Table 1. Incubation times following inoculation of Tg(CerPrP)1536 mice with prions from skeletal muscle and brain samples of CWD-affected deer.

Inocula	Incubation time, mean d ± SEM (n/n0)*	
	Skeletal muscle	Brain
	CWD-affected deer	
H92	360 ± 2 d (6/6)	283 ± 7 d (6/6)
33968	367 ± 9 d (8/8)	278 ± 11 d (6/6)
5941	427 ± 18 d (7/7)	
D10	483 ± 8 d (8/8)	231 ± 17 d (7/7)
D08	492 ± 4 d (7/7)	
Averages	426 d	264 d
	Non-diseased deer	
FPS 6.98	>523 d (0/6)	
FPS 9.98	>454 d (0/7)	>454 d (0/6)
None	>490 d (0/6)	
PBS	>589 d (0/5)	

*The number of mice developing prion disease divided by the original number of inoculated mice is shown in parentheses. Mice dying of intercurrent illnesses were excluded.

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	機構処理欄
				2005. 11. 24	該当なし	

一般的名称	人血清アルブミン	研究報告の公表状況	Ligios C, Sigurdson CJ, Santucciu C, Carcassola G, Manco G, Basagni M, Maestrale C, Cancedda MG, Madau L, Aguzzi A. Nat Med. 2005 Nov;11(11):1137-8.	公表国
販売名(企業名)	赤十字アルブミン20(日本赤十字社) 赤十字アルブミン25(日本赤十字社)			イタリア

研究報告の概要

○スクレイピーおよび乳腺炎に罹患した羊の乳腺におけるPrP^{Sc}

イタリアのサッサリ県で818頭の羊を調査した。7頭は脳、リンパ節、扁桃腺においてPrP^{Sc}が検出され、臨床的に明らかなスクレイピーの症状を呈していた。4頭が乳腺炎とスクレイピーを併発していた。この4頭全ての乳腺においてPrP^{Sc}が検出されたが、乳腺炎を併発していないスクレイピー発症前の羊やスクレイピーを発症した同じ群(n=14)又は他の群(n=1)由来の羊、乳腺炎に罹患しているがスクレイピーへの感染は認められない羊(n=2)においてはPrP^{Sc}は検出されなかった。乳腺の炎症病変部の解析では、PrP^{Sc}のリンパ濾胞部位への集積が認められた。PrP^{Sc}は、乳腺炎による病変部位中の主にCD68+マクロファージおよびFDCsと共局在化していた。

慢性的な炎症とスクレイピーの併発により、PrP^{Sc}が想定外の組織まで拡大して蓄積する可能性が示された。乳房中のPrP^{Sc}濃度の中央値は、脾臓の0.1%、脳の0.05%と算出されたが、乳房のリンパ濾胞は確率的な分布を示しているため、局部的なPrP^{Sc}量には顕著なばらつきが認められた。

本研究ではMaedi-Visnaウィルス(MVV)の血清抗体陽性反応とリンパ濾胞乳腺炎の相関が示された。ヨーロッパの小型反芻動物のほとんどはMVVおよび関連レンチウィルスに感染している。ごく一般的なウィルス感染が原因となるプリオン病感染拡大の可能性が示唆された。MVVは、乳房上皮細胞やマクロファージ中に存在し、羊乳を介して子羊に伝播することが実験的に証明されている。PrPの乳房リンパ濾胞部位のCD68+細胞への蓄積は、乳腺炎の羊の羊乳中への大量のマクロファージの混入も併せ、プリオン感染と分泌器官の炎症の併発が分泌物のプリオン汚染を誘導し、群中におけるプリオンの水平感染の共同因子となり得るのか、という疑問を提起することとなった。

使用上の注意記載状況・その他参考事項等

赤十字アルブミン20
赤十字アルブミン25

血液を原料とすることに由来する感染症伝播等

報告企業の意見	今後の対応
スクレイピーおよび乳腺炎に罹患した羊の乳腺でPrP^{Sc} が検出されたとの報告である。	これまでの疫学研究等では、ヒトにおいて、血漿分画製剤を介してスクレーピーを含む伝達性海綿状脳症(TSE)が伝播するという証拠はない。また異常プリオンがアルブミン製剤の製造工程で効果的に除去されるとの報告もあるが、輸血によりvCJDに感染する可能性が示唆されたことから、今後も情報の収集に努める。

Table 1 Summary of the fluorescence correlation spectroscopy measurements using 10 μM RITA

Protein	Diffusion time ± s.e.m.[a]	Change in diffusion time, percent
No protein	0.063 ± 0.011	—
GST-p53 dN(1–63)	0.356 ± 0.070	465
GST-p53 N(1–100)	0.259 ± 0.020	311
GST-p53(1–393)	0.287 ± 0.043	355
His-p53(1–393)	0.198 ± 0.007	214
His-p53(1–312)	0.111 ± 0.014	74
GST	0.076 ± 0.003	20
GST-EBNA2	0.073 ± 0.017	16

[a]All experiments were performed at least three times.

subject for future research.

Accession codes. BIND identifiers (http://bind.ca): 335735.

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PrP^{Sc} in mammary glands of sheep affected by scrapie and mastitis

To the editor:

Besides colonizing the central nervous system, the infectious agent of transmissible spongiform encephalopathies, termed prion, is predominantly associated with follicular dendritic cells (FDCs) of lymphoid tissues[1,2]. Accordingly, PrP^{Sc}, a protease-resistant isoform of the host protein PrP^{C} representing the main prion constituent, is often detectable in spleen, tonsils, Peyer patches and lymph nodes of infected hosts.

Chronic inflammatory states are accompanied by local extravasation of B cells and other inflammatory cells, which may induce lymphotoxin-dependent maturation of ectopic FDCs. Consequently, scrapie infection of mice suffering from nephritis, hepatitis or pancreatitis induces unexpected prion deposits at the sites of inflammation[3]. This has raised concerns that analogous phenomena might occur in farm animals.

We have investigated this question in a flock of 818 Sarda sheep held in the Sassari region of Italy for production of wool and human foods. The European Surveillance Plan for Transmissible Spongiform Encephalopathies mandates the removal of all sheep of scrapie-susceptible genotypes in scrapie-infected flocks.Of the 818 sheep, 261 had *Prnp* alleles[4] that conferred susceptibility to prion disease. Of the latter, seven had clinically overt scrapie with PrP^{Sc} in brain, lymph nodes and tonsil. All scrapie-sick sheep and 100 randomly chosen healthy sheep were killed, and mammary glands were analyzed histologically. Of these, 10 sheep had lymphocytic mastitis, and four had coincident mastitis and scrapie. Using western blots, immunohistochemistry and histoblots, we detected PrP^{Sc} in mammary glands of all four clinically scrapie-sick sheep with mastitis (Fig. 1a,b), but not in noninflamed mammary glands from presymptomatic or scrapie-sick sheep from the same ($n = 14$) or a different flock ($n = 1$), nor in inflamed mammary glands of scrapie-uninfected sheep ($n = 2$). Within the inflammatory mammary lesions, PrP^{Sc} was found to be associated with lymphoid follicles by immunofluorescent labeling and by paraffin-embedded tissue (PET) blotting (Fig. 1c). PrP^{Sc} colocalized predominantly with $CD68^{+}$ macrophages and FDCs within inflamed mammary glands (Fig. 2a).

We then surveyed a second Sarda flock (272 sheep) located 30 km away from the flock described above. One sheep was found to be

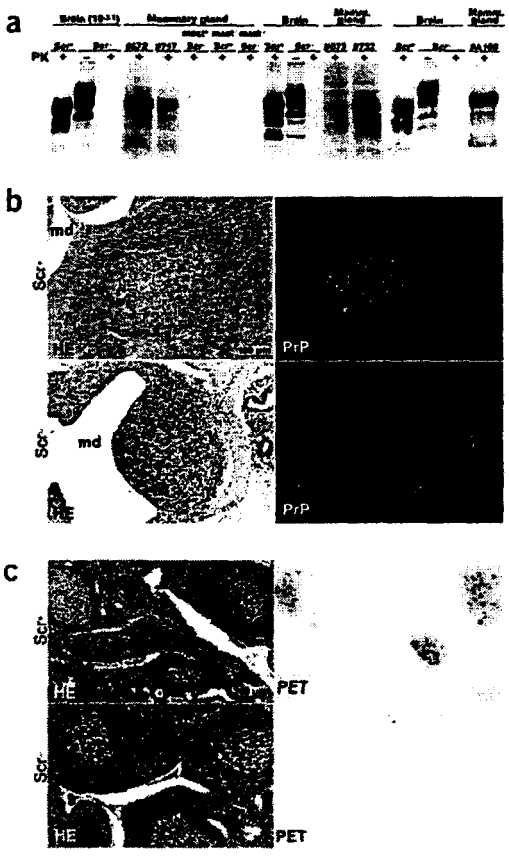

Figure 1 Prion protein in inflamed mammary glands. (**a**) Western blots with a PrP-specific antibody. Lanes 1–3, 9–11, 14–16 from left: native and proteinase K (PK)-digested brain homogenates (diluted 1/1,400) from a scrapie-infected (Scr^{+}) and a scrapie-free sheep (Scr^{-}). Lanes 6–8: mammary glands from a scrapie-free sheep with follicular mastitis (Scr^{-}, $mast^{+}$), a scrapie-positive sheep from a flock with neither MVV seropositivity nor mastitis (Scr^{+}, $mast^{-}$), and a sheep with neither mastitis nor scrapie (Scr^{-}, $mast^{-}$). Each one of five scrapie-infected sheep with mastitis had mammary PrP^{Sc} (lanes 4, 5, 12, 13, and 17). Non-scrapie-infected brain and mammary gland extracts showed no PrP^{Sc} upon PK digestion (lanes 3, 6–8, 11 and 16). (**b**) Mammary gland micrographs from MVV-seropositive sheep with mastitis and coincident scrapie (Scr^{+}), or with mastitis but no scrapie (Scr^{-}). Lymphoid follicles are adjacent to milk ducts (md). Immunofluorescence stains show abundant PrP deposits within mammary lymphoid follicles (arrow) from scrapie-positive but not from scrapie-free sheep. Scale bars, 100 μm. (**c**) PK-treated paraffin-embedded tissue blots of mammary gland sections show punctate PrP^{Sc} deposits colocalizing with lymphoid follicles in scrapie-infected (Scr^{+}), but not in scrapie-free (Scr^{-}) sheep with mastitis. Scale bars, 200 μm.

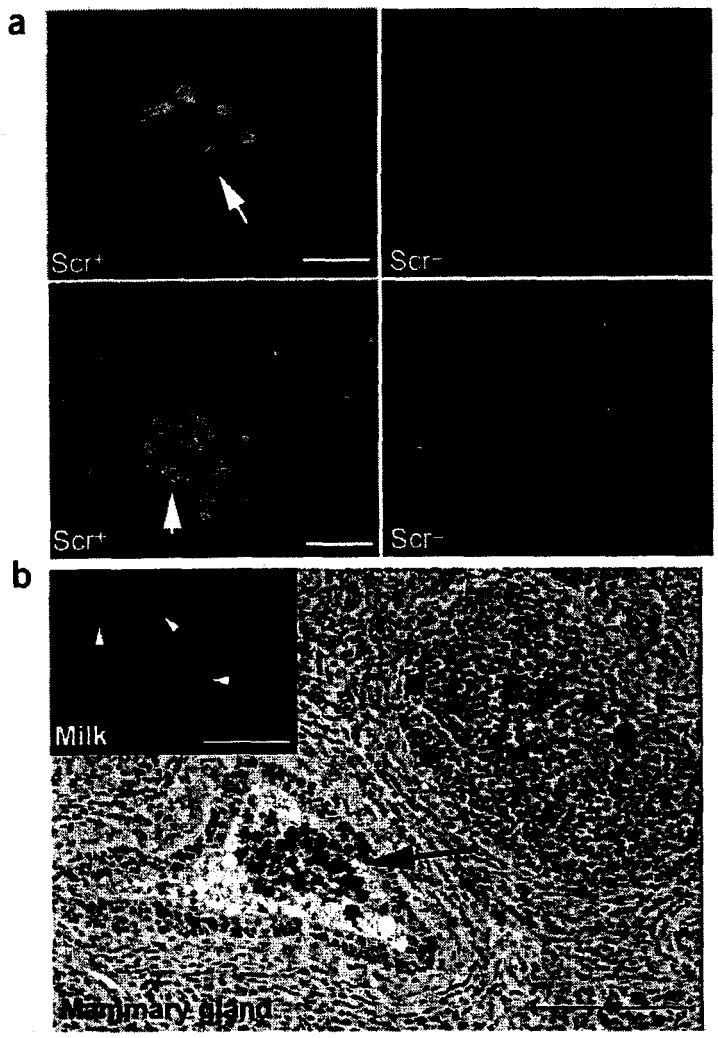

Figure 2 Mammary PrP^{Sc} localizes to macrophages and FDCs. (**a**) Mammary gland from a sheep with coincident mastitis, MVV seropositivity and scrapie (sheep #732). Confocal laser scanning micrographs of lymphoid follicles immunostained for PrP (green), nuclear DNA (blue) and macrophages (red, top panels) or FDC (red, bottom panels). PrP^{Sc} associates with $CD68^+$ macrophages and FDCs in scrapie-positive (Scr^+, arrows) but not in scrapie-free sheep (Scr^-). Scale bars, 6.3 μm (top) and 7.5 μm (bottom). (**b**) $CD68^+$ macrophages (arrow) and degenerating leukocytes within milk ducts and in adjacent lymphoid follicles of an inflamed mammary gland, as well as in milk sediment (inset, arrowheads). Scale bar, 100 μm (mammary gland) or 20 μm (milk cells).

scrapie-sick and was killed: necropsy showed lymphofollicular mastitis and PrP^{Sc} in the brain and tonsil. Again, PrP^{Sc} was present in the mammary gland (**Fig. 1a**). These results indicate that coincidence of natural chronic inflammatory conditions and natural scrapie can expand the deposition of PrP^{Sc} to unexpected tissues of sheep.

By plotting western blot signals against serially diluted scrapie-infected brain and spleen, we determined that the median mammary PrP^{Sc} concentration was 0.1% of that of spleen and 0.05% of brain. But because mammary lymphoid follicles were stochastically distributed, local PrP^{Sc} loads varied markedly. Hence these figures may underestimate PrP^{Sc} in sites of abundant follicles, and overestimate it in sites with few or no follicles.

Common causes of lymphofollicular mastitis in sheep include Maedi-Visna virus (MVV) and mycoplasma[5]. We could not culture mycoplasma from mastitic glands, whereas we found that four of the five sheep with scrapie and mastitis were seropositive for MVV and that the three scrapie-sick sheep without mastitis were seronegative for MVV. In the clinically healthy group, 7 of 10 sheep with mastitis, but only 32 of 90 sheep without mastitis, were seropositive for MVV. Hence, MVV seropositivity correlated with lymphoid follicular mastitis (Fisher exact test, $P = 0.01$) as reported previously[6,7].

MVV and related small-ruminant lentiviruses are endemic in most, if not all, European populations of small ruminants[6]. The above data suggest that common viral infections of small ruminants may enhance the spread of prions. MVV is found within mammary epithelial cells and macrophages[8], and has been experimentally passed to lambs through milk[9]. Milk is believed to represent a major route of transmission for the natural spread of MVV[5]. The PrP deposits in $CD68^+$ cells of mammary lymphoid follicles, in concert with the copious shedding of macrophages into milk of mastitic sheep (**Fig. 2b**)[9,10], raises the question whether coexistence of prion infection and inflammation in secretory organs may lead to prion contamination of secretes, and may represent a cofactor for horizontal prion spread within flocks.

ACKNOWLEDGMENTS

We thank J. DeMartini for discussions and B. Seifert for statistical analysis. This study was supported by grants of the European Union (Apopis), the Swiss National Foundation, the National Competence Center for Research on Neural Plasticity and Repair (to A.A.), Istituto Zooprofilattico Sperimentale della Sardegna 001/02 (to C.L.), US National Institutes of Health K08-AI01802 and the Foundation for Research at the Medical Faculty, University of Zürich (to C.J.S.) and the United States National Prion Research Program (to C.J.S. and A.A.).

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医薬品
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識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2005 年 10 月 25 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			研究報告の公表状況	Ultra-sensitive detection of prion protein fibrils by flow cytometry in blood from cattle affected with bovine spongiform encephalopathy Lothar Trieschmann, Alexander Navarrete Santos, Katja Kaschig, Sandra Torkler, Elke Maas, Hermann Schätzl and Gerald Böhm* BMC Biotechnology 2005, 5:26	公表国 ドイツ	
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
現在、BSE、CJD、CWD、そしてスクレイピーなどの伝染性海綿状脳症(TSE)の確定診断は、死後の病理解剖で病原性プリオン(PrPsc)が検出されることによってのみ下される。一方、臨床症状を発現する前でも、血液中にPrPscが存在する可能性が示唆されており、輸血などによる伝播が問題となっている。しかしPrPscの血中濃度は非常に低く、現在の分析法では検出することが困難である。著者らは、「核」を加えた場合と加えなかった場合のプリオン蛋白単量体の重合化動態の違いを利用して、高感度のプリオン蛋白凝集体の検出法を開発した。凝集の検出はフローサイトメトリーにより行った。BSEを発症した6頭のウシおよび4頭の正常ウシの血清を使用して実験を行った。予め作成した、蛍光標識したプリオン単量体を血清に添加すると、添加しなかった場合と比較して、この単量体が「核」となり新たな凝集塊の形成が促進されることが確認された。この実験系では、発症ウシの血清 6 検体全てにおいて、10^{-8}nM(0.24fg/mL)の低濃度でも合成プリオンの凝集塊が検出された。以上の結果から、本検出法は、少量のBSE陽性の血清と正常血清とを識別する方法として有望であり、プリオン病の発症前診断の手がかりとなるかもしれない。	BYL-2005-0199

報告企業の意見	今後の対応
弊社の血漿分画製剤の製造工程におけるプリオン除去能は4 logを上回ることが確認されている。本論文の実験結果が実用化されれば,プリオンの理論的伝播リスクがさらに低減することが期待される。	現時点で弊社において新たな安全対策上の措置を講じる必要はないと考える。引き続き本方法の実用化およびPrP^{sc}の検出・除去技術に関する関連情報の収集に努める。

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Ultra-sensitive detection of prion protein fibrils by flow cytometry in blood from cattle affected with bovine spongiform encephalopathy

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Published: 04 October 2005

BMC Biotechnology 2005, 5:26 doi:10.1186/1472-6750-5-26

Received: 03 May 2005

Accepted: 04 October 2005

This article is available from: http://www.biomedcentral.com/1472-6750/5/26

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Abstract

Background: The definite diagnosis of prion diseases such as Creutzfeldt-Jakob disease (CJD) in humans or bovine spongiform encephalopathy (BSE) in cattle currently relies on the *post mortem* detection of the pathological form of the prion protein (PrP^{Sc}) in brain tissue. Infectivity studies indicate that PrP^{Sc} may also be present in body fluids, even at presymptomatic stages of the disease, albeit at concentrations well below the detection limits of currently available analytical methods.

Results: We developed a highly sensitive method for detecting prion protein aggregates that takes advantage of kinetic differences between seeded and unseeded polymerization of prion protein monomers. Detection of the aggregates was carried out by flow cytometry. In the presence of prion seeds, the association of labelled recombinant PrP monomers in plasma and serum proceeds much more efficiently than in the absence of seeds. In a diagnostic model system, synthetic PrP aggregates were detected down to a concentration of approximately 10^{-8} nM [0.24 fg/ml]. A specific signal was detected in six out of six available serum samples from BSE-positive cattle.

Conclusion: We have developed a method based on seed-dependent PrP fibril formation that shows promising results in differentiating a small number of BSE-positive serum samples from healthy controls. This method may provide the basis for an *ante mortem* diagnostic test for prion diseases.

Background

A group of fatal transmissible neurodegenerative diseases, including Creutzfeld-Jakob disease (CJD), bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) and scrapie, is caused by an unusual infectious agent that has been termed prion [1]. Prions consist of an aberrant isoform (PrP^{Sc}) of the normal cellular prion protein (PrP^{C}). PrP^{C} is a cell surface glycoprotein expressed in neurons [2] and other cell types [3,4]. The precise physiological function of the cellular prion protein is not known yet. PrP^{Sc} differs from PrP^{C} in its higher content of β-sheet structure [5,6], its partial resistance to protease digestion

[7], and its tendency to form large aggregates [8]. PrP^{Sc} propagates by converting the cellular prion protein to the PrP^{Sc} conformation [9]. PrP^{Sc} aggregates accumulate predominantly in the central nervous system (CNS), and definitive diagnosis of prion diseases currently relies on the *post mortem* detection of PrP^{Sc} in CNS tissue by immunohistochemistry, Western blotting, or ELISA [10]. Transmission studies indicate that prions may also be present in blood, potentially allowing for *ante mortem* diagnosis, but the sensitivity of the currently available analytical methods is insufficient for the detection of the extremely low prion titers that can be expected in body fluids [11].

Here, we report the development of a method based on kinetic differences between seeded and unseeded aggregation of prion protein that allows the detection of PrP aggregates in blood down to attomolar concentrations by flow cytometry.

Results and discussion

Detection of synthetic prion protein aggregates in serum or plasma

Kinetic differences between seeded and spontaneous polymerization of peptide monomers can be used for the detection of amyloid β-protein aggregates in the cerebrospinal fluid of Alzheimer's disease patients [15]. Here, we extend the principle of seeded polymerization to the detection of prion protein aggregates.

While trying to establish conditions for the labeling of synthetic prion protein aggregates with a fluorescently labeled prion protein probe, we observed that the formation of prion protein aggregates proceeds much less efficiently in serum or plasma (not shown) than in PBS (Fig. 1). This inhibition is probably caused by interactions of the prion protein probe with serum proteins.

Next, we found that the addition of preformed prion protein aggregates to plasma can partially overcome this inhibition (Fig. 2). The preformed aggregates presumably function as seeds that facilitate the formation of new aggregates in the inhibitory environment of plasma. The seeds stimulated the formation of prion protein aggregates at all concentrations tested, from 5 nM [120 ng/ml] to 10^{-8} nM [0.24 fg/ml] (Fig 2C). The average ratio of event counts in seeded samples to those in samples without seeds was 6.4. The number of events, however, was not proportional to the seed concentration, but remained relatively constant over the whole concentration range. Thus, the seed-dependent formation of prion protein aggregates can be used to detect extremely low amounts (down to the attomolar range) of spiked prion protein aggregates in blood.

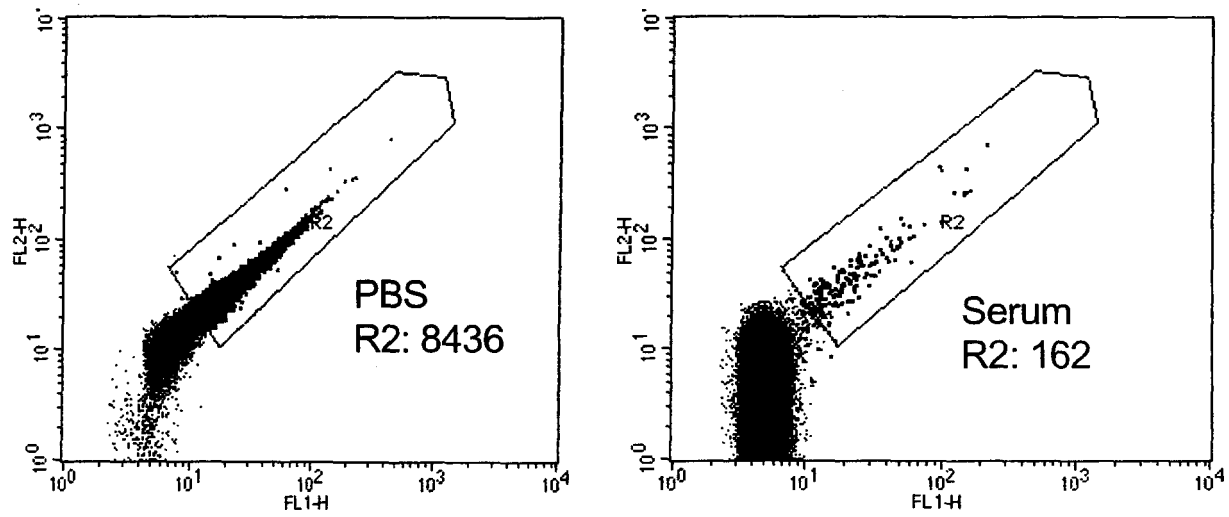

Figure 1

Inhibition of PrP aggregation in serum. FITC-labeled recombinant bovine prion protein (concentration 10 nM) was incubated at 37°C for 20 h with continuous shaking, either in 150 µl PBS (left panel) or in the same volume of serum (right panel), followed by flow cytometry. The measurements are depicted in a Fluorescence 1 (FL1-H) vs. Fluorescence 2 (FL2-H) dot-plot. The number of counts in the area containing specific signals (R2) is given in the figures. Aggregate formation in serum is strongly inhibited.

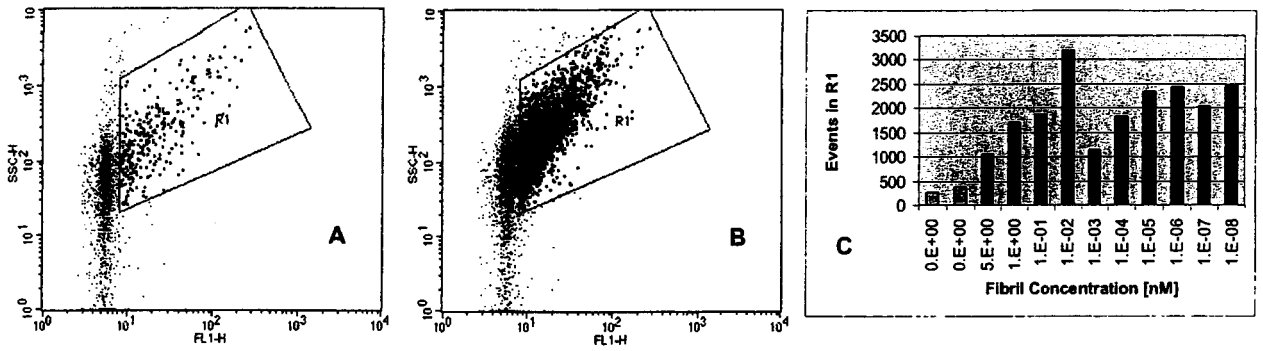

Figure 2
Seed-dependent PrP aggregate formation in plasma. FITC-labeled recombinant prion protein (5 nM) was incubated in plasma as described in the methods section for 20 h either in the absence (panel A) or presence (panel B) of 10^{-8} nM PrP aggregates. Panel C: quantification of measurements shown in A and B, and of measurements (not shown) with different seed concentrations. The measurements are depicted in a Fluorescence 1 (FL1-H) vs. Side-Scatter (SSC) dot-plot. Aggregate formation (signal in region R1) was strongly enhanced by all seed concentrations tested, from 5 nM to 10^{-8} nM.

Analysis of serum from clinical-stage, BSE-positive cattle
Studies demonstrating the transmission of prion diseases by blood transfusion suggest that prions are present in the blood of afflicted animals and people, even at pre-symptomatic stages of the disease [16-18]. We used the method of seed-dependent fibril formation to analyze serum from six confirmed cases of clinical-stage, BSE-positive cattle and four controls. Based on the spiking experiments described above, our hypothesis was that any PrP^{Sc} aggregates present in serum may act as seeds for the formation of easily detectable amounts of labeled PrP aggregates, whereas in the absence of seeds the formation of PrP aggregates would be inhibited. The serum samples from BSE-positive cattle and controls from healthy cattle were incubated with 10 nM of a FITC-labeled bovine PrP probe at 37°C for 20 h with continuous shaking, followed by analysis in a flow cytometer. All six BSE-samples could be clearly distinguished by a population of events that was absent in the controls (Fig. 3A–J, green dots in region R3; quantification in fig. 3K).

Conclusion
We have developed a method based on seed-dependent PrP fibril formation that shows promising results in differentiating a small number of BSE-positive serum samples from healthy controls. More samples need to be tested in order to validate its potential as an *ante mortem* diagnostic test for BSE and other prion diseases.

Methods
Biological fluids
Serum samples from six confirmed cases of BSE in cattle and four control animals were obtained from BFAV, Insel Riems, Germany. Control plasma was obtained from a blood bank.

Labeling of prion protein
Recombinant full-length bovine PrP was produced as described previously [12,13]. The purified protein was labeled with a FITC-labeling kit (Roche) according to the manufacturer's instructions.

Preparation of fibrils from recombinant prion protein
25 μM of unlabeled bovine prion protein in PBS containing 0.2 % SDS was incubated for 10 min at room temperature, followed by a twentyfold dilution with PBS. For fibril formation, the diluted reaction mixture was incubated for 48 h at room temperature [14].

PrP fibril formation in serum or plasma
Recombinant FITC-labeled bovine prion protein was incubated in 150 μl serum or plasma at a concentration of 5 or 10 nM for 5–10 min. at 20°C, shaking at 550 rpm in an Eppendorf thermomixer, followed by an increase of the temperature to 37°C h at constant shaking speed. The incubation was continued for 20 h. Samples were then analyzed by flow cytometry.

Flow cytometry
Analysis of the samples was carried out on a FACSVantage flow cytometer (BD Biosciences) at room temperature, measurement time was 30 sec per sample.

Authors' contributions
LT participated in the design of the study, carried out the measurements and drafted the manuscript. ANS participated in the analysis of the data. EM prepared the recom-

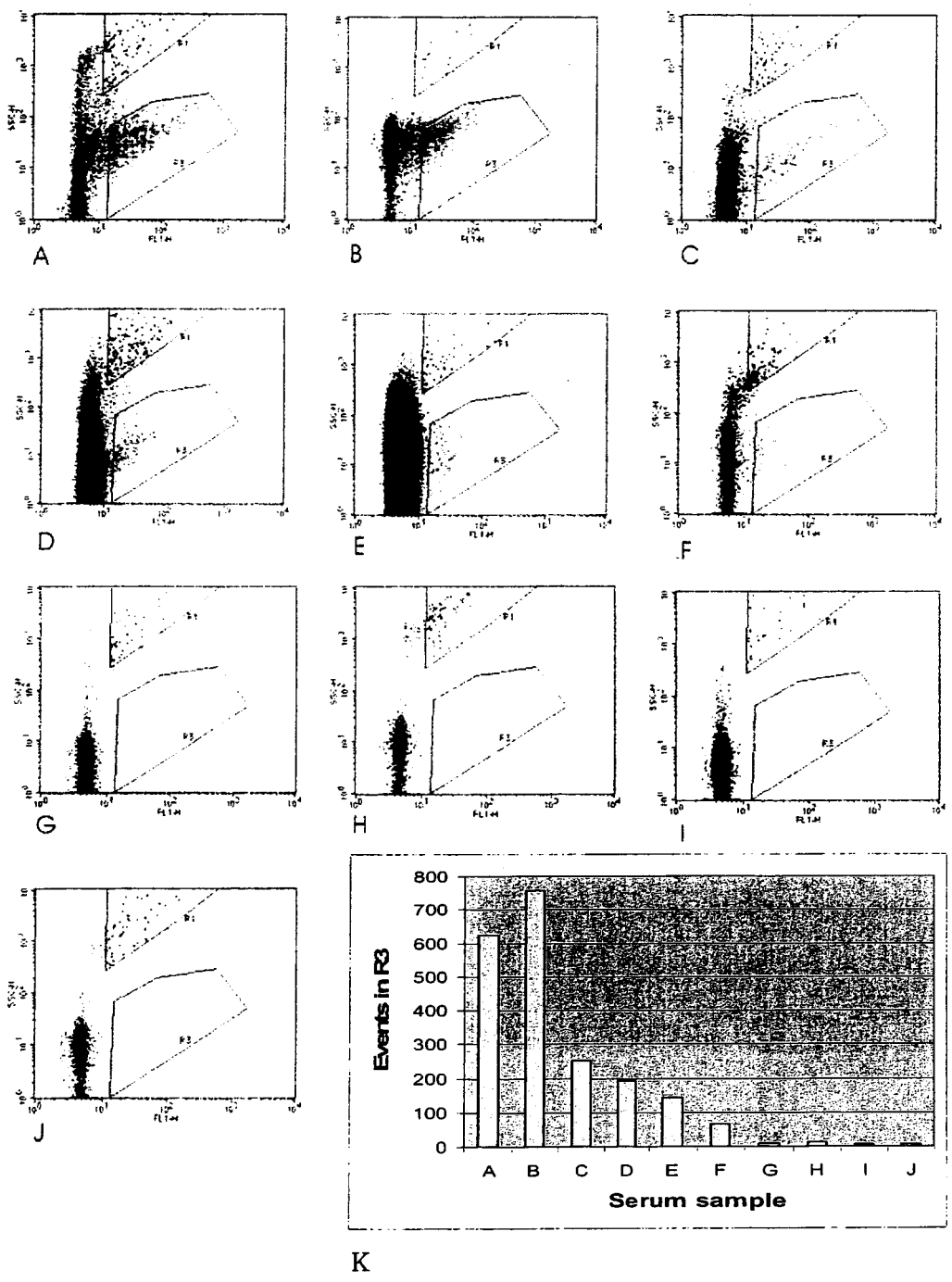

Figure 3
Analysis of serum from BSE-positive cattle. FITC-labeled recombinant prion protein (10 nM) was incubated in 150 µl of the serum samples as described in the methods section and analyzed by flow cytometry. The measurements are shown in a Fluorescence 1 (FL1-H) vs. Side-Scatter (SSC) dot-plot. All six BSE-samples (A-F) can be differentiated from the controls (G-J) by a population of events in region R3 (green dots). Panel K: Quantification of measurements shown in panels A-J.

binant protein. KK and ST were also involved in protein expression and purification. HS participated in the design and coordination of the study. GB conceived of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was kindly supported by grant No. 0312711A from the BMBF (Bundesministerium für Bildung und Forschung) in the context of the German National TSE Research Platform. The authors gratefully acknowledge the help of the TSE Research Platform, Munich, and the BFAV Riems, Germany, with respect to the kind gift of biological material in the context of this study.

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				2005. 12. 20	該当なし	

一般的名称	(製造承認書に記載なし)	研究報告の公表状況	Peretz D, Supattapone S, Giles K, Vergara J, Freyman Y, Lessard P, Safar JG, Glidden DV, McCulloch C, Nguyen HO, Scott M, Dearmond SJ, Prusiner SB. J Virol. 2006 Jan;80(1):322-31.	公表国
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研究報告の概要

○酸性ドデシル硫酸ナトリウムによるプリオンの不活化
酢酸中の分岐ポリアミン・デンドリマーにプリオンを曝露するとプロテアーゼ感受性になるという発見(S. Supattapone, H. Wille, L. Uyechi, J. Safar, P. Tremblay, F. C. Szoka, F. E. Cohen, S. B. Prusiner, and M. R. Scott, J. Virol. 75:3453-3461, 2001)を受けて、弱酸における酸性ドデシル硫酸ナトリウム(SDS)によるプリオンの不活化の検討を行った。ゴールデンハムスターの脳ホモジネート中のSc237プリオンを室温で1% SDS及び0.5% 酢酸に曝露したところ、プリオン力価が約107分の1に減少した。しかし、バイオアッセイに用いたハムスターすべてが最終的にプリオン病を発症した。SDS及び酢酸のさまざまな濃度について実験を行ったところ、曝露期間と温度が相乗的に作用し、これによりハムスターのSc237プリオンとヒトの孤発性クロイツフェルト・ヤコブ病(sCJD)プリオン双方の不活化が起こった。脳ホモジネート中のプリオン及びステンレス鋼線材に付着したプリオンの不活化を、遺伝子組み換えマウスのバイオアッセイで評価した。sCJDプリオンは、不活化に対してSc237より10万倍以上の耐性があり、齧歯類のプリオンで評価されている不活化の手順がヒトのプリオンの不活化に適用できないことが示された。脳ホモジネート中のプリオン力価を有意に減少させた手順には、ステンレス鋼線材に付着したプリオンにはわずかな影響しか与えないものもあった。15分間のオートクレーブと酸性SDSの併用により、ステンレス鋼線材に付着したヒトsCJDプリオンが除去された。この知見は、手術器具、またその他の医療機器、歯科用機器のプリオン不活化に適した非腐食性システムの基礎となる。

報告企業の意見	今後の対応
15分間のオートクレーブと酸性ドデシル硫酸ナトリウムの併用により、ステンレス鋼線材に付着したヒトsCJDプリオンが除去されたとの報告である。	今後も引き続き、プリオン病に関する新たな知見及び情報の収集に努める。

使用上の注意記載状況・その他参考事項等

合成血「日赤」
照射合成血「日赤」

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

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JOURNAL OF VIROLOGY, Jan. 2006, p. 322–331 Vol. 80, No. 1
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.1.322–331.2006
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Inactivation of Prions by Acidic Sodium Dodecyl Sulfate

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Received 14 March 2005/Accepted 16 September 2005

Prompted by the discovery that prions become protease-sensitive after exposure to branched polyamine dendrimers in acetic acid (AcOH) (S. Supattapone, H. Wille, L. Uyechi, J. Safar, P. Tremblay, F. C. Szoka, F. E. Cohen, S. B. Prusiner, and M. R. Scott, J. Virol. 75:3453–3461, 2001), we investigated the inactivation of prions by sodium dodecyl sulfate (SDS) in weak acid. As judged by sensitivity to proteolytic digestion, the disease-causing prion protein (PrP^{Sc}) was denatured at room temperature by SDS at pH values of ≤4.5 or ≥10. Exposure of Sc237 prions in Syrian hamster brain homogenates to 1% SDS and 0.5% AcOH at room temperature resulted in a reduction of prion titer by a factor of ca. 10^7; however, all of the bioassay hamsters eventually developed prion disease. When various concentrations of SDS and AcOH were tested, the duration and temperature of exposure acted synergistically to inactivate both hamster Sc237 prions and human sporadic Creutzfeldt-Jakob disease (sCJD) prions. The inactivation of prions in brain homogenates and those bound to stainless steel wires was evaluated by using bioassays in transgenic mice. sCJD prions were more than 100,000 times more resistant to inactivation than Sc237 prions, demonstrating that inactivation procedures validated on rodent prions cannot be extrapolated to inactivation of human prions. Some procedures that significantly reduced prion titers in brain homogenates had a limited effect on prions bound to the surface of stainless steel wires. Using acidic SDS combined with autoclaving for 15 min, human sCJD prions bound to stainless steel wires were eliminated. Our findings form the basis for a noncorrosive system that is suitable for inactivating prions on surgical instruments, as well as on other medical and dental equipment.

Prions are infectious proteins that cause fatal neurodegenerative illnesses, including Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE), and scrapie in sheep (37, 60, 97). In mammals, prions are comprised solely of the disease-causing isoform of the prion protein (PrP), designated PrP^{Sc}. PrP^{Sc} is formed from the cellular precursor PrP^{C} by a process involving a profound conformational change. While PrP^{C} is a protein with three α-helices and little β-sheet, PrP^{Sc} is rich in β-sheet structure. It seems likely that the infectious prion monomer consists of a trimer of PrP^{Sc} molecules based on an ionizing radiation target size of 55 kDa and electron crystallography studies (3, 7, 30, 99). Limited proteolysis of PrP^{Sc} results in N-terminal truncation to form PrP 27-30, which retains infectivity and polymerizes into amyloid fibrils (49, 67). Electron crystallography combined with molecular modeling suggests that both PrP^{Sc} and PrP 27-30 contain a β-helix (30, 99). The conformation and extraordinarily small size of the prion are probably responsible for its extreme resistance to inactivation.

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Reports in the mid-1960s on the resistance of prions to inactivation by both ionizing and UV radiation served to accentuate the mysterious nature of the infectious agent causing scrapie of sheep (2, 3). Two decades earlier, the resistance of the scrapie agent to inactivation by formalin was recognized when more than 1,500 sheep, immunized against looping-ill virus with a formalin-treated vaccine contaminated by the scrapie agent, developed scrapie several years after vaccination (29). With the transmission of the scrapie agent to mice and later Syrian hamsters (16, 46), studies were undertaken to define conditions for inactivation. The results of numerous studies designed to probe the molecular nature of the scrapie agent and define conditions for inactivation concluded that protein denaturants were effective at reducing infectivity titers but that complete inactivation required extremely harsh conditions, such as 5 h of autoclaving at 134°C or treatment with 2 N NaOH (65, 66). It is important to note that these conditions, on which current guidelines are based, were determined for the rodent strains, before it was known that prion strains may exhibit different stabilities to denaturation by heat, as well as chaotropes (56, 91). Defining conditions for inactivation of prions is an important undertaking in view of the human forms of prion disease that were elucidated by studies demonstrating the experimental transmission of prions from patients who died of kuru or CJD to apes and monkeys (26, 27). Radiation inactivation of CJD prions and studies of human PrP argue that human prions, like those causing scrapie and BSE, are resistant to inactivation (10, 28). More recently, the number of cases of iatrogenic CJD; the transmission of BSE prions from

cattle to humans, causing variant (v) CJD (97, 98); and the probable transmission of vCJD by blood transfusion (45, 55) highlight the pressing need for effective prion decontamination.

In the course of studies on the expression of PrP genes in prion-infected cultured cells, we found that branched polyamine dendrimers rendered PrP^{Sc} susceptible to degradation (83). This enhanced susceptibility to degradation could be mimicked in vitro by incubating prions with polyamine dendrimers at pH ~3.5 (84). Intrigued by the ability of weak acids such as acetic acid (AcOH) in combination with dendrimers to render prions susceptible to proteolytic degradation, we explored prion stability upon exposure to a variety of protein denaturants under weakly acidic conditions. Of all the detergents and chaotropes examined, sodium dodecyl sulfate (SDS) combined with AcOH proved to be the most potent reagent for the inactivation of prions. This finding was unexpected since SDS at neutral pH exhibits only a modest ability to inactivate prions in our experience (63). The experiences of others are noteworthy: 3% SDS at neutral pH has been reported to destroy prion infectivity in brain homogenates when samples were boiled or autoclaved (36, 87, 88). However, prion infectivity in macerated brain samples survived boiling for 15 min in 5% SDS at neutral pH (90). These findings suggest that SDS solutions at neutral pH, even when exposed to high temperatures, cannot be used for the complete inactivation of prion infectivity.

As described here, acidic SDS was superior to all other protein denaturants examined. The PrP^{Sc} molecule or a higher-order multimer such as a trimer is susceptible to denaturation by acidic SDS. To study the inactivation of prions by acidic SDS, Sc237 and sCJD prions from Syrian hamsters and humans, respectively, were used. Sc237 prions originated in sheep with scrapie and were isolated on passage from rats to Syrian hamsters (46). sCJD prions were from a patient who did not have any *PRNP* gene mutations and appeared to have developed prion disease spontaneously. Both immunoblotting and bioassays in rodents were used to assess the inactivation of prions in brain homogenates by acidic SDS, as well as those adhering to a steel surface (100). Our studies identified conditions under which it is possible to inactivate all detectable prion infectivity by a combination of acidic SDS and 15 min of autoclaving.

MATERIALS AND METHODS

Inocula. sCJD was confirmed by histopathology, immunohistochemistry, and detection of human PrP^{Sc} by Western blotting. Genomic sequencing of the open reading frame revealed no mutations and methionine homozygosity at position 129. The Sc237 hamster prion strain was a gift from Richard Marsh and was repeatedly passaged in golden Syrian hamsters (LVG:Lak) purchased from Charles River Laboratory (Wilmington, MA).

Preparation of brain homogenates and acidic buffers. Crude brain homogenates (10% [wt/vol]) in calcium- and magnesium-free phosphate-buffered saline (PBS) were prepared by repeated extrusion through syringe needles of successively smaller size, as previously described (78). Nuclei and debris were removed by centrifugation at 1,000 × *g* for 5 min. Incubations of brain homogenates with various solutions were performed with continuous shaking at 100 cycles/min. Glycine buffer was made as a 1 M stock titrated to pH 3.0, whereas AcOH and peracetic acid were added directly without adjustment. In other experiments, 10% (wt/vol) brain homogenates were prepared in calcium- and magnesium-free PBS with two 3.2-mm stainless steel beads using the Mini-BeadBeater-8 apparatus (BioSpec, Bartlesville, OK) for two cycles of 45 s each and then placed on ice. Homogenates were either precleared at 500 × *g* for 5 min (see Fig. 2B) or used without centrifugation, and the protocol was performed without shaking (see Fig. 1C and 2A and Table 1 to Table 4). The final pH value for each sample was measured directly on parallel, uninfected samples with a calibrated pH electrode (Radiometer, Copenhagen, Denmark) during each experiment and is provided in the appropriate figure legends.

PrP^{Sc} detection by immunoblotting. PrP^{Sc} in neutralized samples was measured by limited proteinase K (PK) digestion and immunoblotting as described previously (83). After incubations, an equal volume of 4% Sarkosyl–100 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; pH 7.5)–200 mM NaCl was added to neutralize each sample. Protease digestion was performed with 20 μg of PK/ml (Invitrogen, Carlsbad, CA) for 1 h at 37°C. Digestions were terminated by the addition of 8 μl of 0.5 M phenylmethylsulfonyl fluoride in absolute ethanol (Roche, Indianapolis, IN). Digested samples were then mixed with equal volumes of 2× SDS sample buffer. All samples were boiled for 5 min prior to electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 1.5-mm 12% polyacrylamide gels (39). After electrophoresis, Western blotting was performed as previously described (78). Membranes were blocked with 5% nonfat milk protein in PBST (calcium- and magnesium-free PBS plus 0.1% Tween 20) for 1 h at room temperature. Blocked membranes were incubated with 1 μg of recombinant, humanized antibody fragments (Fab) D13 (Fig. 1 and 2) or D18 (Fig. 1B)/ml. After incubation with the primary Fab, membranes were washed 3 × 10 min in PBST, incubated with horseradish peroxidase-labeled, anti-human Fab secondary antibody (ICN) diluted 1:5,000 in PBST for 45 min at room temperature, and washed again four times for 10 min each time in PBST. After enhanced chemiluminescent (ECL) detection (Amersham Bioscience, Piscataway, NJ) for 1 to 5 min, blots were sealed in plastic covers and exposed to ECL Hypermax film (Amersham). Films were processed automatically in a Konica film processor.

Preparation and bioassay of prion-coated stainless steel wires. Four-millimeter segments of 3-0 stainless steel suture wire (Ethicon, Cornelia, GA) were coated with prions and bioassayed by a modification of a procedure described previously (100). Wire segments were incubated with 10% prion-infected brain homogenate in PBS at room temperature for 16 h in a 10-cm petri dish, washed five times for 10 min each time at room temperature with PBS, followed by a 10-min wash with H_2O, and air dried in a ducted class II, type B2 biosafety cabinet (Baker, Sanford, ME) overnight. Coated wires (10 to 15) were incubated with 1 ml of H_2O, SDS, AcOH, or an SDS-AcOH solution at different temperatures for various durations. After incubation, wires were washed briefly in PBS and implanted into the right cerebral hemisphere. Mice were premedicated with buprenorphine hydrochloride (Buprenex; Reckitt Benckiser Healthcare, Berkshire, United Kingdom), anesthetized using isoflurane (AErrane; Baxter Healthcare, Deerfield, IL), and kept immobilized in a stereotaxic apparatus. A 1-cm skin incision was performed under aseptic conditions and a 0.9-mm bore hole was drilled through the skull, ca. 1 mm caudal and 1.2 mm right of the skull reference point bregma. The stainless steel wire was inserted into the brain with forceps. The skin was then closed by using surgical glue (Nexaband; Abbott Laboratories, Abbott Park, IL). The wires remained embedded in the brains of the mice for the duration of the experiment.

Bioassay for prion infectivity in brain homogenates. Brain homogenates were diluted 1:10 into sterile, calcium- and magnesium-free PBS plus 5 mg of bovine serum albumin/ml. Brain homogenates treated with various solutions, at final concentrations of 2.5%, were diluted 1:10 (see Tables 1 and 3) or 1:25 (see Tables 2 and 4). New, sterile, individually packaged needles, syringes, and tubes were used. All work was carried out in laminar flow hoods to avoid cross-contamination.

Brain homogenates containing hamster Sc237 prions were bioassayed in hamsters or transgenic (Tg) mice expressing Syrian hamster (SHa) PrP, designated Tg7 mice. Brain homogenates containing human sCJD prions were bioassayed in mice expressing a chimeric mouse-human PrP transgene designated MHu2M(M165V, E167Q), in which the most rapid incubation times are ca. 120 days (38); for simplicity, these mice are designated Tg22372 mice. For negative controls, brain homogenates of uninoculated $Prnp^{0/0}$ mice were used. Hamsters and weanling mice were inoculated intracerebrally with 50 and 30 μl, respectively, of diluted samples. Inoculation was carried out with a 26-gauge, disposable hypodermic needle inserted into the right parietal lobe. After inoculation, mice were examined daily for neurologic dysfunction. Standard diagnostic criteria were used to identify animals affected by prion disease (13, 62). Animals whose deaths were imminent were sacrificed, and their brains were removed for histological and biochemical analysis.

Survival analysis. Prion incubation periods in experimental models have been reported historically as the mean incubation period ± the standard error of the mean. This approach assumes that the data are normally distributed, which is a reasonable approximation for high-titer samples. When prion titers are low, as in prion inactivation studies, the distribution of incubation periods becomes asymmetric, and not all animals succumb to disease. In such cases, the calculation of