別紙様式第2-1

No. 11

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	総合機構処理欄
				2009. 2. 18	該当なし	
一般的名称	乾燥濃縮人血液凝固第Ⅷ因子		研究報告の公表状況	Health Protection Agency, 2009 Feb 17. Available from: http://www.hpa.org.uk/webw/HPAweb&HPAwebStandard/HPAweb_C/1234859690542?p=1231252394302	公表国 英国	
販売名(企業名)	クロスエイトM250(日本赤十字社) クロスエイトM500(日本赤十字社) クロスエイトM1000(日本赤十字社)					

研究報告の概要

○血友病患者の剖検時にvCJD異常プリオンタンパク質が発見された
vCJDとは関係のない疾患により死亡した血友病患者(年齢70歳以上)の剖検時に、患者の脾臓からvCJDの異常プリオンタンパク質感染の証拠が見つかった。この患者は、生前vCJD及び神経学的症状は示していなかった。
英国健康保護局は、英国血友病センター医師会と共同し、現在詳細調査中であるこの予備情報が出血性疾患患者すべてに確実に伝わるよう尽力しているが、この新たな知見により血友病患者の看護や治療の方法が変わることはない。
伝播経路の調査は継続中であり最終的な見解はまだ得られていない。
当該患者は、vCJDに関する血液安全性改善措置が導入された1999年以前に、英国内で供血された凝固因子製剤による治療を受けたことが判明しており、その中に供血の6ヶ月後にvCJDの症状を発現した供血者由来血漿から製造された第VIII因子製剤1バッチが含まれていた。
血友病患者または血漿分画製剤の治療を受けた患者にvCJD異常プリオンタンパク質が見つかったのはこれが初めてである。
血友病患者は、すでに「公衆衛生上vCJDリスクを有する状態」に分類されることが医師から知らされているが、リスクの状態が変更されるものではない。
この新たな知見は、これまで理論上のリスクであったものが、血漿分画製剤を投与された特定の個人に対する現実のリスクとなる可能性を示すものと考えられるが、当該リスクはまだ非常に低いであろうと考えられる。
1999年以降、凝固因子製剤製造に英国内の血漿は使用されておらず、必要な患者には遺伝子組換製剤が使用されている。

使用上の注意記載状況・その他参考事項等

クロスエイトM250
クロスエイトM500
クロスエイトM1000

血液を原料とすることに由来する感染症伝播等
vCJD等の伝播のリスク

海外症例報告:
2009年03月05日付3-08000044

報告企業の意見	今後の対応
英国でvCJDとは関係のない疾患により死亡した血友病患者の剖検時に、初めてvCJDの異常プリオンタンパク質感染の証拠が見つかり、当局はすべての出血性疾患患者への情報提供と伝播経路の調査を実施しているとの報告である。	プリオン病の原因とされる異常プリオンが分画製剤製造工程で効果的に除去されるとの成績と併せて、これまでの疫学研究では如何なるプリオン病も、血漿分画製剤を介して伝播するという証拠は無かった。しかし、原因が特定されていないものの、本報告で初めて、第Ⅷ因子製剤を介してvCJDに感染する可能性が示唆された。引き続きプリオン病に関する新たな知見及び情報を収集するとともに、血漿分画製剤の製造工程における病原因子の除去・不活化技術の向上に努める。 なお、日本赤十字社は、CJD、vCJDの血液を介する感染防止の目的から、献血時に過去の海外渡航歴(旅行及び居住)、CJDの既往歴(本人、血縁者)、hGH製剤投与の有無を確認し、該当するドナーを無期限に献血延期としている。

27

MedDRA/J Ver.11.0.1

Health Protection Agency

Protecting people
Preventing harm
Preparing for threats

JRC2009T-007

vCJD abnormal prion protein found in a patient with haemophilia at post mortem

17 February 2009

Evidence of infection with the agent (abnormal prion protein) that causes variant Creutzfeldt-Jakob Disease (vCJD) has been found at post mortem in the spleen of a person with haemophilia.

The patient, who was over 70 years old, died of a condition unrelated to vCJD and had shown no symptoms of vCJD or any other neurological condition prior to his death. The vCJD abnormal prion protein was only identified during post mortem research tests.

The Health Protection Agency is working with the UK Haemophilia Centre Doctors Organisation to ensure all patients with bleeding disorders are made aware of this preliminary information which is being further investigated. This new finding will not change the way patients with haemophilia are cared for or treated.

A final view as to how vCJD abnormal prion protein was transmitted to this haemophilia patient has yet to be reached because investigations are continuing to determine the most likely route of transmission. It is known that the patient had been treated with several batches of UK sourced clotting factors before 1999, which is when measures to improve the safety of blood in relation to vCJD were introduced. The patient's treatment had included one batch of Factor VIII that was manufactured using plasma from a donor who went on to develop symptoms of vCJD six months after donating the plasma in 1996.

This is the first time that vCJD abnormal prion protein has been found in a patient with haemophilia, or any patient treated with plasma products. This new finding, however, does not change the public health vCJD 'at risk' status of patients with bleeding disorders.

Haemophilia patients have previously been informed by their doctors of their possible increased risk of exposure to vCJD via clotting factors. In 2004 all patients with bleeding disorders who had been treated with UK-sourced pooled plasma products between 1980 and 2001 were told that, owing to potential vCJD infectivity from these products they were to be classified as at-risk of vCJD for public health purposes.

Professor Mike Catchpole, Director of the Health Protection Agency's Centre for Infections, said:

"This new finding may indicate that what was until now a theoretical risk may be an actual risk to certain individuals who have received blood plasma products, although the risk could still be quite low. We recognise that this finding will be of concern for persons with haemophilia who will be awaiting the completion of the ongoing investigations and their interpretation.

The priority is to ensure that patients are informed of this development and have access to the latest information and specialist advice from their own haemophilia centre doctor as soon as possible.

"This finding does not change our understanding of the risk from vCJD for other people in any specific way. But it does reinforce the importance of the precautionary measures that have been taken over the years.

"Since the risk of vCJD transmission through blood was first considered, a number of precautionary measures have been introduced to minimise the risk from the UK blood supply. UK plasma has not been used for the manufacture of clotting factors since 1999 and synthetic clotting factors are provided for all patients for whom they are suitable."

Ends

Notes for editors

1) The post-mortem tests were carried out as part of a research study jointly coordinated by the UK Haemophilia Centre Doctors Organisation and the National CJD Surveillance Unit. The study was commissioned in 2001 and is ongoing.

2) The likelihood of a person who is infected with the vCJD abnormal prion protein going on to develop symptoms of the disease is uncertain and may depend on individual susceptibility. It is possible that infected individuals may never develop symptoms.

3) Haemophilia is a genetic blood condition in which an essential clotting factor is either partly or completely missing. This causes a person with haemophilia to bleed for longer than normal. Treatment for haemophilia is usually by replacing the missing clotting

factor (factor VIII) through regular injections which helps the blood to clot and minimises the likelihood of long term joint damage.

4) In 2004 all patients with bleeding disorders who had been treated with UK-sourced pooled plasma products (e.g. clotting factors for individuals with haemophilia) between 1980 and 2001 were told that, owing to potential vCJD infectivity from these products, they would be classified as at-risk of vCJD for public health purposes.

The start date of 1980 is thought to be the earliest date the agent (abnormal prion protein), that causes BSE in cattle and vCJD in humans, could have entered the food chain. The end date of 2001 is the last possible expiry date of any product manufactured by UK fractionators that had been sourced from UK donors up until 1998.

5) The government introduced a number of measures from 1997 onwards to safeguard blood and plasma supplies.

- Since 1997 all cases of vCJD that are reported to the National CJD Surveillance Unit and diagnosed as having 'probable' vCJD, result in a search of the UK Blood Services blood donor records. If the patient has donated blood, any unused parts of that blood are immediately removed from stock. The fate of all used components of blood from the donor is traced, and surviving recipients informed of their risk.
- In July 1998, the Department of Health announced that plasma for the manufacture of blood products, such as clotting factors, would be obtained from non-UK sources.
- Since October 1999, white blood cells (which may carry the greatest risk of transmitting vCJD) have been removed from all blood used for transfusion.
- In August 2002 the Department of Health announced that fresh frozen plasma for treating babies and young children born after 1 January 1996 would be obtained from the USA, extended to all children under 16 years of age (Summer 2005).
- In December 2002, the Department of Health completed its purchase of the largest remaining independent US plasma collector, Life Resources Incorporated. This secures long-term supplies of non-UK blood plasma for the benefit of NHS patients.
- Since April 2004, blood donations have not been accepted from people who have themselves received a blood transfusion in the UK since 1980. This has been extended to include apheresis donors and donors who are unsure if they had previously had a blood transfusion (August 2004).
- Since late 2005, blood donations have not been accepted from donors whose blood was transfused to patients who later developed vCJD.
- The UK Blood Services continue to promote the appropriate use of blood and tissues and alternatives throughout the NHS.

6) Specialist advice and care concerning vCJD is available from:

The National CJD Surveillance Unit, based at the Western General Hospital Edinburgh: www.cjd.ed.ac.uk. The NHS National Prion Clinic, based at The Hospital for Neurology and Neurosurgery, Queen Square, London http://www.nationalprionclinic.org/

7) For further information about vCJD go to:
www.hpa.org.uk/cjd
http://www.hpa.org.uk/vcjdplasmaproducts
http://www.dh.gov.uk/PolicyAndGuidance/HealthAndSocialCareTopics/CJD/fs/en
http://www.blood.co.uk/
http://www.cjd.ed.ac.uk
http://www.nationalprionclinic.org/

8) For Health Protection Agency media enquiries please contact the Agency's Centre for Infections Press Office on:

Kate Swan	020 8327 7097
Alexandra Baker	0208 327 7098
Louise Brown	020 8327 7080
George Fletcher	020 8327 6690

Last reviewed: 17 February 2009

別紙様式第4

医薬品　研究報告　調査報告書

識別番号・報告回数	F	第1報	報告日 2009年03月04日	第一報入手日 2009年02月18日	新医薬品等の区分 該当なし	機構処理欄
一般的名称	1. 乾燥濃縮人血液凝固第８因子（6343406） 2. ルリオクトコグアルファ（遺伝子組換え）（6343432） 3. 乾燥人血液凝固因子抗体迂回活性複合体（6343414）			http://www.hpa.org.uk/webw/HPAweb&HPAwebStandard/HPAweb_C/1234859690543?p=1231252394302	公表国 イギリス	
販売名（企業名）	1. ヘモフィルM（634340612）（Baxter） 2. リコネイト（634343201） 3. ファイバ（634341401） 4. ガンマガード（634342002） 5. プラズマプロテインフラクション（634342204）	研究報告の公表状況				
研究報告の概要	（概要）：イギリスのおける血友病患者でのvCJDのリスクに関する報告 HPA（英国 Health Protection Agency）から、感染に対する規制が導入される以前に血漿分画製剤を投与された70歳代の血友病患者において、後死によりvCJD感染が報告された。他の死因や症状はなかったとHPAは報告している。 この血友病患者においてvCJD異常性プリオン蛋白質がどのように感染したかについての最終の評価はまだであるが、vCJDに対する安全性を確保する法案が導入された1999年以前、この患者が、1996年に血漿を提供し６カ月後にvCJDの症状を発現したドナーの血漿から生産されたバッチの第VIII因子製剤で治療されていたことは知られている。本報告は、vCJD異常性プリオン蛋白質が、初めて血友病患者において見いだされた、あるいは血漿分画製剤を使用された患者での最初の報告である。凝固因子製剤によるvCJDの感染のリスクは、医師によって血友病患者へ伝えられており、2004年にはすでに1980から2001年の間に英国の血漿から生産された製剤で治療されていた血友病患者において、vCJD感染のリスクがあると言われていた。この新しい調査結果は、今まで理論的なリスクであったものが、リスクはまだ非常に小さいものであるが、血漿分画製剤で治療された患者にとって、実際のリスクがあることを示す。血液を通しての vCJD感染のリスクが最初に評価されていたときから、多くの予防措置が英国の血液の供給からリスクを最小にするために導入されている。英国の血漿が1999年から凝固因子製剤の製造のために使われておらず、合成された凝固因子製剤が患者に提供されている。					使用上の注意記載状況・その他参考事項等 [使用上の注意記載事項] ヘモフィルM：記載なし リコネイト：現在までに本剤の投与によりvCJD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、本剤の添加物である人血清アルブミンの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。 ファイバ、ガンマガード、プラズマプロテインフラクション、プミネート：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				
	当該事象は、血漿分画製剤を投与された血友病患者で初めて報告されたものである。患者は、供血後にvCJDを発症したドナーから製造された血漿分画製剤を投与されていたことから、血漿分画製剤との関連を否定できないと考える。 なお、本報告で使用された製剤は、非加熱製剤の可能性が高いと考える。また、ヘモフィルMは承認を有しているが、本邦の市場には流通していない。リコネイトについても、販売を中止し本邦において、現在流通していない。ファイバ、ガンマガード、プラズマプロテインフラクション、プミネートについては、当該報告と採血国も異なり、また、これまでにvCJD感染の報告もなく、感染のリスクは低いと考える。	今後も同様の情報収集に努める。				

28

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
一般的名称	4. 乾燥イオン交換樹脂処理人免疫グロブリン（6343420） 5. 加熱人血漿たん白（6343422） 6. 人血清アルブミン（6343410）	研究報告の公表状況		公表国	
販売名（企業名）	6. ブミネート（634341005）				

研究報告の概要		使用上の注意記載状況・その他参考事項等

報告企業の意見	今後の対応

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Health Protection Agency

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vCJD abnormal prion protein found in a patient with haemophilia at post mortem

17 February 2009

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"Since the risk of vCJD transmission through blood was first considered, a number of precautionary measures have been introduced to minimise the risk from the UK blood supply. UK plasma has not been used for the manufacture of clotting factors since 1999 and synthetic clotting factors are provided for all patients for whom they are suitable."

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(factor VIII) through regular injections which helps the blood to clot and minimises the likelihood of long term joint damage.

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7) For further information about vCJD go to:
www.hpa.org.uk/cjd
http://www.hpa.org.uk/vcjdplasmaproducts
http://www.dh.gov.uk/PolicyAndGuidance/HealthAndSocialCareTopics/CJD/fs/en
http://www.blood.co.uk/
http://www.cjd.ed.ac.uk
http://www.nationalprionclinic.org/

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Louise Brown	020 8327 7080
George Fletcher	020 8327 6690

Last reviewed: 17 February 2009

別紙 3-1

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		回	報告日 年 月 日	第一報入手日 2009 年 3 月 4 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				Decontamination of prion protein (BSE301V) using a genetically engineered protease		公表国	使用上の注意記載状況・その他参考事項等
販売名（企業名）		研究報告の公表状況		Dickinson, J. et al., J. Hosp. Infect. 72; 65-70, 2009		英国	BYL-2009-0371
研究報告の概要	英国健康保護局は，手術器具の汚染除去に適すると考えられる新規のプリオン不活化法を開発する試みとして，Bacillus lentus，サブチリシン遺伝子を変異させて得られたアルカリプロテアーゼである MC3（または PrionzymeTM）の使用について説明している。この研究プログラムは，散発的クロイツフェルト・ヤコブ病（CJD）が神経外科手術で伝播するという所見によって進められた。新規変異株は野生型サブチリシンのアルカリプロテアーゼ中，3 つのアミノ酸が置換されており，より高い熱安定性ならびにより速い触媒速度を示す。著者らは本稿において，プリオンに感染したマウスの脳ホモジネートを MC3 とともに pH 8，10 および 12 で in vitro でインキュベートしたところ，すべての免疫反応性 PrP^{Sc}（感染性プリオンタンパク質）が完全に分解されたことをウェスタンブロット法で確認した。同条件下においては部分的にしか分解しないプロテイナーゼ K と比較すると，MC3 はより高い有効性を示した。これらの結果は in vivo の実験によって裏付けられており，この実験では MC3 で分解した感染性マウス脳ホモジネート（iMDH）でスパイクしたマウスの 66.6%が 18 ヵ月を超えて生存し（平均インキュベート期間 447±154 日間），一方 10^{-8} に希釈した iMDH をスパイクしたマウスの生存率は 30.4%（326±162 日間）であった。この有意差は 7 log 超のプリオンクリアランスに相当する。これに対して，プロテイナーゼ K で分解した iMDH をスパイクしたマウスの全生存率は 22.7%（327±151 日）と低かった。著者らは，アルカリ条件下における MC3 による分解は，簡単かつ安全な汚染除去法であり，医療器具に対し非破壊的であることから，医療上の管理および手術器具の汚染除去において広範に適用可能であると結論付けている。				
報告企業の意見	この新規手法は，手術器具のプリオン汚染除去に関する興味深いものであり，また，適用された場合は，集団における外科的伝播のリスクならびに比較的高い有病率（英国など）を低減させるのに寄与すると考えられる。しかしながら，このような技術が血漿分画製剤の製造工程においてプリオンに対する病原体除去・不活化能力をさらに向上させることができるか否かは未だ不明である。						
今後の対応	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。 今後，本手法の血漿分画製剤の製造工程への適用に関する情報収集に努める。						

29

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Decontamination of prion protein (BSE301V) using a genetically engineered protease

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KEYWORDS
Bacillus lentus subtilisin; Bovine spongiform encephalopathy; Inactivation processes; Variant Creutzfeldt–Jakob disease

Summary A previous study has demonstrated the potential of alkaline proteases to inactivate bovine spongiform encephalopathy (BSE301V). Here we explored the use of MC3, a genetically engineered variant of *Bacillus lentus* subtilisin. MC3 was used to digest BSE301V infectious mouse brain homogenate (iMBH). MC3 eliminated all detectable 6H4-immunoreactive material at pH 10 and 12; however, Proteinase K was only partially effective at pH 12. When bioassayed in VM mice, MC3- and Proteinase K-digested iMBH gave respectively 66.6% and 22.7% survival rates. Using a titration series for disease incubation, this equates to a >7 log reduction in infectivity for MC3 and >6 log reduction for Proteinase K. This study demonstrates the potential for thermostable proteases to be developed as effective inactivation processes for prion agents in healthcare management.

Introduction

The emergence of bovine spongiform encephalopathy (BSE) in cattle, believed to have given rise to variant Creutzfeldt–Jakob disease (vCJD) in humans, has generated a number of public health issues. Although any residual risk of eating BSE-contaminated food has been minimised, there continues to be a threat of onward transmission by iatrogenic routes.

Defining the levels of risk of transmission of human prion disorders via surgery, transplant or transfusion has proved difficult due to the unique nature of these agents, uncertainties about the underlying prevalence of the disease and the absence of ante-mortem diagnostic methods. The number of clinical vCJD cases remains low: 167 (dead and alive) definite or probable cases of vCJD in the UK with a further 43 cases worldwide to June 2008 [University of Edinburgh's National Creutzfeldt–Jakob Disease Surveillance Unit (NCJDSU) website]. All clinical cases have been restricted to a single genotype, being homozygous for methionine at codon 129 of the prion protein gene (*PRNP*). Further cases of potential infection, with no known clinical disease, have been identified in the two remaining genotypes: two valine homozygotes from a retrospective study looking at appendix samples and a heterozygote case of blood transfusion related transmission.[1–3] Studies suggest that clinical manifestation in these genotypes may require extended incubations and possibly present with different clinical symptoms.[4,5]

The estimation of transmission risk via vCJD is difficult as certain aspects of its presentation differ significantly from the historical forms of CJD. PrP^{Sc}, a biomarker for prion infectivity, has been found at much higher levels in vCJD-infected lymphoid and peripheral nervous tissue than sporadic CJD (sCJD) infected tissue.[6,7] This suggests that the iatrogenic transmission of vCJD is more likely than sCJD, which is supported by the identification of four cases of transfusion-related vCJD.[3,8,9] However, there are well-documented cases of transmission of sCJD via neurosurgery, transplant of dura mater and human growth hormone.[10–14] Some studies have also suggested that an increased rate of sCJD is associated with an increase in the number of surgical events an individual may have undergone.[15,16] In addition, PrP^{Sc} signal has been shown in sCJD-infected skeletal muscle and adrenal gland, suggesting that infectivity may be more widespread than originally thought.[17,18]

Given the high levels of uncertainty, validated methods for the decontamination of surgical instruments are urgently required. It is widely accepted that autoclaving only partially inactivates TSE agents.[19,20] The World Health Organization (WHO) recommends extended treatment with high concentrations of sodium hypochlorite or sodium hydroxide but these are not suitable for many applications, including routine decontamination of surgical instruments.[21] We report here an extension of our previous work using genetically engineered alkaline proteases in an inactivation model relevant to treatment of surgical instruments.

Methods

Reagents and models

Proteinase K was supplied by Finnzymes (Espoo, Finland) and MC3 ('Prionzyme™') by Danisco US Inc., Genencor Division (Rochester, NY, USA). Preparation of BSE301V mouse brain homogenate, analysis by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis, western blotting and bioassays were carried out as previously described.[22] Animal studies were conducted in compliance with current UK Home Office regulations and licences.

Assessment of the subtilisin variants

The kinetic parameters k_{cat}, K_M, and k_{cat}/K_M were measured by hydrolysis of succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (AAPFpna).[23] To assess thermal stability, purified enzyme (15 μg/mL in 0.1 mol/L glycine, 0.01% Tween-80, pH 10.0) ± 50 mmol/L $CaCl_2$ was incubated at 10 °C for 5 min, 10 °C to 60 °C over 1 min and 60 °C for 20 min, then placed on ice for 10 min and enzyme activity measured by hydrolysis as above.

Results

Protease digestion of infectious MBH (IMBH)

Digestion of BSE301V iMBH by MC3 was assessed at different pH values. At all pH values tested, MC3 was able to reduce the levels of 6H4-reactive material as detected by western blot (Figure 1). The western blots showed the presence of the characteristic triple bands corresponding to the unglycosylated, monoglycosylated and diglycosylated forms of PrP^{Sc}. Bands were fully digested at pH 8, 10 and 12: this is in line with previous results showing that alkaline pH is critical to effective digestion of PrP^{Sc} by subtilisin-type proteases.[22] At pH 4, PrP^{Sc} is only partially digested, whereas greater digestion is observed at pH 2 and 6, suggesting that the conformation at pH 4 may be especially resistant to proteolysis. Proteinase K was relatively poor at eliminating PrP^{Sc} with a smear of material evident even at pH 12, partial digestion of PrP^{Sc} at pH 8 and 10, resolution to PrP^{Sc} at pH 6 and little or no effect at pH 2 and 4 (Figure 1). Digestions of iMBH with MC3 at 50, 60, 70 and 80 °C were analysed by western blot; digestion at 60 °C appeared to be most effective (data not shown).

Assessment of MC3-digested iMBH by bioassay gave a highly significant reduction in the overall levels of infectivity compared with our previous study (Figure 2). Of the mice challenged with MC3-digested iMBH, 66.6% survived to the end of the study with a mean incubation of 447 ± 154 days (assuming that all animals had been culled at 18

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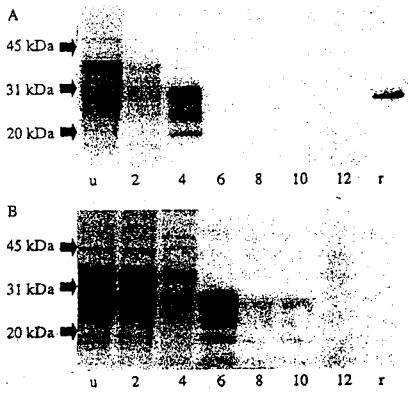

Figure 1 Degradation of 6H4-immunoreactive material by proteases under alkaline conditions. Infectious mouse brain homogenate (iMBH) dialysed against pH buffer indicated, digested for 30 min at 60 °C with 2 mg/mL MC3 (A) and Proteinase K (B). Digestion was assessed by western blot with 6H4. Lane r: recombinant PrP (Prionics); lane u: undigested iMBH in PBS.

months post challenge). This compares with 29.2% (315 ± 173 days) and 30.4% (326 ± 162 days) survival for 10^{-7} and 10^{-8} dilutions of iMBH respectively.[22] The incubation period (447 ± 154 days) is significantly different from the 10^{-8} dilution (326 ± 162 days), equating to a >7 log clearance, as assessed by Kaplan–Meier survival analysis ($P = 0.0195$). Data from our previous study show incubation of iMBH with pH 12 buffer resulted in a reduction in infectivity of ~1 log from 135.8 ± 5.59 to 142.8 ± 9.62 for an equivalent dilution of iMBH. Extended incubation periods of 327 ± 151 days were also obtained for Proteinase K digestion although there was a lower overall survival rate of 22.7%. These values were not significantly different from the 10^{-8} dilution ($P = 0.7971$). Histology was carried out to confirm disease pathology and showed complete correlation between clinical endpoint and signs of disease in the brain (results not shown).

Relevance of the genetic modifications to MC3 and its ability to digest prion material

MC3, a proprietary alkaline protease, represents a genetically engineered variant of the *Bacillus lentus* subtilisin with amino acid changes N76D/

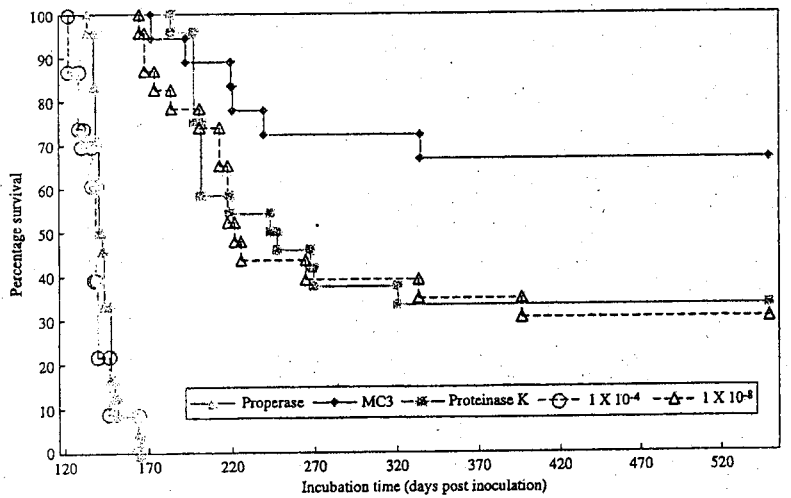

Figure 2 Bioassay survival curves of protease-treated BSE-301V infectious mouse brain homogenate (iMBH) in VM mice. Percentage survival of mice was assessed following protease digestion of 10% BSE301V iMBH. The values were compared to a titration of iMBH in naive MBH (nMBH) as published previously.[22]

Table 1 Thermal stability and enzymatic characteristics of engineered proteases[a]

	Thermal stability ($T_{1/2}$ min)[b] (% of Purafect)	Enzyme kinetic analysis		
		k_{cat} (1/s)	K_M (M)	k_{cat}/K_M
Purafect	100	170	0.78	2.20E + 05
Properase	100	435	1.89	2.30E + 05
MC3	460	830	1.6	5.20E + 05

[a] Measured by hydrolysis of succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide at pH 8.6, 25 °C.
[b] pH 10 buffer.

S103A/V104I.[24] The effects of the three amino acid modifications in MC3 were compared with both the parent enzyme (wild type *Bacillus lentus* subtilisin; Purafect) and Properase (Table I). At pH 10, MC3 shows significantly improved thermal stability with the half-life of the enzyme extended by >4.5-fold compared with Purafect and Properase. Enzyme kinetics were measured by hydrolysis of the substrate AAPFpna. MC3 showed significantly improved catalytic properties with a higher catalytic rate (k_{cat}/K_M) of 5.2×10^5 compared with 2.3×10^5 for Properase and 2.2×10^5 for Purafect.

Discussion

This study demonstrates the ability of genetically engineered proteases, selected for improved stability and catalytic properties at alkaline pH, to digest prion material. We have reduced BSE301V infectivity from ~3 log with Properase in our previous study to >7 log with MC3. This compares favourably with established decontamination/disinfection processes and is beyond the 5 log reduction recommended by the Spongiform Encephalopathy Advisory Committee (SEAC) on its website. MC3 can be easily introduced into existing healthcare practice and avoids the harsh conditions for instruments, operators and the environment that are associated with the WHO guideline conditions of 20 000 ppm active chlorine or 1 mol/L sodium hydroxide. The alkaline pH required for inactivation is comparable with other alkaline prion inactivating cleaners on the market (neodisher® Septo-Clean, Serchem Delta) and is compatible with stainless steel instruments. The 30 min contact time of the enzyme with iMBH was selected on the basis of compatibility with a pre-soak process for the final product.

Since the publication of our earlier study, several other groups have described approaches to the inactivation of prions including further protease-based, detergent-based and gas-phase inactivants.[20,25–35] Comparing data between these models is extremely difficult as a variety of TSE agents, animal models and challenge regimes have been used to assess decontamination. The relevance of different scrapie strains to the inactivation of human prion agents (and BSE) has been questioned, given their lower stability to thermal, chemical and enzymatic denaturation.[20,36,37] Given this uncertainty it would seem most appropriate to use a high titre TSE agent that is directly relevant to human disease in an animal model with no transmission barrier. At the moment such models are limited to the type of strain used here, a murine passaged BSE strain as a model for BSE and vCJD, or vCJD/sCJD in a human transgenic murine model.

Many inactivation studies have used the wire implant model as pioneered by Weissman and colleagues.[38] Although these studies are clearly directly relevant for assessing the effectiveness of prion removal from steel surfaces, they may be limited in their ability to show large reduction values due to the limited volume of material on the wire. Titration curves for 263K scrapie on wires consistently show a very rapid decrease in infectivity at ~4–6 log dilutions compared to the 8 log dilutions typical of this agent in solution.[30,31] Limited data using wire implants in overexpressing transgenic mouse models offer no improvement in sensitivity.[25,33] SEAC has highlighted on its website the need for a standardised model which can be used to compare the efficacy of decontamination technologies. The guidance suggests that a suitable model should mimic the clinical situation, for which the decontamination technology is to be used, as closely as possible. SEAC recognises that infectivity bound to a metal surface, particularly material that is dried on, may represent a tougher challenge than infectivity in solution. However, the guidance also suggests that any decontamination process should demonstrate a reduction in infectivity of ≥5 log, to offer an appreciable improvement over existing practices. In reality this may mean that two models need to be employed: a wire model to demonstrate clinical efficacy and an 'in-solution' model to ensure sufficient dynamic range.

The improved thermal stability and increased catalytic rate of MC3 have clearly contributed to its ability to inactivate prions. The ability of proteases to degrade prions appears to be dependent on the use of reaction conditions or additives that open up the structure of the infectious molecule to allow access to the peptide bonds. Based on previous results we continued to use alkaline conditions as this appeared to be generally more efficient at allowing protease digestion. This was supported by bioassay results showing a reduced log inactivation when experimentally the pH dropped below pH 12 (results not shown). Other groups have used detergent, principally SDS, usually in the presence of heat to effect similar conformational changes to promote protease digestion.[27,28,39]

There are many examples of the use of genetic engineering to enhance the properties of naturally occurring enzymes, and subtilisin-type proteases have been among the foremost of those modified.[22] Properase and MC3 are all engineered versions of the *B. lentus* subtilisin backbone and were selected for these studies on the basis of their stability and activity at alkaline pH. Clearly such an approach has applications in healthcare management with the methods being simple and safe to use and non-destructive to medical instruments.

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Conflict of interest statement

The views expressed in the publication are those of the authors and not necessarily those of the Health Protection Agency or any other funding body.

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References

1. Ironside JW, Bishop MT, Connolly K, *et al.* Variant Creutzfeldt–Jakob disease: prion protein genotype analysis of positive appendix tissue samples from a retrospective prevalence study. *Br Med J* 2006;332:1186–1188.
2. Hilton DA, Ghani AC, Conyers L, *et al.* Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. *J Pathol* 2004;203:733–739.
3. Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* 2004;364:527–529.
4. Bishop MT, Hart P, Aitchison L, *et al.* Predicting susceptibility and incubation time of human-to-human transmission of vCJD. *Lancet Neurol* 2006;5:393–398.
5. Wadsworth JD, Asante EA, Desbruslais M, *et al.* Human prion protein with valine 129 prevents expression of variant CJD phenotype. *Science* 2004;306:1793–1796.
6. Head MW, Ritchie D, Smith N, *et al.* Peripheral tissue involvement in sporadic, iatrogenic, and variant Creutzfeldt–Jakob disease: an immunohistochemical, quantitative, and biochemical study. *Am J Pathol* 2004;164:143–153.
7. Herzog C, Sales N, Etchegaray N, *et al.* Tissue distribution of bovine spongiform encephalopathy agent in primates after intravenous or oral infection. *Lancet* 2004;363:422–428.
8. Llewelyn CA, Hewitt PE, Knight RS, *et al.* Possible transmission of variant Creutzfeldt–Jakob disease by blood transfusion. *Lancet* 2004;363:417–421.
9. 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;368:2061–2067.
10. Bernoulli C, Siegfried J, Baumgartner G, *et al.* Danger of accidental person-to-person transmission of Creutzfeldt–Jakob disease by surgery. *Lancet* 1977;1:478–479.
11. Brown P, Preece M, Brandel JP, *et al.* Iatrogenic Creutzfeldt–Jakob disease at the millennium. *Neurology* 2000; 55:1075–1081.
12. Anonymous. Leads from the MMWR. Update: Creutzfeldt–Jakob disease in a patient receiving a cadaveric dura mater graft. *J Am Med Assoc* 1987;258:309–310.
13. Brooke FJ, Boyd A, Klug GM, Masters CL, Collins SJ. Lyodura use and the risk of iatrogenic Creutzfeldt–Jakob disease in Australia. *Med J Aust* 2004;180:177–181.
14. Swerdlow AJ, Higgins CD, Adlard P, Jones ME, Preece MA. Creutzfeldt–Jakob disease in United Kingdom patients treated with human pituitary growth hormone. *Neurology* 2003;61:783–791.
15. Collins S, Law MG, Fletcher A, Boyd A, Kaldor J, Masters CL. Surgical treatment and risk of sporadic Creutzfeldt–Jakob disease: a case–control study. *Lancet* 1999; 353:693–697.
16. Ward HJ, Everington D, Croes EA, *et al.* Sporadic Creutzfeldt–Jakob disease and surgery: a case–control study using community controls. *Neurology* 2002;59:543–548.
17. Peden AH, Ritchie DL, Head MW, Ironside JW. Detection and localization of PrPSc in the skeletal muscle of patients with variant, iatrogenic, and sporadic forms of Creutzfeldt–Jakob disease. *Am J Pathol* 2006;168:927–935.
18. Herzog C, Riviere J, Lescoutra-Etchegaray N, *et al.* PrPTSE distribution in a primate model of variant, sporadic, and iatrogenic Creutzfeldt–Jakob disease. *J Virol* 2005;79: 14339–14345.
19. Taylor D. Practical aspects of decontamination of the unconventional transmissible agents that cause sporadic and variant Creutzfeldt–Jakob disease and other similar human diseases. *Ig Sanita Pubbl* 2004;60:141–150.
20. Peretz D, Supattapone S, Giles K, *et al.* Inactivation of prions by acidic sodium dodecyl sulfate. *J Virol* 2006;80:322–331.
21. Anonymous. WHO infection control guidelines for transmissible spongiform encephalopathies. Report of a WHO Consultation, Geneva, Switzerland, 23–26 March 1999. HO/CDS/CSR/APH/2000.3. Geneva: WHO.
22. McLeod AH, Murdoch H, Dickinson J, *et al.* Proteolytic inactivation of the bovine spongiform encephalopathy agent. *Biochem Biophys Res Commun* 2004;317:1165–1170.
23. Bonneau PR, Graycar TP, Estell DA, Jones JB. Alteration of the specificity of subtilsin BPN′ by site-directed mutagenesis in its S_1 and S_1' binding sites. *J Am Chem Soc* 1991; 113:1026–1030.
24. Graycar TP, Bott RR, Caldwell RM, *et al.* Altering the proteolytic activity of subtilisin through protein engineering. *Ann NY Acad Sci* 1992;672:71–79.
25. Lawson VA, Stewart JD, Masters CL. Enzymatic detergent treatment protocol that reduces protease-resistant prion protein load and infectivity from surgical-steel monofilaments contaminated with a human-derived prion strain. *J Gen Virol* 2007;88:2905–2914.
26. Muller-Hellwig S, Groschup MH, Pichner R, *et al.* Biochemical evidence for the proteolytic degradation of infectious prion protein PrPsc in hamster brain homogenates by foodborne bacteria. *Syst Appl Microbiol* 2006;29:165–171.
27. Yoshioka M, Murayama Y, Miwa T, *et al.* Assessment of prion inactivation by combined use of Bacillus-derived protease and SDS. *Biosci Biotechnol Biochem* 2007;71:2565–2568.
28. Jackson GS, McKintosh E, Flechsig E, *et al.* An enzyme-detergent method for effective prion decontamination of surgical steel. *J Gen Virol* 2005;86:869–878.
29. Tsiroulnikov K, Rezaï H, Bonch-Osmolovskaya E, *et al.* Hydrolysis of the amyloid prion protein and nonpathogenic meat and bone meal by anaerobic thermophilic prokaryotes and streptomyces subspecies. *J Agric Food Chem* 2004;52: 6353–6360.
30. Fichet G, Comoy E, Dehen C, *et al.* Investigations of a prion infectivity assay to evaluate methods of decontamination. *J Microbiol Methods* 2007;70:511–518.
31. Lemmer K, Mielke M, Kratzel C, *et al.* Decontamination of surgical instruments from prions. II. In vivo findings with a model system for testing the removal of scrapie infectivity from steel surfaces. *J Gen Virol* 2008;89:348–358.
32. Fichet G, Comoy E, Duval C, *et al.* Novel methods for disinfection of prion-contaminated medical devices. *Lancet* 2004;364:521–526.
33. Fichet G, Antloga K, Comoy E, Deslys JP, McDonnell G. Prion inactivation using a new gaseous hydrogen peroxide sterilisation process. *J Hosp Infect* 2007;67:278–286.
34. Baxter HC, Campbell GA, Whittaker AG, *et al.* Elimination of transmissible spongiform encephalopathy infectivity and decontamination of surgical instruments by using radio-frequency gas-plasma treatment. *J Gen Virol* 2005; 86:2393–2399.
35. Yan ZX, Stitz L, Heeg P, Pfaff E, Roth K. Infectivity of prion protein bound to stainless steel wires: a model for testing decontamination procedures for transmissible spongiform encephalopathies. *Infect Control Hosp Epidemiol* 2004;25: 280–283.
36. Taylor DM, Fernie K, Steele PJ, McConnell I, Somerville RA. Thermostability of mouse-passaged BSE and scrapie is independent of host PrP genotype: implications for the nature of the causal agents. *J Gen Virol* 2002;83:3199–3204.
37. Kuczius T, Groschup MH. Differences in proteinase K resistance and neuronal deposition of abnormal prion proteins characterize bovine spongiform encephalopathy (BSE) and scrapie strains. *Mol Med* 1999;5:406–418.
38. Zobeley E, Flechsig E, Cozzio A, Enari M, Weissmann C. Infectivity of scrapie prions bound to a stainless steel surface. *Mol Med* 1999;5:240–243.
39. Langeveld JP, Wang JJ, Van de Wiel DF, *et al.* Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. *J Infect Dis* 2003;188:1782–1789.