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一般的名称			研究報告の公表状況	Species barrier for chronic wasting disease by in vitro conversion of prion protein. Li, L. et al, Biochem. Biophys. Res. Com. 364(4), 796-800 (2007)	公表国 カナダ	
販売名（企業名）						

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
本稿の著者らは，慢性消耗性疾患（北米シカに影響を及ぼす伝染性海綿状脳症）は，in vitro アッセイにおいてある特定の条件下で種の壁をすり抜けて感染することを明らかにした。本アッセイは，異種動物からの正常な脳ホモジネート（正常 PrP^{C}）を基質として，エルク（ヨーロッパヘラジカ）の異常プリオンタンパク質（PrP^{Sc}）とともにインキュベートするものである。標準の条件（pH 7.4）下では，エルク（ヨーロッパヘラジカ）PrP^{Sc} は同種系列〔トナカイ，ムース（アメリカヘラジカ），カリブー及びエルク（ヨーロッパヘラジカ）〕の PrP^{C} をタンパク質分解酵素耐性アイソフォームへと変換させたが，異種 PrP^{C}（ヒト，マウス，ヒツジ，ウシ，ハムスター）については，PrP^{C} のタンパク質配列が全ての種で 90%以上保持されているにもかかわらずタンパク質分解酵素耐性アイソフォームへ変換されたものは僅かであった。しかしながら，低 pH（3.5）による部分変性の条件下では，PrP^{Sc} によるタンパク質分解酵素耐性アイソフォームへの変換は全ての種で劇的に増大した。これより，基質の部分変性によって構造上の変化が起こり，遠隔種間の種の壁を越えることが示唆される。	BYL-2008-0303

報告企業の意見	今後の対応
異常プリオン PrP^{Sc} によるアイソフォーム変換への感度および耐性は，基質である PrP^{C} の立体構造が重要であるとしているが，生物学的な関連性については疑問が残る。	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。

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Species barriers for chronic wasting disease by *in vitro* conversion of prion protein

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Abstract

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy that can affect North American cervids (deer, elk, and moose). Using a novel *in vitro* conversion system based on incubation of prions with normal brain homogenates, we now report that PrP^{CWD} of elk can readily induce the conversion of normal cervid PrP (PrP^{C}) molecules to a protease-resistant form, but is less efficient in converting the PrP^{C} of other species, such as human, bovine, hamster, and mouse. However, when substrate brain homogenates are partially denatured by acidic conditions (pH 3.5), PrP^{CWD}-induced conversion can be greatly enhanced in all species. Our results demonstrate that PrP^{C} from cervids (including moose) can be efficiently converted to a protease-resistant form by incubation with elk CWD prions, presumably due to sequence and structural similarities between these species. Moreover, partial denaturation of substrate PrP^{C} can apparently overcome the structural barriers between more distant species.

Keywords: CWD; PrP^{C}; PrP^{Sc}; *In vitro* conversion; Species barrier

Chronic wasting disease (CWD) is a cervid form of transmissible spongiform encephalopathy (TSE) or prion disease. CWD's rapid spread from' Colorado to other states [1,2], to Canadian provinces (Alberta, Saskatchewan) [1] and to Korea [2,3] has raised concerns about its species tropism [4–6]. CWD has been transmitted to cattle via intracerebral inoculation [7], and to other animals, including ferrets, mink, and goats [8,9]. Reports documenting CWD prions in the muscle [10,11], blood, and saliva [12] of infected cervids, have heightened interest in the disease by public health agencies [13].

CWD and other TSEs are believed to be due to the template-directed accumulation of disease-associated prion protein, generically designated PrP^{Sc}. PrP^{C} in brain homogenates can be converted to a protease-resistant form by incubation with PrP^{Sc} "seeds" which are thought to recapitulate the template-directed misfolding of prion protein in disease [14,15], including protein misfolding cyclic amplification (PMCA) [15]. We have previously reported that partially denatured human brain PrP^{C} (which may mimic a PrP conversion intermediate [16]) is a superior substrate for templated *in vitro* conversion compared with untreated PrP^{C} in an incubation-shaking assay that does not utilize PMCA sonication [17].

Materials and methods

Reagents and antibodies. Proteinase K (PK) was purchased from Invitrogen. Mouse monoclonal antibody 6H4 was from Prionics Co. (Zürich, Switzerland). Horseradish peroxidase-conjugated sheep anti-

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mouse antibody was purchased from Amersham Biosciences. All other chemicals were purchased from Sigma unless specified otherwise.

Brain tissues and homogenate preparation. All brain samples were obtained from the disease control and surveillance programs of the Canadian Food Inspection Agency (CFIA) and were harvested within 24 h of death. The normal brain tissue was determined to be free of neurological disorders on the basis of neuropathological examination. The presence of PrP^{Sc} in brain tissue from an elk with clinical chronic wasting disease (CWD) was confirmed by immunohistochemistry and PK resistance on immunoblotting analysis. All tissues were frozen immediately after collection and stored at −80 °C. Ten percent (w/v) brain homogenates were prepared in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 10 mM Tris–HCl, pH 7.5) as previously described [17].

Preparation of acid/GdnHCl-treated PrP^{C}. The preparation was followed as previously described [17], in brief, 100 µl of 10% brain homogenate was mixed with an equal volume of 3.0 M guanidine hydrochloride GdnHCl (final concentration of 1.5 M) in PBS at pH 7.4 or pH 3.5 adjusted with 1 M HCl, and incubated for 5 h at room temperature with shaking. After that, samples were precipitated with methanol and resuspended in 100 µl of PBS (pH 7.4) with 0.05% SDS, 0.5% Triton X-100.

In vitro conversion of acid/GdnHCl-treated PrP^{C}. In vitro conversion was performed in a 50 µl volume of the appropriate test substrate material (49 µl of normal brain homogenate + 1 µl CWD brain homogenate in a 1:50 dilution as the prion template). The sample was then incubated in a thermomixer at 37 °C for 12 h with shaking. After PK digestion and boiling in the loading buffer, the samples were subjected to SDS–PAGE and immunoblotting.

Proteinase K resistance and immunoblotting. To determine the PK-resistance of the PrP, 20 µl of the sample was incubated with PK at 100 µg/ml for 1 h at 37 °C, and the digestion reaction was terminated by addition of PMSF to 2 mM of final concentration. Proteins were separated by NuPAGE 4–12% pre-cast Bis–Tris gel (Invitrogen) and electrotransferred onto PVDF membranes. 6H4 was used as primary antibody (1:5000) and horseradish peroxidase-conjugated sheep anti-mouse IgG as secondary antibody. The proteins were visualized by enhanced chemiluminescence + Plus (ECL + Plus, Amersham Biosciences), the blots were scanned and were analyzed by Quantity One (Bio-Rad) software. At least eight experiments were performed on each species.

Results and discussion

Sequence alignment of prion protein

CWD appears to be freely transmitted among susceptible species of cervids by direct or environmentally medi-

A

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          1                                                  50
Rangifer  MVKSHIGSWI LVLFVAMWSD VGLCKKRPKP GGGWNTGGSR YPGQGSPGGN
Elk       MVKSHIGSWI LVLFVAMWSD VGLCKKRPKP GGGWNTGGSR YPGQGSPGGN
Moose     MVKSHIGSWI LVLFVAMWSD VGLCKKRPKP GGGWNTGGSR YPGQGSPGGN

          51                                                 100
Rangifer  RYPPQGGGGW GQPHGGGWGQ PHGGGWGQPH GGGWGQPHGG GGWGQGGTHS
Elk       RYPPQGGGGW GQPHGGGWGQ PHGGGWGQPH GGGWGQPHGG GGWGQGGAHS
Moose     RYPPQGGGGW GQPHGGGWGQ PHGGGWGQPH GGGWGQPHGG GGWGQGGTHS

          101                                                150
Rangifer  QWNKPSKPKT NMKHVAGAAA AGAVVGGLGG YMLGSAMSRP LIHFGNDYED
Elk       QWNKPSKPKT NMKHVAGAAA AGAVVGGLGG YMLGSAMSRP LIHFGNDYED
Moose     QWNKPSKPKT NMKHVAGAAA AGAVVGGLGG YMLGSAMSRP LIHFGNDYED

          151                                                200
Rangifer  RYYRENMYRY PNQVYYRPVD QYNNQNTFVH DCVNITVKQH TVTTTTKGEN
Elk       RYYRENMYRY PNQVYYRPVD QYNNQNTFVH DCVNITVKQH TVTTTTKGEN
Moose     RYYRENMYRY PNQVYYRPVD QYNNQNTFVH DCVNITVKQH TVTTTTKGEN

          201                                                250
Rangifer  FTETDIKMME RVVEQMCITQ YQRESQAYYQ RGASVILFSS PPVILLISFL
Elk       FTETDIKMME RVVEQMCITQ YQRESQAYYQ RGASVILFSS PPVILLISFL
Moose     FTETDIKMME RVVEQMCITQ YQRESQAYYQ RGASVILFSS PPVILLISFL

          251  256
Rangifer  IFLIVG
Elk       IFLIVG
Moose     IFLIVG
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Fig. 1. Prion protein amino acid sequence alignment. (A) Prion protein sequence alignment of caribou/reindeer (rangifer), elk and moose. Protein sequences of PrP^{C} in cervid group are highly conserved, except for one amino acid polymorphism boxed in grey. (B) Prion protein sequence alignment of elk and other species (hamster, human, mouse, bovine, and sheep). PrP is >90% conserved.

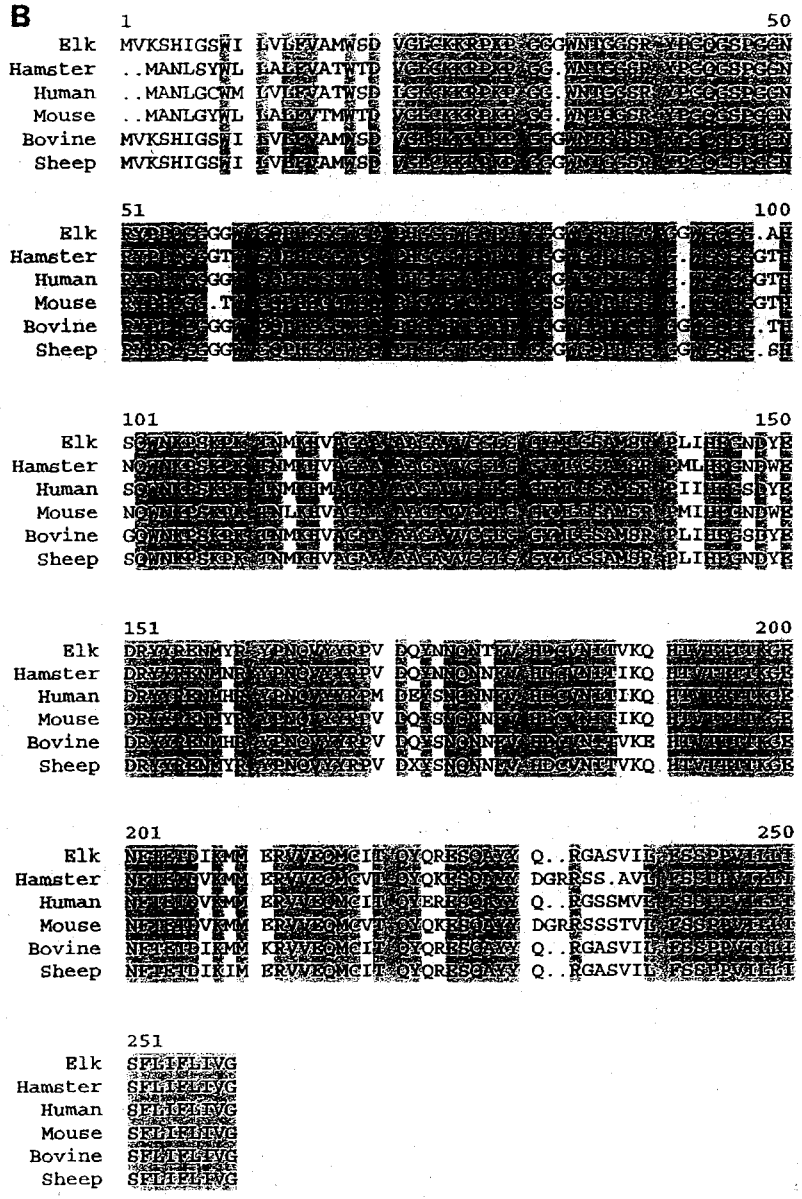

Fig. 1 (*continued*)

ated horizontal contact [5,9,18,19]. We aligned the amino acid sequences from species of cervid species which were used in the experiment: elk (*Cervus elaphus*; GenBank Accession No. CAA70902) reindeer/caribou, (*Rangifer tarandus*; GenBank Accession No. AAZ81477—reindeer is the European name for wild caribou), and moose (*Alces alces*; GenBank Accession No. AAZ81479) (Fig. 1A). The protein sequence of these three cervid species is highly conserved, with only one amino acid polymorphism reported in GenBank. We also aligned the amino acid sequences of elk with other species, such as hamster, human, mouse, bovine, and sheep, which reveals that the protein sequence of PrP^{C} is more than 90% conserved (Fig. 1B).

In vitro conversion of various species with CWD prion template

Normal brain homogenates from elk, reindeer, moose, caribou, human, hamster, mouse, bovine, and sheep, which were incubated with CWD-affected elk brain "seeds", were tested for conversion to a protease-resistant PrP isoform (Fig. 2) as previously described for human CJD *in vitro* conversion [17]. As a negative control, *Prnp* null mouse brain showed no signal corresponding to PK-resistant PrP^{Sc} (Fig. 2, K/O mouse bar). Partial denaturation of normal brain homogenates induced by exposure to low pH and guanidine enhanced *in vitro* conversion to PK-resistant PrP^{Sc} (Fig. 2) has been previously reported for the human

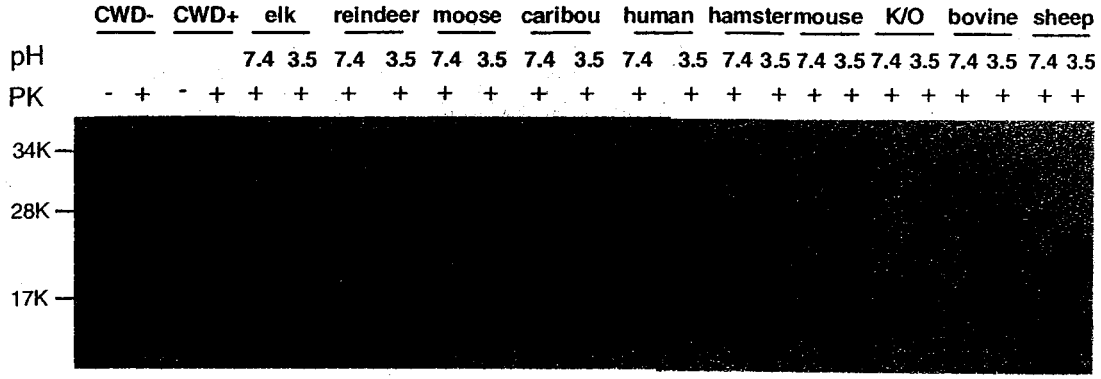

Fig. 2. *In vitro* conversion of treated PrP in the presence of PrP^{Sc} from CWD elk brain. Immunoblots of the PK-resistant PrP isoforms with 6H4 antibody. Samples were treated with GdnHCl and incubated in PBS (pH 7.4) with 0.05% SDS and 0.5% Triton X-100, at 37 °C for 12 h with shaking in the presence of trace amount of elk PrP^{Sc}. CWD−, normal elk brain homogenate as control; − and + indicates the PK treatment. CWD+, elk CWD brain homogenate as a control. The rests are the amplification of PrP^{Sc} in the different species, using elk CWD as seed, treated or untreated with acid (pH 7.4 or pH 3.5).

system [17]. All samples of normal brain contained PrP, which was sensitive to PK digestion (elk shown in Fig. 2, other species not shown). Five microliters of CWD brain homogenate was barely visible after PK digestion (Fig. 2), which was 25-fold greater than the dilution-adjusted CWD seed used in conversion system, excluding artifact from input PrP^{Sc}. Bands of the PK-resistant PrP^{Sc} form were present at ~21 kDa in all the species under acidic conditions (pH 3.5), except for the *Prnp* null mouse (Fig 2). However, PK-resistant PrP^{Sc} was poorly generated in some species in which the brain homogenates were treated under neutral conditions (pH 7.4), such as in human, hamster, mouse, bovine, and sheep. For homogenates treated at neutral pH (pH 7.4), the progression from most susceptible to least susceptible was: elk, reindeer > moose > caribou > hamster > human, bovine, sheep > mouse, with no detected conversion in *Prnp* null mouse brain.

PrP conversion efficiency enhancement by partial denaturation

Treatment of substrate brain with acidic pH (pH 3.5) enhanced PrP^{CWD}-induced conversion of all species, except *Prnp* null mice as expected (Fig. 3A). If the conversion of partially denatured PrP can be considered to be the maximum achievable conversion, the ratio of conversion of brain homogenates treated at pH 7.4 relative to pH 3.5 may provide a "conversion efficiency ratio" (CER) for that species. The comparative CER within different species is shown in Fig. 3B. Notably, some cervid species showed variability in crude conversion efficiency of native and denatured substrate, despite similar (or even identical) PrP amino acid sequences (e.g., caribou and reindeer). Although individual assays might vary for trivial reasons such as slightly differing concentration of brain homogenate, the adjusted CER seems to indicate all cervids display similar substrate conversion efficiency as expected from their evolutionary proximity. The CER analysis also

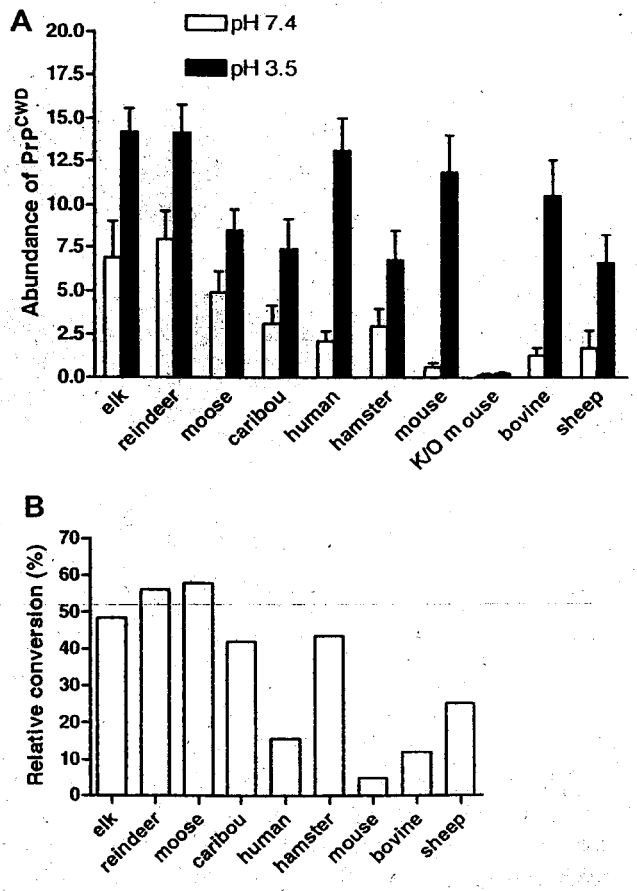

Fig. 3. (A) The immunoblots as in Fig. 2 were examined by densitometry to determine the ratio of neutral (pH 7.4) and acidic (pH 3.5) forms of PrP^{Sc} using Quantity One software (Bio-Rad). (B) Conservation efficiency ratio of native and denatured PrP substrate.

appears to show that hamster segregates with the cervids. Although Syrian hamsters were initially deemed resistant to CWD, a recent publication demonstrates that CWD can be transmitted and adapted to hamsters [20].

Measurement of species barriers by in vitro conversion assays

A number of studies have been published on the PrP^{Sc}-induced conversion of PrP^{C} [14,15,21–25]. However, in these assays require molecular cloning to obtain recombinant PrP of different species, derived from cells in culture that may not possess brain-specific PrP posttranslational modifications, and/or brain molecules which may facilitate PrP isoform conversion. Furthermore, it now appears that PMCA may trigger stochastic generation of PrP^{Sc} *de novo* [15], which may render this technique unsuitable for determining species barriers of prion infection.

Substrate denaturation and human health

We confirm with multiple species that acid/GdnHCl-treated brain PrP^{C} is a superior substrate for *in vitro* conversion than untreated PrP^{C}, possibly by overcoming conformational barriers in partial denaturation of substrate PrP^{C}. PrP conversion in scrapie-infected neuroblastoma cells is believed to occur in endosomes, a low-pH and reducing environment [26]. The non-ruminant stomach possesses a low pH lumen, and PrP^{C} is expressed in this organ [27]. Such acidic (denaturing) organ or cellular organellar environments might also promote CWD transmission to non-cervid species, including humans.

Acknowledgments

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識別番号・報告回数			報告日	第一報入手日 2007 年 12 月 10 日	新医薬品等の区分	厚生労働省処理欄
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研究報告の概要

ヒトおよび反芻動物における伝達性海綿状脳症(TSE)の診断は死後の脳組織中のプロテアーゼ抵抗性の宿主糖タンパク質 PrP の検出に依存している。この異常なアイソフォーム(PrP^{Sc})が組織中に存在することは TSE の感染性が存在することを示すものとされている。
本研究は、PrP^{Sc} のレベルが低いか、もしくは検出されない動物の TSE 疾患の臨床的および空胞化徴候を示す脳組織内に、高タイターの TSE 感染性が存在しうることを明確に示している。本研究は PrP^{Sc} のレベルと感染価との間の相関性に疑問を投げかけるものであり、プロテアーゼ K 抵抗性の PrP をほとんどもしくは全く含まない組織が感染源となりうること、および高タイターの TSE 感染性を有しうることを示すものである。
従って、プロテアーゼ抵抗性の PrP^{Sc} を感染性の唯一の尺度としてそれに依存することは、場合によっては診断しようとするサンプルの生物学的特性を著しく過小評価し、そのことによって TSE を防止し根絶しようとする努力を減弱させる可能性がある。

報告企業の意見	今後の対応
PrP^{Sc} のレベルが低いか、もしくは検出されない動物の TSE 疾患の臨床的および空胞化徴候を示す脳組織内に、高タイターの TSE 感染性が存在するとの報告である。 これまで血漿分画製剤によってvCJD、スクレイピー及びCWDを含むプリオン病が伝播したとの報告はない。しかしながら、万一vCJD感染者の血漿が本剤の原料に混入した場合には、製造工程においてプリオンを低減し得るとの報告があるものの、製剤から伝播する可能性を完全には否定し得ない。そのため、弊社の血漿分画製剤の製造工程におけるTSE感染性低減に関する検証実験を加速し、自社データを早期に取得し、工程評価を行い、必要に応じて工程改善を実施する予定である。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

使用上の注意記載状況・その他参考事項等

代表として献血ヴェノグロブリン-IH ヨシトミの記載を示す。
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。

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High Titers of Transmissible Spongiform Encephalopathy Infectivity Associated with Extremely Low Levels of PrP^{Sc} *in Vivo*[*] [S]

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Diagnosis of transmissible spongiform encephalopathy (TSE) disease in humans and ruminants relies on the detection in post-mortem brain tissue of the protease-resistant form of the host glycoprotein PrP. The presence of this abnormal isoform (PrP^{Sc}) in tissues is taken as indicative of the presence of TSE infectivity. Here we demonstrate conclusively that high titers of TSE infectivity can be present in brain tissue of animals that show clinical and vacuolar signs of TSE disease but contain low or undetectable levels of PrP^{Sc}. This work questions the correlation between PrP^{Sc} level and the titer of infectivity and shows that tissues containing little or no proteinase K-resistant PrP can be infectious and harbor high titers of TSE infectivity. Reliance on protease-resistant PrP^{Sc} as a sole measure of infectivity may therefore in some instances significantly underestimate biological properties of diagnostic samples, thereby undermining efforts to contain and eradicate TSEs.

The transmissible spongiform encephalopathy (TSE)[4] diseases (also known as prion diseases) are infectious, fatal neurodegenerative diseases of animals, which include Creutzfeldt-Jacob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in cattle. The true identity of the infectious agent responsible for these diseases is not known. However, it has been proposed that TSE disease is caused by an abnormal form of the host glycoprotein, PrP (1). The abnormal, disease-associated form of the protein (PrP^{Sc}), is partially protease-resistant and detergent-insoluble unlike the normal cellular conformer (PrP^{C}), and is seen to accumulate in diseased tissues. The prion hypothesis predicts that PrP^{Sc} alone is the infectious agent of TSE and is able to induce the conversion of endogenous PrP^{C} into the abnormal form during disease (2).

Most human TSE diseases are familial or sporadic, but disease can also be acquired by surgical intervention (3) or blood transfusion from infected individuals (4–9), or possibly from the consumption of BSE-infected meat products, the presumed cause of variant CJD (vCJD) (10). The extent to which vCJD infection in particular is present in the United Kingdom population is unknown, but recent research has suggested there may be a higher rate of subclinical or preclinical vCJD than previously thought in different human PrP genotypes (7, 11–13). Although BSE is declining in the United Kingdom, cases have now been observed in cattle in countries that have not previously reported BSE. It is also unknown whether the agent responsible for BSE has re-entered the human food chain following transmission to sheep. For these reasons a high level of active and passive surveillance of ruminants is required at slaughter to monitor and prevent TSE-infected material from entering the human food chain. The introduction of ante-mortem surveillance in the human population is also critical to prevent the human-to-human transmission of vCJD by blood transfusion or surgical procedures. This will be of particular importance if subclinical disease proves to be a significant risk in vCJD transmission (12, 13).

Positive identification of TSE infectivity can only be demonstrated conclusively by transmission of disease to laboratory animals. Such assays are time-consuming, due to long incubation times, and expensive, and are therefore not suitable for the rapid diagnosis of all ante- or post-mortem samples. Current diagnostic tests instead rely on the detection of disease-associated PrP^{Sc} in samples taken from brain post-mortem. The development of ante-mortem diagnostic tests is also being based around more sensitive assays for PrP^{Sc}. Several diagnostic tests are available commercially, and most require proteinase K (PK) treatment of tissue homogenates to isolate disease-specific PK-resistant PrP^{Sc} (PrP-res). It has not yet been definitively proven that PrP^{Sc} is the TSE infectious agent, and whether it is present in all infected tissues. Studies using 263K hamster scrapie have shown a strong correlation between PrP-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.

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[4] The abbreviations used are: TSE, transmissible spongiform encephalopathy; CJD, Creutzfeldt-Jacob disease; vCJD, variant Creutzfeldt-Jacob disease; PK, proteinase K; GSS, Gerstmann Sträussler Scheinker; CDI, conformation-dependent immunoassay; IP, immunoprecipitation; IHC, immunohistochemistry; mAb, monoclonal antibody; BSE, bovine spongiform encephalopathy; PrP-res, PK-resistant PrP^{Sc}; $sPrP^{Sc}$, PK-sensitive form of PrP^{Sc}; ELISA, enzyme-linked immunosorbent assay; d/n, ratio of denatured to native signal; Wt, wild-type.