IV. 研究成果の刊行物・別刷

牛海綿状脳症，変異クロイツフェルト・ヤコブ病

POINT
- ▶感染因子“プリオン”の感染により生じる致死性神経変性疾患。
- ▶BSE（牛海綿状脳症）がヒトに感染してvCJD（変異クロイツフェルト・ヤコブ病）が発生した。
- ▶vCJDは輸血により伝播する。
- ▶管理措置が有効に機能し，世界的にBSEの発生は収束している。

1 病　名

牛海綿状脳症（bovine spongiform encephalopathy：BSE），変異クロイツフェルト・ヤコブ病（variant Creutzfeldt-Jakob disease：vCJD）。

2 定　義

BSEはウシのプリオン病で，BSEプリオンの感染により生じる，神経細胞および神経網の空胞化，アストログリオーシス，およびミクログリアの増生を特徴とする致死性神経変性疾患。食を介してBSEプリオンがヒトに感染した結果，それまで知られていたヒトのプリオン病である，孤発性あるいは家族性のクロイツフェルト・ヤコブ病（Creutzfeldt-Jakob disease：CJD）とは病型の異なるvCJDが発生した。

3 概　要

プリオン病は，その原因により，感染性，遺伝性，特発性（原因不明），の3種に分類される（表1）。ヒトのプリオン病には獲得性（感染性），遺伝性，および特発性の3種がある。このうち，特発性に分類される孤発性CJD（sporadic CJD：sCJD）が85%を占める。

BSEは1980年代半ばに英国で出現し，大流行した。その後，欧州，北米およびアジアへ拡散した。1996年に，BSEがヒトに感染した結果発生したと考えられるvCJDの存在が報告された[1]。BSEの起源は，ヒツジのスクレイピーが原因であるとする説と，元来ウシに存在していた病気とする説があるが結論は出ていない。図1にBSE出現後の動物種を越えた感染拡大を示す。

BSEは1985年頃から英国で発生し，発生数は1992年にピークに達した（図2）。英国では1988年7月に，反芻動物由来の肉骨粉を反芻動物に与えることを禁止する飼料規制を導入した。6年が経過した1994年以降，BSEの発生は減少に転じた。欧州諸国では2000年以降BSE牛の数が著しく増加したが，2003～2004年をピークにBSEの発生数は減少している（図2）。わが国でも2002年2月以降に生まれたウシでBSE牛は摘発されていない。

vCJDはこれまで英国で177例，英国以外で52例の報告がある（表2）。英国での発生は2000年をピークに減少している（図2）。わが国でも欧州滞在歴がある症例が1例報告されている。

4 病　因

プリオン病の病原体“プリオン”は，蛋白質から構成され，病原体ゲノムとなる核酸はない。一個の感染粒子の形態も不明である。プリオンの主要構成要素である異常型プリオン蛋白質（PrP^{Sc}）は，宿主遺伝子*PrP*にコードされる正常型プリオン蛋白質（PrP^{C}）の構造異性体であり，アミノ酸配列はPrP^{C}と同じである。“Sc”はscrapie（スクレイピー）に，“C”は細胞のcellularに由来する。

PrP^{C}は中枢神経系組織で発現が高いが，生命維持に必須ではない。プリオン病に罹患したヒトや動物の脳組織にはPrP^{Sc}が蓄積する。PrP^{Sc}はPrP^{C}と高次構造

表1 動物とヒトのプリオン病

動物のプリオン病	動物種	原因など
スクレイピー	ヒツジ，ヤギ	感染（自然状態）
慢性消耗病（CWD）	シカ，エルク	感染（自然状態）
牛海綿状脳症（BSE）	ウシ	感染（汚染飼料）
伝達性ミンク脳症（TME）	ミンク	感染（汚染飼料）
猫海綿状脳症（FSE）	猫科動物	感染（汚染飼料）
ヒトのプリオン病	**分類**	**原因など**
クロイツフェルト・ヤコブ病（CJD）		
孤発性 CJD（sCJD）	特発	偶発的（宿命）
家族性 CJD（fCJD）	遺伝	PrP 遺伝子の変異
医原性 CJD（iCJD）	感染（獲得性）	汚染硬膜移植等
変異 CJD（vCJD）	感染（獲得性）	BSE
ゲルストマン・ストライスラー症候群（GSS）	遺伝	PrP 遺伝子の変異
致死性家族性不眠症（FFI）	遺伝	PrP 遺伝子の変異
クールー（Kuru）	感染（獲得性）	宗教的食人儀式

プリオン病は，その原因により感染性，遺伝性，および特発性に分類される。動物のプリオン病はすべて感染が原因と考えられている。ヒトのプリオン病には3種の原因のものが存在する。

CWD：chronic wasting disease，BSE：bovine spongiform encephalopathy，TME：transmissible mink encephalopathy，FSE：feline spongiform encephalopathy，CJD：Creutzfeldt-Jakob disease，sCJD：sporadic CJD，fCJD：familial CJD，iCJD：iatrogenic CJD，vCJD：variant CJD，GSS：Gerstnamm Sträussler syndrome，FFI：fatal familial insomnia　（筆者作成）

が異なる。PrP^{C}がαヘリックスの割合が高いのに対し，PrP^{Sc}はβストランドの割合が高い。そのためPrP^{Sc}は凝集体を形成し，蛋白分解酵素（proteinase K：PK）抵抗性（図3）や不溶性となる。実際には，PrP^{Sc}にはPKに感受性と抵抗性の画分があり，凝集体も，小さなオリゴマーから大きな凝集体までを含むヘテロな集団である。

図4に，PrP^{Sc}の細胞内産生機構を示す。プリオン感染細胞では，細胞内膜輸送に関わる細胞内小器官でPrP^{Sc}が検出される[2]。PrP^{Sc}は主に細胞膜上およびendocytic-recycling経路にある細胞内小器官で産生される。特に，エンドソーマルリサイクリングコンパートメントはPrP^{Sc}産生に関わる主要な細胞内小器官の一つと考えられる。

5 動物の感染症

BSEの潜伏期は平均4～8年である。英国におけるBSEの大流行は，1980年頃にレンダリング工程（肉骨粉の製造工程）がバッチ方式から連続方式に代わったことによりプリオンが完全に不活化されず肉骨粉に残存し，そのような肉骨粉を代用乳・人工乳に添加して給餌したことに起因する。

経口ルートで侵入したプリオンは，消化管からパイエル氏板などの消化管付随リンパ濾胞の上皮に存在するM細胞から体内に取り込まれる。体内に取り込まれたプリオンは，末梢神経へと移行し，副交感神経系（迷走神経）を経て延髄に至る経路と，交感神経系（内臓神経）を経て脊髄胸腰部に至る経路で，中枢神経系に侵入する。BSEの最少感染量は，BSE感染牛脳1 mg以下と非常に少ない[3]。

BSE病原体の体内分布は神経系組織に限局している。中枢神経系組織，背根神経節，三叉神経節，座骨神経などの末梢神経，および回腸遠位部などで感染性が確認されている。自然状態でウシ間の水平感染は起こらない。

BSEの初期症状としては音に対する異常反応，不安

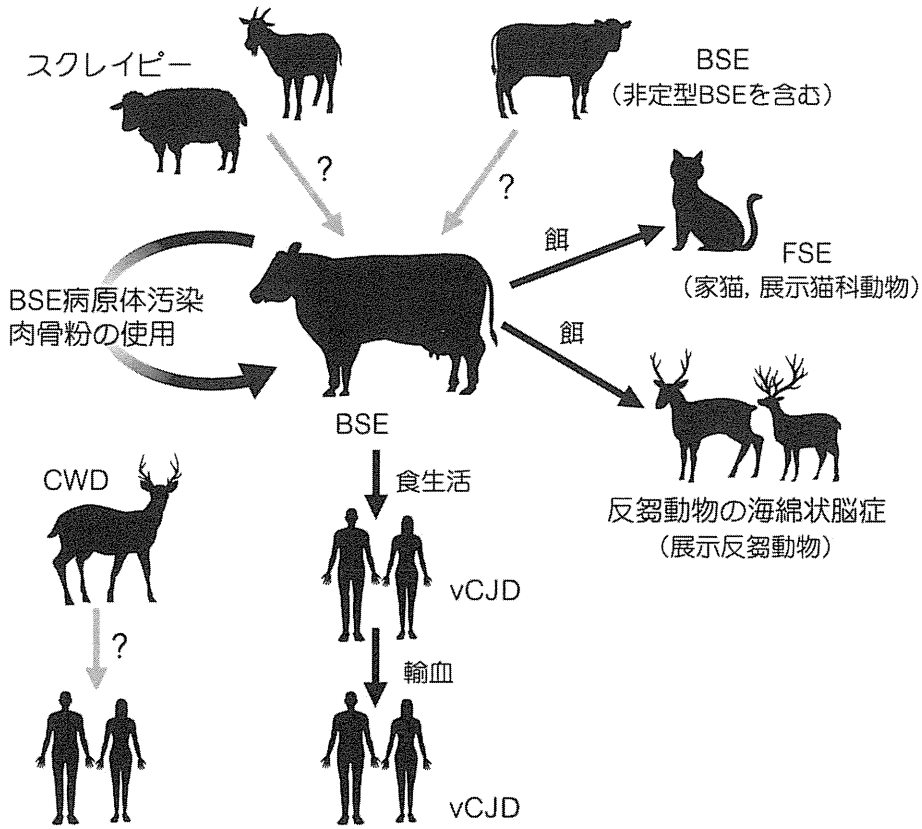

図1　種を越えた感染拡大と感染経路

BSE の起源は不明であるが，BSE の感染拡大は BSE 病原体に汚染された肉骨粉の使用が原因である。BSE は猫科動物，展示反芻動物に感染が拡大した。ヒトにも感染が拡大して vCJD が発生した。CWD がヒトに伝播するかは明らかでない。

（筆者作成）

動作，持続的に鼻をなめる，などの行動異常が観察される。その後，起立時の後肢開脚，ふらつき歩行などの運動失調を経て，起立不能へと症状が進行し，死に至る。神経病理組織学的検査による神経細胞および神経網の空胞化とアストログリオーシスの確認，ELISA，ウエスタンブロット，あるいは免疫組織化学による PrP^{Sc} の検出により確定診断する（図5，6）。

英国で発生して欧州，北米，日本に感染が広がった BSE を“定型 BSE”と呼ぶ。一方，2004 年以降，定型 BSE とは病原体の性状が異なる，“非定型 BSE”が，欧州，北南米，および日本で，主に 8 歳以上の高齢牛で見つかっている。非定型 BSE は，ヒトの孤発性 CJD のように，高齢牛で自然発生する BSE の可能性が指摘されている。

6　ヒトの感染症

プリオン病は長い潜伏期の後発症し，一度発症すると，亜急性に進行して死に至る致死性の神経変性疾患で有効な治療法はない。英国での vCJD の発生は一つのピークを過ぎた（図2）。これらの患者は PrP のコドン 129 が Met/Met のホモであり，vCJD に感受性が高い集団である。今後 PrP コドン 129 が Met/Val，あるいは Val/Val を持つヒトで vCJD が発生するかどうか注視する必要がある。

sCJD と異なり，vCJD では中枢神経系以外に扁桃や盲腸の粘膜下リンパ濾胞など末梢のリンパ系組織でも PrP^{Sc} が検出される。英国における盲腸 PrP^{Sc} 検出の遡及研究では，数千人から数万人の vCJD キャリアが存

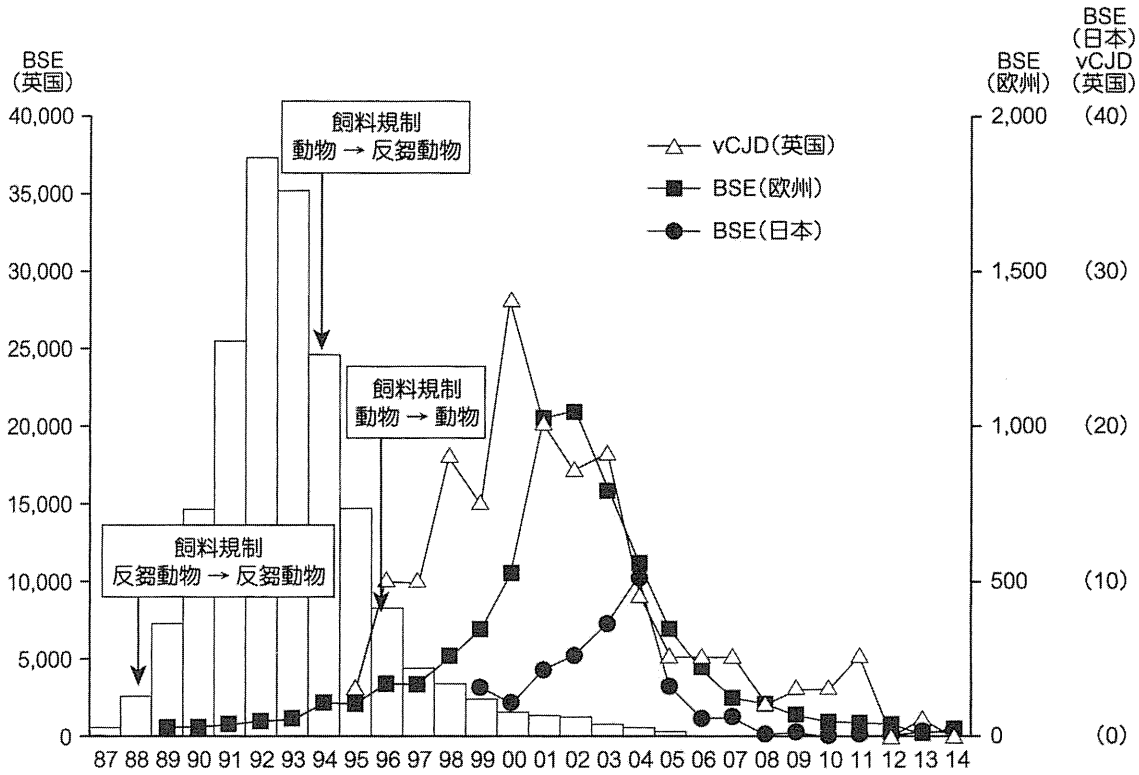

図2 英国，欧州，および日本における BSE 発生数の経時変化と英国における vCJD 患者数の変化
棒グラフは英国における BSE 発生数，△は英国における vCJD 発生数，■は欧州における BSE 発生数，●は日本における BSE 発生数を示す。（筆者作成）

表2 vCJD 患者数

国	患者数（人）
イギリス	177
フランス	27
アイルランド	4
イタリア	2
アメリカ	4
カナダ	2
サウジアラビア	1
日本	1
オランダ	3
ポルトガル	2
スペイン	5
台湾	1

vCJD の発生は BSE が大流行した英国で最も多く，これまでに英国で 177 例，次いでフランスで 27 例の報告がある。英国以外では計 52 例の報告がある。わが国でも欧州滞在歴がある症例が 1 例確認されている。
（www.cjd.ed.ac.uk〔2015 年 2 月 13 日〕を基に筆者作成）

在するとの推測もある[4]。vCJD は輸血により伝播する（図 1）。

vCJD の発症年齢（12 ～ 74 歳〔平均 26 歳〕）は sCJD（日本：32 ～ 94 歳〔平均 68 歳〕）に比べると若く，発症後の経過も 6 ～ 39 カ月（平均 13.0 カ月）と，sCJD と比較して緩徐である。初発症状は抑うつ，無気力，不安などの精神症状であり，神経症状としては上下肢の疼痛性感覚異常が認められる[5]。末期には興奮，偏執，方向感覚の喪失などの精神症状が出現する。sCJD で高率に認められる周期性同期性放電は，vCJD の有症期後期にまれにみられるのみである。神経病理学的特徴として，vCJD の患者の脳では，florid plaque と呼ばれる，花弁状の PrP プラークが認められる。PrP^{Sc} は，Parchi らの分類による 2b 型[6]（Collinge らの分類による 4 型）を示す。MRI は vCJD の診断に有効で，FLAIR 画像や T2 強調画像で，pulvinar sign と呼ばれ

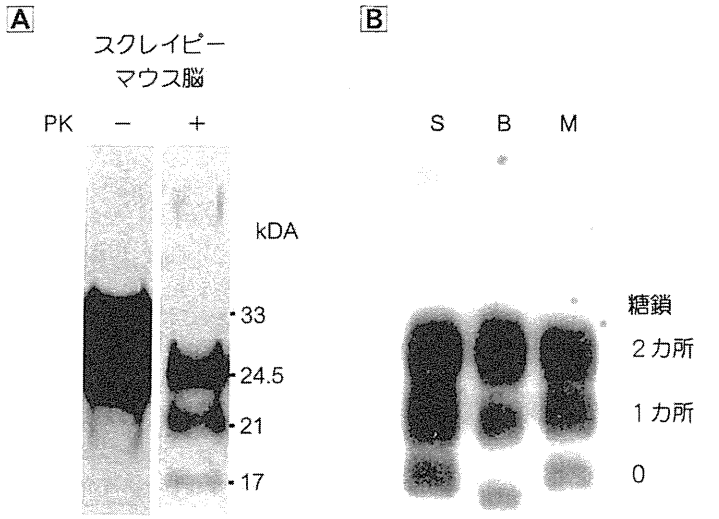

図3 プリオン蛋白（PrP^{Sc}）のSDSポリアクリルアミドゲル電気泳動

A：スクレイピー感染マウス脳をproteinase K（PK）消化前（−）と後（+）をSDS-ポリアクリルアミドゲル（12%）で電気泳動し，抗プリオン蛋白抗体B103を用いたウエスタンブロット法により検出した。消化前は33 kDa以下の不明瞭なスメア状のバンドが，消化後は24.5 kDa以下，糖鎖の結合数の違いにより明瞭な3本のバンドとなる。

B：スクレイピー感染羊（S），BSE感染牛（B）およびスクレイピー感染マウス（M）の脳をPK消化後泳動し，Aと同様に異常プリオン蛋白を検出した。種による分子量の差がいくらか認められるが，いずれも3本のバンドを形成する。

（筆者提供）

（カラー図譜21頁）

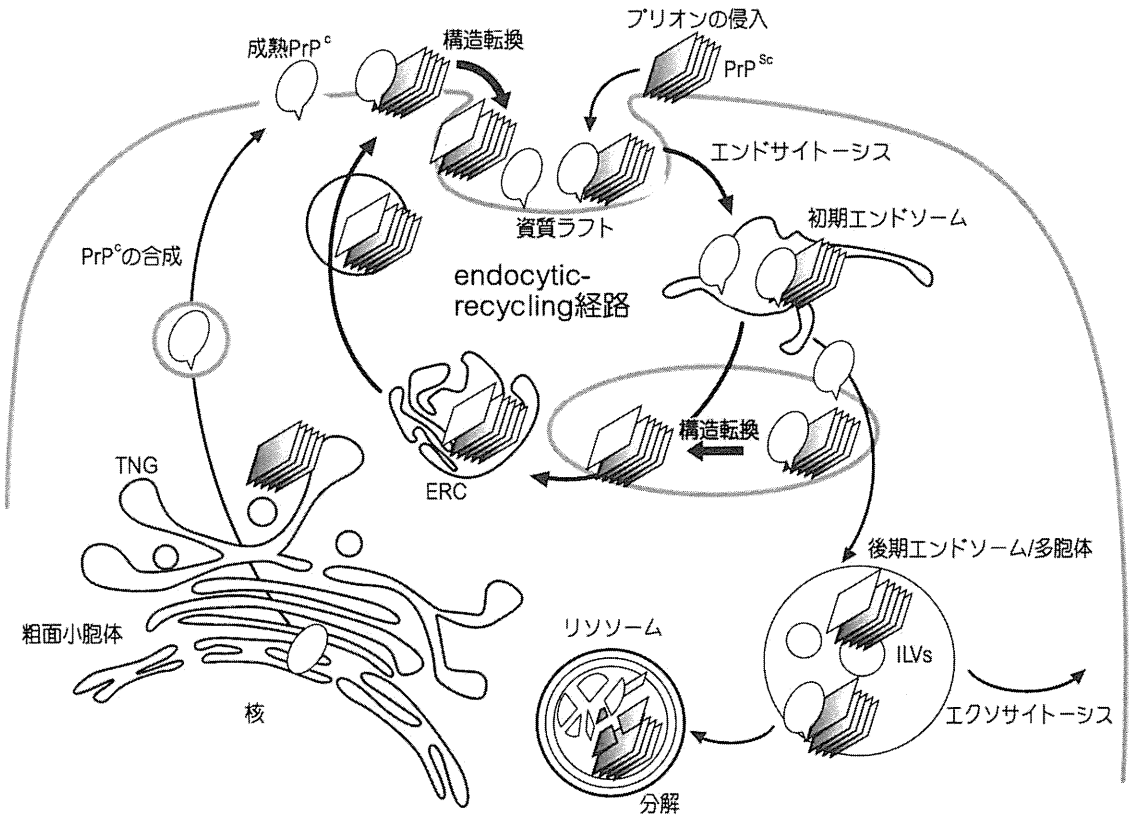

図4 PrP^{Sc}の細胞内産生機構

PrP^{Sc}は細胞内小胞輸送に関わる細胞内小器官に存在する。PrP^{Sc}は主に，細胞膜上およびendocytic-recycling経路にある細胞内小器官で産生される。

ERC：エンドソーマルリサイクリングコンパートメント，TGN：トランスゴルジネットワーク

（筆者作成）

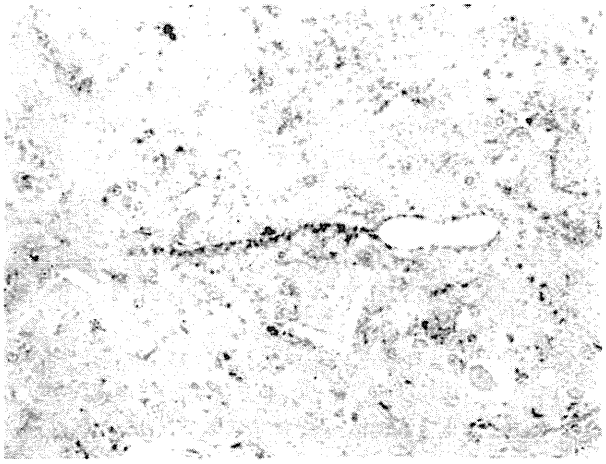

図5 抗プリオン蛋白抗体を用いたBSEの免疫組織化学（中脳の空胞辺縁および神経網の陽性反応）
ニューロピルの空胞変性とその空胞辺縁および神経網に，プリオンの蓄積が免疫反応陽性として証明される。
（帯広畜産大学家畜病理学教室 古岡博士提供）
（カラー図譜21頁）

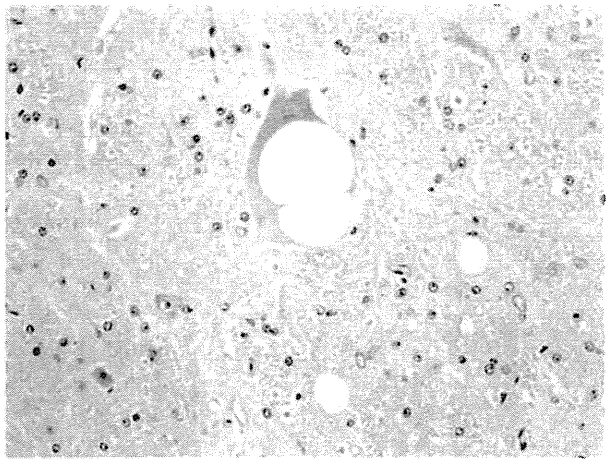

図6 BSEの病変（ヘマトキシリン・エオシン染色）
中脳にみられた神経細胞細胞質およびニューロピルの空胞変性。
（帯広畜産大学家畜病理学教室 古岡博士提供）
（カラー図譜21頁）

る視床枕での両側性の高信号が認められる。

vCJD以外の獲得性プリオン病として，クールーと医原性CJDがある。わが国では，プリオン汚染脳硬膜の使用による医原性CJDが大きな薬害問題となり，症例は140例を超えた。当該脳硬膜が使用されていたのは1991年までであるが，20年以上を経過した今日でも，まれに発生がある。パプアニューギニアのフォア族で発生していたクールーは宗教的な食人儀式の廃止により発生は減少したが，潜伏期は長い例で50年を超える可能性もある。

7 教　訓

BSE対策は，食の安全・安心の担保，治療法のない致死性疾患であること，病原体の不活化が難しいことなどから，実際のリスクに比して厳しい管理措置がとられてきた。定型BSEの発生は収束し，管理措置が緩和されている。非定型BSE，ヒツジのスクレイピーは存在することから，BSE再興の悪夢を阻止するには，適切な管理措置の維持が必須である。

（堀内 基広）

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Structural conservation of prion strain specificities in recombinant prion protein fibrils in real-time quaking-induced conversion

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EXTRA VIEWS

Structural conservation of prion strain specificities in recombinant prion protein fibrils in real-time quaking-induced conversion

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ABSTRACT. A major unsolved issue of prion biology is the existence of multiple strains with distinct phenotypes and this strain phenomenon is postulated to be associated with the conformational diversity of the abnormal prion protein (PrP^{Sc}). Real-time quaking-induced conversion (RT-QUIC) assay that uses *Escherichia coli*-derived recombinant prion protein (rPrP) for the sensitive detection of PrP^{Sc} results in the formation of rPrP-fibrils seeded with various strains. We demonstrated that there are differences in the secondary structures, especially in the β-sheets, and conformational stability between 2 rPrP-fibrils seeded with either Chandler or 22L strains in the first round of RT-QUIC. In particular, the differences in conformational properties of these 2 rPrP-fibrils were common to those of the original PrP^{Sc}. However, the strain specificities of rPrP-fibrils seen in the first round were lost in subsequent rounds. Instead, our findings suggest that nonspecific fibrils became the major species, probable owing to their selective growth advantage in the RT-QUIC. This study shows that at least some strain-specific conformational properties of the original PrP^{Sc} can be transmitted to rPrP-fibrils *in vitro*, but further conservation appears to require unknown cofactors or environmental conditions or both.

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KEYWORDS. fibril, prion, real-time quaking-induced conversion (RT-QUIC), recombinant prion protein (rPrP), strain, transmission

IN VITRO *CONVERSION OF RECOMBINANT PrP INTO THE PROTEINASE K (PK) RESISTANT AMYLOID FIBRILS*

Prion diseases, or transmissible spongiform encephalopathies, are infectious and fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease in humans, and scrapie and bovine spongiform encephalopathy in animals. The infectious agent, prion, is assumed to be formed mainly or exclusively by abnormal prion protein, designated PrP^{Sc}, which is partially protease-resistant[1] and a β-sheet-rich conformer,[2,3] frequently resulting in amyloid fibril formation. Although the pathogenesis has not been clarified fully, it is widely accepted that prion disease occurs through autocatalytic conformational conversion of the ubiquitous normal form of prion protein (PrP^{C}) to PrP^{Sc} in a "protein only" manner.[4]

Studies using *Escherichia coli*-derived purified recombinant PrP (rPrP) has contributed to solving the controversial protein-only hypothesis. It has been demonstrated that rPrP fibrils (rPrP-fibrils) formed *in vitro* cause the accumulation of PrP^{Sc} in the brains of PrP-overexpressing transgenic (Tg) mice[5–7] and some wild-type hamsters.[8] These studies suggest that rPrP can be converted into a PrP^{Sc}-like form *in vitro*; however, the infectious titers seem to be much lower than that of authentic PrP^{Sc}. In contrast, prion infectivity could be propagated when brain-derived PrP^{C} or baculovirus-derived PrP^{C} was used as substrates for protein misfolding cyclic amplification (PMCA) in the presence of certain cofactors such as nucleic acids.[9,10] Relatively high levels of prion infectivity was demonstrated by injection of PK-resistant rPrP-fibrils generated by unseeded PMCA in the presence of 1-palmitoyl-2-oleoylphosphatidylglycerol and total liver RNA into wild-type mice. Subsequently, these mice developed prion disease with an incubation period of approximately 150 days.[11] However, other group failed to show infectivity of rPrP-fibrils generated by the same methods.[12]

TRANSMISSION OF CONFORMATIONAL PROPERTIES OF PRION STRAINS TO rPrP-FIBRILS IN RT-QUIC

Prion is known to provide extensive strain diversity showing different phenotypic and pathological states in mammalian species. The strain-specific characteristics can usually be serially passaged stably in the same species. Furthermore, PrP^{Sc} generated by PMCA using brain homogenate from normal animals as a source of PrP^{C} (BH-PMCA) seeded with different mouse prion strains retained the strain-specific properties, such as incubation time, neuropathology, and biochemical characteristics from original PrP^{Sc}.[13] This result indicates that the intracellular mechanisms and cell-to-cell transmission are dispensable for the maintenance and propagation of strain characteristics. The finding that PrP^{Sc} from different strains have distinct secondary structures and biochemical properties supports the notion that prion strains are manifested by conformational variations of the PrP^{Sc}.[14] For example, strain-dependent differences in β-sheet-rich structures of PrP^{Sc} have been demonstrated by infrared spectroscopy.[15–18] In addition, the conformational stability of PrP^{Sc} differed among prion strains, as demonstrated by guanidine hydrochloride denaturation assay followed by protease digestion.[19,20] However, the mechanistic relationship between PrP^{Sc} conformational differences and the molecular basis of prion strains remains poorly understood.

The recently developed "real-time quaking-induced conversion" (RT-QUIC) is a sensitive

prion detection method, in which intermittent shaking enhances the conversion of soluble rPrP into amyloid fibrils in the presence of PrP^{Sc}.[21] Recent studies show that RT-QUIC assays allow highly sensitive detection of PrP^{Sc} in most species and strains, including Creutzfeldt-Jakob disease in humans,[21–24] scrapie in rodents,[25,26] and chronic wasting disease in cervids.[27]

We generated the amyloid fibrils seeded with 100 pg of PrP^{Sc} derived from either the Chandler or 22L strain in the first round of RT-QUIC (1^{st}-rPrP-fib^{Sc}).[28] Spontaneous formation of rPrP-fibrils (rPrP-fib^{spon}) was observed by decreasing the concentration of rPrP, because there was an inverse correlation between the rate of fibril formation and the concentration of rPrP. Previous studies using FTIR and hydrogen/deuterium exchange have shown that there are structural differences between PrP^{Sc}-seeded and spontaneous rPrP-fibrils generated by PMCA.[29,30] We found that the PK-**resistant** band pattern, structural morphology, secondary structure, and conformational stability distinguish 1^{st}-rPrP-fib^{Sc} from rPrP-fib^{spon}. Although there were no differences in the PK-**resistant** band pattern and structural morphology between Chandler-seeded (1^{st}-rPrP-fib^{Ch}) and 22L-seeded rPrP-fibrils (1^{st}-rPrP-fib^{22L}), we observed significant differences in the secondary structure and conformational stability between strains. FTIR analysis showed that native rPrP had an abundance of α-helical structures, whereas 1^{st}-rPrP-fib^{Ch} and 1^{st}-rPrP-fib^{22L} were substantially enriched in β-sheets. While the 1^{st}-rPrP-fib^{Ch} was characterized by a major band at 1624 cm^{-1} in the β-sheet region of second-derivative spectra, the 1^{st}-rPrP-fib^{22L} was characterized by 2 absorbance bands at 1629 and 1617 cm^{-1}, indicating that there were conformational differences in β-sheet structures between the 2 1^{st}-rPrP-fib^{Sc}. Similarly, purified Chandler-PrP^{Sc} from brains of mice displayed the spectrum with a peak at 1630 cm^{-1}, whereas purified 22L-PrP^{Sc} had 2 major maxima at 1631 and 1616 cm^{-1}, as previously reported. Thus, the differences in β-sheet spectrum shape between strains were common to both PrP^{Sc} and 1^{st}-rPrP-fib^{Sc}. The conformational stability of 1^{st}-rPrP-fib^{22L} was significantly lower than that of 1^{st}-rPrP-fib^{Ch}, as with Chandler- and 22L-PrP^{Sc}. Furthermore, wild-type mice inoculated with the 1^{st}-rPrP-fib^{Sc} showed an increased attack rate and a significantly shorter survival period compared with those inoculated with mock preparations. The infectious titers (per 40 μl) of 1^{st}-rPrP-fib^{Ch} and 1^{st}-rPrP-fib^{22L} were estimated to be 407.2 $\pm$ 226.6 and 1067.0 $\pm$ 678.7 LD_{50}, respectively, whereas the titers of Chandler and 22L prion were 20.2 and 28.9 LD_{50} units/40 pg of PrP^{Sc}, respectively, indicating that QUIC reaction in the first round resulted in a 20- to 37-fold increase in the infectious titer. These results suggest that strain features of PrP^{Sc} can be transmitted to rPrP-fibrils in a simple system solely consisting of pure rPrP. However, it is clear that the conformation of 1^{st}-rPrP-fib^{Sc} is not identical to that of authentic PrP^{Sc}. It should be noted that the degrees of vacuolation of mice inoculated with 1^{st}-rPrP-fib^{Sc} were significantly lower in the hippocampus and cerebellum than those of inoculated with mock preparations. The different lesion profiles may result from the conformational differences between 1^{st}-rPrP-fib^{Sc} and authentic PrP^{Sc}.

WHAT IS REQUIRED FOR MAINTAINING STRAIN-SPECIFIC CONFORMATIONS?

We found that the strain-specific conformational features and the infectivity disappeared in rPrP-fibrils during and after the second round,[28] suggesting that RT-QUIC has the limitation of technology with respect of reproducing the prion propagation. One possible reason for the loss of the prion strain-specific traits is that *E. coli*-derived rPrP lacks post-translational modifications. PrP bears 2 N-linked glycosylation sites at amino acids 180 and 196 that can produce di-, mono-, and unglycosylated forms. PrP^{Sc} has varying degrees of glycosylation among strains[14,31,32] and therefore the glycosylation pattern is postulated to confer strain specificity. Studies using Tg mice expressing glycosylation site mutants revealed that the strain properties of strain 79A were altered by the glycosylation state of PrP^{C}, but the strain

properties of strains ME7 and 301C were not affected.[33] Moreover, the glycosylation-deficient PrP^C as a substrate of PMCA by treatment with PNGase F did not affect strain-dependent neurotropisms in the 2 murine strains RML and 301C.[34] Furthermore, the cell tropisms determined by the cell panel assay were altered in strains RML, 139A, 79A, and ME7 but not in strain 22L when the strains were propagated in Tg mice expressing PrP devoid of a GPI anchor.[35] These results suggest that the necessity of glycans and the GPI-anchor for the transmission and preservation of strain-specific properties is dependent on the strains.

An additional reason for the loss of strain specificity from rPrP-fibrils might be because of a decrease in cofactor(s) over serial passages. Strains have been reported to differ in their RNA requirements for propagation in BH-PMCA, although RNA is not essential for maintaining strain-specific characteristics in mice.[36] Moreover, another study showed that phosphatidylethanolamine is a cofactor required for the propagation of prion infectivity in seeded rPrP-PMCA but not for the transmission of strain-specific properties, because 3 different prion strains changed into a single new strain after the serial passages of rPrP-PMCA reactions.[37,38] Thus, crucial cofactors or environmental conditions for maintaining strain-specific properties remain to be determined.

We observed the "nonspecific rPrP-fibrils" displayed no strain-specific differences in IR spectra and conformational stability after 5 serial rounds of RT-QUIC, which have the ability to cause the conversion of rPrP but failed to induce clinical signs of prion disease in the wild-type mice.[28] Additionally, we found that the β-sheet spectra of rPrP-fibrils generated in the presence of small amount (1 pg) of PrP^{Sc} or generated at pH4 in the first round were similar to nonspecific rPrP-fibrils.[28] These observations raise the possibility that nonspecific rPrP-fibrils lacking prion infectivity can be generated even in the first round and may interrupt the formation of the fibrils with strain-specific conforma-

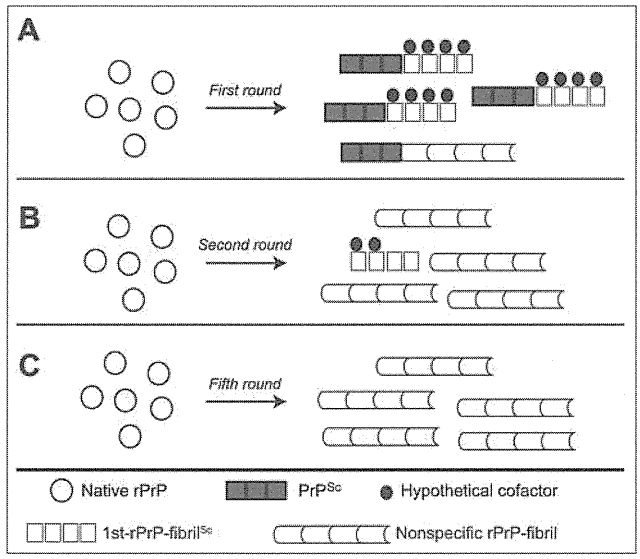

FIGURE 1. Hypothetical models for the formation of rPrP-fibrils in sequential RT-QUIC reactions. (**A**) The formation of 1^{st}-rPrP-fib^{Sc} is induced predominantly in the presence of hypothetical cofactors and brain-derived PrP^{Sc} in the first round. However, a small amount of nonspecific rPrP-fibrils may be concomitantly generated. (**B**) In the second round, the nonspecific rPrP-fibrils become predominant because of the paucity of hypothetical cofactors and/or a selective growth advantage of nonspecific fibrils. (**C**) The formation of nonspecific rPrP-fibrils occupies almost the whole reaction in the fifth round.

tions, because of a selective growth advantage of nonspecific fibrils (**Fig. 1**). Of note, the formation of quasi-species that is consisting of a variety of conformational variants has been reported in prion-infected cultured cells under different environmental conditions.[39,40] Furthermore, different prion strains can interfere with each other, and this is known as prion strain interference.[41–44] The competition for substrates among the variants is thought to act as a selection pressure in Darwinian evolution and to cause the phenomenon of prion strain interference. Previous work showed that 2 conformational variants of rPrP-fibrils are mutually exclusive and compete for monomeric rPrP as a substrate in the rPrP-PMCA.[29] Likewise, competitive amplification of 2 prion strains was observed in BH-PMCA.[45] We postulate that PrP^{Sc} predominantly leads to strain-specific conformational conversion of rPrP, particularly in the presence of hypothetical cofactors, while some quantity of nonspecific fibrils could be generated simultaneously in the first round (**Fig. 1A**). The conditions of subsequent rounds would favor growth of nonspecific species (**Fig. 1B and C**). The fact that prion infectivity was often diminished in serial rPrP-PMCA[46] or BH-PMCA[47–49] support the hypothesis that the amplification of nonspecific rPrP fibrils is accelerated by certain conditions. Further studies are needed to ascertain the key factors responsible for maintaining the infectious and strain-specific conformations *in vitro*.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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RESEARCH ARTICLE

Rapid and Quantitative Assay of Amyloid-Seeding Activity in Human Brains Affected with Prion Diseases

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Abstract

The infectious agents of the transmissible spongiform encephalopathies are composed of amyloidogenic prion protein, PrP^{Sc}. Real-time quaking-induced conversion can amplify very small amounts of PrP^{Sc} seeds in tissues/body fluids of patients or animals. Using this *in vitro* PrP-amyloid amplification assay, we quantitated the seeding activity of affected human brains. End-point assay using serially diluted brain homogenates of sporadic Creutzfeldt–Jakob disease patients demonstrated that 50% seeding dose (SD_{50}) is reached approximately 10^{10}/g brain (values varies $10^{8.79–10.63}$/g). A genetic case (GSS-P102L) yielded a similar level of seeding activity in an autopsy brain sample. The range of PrP^{Sc} concentrations in the samples, determined by dot-blot assay, was 0.6–5.4 μg/g brain; therefore, we estimated that 1 SD_{50} unit was equivalent to 0.06–0.27 fg of PrP^{Sc}. The SD_{50} values of the affected brains dropped more than three orders of magnitude after autoclaving at 121°C. This new method for quantitation of human prion activity provides a new way to reduce the risk of iatrogenic prion transmission.

Introduction

Human prion diseases (HPD) are neurodegenerative diseases caused by accumulation of amyloidogenic prion protein (PrP^{Sc}), which is generated from the cellular prion protein (PrP^{C}) via a conformational change. PrP^{Sc} is detected not only in neuronal tissues, but also in lymphoid tissues (e.g., spleen, tonsil, lymph node) [1, 2] and muscles of some sporadic Creutzfeldt—Jakob disease (CJD) (sCJD) patients [1]. In cases of variant CJD, PrP^{Sc} has been detected in blood, rectum, adrenal gland, and thymus [3]. Accidental iatrogenic transmission of prion has occurred due to use of human growth hormone [4], dura mater grafts [5], corneal grafts [6],

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and blood [7–9] from HPD patients or carriers. Infectivity mainly resides in the neural tissues of sCJD patients, whereas prion infectivity in extraneural tissue is low, and it is difficult to estimate the exact infectivity. Transmission studies of human prion, conducted in chimpanzees and other primates [10–14], demonstrated that it takes more than 12 months to develop the syndrome. Normal rodents infected with human prion require prolonged incubation times to develop illness because of the so-called species barrier. Since then, several lines of transgenic mice expressing human prion protein (or human—mouse chimeric protein) have been produced, and some of them exhibited abbreviated incubation times of 110 days following infection with human prion [15]. Quantitation of infectivity of tissue from a patient with HPD can be achieved by animal bioassay using humanized mice [16, 17]; however, these mice have different susceptibilities to human prion strains [18–20], and the assays are still highly time-consuming and costly.

Previously, we developed the real-time quaking-induced conversion (RT-QUIC) assay for detection of very small amounts of abnormal PrP in tissue and body fluids. This technique provides a highly sensitive means for detecting prion-seeding activity using human recombinant PrP as a substrate [21]. Recent studies showed that seeding activity *in vitro*, determined by end-point RT-QUIC, parallels the infectivity of prion-containing animal specimens [22, 23]. Moreover, these studies demonstrate that RT-QUIC is more sensitive than bioassay. Although bioassay is the only tool currently available for determining the known infectivity of human prion, in the future it will be possible to replace LD_{50} (50% lethal dose) with SD_{50} (50% seeding dose). To define the distribution of infectivity in human bodies, we applied end-point RT-QUIC to evaluation of human prion seeding activity in brains from patients with human prion disease.

Materials and Methods

Patients

Sporadic CJD was diagnosed according to Parchi's classification [24], i.e., based on the genotype at codon 129 of *PRNP* gene (methionine homozygous [MM], valine homozygous [VV], or heterozygous [MV]), and the physicochemical properties of PrP^{Sc}. Autopsy brains from 10 patients with prion diseases were subjected to study after histopathological confirmation of the clinical diagnosis. There were six cases of sCJD MM1 and a single case each of the MM2-cortical form, the MM2-thalamic form, MV2, and Gerstmann—Sträussler—Scheinker syndrome (GSS) associated with a mutation of Pro to Leu at codon 102 of *PRNP* (P102L) (Table 1).

Two brain specimens were used as control. One of the donors suffered dementia with Lewy bodies (DLB), and in the other case the cause of death was dissecting aortic aneurysm without any brain damage. Written informed consent to participate in the study was given by all patients' families. The protocol for investigation was approved by the Ethics Committee of Nagasaki University Hospital (ID: 10042823), and the study was registered with the University Hospital Medical Information Network (ID: UMIN000003301). The protocol was also granted ethical approval for the use of brain tissues by the Japan Surveillance Unit for human prion diseases. Analysis of the *PRNP* gene was conducted by Dr. Kitamoto of the Japan Surveillance Unit.

Brain homogenate preparation and heat treatment

All samples were taken from frontal cortex and stored at -80°C. Brain tissues were homogenized at 10% (w/v) in ice-cold phosphate-buffered saline (PBS) supplemented with a protease inhibitor mixture (Roche, Mannheim, Germany) using a multi-bead shocker (Yasui Kikai,

Table 1. Summary of patients with prion disease.

Patient number	Sex	Age at death (years)	codon 129	WB type	mutation	log SD_{50}/ g brain (Mean ± S.D.)	PrP-res/brain (μg/g)
1	male	73	MM	1	-	10.07 ± 0.19	1.1
2	male	64	MM	1	-	10.63 ± 0.43	5.4
3	male	70	MM	1	-	10.08 ± 0.58	3.3
4	male	74	MM	1	-	9.92 ± 0.59	1
5	female	75	MM	1	-	9.96 ± 0.44	1.1
6	female	64	MM	1	-	9.71 ± 0.40	0.6
7	female	43	MM	-	P102L	10.13 ± 0.25	1.1
8	male	69	MV	2	-	10.21 ± 0.26	0.9
9*	male	35	MM	2	-	10.08 ± 0.36	2.3
10**	male	67	MM	2	-	8.79 ± 0.26	N.D

Clinical data and the SD_{50} concentrations in the brain homogenates from patients with prion disease.

*: MM2 cortical form

**: MM2 thalamic form. N.D.: not detected. MM: methionine homozygosity at codon 129 of *PRNP*. MV: methionine/valine heterozygosity at codon 129 of *PRNP*. S.D.: standard deviation. P102L: Pro-to-Leu point mutation at codon 102 of *PRNP*. WB: Western-blotting assay

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Osaka, Japan), and stored at -80°C. Aliquots of 10% brain homogenates were inactivated by autoclaving (SX700HY, TOMY, Tokyo, Japan) at 121°C for 20–60 min.

RT-QUIC

Purification of recombinant human PrP (rHuPrP: residues 23–231, codon 129M) was performed as previously described [25]. After purification, rHuPrP was stored at -80°C. Brain homogenates (BHs) (10% [w/v]) were serially diluted (10-fold) with artificial cerebrospinal fluid (A-CSF) containing 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 ng/ml BSA, and 0.05% glucose. rHuPrP, suspended in 95 μl of RT-QUIC buffer (500 mM NaCl, 50 mM PIPES pH7.0, 10 μM Thioflavin T (ThT), and1 mM EDTA), was loaded into each well of a 96-well plate and mixed with 5 μl of brain sample, and then the assay was monitored for 53 h. Four to eight replicates of each diluted sample were measured. The SD_{50} was calculated by the Spearman—Kärber method [23]. We arbitrarily designated positive reactions as those with fluorescence intensities more than double of the average of negative controls.

Dot blotting

Dot blotting was performed to determine the amount of PrP-res in brain homogenates. BHs (10% [w/v]) were incubated with 20 μg/ml proteinase K for 30 min at 37°C. Protease was inactivated by the addition of 2 mM Pefabloc sc (Shigma-Aldrich, Buchs, Switzerland). rHuPrP was used to generate a standard curve; the concentrations of unknown samples were determined by interpolation on the graph. Samples (Human recPrP and 10% BHs) were blotted onto nitrocellulose membrane (GE Healthcare, Freilburg, Germany). PrP-res on the membrane was denatured with 3 M GdnSCN. After blocking with 5% skim milk in TBST (10 mM Tris-HCl [pH 7.8], 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, the membrane was incubated overnight at 4°C with the primary antibody (AB) (6H4, Prionics, Zürich, Switzerland 1:5000) in 1% skim milk in TBST; after washing, the membrane was incubated for 1 h at room temperature with the secondary AB (anti-mouse IgG HRP, GE Healthcare, Buckinghamshire, UK, 1:5000) in 1% skim milk in TBST. Quantitative detection of PrP-res was performed using a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

Results

End-point RT-QUIC revealed high seeding activities in human brains affected with sporadic and genetic prion diseases.

We first analyzed patient no.1 (patient 1, sCJD MM-1). Brain homogenate was diluted from 5×10^{-5} to 5×10^{-9} and subjected to end-point RT-QUIC assay to quantitate seeding activity. The fluorescence of ThT was elevated at dilutions from 5×10^{-5} to 5×10^{-8}, then at 5×10^{-9} dilution there was yielded no reaction, as did a negative controls (non-CJD BH, DLB-BH, and A-CSF) (S1 Fig). The percentage of positive reaction decreased in a sigmoidal curve within the dilution range and the SD_{50} was calculated (Fig 1a). We were successfully able to analyze eight other sporadic CJDs and a genetic case, GSS-P102L; in all of these cases, the SD_{50} values were similar (it reached around 10 $\log_{10}$ SD_{50}/g of brain) (Figs 1b, 2 and Table 1).

1 SD_{50} unit is equivalent to 0.12 fg of PrP-res

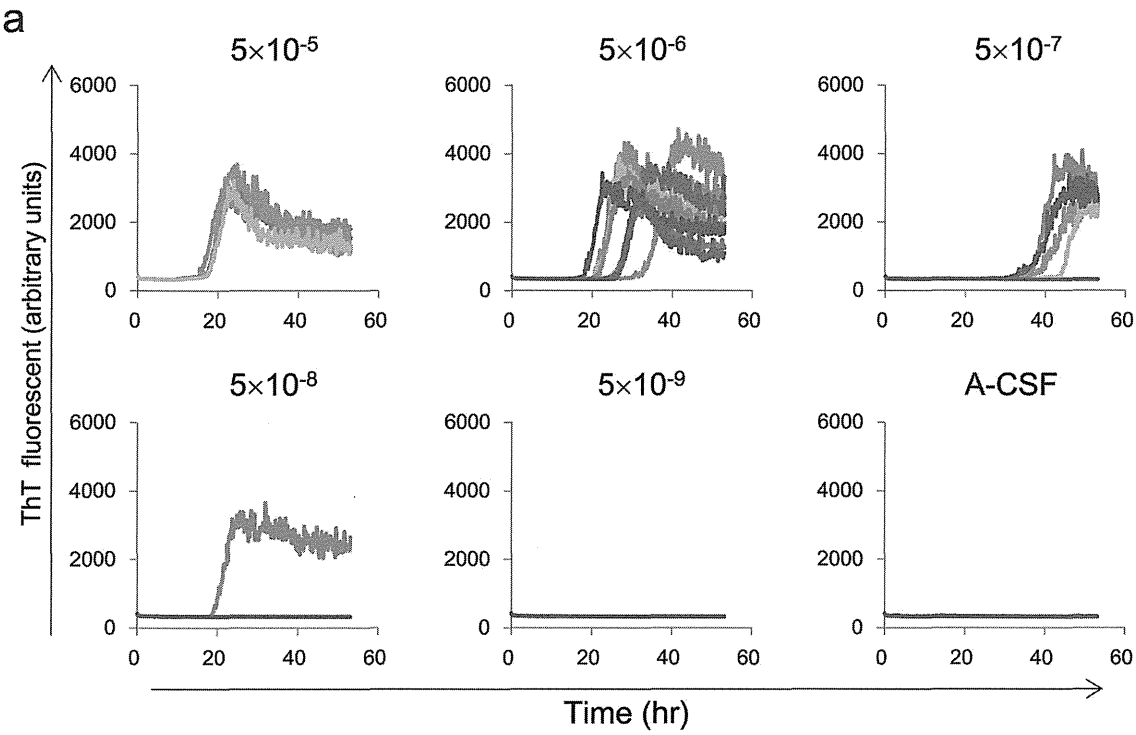
We quantified PrP-res in the brain samples by dot blotting. Based on the signals of recombinant human PrP (rHuPrP) (Fig 3a and 3b), the linearity of the standard curve was observed in the range of 0.39–12.5 ng protein (R^2 = 0.9899), we determined the amount or the concentration of PrP-res (Fig 3c and Table 1). The concentrations of PrP-res in BH samples were in the range 0.6–5.4 μg/g (PrP-res/brain). In those samples, MM1 (patient 2) had the highest level of PrP-res (5.4 μg/g), whereas the MM2 thalamic form (patient 10) had the lowest level, below the detection limits. Similarly, in terms of the value of SD_{50}, patient 2 was the highest and patient 10 was the lowest (Table 1). In cases of CJD-MM1, SD_{50} was linearly correlated with the level of PrP-res (R^2 = 0.8173) (Fig 3d) and 1 SD_{50} unit corresponded to 0.12 femto gram of PrP-res.

The lag time of RT-QUIC reaction are well correlated to amount of seeds but not applicable for quantitative assay instead of SD_{50}

RT-QUIC reactions exhibited elevation of ThT fluorescence after a lag phase of 15–18 h after seeding with a 5×10^{-5} diluted brain sample, and the duration of the lag phase increased along with higher dilutions. As a recent study by others has suggested a correlation between concentration of prion and lag time of RT-QUIC [26], we performed RT-QUIC in combination with three-fold serial dilution to confirm that the lag time can be used as a quantitative parameter instead of SD_{50} (Fig 4a). The correlation coefficient obtained from linear regression between PrP-res and lag time was lower than that obtained by polynomial regression between these two parameters y=-1.733ln(x)+6.8538 (R^2 = 0.9596). Of note, there was little difference among the higher concentration specimens (SD_{50} value of $10^{4.2}$ to $10^{2.8}$). On the other hand, lower concentration of brain specimens (SD_{50} value of $10^{1.3}$ to $10^{0.4}$) had larger standard deviations (Fig 4b), resulting huge overlap of data between different dilutions. We also observed inhibition of the RT-QUIC reaction seeded with 5 μl of seed containing 1 to 0.1% (SD_{50} value of $10^{5.6}$ to $10^{4.7}$) brain tissue. Taken together, although the good correlation was observed between seeding activity and lag time, these data suggest the limitations on the use of "lag time" as a parameter for prion activity under our experimental condition.

End-point QUIC can quantitatively evaluate the effect of treatment on human prion directly.

Next, we tested the RT-QUIC assay to evaluate decontamination methods. After autoclave treatment, brain homogenates from patients 3–5 and 8–10 were subjected to end-point RT-QUIC. Heat treatment at 121°C for 20 and 60 min decreased the SD_{50} by 2.25 and 3.88 orders of magnitude, respectively (Fig 5b). Fig 5a shows that brain from CJD (MM1) subjected to



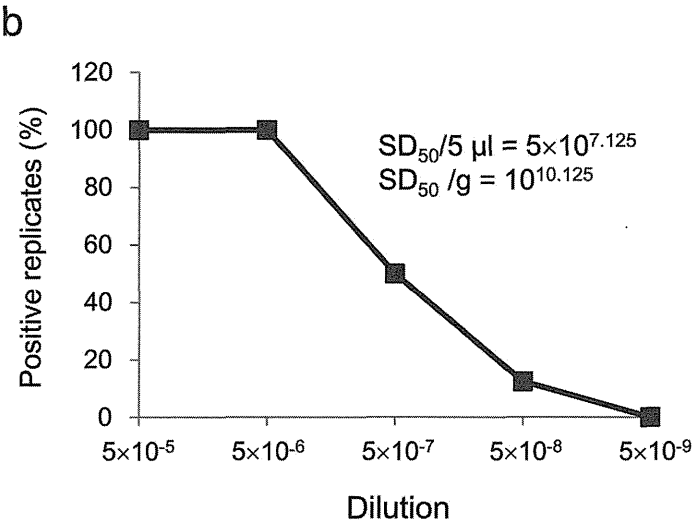


Fig 1. Quantitation of seeding activity in brain tissue from a sporadic CJD patient using end-point RT-QUIC. (a) BH (Pt 1) was diluted (5×10^{-5} to 5×10^{-9}) and subjected to RT-QUIC reaction containing human recPrP substrate. The fluorescence of ThT was elevated at dilutions from 5×10^{-5} to 5×10^{-8}. The 5×10^{-9} dilution yielded no reaction, as did a negative control consisting of A-CSF. (b) The percentage of positive reaction decreased in a sigmoidal curve within the dilution range when BH was used as the seed. The SD_{50} was calculated using the Spearman—Kärber method.

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