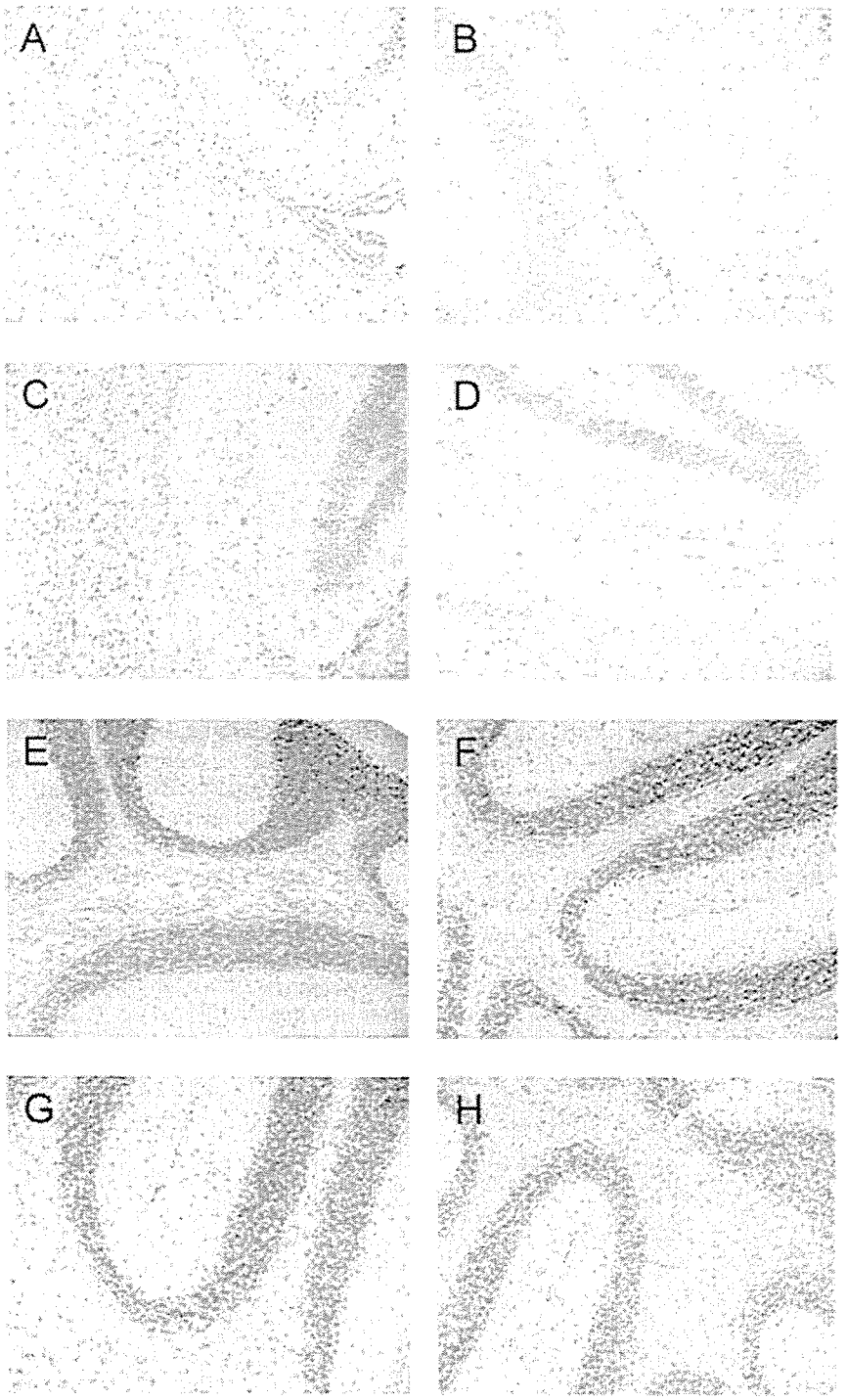

FIG. 2. Histological features of brain tissues infected with prion strains. Sections of the cerebrum (A to D) and cerebellum (E to H) of mice inoculated with Fukuoka-1/brain (A and E), Chandler/brain (B and F), Fukuoka-1/GT1-7 (C and G), and Chandler/GT1-7 (D and H) were stained with hematoxylin and eosin.

compared by immunoblotting. As shown in Fig. 4a, without PK treatment, the IBL-N antibody raised against N-terminal peptides of PrP visualized PrPs both in the noninfected brain and in GT1-7 cells. However, glycosylated components, a diglycosylated band in particular, of the latter migrated much more slowly, indicating that PrP in GT1-7 cells was more heavily glycosylated. Moreover, migration patterns of unglycosylated PrPs from the two sources also looked different: that in GT1-7 cells migrated a little faster. In the tissues and cells infected with Chandler and Fukuoka-1 strains, these host-specific char-

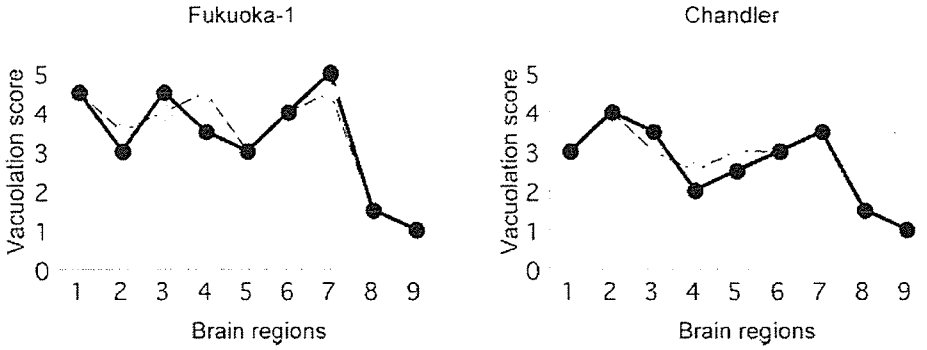

FIG. 3. Region profiles of vacuolation scores in infected mouse brain tissues. Scores were established based on the pattern, size, and density of vacuoles in the tissue using standard criteria with zero for none and five for maximum vacuolation. The pattern of vacuolation was examined in 9 areas, namely the midbrain (1), hypothalamus (2), thalamus (3), hippocampus (4), paraterminal body (5), posterior cortex (6), cerebellar medulla (7), cerebellar granular layer (8), and cerebellar molecular layer (9). Closed and open symbols indicate brain sections infected with brain homogenates and GT1-7 cell lysates, respectively. Each plot indicates the average score of sections from two mice.

acteristics of the PrP structure were essentially preserved. To confirm the difference in the migration patterns between unglycosylated PrPs from brain tissues and GT1-7 cells, Asn-linked glycosylation was completely removed by PNGase F treatment before immunoblotting. As shown in Fig. 4b, regardless of the presence or absence of prion infection, unglycosylated PrP from GT1-7 cells always migrated faster than that from brain tissues.

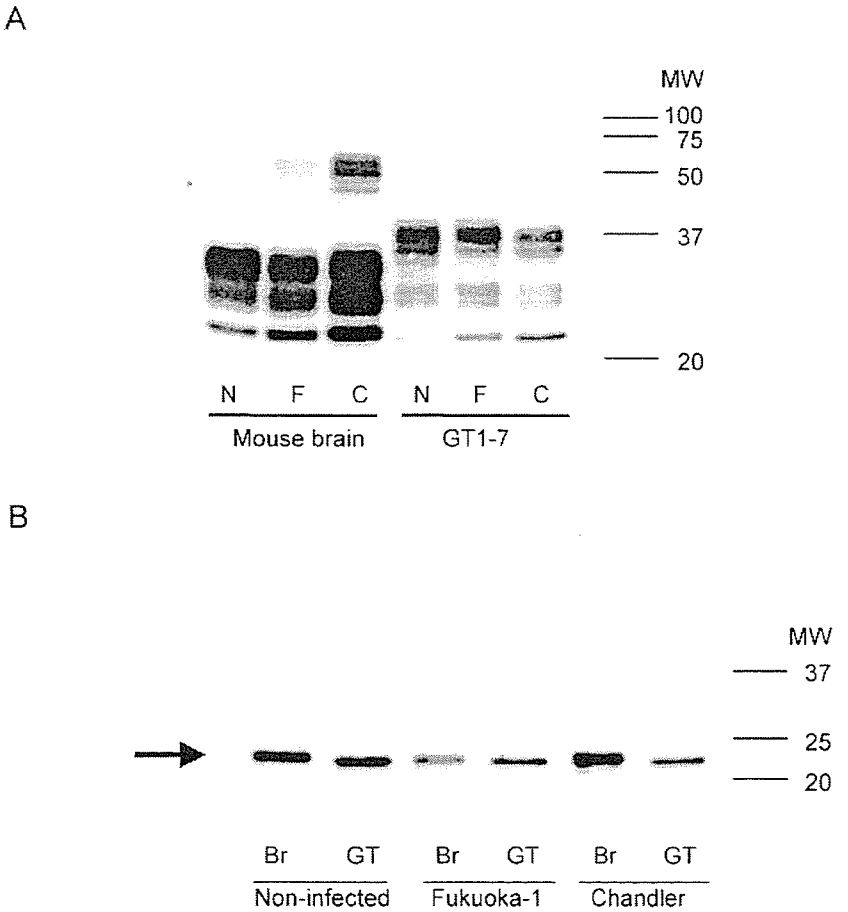

FIG. 4. Detection of PrP in brain homogenates and GT1-7 lysates without PK treatment. (A) Noninfected (N) brain homogenates and GT1-7 cell lysates and those infected with Fukuoka-1 (F) or Chandler (C) prions were subjected to immunoblotting with the IBL-N antibody against N-terminal PrP peptides. (B) The mobilities of nonglycosylated PrPs (arrow) from the brain tissues (Br) and GT1-7 cells (GT) were compared on an immunoblot with the IBL-N antibody after deglycosylation by PNGase F treatment. MW, molecular weight.

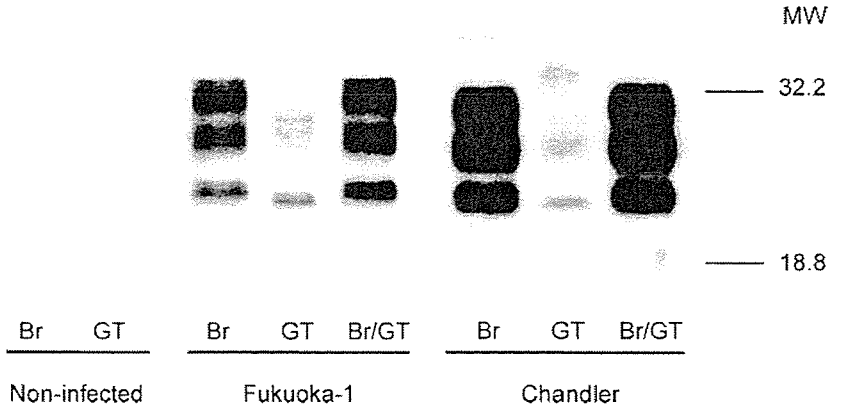

FIG. 5. Immunodetection of PK-resistant PrP in brain homogenates and GT1-7 lysates infected with prion strains. After treatment with PK, noninfected brain homogenates (Br) and GT1-7 cell lysates (GT) and those infected with Fukuoka-1 or Chandler prions were subjected to immunoblotting with polyclonal mouse antisera against PrP. The brain homogenates from terminal-stage mice inoculated with the lysates of GT1-7 cells infected with Fukuoka-1 or Chandler prions (Br/GT) were also analyzed. MW, molecular weight.

Strain-dependent differences in the biochemical aspects of PrP^{Sc}. PK completely digested PrP in the noninfected tissues and cells, while the resistant components (PrP^{Sc}) in the infected tissues and cells were visualized by polyclonal antiserum raised against a recombinant PrP (Fig. 5). Again, diglycosylated PrP^{Sc} in GT1-7 cells migrated to a much higher molecular mass, and its unglycosylated component migrated faster than PrP^{Sc} in the brain tissues. Of importance, PrP^{Sc} developed in the brain tissues of mice inoculated with the infected GT1-7 cell lysates had migration patterns that were indistinguishable from those of PrP^{Sc} in the original brain tissues. PNGase F treatment confirmed the different migration pat-

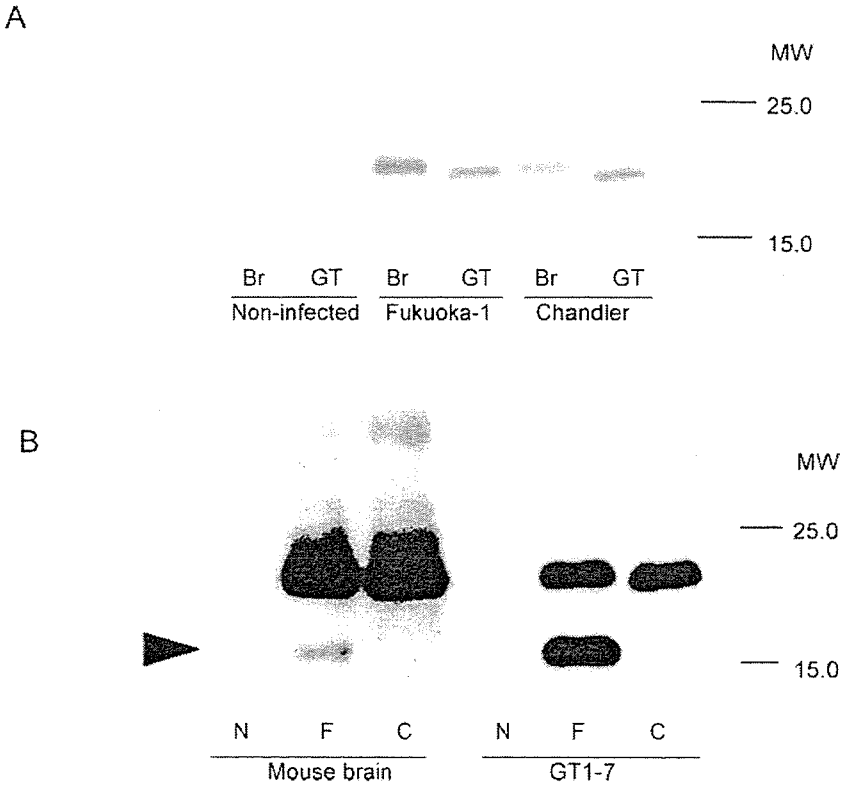

FIG. 6. Differences in the mobility of PK-resistant PrP between hosts and between strains. (A) After successive treatments with PK and PNGase F, mobilities in a gel of nonglycosylated PrP^{Sc} from the brain tissues (Br) and GT1-7 cells (GT) were compared on an immunoblot with the M20 antibody against C-terminal PrP peptides. (B) Mobilities of nonglycosylated PrP^{Sc} generated by Fukuoka-1 (F) and Chandler (C) strains are directly compared by immunoblotting with the M20 antibody. PrP in noninfected tissues and cells (N) is completely digested by PK. The arrowhead indicates the 13-kDa PrP fragment specifically found in the Fukuoka-1-infected tissues and cells. MW, molecular weight.

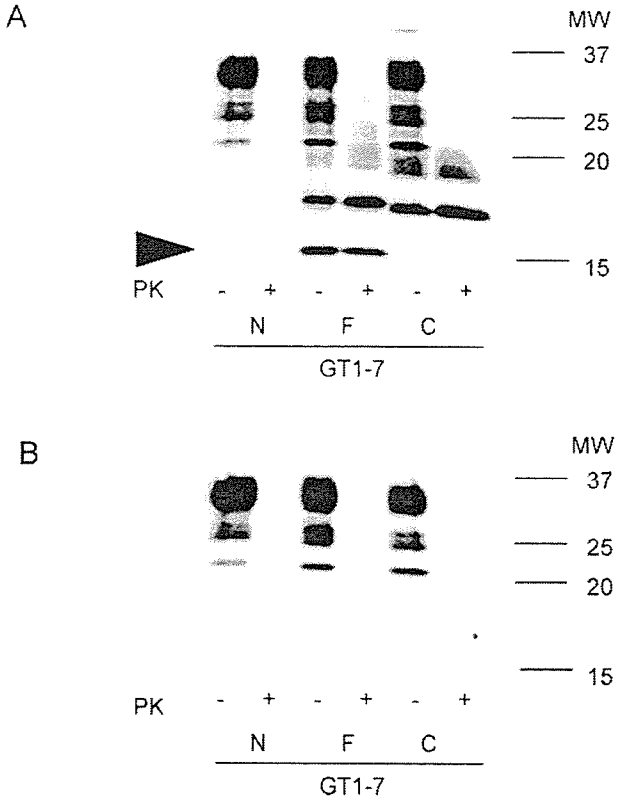

FIG. 7. The 13-kDa PrP fragment is detectable in Fukuoka-1-infected GT1-7 cells even without PK treatment. The cell lysates from noninfected (N) GT1-7 cells and those infected with the Fukuoka-1 (F) or Chandler (C) strain, with (+) or without (−) PK treatment, were subjected to immunoblotting using M20 (A) or IBL-N (B) anti-PrP antibodies. MW, molecular weight.

terns of unglycosylated PrP^{Sc} between the hosts (Fig. 6a). On the other hand, when migration patterns of PrP^{Sc} were directly compared between the strains in the same host on an immunoblot, Fukuoka-1-derived unglycosylated PrP^{Sc} clearly migrated more slowly than the Chandler-derived version (Fig. 6b). These findings strongly suggested that both host-specific and strain-specific factors are involved in the determination of the mobility of PK-resistant unglycosylated PrP^{Sc} in gel. Interestingly, in this immunoblotting, the M20 anti-C-terminal PrP antibody clearly visualized at a low molecular size, ~13 kDa, a component of PrP^{Sc} in Fukuoka-1-infected, but not Chandler-infected, GT1-7 cells (Fig. 5b). A faint but significant band of the 13-kDa fragments was also detectable in the Fukuoka-1-infected brain tissue. Since this fragment was similarly detectable even before the PK treatment and recognized by C-terminal (M20) but not N-terminal (IBL-N) PrP antibodies (Fig. 7), it is likely to be a C-terminal PrP fragment lacking a PK cleavage site.

DISCUSSION

Passage through the neuronal cell cultures of two prion strains, Chandler and Fukuoka-1, did not affect the biological characteristics, including clinical signs, incubation periods, and pathological findings, in the inoculated mice. Carryovers of the original strains into the cultures were unlikely, since the infected cells were cultured for more than 30 passages since the initial ex vivo challenges, assuming that residuals of original brain homogenates in the cultures would be diluted far greater than 10 orders of magnitude. We used a mouse neuronal cell line, GT1-7, which expresses a large amount of PrP^{C} and is highly permissive for replication of the agent (15, 18). Infected GT1-7 cells persistently produced PrP^{Sc} for more than 30 passages without subcloning and maintained high infectious titers of Fukuoka-1 and Chandler at the levels of $10^{5.3}$ and $10^{6.5}$ LD_{50} units/10^{7} cells, respectively. The high degree of competence in prion replication allowed us to quantify infectious titers in the cultured cells by end-point assay. Although data are not shown, we have found that the biological characteristics of the two strains, including clinical signs and incubation periods in the inoculated mice, are also conserved in other two neuronal cell lines, N2a58 (18) and 1C11 (17), which are permissive to various prion strains (16, 18; N. Nishida, unpublished). These findings are consistent with those of previous reports indicating that strain phenotypes did not change during several passages in cultured cells (1, 5).

Western blotting identified some differences in the biochemical features of PrP^{Sc} between the brain tissue and GT1-7 cells. The degrees of glycosylation of PrP^{Sc} derived from the two strains in GT1-7 cells were clearly higher than those in the brain tissues. A similar difference was observed before PK treatment even between the noninfected cells and tissues, suggesting the involvement of host cell factors rather than the strains. In some conditions, the degree of glycosylation (a ratio of glycoforms) of PrP^{Sc} is an important signature of the prion strain (5, 8). However, in our experimental models, it is largely determined by the hosts, presumably due to differences in the enzymatic activities involved in glycosylation or the trafficking pathway of de novo-synthesized PrP^{C}. Host cell- or tissue-determined PrP^{Sc} glycoforms have also been reported by others (31, 36). The mobility of unglycosylated PrP^{Sc} fragments in gel was also distinguishable between the brain tissues and GT1-7. This possibly reflected PK cleavage site heterogeneities due to the difference in the conformation of PrP^{Sc} or an artifact of experimental conditions such as pH (20). However, it is noteworthy that the size difference was also the case for PrP without PK treatment even between the noninfected tissues and cells, arguing against a difference in the PK cleavage sites. Sequencing of PrP cDNAs amplified by reverse transcription-PCR from the brain tissues and GT1-7 cells confirmed that their primary structures were identical (data not shown). A previous study identified, by use of mass spectrometry, six different glycosylphosphatidylinositol (GPI) glycoforms with molecular masses ranging from 2,670 to 3,285 Da in PrP^{Sc} purified from infected hamster brains as well as partially purified PrP^{C} (33). The presence of tissue-specific differences in the GPI composition was also suggested (12). Although the involvement of some difference in the PrP conformation preserved even in a denatured condition cannot be ruled out, a more likely explanation is that it is due to heterogeneity in the composition of GPI moieties. Precise mechanisms for the diversity in PrP structures among the hosts await elucidation, but these structural features are unlikely to affect the strain phenotype, which is shared by the hosts.

It has been hypothesized that the strain-specific conformation of PrP^{Sc} determines the pathological features and func-

tions as a template during pathogenic structural conversion of PrP^{C} to PrP^{Sc} in affected brain tissues (25). The present study also revealed evidence that some strain-specific features of the PrP^{Sc} band pattern on an immunoblot were conserved in the cultured cells. The unglycosylated PK-resistant PrP^{Sc} fragment derived from Fukuoka-1 always migrated more slowly than the Chandler-derived version accumulating in the same host, either in brain tissues or GT1-7 cells. So far, many prion strains, including those of human, sheep, and mink origin, have been characterized by the size of the PrP^{Sc} core fragment generated by PK (2–4, 24). In most cases, the size difference due to the diverse cleavage sites of PK is presumed to be a consequence of the extent of the β-sheet structure. However, Arjona et al. reported that identical PrP^{Sc} band patterns could be observed in GT1-7 cells infected with distinct CJD strains, FU and SY, but were different from those in brain tissues and N2a cells (1). This indicated that the conformational divergence of PrP^{SC} does not necessarily alter strain characteristics. We also demonstrated host-determined divergence, such as glycosylation patterns, which did not affect the biological characteristics of prion strains. Furthermore, the possible involvement of a putative agent or agent-induced factors other than the PrP^{Sc} conformation itself is not precluded. It would be of value to search for such factors that affect the mobility of PrP^{Sc} core fragment by using the cell culture model.

Strikingly, the small 13-kDa PrP^{Sc} fragment detected in Fukuoka-1-infected tissues and cells was not seen in Chandler-infected materials. It is likely that the 13-kDa. Fragment is strain-specific, since Fukuoka-1 but not Chandler resulted in the band not only in GT1-7 cells but also in N2a58 and 1C11 cells (N. Nishida, unpublished). It was PK resistant but detectable before PK treatment, suggesting a lack of PK cleavage sites and the involvement of endogenous proteolytic processes. It is possible that certain environmental factors, for instance, pH and metal ion concentration, influenced by the strain might alter the catalytic activities, but it is also possible that the Fukuoka-1-specific PrP^{Sc} conformation could allow endogenous proteases to access and catalyze the full-length PrP^{Sc}. Although both the Chandler and Fukuoka-1 strains are mouse-adapted ones, the former is of scrapie origin and the latter was derived from a GSS patient carrying the P102L mutation. One group previously demonstrated a similar 13-kDa fragment in the brain tissues from five of seven P102L GSS patients, and this fragment was immunoreactive to a C-terminal PrP antibody but not to 3F4 monoclonal antibody, indicating that it was N-terminally truncated beyond residue 112 (22). It would be intriguing to see whether the specific PrP^{Sc} conformation determined by a particular genetic mutation in the human brain tissues has been conserved during successive transmission to mouse brains and cultured cells lacking such a mutation. Some other reports demonstrated that PrP^{Sc} derived from F198S GSS and CJD also displayed patterns of endogenous proteolysis characteristic of each disorder, leading to distinct sets of PrP^{Sc} fragments (12). It is conceivable that different PrP^{Sc} fragments may exhibit unique biological and pathological consequences in the CNS. The most important pathological consequence of prion strains is the difference in the distribution of vacuolar degeneration among the CNS regions of affected animals. A possible explanation is that each prion strain possesses its own cell tropism. The investigation of cell tropism requires the stable infection of a single cell type which is permissive to more than one strain. In our preliminary experiments using the cell culture models, some strains revealed differential tropism among the cell types examined (N. Nishida, unpublished).

In conclusion, we demonstrated here that the prion strains tested conserved their biological characteristics following cell culture, and the accumulated PrP^{Sc} reproduced some specific features of its band pattern on an immunoblot. However, the molecular basis for conformational divergence of PrP^{Sc} is still enigmatic, and whether or not there is a causal relationship between the PrP^{Sc} conformation and strain phenotype remains to be concluded. Our cell culture models allow the analysis of trafficking and metabolism of PrP, i.e., posttranslational cleavage, glycosylation, recycling, and degradation, etc., which will provide a new insight into the understanding of the molecular basis of prion strains.

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Inhibition of prion propagation in scrapie-infected mouse neuroblastoma cell lines using mouse monoclonal antibodies against prion protein

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Abstract

We screened six mouse monoclonal antibodies (mAbs) against prion protein (PrP), which were previously established in our laboratory, for inhibitory activity against PrP^{Sc}-accumulation in scrapie-infected cell lines and identified two mAbs, 3S9 and 2H9, as possessing this inhibitory activity. mAb 3S9 recognized an epitope including 154th tyrosine in the helix 1 region of PrP, while mAb 2H9 recognized a discontinuous region that included helix 1. In three scrapie-infected cell lines infected with different mouse-adapted scrapie strains, mAb 3S9 strongly inhibited accumulation of PrP^{Sc}, while mAb 2H9 moderately inhibited accumulation of PrP^{Sc}, indicating that inhibition of prion propagation by mAbs may be dependent on PrP^{Sc} characteristics. Furthermore, mAb 3S9 completely excluded PrP^{Sc} from these cell lines. These results suggest that mAbs 3S9 and 2H9 might be useful for clarifying the mechanisms of prion propagation and prevention by PrP-specific antibodies, and for tracing the conversion of PrP^{C} to other PrP^{Sc} isoforms.

Keywords: PrP-specific antibody; Inhibition of prion accumulation; Helix 1 of PrP; 154th tyrosine of PrP; Prion strain

Transmissible spongiform encephalopathies (TSEs) are so-called prion diseases and comprise a group of fatal neurodegenative disorders, including scrapie in sheep and goats, bovine spongiform encephalopathy in cattle, and Creutzfeldt–Jakob disease in humans [1]. These diseases share the accumulation of a pathogenic isoform of prion protein (PrP^{Sc}) in the central nervous system as a common feature and no effective therapy against prion diseases currently exists. The PrP^{Sc} is converted from the host-encoded cellular isoform of PrP (PrP^{C}) by post-translational modifications. Although the two prion isoforms have identical amino acid sequences [2], their biological and biochemical properties differ. PrP^{Sc} is insoluble in detergents and is partially resistant to proteinase K (PK) digestion, whereas PrP^{C} is readily soluble under nondenaturing conditions and is completely digested by PK [3–5]. The mechanism by which PrP^{C} is converted to PrP^{Sc} remains unclear.

PrP-specific antibodies are useful in understanding both the structure of PrP [6,7] and in tracing the conformational changes from PrP^{C} to PrP^{Sc} [8,9]. In addition, PrP-specific antibodies can be used in the diagnosis of prion diseases in experimental and domestic animals as well as therapeutic approaches of Creutzfeldt–Jakob disease in humans [10–13]. Recent reports indicate that

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some PrP-specific antibodies can inhibit prion propagation and exclude PrP^{Sc} both in vitro and in vivo [14]. Therefore, therapeutic approaches using mAbs are being explored. However, the prevention mechanisms by which PrP-specific antibodies function remain unclear, and few inhibitory mAbs have been reported [8,9,14–18]. The generation and identification of PrP-specific antibodies are thus important in structural studies, diagnosis, and therapeutic approaches.

In this study, we investigated whether six mouse mAbs against PrP established in our laboratory inhibit prion propagation in scrapie-infected cell lines. We report here that two mAbs, 3S9 and 2H9, inhibited the conversion of PrP^{C} to PrP^{Sc} and that one of these mAbs completely excluded PrP^{Sc} from scrapie-infected cells.

Materials and methods

Cell lines. SP2/0-Ag14 (SP2) [19] was used as the myeloma cell line partner in the cell fusion experiment. SP2 was maintained in Iscove's modified Dulbecco's medium (IMDM, Invitrogen, USA) containing 10% fetal bovine serum (FBS, Sigma, USA) in a 5% CO_2 incubator at 37 °C. The scrapie-infected mouse neuroblastoma cell lines N2a/22L [20], N2a/Chandler [20], and N2a/Fukuoka were used to investigate the inhibition of prion propagation by PrP-specific antibodies. These cell lines are persistently infected with three mouse-adapted scrapie strains (22L, Chandler and Fukuoka-1) having the conserved biological and biochemical characteristics of the original prion strain [21]. N2a/22L cells possessed much higher levels of PrP^{Sc} than the other two cell lines. These cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) containing 10% FBS in a 5% CO_2 incubator at 37 °C.

Recombinant PrP. Recombinant Hu122–230 (codons 122–230 of human PrP) was kindly supplied by Dr. Kitamoto (Tohoku University, Japan). Recombinant Ha23–231 (codons 23–230 of hamster PrP) was kindly supplied by Dr. Horiuchi (Hokkaido University, Japan). Recombinant PrPs, Mo121–231 (codons 121–231 of mouse PrP), Sh125–234 (codons 125–234 of sheep PrP), and Bo133–241 (codons 133–241 of bovine PrP), were generated as described previously [22]. Briefly, recombinant PrPs were expressed with pET22b (Novagen, Germany) and then purified by using nickel ion-charged Chelating Sepharose Fast Flow (Amersham Biosciences, USA) and HiPrep Sephacryl S-100 HR (Amersham Biosciences, USA) according to the manufacturer's instructions.

Mouse PrP deletion mutants (mouse PrP: codons 121–231, 131–231, 141–231, 151–231, 161–231, 171–231, 181–231, 191–231, 201–231, 121–221, 121–212, 121–201, 121–191, 121–181, 121–171, 121–163, and 121–151) were generated with pGEX-6P-1 (Amersham Biosciences, USA) in order to synthesize PrP as a glutathione *S*-transferase (GST) fusion protein. Briefly, DNA fragments of deletion mutants were amplified by PCR using primer sets as indicated in Table 1. Amplified fragments were digested with *Bam*HI and *Xho*I, and cloned into the *Bam*HI and *Xho*I sites of pGEX-6P-1. These expression plasmids were transformed into *Escherichia coli* BL21 (DE3) (Novagen, Germany). Protein expression was induced by addition to 0.1 mM isopropylthio-β-D-galactoside. Bacterial cells were collected and sonicated. Deletion mutants were purified with glutathione–Sepharose 4B beads (Amersham Biosciences, USA) according to the manufacturer's instructions.

Concentrations of these recombinant PrPs were measured by BCA Protein Assay Reagent Kit (Pierce, USA) according to the manufacturer's instructions. Hu122–230 and Sh125–234 were used as immunogens to generate mAbs. Recombinant PrPs including in Hu122–230 and Sh125–234 were used as antigens for enzyme-linked immunosorbent assay (ELISA). Deletion mutants were subjected to mAb epitope analysis.

Monoclonal antibodies. Monoclonal antibodies (mAbs) specific to PrP were generated using cell fusion technology. Briefly, 6-week-old $PrP^{0/0}$ mice [23] were immunized intraperitoneally with 100 μg Hu122–230 or Sh125–234 in 0.1 ml phosphate-buffered saline (PBS) together with an equal volume of alum solution. Subsequent immunization was carried out every 3 weeks. Three days after the final injection, spleen cells from the immunized $PrP^{0/0}$ mice were fused with the SP2 cells using 50% (wt/vol) polyethylene glycol 1500 (Roche Diagnostics, Switzerland) and were selected in hypoxanthin aminopterin thymidine (HAT) medium. Hybridoma culture supernatants were screened by ELISA using recombinant PrPs as immunogens. The ELISA procedure is described below. Hybridomas secreting antibodies against PrP were cloned by limiting dilution. The mAbs were purified from mouse ascites and were used in subsequent experiments. Protein concentration was measured with a BCA Protein Assay Reagent Kit (Pierce, USA) according to the manufacturer's instructions. The isotypes of the mAbs were determined using a Mouse monoclonal isotyping kit (Amersham Biosciences, USA) according to the manufacturer's instructions.

Four mAbs, 22L/2H9 (2H9), 22L/2H12 (2H12), 22L/1A3 (1A3), and 22L/8H12 (8H12), specific for PrP generated previously [22], were used in epitope analysis and inhibition assay for prion propagation.

ELISA. ELISA plates (Nunc, USA) were coated with 50 μl/well of 2.5 μg/ml of recombinant PrPs (Hu122–230, Mo121–231, Ha23–231, Sh125–234, and Bo133–241) in PBS at 4 °C overnight. Plates were blocked with 380 μl/well of 25% (vol/vol) BlockAce (Yukijirushi, Japan) in PBS at 37 °C for 1 h. After washing with PBS containing 0.05% (vol/vol) Tween 20 (PBS-T), hybridoma culture supernatants were added (50 μl/well) and plates were incubated at 37 °C for 1 h. After washing with PBS-T, HRP-labeled goat anti-mouse IgA + IgG + IgM (H + L) (Kirkegaard and Perry Laboratories, USA) was added

Table 1
Primers for generation of deletion mutants

Sense primer	Anti-sense primer
MoPrP121-F: 5′CGGGATCCGTGGGGGGCCTTGG3′	MoPrP231-R: 5′CCCTCGAGGCTGGATCTTCTCC3′
MoPrP131-F: 5′CGGGATCCAGCGCCGTGAGCAG3′	MoPrP221-R: 5′CCCTCGAGGGACTC CTTCTGG3′
MoPrP141-F: 5′CGGGATCCGGCAACGACTGGGAGGA3′	MoPrP212-R: 5′CCCTCGAGCATCTG CTCCACCA3′
MoPrP151-F: 5′CGGGATCCGAAAACATGTACCGCTAC3′	MoPrP201-R: 5′CCCTCGAGATCGGT CTCGGTGA3′
MoPrP161-F: 5′CGGGATCCTACTACAGGCCAGTGG3′	MoPrP191-R: 5′CCCTCGAGGGTGGTGGTGACC3′
MoPrP171-F: 5′CGGGATCCCAGAACAACTTCGTG3′	MoPrP181-R: 5′CCCTCGAGGATACTTACGCAGTCGT3′
MoPrP181-F: 5′CGGGATCCATCACCATCAAGCAG3′	MoPrP171-R: 5′CCCTCGAGCTGGTTGCTGTACTGATCC3′
MoPrP191-F: 5′CGGGATCCACCACCAAGGGGGAGA3′	MoPrP163-R: 5′CCCTCGAGCCTGTAGTACACTTGGTTAGG3′
MoPrP201-F: 5′CGGGATCCGATGTGAAGATGATGG3′	MoPrP151-R: 5′CCCTCGAGTTCACGGTAGTAGCGGTCCT3′

Primer sets of deletion mutants on N-terminal: sense primers + MoPrP231-R. Primer sets of deletion mutants on C-terminal: MoPrP121-F + anti-sense primers.

(50 μl/well) as a detection antibody and plates were incubated at 37 °C for 1 h. After washing with PBS-T, *o*-phenylenediamine sulfate (Sigma, USA) was added as a substrate and optical density was then measured at 490 nm.

Preparation of PrP^C and PrP^{Sc} for Western blotting. Brain tissues from different animals (mouse, sheep, and cow) were homogenized in 9 volumes of lysis buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate] containing Complete Protease Inhibitor Cocktail Set (Roche Diagnostics, Switzerland). Homogenates were centrifuged at 800*g* for 5 min at 4 °C and the supernatants were used as a source of PrP^C. Protein concentration of the supernatant was then measured using a BCA Protein Assay Reagent Kit (Pierce, USA). PK-treated materials were prepared using brain tissues from scrapie-infected mice and the scrapie-infected mouse neuroblastoma cell lines N2a/22L, N2a/Chandler, and N2a/Fukuoka [20,23]. These brain homogenates and cell lysates were treated with 20 μg/ml PK for 40 min at 37 °C. Digestion was stopped with 1 mM Pefabloc Sc (Roche Diagnostics, Switzerland) and the materials were centrifuged at 1,00,000*g* for 1 h at 25 °C. After removing the supernatant, the pellet was resuspended in 30 μl Laemmli buffer, followed by incubation at 55 °C and boiling for 10 min. PrP^C and PrP^{Sc} were then subjected to Western blotting.

Western blotting. PrP^C and PrP^{Sc} were separated by SDS–PAGE on 13.5% polyacrylamide gel and were then transferred to an Immunblot PVDF membrane (Bio-Rad, USA) by electroblotting at 300 mA for 2 h. Membranes were blocked with blocking buffer (8% (wt/vol) skim milk, 0.2% (vol/vol) Tween 20, and 2.5 mM EDTA in PBS) at room temperature for 1 h and then incubated for 1 h at room temperature with the anti-PrP mAbs in washing buffer (1% (wt/vol) skim milk, 0.2% (vol/vol) Tween 20, and 2.5 mM EDTA in PBS). Membranes were washed in washing buffer with agitation, and then incubated at room temperature for 1 h with HRP-labeled Goat Anti-Mouse IgA + IgG + IgM (H + L) (Kirkegaard and Perry Laboratories, USA) in washing buffer. After membranes were washed, the blots were developed with ECL plus Western blotting detection reagents (Amersham Biosciences, USA) and detected by LAS-3000 lumino image analyzer (Fujifilm, Japan).

Epitope analysis. The PrP epitopes recognized by mAbs were determined by Western blotting using mouse PrP deletion mutants (mouse PrP: codons 121–231, 131–231, 141–231, 151–231, 161–231, 171–231, 181–231, 191–231, 201–231, 121–221, 121–212, 121–201, 121–191, 121–181, 121–171, 121–163, and 121–151). Western blotting was as described above. As primary antibodies, six mAbs were used; 17H5, 3S9, 2H9, 2H12, 1A3, and 8H12. Anti-GST Antibody (Amersham Biosciences, USA) was used for detection of GST fusion PrPs as a control. As second antibodies, HRP-labeled goat anti-mouse IgA + IgG + IgM (H + L) (Kirkegaard and Perry Laboratories, USA) and HRP-labeled rabbit anti-goat IgG (H + L) (Kirkegaard and Perry Laboratories, USA) were used.

Inhibition analysis of PrP^{Sc} accumulation in prion-infected cell lines using mAbs. The scrapie-infected mouse neuroblastoma cell lines, N2a/22L, N2a/Chandler, and N2a/Fukuoka, were each seeded (2×10^5 cells/dish) in 60-mm dishes containing 3 ml of 10% FBS–DMEM. Cells were cultured for 4 days in the absence or presence of 10 μg/ml mAbs. After culture, cells were lysed according to the procedure described above. Western blotting analysis was performed as described above, with the following exceptions. Detection of PrPs (PK-treated and -untreated PrPs) was performed using HUC2-13 specific for the N-terminal of PrP [24] or HUNN1 specific for the PK cleavage site [25]. As a detection antibody, HRP-labeled goat anti-chicken IgG (H + L) (Kirkegaard and Perry Laboratories, USA) was used.

In order to determine the dose-dependency of mAbs in the inhibition of PrP propagation, N2a/Chandler cells were seeded (2×10^5 cells/dish) in 60-mm dishes containing 3 ml of 10% FBS–DMEM. Cells were cultured for 4 days in the presence or absence of 0.0032–10 μg/ml mAbs. After culture, cells were lysed and analyzed by Western blotting according to the procedure described above. The intensity of immunostained bands on Western blotting was quantified using Science Lab 2001 Image Gauge software (Fujifilm, Japan).

Disappearance of PrP^{Sc} from prion-infected cells by anti-PrP mAbs. N2a/Chandler or N2a/22L cells were seeded (2×10^5 cells/dish) in 60-mm dishes containing 3 ml of 10% FBS–DMEM. Cells were cultured for 4 days in the presence of 10 μg/ml of mAbs. After cultivation for 4 days, cells were passaged into two dishes at 2×10^5 cells/dish. One group was used for Western blotting, the other was serially treated with identical mAbs. Cells were treated with 10 μg/ml of mAbs for 4–16 days. Every 4 days, the cells were passaged. After each treatment with mAbs, cells were further incubated in the absence of mAbs for 4–16 days. Cells were then lysed and analyzed according to the procedure described above.

Results

Monoclonal antibodies

The six mouse mAbs (17H5, 3S9, 2H9, 2H12, 1A3, and 8H12) used in this study were generated by immunizing $PrP^{0/0}$ mice with recombinant PrPs or scrapie-infected mouse neuroblastoma cell lines [22]. Of the mAbs, 17H5 and 3S9 were newly generated in this study. We further examined the PrP epitopes recognized by the mAbs, and Western blotting was performed using mouse PrP deletion mutants (Fig. 1). mAb 17H5 reacted with mutants lacking 171 N-terminal residues and 221 C-terminal residues. mAb 3S9 reacted with mutants lacking 141 N-terminal residues and 161 C-terminal residues. mAbs 2H9 and 2H12 reacted with mutants lacking 151 N-terminal residues and 221 C-terminal residues. mAbs 1A3 and 8H12 reacted with mutants lacking 141 N-terminal residues and 221 C-terminal residues. These results indicate that the epitopes recognized by mAbs 17H5 and 3S9 were located at residues 171–221 and 141–161, respectively. The epitopes recognized by mAbs 2H9 and 2H12 were located at residues 151–221, and those recognized by mAbs 1A3 and 8H12 were located at residues 141–221. The characteristics of the six mAbs are shown in Table 2.

Inhibition of PrP-propagation by mAbs

In order to identify mAbs that inhibit accumulation of PrP^{Sc} in cultured cells, inhibition studies of prion propagation were performed using scrapie-infected neuroblastoma cell lines N2a/22L, N2a/Chandler, and N2a/Fukuoka. mAbs 3S9 and 2H9 induced decreases in the amount of PK-treated PrP^{Sc} in these cell lines, whereas mAb 17H5 had no effect on the amount of PK-treated PrP^{Sc} (Fig. 2). The other mAbs, 2H12, 1A3, and 8H12, had no effect on the amount of PK-treated PrP^{Sc}, as observed with mAb 17H5 (data not shown). Furthermore, the amounts of PK-untreated PrP were not affected by treatment with mAbs. mAbs 3S9 and 2H9 inhibited the accumulation of PrP^{Sc} in these cell lines. Interestingly, mAb 3S9 was particularly effective at

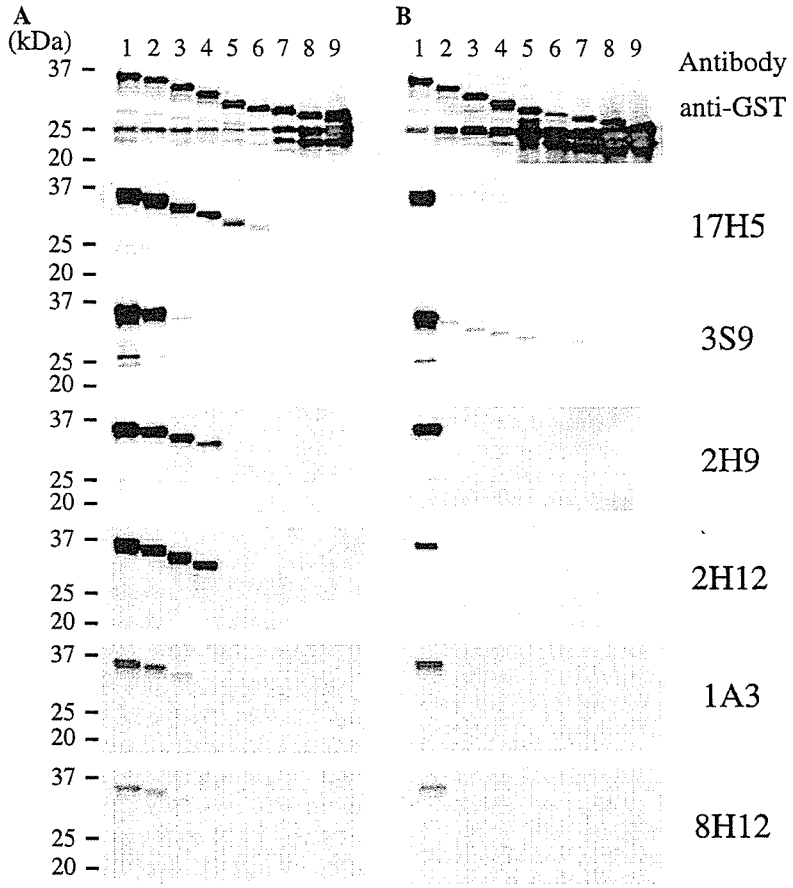

Fig. 1. Epitope mapping of PrP recognized by PrP-specific antibodies. (A) Western blotting profiles using N-terminal deletion mutants. Lanes 1–9 show amino acid residues of PrP, lane 1; 121–231, 2; 131–231, 3; 141–231, 4; 151–231, 5; 161–231, 6; 171–231, 7; 181–231, 8; 191–231, and 9; 201–231. (B) Western blotting profiles using C-terminal deletion mutants. Lanes 1–8 show amino acid residues of PrP, lane 1; 121–221, 2; 121–212, 3; 121–201, 4; 121–191, 5; 121–181, 6; 121–171, 7; 121–163, and 8; 121–151. Lane 9 shows GST alone. Anti-GST antibody was used for detection of deletion mutants (GST fusion proteins) as a control. Molecular masses are indicated on the left.

Table 2
Characteristics of mouse monoclonal antibodies against PrP

mAbs	Isotype	Immunogen	Epitope	Reference
3S9	IgG_1	Recombinant sheep PrP	141–161	—
17H5	IgG_1	Recombinant human PrP	171–221	—
22L/2H9	IgG_1	Scrapie-infected mouse	151–221	Nakamura et al. [22]
22L/1A3	IgG_1	Neuroblastoma cell lines	141–221	
22L/2H12	IgG_1		151–221	
22L/8H12	IgM		141–221	

inhibiting accumulation of PrP^{Sc} in all cell lines, whereas mAb 2H9 markedly inhibited accumulation of PrP^{Sc} in N2a/Chandler and N2a/Fukuoka cells, but did so slightly in N2a/22L.

In order to compare inhibition activity, the dose-dependency of the mAbs was examined. N2a/Chandler cells were incubated for 4 days with various concentrations of mAbs ranging from 0.0032 to 10 μg/ml. Dose-dependent inhibition was determined using the mAb concentration at which 50% inhibition of PrP^{Sc} levels was seen (50% inhibitory concentration (IC_{50})). When compared with PrP^{Sc} in untreated cells, PrP^{Sc} levels in treated cells was reduced in a dose-dependent manner. The IC_{50} values obtained for mAbs 3S9 and 2H9 were 0.08 μg/ml (0.6 nM) and 1.2 μg/ml (8.4 nM), respectively (Fig. 3). The inhibition activity of mAb 3S9 was stronger than the activity of mAb 2H9.

In order to investigate whether it is possible to completely exclude PrP^{Sc} from N2a/Chandler and N2a/22L cells using mAbs, these cell lines were treated with

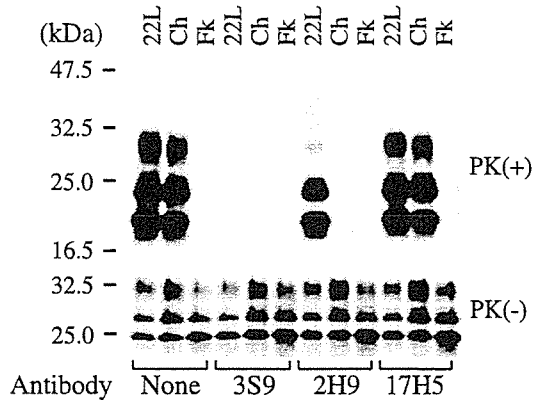

Fig. 2. Inhibition of prion propagation in scrapie-infected cell lines N2a/22L (22L), N2a/Chandler (Ch), and N2a/Fukuoka (Fk) by PrP-specific antibodies. PrP treated with or without PK was detected by Western blotting using HUC2-13 specific for the N-terminal of PrP and HUNN1 specific for the PK cleavage site of PrP. Molecular masses are indicated on the left.

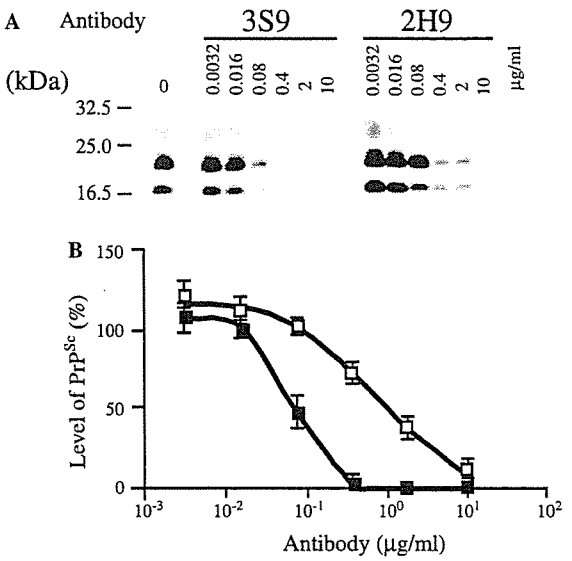

Fig. 3. Dose-dependency of mAbs 3S9 and 2H9 on the inhibition of PrP^{Sc} accumulation in N2a/Chandler cells. (A) Western blotting profiles of PrP^{Sc} from N2a/Chandler cells using mAbs 3S9 and 2H9. N2a/Chandler cells were cultured for 4 days with various concentrations of mAb. Cells were lysed and then digested with PK. Levels of PrP^{Sc} in the cells were determined by Western blotting using mAb HUNN1, which is specific for the PK cleavage site of PrP. Molecular masses are indicated on the left. (B) Inhibition curve for mAbs 3S9 and 2H9. Levels of PrP^{Sc} given as 100% correspond to the intensity of PrP^{Sc} bands in the absence of mAbs, while 0% represents undetectable levels of PrP^{Sc}. Each square (closed squares, 3S9; open squares, 2H9) represents means ± SD from at least three independent experiments.

10 μg/ml mAbs for 4, 8, 12 or 16 days. After treatment, cells were further incubated in the absence of mAbs for 4, 8, 12 or 16 days. PrP^{Sc} in N2a/Chandler cells treated with mAb 3S9 for 4 days was reduced to non-detectable levels, but gradually recovered on incubation in the absence of mAb (Fig. 4A). However, PrP^{Sc} in cells treated with mAb 3S9 for 8 days remained at non-detectable levels after 12 days in the absence of mAb. mAb 2H9 was also able to reduce and exclude PrP^{Sc} from N2a/Chandler cells after 16 days of treatment with mAb. These two mAbs reduced accumulation of PrP^{Sc} in N2a/22L cells, and mAb 3S9 was able to continue excluding PrP^{Sc} from cells for 12 days after incubation (Fig. 4B). In addition, cells treated with mAb 3S9 for 8 days did not express PrP^{Sc} in the absence of mAb, even after 1 year of culture (data not shown). These results indicate that mAbs enable complete exclusion of PrP^{Sc} from infected cell lines following continuous treatment with mAb.

Reactivity of mAbs against PrP

The reactivity of inhibitory mAbs, 3S9 and 2H9, against PrP^{C} and PrP^{Sc} was investigated by Western blotting. mAb 3S9 recognized the mouse and sheep PrPs, while mAb 2H9 recognized mouse PrP. mAbs 3S9 and 2H9 recognized the PK-treated PrP^{Sc} (Fig. 5). mAb 3S9 strongly recognized three glycoforms of PrP from scrapie-infected mouse brain and N2a/22L cells, whereas mAb 2H9 failed to recognize the di-glycosylated form of PrP^{Sc} from N2a/22L cells.

The reactivity of inhibitory mAbs was also confirmed by ELISA using recombinant human, mouse, hamster, sheep, and bovine PrPs. mAb 3S9 recognized mouse and sheep PrPs, while mAb 2H9 recognized mouse and hamster PrPs (Table 3).

Discussion

Antibodies against PrP are indispensable in the diagnosis of prion diseases in humans and animals. The results of recent reports [8,9,14–18] have shown that certain PrP-specific mAbs may be useful in therapeutic approaches for prion diseases. Research into mAbs against PrP is thus important in the diagnosis and treatment of prion diseases.

To identify mAbs that prevent the PrP^{Sc} accumulation in prion-infected cell cultivation, we screened various mAbs against the C-terminal portion of PrP. We found that two mAbs, 3S9 and 2H9, inhibited conversion of PrP^{C} to PrP^{Sc} (Fig. 2–4). These mAbs recognized different epitopes; the epitopes recognized by 3S9 and 2H9 were located in residues 141–161 and 151–221, respectively (Fig. 1). Epitopes recognized by mAbs that inhibit prion propagation have been classified into four regions; 59–89, 90–109, 144–156, and 225–231 [8,9,14–18]. It is thought that residues 144–156 (helix 1 region) are particularly important in the conversion of PrP^{C} to PrP^{Sc} [8,9,26,27].

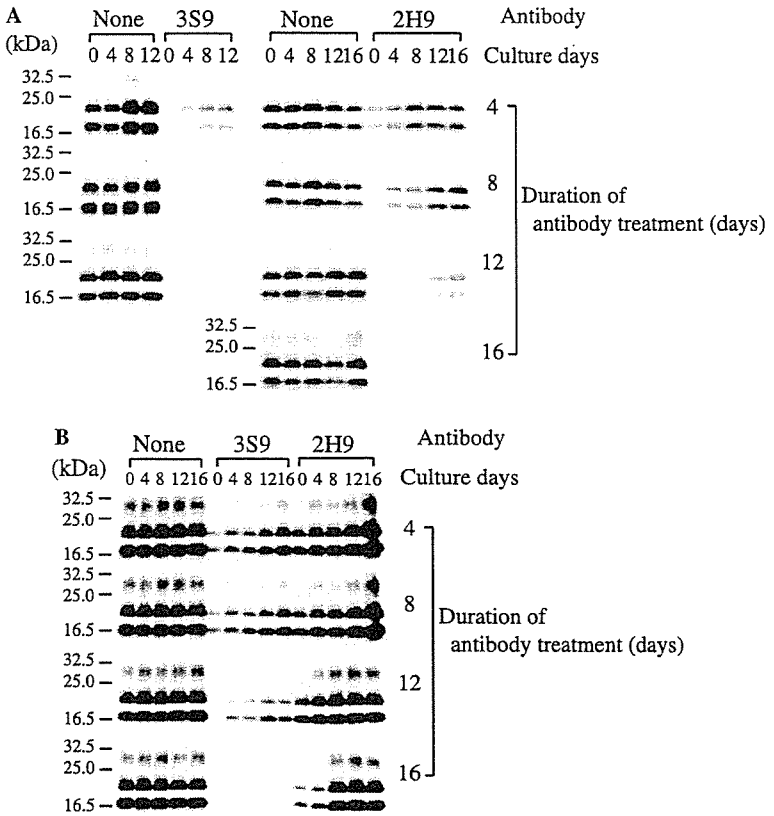

Fig. 4. Disappearance of PrP^{Sc} from prion-infected cells treated with mAbs 3S9 and 2H9. (A) N2a/Chandler cells were cultured for 4, 8, and 12 (and 16) days with or without 10 μg/ml of 3S9 or 2H9. (B) N2a/22L cells were cultured for 4, 8, 12, and 16 days with or without 10 μg/ml of 3S9 or 2H9. Cells were lysed and then digested with PK. Levels of PrP^{Sc} in cells were determined by Western blotting immediately after antibody treatment was initiated (day 0) or after cell cultivation for 4, 8 or 12 (or 16) days (day 4, 8, 12 or 16) in the absence of antibody. PrP^{Sc} was detected by mAb HUNN1, which is specific for the PK cleavage site of PrP. Molecular masses are indicated on the left.

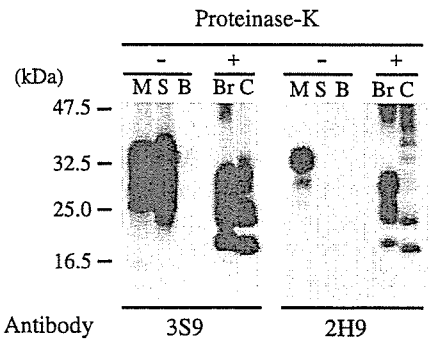

Fig. 5. Western blotting profiles of mammalian PrP^{C} and PK-treated PrP^{Sc} using inhibitory mAbs identified in this study. Mammalian PrP^{C} are brain homogenates from normal mouse (M), sheep (S), and cow (B), respectively. PK-treated PrP^{Sc} are from scrapie infected-mouse brain (Br) and N2a/22L cells (C). The designation, + or −, indicates whether PK was applied to the sample. Molecular masses are indicated on the left.

Table 3
Reactivity of anti-PrP mAbs against mammalian PrPs

mAb (Isotype)	Mouse	Hamster	Human	Sheep	Cow
3S9 (IgG1)	+	−	−	+	−
2H9 (IgG1)	+	+	−	−	−

+, reactive (OD value: >0.1); −, non-reactive (OD value: <0.1).

mouse	141	GNDWEDRYYRENMYRYPNQVY	161
hamster	141	GNDWEDRYYRENMNRYPNQVY	161
human	141	GSDYEDRYYRENMHRYPNQVY	161
sheep	141	GNDYEDRYYRENMYRYPNQVY	161
cow	141	GSDYEDRYYRENMHRYPNQVY	161

Fig. 6. Alignment of amino acids of the PrP epitope recognized by mAb 3S9. Residue numbers are indicated on the left and right. The helix 1 region of PrP is underlined, and the arrowheads indicate non-conserved amino acids in this region.

Most of the inhibitory mAbs reported previously, 6H4 [8], D18 [9], ICSM18 [14], ICSM17 [15], SAF61 [16,18], and SAF53 [18], recognized epitopes in the helix 1 region. This region is highly conserved among mammalian PrPs, and many inhibitory mAbs recognize epitopes common to all mammalian PrPs in the helix 1 region. The epitope recognized by mAb 3S9 is also located on helix 1 of PrP^{C} as well as other mAbs that

prevent PrP^{Sc} propagation. However, species specificity of PrP recognition by this mAb was different from other inhibitory mAbs.

On Western blot and ELISA, mAb 3S9 recognized mouse and sheep PrPs (Fig. 5 and Table 3). Three amino acid residues between 141 and 161 (142, 144, and 154) are not conserved among mammalian PrPs (Fig. 6). Amino acid 154 (Tyr) is the only residue that is common to mouse and sheep PrPs in this region. The results indicate that the epitope of mouse and sheep PrPs recognized by mAb 3S9 includes residue 154 (Tyr), and that mAb 3S9 recognizes different residues than other mAbs [8,9,14,16–18] having epitopes in the helix 1 region. These facts suggest that many amino acids important to prion propagation are present in the helix 1 region, that common amino acids recognized by these mAbs may be significant for prion propagation, and that residue 154 (Tyr) is required for the strong inhibition of prion propagation by mAb 3S9.

The fact that the epitope recognized by mAb 2H9 is located in residues 151–221 suggests that mAb 2H9 may recognize a discontinuous epitope. Inhibition of prion propagation by mAb 2H9 may be dependent on the recognition of amino acids in the helix 1 region or another region of PrP.

Two inhibitory mAbs identified in this study, 3S9 and 2H9, inhibited conversion of PrP^{C} to PrP^{Sc} in both N2a/Chandler and N2a/22L cell lines (Fig. 4). One of these, 3S9, effectively inhibited prion propagation in N2a/22L cells expressing PrP^{Sc} at high levels. Furthermore, mAb 3S9 was able to completely exclude PrP^{Sc} from N2a/22L cells with continuous treatment. The effects of inhibitory antibodies reported previously were determined in ScN2a cells expressing low levels of PrP^{Sc}. Because the levels of PrP^{Sc} are much higher in N2a/22L cells than in ScN2a cells [20], the results obtained here show that the inhibitory activity of mAb 3S9 was high. Interestingly, mAb 2H9 markedly inhibited accumulation of PrP^{Sc} in N2a/Chandler, but did so slightly in N2a/22L cells (Fig. 2). Recent reports [21] have shown that the characteristics of prion strains were conserved in persistently infected cell lines and that the characteristics of PrP^{Sc} depend on both the host cell type and the strains used for infection. Indeed, the band patterns of PrP^{Sc} were slightly different among the cell lines (Fig. 2). Therefore, the results suggest that differences in inhibition efficiency by mAb 2H9 may be due to the expression levels or characteristics of PrP^{Sc} in the two cell lines.

A recent paper has suggested that the inhibition of prion propagation by mAbs was caused by degradation of PrP^{C} [16]. However, the fact that levels of PK-untreated PrP were not affected by treatment with mAbs (Fig. 2) indicates that inhibition of prion propagation by mAbs 3S9 and 2H9 is unrelated to degradation of PrP^{C}. Inhibition of prion propagation by mAbs 3S9 and 2H9 was probably due to inhibition of the formation of molecular complexes between PrP^{C} and PrP^{Sc}, as suggested in previous studies [8,9]. Although the amount of PrP^{Sc} in N2a/22L cells treated with mAb for 4 days decreased slightly, the amount did not increase for 4 days after treatment with mAb 2H9 (Fig. 4B). This result suggests that the conversion of PrP^{Sc} from PrP^{C} occurs very slowly.

The inhibitory mAbs, 3S9 and 2H9, identified in this study may be useful for clarifying the mechanisms by which prion propagation is inhibited by PrP-specific antibodies and may be valuable in the search for variations in PrP^{Sc}. At present, the reasons for differences in inhibition activity between the two mAbs are not clear. Further experiments to clarify these differences are underway.

Acknowledgments

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Involvement of the peripheral nervous system in human prion diseases including dural graft associated Creutzfeldt–Jakob disease

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PAPER

Involvement of the peripheral nervous system in human prion diseases including dural graft associated Creutzfeldt–Jakob disease

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Objective: To investigate abnormal prion protein (PrP) deposition in the peripheral nervous system (PNS) in human prion diseases.
Methods: Eight patients with prion diseases were examined: three with sporadic Creutzfeldt–Jakob disease (sCJD), two with dural graft associated CJD (dCJD), one with Gerstmann–Sträussler–Scheinker disease (GSS) with a PrP P102L mutation (GSS102), and two with a P105L mutation (GSS105). An atypical case of sCJD with PrP plaques in the brain presented clinically with peripheral neuropathy, and showed demyelination in 12% of the teased fibres of the sural nerve. The PNS was investigated by immunohistochemical and western blotting analyses of PrP.
Results: In immunohistochemical studies, granular PrP deposits were detected in some neurones of dorsal root ganglia and a few fibres of peripheral nerves and spinal posterior roots in one sCJD and two dCJD patients, but not in GSS102 or GSS105 patients. The atypical case of sCJD with peripheral neuropathy showed no obvious PrP deposition in the nerves. Western blotting analysis of the PNS from the dCJD patients revealed a small amount of protease K resistant PrP in the dorsal root ganglia and peripheral nerves.
Conclusions: Abnormal PrP deposition occurs in the dorsal root ganglia and peripheral nerves in sCJD and dCJD. The PrP deposits in the PNS are not correlated with clinical manifestation of peripheral neuropathy in CJD.

Human prion diseases, including Creutzfeldt–Jakob disease (CJD) and Gerstmann–Sträussler–Scheinker disease (GSS), are transmissible and genetic diseases, and involve predominantly the central nervous system (CNS), which shows spongiform changes and abnormal prion protein (PrP) deposits. Some patients with familial and sporadic CJD (sCJD) and GSS show fasciculation, areflexia, glove and stocking type sensory disturbance, and amyotrophy, implying the presence of peripheral neuropathy or motor neuronopathy.[1–7] Electrophysiological and histological studies supported these clinical signs or subclinical peripheral involvement, and indicated the presence of demyelinating or axonal neuropathy, or loss of anterior horn cells in the spinal cord in human prion diseases.[1–4 8–10] However, PrP deposition in the peripheral nervous system (PNS) has not been fully explored in previous studies.[1–4 8–10] In addition, it remains unclear whether peripheral neuropathy is correlated with abnormal PrP deposition in the PNS of patients with CJD and GSS. In the present study, we carried out immunohistochemical and western blotting analyses of PrP in the PNS in sCJD, dural graft associated CJD (dCJD), and GSS, and we discuss the mechanisms of the PNS involvement in human prion diseases.

METHODS

Patients

We studied eight patients with necropsy confirmed prion diseases: three with sCJD, two with dCJD,[11 12] one with GSS with a PrP P102L mutation (GSS102), and two with a PrP P105L mutation (GSS105) (table 1). One patient (patient 3) with sCJD was an atypical case as PrP plaques were detected in the CNS.[13] Patient 3 presented clinically with dementia, parkinsonism, and pyramidal signs, but no paroxysmal periodic discharges on EEG.[13] In addition, patient 3 showed myokymia, hyporeflexia in the lower extremities, and decreased nerve conduction velocities without conduction block, indicating the presence of peripheral neuropathy. Of the two patients with dCJD,[11 12] one (patient 5) had atypical clinicopathological features showing PrP plaque formation in the brain,[11] but had no evidence of peripheral neuropathy. Patient 6 presented classic clinicopathological manifestations of GSS102, including progressive ataxia and dementia, and also showed areflexia in the lower extremities and painful dysaesthesia.[5] The other patients had no clinical signs of peripheral neuropathy.

None of the patients with sCJD or dCJD had mutations in the PrP gene. With regard to polymorphic sites in the PrP gene, codon 129 was methionine/valine in only two cases of GSS105 (patients 7 and 8),[14] and the other patients were homozygous for methionine. All patients were homozygous for glutamic acid in codon 219. The patients with sCJD and dCJD showed a type 1 pattern on western blotting analyses of the protease K resistant PrP (PrP^{Sc}) in the brain.

Neuropathology

Spinal cords, dorsal root ganglia, spinal roots at several levels, peripheral nerves (mainly sciatic and femoral), and quadriceps femoris muscles were sampled at necropsy. Sections from the PNS were stained by routine techniques, including Klüver–Barrera and Bodian. The sural nerve of patient 3 was embedded in Epon and stained with toluidine blue; teased fibres were also analysed in the sural nerve.

Abbreviations: CJD, Creutzfeldt–Jakob disease; dCJD, dural graft associated CJD; GSS, Gerstmann–Sträussler–Scheinker disease; PNS, peripheral nervous system; PrP, prion protein; sCJD, sporadic CJD

Table 1 Summary of the patients

Patient	Age (y)/sex	Diagnosis	Clinical course	Clinical signs of peripheral neuropathy	Patterns of PrP deposits in CNS	PrP gene analysis		
						Mutation*	Codon129†	Codon219†
1	60/F	sCJD	4 m	–	Synaptic	–	M/M	E/E
2	70/M	sCJD	11 m	–	Synaptic	–	M/M	E/E
3	76/F	sCJD (atypical)	11 m	Myokymia, hyporeflexia, decrease in NCV	Synaptic>plaque	–	M/M	E/E
4	69/F	dCJD	6 m	–	Synaptic	–	M/M	E/E
5	42/M	dCJD (atypical)	13 m	–	Synaptic>plaque	–	M/M	E/E
6	38/F	GSS102	3 y	Dysaesthesia, areflexia in the legs	Plaque>synaptic	P102L	M/M	E/E
7	58/F	GSS105	8 y	–	Plaque>synaptic	P105L	M/V	E/E
8	53/M	GSS105	11 y	–	Plaque>synaptic	P105L	M/V	E/E

*L, leucine; P, proline.
†E, glutamic acid; M, methionine; V, valine.
dCJD, dural graft associated Creutzfeldt–Jakob disease; F, female; GSS, Gerstmann–Sträussler–Sheinker disease; m, months; M, male; NCV, nerve conduction velocity; PrP, prion protein; sCJD, sporadic Creutzfeldt–Jakob disease; y, years.

PrP immunohistochemistry

Immunohistochemical studies to detect PrP deposits were carried out using a mouse monoclonal antibody 3F4 (Senetek, Maryland Heights, Missouri, USA; 1:500) after hydrolytic autoclaving of formalin fixed, paraffin embedded sections 8 μm thick.[15] The same specimens as were examined morphologically were studied by PrP immunohistochemistry. 3F4 is a well characterised antibody that recognises an epitope corresponding to amino acids 109 to 112 of human PrP.[16] To confirm specificity of the immunohistochemical reaction, an antigen absorption test was carried out simultaneously using a peptide of 106–115 of human PrP. From patient 3, the femoral nerve was examined for PrP immunohistochemistry. In addition, we used another anti-PrP antibody, anti-PrP-95, which recognises residues 95 to 108 of PrP[17]; the results of PrP immunohistochemistry were shared by both 3F4 and anti-PrP-95.

Western blotting analysis

The dorsal root ganglia tissues from patients 1, 4, and 5, and the femoral nerve and quadriceps femoris muscle tissues from patients 3 and 5 were homogenised by a procedure including a collagenase digestion step to improve detection of PrP^{Sc},[18] and western blotting analysis of PrP^{Sc} was undertaken with 3F4.[19]

RESULTS

Neuropathology

No neuronal loss or histological abnormalities were observed in any PNS tissues from patients 1, 2, or 4–8, or in the femoral nerve from patient 3. The sural nerve from patient 3 showed no apparent loss of myelinated fibres, but demyelination was seen in 12% of all the teased fibres.

PrP immunohistochemistry

The results of PrP immunohistochemistry are shown in table 2.

Spinal cord

Granular PrP deposits were observed in the posterior horns of the spinal cords, particularly in the substantia gelantinosa, of all patients with sCJD and dCJD (patients 1 to 5), and of one with GSS102 (patient 6) (fig 1, panels A and B). There were no granular deposits of PrP in the spinal cords of two subjects with GSS105 (patients 7 and 8). Only patient 6 with GSS102 and patient 8 with GSS105 showed a few PrP positive plaques in the spinal posterior horns (fig 1), as reported previously.[5]

Dorsal root ganglia

Granular PrP deposits were detected inside and on the cell surfaces of some dorsal root ganglia neurones in one of two sCJD patients examined (patient 2), and in both of the dCJD cases (patients 4 and 5) (fig 2). Patient 1 with sCJD, patient 6 with GSS102, and patients 7 and 8 with GSS105 showed no PrP deposits in the dorsal root ganglia.

Spinal roots

Patients 2, 4, and 5—who had PrP deposits in dorsal root ganglia—showed PrP immunoreactivity in some of the spinal roots (fig 3A). No PrP deposits were detected in the spinal roots in the patients who showed no PrP deposits in the dorsal root ganglia.

Peripheral nerves

PrP was detected in the peripheral nerves, particularly in the axons of some nerves, from patients 4 and 5 (fig 3B). Patients 4 and 5 also showed PrP deposition in the dorsal root ganglia

Table 2 Results of immunohistochemical analysis for prion protein deposition

Patient	Age (y)/sex	Diagnosis	Spinal cord		Dorsal root ganglia	Roots	Nerves	Muscles
			Synaptic type	Plaque type				
1	60/F	SCJD	+ (PH)	–	–	–	–	–
2	70/M	SCJD	+ (PH)	–	+	+	NE	NE
3	76/F	SCJD	+ (PH)	–	NE	–	–	–
4	69/F	dCJD	+ (PH)	–	+	+	+	NE
5	42/M	dCJD	+ (PH)	–	+	+	+	–
6	38/F	GSS102	+ (PH)	+	–	–	–	NE
7	58/F	GSS105	–	–	–	–	–	NE
8	53/M	GSS105	–	+	–	–	–	–

dCJD, dural graft associated Creutzfeldt–Jakob disease; GSS, Gerstmann–Sträussler–Sheinker disease; GSS102, GSS with a P102L mutation; GSS105, GSS with a P105L mutation; NE, not examined; PH, posterior horn; sCJD, sporadic Creutzfeldt–Jakob disease.

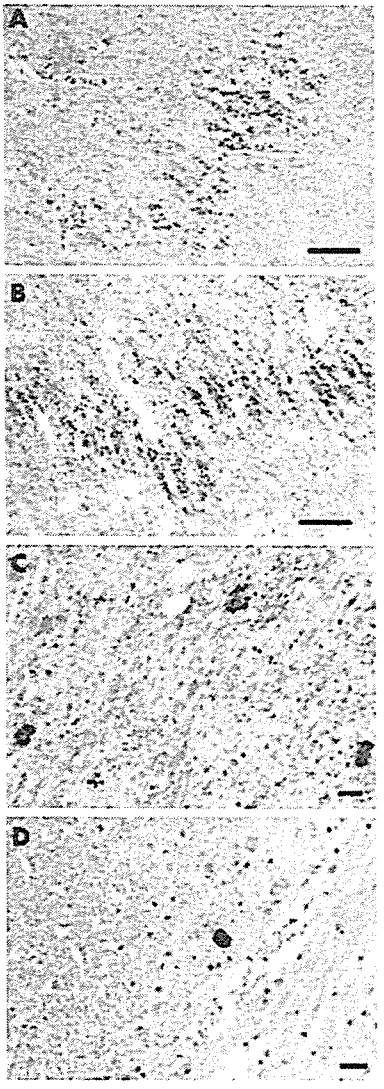

Figure 1 Immunohistochemical findings in the substantia gelantinosa in spinal posterior horns using an anti-prion protein (PrP) monoclonal antibody, 3F4. Granular PrP deposits were detected in patient 2 with sporadic Creutzfeldt–Jakob disease (CJD) (panel A), and patient 4 with dural graft associated CJD (panel B). (C) Patient 6 with Gerstmann–Sträussler–Scheinker disease (GSS) having a P102L mutation showed both granular PrP deposits and a few PrP plaques. (D) Patient 8 with GSS having a P105L mutation showed only plaque type PrP deposits. Scale bars in panels A and B, 100 μm. Scale bars in panels C and D, 20 μm.

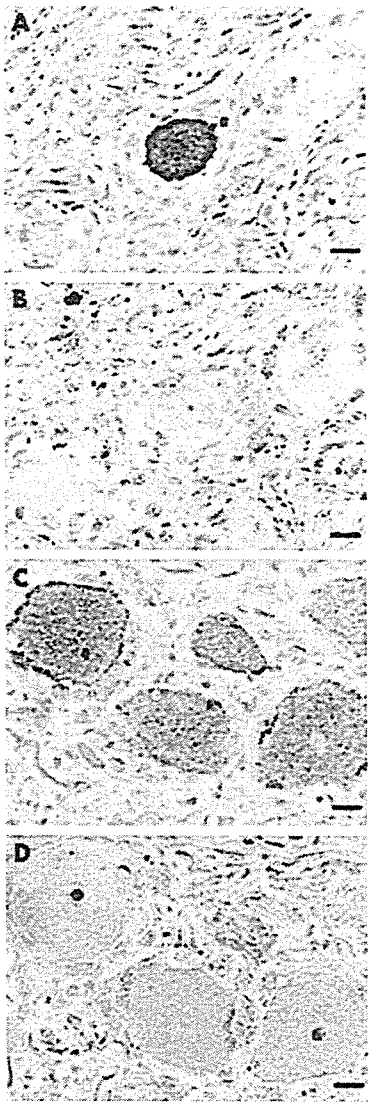

Figure 2 Immunohistochemical findings of dorsal root ganglia using an anti-prion protein (PrP) monoclonal antibody, 3F4, in patient 2 with sporadic Creutzfeldt–Jakob disease (CJD) (panels A and B), and in patient 5 with dural graft associated CJD (panels C and D). PrP deposits were detected in some dorsal root ganglia cells from patient 2 (A) and from patient 5 (C). PrP immunoreactivity was abolished by pre-absorption of the antibody with a peptide corresponding to residues 106 to 115 of human PrP (B and D). Scale bars, 100 μm.

and the spinal roots. Patients 3 and 6 with clinical signs of peripheral neuropathy showed no PrP deposits in the peripheral nerves.

Muscles

None of the muscles examined from patients 1, 3, 5, and 8 showed PrP immunoreactivity.

Western blotting analysis

PrP^{Sc} with a type 1 pattern was detected on western blotting analysis of the dorsal root ganglia, and was seen at a very low level in the peripheral nerve from patient 5 with dCJD, but not in the others (fig 4). The femoral nerve and quadriceps femoris muscle from patient 3 showed no PrP^{Sc} positive bands (data not shown).

DISCUSSION

Our immunohistochemical studies indicated that PrP deposition occurred in the dorsal root ganglia and peripheral nerves in sCJD and dCJD. Further, western blotting analysis revealed PrP^{Sc} in the dorsal root ganglia and the peripheral nerves in dCJD.

Extensive accumulation of PrP^{Sc} in the dorsal root ganglia and autonomic ganglia has been reported in variant CJD (vCJD).[20–22] In non-iatrogenic CJD, some immunohistochemical studies have revealed PrP deposits in the PNS.[21 23 24] A patient with possibly familial CJD showed PrP immunoreactivity in the posterior root nerve fibres in an adaxonal location, although western blotting analysis was not undertaken in this case.[23] In a valine homozygous type-1 (VV1) case of sporadic CJD, linear PrP deposits were detected in the

posterior roots and along nerve fibres of both myelinated and unmyelinated fibres in the sciatic and peroneal nerves.[24] However, western blotting analysis showed the absence of PrP^{Sc} in this VV1 case of sCJD.[24] With regard to sCJD patients except for VV1 cases, three of six patients showed PrP deposits in the dorsal root ganglia, while no PrP deposition has been found in the peripheral nerves of any patients.[21] Our results support those of previous reports indicating the presence of PrP deposition in the dorsal root ganglia and peripheral nerves in sCJD.[21 24] However, patient 2 in the present study is the first case of methionine homozygous type-1 (MM1) sCJD showing PrP deposits in the PNS, because the previous report did not refer to the PrP phenotypes of the sCJD patients.[21] There was no significant correlation between the duration of the clinical course and the presence of PrP deposits.

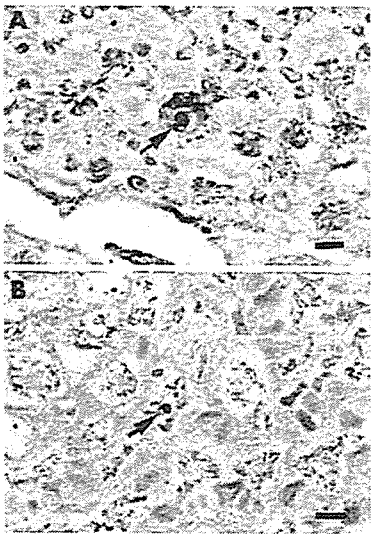

Figure 3 Immunohistochemical findings in a posterior root from patient 2 with sporadic Creutzfeldt–Jakob disease (CJD) (A) and a peripheral nerve from patient 5 (B) with dural graft associated CJD using an anti-prion protein (PrP) monoclonal antibody, 3F4. Some nerve fibres, particularly in the axons surrounded by myelin sheaths, showed PrP immunoreactivity (arrows). Scale bars, 1 μm.

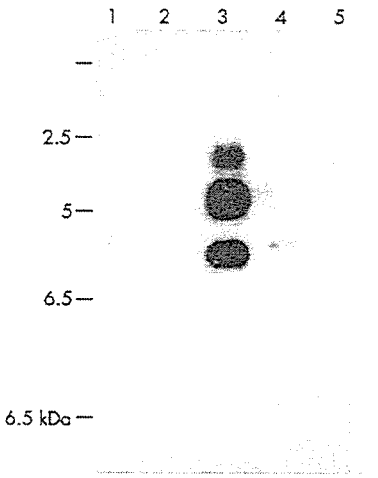

Figure 4 Western blotting analysis using 3F4 of protease K resistant fractions from dorsal root ganglia (DRG) of patient 1 (lane 1), DRG of patient 4 (lane 2), and DRG (lane 3), peripheral nerve (lane 4), and skeletal muscle (lane 5) of patient 5. No bands were detected in the DRG of patient 1 with sporadic Creutzfeldt–Jakob disease (CJD), or patient 4 with dural graft associated CJD (dCJD) (lanes 1 and 2). In patient 5 with dCJD, the DRG tissue showed significant immunoreactivity with 3F4 (lane 3), and the peripheral nerve showed faint immunoreactivity (lane 4). The skeletal muscle from patient 5 showed no immunoreactivity (lane 5).

Our results indicated a novel finding regarding the presence of PrP^{Sc} in the dorsal root ganglia and peripheral nerves in dCJD. For iatrogenic CJD, PrP deposits have been reported only in the spinal cords of growth hormone related cases and of a patient with dCJD, but not in the PNS.[25 26] Another earlier study showed no PrP^{Sc} deposits in the dorsal root ganglia or the peripheral nerves of a patient with growth hormone related CJD.[21]

With regard to GSS (GSS102 and GSS105), no PrP deposits were found in the dorsal root ganglia or nerves in the present study, although the posterior horns of the spinal cord were affected by PrP.[5 14] Our results suggest that there are differences in PrP accumulation in the PNS between CJD and GSS. In skeletal muscles, we found no PrP immunoreactivity or PrP^{Sc} accumulation by western blotting analysis. These results differ from those of a recent report, which showed the presence of PrP^{Sc} in skeletal muscle from eight of 32 patients with sCJD.[27] However, our procedure of PrP^{Sc} analysis with collagenase digestion of samples[18] was different from the one in this previous study which used precipitation with phosphotungstic acid,[27] and our negative results were limited to only four patients—two with sCJD, one with dCJD, and one with GSS105.

PrP deposition is observed in the PNS in natural[28 29] and experimental scrapie,[30–32] bovine spongiform encephalitis,[33] and variant CJD (vCJD).[20–22] These previous studies suggest that the PNS can be a neural pathway to the CNS following oral exposure to a prion agent. The strong PrP immunopositivity (consisting of PrP^{Sc}) of the autonomic and dorsal root ganglia in vCJD may also support a peripheral pathogenesis.[20–22] However, our results showed PrP^{Sc} deposits in the PNS in dCJD. dCJD might be caused by direct attachment of contaminated cadaveric dura mater to the brain surface, because some patients with dCJD are associated with initial clinical manifestations[12] or abnormalities on magnetic resonance imaging in the dura mater grafted sites (Satoh T, personal communication). These findings suggest that PrP deposits in the PNS do not necessarily provide evidence of peripheral pathogenesis in human prion diseases.

Our results also imply that the PrP deposits in the PNS are not necessarily correlated with clinical presentations of peripheral neuropathy in CJD. In the present study, two patients—patient 3 with sCJD and patient 6 with GSS102—presented with clinical manifestations suggesting involvement of the PNS. Patient 6 had dysaesthesia and areflexia in the lower extremities, which could have reflected involvement of the posterior horn with PrP deposits. Patient 3 showed myokymia, hyporeflexia in the lower extremities, decreased conduction velocities in nerve conduction studies, and some demyelination in the teased fibres. Nerve conduction studies in some CJD patients showed decreases in conduction velocity,[1–3 6 8] multifocal conduction block,[1] low occurrence of F waves,[7] abnormalities of sensory evoked potentials,[7] diminished compound muscle action potential and sensory nerve action potential, and prolonged distal latency.[2] Fibrillation, fasciculation, and polyphasic motor unit potentials have also been reported in electromyograms in patients with CJD.[1 8] The results of histological studies have supported these electrophysiological abnormalities in only a few patients.[1 3 8] In addition, no PrP immunoreactivity was detected in peripheral nerves from CJD patients with histologically confirmed demyelination, which was

consistent with the findings in patient 3 in the present study.[1 3 9] It is unlikely that peripheral neuropathy reported in some CJD patients[1–10] is an incidental finding unrelated to CJD. We speculate that peripheral neuropathy in CJD may not be caused directly by abnormal PrP accumulation in the PNS. Transgenic mice overexpressing wild-type PrP showed polyneuropathy, although PrP deposits were not examined in the PNS.[34] It is possible that abnormal metabolism of PrP in the PNS without apparent accumulation of PrP^{Sc} may be responsible for peripheral neuropathy in CJD, although the precise mechanism remains to be determined. Further clinical and experimental studies are necessary to elucidate the pathomechanism underlying peripheral neuropathy in CJD.

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