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Biochemical and Biophysical Research Communications 335 (2005) 197–204
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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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Received 12 July 2005
Available online 25 July 2005

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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doi:10.1016/j.bbrc.2005.07.063

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

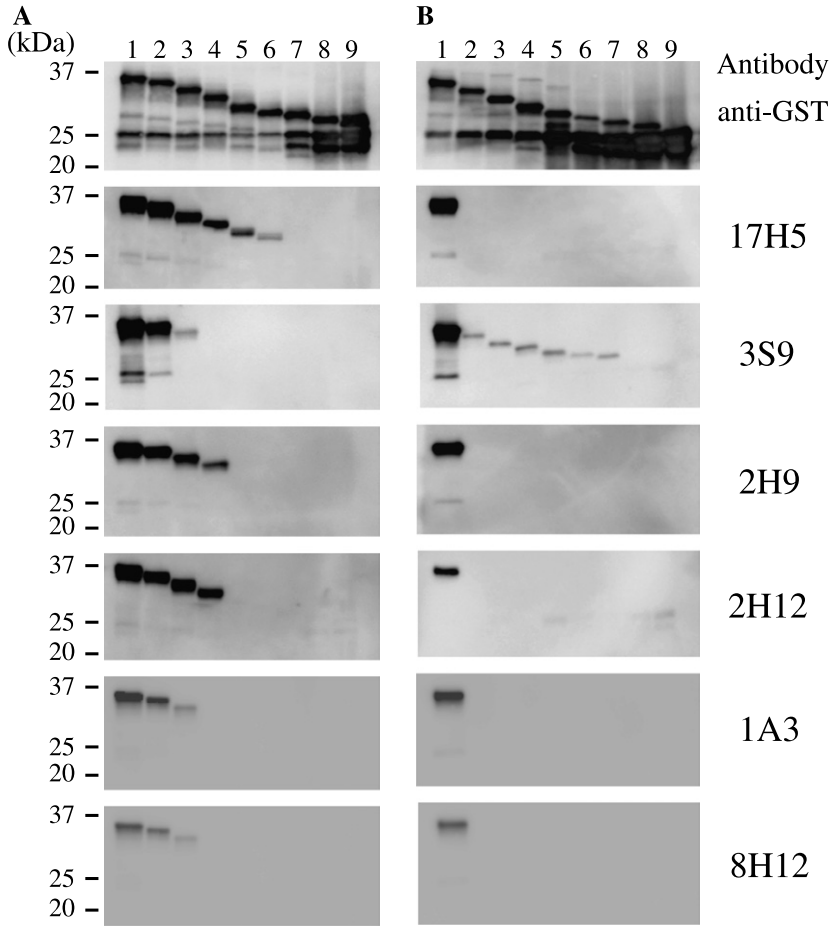

Fig. 1. Epitope mapping of PrP recognized by PrP-specific antibodies. (A) Western blotting profiles using N-terminal deletion mutants. Lanes 1–9 show amino acid residues of PrP, lane 1; 121–231, 2; 131–231, 3; 141–231, 4; 151–231, 5; 161–231, 6; 171–231, 7; 181–231, 8; 191–231, and 9; 201–231. (B) Western 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

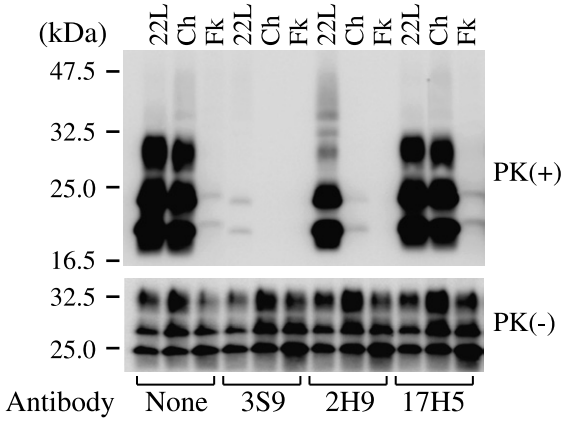

Fig. 2. Inhibition of prion propagation in scrapie-infected cell lines N2a/22L (22L), N2a/Chandler (Ch), and N2a/Fukuoka (Fk) by PrP-specific antibodies. PrP treated with or without PK was detected by Western blotting using HUC2-13 specific for the N-terminal of PrP and HUNN1 specific for the PK cleavage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

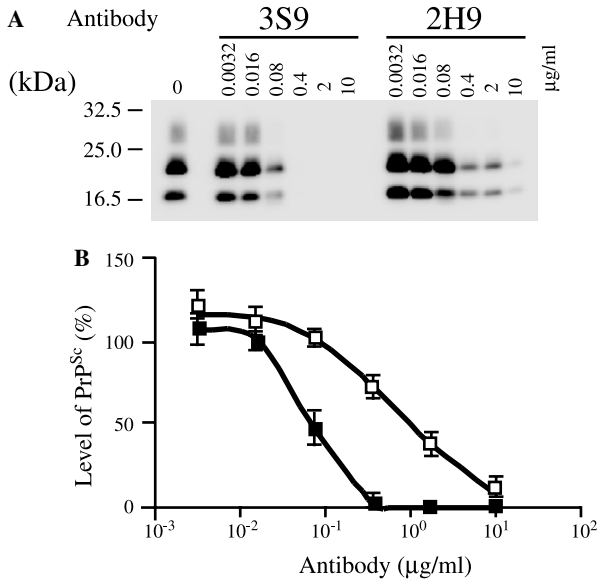

Fig. 3. Dose-dependency of mAbs 3S9 and 2H9 on the inhibition of PrP^{Sc} accumulation in N2a/Chandler cells. (A) Western blotting profiles of PrP^{Sc} from N2a/Chandler cells using mAbs 3S9 and 2H9. N2a/Chandler cells were cultured for 4 days with various concentrations of mAb. Cells were lysed and 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].

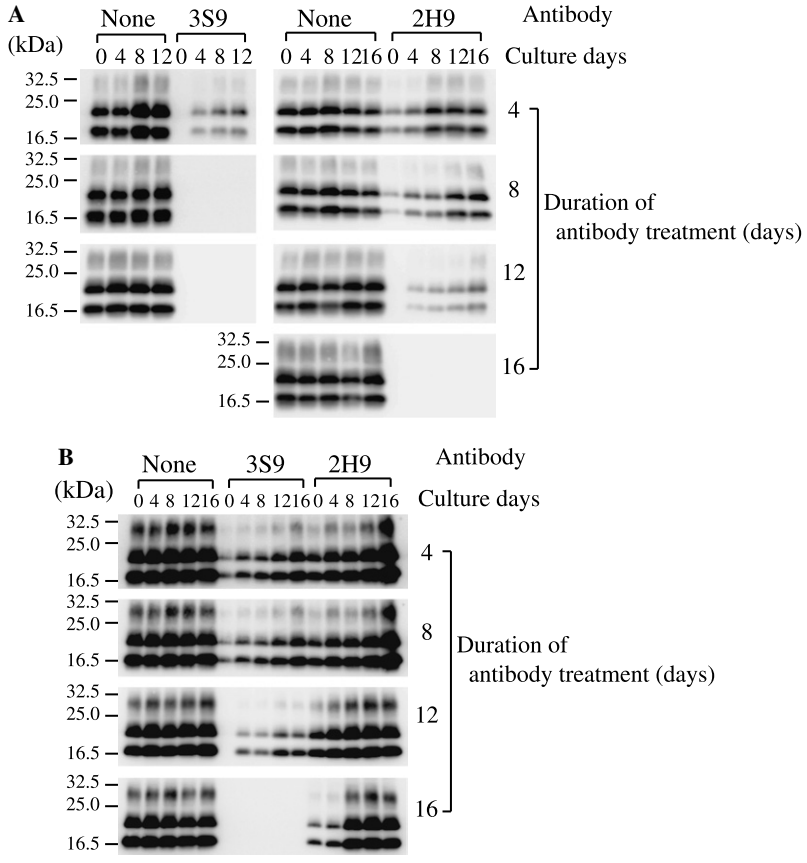

Fig. 4. Disappearance of PrP^{Sc} from prion-infected cells treated with mAbs 3S9 and 2H9. (A) N2a/Chandler cells were cultured for 4, 8, and 12 (and 16) days with or without 10 μg/ml of 3S9 or 2H9. (B) N2a/22L cells were cultured for 4, 8, 12, and 16 days with or without 10 μg/ml of 3S9 or 2H9. Cells 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.

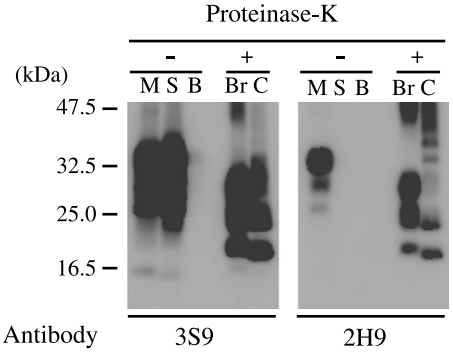

Fig. 5. Western blotting profiles of mammalian PrP^{C} and PK-treated PrP^{Sc} using inhibitory mAbs identified in this study. Mammalian PrP^{C} are brain homogenates from normal mouse (M), sheep (S), and cow (B), respectively. PK-treated PrP^{Sc} are from scrapie infected-mouse brain (Br) and N2a/22L 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).

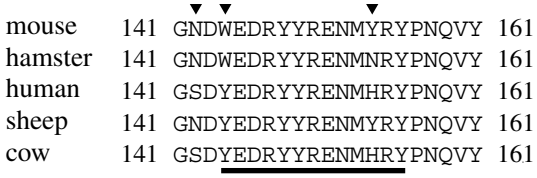

Fig. 6. Alignment of amino acids of the PrP epitope recognized by mAb 3S9. Residue numbers are indicated on the left and right. The helix 1 region of PrP is underlined, and the arrowheads indicate non-conserved amino acids in this region.

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

We thank Dr. Kitamoto (Tohoku University, Japan) for his gift of recombinant human PrP and Dr. Horiuchi (Hokkaido University, Japan) for his gift of recombinant hamster PrP. This work was partly supported by a Grant-in-Aid for the Ministry of Agriculture, Forestry and Fisheries, Japan.

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