Articles

Reduction in infectivity of endogenous transmissible spongiform encephalopathies present in blood by adsorption to selective affinity resins

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Lancet 2006; 368: 2226–30

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Summary

Background Transmissible spongiform encephalopathies (TSE) can be contracted through blood transfusion. Selective adsorption of the causative agent from donated blood might be one of the best ways of managing this risk. In our study, affinity resin L13, which reduces brain-derived infectivity spiked into human red blood cell concentrate by around 4 $\log_{10}ID_{50}$, and its equivalent, L13A, produced on a manufacturing scale, were assessed for their ability to remove TSE infectivity endogenously present in blood.

Methods 500 mL of scrapie-infected hamster whole blood was leucoreduced at full scale before passage through the affinity resins. Infectivity of whole blood, leucoreduced whole blood (challenge), and the recovered blood from each flow-through was measured by limiting dilution titration.

Findings Leucoreduction removed 72% of input infectivity. 15 of 99 animals were infected by the challenge, whereas none of the 96 or 100 animals inoculated with the final flow-throughs from either resin developed the disease after 540 days. The limit of detection of the bioassay was 0·2 infectious doses per mL. The overall reduction of the challenge infectivity was more than 1·22 $\log_{10}ID$. The results showed removal of endogenous TSE infectivity from leucoreduced whole blood by affinity ligands. The same resins adsorb normal and abnormal prion protein from human infections with variant, sporadic, and familial Creutzfeldt-Jakob disease, in the presence of blood components.

Interpretation TSE affinity ligands, when incorporated into appropriate devices, can be used to mitigate the risks from TSE-infected blood, blood products, and other materials exposed to TSE infectivity.

Introduction

Experimental and natural transmissible spongiform encephalopathy (TSE) diseases can be contracted via blood transfusion (Rohwer Laboratory, ongoing research).[1–6] There have been three known transfusion transmissions of variant Creutzfeldt-Jakob disease (vCJD) in humans from a small group of known pre-symptomatic donors, indicating high infection efficiency. A survey of surgically removed tissues estimated the number of individuals infected with vCJD in the UK population to be around 4000, assuming 100% ascertainment. This prevalence is greater than that estimated from the 199 symptomatic cases recognised to date and forewarns that blood donations from presymptomatic individuals present a serious threat of human to human transmission.[3–5,7,8] To reduce this risk, deferral of potentially exposed donors has been implemented in many countries.[9] However, this option is not available for highly exposed populations, such as in the UK. Although testing individual donations is desirable, the technical challenges for such a test remain formidable.[10,11] Most of these obstacles can be circumvented by appropriately designed and validated removal strategies, which in the end might also provide greater protection from these low-titre, long incubating agents than is even theoretically possible by diagnostic strategies.

To obtain highly specific ligands that bind the prion protein (PrP), we screened millions of ligands from combinatorial libraries[12,13] and other materials for selective binding to both normal (PrP^{c}) and abnormal (PrP^{res}) prion proteins from humans, hamsters, and other species[14,15] in the presence of blood and blood components. Ligands and their resin supports were further selected for lack of negative effects on red blood cells and plasma proteins. Strong binding to human PrP was confirmed using tissue from people with sporadic CJD (sCJD) and vCJD, as well as mouse-adapted familial CJD (fCJD).

The best resins were further assessed in column format for binding normal human PrP^{c} prepared from human platelets, abnormal human PrP^{res} from sCJD brain homogenates spiked into human red blood cell concentrates, and PrP from normal and scrapie-infected hamster brain homogenates spiked into human red blood cells. From this analysis seven resins were chosen for infectivity studies. Each was challenged in column format with a unit of human red blood cells spiked with hamster brain homogenate infected with 263K scrapie. Three resins reduced the spiked infectivity by around 4 $\log_{10}ID_{50}$. More than 10^{9} infectious doses of spiked infectivity were bound, which is at least six orders of magnitude greater than the infectivity expected for a unit of leucoreduced red blood cell concentrate from an

infected donor.[16] Nevertheless, a small fraction (1:10000) of the brain infectivity could not be removed,[16] raising the question of whether the endogenous TSE infectivity present in blood might be in this capture-resistant form. To address this question and to satisfy other reservations about the relevance of brain-derived infectivity spikes for blood studies, the resin that proved most effective in the spiking study, L13, was tested for its ability to remove TSE infectivity from leucoreduced, scrapie-infected blood.

Methods

We obtained blood from around 130 symptomatic hamsters infected by the intracranial route with a low dose of the 263K strain of scrapie as previously described.[17] The blood was pooled to produce a human-size unit (505 mL) and kept at ambient temperature throughout the study. A sample (15 mL) of whole blood was removed for titration. The remaining blood was leucoreduced with a commercial Pall WBF2 filter (Pall Corporation, Port Washington, NY, USA) following the manufacturer's instructions. The reduction in hamster white blood cell concentration was confirmed to be around 3 $\log_{10}$ (to 25 cells per µL).[17] A 15 mL sample of leucoreduced blood (challenge) was removed for titration.

Two ligands immobilised on the same type of chromatographic resin support were tested: L13, previously described,[16] and L13A, a version of the same resin produced at manufacturing scale. Roughly 50 mL of the leucoreduced hamster infected blood was applied to 1 mL of each resin packed in disposable columns (PIKSI kit, ProMetic Biosciences, Cambridge, UK). The column flow rate was controlled with a peristaltic pump (Rainin Instrument, Woburn, MA, USA) at 0·5 mL/min. Each resin was divided between two chromatography columns, which were challenged in series by a common pool of leucoreduced, scrapie-infected hamster whole blood. For each resin, the flow-through from column 1 (flow-through 1) was used to challenge column 2 (flow-through 2). The column-in-series strategy was used, as in the brain spike study,[16] with two objectives: to confirm the volume of resin needed to clear the infectivity and to assess the intrinsic susceptibility of the infectivity to removal. When infectivity is present in flow-through 1 but not in flow-through 2, all of the infectivity can be adsorbed but two column volumes are needed. If the infectivity that escaped column 1 also escaped column 2, we could conclude that there is a form of infectivity that is not captured by the resin.

Samples of the blood before and after leucoreduction and samples of the flow-throughs 1 and 2 of both resins were titred. For each resin, the first flow-through was collected in a single fraction, mixed thoroughly, and 12 mL removed for titration. The remainder was applied to an identical second column and the flow-through 2 again collected in a single fraction, mixed thoroughly, and stored for titration.

Titrations were by the limiting dilution method.[17] This method is a precise and sensitive way to measure TSE infectivity in low-titre samples such as blood. About 10 mL of each sample was ultrasonicated at 40% full power in four 15 s pulses on ice (Sonics and Material, Vibra cell, VCX 750 and stepped microprobe, Newtown, CT, USA), before inoculation of weanling golden Syrian hamsters with 50 µL per animal. The titre was calculated as previously described.[17]

Whole blood was inoculated into about 50 animals, whereas all other samples were inoculated into roughly 100 animals. Animals that died at the time of inoculation or before 21 days' incubation were removed from the total count, since the short incubation time was not consistent with scrapie infections. Altogether, this loss represented about 10% of the total animals inoculated and is due to toxic consequences of the intracranial inoculation of blood. Animals were isolated when they showed the first symptoms of scrapie and were subsequently observed daily. The brains of all animals that contracted scrapie or died intercurrently or were terminated for unrelated causes were collected. At 544–45 days after inoculation, all animals still alive were killed and the brain of each animal was removed.

Every brain was tested for the presence of the proteinase K-resistant protein, PrP^{res}. Individual brains were homogenised in phosphate buffered saline pH 7·2 to 10% weight per volume with a MediFASTH homogeniser (Consul AR, Villeneuve, Switzerland). The assay was done according to a previously described protocol[16] with the following modifications: in one 100 µL sample, proteinase K was added at 0·1 mg/mL final concentration, and in another 100 µL sample the enzyme was replaced with phenylmethylsulphonyl fluoride at 1·7 mg/mL final concentration. Both reaction mixtures contained 2% sodium dodecyl sulphate (SDS). The reaction mixtures were incubated at 37°C for 20 min followed by 100°C for 10 min to stop the digestion and denature the PrP^{res}. The samples were diluted 100-fold in DELFIA (Dissociation Enhanced Lanthanide Fluorescence Immuno Assay)[18] assay buffer (PerkinElmer, Wellesley, MA, USA) before testing for the presence of PrP^{res} with purified 3F4 (Signet, Dedham, MA, USA) as the capture antibody, and purified 7D9 (Signet) labelled with Europium as the detection antibody. Antibody labelling was done according to the manufacturer's instructions (PerkinElmer) and the molar ratio of Europium:7D9 antibody was 7·4:1. Animals that died intercurrently and all animals with inconclusive DELFIA results were assayed for the presence of PrP^{res} by western blot with 3F4 monoclonal antibody.[16]

Role of the funding source

This work was funded by Pathogen Removal and Diagnostic Technology (PRDT) Inc and by MacoPharma, a worldwide manufacturer of in-line blood filters and blood collection sets. PRDT is a joint venture of the American National Red Cross and ProMetic BioSciences.

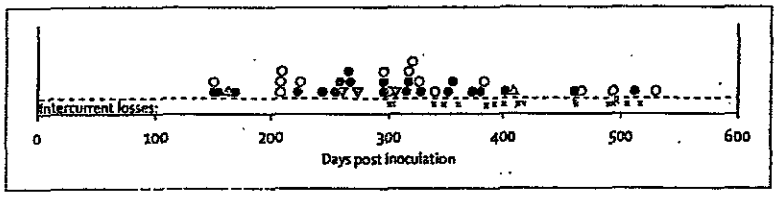

Figure: Distribution of incubation times for all infections in the study
598 animals were inoculated. Animals that died from complications during inoculation or before 21 days following inoculation were excluded from the calculations (around 10%). Only animals that became infected with scrapie or died intercurrently of unrelated causes are plotted (58 of 540 that survived past 21 days). 89% of the 540 experimental animals survived without infection to the end of the experiment at 540 days. Whole blood before leucoreduction (black circles), leucoreduced whole blood (white circles), L13 flow-through 1 (down triangles) and L13A flow-through 1 (up triangles), intercurrent losses unrelated to scrapie (x).

PRDT was involved at all levels of this study: in the design and execution of the study, the interpretation of data, the decision to submit the paper for publication, and in the writing of the manuscript. MacoPharma supported this study financially. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

Results

The incubation times of each infection in this study are shown in the figure. The total volume and number of animals inoculated for each sample, and the number of animals that contracted scrapie, are reported in the table along with the calculated titres and standard deviations of each sample. The brains of all animals in the study were analysed for PrP^{res} to confirm the clinical scoring and to discover incubating infections at the time of termination. Only one animal (challenge group), which died of intercurrent causes at 530 days after inoculation, was detected by western blot to be incubating scrapie. All other animals that were clinically normal were negative for PrP^{res} by DELFIA or western blot, while all symptomatic animals had PrP^{res} in their brains.

In this study, leucoreduction removed 72% of the infectivity present in whole blood compared with an earlier study, in which only 42% infectivity was removed.[9] Two of 99 animals inoculated with flow-through 1 of L13 resin were infected, as were three of 99 animals inoculated with flow-through 1 of L13A resin. This result corresponded to a decrease in titre from 3·3 (SD 0·8) ID/mL (leucoreduced whole blood) to 0·4 (0·3) and 0·6 (0·4) ID/mL for L13 and L13A, respectively (table). By contrast, none of the 100 and 96 animals inoculated with flow-through 2 of either resin developed the disease during 540 days of incubation. Thus, there was complete removal of the 16 ID present in the 4·95 mL of challenge inoculated. For the entire processed volume (table), infectivity was reduced to below the limit of detection of the assay, which is one infection in 5 mL inoculated or 0·2 (0·2) ID/mL.

The $\log_{10}$ of infectivity reduction calculated for each filtration step individually and cumulatively are reported in the right-hand columns of the table. The first passage reduced the infectivity in the challenge by 0·91 $\log_{10}$ID and 0·73 $\log_{10}$ID for L13 and L13A, respectively. The infectivity that passed the first column was captured by the second column. Residual infectivity, if any, was below the limit of detection. The cumulative infectivity reduction for the two columns was greater than 1·22 $\log_{10}$ID.

Discussion

Blood-associated TSE infectivity presents unique challenges to risk management absent from other blood-borne pathogens. The low concentration of infectivity, although difficult to detect, has nevertheless been sufficient for efficient transfusion transmission of these fatal diseases. People with certain genotypes could harbour the infection in a transmissible but undetectable form for decades. The form that the infectivity takes in

	Volume processed (mL)	Total volume inoculated (mL)	Total animals assayed	Total animals infected	Calculated titre		Total measured infectivity			Reduction factor*	
					Titre† (ID/mL)	SD‡ (ID/mL)	Challenge (ID)	Unbound (ID)	Removed (ID)	Stepwise ($\log_{10}$ID)	Cumulative ($\log_{10}$ID)
Whole blood	490	2·35	47	21§	11·8	2·2					
Leucoreduced blood	490	4·95	99	15	3·3	0·8	59	16	42	0·56	
Challenge		4·95	99	15	3·3	0·8					
L13 flow-through 1	51	4·95	99	2	0·4	0·3	16	2	14	0·91	
L13 flow-through 2	40	4·80	96	0	<0·2¶	0·2	2	0	>2	0·31	>1·22
L13A flow-through 1	50	4·95	99	3	0·6	0·4	16	3	13	0·73	
L13A flow-through 2	38	5·00	100	0	<0·2¶	0·2	3	0	>3	>0·49	>1·22

*Reduction factors are calculated as $\log_{10}$(challenge titre)–$\log_{10}$titre remaining after reduction step or when there is no remaining infectivity, the titre at the limit of detection). †$Titre_{max}$=[–ln [(total animals assayed–total animal infected)/(total animals assayed)]]×[(1 ID/50uL)×(1000 uL/mL)]. ‡SD_{max}=[($titre_{max}$)/(total volume $inoculated_{max}$)]$^{1/2}$. §Less than half as much volume was inoculated for this sample as the others. ¶Limit of detection in a limiting dilution titration is a single infection in the cohort inoculated. For a cohort, c, the probability of infection by a single inoculation at the limit of detection titre is 1/c. Probability of no infections, P (0), at that titre is (1–1/c). Probability of no infections in an entire cohort inoculated at the limit of detection titre is $P_c(0)=[P(0)]^c$. Probability of observing at least one infection at a given P (0) is $P_c(\geq 1)=1-P_c(0)$. For no infections in 100 inoculations of 50 uL, there is a minimum probability of 0·63 that the concentration of infectivity is ≤0·20 ID/mL, the limit of detection, and a minimum probability of 0·90 that it is ≤0·46 ID/mL. Since the actual titre is unknown and could be far less than the limit of detection, these values represent upper limits on the actual titre.

Table: Distribution of infectivity in whole blood, challenge, and resin flow-through

blood is unknown but might be different from that associated with brain. No pathogen-specific antibodies have been identified for diagnosis, and infection-associated forms of the prion protein constitute only a minuscule proportion of the total prion protein present in blood, thereby presenting a formidable challenge to detection.

Selective adsorption of TSE infectivity from donated blood might be one of the best ways of managing this risk. Ligands that bind all forms of the prion protein with high affinity can still remove trace amounts of infection-associated forms in the presence of much higher concentrations of normal forms. Binding will take place even at the earliest times in the infection when the concentration of infectivity is below the limit of detection of diagnostic assays. Finally, if every donation is treated, the protection might be more comprehensive than from a diagnostic test.

In this study, powerful affinity ligands, selected from libraries of millions of compounds and materials, were tested for their ability to remove the low amounts of TSE infectivity that are naturally present in the blood of infected individuals. After increasingly stringent screening, resin L13 was selected for assessment with endogenous blood infectivity. Optimisation to facilitate manufacturing of this resin produced L13A. The two resins are identical in terms of ligand chemistry and matrix, and are functionally equivalent.

Universal leucoreduction has been implemented in several countries, including the UK, in part to remove white cell-associated TSE infectivity. However, we previously showed that leucoreduction removed less than half of blood-borne TSE infectivity.[9] Other evidence suggests that the remaining infectivity is in plasma.[9] To further manage TSE risk, the UK no longer fractionates its domestic plasma. That leaves the bulk of the residual risk with red blood cell concentrate, which is by far the most frequently used blood component, but one for which there is no alternative to domestic sourcing. Red blood cells are for this reason in most urgent need of risk mitigation and have been the first objective for TSE removal. However, because of the low and variable amounts of plasma in leucoreduced red blood cell concentrates, the infectivity in a red blood cell preparation from hamsters might be insufficient to show a meaningful level of removal. Instead, leucoreduced whole blood from hamsters infected with 263K scrapie was used to challenge resins L13 and L13A. Hamster 263K scrapie has consistently produced blood infectivity titers of 10 (SD 2) ID/mL during symptomatic disease (Rohwer Laboratory, unpublished).[10] Since leucoreduced whole blood retains its full complement of plasma and red blood cells, and since removal was to the limit of detection, the same resins would probably also remove endogenous infectivity from plasma, plasma derivatives, and red blood cell concentrates.

The main objectives of this study were to confirm that the volume of resin needed to capture all measurable blood-borne infectivity, as deduced from earlier experiments, was correct, and to make sure that the infectivity in blood was in a form that could be adsorbed by the resins. In an earlier study, one part per 10 000 of the infectivity in a brain spike was not removable by any resin tested.[6] In this study, resins in the proportion of 2 mL to 50 mL leucoreduced blood removed the endogenous, blood-associated infectivity to below the limit of detection (0·2 ID/mL). With a challenge of 3·3 ID/mL of leucoreduced whole blood, we were able to show a greater than 1·22 $\log_{10}$ID reduction. All of the infectivity in the roughly 5 mL volume inoculated was removed before the final flow-through of both resins. Because there were no infections, we can calculate from the Poisson distribution that there is a 63% minimum probability that the concentration of infectivity is 0·20 ID/mL or less, ie, the limit of detection, and a 90% minimum probability that it is 0·46 ID/mL or less. Since the actual titre is unknown and could be far less than the limit of detection, these values represent upper limits on the actual titre. In this experiment, the endogenous infectivity in a full unit of leucoreduced whole blood (450 mL) would be 1485 ID. Passage through the resin would reduce the infectivity to, at maximum, 90 remaining infectious doses. This corresponds to, at minimum, 1395 infectious doses removed.

The reduction efficiency of the resin differed for endogenous blood-associated infectivity and spiked brain-derived infectivity. Between 1 and 2 mL of resin were needed to capture the 165 ID from 50 mL of leucoreduced hamster blood versus 20–30 mL of the same resin to capture several million infectious doses from brain spiked in a 330 mL unit of human red blood cell concentrate.[6] Important differences between the studies include the source of infectivity—blood versus brain; the protein environment—human red blood cell concentrates versus hamster whole blood; and the flow rate—gravity versus pump. Both approaches provided valuable information and our experience emphasises the value of experiments that use both methods.

The figure shows that symptomatic disease in limiting dilution titration assays occurs randomly from 120 days to more than 500 days, and only rarely after 500 days incubation.[9] Titre cannot be deduced from incubation times, and premature termination would lead to overestimates of removal capabilities and underestimates of titre. However, extending the endpoint beyond 540 days is not beneficial, since new infections are rare, whereas age-related mortality increases greatly (Rohwer Laboratory, unpublished).

In a previous study, leucoreduction removed around 42% of the infectivity in hamster whole blood[9] compared with 72% in this study following the same protocol. Both filtrations used human-sized units of whole hamster blood and both filters worked to specification for removal of white blood cells. The differences in infectivity removal could indicate natural variability between blood pools, filters, stability of white blood cell associations, or subtle

differences in execution. Differences we are aware of include: the collection interval—7 h in the first experiment and 4 h in the second; and the filtration time—about 20 min in the first filtration and around 11 min in the second.[P] Neither filtration removed sufficient infectivity to eliminate the risk. Thus, a strong TSE blood safety programme cannot rely only on leucoreduction. We note also that the 30% difference between these two results could not have been detected by any other means than limiting dilution titration.

Resins L13 and L13A, which were indistinguishable in these studies, adsorb endogenous TSE infectivity from leucoreduced whole blood. Both resins capture PrP^{res} from infected human and hamster brain diluted into human whole blood and plasma, and PrP^{c} from human whole blood and plasma (data not shown). These protein-based data from people and hamsters, together with this study showing removal of endogenous infectivity from hamster blood, makes adsorption of TSE infectivity from human blood, plasma, and red blood cell concentrates by these resins highly probable. Resin L13A has been incorporated into a filter device format, P-CAPT (MacoPharma, Lille, France), for reduction of human endogenous TSE infectivity in leucoreduced red blood cell concentrates.

Contributors

The overall design and execution of the experiment, including management of the logistics and all the infectivity work, was by L Gregori, B C Lambert, and R G Rohwer with the assistance of the staff of the Molecular Neurovirology Laboratory. P V Gurgel, R G Carbonell, S J Burton, and D J Hammond provided expertise on resins, combinatorial peptide and polymer libraries, and chromatography. J T Lathrop and D J Hammond supervised the blood studies. P Edwardson coordinated the efforts of several laboratories to the final goal of this study.

Conflict of interest statement

R G Rohwer, R G Carbonell, and D J Hammond are cofounders and part owners of Pathogen Removal and Diagnostics Technologies, which sponsored this study together with MacoPharma. S J Burton, P V Gurgel, and P Edwardson are employees of ProMetic BioSciences, a joint venture partner in the development of the removal resin. D J Hammond and J T Lathrop are employees of the American National Red Cross, also a joint venture partner in the development of the resin. L Gregori and B C Lambert receive contract support from Pathogen Removal and Diagnostics Technologies for these and other studies on TSE removal resins.

Acknowledgments

The authors thank the ABSL 3 animal facility staff at the Veterans Affairs Medical Center for the excellent animal care. This work was funded by Pathogen Removal and Diagnostic Technologies, and by MacoPharma.

References

1 Houston F, Foster JD, Chong A, Hunter N, Bostock CJ. Transmission of BSE by blood transfusion in sheep. *Lancet* 2000; 356: 999–1000.

2 Hunter N, Foster J, Chong A, et al. Transmission of prion diseases by blood transfusion. *J General Virol* 2002; 83: 2897–905.

3 Llewelyn CA, Hewitt PE, Knight RS, et al. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet* 2004; 363: 417–21.

4 Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* 2004; 364: 527–29.

5 Wroe SJ, Pal S, Siddique D, et al. Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. *Lancet* 2006; 368: 2061–67.

6 Brown P, Cervenakova L, McShane LM, Barber P, Rubenstein R, Drohan WN. Further studies of blood infectivity in an experimental model of transmissible spongiform encephalopathy, with an explanation of why blood components do not transmit Creutzfeldt-Jakob disease in humans. *Transfusion* 1999; 39: 1169–78.

7 Hilton DA, Ghani AC, Conyers L, et al. Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. *J Pathol* 2004; 203: 733–39.

8 The National Creutzfeldt-Jakob Disease Surveillance Unit, Edinburgh, Scotland. Variant Creutzfeldt-Jakob disease current data (October 2006). http://www.cjd.ed.ac.uk/vcjdworld.htm (accessed October, 2006).

9 US Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research. Guidance for Industry: revised preventive measures to reduce the possible risk of transmission of Creutzfeldt-Jakob Disease (CJD) and Variant Creutzfeldt-Jakob Disease (vCJD) by blood and blood products, January 2002. http://www.fda.gov/cber/guidelines.htm (accessed October, 2006).

10 Brown P. Blood infectivity, processing and screening tests in transmissible spongiform encephalopathy. *Vox Sang* 2005; 89: 63–70.

11 Minor PD. Technical aspects of the development and validation of tests for variant Creutzfeldt-Jakob disease in blood transfusion. *Vox Sang* 2004; 86: 164–70.

12 Furka A, Sebestyen F, Asgedom M, Dibo G. General method for rapid synthesis of multicomponent peptide mixtures. *Intl J Pept Protein Res* 1991; 37: 487–93.

13 Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ. A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* 1991; 354: 82–84.

14 Patent: Prion protein ligands and methods of use. Hammond D, Lathrop J, Cervenakova L, Carbonell R; WUO 2004/050851A2, filed 12/3/03.

15 Baumbach GA, Hammond DJ. Protein purification using affinity ligands deduced from peptide libraries. *BioPharm* 1992; 24–35.

16 Gregori L, Lambert BC, Gurgel PV, et al. Reduction of TSE infectivity from human red blood cells using prion protein affinity ligands. *Transfusion* 2006; 46: 1152–61.

17 Gregori L, McCombie N, Palmer D, et al. Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood. *Lancet* 2004; 364: 529–31.

18 MacGregor I, Hope J, Barnard G, et al. Application of a time-resolved fluoroimmunoassay for the analysis of normal prion protein in human blood and its components. *Vox Sang* 1999; 77: 88–96.

19 Brown P, Rohwer RG, Dunstan BC, MacAuley C, Gajdusek DC, Drohan WN. The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. *Transfusion* 1998; 38: 810–16.

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	機構処理欄
				2007. 1. 22	該当なし	

一般的名称	(製造承認書に記載なし)	研究報告の公表状況	Franscini N, Gedaily AE, Matthey U, Franitza S, Sy MS, Burkle A, Groschup M, Braun U, Zahn R. PLoS ONE 1(1): e71. 2006 Dec 20.	公表国
販売名(企業名)	合成血「日赤」(日本赤十字社) 照射合成血「日赤」(日本赤十字社) 合成血-LR「日赤」(日本赤十字社) 照射合成血-LR「日赤」(日本赤十字社)			米国

研究報告の概要 709

○乳汁中のプリオンタンパク質
【背景】プリオンは、中枢神経系に蓄積した後に伝達性海綿状脳症(TSE)を引き起こすことが知られている。プリオンは体液中にも存在し、輸血によりプリオンが感染する可能性があるというエビデンスが増えつつある。体液中のタンパク質性病原体濃度の低さと当該病原体の潜伏期間の長さが、疫学的分析並びに伝播の推定(すなわちヒトへの感染リスクの推定)を困難にしている。この感染性病原体をモニターするための感度の高い方法はなく、これは特に食品医薬品業界では問題である。
【方法/主な知見】プリオンタンパク質に特異的かつ高親和性で結合する吸着マトリックス、Alicon PrioTrap®を開発した。これを用いてヒト、ウシ、ヒツジ、ヤギの乳汁中のプリオンタンパク質(PrPC、プリオンPrPScの前駆体)が特定できた。PrPCの絶対量には種間差があった(ヒツジの乳中でμg/Lレンジ～ng/Lレンジ)。PrPCは、均質化し低温度殺菌した市販ミルクからも認められ、超高温処理を施しても内因性PrPCの濃度はわずかにしか減少しなかった。
【結論/有意性】乳腺炎に罹患したスクレイピー感染ヒツジの乳腺中のプリオン複製の証拠を示す最近の研究を考えれば、乳汁中にPrPCが出現することは、TSE感染動物の乳汁がPrPScの感染源となる可能性を示唆する。

使用上の注意記載状況・その他参考事項等

合成血「日赤」
照射合成血「日赤」
合成血-LR「日赤」
照射合成血-LR「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

報告企業の意見	今後の対応
ヒト、ウシ、ヒツジ、ヤギの乳汁及び均質化し低温度殺菌した市販ミルクからPrPCが検出されたとの報告である。	今後も引き続き、プリオン病に関する新たな知見及び情報の収集に努める。

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PLoS one

Prion Protein in Milk

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Background. Prions are known to cause transmissible spongiform encephalopathies (TSE) after accumulation in the central nervous system. There is increasing evidence that prions are also present in body fluids and that prion infection by blood transmission is possible. The low concentration of the proteinaceous agent in body fluids and its long incubation time complicate epidemiologic analysis and estimation of spreading and thus the risk of human infection. This situation is particularly unsatisfactory for food and pharmaceutical industries, given the lack of sensitive tools for monitoring the infectious agent. ***Methodology/Principal Findings.*** We have developed an adsorption matrix, Alicon PrioTrap®, which binds with high affinity and specificity to prion proteins. Thus we were able to identify prion protein (PrP^C)–the precursor of prions (PrP^{Sc})–in milk from humans, cows, sheep, and goats. The absolute amount of PrP^C differs between the species (from µg/l range in sheep to ng/l range in human milk). PrP^C is also found in homogenised and pasteurised off-the-shelf milk, and even ultrahigh temperature treatment only partially diminishes endogenous PrP^C concentration. ***Conclusions/Significance.*** In view of a recent study showing evidence of prion replication occurring in the mammary gland of scrapie infected sheep suffering from mastitis, the appearance of PrP^C in milk implies the possibility that milk of TSE-infected animals serves as source for PrP^{Sc}.

Citation: Franscini N, Gedaily AE, Matthey U, Franitza S, Sy M-S, et al (2006) Prion Protein in Milk. PLoS ONE 1(1): e71. doi:10.1371/journal.pone.0000071

INTRODUCTION

Prion protein was detected in attempts to identify the infective agent of TSE [1,2]. The finding that prion protein is present in normal and TSE-infected brain at similar levels [3,4] suggests that the "cellular" prion protein (PrP^C) constitutes a precursor of the "scrapie" prion protein (PrP^{Sc}) causing TSE such as bovine spongiform encephalopathy (BSE) in cattle or Creutzfeldt-Jakob disease (CJD) in humans. There is convincing evidence that the transition from precursor protein to infectious prion is due to a major conformational transition [5].

Prion protein is highly conserved among mammals [6]. It is primarily synthesized in cells of the central nervous system [7], but is also abundantly expressed in several peripheral tissues [8,9]. An amino-terminal signal sequence targets prion protein to the endoplasmatic reticulum, where it transits the Golgi and ultimately reaches the external surface of the cell membrane [10]. There it is attached to a carboxy-terminal glycosyl phosphatidylinositol anchor [11]. The mature bovine protein of 217 amino acids contains two consensus acceptor sites for addition of N-linked polysaccharides [12].

Prion proteins (PrP^C and PrP^{Sc}) have been detected in the cellular fraction of blood [13–17], but so far not in milk [18–21]. Considering that milk and milk products represent a major component of human nutrition it seems of particular importance to analyze milk for the presence of prion proteins. A first step in this direction is to determine the amount of PrP^C in milk of healthy animals. If milk contains a significant amount of PrP^C, this could indicate that PrP^{Sc} might be present in so far undetectable amounts in milk of TSE infected animals. However, the high concentration of total protein (about 40 mg/ml) and the high amount of lipids (about 35 mg/ml) in the milk make prion protein analysis by common biochemical methods demanding. We have therefore developed an adsorption matrix, Alicon PrioTrap®, which binds with high affinity and specificity to prion proteins PrP^C and PrP^{Sc}. The exceptional binding properties of Alicon PrioTrap® result from hydrophilic and hydrophobic surface clusters that recognize different prion protein epitopes, allowing quantitative enrichment of extreme low quantity of prion proteins in body fluids and in biological tissues.

RESULTS

The detection of native PrP^C after enrichment from 10 ml milk from cow, sheep, goat, and human using Alicon PrioTrap® is shown in figure 1. In cow milk three PrP^C isoforms are observed with an apparent molecular mass of about 34 kD, 30 kD, and 27 kD corresponding to diglycosylated, monoglycosylated, and unglycosylated PrP^C, respectively. In some preparations monoglycosylated PrP^C appears as a double band, indicating that the two glycosylation sites may be linked to different carbohydrates. The apparent molecular mass of unglycosylated PrP^C is slightly higher when compared to a recombinant bovine PrP(25–241) standard at 26 kD, indicating that native PrP^C in milk contains a glycosyl phosphatidylinositol anchor [11]. About the same distribution of PrP^C isoforms is observed for sheep, goat, and human milk, although the total amount of native PrP^C significantly differs between the species. The relative ratio of sheep/cow/goat/human PrP^C is estimated at 100/20/4/1. From experiments performed on sequential incubations with Alicon PrioTrap® the total concentration of PrP^C in fresh cow milk can be estimated to be about 200 pg/ml. Taking into account the

Academic Editor: Matthew Baylis, University of Liverpool, United Kingdom

Received October 19, 2006; Accepted November 6, 2006; Published December 20, 2006

Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

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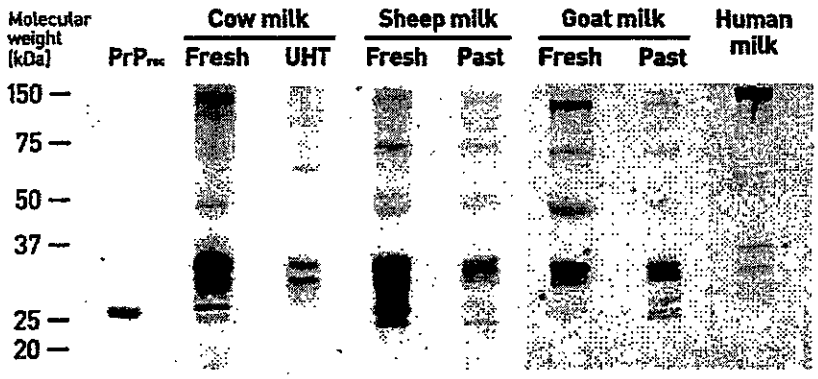

Figure 1. Detection of native PrP^C in milk of human and animals. A volume of 10 ml fresh, ultra-high temperature treated (UHT) and pasteurised (Past) milk were enriched for PrP^C using Alicon PrioTrap® technology. Concentrated PrP^C was analyzed by Western Blotting using PrP—mab 8B4 (Alicon AG). The molecular weight markers are indicated. Recombinant bovine PrP(25—241) (Alicon AG) was used as a standard.
doi:10.1371/journal.pone.0000071.g001

relative ratios of PrP^C in milk of different species, fresh sheep milk and goat milk contain about 1 ng/ml and 40 pg/ml PrP, respectively. Human breast milk contains less than 10 pg/ml PrP^C. The concentration of PrP^C in Swiss off-the-shelf milk is reduced when compared to fresh milk, but prion protein can clearly be detected (Figure 1). About the same concentration of PrP^C was measured for organic farm milk and non-organic farm milk as well as for pasteurized and ultra-high temperature (UHT) treated milk (data not shown).

To confirm specificity of immunochemical detection of PrP^C in milk, we compared different anti-PrP monoclonal antibodies, which are directed against non-overlapping epitopes (Figure 2): PrP—mab 8B4 binds to residues 37—44 of mouse PrP [22]; mAB 6H4 targets residues 144—152 [23]; and PrP—mab 8H4 binds to residues 175—185 [24]. The three antibodies recognize the same proteins and thus confirm the presence of PrP^C in milk. In control experiments, with non-PrP antibodies, *e.g.*, anti-Tau protein monoclonal antibody (Chemicon International) (Figure 2) and anti-Aβ monoclonal antibody (Calbiochem, Germany; data not shown), none of the PrP^C isoforms was detected, thus confirming binding specificity of the anti-PrP monoclonal antibodies. An interesting observation with regard to antibody 8B4 is its "clear" detection profile when compared to 6H4 and 8H4 antibodies. This can be rationalized by 8B4 not recognizing a variety of carboxy-terminal fragments of milk PrP^C, which appear as smear in the Western Blot.

We further compared the glycoforms of native prion protein in cow milk with those of bovine brain, a tissue where prion protein expression is well characterized. The glycoforms were identified by digestion with PNGase (Figure 3), an enzyme that cuts off oligosaccharides from N-linked glycoproteins, *e.g.*, the two N-linked sugars of PrP^C [12]. After partial cleavage with PNGase the upper PrP-isoform in the Western Blot representing diglycolysated PrP^C (34 kD) disappears in favour of monoglycosylated (30 kD) and nonglycosylated PrP^C (27 kD). In parallel, there seems to be a small shift from the higher molecular weight monoglycosylated form to the lower molecular weight form. A slight downshift of the monoglycosylated PrP^C is also observed for brain homogenate after PNGase treatment (Figure 3). The diglycosylated PrP^C isoforms differ slightly in molecular mass, indicating that carbohydrate structure of PrP^C in milk and brain may not be identical. More stringent reaction conditions result in complete truncation of carbohydrates from PrP^C. Most importantly, the apparent molecular masses of nonglycosylated PrP^C in milk exactly matches with that of the corresponding PrP^C in brain homogenate.

Alicon PrioTrap® can also be applied for elimination of prion protein from milk. As shown in Figure 4, after the first treatment of 10 ml milk with Alicon PrioTrap® more than 95% of endogenous PrP^C was already removed, and after the second treatment PrP^C was completely eliminated. However, the overall protein concen-

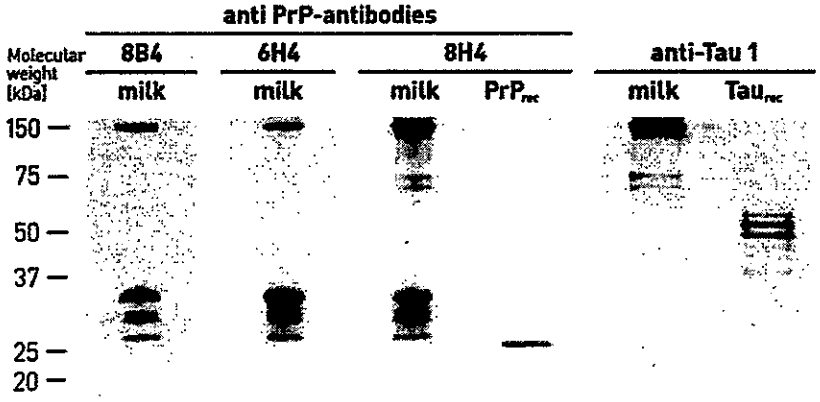

Figure 2. Specific binding of anti-PrP monoclonal antibodies to milk PrP^C. Various anti-PrP monoclonal antibodies were used for detection of PrP^C in fresh cow milk. A Tau-1 protein-specific monoclonal antibody was used as a negative control. Recombinant bovine PrP(25—241) (Alicon AG) and recombinant Tau-1 protein (Chemicon International) were used as a standard.
doi:10.1371/journal.pone.0000071.g002