別紙様式第2-1

医薬品 研究報告 調査報告書

No. 18

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
				2006. 3. 25	該当なし	
一般的名称	解凍人赤血球濃厚液		研究報告の公表状況	Yamada M; Variant CJD Working Group, Creutzfeldt-Jakob Disease Surveillance Committee, Japan. Lancet. 2006 Mar 11;367(9513):874.	公表国 日本	
販売名(企業名)	解凍赤血球濃厚液「日赤」(日本赤十字社) 照射解凍赤血球濃厚液「日赤」(日本赤十字社)					

研究報告の概要

○変異型クロイツフェルト・ヤコブ病(vCJD)日本初の症例は周期性脳波を示していた

2004年2月、50歳の日本人男性の症例がCJDサーベイランス委員会に報告された。患者は1990年代前半に英国に24日間、フランスに3日間、vCJDの報告がない欧州の他の国に2週間滞在していた。手術歴や輸血歴はなく、プリオン病の家族歴もなかった。2001年6月、48歳の時に漢字を書くことが困難になった。2001年10月、興奮性、人格変化、記憶障害など精神症状を発現、後に異常感覚、運動失調、認知症、異常行動が見られた。2002年8月のMRIでは、視床部にわずかに高信号が認められた。2003年1月、認知症、運動失調、過反射を示した。MRI画像は、視床部に対称性の高信号域を認めた。脳波画像(EEG)は徐波化を示したが、孤発性クロイツフェルト・ヤコブ病(sCJD)に特徴的な周期性同期性放電(PSD)は認められなかった。脳脊髄液の14-3-3蛋白質は陽性だった。コドン129(メチオニン/メチオニン)と219(グルタミン酸/グルタミン酸)に変異はなかった。この時点ではvCJD可能性例の診断基準を満たしていた。運動機能と認知機能は急速に低下した。

2003年12月、無動性無言、ミオクローヌス、錐体路徴候を示した。MRI像は、尾状核、被殻、視床、大脳皮質に高信号を認めた。信号強度は視床より尾状核と被殻の方が高かった。EEGはPSDを示しsCJDの可能性例と診断された。2004年12月、患者は肺炎で死亡した。剖検結果はvCJDの特徴を示した。前頭葉に花弁状空胞が見られ、プロテアーゼ抵抗性PrPは、Collingeの4型PrP^{Sc}、すなわちParchiの2B型だった。

これは日本初のvCJD確定例である。進行性の神経精神疾患はvCJDを示したが、罹病期間は通常(中央値14カ月)よりはるかに長かった。これまで、vCJDで視床枕徴候が陽性から陰性になった症例は複数報告があったが、PSDを伴うvCJD症例は初めてである。この症例は、PSDによってvCJDの可能性が除外されないことを示しており、生前診断でvCJDを見逃さないため、WHOのvCJDの定義の修正を提案する。今回の患者がどこで病原体に暴露したのかは明らかではない。患者の渡英時、英国でのBSE流行は続いており、このとき暴露の可能性がある食品を食べたことは確認されているが、フランスや他の国、または日本で感染した可能性も否定できない。英国で感染したと仮定すると、暴露から発症までの潜伏期間は11. 5年となる。

報告企業の意見	今後の対応
変異型クロイツフェルト・ヤコブ病(vCJD)日本初の症例は周期性脳波を示し、sCJDの可能性例と診断されたが、剖検結果はvCJDの特徴を示していたとの報告である。	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。

使用上の注意記載状況・その他参考事項等

解凍赤血球濃厚液「日赤」
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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

(12)

Case Report

The first Japanese case of variant Creutzfeldt-Jakob disease showing periodic electroencephalogram

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Lancet 2006; 367: 874

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In February, 2004, a 50-year-old Japanese man was referred to our Creutzfeldt-Jakob disease (CJD) surveillance committee. In the first half of 1990, the patient had spent about 24 days in the UK, 3 days in France, and 2 weeks in other European countries where variant CJD (vCJD) has not been reported. He had no history of surgery or blood transfusion, or a family history of prion disease. In June, 2001, aged 48 years, he had difficulty in writing Chinese characters. In October, 2001, he showed mental symptoms, such as irritability, personality changes, and memory impairment, followed by painful dysaesthesia in the legs, ataxia, dementia, and abnormal behaviour. Retrospective review of an MRI taken in August, 2002, showed slight hyperintensity in the thalamus. In January, 2003, he showed dementia, ataxia, and hyperreflexia. Brain MRI at that time showed symmetrical hyperintensity of the thalamus. Electroencephalogram (EEG) showed diffuse slowing, but no periodic synchronous discharges (PSD). The cerebrospinal fluid was positive for 14-3-3 protein. Analysis of the prion protein (PrP) gene showed no mutation, methionine/methionine at codon 129, and glutamic acid/glutamic acid at codon 219. He showed rapid deterioration of both motor and cognitive function.

In December, 2003, he developed akinetic mutism, myoclonus, and pyramidal signs. Brain MRI showed hyperintensity in the caudate, putamen, thalamus, and cerebral cortex, with higher intensity in the caudate and putamen than the thalamus. EEG suggested the presence of PSD (figure, A). The diagnosis of probable sporadic CJD was supported by EEG and MRI findings.[1] He died of pneumonia in December, 2004. Autopsy showed findings characteristic of vCJD, including florid plaques (figure, B) and the Parchi type 2B or Collinge type 4 pattern of protease-resistant PrP (not shown).

This is the first Japanese case of definite vCJD. The progressive neuropsychiatric disorder was consistent with vCJD, although the illness duration was unusually long compared with most vCJD cases reported to date (median 14 months).[2] The findings 19 months after onset of symptoms, showing the pulvinar sign on MRI and the absence of PSD on EEG, together with the clinical features, fulfilled the criteria of probable vCJD.[3] However, 30 months after onset, PSD appeared on EEG, and the pulvinar sign on MRI disappeared following an increase in intensity of other grey matter nuclei, fulfilling the criteria of probable sCJD.[3] There have been no previous reports of PSD on EEG in vCJD, although conversion of a positive to negative pulvinar sign on MRI has been described in a few vCJD patients.[4] Our case shows that PSD does not exclude the possibility of vCJD. We suggest revision of the WHO vCJD case definition[3] to prevent missing cases of vCJD. It is unclear when our patient was exposed to the infective agent. The BSE outbreak in the UK was still increasing when he visited the UK, and it was confirmed that he ate food containing mechanically recovered meat that may be associated with contamination with BSE agent from nervous tissue;[5] however, exposure in France, other European countries, and Japan cannot be excluded. If he was exposed to the BSE agent in the UK (exposure just once would be sufficient to cause vCJD), we calculate the incubation period between such pinpoint exposure and onset of vCJD to be 11·5 years.

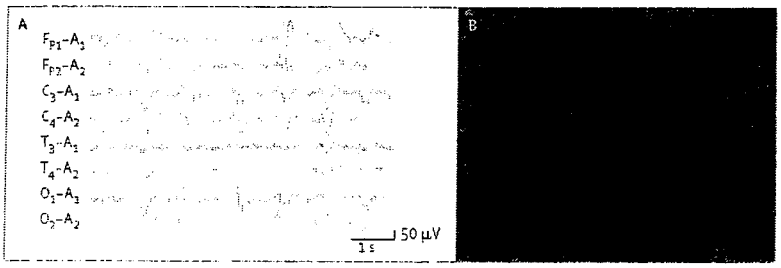

Figure: (A) EEG in August, 2004, 39 months after onset, showing PSD typical of sporadic CJD. (B) The frontal cortex showing florid plaques, severe spongiform changes, and neuronal loss (HE, bar=100 μm). PrP immunohistochemistry showed many PrP-positive plaques and PrP deposits with a pericellular pattern (not shown).

Contributors

Following the identification of this first Japanese case of vCJD by the CJD Surveillance Committee, Japan, we constituted the vCJD Working Group in the Committee. To protect the patient's privacy, we have decided to publish this report under the name of the vCJD Working Group, CJD Surveillance Committee, Japan. The corresponding author is the chair of both the vCJD Working Group and the CJD Surveillance Committee.

Acknowledgments

We thank the patient's family for permission to publish this report. The CJD Surveillance Committee is funded by the Ministry of Health, Labour and Welfare, Japan; the funding source had no involvement in the process of publication of this paper.

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3 The revision of the surveillance case definition for variant Creutzfeldt-Jakob Disease (vCJD). Report of a WHO consultation Edinburgh, United Kingdom 17 May 2001 (http://www.who.int/csr/resources/publications/bse/WHO_CDS_CSR_EPH_2001_5/en/) (accessed Nov 30, 2005).

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5 Food Standards Agency. Agency study reports on historic use of mechanically recovered meat in food 1980–1995 (2002) (http://www1.food.gov.uk/news/pressreleases/2002/oct/report_ mrm) (accessed Nov 30, 2005).

医薬品
医薬部外品　研究報告　調査報告書
化粧品

別紙様式第2-1
番号16

識別番号・報告回数			報告日	第一報入手日 2006年4月21日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	①ポリエチレングリコール処理抗破傷風人免疫グロブリン ②乾燥抗破傷風人免疫グロブリン		研究報告の公表状況	The Lancet Neurology 2006; 5: 393-398	公表国 イギリス	
販売名 （企業名）	①テタノブリンーIH（ベネシス） ②テタノブリン（ベネシス）					

研究報告の概要

＜背景＞
輸血を介したvCJD感染の可能性が確認されたことによって、ヒトの間でこの疾患が蔓延することへの懸念が起きている。我々が目指したのは、vCJD及びBSEの感染効率を比較し、人の感受性に関するコドン129遺伝的多型の影響の評価することを可能にするための医原的伝播のモデルを作成することであった。
＜方法＞
マウスPrP遺伝子の交換によってヒト又はウシのPrP蛋白を発現するマウスを作製した。ヒトのPrP遺伝子はコドン129にMM、VV、MVの遺伝型の多様性を有することから、コドン129のMM、VV、MV遺伝型を有するヒトPrPを発現するために、同じ遺伝背景を有する3つの近交系（inbred line）を作製した。マウスにBSE又はvCJDを接種し、疾患の臨床的及び病理学的な徴候を観察した。
＜結果＞
BSEはウシの系には感染したが、ヒトの系には感染しなかった。対照的に、vCJDはヒトの3つの系全てに感染し、各々の遺伝型で病理学的特徴が異なり、感染効率はMM＞MV＞VVの順であった。
＜解釈＞
BSEからヒトへの感染はおそらく、種の壁の存在によって制限を受けている。しかし、vCJDのヒトからヒトへの感染には、実質的に壁が低くなっているように見える。さらに、全ての個人は、コドン129の遺伝型に関係なく、輸血のようなルートによって、vCJDの2次感染が起こりやすい可能性がある。潜伏期間の長い疾患はこれらのモデルによって予測ができるが、このモデルは、病気の感染を更に拡げる危険性をあることを示しており、これにより重大な公衆衛生の問題を提示している。

報告企業の意見	今後の対応
トランスジェニックマウスを用いたvCJD感染実験により、vCJDはコドン129がMM、VV、MVの全ての遺伝型が感染し、VV及びMVはMM型よりも潜伏期間が長いために、輸血のようなルートによって、vCJDの感染を更に拡げる危険性を示唆した報告である。 これまで血漿分画製剤によってvCJDを含むプリオン病が伝播したとの報告はない。しかしながら、万一vCJD感染者の血漿が本剤の原料に混入した場合には、製造工程においてプリオンを低減し得るとの報告があるものの、製剤から伝播する可能性を完全には否定し得ない。そのため、弊社の血漿分画製剤の製造工程におけるTSE感染性低減に関する検証実験を加速し、自社データを早期に取得し、工程評価を行い、必要に応じて工程改善を実施する予定である。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

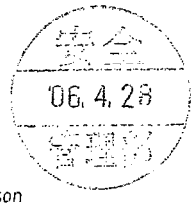
使用上の注意記載状況・その他参考事項等

代表としてテタノブリンーIHの記載を示す。

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

(13)

Predicting susceptibility and incubation time of human-to-human transmission of vCJD



M T Bishop, P Hart, L Aitchison, H N Baybutt, C Plinston, V Thomson, N L Tuzi, M W Head, J W Ironside, R G Will, J C Manson

Summary

Background Identification of possible transmission of variant Creutzfeldt-Jakob disease (vCJD) via blood transfusion has caused concern over spread of the disease within the human population. We aimed to model iatrogenic spread to enable a comparison of transmission efficiencies of vCJD and bovine spongiform encephalopathy (BSE) and an assessment of the effect of the codon-129 polymorphism on human susceptibility.

Methods Mice were produced to express human or bovine prion protein (PrP) by direct replacement of the mouse *PrP* gene. Since the human *PrP* gene has variation at codon 129, with MM, VV, and MV genotypes, three inbred lines with an identical genetic background were produced to express human PrP with the codon-129 MM, MV, and VV genotypes. Mice were inoculated with BSE or vCJD and assessed for clinical and pathological signs of disease.

Findings BSE was transmitted to the bovine line but did not transmit to the human lines. By contrast, vCJD was transmitted to all three human lines with different pathological characteristics for each genotype and a gradation of transmission efficiency from MM to MV to VV.

Interpretation Transmission of BSE to human beings is probably restricted by the presence of a significant species barrier. However, there seems to be a substantially reduced barrier for human-to-human transmission of vCJD. Moreover, all individuals, irrespective of codon-129 genotype, could be susceptible to secondary transmission of vCJD through routes such as blood transfusion. A lengthy preclinical disease is predicted by these models, which may represent a risk for further disease transmission and thus a significant public-health issue.

Lancet Neurol 2006; 5: 393–98

Published Online
March 27, 2006
DOI: 10.1016/S1474-4422(06)70413-6

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Introduction

After the identification of variant Creutzfeldt-Jakob disease (vCJD) in 1996,[1] there have been many attempts to estimate the extent of the UK epidemic. Many individuals are likely to have been exposed to bovine spongiform encephalopathy (BSE) material through their diet; however, there have been only 161 cases of the disease in the UK. The predicted total number of future cases has ranged from the low hundreds[2] to hundreds of thousands.[3] However, findings from a retrospective immunocytochemical study that aimed to detect prion protein (PrP) in appendix and tonsil specimens suggested a prevalence of BSE infection of 237 per million people in the UK.[4] DNA sequence analysis of the PrP gene (*PRNP*) in vCJD has shown that 100% of tested cases are homozygous for methionine at the codon-129 polymorphism compared with about 40% of the general white population and about 70% of sporadic CJD cases. The methionine homozygous genotype (MM) has been included as a limiting variable in most mathematical predictions of the size of the epidemic.[2,3] Identification at autopsy of preclinical vCJD infection in a methionine/valine (MV) heterozygous individual who had received a transfusion of red cells from a donor who later died of vCJD, was the first indication that MM might not be the only susceptible genotype.[5]

Polymorphisms and mutations in *PRNP* in various species can affect disease susceptibility, although the precise mechanisms by which these effects are mediated have not been established.[6,7] Codon 129 of the human *PRNP* gene has been shown to affect the clinicopathological phenotype of disease in CJD and fatal familial insomnia.[8–13] Heterozygosity at *PRNP* codon 129, when compared with homozygous individuals, has been reported to lengthen incubation times in iatrogenic CJD cases associated with growth hormone treatment, and in kuru,[9,14] whereas valine homozygosity (VV) has been proposed to be protective for both BSE and vCJD transmission in studies that used murine models overexpressing human PrP.[15] At a molecular level, the biophysical properties of PrP refolding into the disease associated form (PrP^{Sc}) have been shown to be affected by the codon-129 genotype, with the methionine variant having an increased propensity to form PrP^{Sc}-like structures.[16]

We sought to analyse the transmission characteristics of BSE and vCJD to four inbred lines of transgenic mice after intracerebral inoculation with brain homogenate from cases of vCJD and BSE. We then aimed to use these models to address the apparent low level of vCJD in the human population resulting from exposure to BSE and to predict the potential for human-to-human spread of vCJD and the susceptibility of different genotypes in the human population.

Methods

Transgenic mice

Details of how the gene-targeted transgenic lines were created are supplied as supplementary information

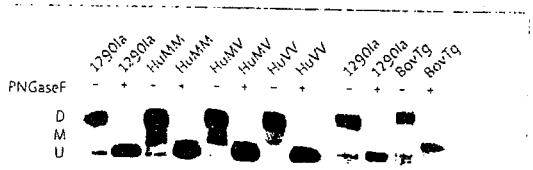

Figure 1: Western blot of brain extract from uninoculated mice showing that PrP^{c} is detected with equivalent electrophoretic mobility and glycoform ratio in all three human transgenic lines
D=diglycosylated PrP^{c} band; M=monoglycosylated PrP^{c} band; U=unglycosylated PrP^{c} band. In the BovTg line, a deglycosylated band is detected of increased molecular weight due to the additional N-terminal octapeptide repeat motif. Protein levels are similar to the wildtype line used in generating the transgenics (1290Ia). Glycosylation is confirmed by the reduction to a single band after deglycosylation with the enzyme PNGaseF. The anti-PrP antibody 7A12 was used for the HumTg blot as it will react with both murine and human PrP, and 8H4 was used for the BovTg blot.

See Online for webappendix

(webappendix). Transgenic mice were anaesthetised with halothane and then injected with 0·02 mL of brain homogenate into the right cerebral hemisphere. The vCJD tissue homogenate (at 10^{-2} dilution) was supplied by the UK National Institute for Biological Standards and Control (Code NHBY0/0003). BSE-infected cattle brain (Veterinary Laboratories Agency, reference BBP 12/92) was prepared by maceration of the tissue in sterile saline to a dilution of 10^{-1}. From 100 days they were scored each week for signs of disease.[17] Mice were killed by cervical dislocation whether they had clinical signs of transmissible spongiform encephalopathy (TSE) or another non-specific disorder. The brain was recovered at post mortem. Half the brain was snap-frozen in liquid nitrogen for biochemical analysis and the remaining half was fixed for histology.

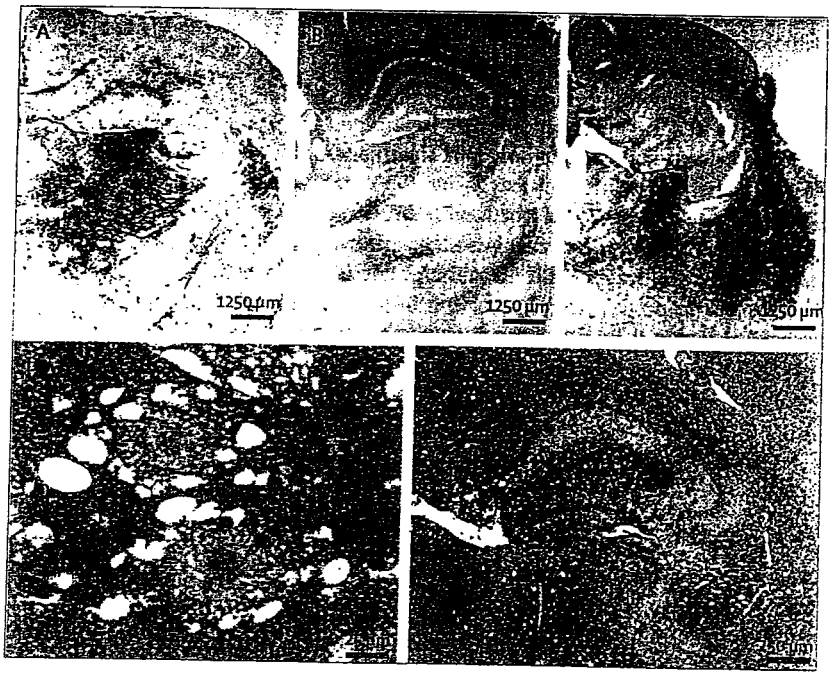

Figure 2: Immunocytochemistry of histological sections with anti-PrP antibody 6H4 showing the cortex, hippocampal, and thalamic regions of the mouse brain with PrP detection (brown)
A–D: Human transgenic mice with vCJD inoculum. A: HuMM mouse 693 days post inoculation. B: HuMV mouse 707 days post inoculation. C: HuVV mouse 693 days post inoculation. D: Florid plaques found in the hippocampus of the HuMM mouse in panel A. Each plaque has an eosinophilic core with a paler halo and is surrounded by a ring of vacuolation (haematoxylin and eosin stain). E: Hippocampal region of a BovTg mouse inoculated with BSE. PrP is deposited in a more diffuse/granular form with occasional plaques.

Procedures

Immunocytochemical detection of disease-associated PrP (PrP^{Sc}) deposits in the brain is a key pathological marker of TSE transmission, and variation in location and morphology of PrP^{Sc} deposits can be affected by both the strain of TSE agent and by the host PrP.[7,18] After fixation in 10% formal saline, brains were treated for 1·5 h in 98% formic acid (to reduce the titre of infectivity for safety reasons), cut transversely into four sections, and embedded in paraffin. We used the Vectastain Elite ABC Kit (Vector Labs, UK) with overnight primary antibody incubation (6H4 at 1:2000; Prionics, Switzerland) for PrP detection. Identification of antibody binding was through deposition of 3,3′-diaminobenzidine chromogen via a horseradish peroxidase reaction. The BSE-inoculated human transgenics were also studied using the Catalysed Signal Amplification kit (DAKO K1500). This kit uses the same principles as the Vector Labs kit, but has an additional step, which amplifies the final detected signal and therefore improves sensitivity.

Scoring of the abundance and location of TSE-associated vacuolation in grey and white matter of the brain is routinely used for diagnosis and strain classification in non-transgenic mice[17,19] and was used to assess all the mice in this study. TSE-related vacuolation was assessed at nine grey-matter regions and three white-matter regions to produce a lesion profile, as previously described.[20,21]

Analysis

Frozen brain samples from the human transgenic mice were homogenised in 0·9% saline to give a 10% suspension. This material was cleared by centrifugation and the supernatant treated with 0·05 g/L proteinase K for 1 h at 37°C, as previously described in detail.[22] The digested product was denatured then loaded onto a 10% Bis/Tris NuPAGE Novex gel (Invitrogen, UK). After electrophoresis the gel was blotted onto polyvinylidine difluoride (PVDF) membrane. We used the ECL+ technique (Amersham Biosciences, UK) with primary antibody 6H4 (Prionics, Switzerland) at 1:40000 and an anti-mouse IgG peroxidase-linked secondary (Amersham Biosciences, UK) at 1:40000 for the detection of PrP. Chemiluminescence was captured on radiographic film. Samples prepared for figure 1 were digested overnight at 37°C with 500 units of PNGaseF (New England Biolabs, UK) and not with proteinase K; the primary antibody was 7A12.[23]

Frozen brain samples from the bovine transgenic mice were homogenised in an NP40 buffer (0·5% v/v NP40,

0·5% w/v sodium deoxycholate, 0·9% w/v sodium chloride, 50mM Tris-HCl pH 7·5) to give a 10% suspension. This material was cleared by centrifugation and the supernatant digested with PNGaseF. The products were denatured then loaded onto a 12% Novex Tris/Glycine gel (Invitrogen, UK). After electrophoresis the gel was blotted onto PVDF membrane. PrP was identified with the SuperSignal West Dura chemiluminescence detection kit (Pierce, UK) with primary antibody 8H4[4] at 1:20000 and an anti-mouse IgG peroxidase-linked secondary (Jackson Immuno Research Laboratories, UK) at 1:10000. Images were captured on radiographic film and with a Kodak 440CF digital imager (figure 1).

Role of the funding source

The sponsors of this study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We first investigated the potential effects of the species barrier between BSE and human beings and any alteration in that barrier once BSE had passed through people in the form of vCJD. We then investigated the effect of the codon-129 polymorphism on human-to-human transmission of vCJD using gene-targeted inbred mice developed by direct replacement of the murine *PrP* gene for the human gene. These mice produce PrP under the control of the normal regulatory elements for PrP and thus express physiological concentrations of PrP with the correct tissue distribution (figure 1). Three inbred lines with an identical genetic background were produced to express human PrP with the codon-129 MM, MV, and VV genotypes (designated HuMM, HuMV, and HuVV, respectively). Each line differs by only a single codon in *PRNP* and in all other respects the mice were genetically identical. Additionally, in an identical manner, we produced mice that express bovine PrP to enable direct comparisons to be made not only between transgenic and wild-type mice, but also between each of the transgenic lines.

Typical clinical signs of TSE disease were seen in more than half (15/22) the BovTg mice inoculated with BSE material with a mean incubation period of 551 days (SD 47). These clinical cases were confirmed by a positive test for the presence of TSE vacuolation or PrP^{Sc} deposition by immunocytochemistry. The lesion profiles generated for targeting and degree of vacuolation showed similar patterns for all positive mice. Immunocytochemical data showed PrP^{Sc} deposition mainly in a diffuse and synaptic form, and also as plaque-like structures, frequently associated with areas of spongiform change (figure 2). Deposition was most abundant in the thalamus and hippocampus, but was recorded throughout other regions of the brain. The cerebral cortex showed only occasional plaque-like structures and the cerebellum had only a few areas of PrP^{Sc} deposition limited to the granule cell layer. Further pathological analysis was undertaken on mice that were culled for reasons other than clinical TSE (intercurrent deaths). This analysis showed that all the brains had pathological signs of TSE disease in terms of vacuolation or PrP deposition. Thus, all the bovine transgenic mice (22/22) seemed to be susceptible to BSE infection, although not all developed clinical signs of infection (tables 1 and 2).

HuMM, HuMV, and HuVV mice were inoculated with BSE material and after extensive pathological analysis all were confirmed as negative for TSE transmission (table 1). Mice of each genotype line were inoculated with vCJD material. Two pathologically confirmed clinically positive mice were seen in the HuMM line (at 497 and 630 days post inoculation), one in the HuMV line (at

	Clinically positive	Vacuolation positive	PrP positive*	Negative†
BovTg (n=22)				
0–400	0	3	6	0
401–500	1	1	0	0
501–600	10	11	5	0
>600	4	4	2	0
HuMM (n=18)				
0–400	0	0	0	4
401–500	0	0	0	5
501–600	0	0	0	2
>600	0	0	0	7
HuMV (n=23)				
0–400	0	0	0	3
401–500	0	0	0	6
501–600	0	0	0	4
>600	0	0	0	10
HuVV (n=22)				
0–400	0	0	0	9
401–500	0	0	0	4
501–600	0	0	0	7
>600	0	0	0	2

*Because most mice were positive by both clinical and vacuolation scoring not all mice were tested by immunocytochemistry for PrP deposition. †Negative by clinical or pathological analysis, or positive by clinical scoring but not confirmed by pathology.

Table 1: **Clinical and pathological scoring of BovTg and human transgenic mice, by number of days after BSE inoculation**

	BSE				vCJD		
	BovTg	HuMM	HuMV	HuVV	HuMM	HuMV	HuVV
Susceptibility*	22/22	0/18	0/23	0/22	11/17	11/16	1/16

*Positives confirmed by immunocytochemistry or lesion profile.

Table 2: **Susceptibility to TSE disease comparison of BovTg and human transgenic mice inoculated with BSE or vCJD**

	Clinically positive	Vacuolation positive	PrP positive	Negative*
HuMM (n=17)				
0–400	0	0	2	2
401–500	1	1	1	2
501–600	0	1	3	2
>600	1	4	5	0
HuMV (n=16)				
0–400	0	0	0	0
401–500	0	0	0	0
501–600	0	0	4	3
>600	1	1	7	2
HuVV (n=16)				
0–400	0	0	0	0
401–500	0	0	0	1
501–600	0	0	0	5
>600	0	1	1	9

*Negative by clinical or pathological analysis, or positive by clinical scoring but not confirmed by pathology.

Table 3: Clinical and pathological scoring of human transgenic mice, by number of days after vCJD inoculation

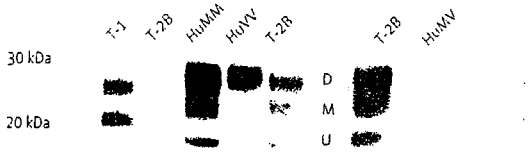

Figure 3: Western blots of brain extract from three transgenic lines inoculated with vCJD
D=diglycosylated PrP^{Sc} band; M=monoglycosylated PrP^{Sc} band; U=unglycosylated PrP^{Sc} band. T-2B corresponds to human vCJD brain homogenate showing the typical PrP^{Sc} type 2B and T-1 corresponds to human sCJD brain homogenate showing the typical PrP^{Sc} type 1 signature. Type 2B and 1 differ in mobility of the unglycosylated band (~19 kDa and ~20 kDa respectively) and the degree of glycosylation (diglycosylated dominant and mono/unglycosylated dominant respectively). All samples were treated with proteinase K. The anti-PrP detection antibody was 6H4. The HuMV and T2-B control blot had to be overexposed as the signal from the HuMV was weak, due to the low levels of PrP^{Sc} seen by immunocytochemistry.

665 days post inoculation), and none in the HuVV line (table 3). HuMM mice were more likely to show disease-associated vacuolation, beginning at around 500 days post inoculation. Six were scored positive and showed similar distribution of vacuolation in the brain, with the highest levels found in the dorsal medulla, thalamus, and cerebellar white matter. By contrast, only a single mouse in each of the HuMV and HuVV groups scored positive for vacuolation at approximately 700 days post inoculation.

Most of the HuMM mice (11/15) showed PrP^{Sc} deposition in most areas of the brain at a relatively early stage (from around 370 days post inoculation), before the vacuolar pathology became evident. From 500 days post inoculation the appearance of vacuolation was accompanied by a significant increase in PrP^{Sc} deposition. By contrast, although PrP^{Sc} deposition was identified in many HuMV mice (11/13), they had little deposition restricted to only a few areas (including the ventrolateral and ventromedial thalamic nuclei and the red nucleus of the mid-brain), even after 700 days post inoculation (figure 2, table 4). Although PrP^{Sc} deposition was clearly present at 581 days, the timing of initial onset of deposition in this line was not established.

Significant levels of PrP^{Sc} deposition were noted in the brain of the subclinical HuVV case. Indeed, these were similar in intensity to those observed in the clinical HuMM cases. Patterns of PrP deposition and plaque formation show differences among the three genotypes, including the presence of florid plaques only in the HuMM mice (table 4).

PrP^{Sc} found in vCJD brain is characterised by a 19 kDa non-glycosylated fragment and the predominance of the diglycosylated form (type 2B).[22] Both biochemical properties of PrP^{Sc} are maintained when vCJD is transmitted to the human transgenic mice, irrespective of their codon-129 genotype (figure 3). Preliminary densitometric analysis suggested that there was an increase in the diglycosylated form in the HuVV mouse compared with the HuMM mouse. Additionally, comparison of PrP^{Sc} from the BSE inoculum and brain material from BovTg mice also confirmed propagation of the predominantly diglycosylated glycoform signature of PrP^{Sc} associated with the BSE/vCJD agent strain (data not shown).

	HuMM	HuMV	HuVV
Vacuolation*	Thalamus (severe); cerebral cortex and hippocampus (mild); cerebellar cortex (minimal)	Thalamus, cerebral cortex, hippocampus, and cerebellar cortex (minimal)	Thalamus and cerebral cortex (severe); hippocampus (mild); cerebellar cortex (minimal)
Plaque formation*	Fibrillary amyloid plaques; florid and non-florid plaques in cerebral cortex and hippocampus; no evidence of plaques in cerebellum	No evidence of amyloid plaques	Amorphous non-fibrillary structures often forming into clusters in cerebral cortex and thalamus
PrP deposition†	Intense staining of plaques in hippocampus and cerebral cortex; plaque-like, pericellular, and amorphous deposits in the hippocampus; synaptic, peri-neuronal, and diffuse perivascular deposits in the thalamus	Occasional small plaque-like deposits and pericellular deposits in the thalamus	Strongly positive large amorphous deposits and clusters of plaques, small plaque-like structures, perivascular aggregates, and sub-pial deposits in the cerebral cortex and thalamus

*Analysed with haematoxilin and eosin staining. †Analysed with immunocytochemical techniques.

Table 4: Comparison of TSE-associated neuropathology in human transgenic mice inoculated with vCJD

Discussion

Although the cattle BSE epidemic in the UK has amounted to more than 180 000 cases since the 1980s, the extent of the human vCJD epidemic has so far remained limited with the total number of cases worldwide currently at 190. One explanation for this apparent discrepancy is that there exists a significant species barrier between cattle and human beings, which limits the susceptibility of the human population to BSE. The data shown here suggest that this could indeed be the case since BSE was readily transmissible to the bovine transgenic mice but not to the human transgenic mice. However, once BSE has passed through human beings in the form of vCJD, the transmissibility of this TSE strain is altered for the human population.

All the human transgenic lines inoculated with BSE were negative for TSE transmission, which suggests that either the human transgenic lines are relatively resistant to transmission of BSE or the incubation time is longer than the length of the experiment (approximately 700 days). BSE transmission previously observed by others, in human transgenic lines overexpressing the human prion protein, could be due to overexpression of the *PrP* gene and may not therefore give a true reflection of the species barrier between BSE and human beings.[15,25,26] This apparent resistance of human transgenic mice to BSE could be explained by a large species barrier and this in turn could explain the low number of vCJD cases in the human population.

vCJD was transmitted to all three human lines with different pathological characteristics for each genotype, and a gradation of transmission efficiency from MM to MV to VV. The greater transmission efficiency in HuMM mice suggests that homozygosity for methionine at codon 129 leads to earlier onset of TSE-related pathological features and clinical disease than for the other two genotypes. The differences in PrP^{Sc} deposition in the HuMM and HuMV lines suggest that the codon-129 polymorphism in human beings is likely to affect the distribution of PrP^{Sc} deposition in the brain. Moreover, the similar numbers that scored positive for PrP deposition in each of the MM and MV groups (11/15 and 11/13 respectively) suggest that the two genotypes might be equally susceptible to vCJD, but with different incubation periods. Titration experiments are needed to fully compare the susceptibility of each line. The single HuVV mouse positive for PrP^{Sc} shows that VV individuals may be susceptible to vCJD with very long incubation times, including a lengthy subclinical phase. Transmission studies from all three genotype mice are now underway to examine the infectious nature of the disease and determine any alterations in the strain characteristics on passage through human transgenic mice. By contrast with published data suggesting that VV individuals cannot propagate the vCJD biochemical phenotype,[15] the data presented here suggest that the PrP^{Sc} type will remain a useful diagnostic feature of secondary vCJD infection irrespective of codon-129 genotype, as has been observed for the two extant cases of transfusion-associated vCJD infection.[5,7]

Transmission of vCJD to the three lines of human transgenic mice indicates that the human population could be at significantly heightened risk of developing disease after iatrogenic exposure to vCJD. Secondary transmission of vCJD has partly removed the cattle-to-human species barrier and has resulted in an agent that can be transmitted from human to human with relative efficiency. Transmission studies in cynomolgus macaques provide further evidence for this agent adaptation as they show reduction in incubation times after serial passage of BSE.[28] Our BSE inoculation at 10^{-1} dilution was compared with vCJD inoculation at 10^{-2} because the latter inoculum was found to be toxic to the mice at 10^{-1}. Use of a higher dose of vCJD inoculum would have maintained or increased the transmission efficiency of vCJD and enhanced the current findings.

Our findings raise concerns relevant to the possibility of secondary transmission of vCJD through blood transfusion, fractionated blood products, or contaminated surgical instruments. For this study mice were injected intracerebrally, whereas the probable human exposure to these agents is by peripheral routes (eg, oral or intravenous), and thus human-to-human exposures might be significantly less efficient. However, it is difficult to know for sure what the practical implications might be in human beings. Peripheral route challenge is in progress; however, BSE transmission studies in primates have shown the intravenous route to be as efficient as the intracerebral route, with an extension of the incubation time.[28]

Although all cases of vCJD up to now have been observed in the MM genotype, this model of human-to-human vCJD transmission suggests that other genotypes are also susceptible. In our experimental setting, all *PRNP* codon-129 genotypes are susceptible to vCJD infection; however, progressive development of pathological TSE features (vacuolation and PrP deposition) is more rapid in the MM-genotype mice. An explanation for this finding might be provided by in-vitro conversion of recombinant human PrP by BSE and vCJD agents, which has shown that PrP with methionine at position 129 is more efficiently converted than PrP with valine, and that conversion by vCJD is significantly more efficient than by BSE.[29] Long incubation periods during which PrP^{Sc} is deposited predicts that, in human beings, infection could be present in all genotypes for a significant period before clinical onset. Incubation periods of more than 30 years have been reported in the human TSE disease kuru.[30]

The possibility that an MV or VV genotype could result in a phenotype distinct from that recognised in vCJD draws attention to the importance of systematic assessment of the clinical, genetic, pathological, and

biochemical features of all human prion diseases. Our findings indicate that for human-to-human vCJD infection it should be assumed that all codon-129 genotype individuals (not just MM) can be infected, that long incubation times can occur, and that a significant level of subclinical disease might be present in the population.

Contributors

MTB, PH, and CP did immunocytochemical and western blot analysis; JCM, NT, HNB, and LA produced the transgenic mouse lines; JWI supplied vCJD case material and reviewed the neuropathology; VT did the mouse inoculations; and MTB, PH, MWH, RGW, JWI, and JCM prepared the manuscript.

Conflicts of interest

We have no conflicts of interest.

Acknowledgments

We thank the Animal Facility staff at IAH/NPU and especially Emma Murdoch, Leanne Frame, Sally Shillinglaw, Tricia Matheson, Becky Greenan, Fraser Laing, and Kris Hogan, under the supervision of V Thomson and Irene McConnell, for their assistance; the pathology division for sectioning the mouse brains and assessing the levels of TSE vacuolation (Sandra Mack, Gillian McGregor, Aileen Boyle, and Anne Suttie); Tricia McBride for reviewing the neuropathology; Margaret Le Grice, Suzanne Lowrie, and Diane Ritchie, under the supervision of Linda McCardle, for technical assistance with immunocytochemistry at the CJDSU; and Alex Peden and Helen Yull, under the supervision of MW Head, for help with western blot methodology and CJD control samples. This study was funded by the UK Department of Health, Defra, and the Medical Research Council.

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一般的名称	①ポリエチレングリコール処理抗破傷風人免疫グロブリン ②乾燥抗破傷風人免疫グロブリン	研究報告の公表状況	PloS Pathogens 2006; 2: e32	公表国 アメリカ
販売名（企業名）	①テタノブリンーIH（ベネシス） ②テタノブリン（ベネシス）			

研究報告の概要

確認されていない環境中の感染性の貯蔵場所が、ヒツジ、シカ及びエルクのプリオン病（TSE）の自然感染の一因となっている。プリオンの感染性は、病気の動物の畜殺及び感染した死骸の腐敗を通して土壌環境に入る可能性がある。廃棄手段として、TSE 汚染のウシ、ヒツジ及びシカを埋めることにより、地表下への意図しない混入が起こる。我々は、ありふれた土壌ミネラルと病気に関係するプリオン蛋白の相互作用を検討することによって、土壌がTSE貯蔵場所として作用する可能性を調査した。
本調査で、2種類の粘土ミネラル（モンモリロナイト及びカオリナイト）、石英及び4種類の無処理土壌サンプルに PrP^{Sc} が吸着することがわかった。加えて、モンモリロナイトと PrP^{Sc} の吸着は強固であり、低pH（pH2.5）並びに高pH（pH11.5）、イオン強度の増加（0.1M又は1.0M NaCl）及び水素結合を切断し土壌鉱物からたんぱく質の分離させるのに有効であるカオトロピック剤（8M尿素及び8Mグアニジン）でも検出可能な PrP^{Sc} を遊離することができず、10%SDS存在下の100℃煮沸のみ PrP^{Sc} を遊離することができた。モンモリロナイトから分離した PrP^{Sc} はN末端で切れていた。また、モンモリロナイトに吸着した PrP^{Sc} は感染実験により感染性を維持していることが確認できた。我々の研究結果は、土壌環境に放出された PrP^{Sc} は生物に利用できる形態で維持され、プリオン病の動物感染を永続させるとともに、他の種をこの感染性病原体に曝露させる可能性があることを示している。

使用上の注意記載状況・その他参考事項等

代表としてテタノブリンーIHの記載を示す。

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

報告企業の意見	今後の対応
土壌環境に放出されたPrP^{Sc}は生物に利用できる形態で維持され、プリオン病の動物感染を永続させることにより、他の種をこの感染性病原体に曝露させる可能性があることを示唆した報告である。 これまで血漿分画製剤によってvCJDを含むプリオン病が伝播したとの報告はない。しかしながら、万一vCJD感染者の血漿が本剤の原料に混入した場合には、製造工程においてプリオンを低減し得るとの報告があるものの、製剤から伝播する可能性を完全には否定し得ない。そのため、弊社の血漿分画製剤の製造工程におけるTSE感染性低減に関する検証実験を加速し、自社データを早期に取得し、工程評価を行い、必要に応じて工程改善を実施する予定である。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

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Prions Adhere to Soil Minerals and Remain Infectious

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An unidentified environmental reservoir of infectivity contributes to the natural transmission of prion diseases (transmissible spongiform encephalopathies [TSEs]) in sheep, deer, and elk. Prion infectivity may enter soil environments via shedding from diseased animals and decomposition of infected carcasses. Burial of TSE-infected cattle, sheep, and deer as a means of disposal has resulted in unintentional introduction of prions into subsurface environments. We examined the potential for soil to serve as a TSE reservoir by studying the interaction of the disease-associated prion protein (PrP^{Sc}) with common soil minerals. In this study, we demonstrated substantial PrP^{Sc} adsorption to two clay minerals, quartz, and four whole soil samples. We quantified the PrP^{Sc}-binding capacities of each mineral. Furthermore, we observed that PrP^{Sc} desorbed from montmorillonite clay was cleaved at an N-terminal site and the interaction between PrP^{Sc} and Mte was strong, making desorption of the protein difficult. Despite cleavage and avid binding, PrP^{Sc} bound to Mte remained infectious. Results from our study suggest that PrP^{Sc} released into soil environments may be preserved in a bioavailable form, perpetuating prion disease epizootics and exposing other species to the infectious agent.

Citation: Johnson CJ, Phillips KE, Schramm PT, McKenzie D, Aiken JM, et al. (2006) Prions adhere to soil minerals and remain infectious. PLoS Pathog 2(4): e32. DOI: 10.1371/journal.ppat.0020032

Introduction

Transmissible spongiform encephalopathies (TSEs, prion diseases) are a group of fatal neurodegenerative diseases that affect a variety of mammalian species and include bovine spongiform encephalopathy (BSE, "mad cow" disease), chronic wasting disease (CWD) of deer and elk, sheep scrapie, and Creutzfeldt-Jakob disease in humans [1]. The agricultural, economic, and social impacts of prion diseases have been intensified by evidence suggesting transmissibility of BSE to humans [2]. The putative infectious agent in these diseases, designated PrP^{Sc}, is a misfolded isoform of the normal cellular prion protein (PrP^{C}). The amino acid sequences of PrP^{Sc} and PrP^{C} are identical [3]; normal and abnormal forms of the protein differ only in conformation. No differences in posttranslational covalent modification have been demonstrated [3]. Circular dichroism and infrared spectroscopy indicate that the disease-specific isoform has a higher β-sheet and lower α-helix content than PrP^{C} [4]. The normal isoform is soluble and primarily monomeric in solution, whereas PrP^{Sc} forms insoluble aggregates.

Sheep scrapie and cervid CWD are unique among TSEs, because epizootics can be sustained by horizontal (animal-to-animal) transmission [5,6]. Routes of natural transmission remain to be clarified, but available evidence indicates that an environmental reservoir of infectivity contributes to the maintenance of these diseases in affected populations [6–8]. The expanding range of CWD (several regions of North America and Korea) increasingly brings domestic livestock, companion animals, and wildlife species into contact with infected animals and carcasses, and shedded TSE agent, raising the possibility of cross-species transmission. This was demonstrated by the recent detection in Colorado, USA, of a free-ranging, CWD-infected moose, a species not previously known to be affected by the disease in the wild [9].

Although other modes of environmental transmission of scrapie and CWD have been proposed (e.g., flesh flies [10], hay mites [11]), several lines of evidence point to soil as a reservoir for TSE infectivity. TSE infectivity exhibits remarkable resistance to inactivation by most chemical agents, radiation, and heat [12] and has been shown to persist after burial in soil for at least 3 y [13]. Anecdotal observations of healthy sheep contracting scrapie after occupying fields previously containing diseased animals have been reported [7,8]. Although these older studies did not account for the genetic susceptibility of the sheep under study, they suggest that scrapie agent can persist in the environment for years. Recent controlled field experiments provide more compelling evidence of the environmental persistence of prions. Miller et al. [14] demonstrated that naïve mule deer could contract CWD

Editor: David Westaway, University of Toronto, Canada

Received December 20, 2005; **Accepted** March 8, 2006; **Published** April 14, 2006

DOI: 10.1371/journal.ppat.0020032

Abbreviations: BH, brain homogenate; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; dpi, days postinoculation; Kte, kaolinite; Mte, montmorillonite; PK, proteinase K; PrP^{C}, normal cellular isoform of the prion protein; PrP^{Sc}, disease-associated prion protein; TSE, transmissible spongiform encephalopathy

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Synopsis

Transmissible spongiform encephalopathies (TSEs) are a group of incurable diseases likely caused by a misfolded form of the prion protein (PrP^{Sc}). TSEs include scrapie in sheep, bovine spongiform encephalopathy ("mad cow" disease) in cattle, chronic wasting disease (CWD) in deer and elk, and Creutzfeldt-Jakob disease in humans. Scrapie and CWD are unique among TSEs because they can be transmitted between animals, and the disease agents appear to persist in environments previously inhabited by infected animals. Soil has been hypothesized to act as a reservoir of infectivity because PrP^{Sc} likely enters soil environments through urinary or alimentary shedding and decomposition of infected animals. In this manuscript, the authors test the potential for soil to serve as a reservoir for PrP^{Sc} and TSE infectivity. They demonstrate that PrP^{Sc} binds to a variety of soil minerals and to whole soils; they also quantitate the levels of protein binding to three common soil minerals and show that the interaction of PrP^{Sc} with montmorillonite, a common clay mineral, is remarkably strong. PrP^{Sc} bound to Mte remained infectious to laboratory animals, suggesting that soil can serve as a reservoir of TSE infectivity.

when housed in paddocks previously inhabited by infected animals or containing decomposed infected carcasses.

TSE agents directly enter the environment when carcasses of infected animals decompose [13], through alimentary shedding of the agent from gut-associated lymphoid tissue [15,16], or from urinary excretion from infected, nephritic animals [17]. Furthermore, bovine, sheep, and deer TSE agents have been introduced to soil environments through the burial of diseased carcasses and other infected material [18]. Animals ingest soil both deliberately and incidentally [19]. Cattle, deer, sheep, and other animals can consume hundreds of grams of soil daily [20,21]. Taken together, these data support the notion that PrP^{Sc}-contaminated soil may allow intraspecies TSE transmission and enhance the likelihood of spread to other species. As a first step toward understanding the role of soil as a reservoir of TSE infectivity, we investigated the binding of PrP^{Sc} to common soil minerals and whole soils and examined the infectivity of mineral-bound prions.

Results

Binding of PrP^{Sc} to Soil Minerals

We examined the sorption of purified PrP^{Sc} to three common soil minerals (Table S1): quartz, montmorillonite (Mte, an expandable layered silicate clay mineral), and kaolinite (Kte, a nonexpandable phyllosilicate mineral). Quartz of two particle sizes was employed in sorption experiments: fine sand (hydrodynamic diameter [d_h] = 125–250 μm), representing quartz concentrated in the sand and silt fractions of soils, and microparticles (d_h = 1–5 μm), representing quartz present in the coarse clay fraction [22]. Purified PrP^{Sc} (~0.2 μg) was introduced into aqueous suspensions (pH 7.0) of each soil mineral and subjected to 2-h mixing. Unbound PrP^{Sc} was separated from bound protein by centrifugation through a 750-mM sucrose cushion. Bound and unbound fractions were analyzed by SDS-PAGE and immunoblotting.

The extent of PrP^{Sc} sorption differed among the mineral particles examined. All detectable PrP^{Sc} adsorbed to the expandable clay mineral Mte (Figure 1A). X-ray diffraction analysis provided no evidence that PrP^{Sc} entered Mte interlayer spaces (Mte d_{001} spacings were 1.22 nm and 1.47 nm before and after PrP^{Sc} adsorption, respectively); prion protein appeared to adsorb to only external clay surfaces. PrP^{Sc} did not associate with an equal mass of fine quartz sand at levels detectable by immunoblotting (Figure 1A). A large degree of PrP^{Sc} binding to the nonexpandable clay mineral Kte was observed when the surface area was matched to that of external Mte surfaces (Figure 1A). The limited association of PrP^{Sc} with fine quartz sand was at least in part attributable to the much smaller specific surface area of these particles as compared to kaolinite and external Mte surfaces (Table S1). When quartz surface area was matched to that of external Mte surfaces, all detectable PrP^{Sc} adsorbed to quartz (Figure 1A).

Adsorption Capacities of Soil Minerals for PrP^{Sc}

The amount of PrP^{Sc} adsorbed to Mte was semiquantitatively assessed by serial dilution of samples to the limit of immunoblotting detection. The dilution at which no detectable immunoreactivity remained provided a basis for comparison with samples lacking immunoreactivity before dilution. PrP^{Sc} desorbed from Mte still exhibited immunoreactivity after 100-fold dilution, indicating that the amount of prion protein adsorbed to Mte exceeded that in samples without immunoreactivity (e.g., unbound PrP^{Sc} in experiments with Mte) by at least two orders of magnitude (Figure 1B). Furthermore, this result suggests that fine quartz sand was saturated by at least 100-fold less PrP^{Sc} ($\leq$ 0.002 μg) than used for sorption experiments (Figure 1A).

To assess the PrP^{Sc}-binding capacity of the other soil minerals, increasing quantities of PrP^{Sc} were added to each mineral. Protein desorbed from mineral particles was serially

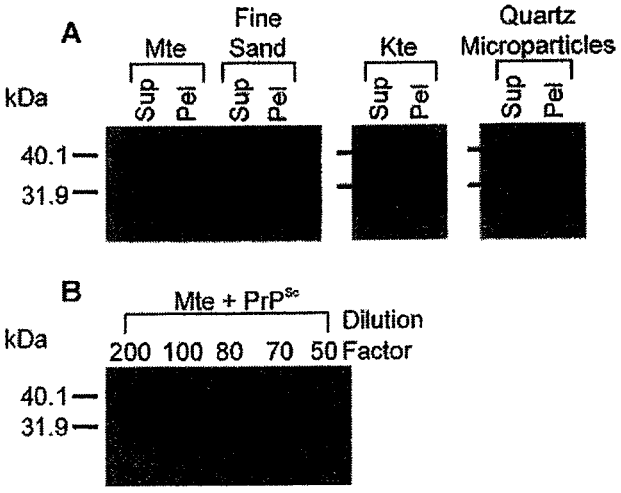

Figure 1. PrP^{Sc} Adsorption to Clay Minerals and Quartz Microparticles Substantially Exceeded That to Fine Quartz Sand

(A) Detectable amounts of PrP^{Sc} adsorbed to Mte and Kte but not to fine quartz sand (d_h = 125–250 μm). PrP^{Sc} desorbed from Mte was of lower molecular mass than the starting material. Adsorption to quartz was observed when quartz microparticles (d_h = 1–5 μm) were employed and surface area was matched to Mte.

(B) Immunoblotting sensitivity was determined by dilution of Mte-adsorbed PrP^{Sc} to the limit of detection. Protein was desorbed from Mte in 50 μl of SDS-PAGE sample buffer at 100 °C and serially diluted. Immunoblots used monoclonal antibody (mAb) 3F4. Pel, PrP^{Sc} associated with pelleted mineral particles; Sup, unbound PrP^{Sc} in supernatant.

DOI: 10.1371/journal.ppat.0020032.g001