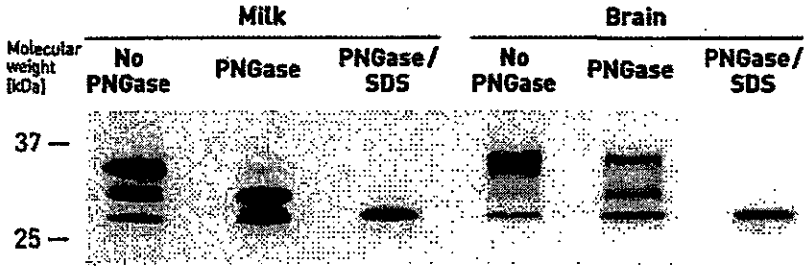

Figure 3. Glycoform patterns of milk and brain PrP^C. Alicon PrioTrap®-enriched PrP^C from cow milk and brain homogenate was treated with PNGase or PNGase/SDS before Western Blotting using PrP–mab 8B4.
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tration (measured by bicinchoninic acid assay, Pierce) was constant with about 40 mg/ml before and after PrP^C elimination. The protein composition of milk as analyzed by SDS PAGE (Figure 4) was not affected either. Prion protein was also completely removed, when milk was spiked with PrP^{Sc} from mouse Rocky Mountain Laboratory (RML) brain homogenate (data not shown). Thus, Alicon PrioTrap® can be used for enrichment and detection of overall prion protein in milk, but also for complete removal of prions.

DISCUSSION

Milk contributes 13% to the worldwide protein supply for humans. World milk production ranges around 500 million tons a year. Before fresh milk reaches the consumer, it is usually homogenized to reduce fat particle size in order to increase digestibility of the milk and heated. Surprisingly, pasteurisation (heating for 30 seconds to 72°C) and ultra-high temperature treatment (heating for 1–4 seconds to 135°C) only leads to a partial reduction of the amount of PrP^C. This supports the observation that PrP^C is highly stable in milk. Thus, the heating procedures used to inactivate DNA-containing pathogens are not sufficient to eliminate endogenous prion proteins.

The presence of PrP^C in blood has been documented [14,15], and is confirmed by our own unpublished observations. To produce one liter of milk, about 400 to 500 liters of blood must pass through the udder of a cow. It is thus possible that the PrP^C found in milk derives from blood cells or, alternatively, has been secreted from glandular epithelial cells. Cell types that have been identified in milk from healthy cows are mainly macrophages, and other leucocytes. However, in our assay cells are completely removed by centrifugation. Therefore, the recovered PrP^C is not cell associated but most likely binds to other proteins or lipids resulting in stable molecular complexes. The fact that milk contains full-length PrP^C, very likely comprising the glycolipid anchor, indicates that prion protein was originally cell-bound and does not represent any of the amino-terminal truncation products of PrP^C known to be released from normal cells under physiological conditions [25]. The detection of such a considerable expression of cell membrane bound or derived PrP^C in milk constitutes one of the key requirements for the generation of infectious prions in the udder of infected animals.

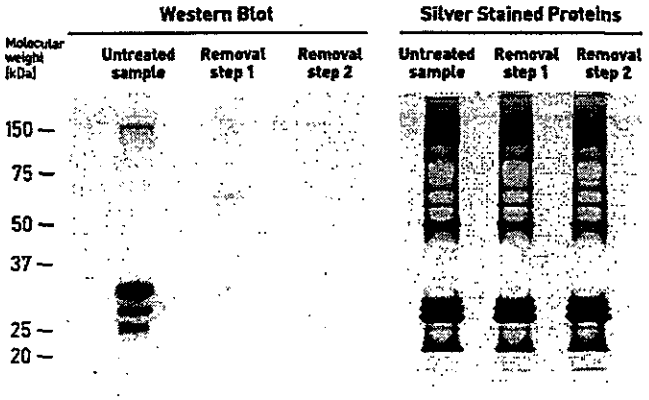

Figure 4. Elimination of PrP^C from milk. PrP^C from 10 ml fresh cow milk was detected as described in figure 1, followed by two consecutive PrP^C removal steps, where the milk supernatant was incubated with Alicon PrioTrap® filtration resin for 30 min before immunochemical detection. The total amount of milk protein was analyzed by Silver Stain analysis.
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Over the last 10 years, scientific groups, risk assessment agencies, and public health organizations have debated the TSE risk for milk and milk products [26,27]. Epidemiological and bioassay data so far available have not provided evidence for milk to harbour prion infectivity and infectious prions have as yet not been detected by bioassays in the milk, colostrum or udder of clinical BSE cases in cow [18–21]. However, a recent statement of the European Food Safety Authority affirmed that based on a number of observations from research data, there are indications that infectivity in the milk from small ruminants can not be totally excluded [28]. Furthermore, the exclusion of animals with mastitis, an inflammation of the mammal gland, being able to destabilize the blood-milk barrier, is considered a measure able to reduce but not to eliminate the potential contamination risk [28]. The rational of this conclusion is confirmed by a recent study showing that in sheep naturally affected with both scrapie and lymphocyte or lymphofollicular mastitis, PrP^{Sc} accumulation was present in lymphoid follicles adjacent to milk ducts [29]. At least in natural sheep scrapie, prion replication can occur following a lymphotropic virus infection in the inflamed mammary gland. This study has not detected PrP^{Sc} or prion infectivity in milk itself. However, since under such inflammatory conditions, the total number of immune cells increases in milk of animals, it might be possible that infectious PrP^{Sc} is also passing through and reaches the milk. In this context milk from such animals could possibly be responsible for the spread of scrapie from the ewes to their offspring in affected sheep or goat flocks. Moreover, sheep and goat milk could also constitute a TSE exposure risk for mammals (humans) consuming these products.

The former Scientific Steering committee of the European Commission and the European Food Safety Authority recommend that research should intensify on the safety of milk of small ruminants with regard to TSE risk. Limited new data are expected to be published in the near future and there is still little research initiated in this area [28]. The Alicon PrioTrap® technology opens a new avenue for studying the biochemical characteristics of prion

protein in milk and thus may contribute to offer a feasible approach to perform an appropriate study on the milk safety with regard to TSE risk.

MATERIALS AND METHODS

Preparation of milk samples

Fresh milk was obtained from healthy individuals and transported at 4°C. Cow UHT milk, sheep and goat pasteurised milk were obtained from the Swiss market. Each sample was prepared from 10 ml milk, centrifuged at 3000× g for 10 min to ensure complete removal of cells.

Preparation of brain homogenate

10% (w/v) bovine brain homogenate was prepared in 100 mM Tris-HCl pH 7.5 containing 2% sodium lauryl sarcosinate. This solution was diluted in 100 mM sodium phosphate buffer pH 8 containing 0.5% NP-40 to obtain 1% (w/v) brain homogenate.

Concentration of milk PrP^C

The milk supernatant was stirred for 30 min in the presence of Alicon PrioTrap®. The resin was centrifuged for 2 min at 2000× g and washed three times at RT with 10 ml washing solution containing 100 mM sodium phosphate buffer pH 8.

Immunochemical PrP^C detection

Concentrated milk and brain PrP^C was denaturated in SDS sample buffer and heated at 70°C for 10 min and at 95°C for 5 min, respectively. Samples were applied to a 12% SDS polyacrylamide gel for electrophoresis and subsequently transferred to a PVDF membrane. The membrane was blocked with 2% ECL Advance™ blocking agent (Amersham) if probed with 8B4 antibody or 1% bovine serum albumin if probed with other antibodies used in this study. 8B4 antibody was incubated at a concentration of 450 ng/ml, 8H4 at 85 ng/ml, 6H4 at 285 ng/ml, and anti-Tau-1 at 50 ng/ml. Sheep anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Amersham) was incubated at a 1/20,000 dilution. The immunoreactivity was visualized by chemiluminescence detection following the manufacture's instruction (ECL Advance Western Blot detection Kit, Amersham).

PNGase treatment of milk and brain PrP^C

For PNGase treatment prion protein extracted from 10 ml cow milk or 10 µl of 1% (w/v) cow brain homogenate was incubated for 12 h at 37°C in buffer containing 100 mM sodium phosphate, 10 mM Tris-HCl, 1% NP-40, 1% MEGA-8, pH 8, and 1.5 units of N-Glycosidase F (Roche). Under more stringent cleavage conditions, proteins were denatured by heating for 10 minutes at 100°C in the presence of 0.5% SDS before treatment with 4 units of N-Glycosidase F.

Total milk protein detection

A milk volume corresponding to 40 µg total protein (1 µl) was heated in SDS loading buffer at 70°C for 10 min. After electrophoresis on a 12% SDS polyacrylamide gel, the proteins were detected by silver staining (SilverSNAP Stain kit II, Pierce).

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Author Contributions

Conceived and designed the experiments: ZR FN EA MU FS RZ NF AE UM SF. Performed the experiments: FN EA MU FS NF AE UM SF. Analyzed the data: ZR FN EA MU FS RZ NF AE UM SF. Contributed reagents/materials/analysis tools: AB MG SM BA GM BU MS UB. Wrote the paper: AB MG ZR FN BA GM BU RZ NF UB.

REFERENCES

1. Bolton DC, McKinley MP, Prusiner SB (1982) Identification of a protein that purifies with the scrapie prion. Science 218: 1309–1311.
2. Prusiner S B, et al. (1982) Further purification and characterization of scrapie prions. Biochemistry 21: 6942–6950.
3. Oesch B, Westaway D, Walchli M, McKinley MP, Kent SB, et al. (1985) A cellular gene encodes scrapie PrP 27–30 protein. Cell 40: 735–746.
4. Basler K, Oesch B, Scott M, Westaway D, Walchli M, et al. (1986) Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. Cell 46: 417–428.
5. Prusiner SB (1982) Novel proteinaceous infectious particles cause scrapie. Science 216: 136–144.
6. Schätzl HM, Da Costa M, Taylor L, Cohen FE, Prusiner SB (1995) Prion protein gene variation among primates. J Mol Biol 245: 362–374.
7. Kretzschmar HA, Prusiner SB, Stowring LE, DeArmond SJ (1986) Scrapie prion proteins are synthesized in neurons. Am J Pathol 122: 1–5.
8. Mobley WC, Neve RL, Prusiner SB, McKinley MP (1988) Nerve growth factor increases mRNA levels for the prion protein and the β-amyloid protein precursor in developing hamster brain. Proc Natl Acad Sci USA 85: 9811–9815.
9. Manson J, West JD, Thomson V, McBride P, Kaufman MH, et al. (1992) The prion protein gene: a role in mouse embryogenesis? Development 115: 117–122.
10. Turk E, Teplow DB, Hood LE, Prusiner SB (1988) Purification and properties of the cellular and scrapie hamster prion proteins. Eur J Biochem 176: 21–30.
11. Stahl N, Borchelt DR, Hsiao K, Prusiner SB (1987) Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell 51: 229–240.
12. Haraguchi T, Fisher S, Olofsson S, Endo T, Groth D, et al. (1989) Asparagine-linked glycosylation of the scrapie and cellular prion proteins. Arch Biochem Biophys 274: 1–13.
13. Cashman NR, Loertscher R, Nalbantoglu J, Shaw I, Kascsak RJ, et al. (1990) Cellular isoform of the scrapie agent protein participates in lymphocyte activation. Cell 61: 185–192.
14. MacGregor I, Hope J, Barnard G, Kirby L, Drummond O, et al. (1999) Application of a time-resolved fluoroimmunoassay for the analysis of normal prion protein in human blood and its components. Vox Sang 77: 88–92.
15. Barclay GR, Houston EF, Halliday SI, Farquhar CF, Turner ML (2002) Comparative analysis of normal prion protein expression in human, rodent, and ruminant blood cells by using a panel of prion antibodies. Transfusion 42: 517–526.
16. Ironside JW, Head MW (2004) Variant Creutzfeldt-Jakob disease: risk of transmission by blood and blood products. Haemophilia 10 Suppl. 4: 64–69.
17. Castilla J, Saa P, Soto C (2005) Detection of prions in blood. Nat Med 11: 982–985.
18. Hadlow WJ, Kennedy RC, Race RE, Eklund CM (1980) Virologic and neurohistologic findings in dairy goats affected with natural scrapie. Vet Pathol 17: 187–199.
19. Bradley R, Wilesmith JW (1993) Epidemiology and control of bovine spongiform encephalopathy (BSE). Br Med Bull 49: 932–959.
20. Taylor DM, Ferguson CE, Bostock CJ, Dawson M (1995) Absence of disease in mice receiving milk from cows with bovine spongiform encephalopathy. Vet Rec 136: 592.
21. Buschmann A, Groschup MH (2005) Highly bovine spongiform encephalopathy-sensitive transgenic mice confirm the essential restriction of infectivity to the nervous system in clinically diseased cattle. J Infect Dis 192: 934–942.
22. Li R, Liu T, Wong BS, Pan T, Morillas M, et al. (2000) Identification of an epitope in the C terminus of normal prion protein whose expression is modulated by binding events in the N terminus. J Mol Biol 301: 567–573.
23. Korth C, Stierli B, Streit P, Moser M, Schaller O, et al. (1997) Prion (PrP^{Sc})-specific epitope defined by a monoclonal antibody. Nature 390: 74–77.
24. Zanusso G, Liu D, Ferrari S, Hegyi I, Yin X, et al. (1998) Prion protein expression in different species: analysis with a panel of new mAbs. Proc Natl Acad Sci USA 95: 8812–8816.
25. Laffont-Proust I, Faucheux BA, Hassig R, Sazdovitch V, Simon S, et al. (2005) The N-terminal cleavage of cellular prion protein in the human brain. FEBS Lett 579: 6333–6337.
26. EC. Scientific Veterinary Committee, Report on the risk analysis for colostrum, milk, and milk products (Document N° VI/8197/96 Version J, Final, 1997).

27. EC. Multidisciplinary Scientific Committee, Opinion on the possible risks related to the use of colostrum, milk and products (1997).
28. Statement of the European Food Safety Authority Scientific Expert Working Group on BSE/TSE of the Scientific Panel on Biological Hazards on the health risks of the consumption of milk and milk derived products from goats (2004). Last updated 11. Juli 2006. Available: http://www.efsa.europa.eu/it/science/biohaz/biohaz_documents/709.html. Accessed 2006 Oct 16.
29. Ligios C, Sigurdson CJ, Santucciu C, Carcassola G, Manco GC, et al. (2005) PrP^{Sc} in mammary glands of sheep affected by scrapie and mastitis. Nat Med. 11: 1137–1138.

医薬品 研究報告 調査報告書

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研究報告の概要

○スクレイピー感染細胞およびクロイツフェルト・ヤコブ病病原体感染細胞では、細胞内に25nmウイルス様粒子が生じる
我々は、プリオンタンパク(PrP)がほとんど存在しないが、高い感染性を持つ脳において直径約25nmのウイルス様粒子を反復して認めたため、高い感染性を持つ細胞株で同様のウイルス様粒子の存在を調べた。スクレイピー22L株に感染した神経芽腫細胞およびFUクロイツフェルト・ヤコブ病病原体に感染した視床下部GT細胞は、直交状に配列した密集する25nmのウイルス様粒子が存在したが、並行対照群ではウイルス様粒子が存在しなかった。in situのこれら粒子は異常PrPアミロイドと関連性がなく、正常および異常細胞内PrPが存在するとされる部位である粗面小胞体膜やアミロイド線維を正確に認識するPrP抗体によって標識されなかった。また、ホルボールエステルはN2a+22L感染細胞の異常PrPゲルバンドの発現量を5倍以上増加させたが、ウイルス様粒子の列数やこれら細胞の感染価は増加しなかった。したがって、25nmの当該感染関連粒子はプリオンではあり得なかった。シナプス分化や神経変性ならびに神経芽腫細胞粗面小胞体膜に存在するレトロウイルスは、粒子産生に必要でなかった。培養細胞における25nm粒子の列は、1968年に初めてスクレイピー感染脳のシナプス領域で報告され、続いて自然発症TSEおよびTSE実験モデルの多くに特定されたものと非常に類似している。この類似した分離ウイルス様粒子が内因性PrP抗体に対する反応性を示さず、感染性が高く、核酸-タンパク質複合体を破壊したときに感染性を失うことと合わせれば、当該25nm粒子が後期にPrP脳病変を誘発するTSEの原因ビリオンである可能性が高い。

報告企業の意見	今後の対応
スクレイピー感染細胞およびクロイツフェルト・ヤコブ病病原体感染細胞では、TSEの原因と考えられる25nmウイルス様粒子が細胞内に生じるとの報告である。	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CJD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本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。

使用上の注意記載状況・その他参考事項等

クロスエイトM250
クロスエイトM500
クロスエイトM1000

血液を原料とすることに由来する感染症伝播等
vCJD等の伝播のリスク

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Cells infected with scrapie and Creutzfeldt–Jakob disease agents produce intracellular 25-nm virus-like particles

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Cells infected with scrapie and Creutzfeldt–Jakob disease agents produce intracellular 25-nm virus-like particles

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We had repeatedly found ~25-nm-diameter virus-like particles in highly infectious brain fractions with little prion protein (PrP), and therefore we searched for similar virus-like particles *in situ* in infected cell lines with high titers. Neuroblastoma cells infected with the 22L strain of scrapie as well as hypothalamic GT cells infected with the FU Creutzfeldt–Jakob disease agent, but not parallel mock controls, displayed dense 25-nm virus-like particles in orthogonal arrays. These particles had no relation to abnormal PrP amyloid *in situ*, nor were they labeled by PrP antibodies that faithfully recognized rough endoplasmic reticulum membranes and amyloid fibrils, the predicted sites of normal and pathological intracellular PrP. Additionally, phorbol ester stimulated the production of abnormal PrP gel bands by >5-fold in infected N2a + 22L cells, yet this did not increase either the number of virus-like arrays or the infectious titer of these cells. Thus, the 25-nm infection-associated particles could not be prions. Synaptic differentiation and neurodegeneration, as well as retroviruses that populate the rough endoplasmic reticulum of neuroblastoma cells, were not required for particle production. The 25-nm particle arrays in cultured cells strongly resembled those first described in 1968 in synaptic regions of scrapie-infected brain and subsequently identified in many natural and experimental TSEs. The high infectivity of comparable, isolated virus-like particles that show no intrinsic PrP by antibody labeling, combined with their loss of infectivity when nucleic acid–protein complexes are disrupted, make it likely that these 25-nm particles are the causal TSE virions that induce late-stage PrP brain pathology.

infection | neuroectodermal cultures | virion ultrastructure | prion amyloid | retrovirus

It is often stated (1) that the transmissible spongiform encephalopathies (TSEs), such as sheep scrapie, human Creutzfeldt–Jakob disease (CJD), and bovine spongiform encephalopathy, are caused by an abnormal "infectious form" of the normal host prion protein (PrP). Abnormal PrP is visualized ultrastructurally as amyloid fibrils or as electrophoretic bands that are relatively resistant to proteinase K digestion (PrP-res). Although host PrP is required for TSE agent susceptibility, much as other specific host proteins are necessary for infection by a variety of viruses, abnormal PrP itself does not fulfill Koch's postulates for an infectious agent (reviewed in ref. 1). For example, it is not invariably present in highly infectious samples such as myeloid microglia (2), and it is not proportional to infectivity in subcellular fractions (3), infected cell cultures (4), or diseased brains (5, 6). Additionally, high PrP-expressing transgenic brains, as well as abnormal recombinant PrPs, have failed to show significant or reproducible infectivity (1). Moreover, despite 25 years of intense study, no "infectious conformation" has been structurally resolved, and the existence of many different TSE agent strains are incompatible with the speculation that different PrP conformations transmit or "encipher" strain specific information (7). Whereas TSE agents breed true despite passage through many different species and cell types with variant PrPs, abnormal PrP-res bands are species and cell-type specific (4). Abnormal PrP appears to be a relatively late pathological response to infection, rather than the causal agent itself (8, 9).

It is also claimed that there is no evidence for virus-like particles in TSEs (7). Yet there have been reports published by many different laboratories demonstrating very similar dense 20- to 35-nm virus-like particles in synaptic regions of TSE-infected brains. These particle arrays were shown first in experimental mouse scrapie in 1968 (10), in natural sheep scrapie by 1971 (11) and, subsequently, in primate and human CJD brain samples (12). These arrays were so virus-like in structure that they were considered to be papovaviruses (13). Similar particles in "paracrystalline" virus-like arrays also were identified in scrapie brain samples from Prusiner's laboratory and considered highly compatible with the 25-nm scrapie agent size as determined by membrane filtration of brain homogenates (14). By 1992, we obtained additional independent evidence for 25-nm spherical infectious particles by examining more purified agent preparations. Field flow sedimentation of highly infectious 120S brain fractions that first had been separated from the majority of abnormal (but uninfectious) PrP displayed virus-like 25- to 30-nm-diameter particles that behaved as 30-nm diameter control spheres; moreover, corresponding dense particles of the same diameter were identified by electron microscopy (EM) in this infectious preparation (15). Notably, these virus-like particles did not bind PrP antibodies, unlike the cosedimenting residual fluffy and amyloid fibril PrP (1). Treatment with low concentrations of SDS removed residual PrP from these particles but did not reduce their infectivity or size. In contrast, disruption of nucleic acid–protein complexes destroyed >99.5% their infectivity (16). Thus, there has always been good evidence for 25-nm virions in TSEs. A viral protein–nucleic acid complex is, moreover, the most parsimonious way to explain TSE strains and many other biologic properties of these agents (1).

With the establishment of high-titer CJD and scrapie agent-infected cell lines (4, 17), it became possible to test whether virus-like particles similar to those seen in more purified infectious brain preparations could be visualized in intact cells. Although these infected cell lines are of neural lineage, they do not develop synaptic structures and do not show the degenerative changes of TSE-infected brain. Thus, the presence of these virus-like particles in infected cells would show that synaptic and neurodegenerative processes are not required for their production. Because the infected cells used here also display large amounts of intracellular PrP amyloid without apparent ill effects (4), the relation of virus-like particles to the accumulation of

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The authors declare no conflict of interest.

Abbreviations: CB, cacodylate buffer; CJD, Creutzfeldt–Jakob disease; IAP, intracisternal A particle retrovirus; PMA, phorbol myristate acetate; PrP, prion protein; RER, rough endoplasmic reticulum; TSE, transmissible spongiform encephalopathy.

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