告の公表状況	Prion removal effect of a specific affinity ligand introduced into the manufacturing process of the pharmaceutical quality solvent / detergent (S/D)-treated plasma OctaplasLG. A. Neisser-Svaeet et al, Vox Sanguinis 97, 226-233. 93. (2009).		

研究報告の概要

異常プリオン蛋白（PrPSc）と特異的に結合する親和性リガンドを用いて新しいアフィニティクロマトグラフィの技術が開発された。本試験では、変異型クロイツフェルト・ヤコブ病（vCJD）伝播リスクに対する安全性を向上させる目的で、solvent/detergent処理血漿分画製剤オクタプラスの製造過程に本法を導入し、各処理段階におけるPrPSc除去効果を検証した。ハムスター順化スクレイピー263K株感染ハムスターより得られた10%未精製脳ホモジネート（CBH）を精製した。PrPScをミクロソーム／ライソゾーム画分（MIC）とCBH(+IIO)にスパイクし、親和性リガンド固定樹脂カラムに添加した。分画サンプル中のPrPScをウエスタンブロットにて同定・定量した結果、MICスパイクサンプルでのreduction factorは≧3.0 log10相当であった。

MICおよびCBH(+IIO)スパイクサンプルのリガンドゲル1mL当たりのPrPSc除去能は非常に高く、7.3および6.4log10 ID50/mLであった。ウェスタンブロットでは、親和性リガンドゲル1mL当たりのPrPSc除去能は6.4log10であった。0.01%に希釈したCBH(+IIO)のスパイクサンプルは全く検出されなかった。PrPSc除去能は6.4log10 ID50/mLであった。これは製造工程においては約10log10 ID50/カラムに相当する除去率であった。ハムスタースクレイピー263K株、ヒトvCJD株、ヒト散発性CJD株、Gerstmann-Sträussler-Scheinker病マウスFukuoka株由来PrPScに対するリガンド特異性を検討した結果、全ての株由来PrPScがウェスタンブロットによって検出され、ヒト由来PrPScに対するリガンド特異性が示された。本法をオクタプラスの製造過程に導入することは技術的に可能であり、vCJD感染の観点からも本法導入によるオクタプラスの安全域の拡大が期待される。

報告企業の意見	今後の対応
本論文では、効率的に異常プリオン蛋白（PrPSc）除去可能なアフィニティクロマトグラフィが開発されたことが発表されたが、この工程は血漿分画製剤の製造過程に導入することが可能であり、この点で、製造工程における異常プリオン除去能力をさらに向上させることができる可能性が高い。	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。本報で報告されたような、導入が可能と思われるウイルス除去方法に関して今後とも情報収集に努める。

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ORIGINAL PAPER

Prion removal effect of a specific affinity ligand introduced into the manufacturing process of the pharmaceutical quality solvent/detergent (S/D)-treated plasma OctaplasLG®

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Background and Objectives A new chromatographic step for the selective binding of abnormal prion protein (PrP^{Sc}) was developed, and optimization for PrP^{Sc} capture was achieved by binding to an affinity ligand attached to synthetic resin particles. This step was implemented into the manufacturing process of the solvent/detergent (S/D)-treated biopharmaceutical quality plasma Octaplas® to further improve the safety margin in terms of risk for variant Creutzfeldt–Jakob disease (vCJD) transmission.

Materials and Methods Intermediates and Octaplas® final container material, spiked with hamster brain-derived PrP^{Sc}-containing fractions, were used for experiments to establish the feasibility of introducing this novel chromatography step. The binding capacity per millilitre of ligand gel was determined under the selected manufacturing conditions. In addition, the specificity of the ligand gel to bind PrP^{Sc} from human sources was investigated. A validated Western blot test was used for the identification and quantification of PrP^{Sc}.

Results A reduction factor of $\geq 3.0 \log_{10}$ could be demonstrated by Western blotting, utilizing the relevant Octaplas® matrix from manufacturing. In this particular cell-free plasma solution, the PrP^{Sc} binding capacity of the selected gel was very high ($\geq 6 \log_{10} ID_{50}/ml$, equivalent to roughly $10 \log_{10} ID_{50}/$column at manufacturing scale). The gel binds specifically PrP^{Sc} from both animal (hamster and mouse) and human (sporadic and variant CJD) sources.

Conclusion This new single-use, disposable PrP^{Sc}-harvesting gel ensures a very high capacity in terms of removing the pathogenic agent causing vCJD from the new generation OctaplasLG®, in the event that prions can be found in plasma from donors incubating the disease and thereby contaminating the raw material plasma used for manufacturing.

Key words: affinity ligand chromatography, OctaplasLG®, prion safety, PrP^{Sc}, vCJD.

Introduction

In the last few years, four probable transmissions of variant Creutzfeldt–Jakob disease (vCJD) through non-leucocyte depleted red blood cell concentrates in the UK [1–4], as well as the first probable case of vCJD through a plasma-derived factor concentrate [5], have made prion diseases a matter of concern in today's blood therapy.

A number of actions have been implemented by regulatory authorities, such as requiring that all manufacturers of plasma-derived biopharmaceuticals should perform appropriate prion safety evaluations of their product portfolio. Different manufacturing steps have been demonstrated to provide significant removal, either of prion infectivity or the disease-associated marker PrP^{Sc} [6]. Specific affinity ligands designed to bind prions have previously shown a significant capacity to remove PrP^{Sc} and associated infectivity from blood components such as red blood cell concentrates [7–9]. Such specific affinity ligands have until now not been investigated for the removal of PrP^{Sc} in plasma-derived biopharmaceuticals such as Octaplas®.

Octaplas® is the first generation solvent/detergent (S/D)-treated, human, coagulation-active plasma. The production process is straightforward and very reproducible. Cells and cell fragments are removed by a 1.0 μm filtration step at the front-end of the process. The S/D treatment is performed utilizing 1.0% (w/w) tri-n-butyl-phosphate (TNBP) and 1.0% (w/w) Octoxynol-9. TNBP is subsequently removed by oil and Octoxynol-9 by solid phase extraction. Finally, two filtration steps are performed (0.45 and 0.2 μm) to ensure sterility of the final product.

It has already been demonstrated that the current Octaplas® manufacturing process is able to remove $2.5 \log_{10}$ cell-bound and free PrP^{Sc}, when using a chronically infected cell line as spike material, which in itself ensures a good safety margin for this plasma product in terms of prion transmission [10]. The implementation of an additional orthogonal prion removal step would further enhance the safety of Octaplas® in this respect. The company Pathogen Removal and Diagnostic Technologies Inc. (PRDT, NY, USA) has developed a group of ligands, coupled to a standard resin base, which have demonstrated strong affinity for the prion.

The studies reported in this paper were designed to determine the potential for prion removal by a specific affinity ligand implemented into the new generation OctaplasLG® (LG, ligand gel) manufacturing process. To prevent potential interference of the non-homogeneous plasma product (e.g. possibly containing cells and cell debris) with the binding of PrP^{Sc} to the affinity ligand, it was decided to incorporate the new prion removal resin post-cell filtration and S/D treatment, at which point the product is clean from cells and debris that might contain or carry the pathogenic prions. The technical implementation of the ligand resin was performed by Octapharma PPGmbH, Vienna, Austria.

Materials and methods

Spike material preparations

The 263K strain of hamster-adapted scrapie used in the experiments was supplied as a 10% crude brain homogenate (CBH) by the laboratory of Dr Robert G. Rohwer (Baltimore, MD, USA). A microsomal/cytosolic (MIC) fraction was prepared from the 10% CBH following the preparation procedure established for various TSE sub-cellular fractions (the CBH was centrifuged at 10 000 g for 8 min at ambient temperature and the supernatant was separated from the pellet and harvested as the MIC fraction) [11]. For studies on the robustness of PrP^{Sc} removal, the pellet from the above centrifugation was used as the spike [CBH$_{(-MIC)}$] after re-suspended at a ~10% concentration in tris-buffered saline (TBS) or phosphate-buffered saline (PBS). The CBH$_{(-MIC)}$ fraction contained the large membrane fragments and tissue not present in the MIC fraction, which was mostly consistent of more soluble and presumably smaller PrP^{Sc} components.

The studies shown in Figs 2–4, as well as the supporting feasibility studies, were performed with a Sarkosyl-treated spike material. CBH was treated with 0.5% Sarkosyl for 30 min on ice. The solution was centrifuged at 13 000 g for 10 min at room temperature to remove debris. The supernatant (CBH$_{Sark}$) was used as the spike [8].

Determination of PrP^{Sc}

The proteinase K (PK) digestion and Western blot assay used for the detection of PrP^{Sc} were either performed as described by Gregori L et al. [8] or with some minor modifications – where Triton X-100 instead of sodium dodecyl sulphate (SDS) was used as detergent during the PK digestion step, and where the polyacrylamide gel concentration was 12% (Bio-Rad Laboratories, Vienna, Austria) instead of 14% (NuPAGE, Invitrogen Life Science, Carlsbad, CA, USA). The end-point titre of the sample used for reduction factor calculations was determined in a $0.5 \log_{10}$ serial dilution setup and defined as the first dilution where no signal was observed on the Western blot. Samples were processed before PK digestion in order to overcome interference as detailed below.

Western blot validation

The Western blot assay used for determination of prion reduction factors and binding capacity in Tables 1 and 2 was subject to a formal validation following International Conference on Harmonisation (ICH) guidelines to enable an evaluation of the suitability of the assay in terms of assay variability and linearity for use in the clearance studies detailed in this report, and also an evaluation of the limit of detection (LOD) of the assay in comparison with a prion stock of known (defined) bioassay titre. The linearity of the assay is shown in Fig. 1. The regression parameters can be used to convert Western blot titres into infectious titres using the following formula:

$$Titre_{(Bioassay)} = \frac{Titre_{(Western blot)} + 4.5867}{1.0667}$$

This formula was used for calculation of the resin binding capacity in terms of infectious doses.

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Table 1 PrP^{Sc} removal during chromatography with a non-S/D-treated spike. Fifty millilitres of Octaplas® final product was spiked at the indicated spike ratio with a CBH$_{Sark}$ from hamsters infected with hamster-adapted scrapie 263 K strain. After withdrawal of a sample of the spiked start material, the spiked plasma was loaded onto the PRDT column and the flow-through fractions were collected. Following plasma loading and washing of the PRDT column with citrate buffer, the column was washed experimentally with 2 M NaCl, and finally the remaining resin was re-suspended in TBS and tested (column gel)

| Sample | Western blot sample titre from end-point titration [log$_{10}$] | |
	5% CBH$_{Sark}$ spike/ 9·5 ml gel	1% CBH$_{Sark}$ spike/ 1·9 ml gel
Spiked start material	2·5	2·0
Flow-through 0–5 ml	≤ –0·5	≤ –0·5
Flow-through 5–10 ml	≤ –0·5	≤ –0·5
Flow-through 10–20 ml	0·5	1·0
Flow-through 20–50 ml	0·5	1·5
Flow-through 50–95 ml	0·5	·1·5
2 M NaCl wash	2·0	1·5
Column gel	3·5	3·0

Table 2 PrP^{Sc} removal during Octaplas® manufacturing with an S/D-conditioned spike. Approximately 200 ml of crude plasma was spiked at a spike ratio of 1% with the indicated spike materials from hamsters infected with hamster-adapted scrapie 263 K strain. After withdrawal of a sample of the spiked start material, the spiked plasma was processed through a downscaled model of the Octaplas® process from front-end cell and cell-debris filtration, via S/D-treatment, filtration and solid phase extraction until eventually 50 ml of the S/D-treated plasma intermediate after solid phase extraction were loaded onto the 5 ml PRDT column from which the indicated flow-through fractions were collected. Following plasma loading and washing of the PRDT column with citrate buffer, the column was washed experimentally with 2 M NaCl, and finally the remaining resin was re-suspended in TBS and tested (column gel)

| Sample | Western blot sample titre from end-point titration [log$_{10}$] | |
	1% MIC spike	1% CBH$_{(-MIC)}$ spike
Spiked Octaplas® after 1 µm filtration	2·5	2·0
After S/D-treatment, liquid phase extraction and depth-filtration	2·5	1·0
After solid phase extraction After PRDT gel	2·0	1·0
Flow-through 0–0·5 ml	≤ –0·5	≤ –0·5
Flow-through 0·5–5·0 ml	≤ –0·5	≤ –0·5
Flow-through 5·0–10 ml	≤ –0·5	≤ –0·5
Flow-through 10–20 ml	0·5	≤ –0·5
Flow-through 20–50 ml	1·5	0·5
2 M NaCl wash	3·0	2·0
Column gel	2·0	1·5
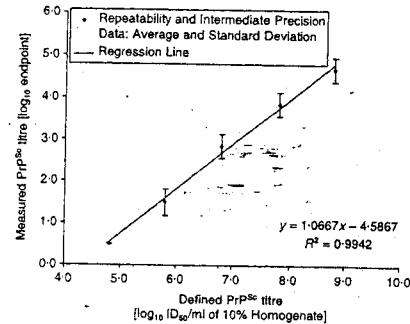
Fig. 1 Linearity of Western blot assay. A plot of Western blot end-point titres obtained from multiple determinations (at least 3) of various dilutions of a hamster-adapted scrapie 263 K prion stock of known (defined) bioassay titre. The limit of detection is 4·5 log ID$_{50}$/ml. The individual standard deviations for samples at each dilution tested was no greater than ±0·25 log$_{10}$.

Interference handling

A direct Western blotting of the samples containing Octaplas® could not be conducted due to the interference from high plasma protein content. To reduce this interference and to enable assaying of the flow-through samples after adsorption by the gel ligand, spiked samples were pre-diluted 3·2-fold (0·5 log$_{10}$) in TBS containing 0·1% bovine serum albumin followed by a centrifugation at 15 558 g for 60 min at ambient temperature. After centrifugation, the supernatant was carefully decanted and the pellet re-suspended in either the same volume of the original spiked sample, or in 1/10th the original volume centrifuged (i.e. 10-fold concentration), achieving an effective concentration of 0·5 log$_{10}$. Recovery within 0·5 log titre as determined by serial dilution Western blot assay of low titre PrP^{Sc} was demonstrated via this procedure in control experiments, as indicated by comparable Western blot end-point titres for the centrifuged samples when compared with a non-centrifuged sample (data not shown).

Regeneration samples containing basic high salt concentration were diluted 0·5 log$_{10}$ and then tested in the Western blot assays. The column gel samples were tested undiluted before analysis by Western blotting (i.e. without centrifugation). The PK digestion was performed *in situ* on the matrix. Following boiling in SDS, the PrP^{Sc} was released from the matrix.

Robustness of the prion reduction step with regard to different spike preparations

Octaplas® was spiked with either MIC or CBH$_{(-MIC)}$ at a 1% spike ratio. The pH of the spiked material was determined and, if necessary, adjusted to a pH of 6·9–7·4.

Following removal of a sample for determination of titre in the spiked start material, the remaining spiked material was loaded onto a prepared ligand resin column (Vantage L11 X250, Millipore, Bedford, MA, USA), which had been equilibrated with water for injection, 20 mM citrate buffer, pH 7·0 containing 140 mM NaCl. The flow rate of the chromatography was adjusted to the necessary contact time (plasma with resin) of ~2 min. Collection of the flow-through began once the ultraviolet (UV) baseline had reached peak absorbance. Following loading of the sample, the column was washed with the citrate buffer used for equilibration, and collection of the flow-through continued until the UV absorbance began to drop. All chromatography-steps were performed at ambient temperature. Samples (flow-through) were collected at various stages of the passage of the spiked start material through the column. An aliquot of each flow-through was stored at ≤ –60°C until tested by Western blotting as indicated above.

Determination of the PrP^{Sc} binding capacity per gel volume

In order to evaluate the PrP^{Sc} binding capacity per millilitre gel, studies were performed using sequential identical columns. In these experiments, 0·01% CBH$_{Sark}$ (final concentration of brain homogenate) was spiked in Octaplas® harvested from routine production. Ten millilitres of this challenge was applied to the first column (0·5 ml bed volume) containing the gel in a Protein Isolation Kit mini-column (PIKSI, ProMetic Life Sciences Inc., Mount Royal, Quebec, Canada). The flow-through from the first column was applied onto the second column – and from the second onto the third. The gel-bound PrP^{Sc} was quantified by densitometric reading of the Western blot signals, and the binding capacity per column and millilitre gel was estimated in comparison to the PrP^{Sc} input level.

Binding of infectious prions from different sources

Leucocyte-reduced human red blood cells in residual plasma spiked with brain homogenate from different transmissible spongiform encephalopathy strains, including hamster scrapie, human vCJD, human sporadic (sp)CJD, and mouse Fukuoka strain Gerstman-Sträussler-Scheinker disease (GSS), were applied in duplicate to the ligand resin in column format.

Calculation of reduction factors

Reduction factors (RF) were calculated as detailed in 'Note for Guidance on the Performance of Virus Clearance Studies' [CPMP/BWP/268/95 (1996)]: $RF = (V_1 \times T_1)/(V_2 \times T_2)$, in which V_1 and T_1 are the volume and titre of the start material – and V_2 and T_2 are the volume and titre of the product fraction, respectively. In logarithmic terms, this equation can be expressed as: $\log_{10}(RF) = [\log_{10}(V_1) + \log_{10}(T_1)] – [\log_{10}(V_2) + \log_{10}(T_2)]$, and the logarithmic reduction factors (LRF) were rounded to one decimal place only after having completed the final calculation.

Results

In preliminary studies, four of the most promising ligands among the many screened by the company PRDT [8,12] were selected for investigating their compatibility with the Octaplas® manufacturing process and its outcome. One of them did not change the biochemical profile of Octaplas® at all, whereas the other three depleted significantly both coagulation factors and inhibitors (data not shown).

Different aspects of prion binding were investigated by using different spike preparations. As unprocessed CBH probably contains all possible infectious modalities, it was used as the starting spike material for the various spike preparations. The MIC preparation has been chosen because it is enriched with the smallest and most soluble forms of PrP^{Sc}. Where the PrP^{Sc} concentration, as determined by Western blot, is theoretically unrelated to the size distribution of the prion aggregates, this spike with small PrP^{Sc} sizes may represent a form of infectivity closer to that assumed to be potentially present in plasma from blood donors than the form present in spikes with large particle sizes.

The CBH from which the microsomal fraction had been removed by centrifugation [CBH$_{(-MIC)}$] was selected to investigate the binding of larger particle size distributions, i.e. those not contained in the MIC fraction. The use of the two spike preparations above provides for a more thorough investigation of the binding properties of the ligand resin than when only CBH is used.

In addition, for some experiments a sarkosyl-solubilized spike was used. Sarkosyl-solubilized prion spike agents have been utilized widely in prion spiking studies, and yield a spike preparation from which the membrane components have been removed – which may mimic very well the nature of our target Octaplas® matrix following the S/D treatment. The use of sarkosyl as opposed to other detergents is a balance between avoiding extremely strong detergents, such as SDS, which may denature the prion aggregate, and using non-ionic detergents that tend to be too weak to provide sufficient solubilization. Where the spike material was solubilized with sarkosyl before spiking, the respective abbreviation for the spike material is appended with the subscripted text 'Sark' (i.e. CBH$_{Sark}$).

Feasibility experiments

In the first set of studies, experiments were performed where a sarkosyl-solubilized spike (i.e. lacking membrane components) was spiked into Octaplas® final product and applied directly onto PRDT columns. Two PRDT columns containing

the ligand resin at two different column volumes, 1·9 ml and 9·5 ml, were challenged with two concentrations of spiked Octaplas®, 1% and 5% spike ratios, respectively. The flow-through sample was collected in fractions as indicated in Table 1 and analysed by Western blot for PrP^{Sc}. Under a high PrP^{Sc} loading (i.e. 5% spike ratio), with 2·5 $\log_{10}$ as the input, a ≤ −0·5 $\log_{10}$ of PrP^{Sc} signal was recovered with a RF of ≥ 3·0 $\log_{10}$ (2·5 $\log_{10}$ minus ≤ −0·5 $\log_{10}$) could be demonstrated for the early flow-through fractions (0–10 ml), utilizing the relevant Octaplas® matrix from manufacturing. We applied the methodology described above (see Interference handling) to remove the Western blot-interfering plasma proteins by assaying the pellet after centrifugation, which resulted in a quantitative recovery of the PrP^{Sc}. Furthermore, this centrifugation step provided 0·5 $\log_{10}$ of PrP^{Sc} concentration and, thus, increased the assay sensitivity. The results indicated that the binding capacity, determined by the volume at which breakthrough occurred, was dependent on the PrP^{Sc} load vs. the amount of affinity ligand in a reproducible manner. Within the accuracy of the assay, the total bound PrP^{Sc} loaded onto the column was quantitatively recovered – either in the experimentally applied 2 M NaCl wash or still bound to the gel.

PrP^{Sc} removal under manufacturing conditions

Further experiments were performed to investigate removal of PrP^{Sc} which had been conditioned via the S/D-treatment, filtration and solid phase extraction steps, which forms the mid-section of the standard Octaplas® manufacturing process. Crude plasma was spiked with hamster brain-derived infectivity and processed using a validated downscale of the manufacturing process, including the front-end cell and cell-debris filtration. Following the final solid phase extraction step, the product was loaded directly onto a PRDT column to investigate PrP^{Sc} removal. Note, that the level of removal observed for the Octaplas® manufacturing process before PRDT removal cannot be compared with that reported in previous publications which used a chronically infected whole cell preparation as spike. This earlier work measured prion removal for the Octaplas® process including cell removal via 1·0 µm filtration, whereas the current studies only addressed potential removal of non-cell associated prions post-1·0 µm filtration. Irrespective of the spike's nature [MIC or $CBH_{(-MIC)}$], an effective PrP^{Sc} removal to below the limit of assay sensitivity was observed in the early flow-through fractions from the column (Table 2). For the $CBH_{(-MIC)}$ spike, a slightly higher loss of spike material was observed for the steps before the column. Although not significant, this finding is consistent with the nature of this spike, which probably contained larger PrP^{Sc} aggregates or PrP^{Sc} associated with membranes fragments large enough to be filtered out. The pattern of breakthrough also demonstrates slight differences between the two spike materials, in

which the MIC spike showed earlier breakthrough than the $CBH_{(-MIC)}$ spike. This result may reflect an earlier saturation of available PrP^{Sc} binding sites by MIC, due to the smaller prion aggregates present in this spike preparation, or it may reflect the higher PrP^{Sc} loading onto the column due to the lower upstream loss of PrP^{Sc} compared to the $CBH_{(-MIC)}$ case. Again, for the early flow-through fractions (0–10 ml), the ≥ 3·0 $\log_{10}$ RF for the whole process (≥ 2·0–2·5 $\log_{10}$ RF by PRDT column) could be demonstrated using the MIC spike and the amount of PrP^{Sc} recovered from the experimental 2 M NaCl wash and gel demonstrate the substantial binding capacity of the affinity ligands. Based on the input of PrP^{Sc} and the sensitivity of the Western blot assay, it was calculated (see Materials and methods) that the PrP^{Sc} removal capacity per millilitre gel was 7·3 and 6·4 $\log_{10}$ 50% infectious dose (ID_{50})/ml resin for the MIC and $CBH_{(-MIC)}$ spike, respectively.

Determination of PrP^{Sc} binding capacity per gel volume

The gel binding capacity for PrP^{Sc} was also investigated utilizing a different study design, in which the PrP^{Sc} bound to the gel was analysed. In these studies, a fixed volume of challenge (10 ml) and a fixed volume of gel (0·5 ml) were used. The challenge concentration was 0·01% CBH_{sark} (final concentration of brain homogenate). The spiked challenge solution was applied to three columns in series. The binding to each column was then evaluated independently via Western blotting. The results (Fig. 2) indicated that the vast majority of the detectable signal was concentrated in column 1. The flow-through from column 1 contained some contamination of PrP^{Sc}, which was visualized as a very weak signal captured by the second gel (< 3% of PrP^{Sc} input), as shown in Fig. 2. In all tests performed, no signal was ever detected in column 3 indicating that all PrP^{Sc} had been removed before this stage. Furthermore, this demonstrated very strong PrP^{Sc} capture was reproducible when different batches of gel were tested (Fig. 3). The quantification by densitometry of the PrP^{Sc} bands recovered from the resin was conducted using a Bio-Rad VersaDoc imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The results indicated that practically all input PrP^{Sc} was detected bound to the resin. We had previously determined that the total ID_{50} in the challenge were $5 \times 10^5 ID_{50}$ based on the infectivity titration of the spike with the bioassay. Thus, in all cases the PrP^{Sc} binding capacity per millilitre gel was found to be in the range of 5×10^5/0·5 ml gel, equivalent to 6·0 $\log_{10}$ ID_{50}/ml resin.

Determination of the gel ligand specificity for PrP^{Sc} from different sources

Figure 4 shows that the resin has the ability to bind infectious prion from all the sources tested, including the human vCJD
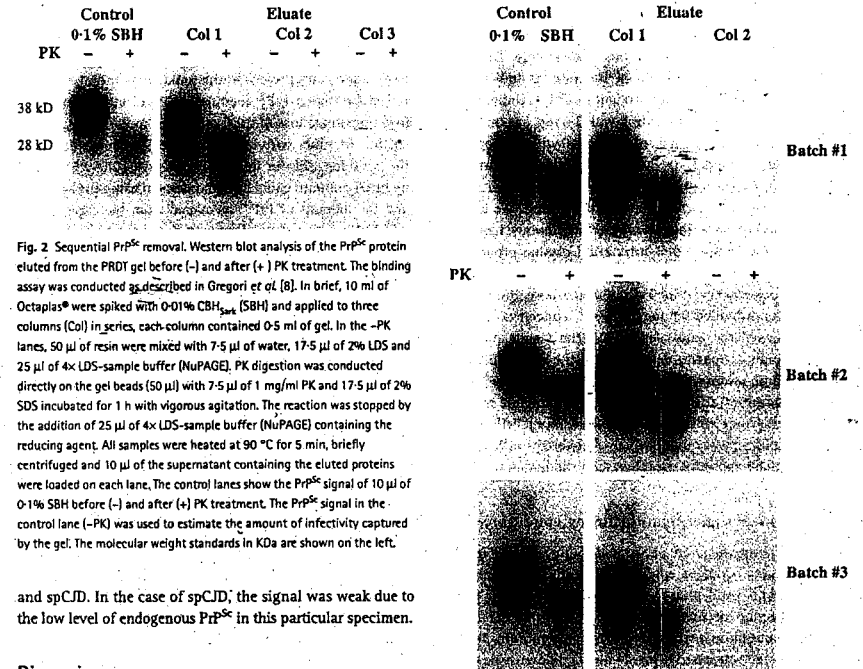
Fig. 2 Sequential PrP^{Sc} removal. Western blot analysis of the PrP^{Sc} protein eluted from the PRDT gel before (−) and after (+) PK treatment. The binding assay was conducted as described in Gregori et al. [8]. In brief, 10 ml of Octaplas® were spiked with 0·01% CBH_{sark} (SBH) and applied to three columns (Col) in series, each column contained 0·5 ml of gel. In the −PK lanes, 50 µl of resin were mixed with 7·5 µl of water, 17·5 µl of 2% LDS and 25 µl of 4× LDS-sample buffer (NuPAGE). PK digestion was conducted directly on the gel beads (50 µl) with 7·5 µl of 1 mg/ml PK and 17·5 µl of 2% SDS incubated for 1 h with vigorous agitation. The reaction was stopped by the addition of 25 µl of 4× LDS-sample buffer (NuPAGE) containing the reducing agent. All samples were heated at 90 °C for 5 min, briefly centrifuged and 10 µl of the supernatant containing the eluted proteins were loaded on each lane. The control lanes show the PrP^{Sc} signal of 10 µl of 0·1% SBH before (−) and after (+) PK treatment. The PrP^{Sc} signal in the control lane (−PK) was used to estimate the amount of infectivity captured by the gel. The molecular weight standards in KDa are shown on the left.

and spCJD. In the case of spCJD, the signal was weak due to the low level of endogenous PrP^{Sc} in this particular specimen.

Discussion

A resin with a ligand, developed by the company PRDT, able to bind and remove PrP^{Sc} quickly and efficiently from plasma during the industrial manufacturing of the Octaplas® product has been identified. A number of studies have been performed investigating the clearance of PrP^{Sc} by this resin under a variety of conditions and utilizing various spike forms. The introduction of this prion binding step provides a robust and effective prion removal step dedicated to improving the prion safety profile of Octaplas® even further, without having a negative impact on the final product quality [13].

Various spike forms and study designs were used in order to evaluate the robustness of the PRDT resin. The resin challenged with CBH, detergent-soluble PrP^{Sc} forms, or homogenates enriched with small or large PrP^{Sc} forms all indicated several log-steps of consistent and reproducible removal (≥ 3·0 $\log_{10}$). The PrP^{Sc} binding capacity of the resin per millilitre gel was shown to be in the region of 6·0–7·3 $\log_{10}$ ID_{50}/ml resin, and effective removal was observed up until the binding capacity of the column was reached. Thus, for the gel volume chosen (3·8 l) for a standard OctaplasLG®

Fig. 3 Reproducibility of PrP^{Sc} removal in sequential set-up. Western blot comparison of PrP^{Sc} binding to three independently manufactured batches of PRDT gel. Ten millilitres of Octaplas® were spiked with 0·01% CBH_{sark} (SHB) and applied to two columns (Col) in series, each column contained 0·5 ml of gel. The samples without (−) and with (+) PK were processed as described in Fig. 2. Ten microlitres of the eluted proteins were loaded on each lane. The control lanes show the PrP^{Sc} signal of 10 µl of 0·1% SHB before (−) and after (+) PK treatment.

batch size (380 l), the total PrP^{Sc} capture is equivalent to at least 9·6 $\log_{10}$ ID_{50}, which is equivalent to 9·4 $\log_{10}$ ID $(ID_{50} \times 0.69)$ [8]. In order to overload this removal capacity, every millilitre of such OctaplasLG® pools would need to contain more than 6900 ID_{50}/ml plasma. Up to 20 ID/ml plasma have been found in relevant rodent models at the clinical stage of disease [14]. Thus, in theory one contaminated single plasmaphaeresis unit of 600 ml would cause a maximum PrP^{Sc} load of 0·03 ID/ml in the OctaplasLG® pool, i.e. the gel capacity exceeds the prion load ≥ 218 500 times (≥ 5·3 $\log_{10}$). Even with as many as 10 (1·6%) contaminated plasma units out of 630 plasmaphaeresis bags in an OctaplasLG® batch, the affinity ligand column is able to remove the total theoretical
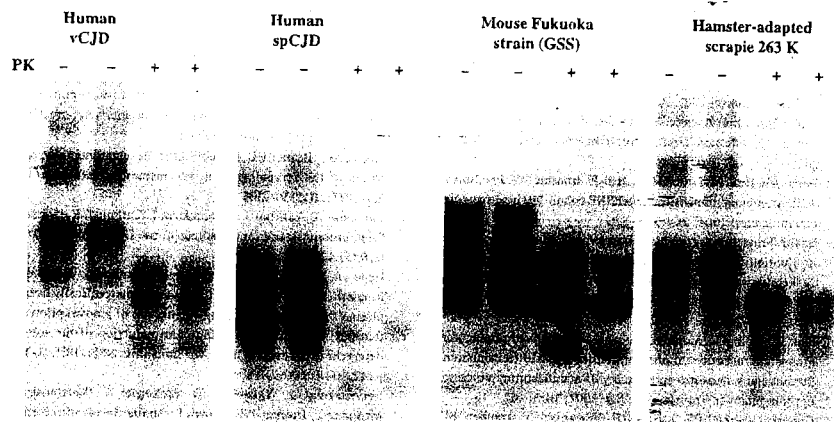
Fig. 4 Binding of PrP^{Sc} derived from various prion diseases. Western blot analysis of PrP^{Sc} binding to PRDT gel. Ten millilitres of human leukoreduced red blood cells in residual plasma were spiked with 1% CBH$_{Sark}$ from a case of variant CJD (vCJD), a case of sporadic CJD (spCJD), a brain pool from mouse-adapted Fukuoka strain (GSS) and 0·1% CBH$_{Sark}$ pool from hamsters infected with hamster-adapted scrapie 263 K strain. Each sample was applied to 0·5 ml of resin in duplicate. Fifty microlitres of each resin (with (+) and without (–) PK treatment) were processed as described in Fig. 2. Ten microlitres of the eluted proteins were loaded on each lane. The exposure time of the film for each sample was adjusted to obtain equivalent signals intensity.

load of PrP^{Sc} with a safety margin higher than 21 850-fold ($\geq 4\cdot3$ log$_{10}$). It is important to confirm the PrP^{Sc} binding demonstrated by Western blotting in these studies by animal infectivity studies. One such bioassay (hamsters) has just been completed successfully and the final result (3·0 log$_{10}$) confirmed the biochemical investigations summarized here (A. Bailey, personal communication). A second animal study is currently ongoing.

In theory, excessive amounts of PrP^C might be able to dislodge PrP^{Sc} that is already bound to the ligand in the gel. Thus, an experiment was performed to address this particular issue (data not shown). The normal concentration of PrP^C in plasma is estimated to be in the order of a few nanogram per millilitre of plasma [15,16]. The study therefore tested the ability of either normal Octaplas® or a solution of commercially available recombinant PrP^C at 2 µg/ml (i.e. close to three orders of magnitude higher than the concentration normally found in plasma) to remove gel-bound PrP^{Sc} from a pre-loaded column. It was concluded from these experiments that the PrP^C concentration expected to be found in the different OctaplasLG® batches would have no significant impact on the ability of the column to retain the gel-bound PrP^{Sc}.

In conclusion, the performed studies confirm a very effective PrP^{Sc} removal effect by the specific affinity ligand tested. The resin will be used in a chromatography step as a single-use resin, i.e. no sanitization and re-use. We have

demonstrated that the introduction of the specific prion removal column into the current Octaplas® manufacturing process is technologically possible and will further improve the safety margin of this product in terms of prion diseases such as vCJD. The new generation Octaplas® will be marketed as OctaplasLG®.

Acknowledgements

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