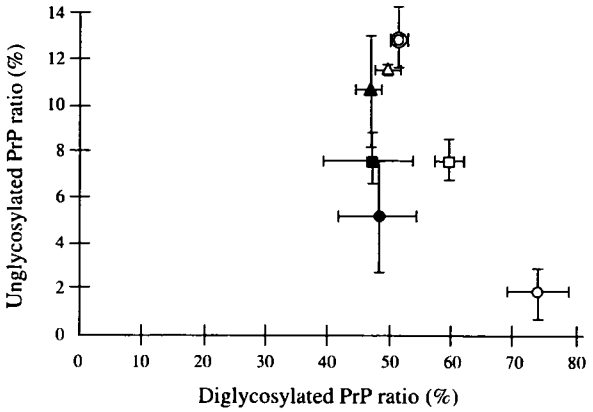

Fig. 5. Glycoform ratio of PrP^{Sc} in animals were plotted with their means and SDs. ○, Hamster (Sc237); □, MH2M (Sc237); △, MHM2 (Sc237); ●, hamster (Obihiro) (first passage); ■, MH2M (Obihiro); ▲, MHM2 (Obihiro); and ◎, mouse (Obihiro). X-axis, diglycosylated PrP band ratio; Y-axis, unglycosylated PrP^{Sc} band ratio

the Obihiro strain and the Sc237 strain. In the MH2M mice, the PrP^{Sc} glycoform ratio changed depending on the prion strain (Fig. 4). Subsequently, the unglycosylated and diglycosylated PrP^{Sc} ratio of each mouse was plotted. The ratio of unglycosylated PrP^{Sc} increased in the following order: hamster, MH2M, MHM2, and mouse. In the MH2M and MHM2 mice, the unglycosylated PrP^{Sc} ratio was constant irrespective of the prion strains. However, the diglycosylated PrP^{Sc} ratio changed depending on the prion strain, and this characteristic was obvious in the MH2M mice (Fig. 5).

Discussion

Prion infection in interspecies transmission has caused a phenomenon of "species barrier." Amino acid substitution in the host PrP and the resulting conformational differences between the invading PrP^{Sc} and host PrP^{C} is thought to be an explanation for this phenomenon. Different PrP glycoform ratios among host species and strain variations may be due to different PrP conformations. To clarify the relationship between scrapie susceptibility and PrP glycoform ratio, hamsters were intracerebrally inoculated with mouse-passaged scrapie prions. The PrP^{Sc} glycoform transition was observed depending on the degree of prion adaptation (Figs. 1, 2). In early passage, the PrP^{Sc} glycoform ratio was similar to that of PrP^{Sc} in the inoculum, and the glycoform ratio gradually shifted to that of the prion-acquired host PrP with prion adaptation. This result indicated that the PrP^{Sc} glycoform ratio was influenced by both the host PrP amino acid sequence and by prion strain characteristics. In early passage, prion characteristics (inoculum) predominantly influenced the PrP^{Sc} glycoform ratio. Following subsequent passages, the glycoform ratio was altered to match the host PrP profile with shortening of the incubation periods. PrP^{Sc} in primary passaged animals might retain most characteristics of the original prion strain.

PrP^{Sc} glycosylation profiles may be indicative of the PrP^{C} glycoform pattern of the brain area in which PrP^{Sc} is formed. PrP^{C} and PrP^{Sc} showed different glycoform patterns depending on the tissue differences [14]. However, our result also showed a similar PrP^{C} glycoform ratio in different brain regions (Fig. 1). To prevent any unexpected influence in different brain areas, we used the medulla region in this experiment.

Investigating the PrP^{Sc} glycoform ratio in primary passaged animals might provide important information regarding prion strain diversities [6]. It has also been reported that MH2M mice inoculated with Sc237 generated a different PrP^{Sc} conformer, resulting in the emergence of a new prion strain [12]. Our result also showed that subsequent passages altered the PrP^{Sc} characteristics. In the case of BSE, pandemic occurrence was observed, and the BSE prion may already have several passage histories among cattle populations. Therefore, it might be difficult to determine the origin of BSE using the PrP^{Sc} glycoform ratio. It has been reported that there were no significant differences among PrP^{Sc} glycoforms of natural scrapie, but that was apparently different from that of BSE in cattle and experimentally BSE-affected sheep [18]. In contrast, 2 different prions, scrapie strain CH1641 and BSE, showed similarities in the PrP^{Sc} glycoform ratio, indicating that it has a limited usefulness in strain differentiation in natural hosts [8]. Many types of amino acid polymorphisms exist among sheep PrP, and some of them have been linked to scrapie susceptibility [1]. PrP^{C} conforma-

tional differences, which depend on the amino acid substitutions, also have to be considered while analyzing the PrP^{Sc} glycoform ratio in sheep scrapie. Recently, an atypical BSE, which showed the accumulation of a different glycoform of PrP^{Sc} with a different molecular weight of PK-resistant PrP, has been reported [2, 4, 20]. To investigate the different phenotype of BSE, it is necessary to clarify the PrP^{Sc} glycomodification mechanisms. In addition, successful transmission is expected to clarify whether these different PrP^{Sc} glycopatterns are linked to the different BSE prion strains.

To clarify the molecular basis of glycoform transition, transgenic mice (MHM2 and MH2M with PrP0/0 background) were used. As reported previously [16], scrapie prion susceptibility was influenced by the third subregion of PrP (131–188). A high-molecular-weight band was predominantly observed in both mouse and hamster PrP^{C}s. However, mouse PrP^{C} showed 3 different bands; in contrast, almost no unglycosylated band existed in hamster PrP^{C}. MHM2 mice, which have amino acid substitutions in PrP at L108M and V111M, expressed PrP^{C} with a mouse-type glycoform ratio, and these mice were susceptible to the mouse-adapted prion. In contrast, MH2M mice, which have 3 other amino acid substitutions (I139M, Y155N, and S170N) in addition to those of MHM2 mice, expressed PrP^{C} with a hamster-type glycoform ratio and were susceptible to the hamster-adapted prion (Table 1). Analysis of transgenic mice revealed that 3 amino acid substitutions located at the subregion of PrP 131–188 may contribute to both prion susceptibility and PrP glycoform ratio.

Glycosylation is initiated in the endoplasmic reticulum (ER). In the ER, glycans play a common role in promoting protein folding, quality control and cellular trafficking, and individual glycosylation patterns; these processes are important for specific functions of the mature glycoproteins that are subsequently processed in the Golgi complex [11]. According to the protein-only hypothesis, the conversion of PrP^{C} to PrP^{Sc} occurs at lipid rafts on the plasma membranes, where PrP^{C} has already been glycomodified in the host tissue. The host-adapted prion generates PrP^{Sc} in a pattern that is similar to host PrP^{C} glycosylation and requires shorter incubation periods. It may be the result of efficient conversion of PrP^{C} to PrP^{Sc}, and glycoform similarity might indicate the degree of prion adaptation. Furthermore, there is a possibility that PrP^{Sc} may directly influence the PrP^{C} glycomodification. Clarifying the mechanisms involved in altering the PrP^{Sc} glycoform ratio might provide insights into the conversion process *in vivo*.

Although PrP^{Sc} of the Obihiro strain showed a similar ratio of diglycosylated PrP^{Sc} within the examined rodent species, the ratio of unglycosylated PrP^{Sc} changed in different animal species. In contrast, in the case of the hamster-passaged prion Sc237, the molecular ratios of both diglycosylated and unglycosylated PrP^{Sc}s changed depending on the host species (Fig. 5). This difference in the PrP^{Sc} glycoform pattern in these transgenic mice also revealed the prion strain diversity.

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References

1. Baylis M, Goldmann W, Houston F, Cairns D, Chong A, Ross A, Smith A, Hunter N, McLean AR (2002) Scrapie epidemic in a fully PrP-genotyped sheep flock. J Gen Virol 83: 2907–2914
2. Biacabe A-G, Laplanche J-L, Ryder S, Baron T (2004) Distinct molecular phenotypes in bovine prion diseases. EMBO Rep 5: 110–114
3. Birkett CR, Hennion RM, Bembridge DA, Clarke MC, Chree A, Bruce ME, Bostock CJ (2001) Scrapie strains maintain biological phenotypes on propagation in a cell line in culture. EMBO J 20: 3351–3358
4. Casalone C, Zanusso G, Acutis P, Ferrari S, Capucci L, Tagliavini F, Monaco S, Caramelli M (2004) Iden-

tification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. Proc Natl Acad Sci USA 101: 3065–3070
5. Caughey BW, Dong A, Bhat KS, Ernst D, Hayes SF, Caughey WS (1991) Secondary structure analysis of the scrapie-associated protein PrP 27–30 in water by infrared spectroscopy. Biochemistry 30: 7672–7680
6. Collinge J, Sidle KC, Meads J, Ironside J, Hill AF (1996) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature 383: 685–690
7. DeArmond SJ, Qiu Y, Sànchez H, Spilman PR, Ninchak-Casey A, Alonso D, Daggett V (1999) PrP^{C} glycoform heterogeneity as a function of brain region: implications for selective targeting of neurons by prion strains. J Neuropathol Exp Neurol 58: 1000–1009
8. Hope J, Wood SCER, Birkett CR, Chong A, Bruce ME, Cairns D, Goldmann W, Hunter N, Bostock CJ (1999) Molecular analysis of ovine prion protein identifies similarities between BSE and an experimental isolate of natural scrapie, CH1641. J Gen Virol 80: 1–4
9. Kimura K, Kubo M, Yokoyama T (2000) Characteristics of prion protein (PrP^{Sc}) in the brains of hamsters inoculated serially with a mouse-passaged scrapie strain. J Comp Pathol 122: 123–130
10. Pan K-M, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ, Cohen FE, Prusiner SB (1993) Conversion of α-helices into β-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci USA 90: 10962–10966
11. Parodi AJ (2000) Protein glucosylation and its role in protein folding. Annu Rev Biochem 69: 69–93
12. Peretz D, Williamson RA, Legname G, Matsunaga Y, Vergara J, Burton DR, DeArmond SJ, Prusiner SB, Scott MR (2002) A change in the conformation of prions accompanies the emergence of a new prion strain. Neuron 34: 921–932
13. Prusiner SB (1991) Molecular biology of prion diseases. Science 252: 1515–1522
14. Russelakis-Carneiro M, Saborio GP, Anderes L, Soto C (2002) Changes in the glycosylation pattern of prion protein in murine scrapie. J Biol Chem 277: 36872–36877
15. Safar J, Roller PP, Gajdusek DC, Gibbs CJ Jr (1993) Conformational transitions, dissociation, and unfolding of scrapie amyloid (prion) protein. J Biol Chem 268: 20276–20284
16. Scott M, Groth D, Foster D, Torchia M, Yang S-L, DeArmond SJ, Prusiner SB (1993) Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. Cell 73: 979–988
17. Somerville RA (1999) Host and transmissible spongiform encephalopathy agent strain control glycosylation of PrP. J Gen Virol 80: 1865–1872
18. Thuring CMA, Erkens JHF, Jacobs JG, Bossers A, Van Keulen LJM, Garssen GJ, Van Zijderveld FG, Ryder SJ, Groschup MH, Sweeney T, Langeveld JPM (2004) Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein. J Clin Microbiol 42: 972–980
19. Vorberg I, Priola SA (2002) Molecular basis of scrapie strain glycoform variation. J Biol Chem 277: 36775–36781
20. Yamakawa Y, Hagiwara K, Nohtomi K, Nakamura Y, Nishijima M, Higuchi Y, Sato Y, Sata T, The Expert Committee for BSE Diagnosis, Ministry of Health, Labour and Welfare of Japan (2003) Atypical proteinase K-resistant prion protein (PrP^{res}) observed in an apparently healthy 23-month-old Holstein steer. Jpn J Infect Dis 56: 221–222
21. Yokoyama T, Itohara S, Yuasa N (1996) Detection of species specific epitopes of mouse and hamster prion proteins (PrPs) by anti-peptide antibodies. Arch Virol 141: 763–769
22. Yokoyama T, Kimura KM, Ushiki Y, Yamada S, Morooka A, Nakashiba T, Sassa T, Itohara S (2001) *In vivo* conversion of cellular prion protein to pathogenic isoforms, as monitored by conformation-specific antibodies. J Biol Chem 276: 11265–11271

特集

異常型プリオン蛋白質への挑戦（前編）

牛への脳内接種法の確立とBSEプリオン実験感染牛を用いた研究

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福田茂夫

3ページ，カラー写真参照

はじめに

牛海綿状脳症（BSE）は中枢神経組織への異常型プリオン蛋白質（PrP^{Sc}）の蓄積と空胞変性病変を特徴とする中枢神経疾患であり，家畜伝染病予防法における法定伝染病である。1986年に英国で初めて確認され，現在までに欧州諸国をはじめ北アメリカ，日本など世界規模で発生がみられる。ヒトの変異型クロイツフェルト・ヤコブ病との関連が強く示唆されているため人獣共通感染症として公衆衛生上大きな問題となっている。国内におけるBSEの発生は2001年9月に初めて確認され，2007年7月までに33例のBSEが発生している。いずれの患畜もBSE特有の臨床症状は確認されておらず，死亡またはと畜後に延髄からPrP^{Sc}が検出されてBSEと診断されている。食肉においてはBSEスクリーニング検査と特定危険部位の除去によりヒトへのBSEプリオン感染リスクは排除されているものの，プリオン自体については未だ解明されていない点が多く，消費者から畜産物への安全・安心を求める声も大きいなか，農場段階やと畜前におけるBSE早期診断技術の確立が強く求められている。

BSEの病態解明や早期診断技術の開発には，BSE罹患牛および牛のBSEプリオン感染組織の精査が必要であるが，BSEプリオン感染牛を生前に発見することが困難であるため，マウスなど実験動物で研究が行われてきた。しかしプリオン感染における「種の壁」や臨床症状，プリオン体内分布などは宿主の影響が大きいと考えられ，BSE研究推進のためにBSEプリオン実験感染牛の作出が必要であった。牛にBSEプリオンを経口投与した場合，BSE発症まで3年以上を要する[1]。これに対し，BSEプリオン感染脳乳剤を直接脳に接種すると，野外発生例と伝播経路の違いがあるものの，感染率が高く発症までの期間が短いことが知られている。しかしながら，脳内接種法によるBSEの牛への伝播試験の報告は少ない[2]。これまでに我々は，早期にBSEプリオン感染牛を確保しBSEの早期診断法の検討および病態を解明するために，牛の脳幹部を標的としたBSEプリオン感染脳乳剤の脳内接種法を考案し，BSEプリオン実験感染牛の作出に成功した。本稿では，牛へのBSEプリオン脳内接種法について解説し，脳内接種によるBSEプリオン実験感染牛を用いたこれまでの研究で得られた知見を紹介する。

【表－1】 BSEの臨床症状検査法

項目	方法
姿勢，行動	牛房内の起立姿勢，対人行動，精神状態などを観察する
歩様，走行姿勢	歩行，走行を促し，歩様や走行姿勢を観察する
聴覚刺激検査	拍手音および金属音に対する反応を観察する
視覚刺激検査	頭上に掲げた白い板などを突然動かし視覚刺激への反応を観察
接触検査	竹製の棒で体表に接触し反応を観察する

脳内接種法の検討

脳幹部を標的としたBSEプリオン感染脳乳剤の脳内接種について，接種方法の検討を行った[3]。2～4カ月齢の子牛の前頭骨は厚さ約7～9㎜程度で筋層も薄く，接種作業が容易にできる。子牛の上顎歯列が水平になるように頭部を保持した場合，角間隆起前縁直下に小脳前縁が位置し，角間隆起前縁1cm鼻側の点から垂直に，また正中線の2cm右側から大脳縦裂を避けて針先を脳幹部に向けて穿刺することとした。今回我々は，18Gカテラン針（針長7cm）を用いることにより，側脳室を通過し，針先が脳幹部・中脳へ到達できることを確認した。前頭骨の貫通は，直径2㎜精密ピンドリルを使用し，また過度の貫通により脳組織を損傷することを防ぐためにピンドリルの刃にステンレスワイヤーを装着した。

2～4カ月齢のホルスタイン種子牛4頭を用い，接種した脳乳剤の脳内分布を検索した。鎮静剤投与および局所麻酔下において，接種部位へ着色した10%正常牛脳乳剤1mLを接種した。接種3～4時間後安楽殺し，投与した脳乳剤の脳内分布を肉眼的に観察した。脳乳剤を接種した子牛は覚醒後に起立し，一般臨床症状に異常はなく，通常の歩行が可能であった。剖検による接種材料の脳内分布検索では，脳幹部に穿刺跡がみられた。また側脳室，第三脳室，中脳水道および第四脳室などが染色され接種材料が脳脊髄液中に拡散していることが観察された（3ページ，写真－1）。

またホルスタイン種子牛1頭に造影剤を脳内接種し，CTスキャンによる接種材料の脳内分布を観察したところ，剖検と同様に18Gカテラン針が中脳に到達していることを確認できた。また接種材料が中脳周囲の脳脊髄液に分布することが観察された（3ページ，写真－2）。

脳乳剤を投与し穿刺針を除去した後，前頭骨の貫通孔より脳脊髄液が漏出することがあった。二次感染および接種した感染性材料による汚染が懸念されたため，φ3×8㎜ステンレススクリューを貫通孔に装着し，脳脊髄液の漏出を封じた。

以上のように，角間隆起から1cm鼻側，正中線から2cm右側の穿刺点とし，ピンドリルによる前頭骨の貫通とカテラン針の脳幹部への穿刺により脳乳剤を接種する方法を考案した。接種された脳乳剤は脳脊髄液中に拡散し，脳室および脳幹部周囲にも分布する。また本法は子牛への影響がなく，処置後に接種試料が体外に漏出しない安全性の高い方法である。

牛へのBSEプリオン実験感染と臨床症状

BSEの診断はPrP^{Sc}を検出することが最も信頼性の高い方法であるが，PrP^{Sc}のほとんどは中枢神経系に存在し，生前の検出は困難である。またBSEプリオン感染に関連し指標となる血液や尿中の物質も現在までに発見されていないことから，BSEの生前診断は中枢神経症状を特徴とする臨床的な変化を観察することが唯一の方法である。そこで我々は，BSEプリオン脳内接種牛を作出し，臨床的変化によるBSEの診断技術について検討した。

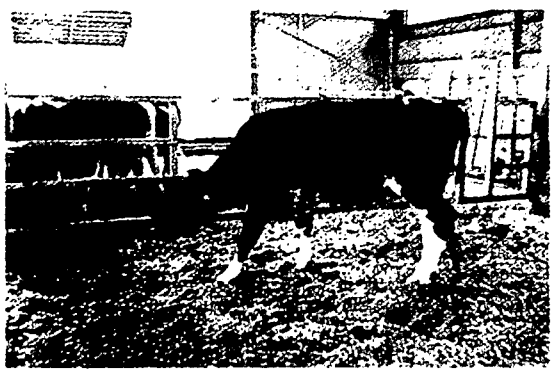
2～4カ月齢のホルスタイン種雌18頭を供試した。BSE感染実験室（動物バイオセーフティ基準2）において脳幹部（中脳）を目標として，前述のカテラン針を用いた脳内接種法により10%BSEプリオン感染脳乳剤1mLを15頭（BSE脳内接種牛）に，BSEプリオン陰性を確認した脳乳剤を3頭に脳内接種した。接種した牛はフリーバーン方式の専用隔離牛舎（動物バイオセーフティー基準1）において飼養した。これらの試験牛のうち，臨床的変化が出現するまで観察した9頭について検討した。

これら9頭について臨床症状検査として，姿勢・行動の

【表－2】 脳内接種によるBSE感染牛の臨床症状の経過

No.	臨床的変化出現の接種後月数(カ月)	異常発見時の症状
1	19	神経質な行動，音に過敏
2	21	起立姿勢，歩様の変化
3	19	音に過剰反応
4	19	歩様の変化
5	22	起立姿勢，歩様の変化
6	22	起立姿勢
7	18	起立姿勢，歩様の変化
8	18	起立姿勢，歩様の変化
9	20	歩様の変化

【写真－3】 頭部を低くし，一方をじっと見つめる姿勢をよくみせる



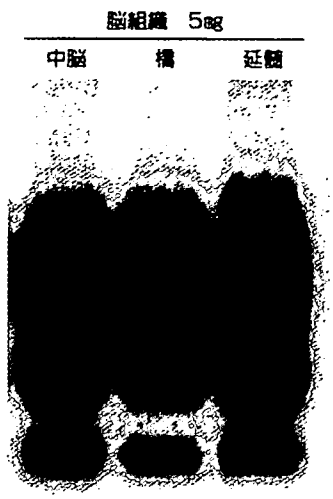
【写真－4】 拍手音に対し，首を振り後ずさりする過剰反応

変化，歩様・走行姿勢，聴覚刺激検査，視覚刺激検査を実施した[4]（表－1）。姿勢・行動の観察は，BSEの臨床症状とされる頭部を低くする等の姿勢や異常行動を発見するため，観察できる程度に離れた位置から，牛の姿勢，対人行動（観察者に対する行動），精神状態（興奮，沈うつ等）などを観察した。歩様・走行姿勢は，牛の運動機能を評価するために，観察者が牛に歩行・走行を促し，歩様や走行姿勢を観察した。聴覚刺激検査は，静かな状態から拍手音または金属音を発生させて，突発的な大きい音への反応を観察した。視覚刺激検査は，白い板などを頭上に掲げて静止し，牛の視野に入っている状態から突然振り下し，突然動くものに対する反応を観察した。接触検査は，竹製の棒（直径2cm，長さ約2m）で牛の頚部，肩部，背部，腹部，臀部および四肢を軽く触り，体表への接触に対する牛の反応を調査した。

9頭は接種22カ月後までに姿勢の異常，歩様異常または音への過剰反応などの臨床的な変化が確認された（表－2）。9頭のうち2頭は，接種18カ月後から音への過剰反応および歩様の変化が観察された。またこれらの個体により現れる変化に差がみられた。9頭すべてにおいて，頻繁に頭部を下げる姿勢をとる姿勢の異常が観察された（写真－3）。鼻を舐めるなどの行動を見せる牛がいた。8頭が速歩を多用する，後肢の動きが硬いなどの歩様や走行姿勢の変化を示した。8頭が拍手音および金属音に対し頭部を振るなど過剰反応を示した（写真－4）。クリップボードの動きに対して後退りや頭部を振るなどの視覚刺激への過剰反応が7頭にみられた。症状が進行した牛のなかには頚部，肩部，胸部を竹製の棒で触ると激しく躯体を動かす牛も1頭みられた。これらの臨床症状は，病理解剖までの2～6カ月間（接種後20～24カ月），ほぼ継続して観察された。

これらの試験において我々は，国内では初めてBSEプリオン感染牛を実験的に作出し，BSEの臨床的変化を観察した。

脳内接種法によりBSEプリオンに感染した牛において接種18カ月後より中枢神経の異常によると思われる音への過剰反応や歩様異常による臨床的変化が出現した。また頭部を下げる姿勢や脚を巻き上げるような歩様をする，鼻を頻繁に舐めるなど異常な行動は，既往の諸外国でのBSE野外発生例の症状に酷似していた。しかしながら，接触への過剰反応を示した個体[4]が1頭で，人への攻撃性を示すような異常な興奮状態を呈する牛[5]がみられなかったなどの野外発生例との違いがみられた。今回作出したBSEプリオン脳内接種牛では，BSE野外発生例とBSEプリオンの侵入経



【写真－5】 BSEプリオン実験感染牛の脳幹部からウエスタンブロット法によりPrP^{Sc}を検出した

路および体内伝播が異なること，または出産，泌乳など生産活動を行っていないなど環境の違いなどの要因が考えられる。出現する症状や程度は，個体により差があったが，PrP^{Sc}の蓄積や神経細胞変性に関連していると考えられる。これらのことからBSEの臨床症状検査により，農場段階あるいはと殺前にBSE発症牛を発見できる可能性が示唆された。

BSEプリオン脳内接種牛のプリオン分布

BSEにおけるPrP^{Sc}は，脳や脊髄，眼や頭部の神経組織，回腸にそのほとんどが存在することが知られている。しかしながら国内のBSE患畜の坐骨神経，腓骨神経および迷走神経[4]，また頚部，腰部および大腿部[7]，の末梢神経系組織において，低レベルであるがPrP^{Sc}が検出されている。BSEにおけるプリオン体内伝播の機序は未だ明らかにされていない。BSE野外例においては，飼料中に混入したBSEプリオンを経口的に摂取し，おそらく回腸遠位部など腸管より体内に進入すると考えられている。その後，延髄に入る迷走神経などから中枢神経組織に到達し，延髄閂部を中心にPrP^{Sc}が増幅すると思われる。BSEプリオン脳内接種牛のPrP^{Sc}の分布についてはこれまでに報告がないことから，我々はプリオンの体内伝播機序を解明するため，BSEプリオン脳内接種牛のPrP^{Sc}の分布について検討している。現在も調査中であるが，これまでにBSEプリオン脳内接種牛の脳および脊髄を含む頭部および脊柱の神経組織，またBSEの臨床的変化のあったBSEプリオン脳内接種牛からは頭部や脊柱の神経組織のほか，星状神経節，腕神経叢，坐骨神経，副腎などからPrP^{Sc}が検出された（写真－5）。扁桃，回腸や筋肉などからPrP^{Sc}は検出されなかった。

おわりに

我が国におけるBSEについては，家畜伝染病予防法や牛海綿状脳症対策特別措置法等で厳正な予防および蔓延防止対策が行われており，今後発生頭数が減少することが期待されている。しかしながら近年，従来のBSEとプリオンの性質が異なる非定型BSEが日本をはじめ各国で相次いで報告されている[8]。いずれの非定型BSEも発生原因が不明で，人をはじめ他の動物への感染性も今後の検討課題となっている。BSEおよびプリオンについて未解明な点が多いことから，と殺牛のBSE検査や死亡牛のサーベイランスなど今後もBSE監視体制とさらなる研究の進展が必要であると思われる。

本研究は，（独）農業・食品特定産業総合研究機構・動物衛生研究所「BSE等動物プリオン病の制圧のための技術開発」および厚生労働科学研究費補助金食品の安全・安心確保推進事業「食を介するBSEリスクの解明等に関する研究」の受託試験として行われた。

参考文献

1）Wells, GAH. et al. : Vet Rec., 142, 103～106（1998）
2）Dawson, M. et al. : Vet Rec., 126, 112～113（1990）
3）Fukuda, F. et al. : Prions-Food and Drug Safety, Springer-Verlag, Tokyo, pp. 183～184（2005）
4）Konold, T. et al. : Vet Rec., 155, 659～666（2004）
5）Braun, U. et al. : Vet Rec., 143, 101～105（1998）
6）Iwamaru, Y. et al. : Prions-Food and Drug Safety, Springer-Verlag, Tokyo, pp. 179～180（2005）
7）Iwata, N. et al. : Jpn. J. Infect. Dis., 59, 100～107（2006）
8）Casalone, C. et al. : PNAS, vol 101, no9, 3065～3070（2004）

特集

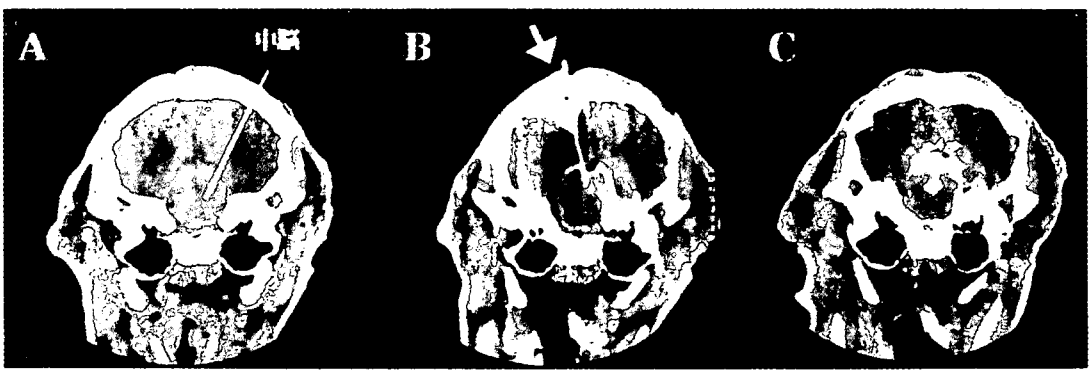
異常型プリオン蛋白質への挑戦（前編）

牛への脳内接種法の確立とBSEプリオン実験感染牛を用いた研究

（本文15ページ参照）

▼北海道立畜産試験場　基盤研究部　遺伝子工学科
福田茂夫

【写真－1】カテラン針を用いた脳内接種により色素で染色された脳組織



A：接種前
B：穿刺したカテラン針（矢印）の先端は脳幹部に達した
C：造影剤および脳乳剤投与後。中脳水道および脳幹部周囲に造影剤が分布した

【写真－2】CTスキャン解析による接種材料の分布（協力：酪農学園大学）

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Species-specificity of a Panel of Prion Protein Antibodies for the Immunohistochemical Study of Animal and Human Prion Diseases

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Summary

Monoclonal antibodies to the prion protein (PrP) have been of critical importance in the neuropathological characterization of PrP-related disease in men and animals. To determine the influence of species-specific amino-acid substitutions recognized by monoclonal antibodies, and to investigate the immunohistochemical reactivity of the latter, analyses were carried out on brain sections of cattle with bovine spongiform encephalopathy, sheep with scrapie, mice infected with scrapie, and human beings with Creutzfeldt-Jakob disease (CJD) or Gerstmann-Sträussler-Sheinker disease (GSS). Immunoreactivity varied between the antibodies, probably as the result of differences in the amino-acid sequence of the prion protein in the various species. Some monoclonal antibodies against mouse recombinant PrP gave strong signals with bovine, ovine and human PrP^{Sc}, in addition to murine PrP^{Sc}, even though the amino-acid sequences determined by the antibody epitope are not fully identical with the amino-acid sequences proper to the species. On the other hand, in certain regions of the PrP sequence, when the species-specificity of the antibodies is defined by one amino-acid substitution, the antibodies revealed no reactivity with other animal species. In the region corresponding to positions 134–159 of murine PrP, immunohistochemical reactivity or species-specificity recognized by the antibodies may be determined by one amino acid corresponding to position 144 of murine PrP. Not all epitopes recognized by a monoclonal antibody play an important role in antigen–antibody reactions in immunohistochemistry. The presence of the core epitope is therefore vital in understanding antibody binding ability.

Keywords: BSE; cattle; CJD; GSS disease; man; mouse; scrapie; sheep

Introduction

Sheep and goat scrapie, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob disease (CJD) (sporadic, iatrogenic, familial and variant forms), Gerstmann-Sträussler-Sheinker disease or syndrome (GSS) and Kuru disease in man are all referred to as prion diseases. A common feature of these diseases is the accumulation of abnormal proteinase-resistant prion protein (PrP^{Sc}), an isoform of the cellular proteinase-sensitive prion protein (PrP^{C}), which occurs as a result of post-translational modification leading to increases in the population of β-sheet conformation in the brain (Prusiner, 1998).

Monoclonal antibodies raised against the prion protein (PrP) have been of critical importance in the neuropathological characterization of PrP-related disease in man and animals (Bodemer, 1999). Numerous monoclonal antibodies (mAbs) for detecting prion proteins in tissue sections have been developed and characterized

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in terms of species-specificity and epitope (Kascsak *et al.*, 1987; Bolton *et al.*, 1991; Piccardo *et al.*, 1998; Zanusso *et al.*, 1998; Van Everbroeck *et al.*, 1999a; Privat *et al.*, 2000). For example, mAb 3F4, whose epitope is mapped between amino acids 109 and 112 of the human prion protein, has been widely used in immunohistochemistry and immunoblotting experiments in human prion disease (Kascsak *et al.*, 1987; Zanusso *et al.*, 1998). This antibody detects the prion protein in man and the hamster but not in the mouse, cow, sheep, Capuchin monkey or squirrel (Zanusso *et al.*, 1998). On the other hand, mAb F89/160.1.5, raised against a synthetic peptide and representing residues 146 to 159 of the bovine prion protein, reacts with the prion protein in human, ovine and bovine tissue (O'Rourke *et al.*, 1998; Van Everbroeck *et al.*, 1999a). Although the prion protein is highly conserved, there is some sequence divergence among species. Possibly a single amino-acid substitution affects epitope recognition by mAb 3F4, in the case of mAb F89/160.1.5 it is possible that the amino-acid sequence recognized is based on the epitope conserved in man and ruminant species.

To determine the effects of species-specific amino-acid substitutions, immunohistochemical analysis with a panel of monoclonal antibodies was undertaken on sections of brain tissue from BSE-infected cattle, scrapie-infected sheep, scrapie-infected mice, and human CJD and GSS cases.

Materials and Methods

Samples

Immunohistochemical analysis was carried out on the following brain tissues, cut coronally at the appropriate level: hippocampus and thalamus from two ICR mice inoculated intracerebrally with the Obihiro strain of scrapie (Shinagawa *et al.*, 1985) and from two negative control mice; medulla oblongata at the level of the obex and spinal cord from three scrapie-affected and two negative control sheep and from three BSE-affected and two control cattle. The disease status of the cattle and sheep was established by histological, immunohistochemical, and Western blot methodology. These samples were fixed in 15% formalin for 48–72 h and embedded in paraffin wax by conventional methods. Tissue blocks containing BSE-affected tissue were treated with 98% formic acid for 1 h to reduce the risk of prion infectivity.

In addition, human post-mortem brain samples were obtained from one patient with sporadic CJD (sCJD) (63-year-old male; codon 129M/M, codon 219E/E) and one with GSS (57-year-old male with PrP P105L mutation; codon 129Val/Val, codon 219E/E). These samples had been fixed in 15% formalin, after which the fixed blocks were immersed in 98% formic acid for 1 h and embedded in paraffin wax. A few tissue sections, including those of the cerebral cortex, prepared from these blocks, were submitted to our laboratory.

Immunohistochemistry (IHC)

Serial tissue sections (4 μm) were placed on silane-coated glass slides (Muto Purechemicals, Tokyo, Japan). After dewaxing, endogenous peroxidases were blocked by incubation in 3% H_2O_2 for 5 min. Six different pretreatment procedures were used, as follows. (1) Pretreatment "FA" (98% formic acid for 5 min). (2) Pretreatment "121DWHA" (hydrated autoclaving at 121 °C, 2 atmospheres [atm] for 20 min in distilled water). (3) Pretreatment "121DWHA/FA" (121DWHA and 98% formic acid for 5 min). (4) Pretreatment "121DWHA/PK" (121DWHA and Proteinase K [0.4 mg/ml; DAKO, California, USA] treatment for 1 min. (5) Pretreatment "135DWHA" (hydrated autoclaving at 135 °C, 3 atm for 20 min in distilled water). (6) Pretreatment "135DWHA/FA" (135DWHA and 98% formic acid for 5 min). The last two methods (5 and 6) were improved hydrated autoclaving methods designed to retrieve PrP^{Sc} immunoreactivity and to be more sensitive than the previous three methods (2–4) for the antibodies reacting with linear epitopes (Furuoka *et al.*, 2004). Because of the limited number of slide sections, sCJD and GSS cases were pretreated by only one, or at most two, of the six methods. After pretreatment, tissue sections were incubated with 10% normal goat or normal horse serum (Nichirei, Tokyo, Japan) for 30 min. The horseradish peroxidase-labelled polymer method (Envision+ kit; DAKO) was used to "visualize" positive antibody binding. The 13 primary antibodies (12 monoclonal and one polyclonal) used are listed in Table 1. Sections were exposed to primary antibodies for 1 h at room temperature. As negative controls, further sections were exposed to each primary antibody without any of the pretreatments. The sections were then incubated with the second antibody for 30 min at room temperature. Positive immunoreactive binding signals were detected with diaminobenzidine (Simple stain DAB; Nichirei). Sections were counterstained with Mayer's haematoxylin. The intensity of specific labelling was scored as follows: 3+, strong; 2+, moderate; +, weak; –, nil.

Results

Histopathology and Immunohistochemistry

The typical lesions and patterns described previously in each species (Fraser and Dickinson, 1968; Wells *et al.*, 1992; Privat *et al.*, 2000; Ryder *et al.*, 2001) were seen. In scrapie-infected mice, neuropil vacuolation was associated with astrogliosis, and microglial proliferation

Table 1
Characteristics of the 13 antibodies (12 monoclonal and one polyclonal [B103]) used in this study

Antibodies	*Epitope*	*Dilution (1 in)*	*Immunogen*	**Species-specificity*				*Source*
				Murine	*Bovine*	*Ovine*	*Human*	
110	56-90	500	Mouse recPrP	+	+	+	+	Dr Horiuchi
132	119-127	200	Mouse recPrP	+	+	+	+	Dr Horiuchi
T1	137-143	200	Mouse recPrP	+	+	+	+	Dr Tagawa
118	137-145	500	Mouse recPrP	+	–	–	–	Dr Horiuchi
31C6	143-151	500	Mouse recPrP	+	–	–	–	Dr Horiuchi
149	147-153	500	Mouse recPrP	+	+	+	–	Dr Horiuchi
43C5	163-169	10 000	Mouse recPrP	+	+	+	–	Dr Horiuchi
147	219-229	200	Mouse recPrP	+	–	–	–	Dr Horiuchi
6H4	155-163	500	Cow recPrP	+	+	+	+	Prionics (Zürich, Switzerland)
F89/160.1.5	146-159	200	Cow recPrP	–	+	+	+	Affinity Bio Reagents (Golden, CO, USA)
3F4	109-112	100	Human recPrP	–	–	–	+	Chemicon (Temecula, CA, USA)
12F10	142-160	200	Human recPrP	–	+	+	+	Cayman Chemical (Ann Arbor, MI, USA)
B103	91-108	500	Cow recPrP	+	+	+	+	Dr Horiuchi

*Species-specificity of antibody summarizes the immunohistochemical reactivity shown in Table 2.

was observed throughout all areas of the brain. Immunohistochemical PrP^{Sc} deposits were distributed diffusely in the cortex, thalamus, and hippocampus.

In the obex region of scrapie-infected sheep, neuropil vacuolation and single or multiple intracytoplasmic vacuoles were found particularly in the dorsal motor nucleus of the vagus nerve (DMNV), which also exhibited particularly intense PrP^{Sc} deposition. Numerous amyloid plaques along blood vessels or plaque-like structures showed PrP^{Sc} accumulations in the reticular formations. Although other nuclei showed immunohistochemical PrP^{Sc} deposits, the intensity was mild. PrP^{Sc} deposition varied in type (fine particulate, coarse particulate, glial-vacuolar, peri-vacuolar).

Due to the subclinical nature of the BSE cases, spongiform lesions (mild) were observed only in the DMNV, and at the periphery of reticular formations. Intense PrP^{Sc} immunoreactivity was also observed at these two locations. Fine or coarse particulate deposits were seen in the olivary nucleus. The hypoglossal nucleus showed positive immunoreactivity, but at a low intensity. In the spinal cord, no obvious vacuolation was observed. Immunohistochemically, however, fine and coarse particulate deposits, and linear and perineuronal labelling were seen in the neuropil of the grey matter. No PrP^{Sc} deposits were observed in untreated sections from the affected animals, or in sections (with or without pretreatment) from control animals.

The sCJD case showed mild to moderate spongiform degeneration in the cerebral cortex, associated with neuronal loss and severe astrogliosis. Immunolabelling, which was confined to the cerebral cortex, was diffuse, synaptic granular or coarse particulate. In the GSS case, the characteristic lesions consisted of numerous multicentric amyloid plaques in the cerebral cortex. Neuronal loss and severe astrogliosis were also observed, but spongiform degeneration was not prominent. All plaques was immunolabelled with PrP antibodies. Diffuse PrP deposits were present in deep layers of the neocortex. Also, large punctate areas of immunoreactivity were observed between nerve cell bodies.

Antibody Reactivity for Different Species

The characteristics of 12 primary monoclonal antibodies and one polyclonal antibody (B103) used in this study are summarized in Table 1. The epitopes of the prion protein recognized by these antibodies are shown in Fig. 1. The immunolabelling results for each pretreatment and antibody are summarized in Table 2. The antibodies used in this study showed patterns of immunoreactive binding that varied with the method of pretreatment. However, the specificity of each antibody in terms of species was well defined, and is shown in Table 1. With all antibodies, FA pretreatment gave weak if any immunolabelling. With most but not all antibodies, 121DWHA/FA pretreatment was more effective than 121DWHA and 121DWHA/PK pretreatment. In general, 135DWHA and 135DWHA/FA were preferable to other methods of pretreatment for antibodies reacting with linear epitopes.

Of eight mAbs for mouse recombinant PrP, three (mAbs 110, 132, and T1) reacted with PrP^{Sc} in all species under some pretreatments (Fig. 2a–d). MAb 149 reacted with murine, bovine and ovine PrP^{Sc}, but showed no immunoreactivity for human PrP^{Sc}. The mAbs 118, 31C6 and 147 exhibited positive immunoreactivity only

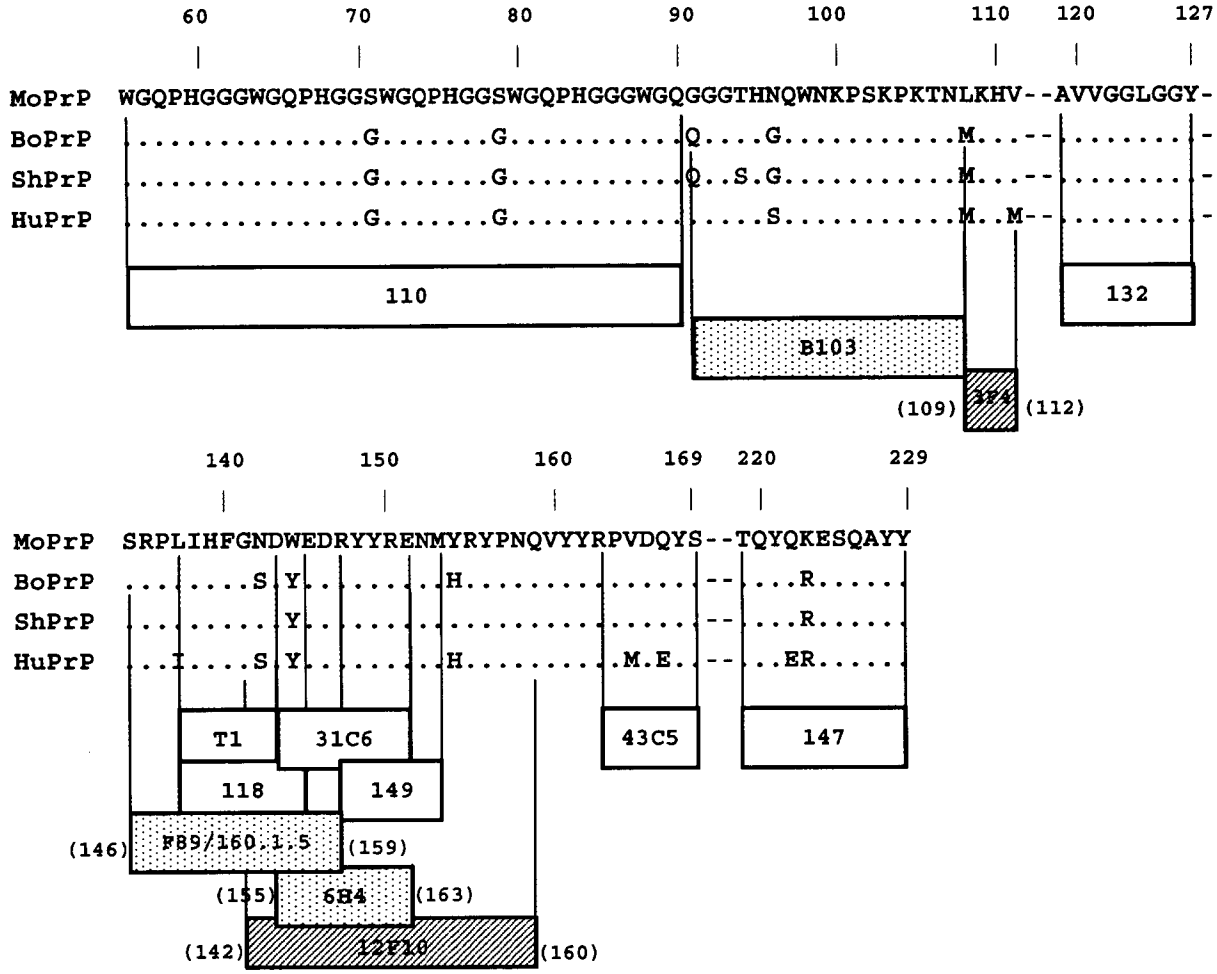

Fig. 1. The locations of the prion protein epitopes recognized by the antibodies used in this study. Amino-acid sequences are based on National Center for Biotechnology Information (NCBI) Protein Databases. MoPrP: amino-acid sequence of murine prion protein (Accession Number NP 035300). BoPrP: amino-acid sequence of bovine prion protein (Accession Number NP 851358). ShPrP: amino-acid sequence of ovine prion protein (Accession Number NP 001009481). HuPrP: amino-acid sequence of human prion protein (Accession Number AAR21603). □, Antibody against recombinant mouse PrP; ⊡, antibody against recombinant cow PrP; ▨, antibody against recombinant human PrP.

with murine PrP^{Sc} and none for bovine, ovine or human PrP^{Sc} (Fig. 2e–h). The mAb 43C5 revealed strong immunoreactivity in all animals, but none in human samples. MAb F89/160.1.5, against recombinant cow PrP, gave immunolabelling invariably, except for murine PrP^{Sc} (Fig. 2i–l). The mAb 6H4 reacted with bovine, ovine, murine and human PrP^{Sc} with 121DWHA/FA or 121DWHA/PK pretreatment, but not with other pretreatments. Polyclonal antibody B103 against recombinant cow PrP gave positive reactions in all species. The mAb 3F4, which recognizes human recombinant PrP, revealed immunoreactivity with PrP^{Sc} in human but not bovine, ovine or murine samples. The mAb 12F10, also raised against human recombinant PrP, showed positive immunoreactivity for human, bovine and ovine PrP^{Sc} but no immunoreactivity for murine PrP^{Sc}. This particular mAb immunolabelled bovine PrP^{Sc} under every pretreatment. However, as with the ovine PrP^{Sc}, pretreatment with 135DWHA and 135DWHA/FA failed to retrieve the antigen.

As shown in Table 2, immunolabelling intensity reflecting the detectable PrP^{Sc} varied with the method of pretreatment (Furuoka *et al.*, 2004). However, there were no obvious differences in labelling patterns.

Discussion

Immunohistochemical demonstration of PrP^{Sc} in tissue sections serves to confirm the diagnosis of prion

Table 2
Immunolabelling results with 13 antibodies after six different pretreatments

Antibody	*Type of specimen*	*Results obtained with the stated pretreatment*					
		FA	*121DWHA*	*121DWHA/FA*	*121DWHA/PK*	*135DWHA*	*135DWHA/FA*
110	M	–	+	2+	3+	3+	3+
	C	–	+	+	2+	3+	2+
	S P/D	+/–	+/+	+/+	2+/2+	3+/3+	3+/3+
	GSS P/D	NE	NE	NE	NE	3+/3+	3+/3+
	CJD	NE	NE	NE	NE	+	+
132	M	–	+	2+	–	2+	2+
	C	–	–	+	–	3+	3+
	S P/D	–/–	+/–	2+/+	–/–	3+/3+	2+/2+
	GSS P/D	NE	NE	NE	NE	2+/+	2+/+
	CJD	NE	NE	NE	NE	+	+
T1	M	–	+	2+	2+	3+	2+
	C	–	+	2+	–	3+	2+
	S P/D	–/–	2+/+	2+/2+	2+/2+	3+/3+	3+/3+
	GSS P/D	NE	NE	NE	NE	3+/3+	3+/3+
	CJD	NE	NE	NE	NE	+	+
118	M	–	–	+	+	+	+
	C	–	–	–	–	–	–
	S P/D	–/–	–/–	–/–	–/–	–/–	–/–
	GSS P/D	NE	NE	–/–	NE	–/–	–/–
	CJD	NE	NE	–	NE	–	–
31C6	M	–	–	2+	+	2+	2+
	C	–	–	–	–	–	–
	S P/D	–/–	–/–	–/–	–/–	–/–	–/–
	GSS P/D	NE	–/–	–/–	–/–	–/–	NE
	CJD	–	NE	–	–	–	–
149	M	–	+	2+	+	3+	+
	C	–	–	2+	–	+	3+
	S P/D	–/–	+/–	2+/+	3+/2+	3+/2+	3+/3+
	GSS P/D	NE	NE	–/–	NE	–/–	NE
	CJD	NE	NE	–	NE	–	NE
43C5	M	–	2+	2+	2+	3+	3+
	C	–	2+	2+	2+	3+	3+
	S P/D	–/–	2+/+	2+/2+	2+/2+	3+/3+	3+/3+
	GSS P/D	–/–	NE	–/–	NE	–/–	NE
	CJD	–	NE	–	NE	–	NE
147	M	–	+	+	–	2+	3+
	C	–	–	–	–	–	–
	S P/D	–/–	–/–	–/–	–/–	–/–	–/–
	GSS P/D	NE	NE	–/–	NE	–/–	NE
	CJD	NE	NE	–	NE	–	NE
6H4	M	–	+	2+	–	–	–
	C	–	+	2+	–	–	–
	S P/D	–/–	+/–	2+/2+	–/–	–/–	–/–
	GSS P/D	NE	NE	+/+	–/–	–/–	–/–
	CJD	–	NE	+	–	–	–
F89/160.1.5	M	–	–	–	–	+	–
	C	+	2+	2+	+	2+	3+
	S P/D	–/–	+/+	2+/2+	2+/2+	3+/2+	3+/3+
	GSS P/D	NE	NE	NE	NE	3+/2+	2+/2+
	CJD	NE	NE	NE	NE	+	+
3F4	M	–	–	–	–	–	–
	C	–	–	–	–	–	–
	S P/D	–/–	–/–	–/–	–/–	–/–	–/–
	GSS P/D	–/–	NE	–/–	+/+	–/–	NE
	CJD	–	NE	–	+	–	NE
12F10	M	–	–	–	–	–	–
	C	–	2+	2+	2+	3+	3+
	S P/D	–/–	–/–	2+/2	2+/2	–/–	–/–

continued on next page

Table 2 (*continued*)

Antibody	*Type of specimen*	*Results obtained with the stated pretreatment*					
		FA	*121DWHA*	*121DWHA/FA*	*121DWHA/PK*	*135DWHA*	*135DWHA/FA*
	GSS P/D	−/−	NE	−/−	+/+	−/−	NE
	CJD	NE	NE	+	NE	−	NE
Bl03	M	−	+	2+	2+	3+	3+
	C	−	+	2+	2+	3+	3+
	S P/D	−/−	+/+	2+/2+	2+/2+	3+/3+	3+/3+
	GSS P/D	NE	NE	NE	NE	+/+	NE
	CJD	NE	NE	NE	NE	+	NE

M, scrapie-affected mouse; C, BSE-affected cow; S, scrapie-affected sheep; GSS, Gerstmann-Sträussler-Sheinker disease; CJD, Creutzfeldt-Jakob disease; NE, not examined; P, plaque type; D, diffuse/synaptic type.
For other abbreviations and for the scoring (− to 3+) of immunolabelling intensity, see Materials and Methods.

diseases (Bodemer, 1999). However, specific pretreatment of paraffin wax sections is necessary to enhance PrP^{Sc} immunoreactivity. Pretreatment methods based on hydrated autoclaving at 121 °C or a combination of hydrated autoclaving (121 °C) with certain chemical reagents (e.g., formic acid or Proteinase K) are now widely used (Kitamoto *et al.*, 1992; Van Everbroeck *et al.*, 1999b; Kovács *et al.*, 2002). Furuoka *et al.* (2004) reported that an improved hydrated autoclaving method (135 °C), enhanced PrP^{Sc} immunoreactivity with antibodies recognizing the linear epitope. The effect of chemical treatments may be to break down the structure of amyloid fibrils and expose the buried epitopes (Doi-Yi *et al.*, 1991; Hashimoto *et al.*, 1992). It is further speculated that hydrated autoclaving contributes to alterations in the three-dimensional structures of PrP, and unravelling the buried epitope caused by PrP aggregation (Kitamoto *et al.*, 1992).

MAbs for mouse recombinant PrP reacted with PrP^{Sc} and PrP^{Sc} in other species provided that these species possessed the same amino-acid sequence in the segment recognized by these antibodies (mAbs 132 and 149). However, the immunoreactivity of mAbs 110, T1, 118, 31C6, 147, F89/160.1.5, 6H4, 3F4 and 12F10 varied when, in heterologous species, the species-specific amino-acid sequence differed. The mAb 110 epitope lies within mouse prion protein residues 56–90, with a species specificity conferred via Ser71 and Ser79. The mouse amino-acid sequence recognized by the mAb T1 epitope showed an overlap with the ovine PrP sequence, but did not fully overlap with the bovine PrP sequence. The mAbs 110 and T1 gave strong immunoreactive signals with bovine, ovine and human PrP^{Sc}, as well as with murine PrP^{Sc}, even though the amino-acid sequence in the epitopes of these antibodies was not completely identical.

On the other hand, mAbs 118 and 31C6 exhibited no immunoreactivity with bovine, ovine or human PrP^{Sc}. The murine amino-acid sequence recognized by mAb 118 recognizes three and two distinct amino-acid sequences in human beings and cattle, respectively, and one such sequence in sheep. One distinct amino-acid region is mapped by mAb 31C6. The mAb F89/160.1.5, which recognizes the 146-157 bovine sequence (O'Rourke *et al.*, 1998), detected bovine, ovine and human PrP^{Sc} but not murine PrP^{Sc}. Similarly, mAb 12F10, raised against human recombinant PrP (which recognizes protein residues 142–160 in the human sequence), produced strong immunolabelling of bovine and ovine PrP^{Sc} but not murine PrP^{Sc}. It appeared that the species-specific epitope in each antibody was defined by one amino-acid substitution, as follows: tryptophan at position 144 of murine PrP in mAbs 118 and 31C6; tyrosine at position 156 of bovine PrP in mAb F89/160.1.5; and tyrosine at position 145 of human PrP in mAb 12F10. Thus, in the region recognized by these antibodies (T1, 31C6, 118, 149, F89/160.1.5, 6H4 and 12F10; corresponding to positions 134–159 of murine PrP), immunohistochemical reactivity or species-specificity associated with the antibodies may be determined by one amino acid corresponding to position 144 of murine PrP. In addition, the results suggested that the relative immunoreactivity of the antibodies was characterized by the core epitope present in the region of amino-acid sequences recognized by the antibody; i.e., the antibody epitope contained a region of high specificity consisting of only a few epitopes, or unable to recognize different conformations.

The mAb 6H4, produced against cattle recombinant PrP and recognizing protein residues 155–163 in the bovine sequence (Korth *et al.*, 1997) (corresponding to positions 143–151 of murine PrP, recognized by mAb 31C6), produced strong labelling of murine and ovine PrP^{Sc} as well as bovine PrP^{Sc}. The mAbs 31C6 and 6H4 shared certain features. For instance, the partial or complete loss of immunoreactivity resulting from PK, 135DWHA or 135DWHA/FA pretreatment suggested that the region recognized by mAbs 31C6 and

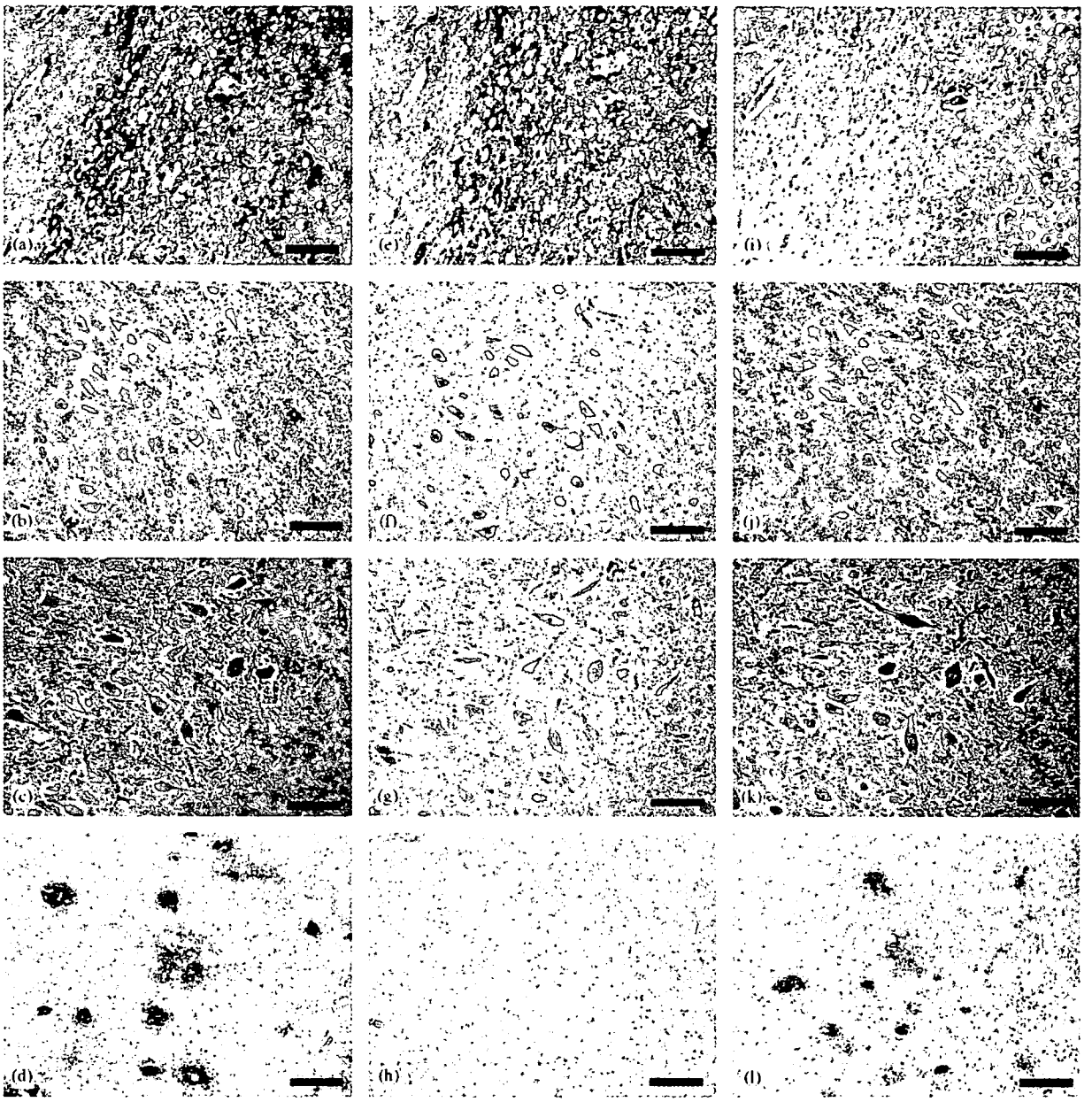

Fig. 2a–l. Immunolabelling of murine, bovine, ovine and human PrP^{Sc} with different antibodies. (a, c and i) Thalamus of scrapie-affected mouse. (b, f and j) DMNV of BSE-affected cow. (c, g and k) DMNV of scrapie-affected sheep. (d, h and l) Cerebral cortex of a GSS case. (a–d) mAb T1. (e–h) mAb 118. (i–l) mAb F89/160.1.5. The mAb T1 reacts with murine, bovine, ovine, and human PrP^{Sc}. The mAb 118 reacts only with murine PrP^{Sc}. The mAb F89/160.1.5 reacts with bovine, ovine and human PrP^{Sc} but not with murine PrP^{Sc}. IHC. Bars, 200 µm.

6H4 may include the labile conformation affected by these three pretreatments. However, the immunohistochemical core epitope of mAb 6H4 appears not to be defined by the substitution of tyrosine for tryptophan, while mAb 31C6 would seem to be species-specific because of the epitope defined by tryptophan at position 144 of murine PrP. The findings suggested that sequence variations recognized by the antibody were not solely responsible for its immunohistochemical specificity. Equally important was the location of the amino-acid substitution in the epitope.

The mAb KG9 against cow recombinant PrP, which recognizes the bovine sequence HRYPN at positions 166–170 (corresponding to positions 154–158 of murine

PrP), reacted with bovine PrP^{Sc} but not sheep PrP^{Sc} (Laffling *et al.*, 2001). On the other hand, mAb L42 raised against an ovine synthetic peptide between positions 145–163 (corresponding to positions 141–159 of murine PrP) reacted with PrP of both cattle and sheep in Western blotting studies (Harmeyer *et al.*, 1998; Vorberg *et al.*, 1999). It is possible that the presence of tyrosine at position 166 of ovine PrP^{Sc} influences the conformation of the relocated KG9 sequence in ovine PrP^{Sc} at the conversion of PrP^{C} to PrP^{Sc} and prevents reaction with the antibody, because mAb KG9 showed similarly low immunoreactivity with neurons of normal sheep and cattle (Laffling *et al.*, 2001). Although it could not be ruled out that the antigen retrieval method failed to unmask the KG9 epitope in PrP^{Sc} in ovine sections as effectively as in bovine sections (Laffling *et al.*, 2001), the existence of the core epitope may explain the differences between the reactivity of mAbs KG9 and L42.

In conclusion, this study demonstrated the immunohistochemical properties (e.g., species-specificity) of newly developed antibodies, and the existence of a core epitope responsible for antibody specificity. Western blotting reactivity and biochemical analysis of the antibodies used in this study will be described elsewhere.

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References

Bodemer, W. (1999). The use of monoclonal antibodies in human prion disease. *Naturwissenschaften*, **86**, 212–220.

Bolton, D. C., Seligman, S. J., Bablanian, G., Windsor, D., Scala, L. J., Kim, K. S., Chen, C. J., Kascsak, R. J. and Bendheim, P. E. (1991). Molecular localization of a species-specific epitope on the hamster scrapie agent protein. *Journal of Virology*, **65**, 3667–3675.

Doi-Yi, R., Kitamoto, T. and Tateishi, J. (1991). Immunoreactivity of cerebral amyloidosis is enhanced by protein denaturation treatments. *Acta Neuropathologica (Berlin)*, **82**, 260–265.

Fraser, H. and Dickinson, A. G. (1968). The sequential development of the brain lesions of scrapie in three strains of mice. *Journal of Comparative Pathology*, **78**, 301–311.

Furuoka, H., Yabuzoe, A., Horiuchi, M., Tagawa, Y., Yokoyama, T., Yamakawa, Y., Shinagawa, M. and Sata, T. (2004). Effective antigen retrieval method for immunohistochemical detection of abnormal isoform of prion proteins in animals. *Acta Neuropathologica (Berlin)*, **109**, 263–271.

Harmeyer, S., Pfaff, E. and Groschup, M. H. (1998). Synthetic peptide vaccines yield monoclonal antibodies to cellular and pathological prion proteins of ruminants. *Journal of General Virology*, **79**, 937–945.

Hashimoto, K., Mannen, T. and Nukina, N. (1992). Immunohistochemical study of kuru plaques using antibodies against synthetic prion protein peptides. *Acta Neuropathologica (Berlin)*, **83**, 613–617.

Kascsak, R. J., Rubenstein, R., Merz, P. A., Tonna-DeMasi, M., Fersko, R., Carp, R. I., Wisniewski, H. M. and Diringer, H. (1987). Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. *Journal of Virology*, **61**, 3688–3693.

Kitamoto, T., Shin, R. W., Doh-ura, K., Tomokane, N., Miyazono, M., Muramoto, T. and Tateishi, J. (1992). Abnormal isoform of prion proteins accumulates in the synaptic structures of the central nervous system in patients with Creutzfeldt-Jakob disease. *American Journal of Pathology*, **140**, 1285–1294.

Korth, C., Stierli, B., Streit, P., Moser, M., Schaller, O., Fischer, R., Schulz-Schaeffer, W., Kretzschmar, H., Raeber, A., Braun, U., Ehrensperger, F., Hornemann, S., Glockshuber, R., Riek, R., Billeter, M., Wüthrich, K. and Oesch, B. (1997). Prion(PrP^{Sc})-specific epitope defined by a monoclonal antibody. *Nature*, **390**, 74–77.

Kovács, G. G., Head, M. W., Hegyi, I., Bunn, T. J., Flicker, H., Hainfellner, J. A., McCardle, L., László, L., Jarius, C., Ironside, J. W. and Budka, H. (2002). Immunohistochemistry for the prion protein: comparison of different monoclonal antibodies in human prion disease subtypes. *Brain Pathology*, **12**, 1–11.

Laffling, A. J., Baird, A., Birkett, C. R. and John, H. A. (2001). A monoclonal antibody that enables specific immunohistological detection of prion protein in bovine spongiform encephalopathy cases. *Neuroscience Letters*, **300**, 99–102.

O'Rourke, K. I., Baszler, T. V., Miller, J. M., Spraker, T. R., Sadler-Riggleman, I. and Knowles, D. P. (1998). Monoclonal antibody F89/160.1.5 defines a conserved epitope on the ruminant prion protein. *Journal of Clinical Microbiology*, **36**, 1750–1755.

Piccardo, P., Langeveld, J. P. M., Hill, A. F., Dlouhy, S. R., Young, K., Giaccone, G., Rossi, G., Bugiani, M., Bugiani, O., Meloen, R. H., Collinge, J., Tagliavini, F. and Ghetti, B. (1998). An antibody raised against a conserved sequence of the prion protein recognizes pathological isoforms in human and animal prion disease, including Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. *American Journal of Pathology*, **152**, 1415–1420.

Privat, N., Sazdovitch, V., Seilhean, D., Laplanche, J. L. and Hauw, J. J. (2000). PrP immunohistochemistry: different protocols, including a procedure for long formalin fixation, and a proposed schematic classification for deposits in sporadic Creutzfeldt-Jacob disease. *Microscopy Research and Technique*, **50**, 26–31.

Prusiner, S. B. (1998). Prions. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 13363–13383.

Ryder, S. J., Spencer, Y. I., Bellerby, P. J. and March, S. A. (2001). Immunohistochemical detection of PrP in the medulla oblongata of sheep: the spectrum of staining in normal and scrapie-affected sheep. *Veterinary Record*, **148**, 7–13.

Shinagawa, M., Takahashi, K., Sasaki, S., Doi, S., Goto, H. and Sato, G. (1985). Characterization of scrapie agent isolated from sheep in Japan. *Microbiology and Immunology*, **29**, 543–551.

Van Everbroeck, B., O'Rourke, K. I. and Cras, P. (1999a). Immunoreactivity of the monoclonal antibody F89/160.1.5 for the human prion protein. *European Journal of Histochemistry*, **43**, 335–338.

Van Everbroeck, B., Pals, P., Martin, J. J. and Cras, P. (1999b). Antigen retrieval in prion protein immunohistochemistry. *The Journal of Histochemistry and Cytochemistry*, **47**, 1465–1467.

Vorberg, I., Buschmann, A., Harmeyer, S., Saalmüller, A., Pfaff, E. and Groschup, M. H. (1999). A novel epitope for the specific detection of exogenous prion proteins in transgenic mice and transfected murine cell lines. *Virology*, **255**, 26–31.

Wells, G. A. H., Wilesmith, J. W. and McGill, I. S. (1992). Bovine spongiform encephalopathy. *Brain Pathology*, **1**, 69–78.

Zanusso, G., Liu, D., Ferrari, S., Hegyi, I., Yin, X., Aguzzi, A., Hornemann, S., Liemann, S., Glockshuber, R., Manson, J. C., Brown, P., Petersen, R. B., Gambetti, P. and Sy, M. S. (1998). Prion protein expression in different species: analysis with a panel of new mAbs. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 8812–8816.

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Characterization of Prion Susceptibility in Neuro2a Mouse Neuroblastoma Cell Subclones

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***Abstract*: In this study, we established Neuro2a (N2a) neuroblastoma subclones and characterized their susceptibility to prion infection. The N2a cells were treated with brain homogenates from mice infected with mouse prion strain Chandler. Of 31 N2a subclones, 19 were susceptible to prion as those cells became positive for abnormal isoform of prion protein (PrP^{Sc}) for up to 9 serial passages, and the remaining 12 subclones were classified as unsusceptible. The susceptible N2a subclones expressed cellular prion protein (PrP^{C}) at levels similar to the parental N2a cells. In contrast, there was a variation in PrP^{C} expression in unsusceptible N2a subclones. For example, subclone N2a-1 expressed PrP^{C} at the same level as the parental N2a cells and prion-susceptible subclones, whereas subclone N2a-24 expressed much lower levels of PrP mRNA and PrP^{C} than the parental N2a cells. There was no difference in the binding of PrP^{Sc} to prion-susceptible and unsusceptible N2a subclones regardless of their PrP^{C} expression level, suggesting that the binding of PrP^{Sc} to cells is not a major determinant for prion susceptibility. Stable expression of PrP^{C} did not confer susceptibility to prion in unsusceptible subclones. Furthermore, the existence of prion-unsusceptible N2a subclones that expressed PrP^{C} at levels similar to prion-susceptible subclones, indicated that a host factor(s) other than PrP^{C} and/or specific cellular microenvironments are required for the propagation of prion in N2a cells. The prion-susceptible and -unsusceptible N2a subclones established in this study should be useful for identifying the host factor(s) involved in the prion propagation.**

***Key words*: Neuro2a, Prion, PrP, Susceptibility**

Transmissible spongiform encephalopathies, so-called prion diseases, are fatal neurodegenerative disorders that include scrapie in sheep and goats, bovine spongiform encephalopathy, and Creutzfeldt-Jakob disease in humans. The major component of causative agent of prion diseases, prion, is thought to be an abnormal isoform of prion protein (PrP^{Sc}). PrP^{Sc} is generated from a normal cellular prion protein (PrP^{C}) by certain post-translational modifications and the process in the conversion of PrP^{C} to PrP^{Sc} is considered to be a central event in pathogenesis of prion diseases (31). PrP^{C} is a sialo-glycoprotein expressed on the cell surface as a glycosyl-phosphatidylinositol anchoring protein (36). Cell biological studies have revealed that mature PrP^{C} on the cell surface acts as a substrate for the PrP^{Sc} biosynthesis, and formation of PrP^{Sc} takes place either at the cell membrane or during the endocytic pathway (3, 8, 37). Depletion of cholesterol inhibits PrP^{Sc} formation in prion-infected cells (2, 38), and the co-existence of PrP^{C} and PrP^{Sc} in lipid rafts or caveolae-like domains suggests that cholesterol- and sphingolipid-enriched membrane microdomains are sites for the interaction between PrP^{C} and PrP^{Sc} (29, 41).

Although PrP^{C} is essential for the propagation of prion and the development of prion diseases (6), other host factors are thought to be involved in the PrP^{Sc} formation, i.e., prion replication. Studies using chimeric

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Abbreviations: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HS, heparan sulfate; LRP/LR, laminin receptor precursor/laminin receptor; MAb, monoclonal antibody; N2a, Neuro2a; PBS, phosphate-buffered saline; PrP, prion protein; PrP^{C}, cellular prion protein; PrP^{Sc}, abnormal isoform of prion protein; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WB, Western blotting.

and point mutants of PrP have suggested that a host factor designated Protein X is required for prion propagation (18, 40), although its identity remains unknown. To date, Bcl-2 (21), laminin receptor precursor/laminin receptor (LRP/LR) (32), neural cell adhesion molecule (35), and several other proteins have been identified as possible counterparts of PrP^{C}. Plasminogen has been reported to bind PrP^{Sc} (11). In addition, a reduction in the level of LRP/LR inhibits PrP^{Sc} formation in prion-infected cells, suggesting that the LRP/LR has a direct or indirect role in prion propagation (22). The biological significance of other proteins in prion propagation remains unclear (34, 35).

Recently, cysteine proteases, such as calpain, and cathepsin B and L were reported to modulate PrP^{Sc} formation (23, 42). Furthermore, inhibitors of c-Abl tyrosine kinase and mitogen-activated protein kinase kinase are reported to accelerate PrP^{Sc} degradation in prion-infected cells (10, 30). Thus, changes in the cellular microenvironment, by interfering with cellular signaling, may also affect prion propagation. These findings suggest the involvement of other host factors for prion propagation. Identification of such host factors and cellular microenvironments involved in prion propagation is of great interest not only for understanding the basic mechanisms of prion propagation but also for finding new therapeutic targets.

Comparative analyses between permissive and non-permissive conditions for prion propagation will facilitate the identification of the host factors involved in prion propagation. In the present study, we established subclones of mouse Neuro2a (N2a) neuroblastoma cells and analyzed their susceptibility to prion. The prion-susceptible and -unsusceptible subclones established in this study should be useful for identifying host factors involved in prion replication.

Materials and Methods

Cell culture and cloning of the cells. N2a cell line (American Type Culture Collection CCL-131, 58th passage at the purchase) was grown in Dulbecco's modified Eagle's medium with high glucose (DMEM; ICN Biomedicals), 10% fetal bovine serum (FBS), and non-essential amino acids. N2a subclones were obtained by limiting dilution.

Inoculation of prion to N2a cells. Mouse prion strain Chandler was propagated in ICR mice (CLEA Japan, Inc.). The brains of mice at the terminal stage of the disease were homogenized in phosphate-buffered saline (PBS) at 10% (w/w) and the homogenates were stored at −30 C until use. The brain homogenate was diluted to 2% with the medium, and 500 μl was added to N2a cells in 60-mm dishes containing 1 ml of medium. After 24 hr, the medium was refreshed, and cells were serially passaged every 3 to 4 days at a 1:10 dilution.

Detection of PrP^{Sc}. Preparation and detection of PrP^{Sc} in the prion-infected cells were carried out as described previously (19, 20) with slight modifications. Cells were lysed in lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA), and small aliquots of the lysates were stored for the determination of the protein concentration. The remaining lysates were digested with 20 μg/ml proteinase K at 37 C for 20 min. The digestion was stopped by the addition of Pefabloc (Roche) to 5 mM. The mixture was adjusted to 0.3% phosphotungstic acid by addition of a 5% solution and then incubated for 30 min at 37 C with constant rotation. PrP^{Sc} was then collected by centrifugation at 20,000 × *g* for 20 min and subjected to SDS-PAGE followed by Western blotting (WB). Blots were probed with monoclonal antibody (mAb) 31C6 (20) and horseradish peroxidase-conjugated sheep $F(ab')_2$ fragment of anti-mouse IgG (Amersham Bioscience). The specific bands were visualized with ECL Western Blotting Detection Reagents (Amersham Bioscience) and a LAS-3000 chemiluminescence image analyzer (Fujifilm). Quantitative analyses of the blots were carried out with Image Reader LAS-3000 version 1.11 (Fujifilm).

Flow cytometry. Flow cytometric analysis was performed as described previously (19).

PrP^{Sc} binding assay. Cells were seeded at 2.5×10^4 cells/well in 6-well plates and grown for 48 hr. Cells were then fed with 500 μl of the fresh medium and inoculated with 250 μl of 0.4 to 2% prion-infected mouse brain homogenate diluted with medium, and kept for 3 hr at either 37 C or on ice with occasional tilting. After the incubation, cells were washed with PBS three times, and bound PrP^{Sc} was detected as described in detection of PrP^{Sc}.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from the cells with TRIzol Reagent (Invitrogen Life Technologies). First strand cDNA was synthesized from the total RNA using a First-Strand Synthesis Kit (Amersham Biosciences) according to the manufacturer's instructions. Real-time *Taq*Man PCR assays were performed to determine the relative quantity of mouse PrP gene expression. Amplification reaction mixtures contained template cDNA, 1X pre-designed set of primers and a *Taq*Man probe targeting the boundary between exons 1 and 2 of the PrP gene (*Taq*Man Gene Expression Assays No. Mm-00448389), and 1X *Taq*-

Man Universal PCR Master Mix (Applied Biosystems) in a final reaction volume of 20 μl. The amplification profile was monitored with an ABI PRISM 7900HT (Applied Biosystems), and the relative quantity was determined by the standard curve method (1) using SDS Plate Utility version 2.1 (Applied Biosystems). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was monitored as an endogenous control using *Taq*Man Rodent-GAPDH Control Reagents (Applied Biosystems).

Cellular cholesterol content of N2a subclones. Cells were seeded in 6-well plates in DMEM containing 10% FBS. After 48 hr, the medium was changed to Opti-MEM (Gibco) and cells were kept for additional 24 hr. Then, cells were washed three times with PBS and lysed with PBS containing 0.1% Triton X-100. The lysates were frozen at −30 C until use. The lysates were clarified by centrifugation at 20,000 × *g* for 15 min at 4 C, and the resulting supernatants were assayed for cholesterol using an Amplex Red Cholesterol Assay Kit (Molecular Probes) according to the manufacturer's instructions. Fluorescence was measured with a fluorescence microplate reader ARVO-SX (Wallac) using excitation at 560 nm and detection at 580 nm.

Stable expression of $MoPrP^C$. Eukaryotic expression vector, pRc/EF-MoPrP (M. H. and A. K. manuscript in preparation), which contains a mouse PrP cDNA expression unit driven by peptide chain elongation factor 1α promoter (27) along with the bacterial aminoglycoside phosphotransferase gene (G418 resistant gene) expression unit, was introduced into N2a cells with FuGENE 6 (Roche). The transfected cells were cultured in the presence of 0.3 mg/ml G418 (Gibco), and G418-resistant cells were selected. The cells were stained with anti-PrP mAb as described for the flow cytometric analysis (19), and cell sorting was performed using an EPICS ALTRA flow cytometer (Beckman Coulter). The cells with fluorescence intensities ranging from 100 to 500 were recovered and cultured with DMEM. Cells passaged more than 3 times were used for prion infection experiments.

Results

Prion-Susceptibility of N2a Subclones

We isolated 31 N2a subclones by limiting dilution and examined them for susceptibility to prion. The N2a subclones were inoculated with scrapie Chandler strain-infected mouse brain homogenates, and prion-susceptibility was determined by the presence of PrP^{Sc} during nine passages after inoculation. Figure 1 shows the representative results for PrP^{Sc} detection in the N2a subclones at the third, sixth, and ninth passages after inoculation. Of 31 N2a subclones, 19 (N2a-2, -3, -5, -6, -7, -17, -21, -22, and -25 in Fig.1) were judged to be prion-susceptible, and the remaining 12 subclones (N2a-1, -4,

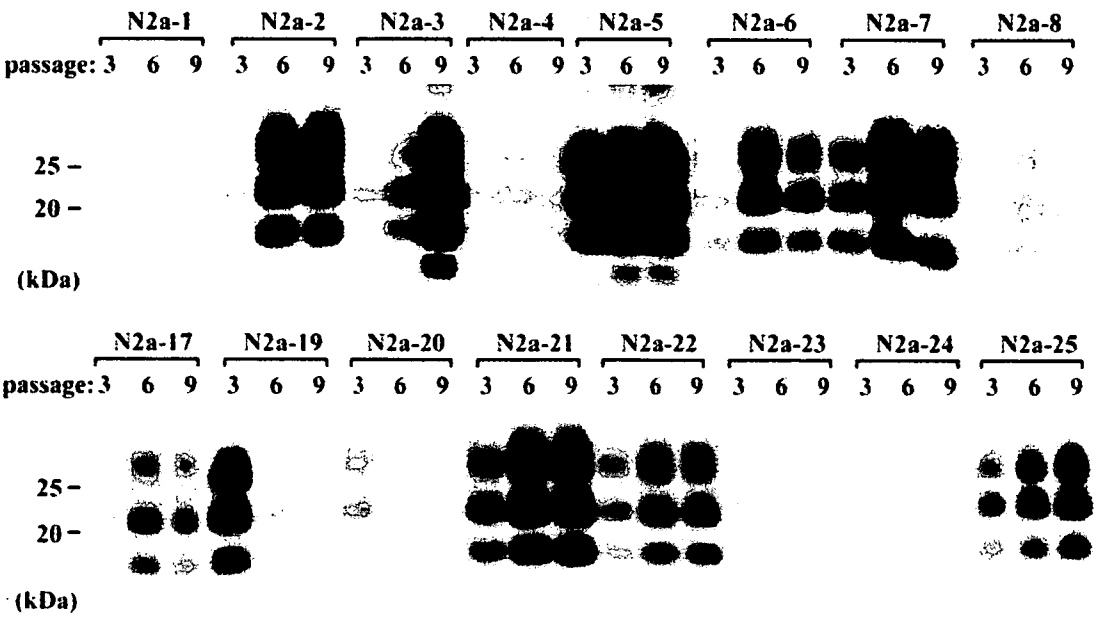

Fig. 1. Detection of PrP^{Sc} in N2a subclones inoculated with prion. N2a subclones were inoculated with 2% brain homogenate from mice infected with Chandler strain, and then consecutively passaged up to nine times. The presence of PrP^{Sc} was examined at the third, sixth, and ninth passages by WB. PrP^{Sc}-enriched sample derived from 0.1 mg of the cell lysates was loaded on each lane, and PrP^{Sc} was detected with mAb 31C6. Results of representative N2a subclones are indicated. Molecular markers are indicated on the left.