inantly established from mice immunized with PrP^{Sc} (9 of 15). Therefore, the differences in the immunodominant regions and predominant immunoglobulin subtypes suggest that the two PrP preparations elicited different type of immune responses, although the two PrPs share primary structure and we used the same immunization procedure. Although PrP^{Sc}-specific antibodies are thought to be an attractive tool for analyzing properties of PrP^{Sc} as well as establishing new diagnostic methods, only one has been previously reported (Korth et al., 1997). Thus, the unique immune response against the PrP^{Sc} fraction suggests that the use of an infectivity-associated PrP^{Sc} fraction as an immunogen may help to generate PrP^{Sc}-specific antibodies. In addition, it is still possible that certain regions are located on the surface of PrP^{Sc} as either a linear epitope or as a PrP^{Sc}-specific discontinuous epitope. Actually, Paramithiotis et al. very recently reported that three amino acid residues, YYR, possibly located in the second β-strand, is not antibody accessible on PrP^{C}, although the region is exposed on the surface of PrP^{Sc} (Paramithiotis et al., 2003). Further generation of mAbs, especially those specific to PrP^{Sc}, will be required for determining the surface structure of PrP^{Sc}.

BSE and vCJD are now global concerns. Because therapeutics for prion diseases are not currently available, elimination of prion-contaminated foodstuff and biomedical materials is essential for preventing further spread of the disease. We have found that some of our mAbs possessed higher sensitivity for detecting bovine PrP^{Sc} than some commercial-based anti-PrP mAbs, including 6H4 (data not shown). Further generation of anti-PrP antibodies with higher affinity and avidity will contribute to enhance the sensitivity of PrP^{Sc} detection methods.

Materials and methods

Plasmid construction

The prokaryotic expression vectors pET22b(+) (Novagen) and pRSETB (Invitrogen) were used in these studies. For the construction of expression plasmids based on pET22b(+), cDNA encoding mouse (Mo) PrP codons 23–231 was amplified by PCR with primers MPrP2 and MPrP3, and genomic DNA encoding sheep (Sh) PrP codons 25–234 was amplified with primers SPrP102 and SPrP103. Amplified fragments were cloned into the *Eco*RV site of pBluescript KS(+) (Stratagene) to confirm nucleotide sequences. The cloned fragments were excised by *Msc*I and *Eco*RI digestion and ligated into the corresponding sites of pET22b(+).

For the construction of expression plasmids based on pRSETB, cDNA encoding MoPrP codons 23–231 was amplified with primers MPrP5 and MPrP3. To express deletion mutants of MoPrP aa 23–167, 23–214, 89–231, and 155–231, we used primer sets of MPrP5 and MPrP9, MPrP5 and MPrP11, MPrP10 and MPrP3, and MPrP12 and MPrP3, respectively, were used for PCR. Hamster (Ha) PrP cDNA encoding codons 23–231, ShPrP gene encoding codons 25–234, and bovine (Bo) PrP cDNA encoding codons 25–242 were amplified with primer sets of MPrP5 and MPrP9, SPrP101 and SPrP102, and BPrP101 and BPrP103, respectively. Amplified fragments were digested with *Bam*HI and *Eco*RI and cloned into the *Bam*HI and *Eco*RI sites of pRSETB. Nucleotide sequences of the cloned PrP gene fragments were confirmed before their expression. To generate the mutant HaPrP containing a single amino acid substitution at codon 179 (Cys to Ala) or 214 (Cys to Ala), we used the ExSite PCR-based site-directed mutagenesis kit (Stratagene) according to the supplier's instructions. Primer sets of HPrP1 and HPrP2, and HPrP3 and HPrP4 were used to introduce the nucleic acid substitution encoding codons 179 and 214, respectively. Primer sequences Primer sequences were as follows: MPrP2, 5′-AATGGCCA AAAAGCGGCCAAAGCCTGGA-3′; MPrP3, 5′-GAGAATTCAGCTGGATCTTCTCCCGTCGT-3′; MPrP5, 5′-AAGGATCC GAAAAAGCGGCCAAAGCCTGG-3′; MPrP9: 5′-GAGAATTC TACTGATCCACTGGCCTG-GTAG-3′; MPrP10, 5′-AAGGATCC GGGCCAAG-GAGGGGGTACCCATAATC-3′; MPrP11, 5′-GAGAAT TC AGACGCACATCTGCTCCACCAC-3′; MPrP12, 5′-AAGGATCC GCGCTACCCTAACCAAGTGTACT-3′; SPrP101, 5′-AAGGATCC GAAGAAGCGACCAA-AACCTGGCGG-3′; SPrP102, 5′-TTGAAT TC AACTTGCCCCCCTTTGGTAATAAG-3′; SPrP103, 5′-AATGGCCA AGAAGCGACCAAAACCTGGCGG-3′; BPrP101, 5′-AAGGATCC GAAGAAGCGAC-CAAAACCTGGAGG-3′; BPrP103, 5′-TTGAAT TCA ACTTGCCCCTCGTTGGTAATAAG-3′; HPrP1, 5′-CAC-GAT*GC*TGTCAACATCACCATCAAG-3′; HPrP2, 5′-CACAAAGTTGTTCTGGTTGTTGTACTG-3′; HPrP3, 5′-AGATG*GC*TACCACCCAGTATCAGAAGG-3′; HPrP4, 5′-GCTCCACCACGCGCTCCATTATCTTG-3′(underlines indicate restriction sites used for cloning, bold indicates stop codons, and italics indicate nucleotide substitutions for the mutation of Cys to Ala).

Expression and purification of recombinant PrP (rPrP)

The expression plasmids based on pRSETB and pET22b(+) were introduced into *E. coli* BL21(DE3)*LysS* and JM109(DE3), respectively. Protein expression was induced by adding isopropylthio-β-D-galactoside to a final concentration at 0.4 mM. Two to four hours after induction, bacterial cells were collected and inclusion bodies were prepared as described elsewhere (Sambrook et al., 1989). The inclusion bodies from BL21(DE3)*LysS* transformed with pRSETB-based expression plasmids were solubilized with 6 M GdnHCl in 20 mM phosphate buffer (pH 7.8). The rPrP was further purified by Ni^{2+}-immobilized metal affinity chromatography (IMAC) using Ni^{2+}-charged Chelating Sepharose Fast flow (Amersham Pharmacia) and a

stepwise elution gradient from pH 4.9 to 4.3 in the presence of 6 M GdnHCl. Inclusion bodies from JM109(DE3) transformed with pET22b(+)-based expression plasmids were solubilized with 8 M Urea and 20 mM Tris–HCl, pH 8.0. Next, the urea concentration was reduced to 6 M, and the mixture was applied to DEAE-Sepharose equilibrated with 6 M Urea and 20 mM Tris–HCl, pH 8.0. The unbound fraction was saved for further purification. The rPrP in the eluate from IMAC and the unbound fraction from DEAE-Sepharose were dialyzed against 10 mM acetate buffer (ranging from pH 4.4 to 3.6). After the dialysis, rPrP containing an intramolecular disulfide bond was purified by reverse-phase HPLC using TSKgel Phenyl-5PW RP (TOSOH) and a 30–50% linear gradient of acetonitrile with 0.05% trifluoroacetic acid. The purified rPrP was lyophilized and dissolved with Mili-Q water at 1 mg/ml and stored at −20 °C.

Purification of PrP^{Sc} and formation of detergent-lipid-protein complexes (DLPC)

A mouse-adapted scrapie Obihiro strain (Shinagawa et al., 1985) was used in this study. ICR/Slc female mice were inoculated intracerebrally with 20 μl of brain homogenate of Obihiro strain infected-mice and were sacrificed under anesthesia when they showed the clinical symptoms of the terminal stage of the disease. PrP^{Sc} was purified from the scrapie-affected mice brains without proteinase K treatment as described by Bolton et al. (1987) with minor modifications (Caughey et al., 1991). Protein concentration was determined by DC protein assay kit (Bio-Rad).

Ten micrograms of purified PrP^{Sc} was suspended in 1.6 ml of DLPC buffer containing 2% Sarkosyl, 0.4% phosphatidylcholine, 150 mM NaCl, and 50 mM Tris–HCl, pH 8.3. The suspension was sonicated for five cycles of 2 s with a Branson Sonifier Contamination-free Ultrasonic Sample Pre-processing System.

Production of monoclonal antibodies

Purified PrP^{Sc}, rMoPrP23–231, or rMoPrP89–231 was mixed with an equal volume of Freund's complete adjuvant and 200 μg of each PrP was inoculated subcutaneously into PrP gene-ablated mice (Yokoyama et al., 2001). After the first immunization, the mice received 100 μg of the same PrP preparation with Freund's incomplete adjuvant twice every 2 weeks. The booster shot was given intraperitoneally with 50 μg of each PrP preparation in PBS. Three days after the booster, splenocytes obtained from immunized mice were fused with P3U1 mouse myeloma cells using polyethylene glycol 1500 (Roche Diagnostic) according to the supplier's instruction, and hybridomas were selected in HAT medium. Hybridoma culture supernatants were screened by ELISA using purified PrP^{Sc} and rMoPrP as described below. The hybridomas secreting mAbs were cloned by limiting dilution. The isotypes of the mAbs were determined using the IsoStrip mouse monoclonal antibody isotyping kit (Roche Diagnostic). Large-scale preparations of mAbs were carried out in INTEGRA CELLine high density culture units (IBS Integra Biosciences). The supernatants harvested from the units were concentrated by precipitation with 50% saturated ammonium sulfate and then purified by size exclusion chromatography with Superdex-200 HR (Amersham Pharmacia Biotech).

ELISA

Ninety-six well plates (MaxiSorp, Nunc) were coated overnight at 4 °C with either 200 ng/well of purified PrP^{Sc} or 100 ng/well of rMoPrP in 50 μl of 20 mM phosphate buffer, pH 7.0. After adsorption, wells were blocked with 5% fetal bovine serum (FBS) in PBS containing 0.1% Tween 20 (PBST) for 2 h at room temperature (r.t.), and then incubated with culture supernatants or antibodies diluted with 1% FBS in PBST for 1 h. After washing with PBST, wells were incubated with 100 μl of 1:2500 diluted HRP-conjugated $F(ab')_2$ fragment anti-mouse Ig (Amersham Bioscience) for 1 h. Finally, antigen–antibody complexes were detected by adding a substrate solution of 100 μg/ml of 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid), 0.04% H_2O_2 in 50 mM citrate-phosphate buffer, pH 4.0, and the absorbance at 405 nm was measured with a microplate reader (Multiscan MS-UV, Labsystems). A ready to use 3,3′,5,5′-tetramethylbenzidine (TMB) was also used as a substrate, and the absorbance at 450 nm was measured for TMB.

Immunoblotting

The preparation of PrP^{Sc} and immunoblotting were carried out as described elsewhere (Grathwohl et al., 1997). The blots were developed with ECL Western blotting detection reagents (Amersham Pharmacia) and immunoreactive proteins were detected with X-ray film.

Pepspots analysis

In these studies, we used pepspots membrane to which an array of 99 overlapping synthetic peptides, corresponding to residues 23–231 of mouse PrP, was covalently attached to a cellulose support via carboxyl termini. Each peptide is 13 amino acid residues long, and there is a two amino acid shift along the mouse PrP amino acid sequence from one peptide to the next. The membrane was blocked with 5% skim milk and 5% sucrose in PBST, and then incubated with culture supernatants of hybridomas as primary antibodies. Bound antibodies were detected using a 1:2500 diluted HRP-conjugated $F(ab')_2$ fragment anti-mouse Ig and an ECL Western blotting detection reagent.

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Cell-surface retention of PrP^C by anti-PrP antibody prevents protease-resistant PrP formation

Chan-Lan Kim,[1] Ayako Karino,[1] Naotaka Ishiguro,[1] Morikazu Shinagawa,[1]† Motoyoshi Sato[2] and Motohiro Horiuchi[1]‡

Correspondence
Motohiro Horiuchi
horiuchi@vetmed.hokudai.ac.jp

Laboratory of Veterinary Public Health[1] and Laboratory of Veterinary Radiology[2], Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

The C-terminal portion of the prion protein (PrP), corresponding to a protease-resistant core fragment of the abnormal isoform of the prion protein (PrP^{Sc}), is essential for prion propagation. Antibodies to the C-terminal portion of PrP are known to inhibit PrP^{Sc} accumulation in cells persistently infected with prions. Here it was shown that, in addition to monoclonal antibodies (mAbs) to the C-terminal portion of PrP, a mAb recognizing the octapeptide repeat region in the N-terminal part of PrP that is dispensable for PrP^{Sc} formation reduced PrP^{Sc} accumulation in cells persistently infected with prions. The 50% effective dose was as low as ~1 nM, and, regardless of their epitope specificity, the inhibitory mAbs shared the ability to bind cellular prion protein (PrP^C) expressed on the cell surface. Flow cytometric analysis revealed that mAbs that bound to the cell surface during cell culture were not internalized even after their withdrawal from the growth medium. Retention of the mAb–PrP^C complex on the cell surface was also confirmed by the fact that internalization was enhanced by treatment of cells with dextran sulfate. These results suggested that anti-PrP mAb antagonizes PrP^{Sc} formation by interfering with the regular PrP^C degradation pathway.

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INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), also called prion diseases, are fatal neurodegenerative diseases and include scrapie in sheep and goats, bovine spongiform encephalopathy and Creutzfeldt–Jakob disease (CJD) in humans. The causative agent of TSEs, often called a prion, is composed mainly of an abnormal isoform (PrP^{Sc}) of the host cellular prion protein (PrP^C). Mice with genetic knockout of the PrP gene are resistant to prion disease (Bueler *et al.*, 1993) and neurons lacking PrP^C expression are resistant to degeneration, regardless of the presence of PrP^{Sc} (Mallucci *et al.*, 2003). Thus, PrP^C is essential for prion propagation and pathogenesis.

Conversion of PrP^C to PrP^{Sc} is believed to involve direct interaction of the two PrP isoforms. Although the molecular mechanism of conversion is not yet fully understood, it is known that mature PrP^C expressed on the cell surface is a substrate for PrP^{Sc} formation, and a process that involves a conformational transformation takes place in subcellular compartments associated with the degradation pathway of PrP^C, including a sphingolipid-rich membrane microdomain, called a lipid raft (Caughey & Raymond, 1991; Naslavsky *et al.*, 1997; Vey *et al.*, 1996).

Because of the emergence of variant CJD and iatrogenic CJD by dura matter transplantation, especially in Japan, the establishment of therapeutics for prion disease is urgently needed. Therapeutics have been directed at the binding of the two PrP isoforms, as well as the process of conformational transformation, since the conversion of PrP^C to PrP^{Sc} is associated with neuronal pathogenicity. To date, many substances have been reported to inhibit PrP^{Sc} formation in cell culture and/or cell-free systems, including amyloid-binding dyes (Caughey & Race, 1992), sulfated glycosaminoglycans (Caughey & Raymond, 1993), tetrapyrrole compounds (Caughey *et al.*, 1998), cysteine protease inhibitors (Doh-Ura *et al.*, 2000), substituted tricyclic derivatives such as chlorpromazine and quinacrine (Doh-Ura *et al.*, 2000; Korth *et al.*, 2001), branched polyamines (Supattapone *et al.*, 1999, 2001), peptides (Chabry *et al.*, 1998; Soto *et al.*, 2000) and conversion-incompetent PrP (Holscher *et al.*, 1998; Horiuchi *et al.*, 2000; Kaneko *et al.*, 1997). Some of these have already been examined *in vivo*. For instance, sulfated glycosaminoglycans and tetrapyrrole compounds were effective when administered at early stages of infection or simultaneously with the scrapie-affected brain inoculum (Ehlers & Diringer, 1984; Ladogana

†Present address: Prion Disease Research Center, National Institute of Animal Health, Kannondai, Tsukuba, Ibaragi, 305-0856, Japan.

‡Present address: Laboratory of Prion Diseases, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan.

et al., 1992; Priola *et al.*, 2000). Polyene antibiotics prolonged the incubation period, even when administered at the middle-late stage of infection (Demaimay *et al.*, 1997), but the effects appeared to depend on the prion strains and host animals studied (Demaimay *et al.*, 1999; Xi *et al.*, 1992). Recently, Doh-Ura and colleagues (2004) showed that intraventricular administration of pentosan polysulfate and quinine prolonged the incubation periods in a prion-infected transgenic mouse model, even at a late stage of infection (Doh-Ura *et al.*, 2004; Murakami-Kubo *et al.*, 2004). Further *in vivo* studies are expected to lead to the establishment of effective therapeutics for prion diseases. However, to achieve more efficient therapeutics, it is essential to elucidate the mechanisms of action and to investigate proper delivery of drugs based on pharmacokinetics.

Anti-PrP antibodies have also been reported to inhibit the formation of PrP^{Sc} in cultured cells and/or cell-free systems (Enari *et al.*, 2001; Horiuchi & Caughey, 1999; Kaneko *et al.*, 1995; Peretz *et al.*, 2001). Transgenic mice expressing an anti-PrP mAb on B cells (Heppner *et al.*, 2001), immunization with recombinant PrP (Sigurdsson *et al.*, 2002) and passive immunization with an anti-PrP mAb (White *et al.*, 2003) antagonized the peripheral inoculation of scrapie-affected brain inoculum. These *in vivo* experiments suggested the possible use of anti-PrP antibodies as a therapy for prion diseases. However, it remains unclear how anti-PrP antibodies can antagonize PrP^{Sc} formation in cells. To address this point, in the current study, we evaluated a panel of anti-PrP mAbs against diverse epitopes for inhibition of PrP^{Sc} formation. We found that a mAb recognizing the octapeptide repeat sequence, a region that is not essential for PrP^{Sc} formation, reduced PrP^{Sc} accumulation in cells persistently infected with prions. Furthermore, our data suggest a possible link between cell-surface retention of PrP^{C} by anti-PrP antibodies and inhibition of PrP^{Sc} formation in cells.

METHODS

Antibodies and chemicals. The properties of anti-PrP mAbs used in this study have been described elsewhere (Kim *et al.*, 2004). The mAb against sarcomeric actin (clone alpha-Sr-1) was purchased from DAKO. Stock solutions of chlorpromazine, dextran sulfate 500 (DS500) and polyethyleneimine were prepared in deionized water, while E-64d was dissolved in DMSO and quinacrine in methanol. Culture medium containing each chemical compound or mAb was prepared freshly for each experiment.

Cell culture. The mouse neuroblastoma cell line Neuro2a (CCL-131; ATCC) was cultured in Dulbecco's modified Eagle's medium (ICN Biomedicals) with 10% fetal bovine serum (FBS) and non-essential amino acids. Mouse neuroblastoma cells persistently infected with prions, originally established by Race *et al.* (1987), were cloned by limiting dilution. Subclone I3/I5-9, which possessed a high level of PrP^{Sc}, was used in this study. I3/I5-9 cells were maintained in Opti-MEM (Invitrogen) containing 10% FBS and cells passaged fewer than 20 times were used for experiments.

Treatment of cells persistently infected with prions and sample preparation. Almost-confluent I3/I5-9 cells in 25 cm^2 flasks were split 1:20 into 35 mm tissue culture dishes. On day 2, the medium was replaced with 3 ml Opti-MEM containing 4% FBS and each test compound or mAb, and the cells were cultured for a further 3 days. For PrP^{C} detection, the cells were washed with PBS and lysed with 300 µl lysis buffer A (1% Zwittergent 3-14, 150 mM NaCl, 50 mM Tris/HCl, pH 7·5) supplemented with protease inhibitors (2 mM EDTA, 1 µg pepstatin ml^{-1}, 2 µg leupeptin ml^{-1}, 2 µM bestatin and 1 µg aprotinin ml^{-1}). After the removal of cell debris by low-speed centrifugation, samples were centrifuged at 45 000 r.p.m. for 30 min at 4 °C using the TLA 100.3 rotor of a Beckman Optima TLX and the resulting supernatants were used as a source of PrP^{C}. For the detection of PrP^{Sc}, cells were lysed with 300 µl lysis buffer B (5 mM EDTA, 0·5% Triton X-100, 0·5% sodium deoxycholate, 150 mM NaCl, 10 mM Tris/HCl, pH 7·5) and kept on ice for 30 min. Cell debris was removed by centrifugation for 5 min at 1000 r.p.m. A portion of the sample (10%) was removed for determination of protein concentration using the DC protein assay (Bio-Rad) and the remaining portions were treated with 20 µg proteinase K ml^{-1} for 20 min at 37 °C. Proteolysis was terminated by the addition of 1 mM Pefabloc (Roche). The samples were then treated with DNase I (100 µg ml^{-1}) and RNase A (5 µg ml^{-1}) for 15 min at room temperature and centrifuged at 70 000 r.p.m. for 2 h at 4 °C using the TLA 100.3 rotor of a Beckman Optima TLX. The resulting pellets were dissolved in SDS-PAGE sample buffer.

SDS-PAGE and immunoblotting. SDS-PAGE was carried out using NuPAGE 12% Bis-tris gels and MOPS-SDS running buffer according to the manufacturer's instructions (Invitrogen). After SDS-PAGE, proteins were transferred on to Immobilon-P PVDF membranes (Millipore) using a Transblot Mini Cell wet-type blotting apparatus (Bio-Rad) and NuPAGE transfer buffer (Invitrogen) at 60 V for 2 h. Immunoreactive proteins were detected using X-ray film as described elsewhere (Kim *et al.*, 2004). For quantitative analysis, immunoreactive proteins were visualized using the Western-Star Protein detection kit (TROPIX) according to the supplier's instructions and processed with an LAS-1000 lumino image analyser (Fujifilm). The intensity of the bands was quantified using Science Lab 98 Image Gauge software (Fujifilm).

Flow cytometric analysis. Adherent cells were treated with ice-cold PBS containing 0·1% collagenase (Wako) and dispersed by pipetting. Cells were washed with 0·5% FBS in PBS (FBS/PBS) and incubated with anti-PrP mAbs diluted with 0·5% FBS/PBS for 30 min on ice. Cells were washed three times with 0·5% FBS/PBS and incubated with 1:2000-diluted Alexa 488-labelled Fab fragment of goat anti-mouse IgG (Molecular Probes) for 30 min. After washing, cells were stained with 5 µg propidium iodide ml^{-1} in 0·5% FBS/PBS for 5 min and analysed using an EPICS XL-ADC flow cytometer (Beckman Coulter). All procedures were carefully carried out under chilled conditions.

Indirect immunofluorescence assay. Cells grown in eight-well slides (Nunc) were fixed with 100% methanol for 20 min at −20 °C. Fixed cells were blocked with 5% FBS/PBS for 30 min at room temperature, after which they were incubated with hybridoma supernatants or mAbs diluted in 1% FBS/PBS for 30 min at room temperature. After washing with PBS, cells were incubated with 1:1000-diluted Alexa 488-labelled Fab fragment of goat anti-mouse IgG for 30 min. Finally, the slides were mounted with PBS containing 50% glycerol and 1% n-propyl gallate (Wako) and examined using a fluorescence microscope equipped with a cooled CCD unit (*Coo*SNAP HQ; Roper).

Cell growth and cytotoxicity. The effect of mAbs on cell growth was analysed using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay (Ishiyama *et al.*, 1996) and cytotoxicity was analysed by lactate dehydrogenase (LDH) release assay using the LDH-Cytotoxic Test (Wako).

RESULTS

Anti-PrP mAbs inhibit PrP^{Sc} accumulation in cultured cells

Several antibodies recognizing regions in the C-terminal portion of PrP have been reported to inhibit PrP^{Sc} accumulation in neuroblastoma cells persistently infected with prions (Enari *et al.*, 2001; Peretz *et al.*, 2001). We recently established a panel of diverse anti-PrP mAbs including those recognizing the octapeptide repeat in the N-terminal region of PrP (Kim *et al.*, 2004). In the current studies, we investigated whether they would effect PrP^{Sc} accumulation in prion-infected neuroblastoma cells. Fig. 1(a) shows the effect of mAbs recognizing linear epitopes on PrP^{Sc} accumulation in I3/I5-9 cells persistently infected with prions. Following a 3-day treatment, only two mAbs reduced PrP^{Sc} accumulation: 31C6, which recognizes aa 143–149 of mouse PrP, and 110, which recognizes PHGGGWG at aa 59–65 and aa 83–89 in the octapeptide repeat. Quantitative analysis revealed that other mAbs did not affect the total amount of PrP^{Sc}, or the ratio of di-, mono- and non-glycosylated PrP^{Sc}.

Flow cytometric analysis showed that mAbs 110 and 31C6 bound PrP^{C} on the cell surface, although the fluorescence intensity of mAb 110 was weaker than that of mAb 31C6 (Fig. 1b, left panel). In contrast, mAbs that had no effect on PrP^{Sc} accumulation did not appear to bind to PrP^{C} on the cell surface (Fig. 1b, right panel). Two other mAbs, 44B1 and 72, which are thought to recognize discontinuous epitopes (Kim *et al.*, 2004), reacted with PrP^{C} on the cell surface (Fig. 1b) and inhibited PrP^{Sc} accumulation (Fig. 2). These results suggested that mAbs that can bind to PrP^{C} on the cell surface have the potential to antagonize PrP^{Sc} accumulation in cells persistently infected with prions.

Fig. 2 shows the dose-dependence of the effect of the anti-PrP mAbs. The four effective mAbs (110, 31C6, 44B1 and 72) reduced the amount of PrP^{Sc} in a dose-dependent manner, although PrP^{Sc} was not completely eliminated following the 3-day treatment. The 50% effective dose (EC_{50}) of mAbs 110, 31C6, 44B1 and 72 was estimated to be 0·2 μg ml^{-1}(1·2 nM), 0·1 μg ml^{-1} (0·7 nM), 0·3 μg ml^{-1} (1·7 nM) and 0·6 μg ml^{-1} (4·1 nM), respectively (Fig. 2b).

Fig. 3 shows the long-term effect of mAbs on PrP^{Sc} formation. Treatment for 6 days with mAb 110, 44B1, 31C6 (Fig. 3) or 72 (data not shown) reduced PrP^{Sc} to an almost undetectable level, and no re-emergence of PrP^{Sc} was observed in the following 6 and 12 days of incubation in the absence of mAbs. On the contrary, mAbs that did not bind to cell-surface PrP^{C} showed little effect on PrP^{Sc} accumulation even after long-term treatment.

The influence of mAbs on cell growth and acute toxicity was examined by WST-1 assay and LDH release assay, respectively. No significant effect on cell growth was observed, even with long-term treatment (5 μg ml^{-1} for 6 days) and mAbs did not demonstrate any acute toxicity (10 μg ml^{-1}) following 2 h of treatment.

Effect of anti-PrP mAbs on total amount of PrP^{C}

Fig. 1(a, lower panel) shows total PrP^{C} in the I3/I5-9 cells treated with mAbs for 3 days. The intensities of PrP^{C} bands were normalized with α-sarcomeric actin on the same blot and PrP^{C} levels relative to cells treated with negative control mAb (P1-284) are indicated at the bottom. Although there was a certain degree of variation, no marked difference was observed in the total amount of PrP^{C}. In contrast, after long-term treatment (6 days), the total amount of PrP^{C} in I3/I5-9 cells treated with mAb 110 or 44B1 appeared to be higher than that with the negative-control mAb or other anti-PrP mAbs (Fig. 3, top right panel). To confirm this further, we repeated the same experiment at least three times for the four inhibitory mAbs, 110, 31C6, 44B1 and 72. Relative PrP^{C} levels in cells treated with these four mAbs were 168±38, 88±23, 183±54 and 103±33%, respectively. These results suggested that the effect of mAbs on PrP^{C} level varied depending on the mAb: mAbs 110 and 44B1 increased total PrP^{C} levels following long-term treatment, while mAbs 31C6 and 72 did not affect the total PrP^{C} level.

Cell-surface localization of the mAb–PrP^{C} complex

The N-terminal portion of PrP, including the octapeptide repeat, is not essential for PrP^{Sc} formation and/or prion propagation (Flechsig *et al.*, 2000; Rogers *et al.*, 1993). The finding that not only the mAbs recognizing the C-terminal part of PrP, such as 31C6 and 44B1, but also mAb 110 inhibited PrP^{Sc} accumulation in the neuroblastoma cells, together with the fact that only the mAbs that bound to cell-surface PrP^{C} showed an inhibitory effect, implied that the mAb–PrP^{C} interaction on the cell surface is essential for inhibition of PrP^{Sc} accumulation. To investigate this further, we analysed the dynamics of anti-PrP mAbs after their binding to the cell surface (Fig. 4). Neuro2a cells were treated with 10 μg mAb 31C6 ml^{-1} for 1 h, after which the cells were cultured for an additional 4 h without mAb. Cells were then harvested and stained with an Alexa 488-conjugated secondary antibody. As a control, cells cultured with mAb 31C6 for 1 h were immediately stained with the secondary antibody. Flow cytometric analysis showed no difference in fluorescence intensity between the two preparations, suggesting that the mAb–PrP^{C} complex remained on the cell surface, even after the additional 4 h culture in the absence of mAb. As I3/I5-9 cells are established by repeated limiting dilution, Neuro2a cells may not be a suitable uninfected control for I3/I5-9 cells. Hence, we carried out the same experiment using I3/I5-9 cells. It is known that elimination of PrP^{Sc} parallels the reduction of prion infectivity. Considering biosafety issues, we used I3/I5-9 cells cured of PrP^{Sc} by long-term treatment with mAb 44B1 for flow cytometric analysis. mAb 31C6 (Fig. 4) and

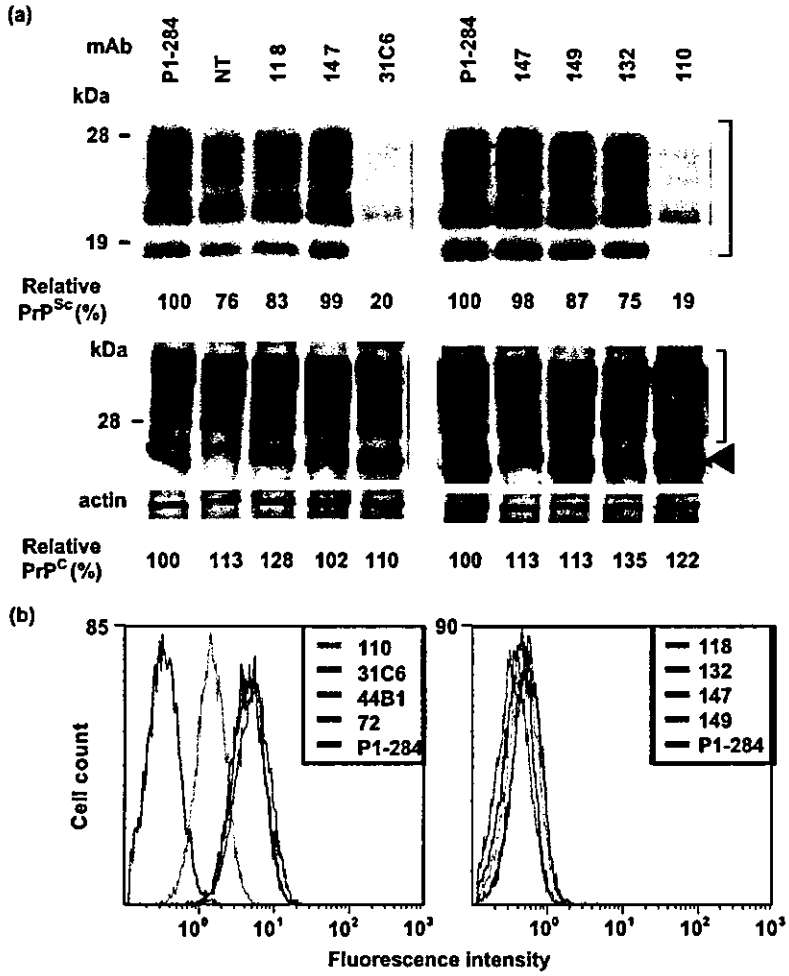

Fig. 1. Inhibition of PrP^{Sc} accumulation in prion-infected I3/I5-9 cells by anti-PrP mAbs. (a) Detection of PrP^{Sc} (upper panels) and PrP^{C} (lower panels). I3/I5-9 cells were cultured for 3 days with 4% FBS in Opti-MEM containing 5 μg mAbs ml^{-1}. The level of PrP^{Sc} in the cells was determined by immunoblot analysis using mAb 44B1. Antibodies added to the culture are indicated above the panels. mAb P1-284 against feline panleukopenia virus was used as a control for non-specific effects. For detection of PrP^{Sc}, the load volume of each sample was adjusted based on the protein concentration of the corresponding cell lysate that had not been treated with proteinase K. For quantitative analysis of PrP^{Sc}, the three PrP^{Sc} bands indicated by a square bracket (right-hand side, upper panels) were grouped together. To check the ratios of the three PrP^{Sc} bands, each was selected separately. For PrP^{C}, the PrP^{C} bands indicated by a square bracket (right-hand side, lower panels) were quantified. The bands indicated by the arrowhead were excluded from the quantitative analysis, as they overlapped with immunoglobulin light chains that were detected by secondary antibodies. The blot used for PrP^{C} detection was also probed with anti-sarcomeric actin mAb for normalization. The levels of PrP^{Sc} and PrP^{C} relative to cells treated with negative-control mAb (P1-284) are indicated below the panels. NT, cells cultured without mAbs. Molecular mass markers are shown in kDa on the left. Epitopes for mAbs were as follows: 110, aa 56–89; 132, aa 119–127; 118, aa 137–143; 31C6, aa 143–149; 149, aa 147–151; 147, aa 219–229 (Kim *et al.*, 2004). (b) Binding of mAbs to the surface of Neuro2a cells examined by flow cytometry. The left panel shows mAbs that bound to the cell surface, while the right panel shows mAbs that did not bind. mAb P1-284 was used as a control for non-specific binding.

the three other inhibitory mAbs, 110, 44B1 and 72 (data not shown), showed the same retention of mAb–PrP^{C} complexes as observed with Neuro2a cells.

To confirm further the retention of mAb–PrP^{C} complexes on the cell surface, Neuro2a and I3/I5-9 cells were cultured for 1 h with mAbs 110, 31C6, 44B1 and 72, and, in some cases, the cells were cultured for an additional 4 h with mAb-free medium. The cells were then fixed with ice-cold methanol and mAb–PrP^{C} complexes were detected using secondary antibody (Fig. 5). All mAbs bound to the cell surface (Fig. 5a–e) and membrane staining could be detected, even after 4 h incubation in the absence of mAbs (Fig. 5f–j). To characterize further the retention of

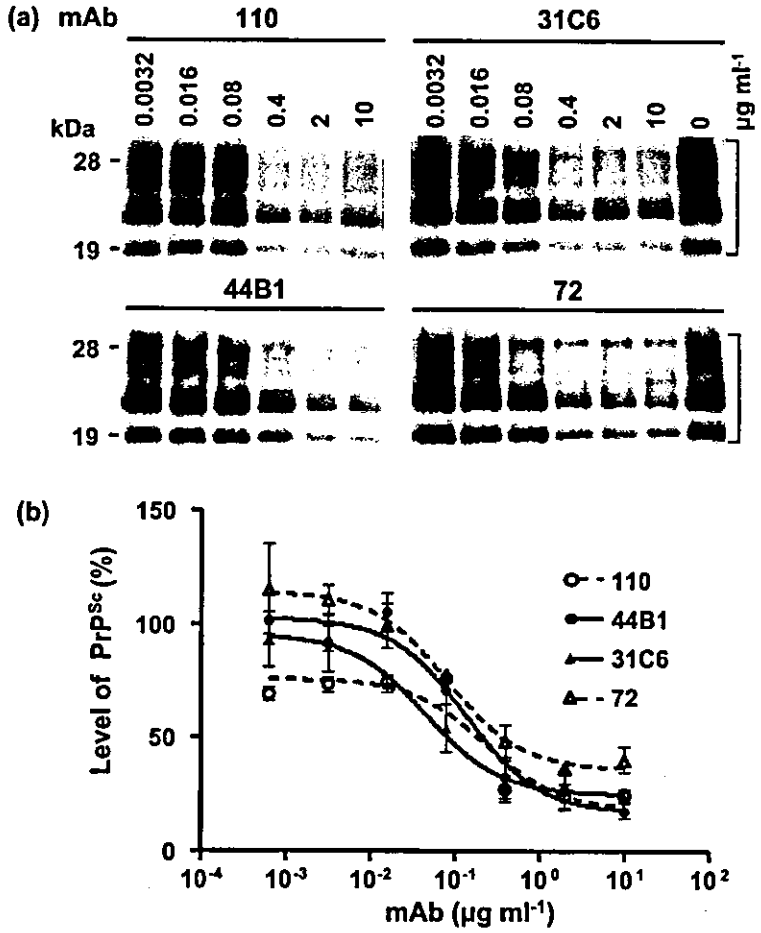

Fig. 2. Dose-dependent inhibition of PrP^{Sc} accumulation by anti-PrP mAbs. (a) Representative results from immunoblotting. I3/I5-9 cells were cultured for 3 days with various concentrations of mAb as indicated above the panels. The level of PrP^{Sc} was determined by immunoblot analysis using mAb 44B1. (b) Dose–response curve. The intensity of the PrP^{Sc} bands in the blots was quantified using an LAS-1000 lumino image analyser. The PrP^{Sc} level in the absence of mAbs was assigned a value of 100% in each experiment. The graph shows means ± SD from at least three independent experiments. EC_{50} values were estimated using GraphPad PRISM (GraphPad Software).

mAb–PrP^C complexes on the cell surface, we examined the effect of DS500, which is reported to accelerate PrP^C endocytosis (Shyng *et al.*, 1995). Following treatment with DS500, the mAb–PrP^C complexes on the cell surface were internalized and detected as intracellular granules (Fig. 5k–o). These results demonstrated that the mAbs bound to the cell-surface PrP^C remained there, regardless of their epitope specificity.

Effect of other compounds on PrP^C expression

Our results indicated a possible link between cell-surface retention of PrP^C by anti-PrP antibodies and the inhibition of PrP^{Sc} formation in cells, and suggested that the mAb treatment altered the total amount of PrP^C at least for mAbs 110 and 44B1. In order to examine whether compounds that inhibit PrP^{Sc} accumulation in prion-infected cells affect PrP^C level in the cells, we tested DS500, E-64d, quinacrine, chlorpromazine and polyethyleneimine. We confirmed that these compounds inhibited PrP^{Sc} accumulation in I3/I5-9 cells (data not shown). Using the concentrations at which these compounds caused >90% inhibition, we examined their effects on cellular levels of PrP^C following a 3-day treatment (Fig. 6a). Immunoblot analysis revealed that only DS500 reduced the PrP^C level (to ~30% that of untreated cells) among the compounds tested. Flow cytometric analysis with mAb 110 (Fig. 6b) confirmed that DS500 reduced the level of cell-surface PrP^C.

Since sulfated glycosaminoglycans like DS500 may bind to the N-terminal region of PrP^C (Pan *et al.*, 2002), the reduction in fluorescence intensity may be due to blocking of mAb 110 binding. For this reason, we used mAbs 31C6 and 44B1 to detect PrP^C instead of mAb 110. Table 1 shows the mean relative amount of PrP^C on the cell surface calculated from at least three independent experiments. Regardless of the mAb used for detection, DS500 reduced the PrP^C level to ~50% of the untreated control. No significant change in cell-surface expression of PrP^C was observed with the other compounds tested.

DISCUSSION

Anti-PrP antibodies that react with the C-terminal portion of PrP inhibit PrP^{Sc} formation in cultured cells (Enari *et al.*, 2001; Peretz *et al.*, 2001). One explanation for the inhibitory effect of these antibodies is that the binding of mAb to the corresponding epitope on PrP^C directly inhibits PrP^C–PrP^{Sc} interaction by occupying their binding domains. Fab D18, the most effective mAb reported by Peretz *et al.* (2001), reacts with the region spanning aa 132–156 in mouse PrP. In this study, we examined three mAbs

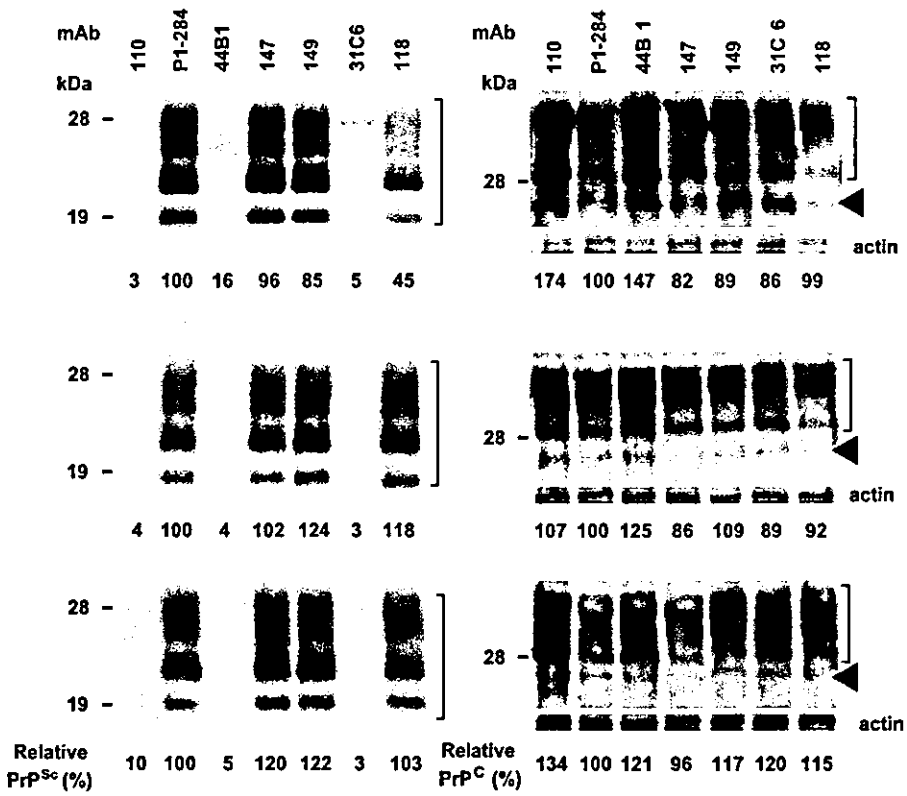

Fig. 3. Clearance of PrP^{Sc} by long-term antibody treatment. I3/I5-9 cells were cultured for 6 days with 5 μg mAb ml^{-1} (top panels). After withdrawal of the mAb, cells were cultured for an additional 6 (middle panels) or 12 (bottom panels) days in the absence of mAb. Quantitative analysis was carried out as described in the legend to Fig. 1 and relative PrP^{Sc} (left panels) and PrP^{C} (right panels) levels are indicated below the corresponding images.

recognizing epitopes within this region, but only mAb 31C6, which recognizes aa 143–149, displayed inhibitory activity. The remaining mAbs, 118 and 149, which bind adjacent epitopes aa 137–143 and aa 147–151, respectively, did not inhibit PrP^{Sc} formation in the cells. The main difference among these three mAbs was their ability to bind mature PrP^{C}; only mAb 31C6 bound PrP^{C} on the cell surface. Although it is well known that the N-terminal portion of PrP, including the octapeptide repeat, is not essential for prion propagation and/or PrP^{Sc} formation (Flechsig *et al.*, 2000; Rogers *et al.*, 1993), mAb 110, which recognizes the sequence in the octapeptide repeat, also antagonized PrP^{Sc} formation. This implied that there are mechanisms of inhibition other than blocking of the specific epitopes. Indeed, four of eight anti-PrP mAbs recognizing different epitopes inhibited PrP^{Sc} formation, suggesting that a common feature of the inhibitory mAbs is their ability to bind PrP^{C} on the cell surface. Taken together, our results suggest that inhibition of PrP^{Sc} formation by mAbs depends on their binding to mature PrP^{C} on the

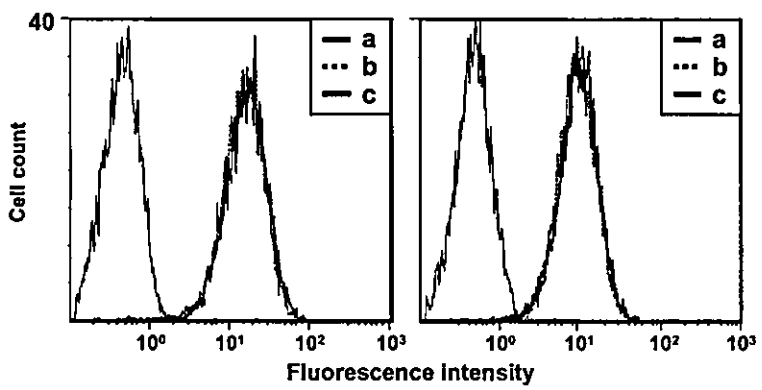

Fig. 4. Retention of mAb–PrP^{C} complexes on the cell surface. Neuro2a (left panel) or I3/I5-9 cells cured of PrP^{Sc} by mAb treatment (right panel) were cultured for 1 h in the presence of 10 μg negative control mAb P1-284 (a) or mAb 31C6 (b, c) ml^{-1}. Cells were harvested immediately and stained with Alexa-488-conjugated secondary antibody (a, b). Alternatively, after the removal of mAb, the cells were cultured for an additional 4 h in the absence of mAb and then harvested and stained with the secondary antibody (c).

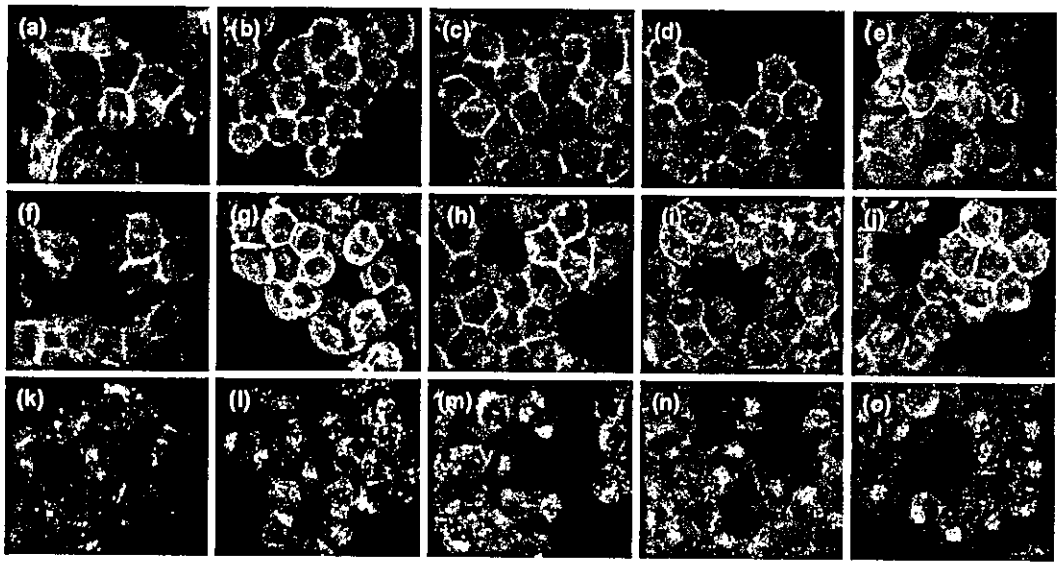

Fig. 5. Internalization of mAb–PrP^C complexes by treatment with DS500. Neuro2a cells (a, f and k) and I3/I5-9 cells (b–e, g–j, l–o) were cultured for 1 h with mAb 110 (b, g and l), 31C6 (a, f and k for Neuro2a cells; c, h and m for I3/I5-9 cells), 44B1 (d, i and n) or 72 (e, j and o). After removal of the mAb, cells were washed with ice-cold PBS and fixed with ice-cold methanol (a–e). Alternatively, after removal of mAb, cells were cultured with mAb-free medium for 4 h and fixed with ice-cold methanol (f–j). For DS500 treatment (k–o), after removal of mAb, cells were cultured for 3 h in mAb-free medium and then treated for 1 h with 25 μg DS500 ml^{-1}, after which they were fixed with ice-cold methanol. The fixed cells were directly stained with Alexa-488-conjugated secondary antibody to detect bound anti-PrP mAb.

cell surface rather than their binding to specific epitopes. On the other hand, transient interaction between the flexible N-terminal region and the second α-helix in the C-terminal globular domain has been postulated (Zahn *et al.*, 2000), and antibody binding to the N terminus of PrP prevents binding of C terminus-specific mAb (Li *et al.*, 2000). Hence, it cannot be excluded that binding of mAb 110 to the octapeptide repeat might sterically influence a particular domain involved in binding to PrP^{Sc}.

Although the cell-surface binding of mAb 110 was lower than that of the other mAbs (Fig. 1b, left panel), it inhibited PrP^{Sc} formation as efficiently. This may be explained by the presence of an 18 kDa N-terminally truncated PrP^C. This truncated PrP^C fragment is produced by cleavage of PrP^C around residue 112 during the recycling process (Chen *et al.*, 1995) so that it is not recognized by mAb 110. Recently, Mishra *et al.* (2002) reported that the N-terminally truncated form comprised as much as 40–50 % of PrP^C on the cell surface. This could account for the lower signals obtained using mAb 110. Because N-terminally truncated PrP^C is unlikely to act as a substrate for prion propagation and/or PrP^{Sc} formation (Lawson *et al.*, 2001; Weissmann, 1999), the binding of mAb 110 to PrP^C possessing the N-terminal portion is apparently sufficient for the inhibition of PrP^{Sc} formation.

In this work, we have demonstrated both quantitatively and qualitatively that mAbs that bind to cell-surface PrP^C remain attached to the membrane, even after withdrawal of the mAbs from the culture medium. This suggests that the mAb–PrP^C complex on the cell surface is not preferentially internalized into the cell. Mature PrP^C expressed on the cell surface is thought to be internalized via either clathrin-coated or -uncoated vesicles from which it enters the degradation pathway (Peters *et al.*, 2003; Shyng *et al.*, 1994; Sunyach *et al.*, 2003). Because PrP^{Sc} formation is believed to take place in the subcellular compartments that include cell membrane during the degradation pathway (Borchelt *et al.*, 1992; Caughey & Raymond, 1991), it is possible that mAb treatment could interfere with the regular PrP^C metabolism simply by retaining it on the cell surface. We suspected that the cell-surface retention of PrP^C would result in an increase in total PrP^C. Actually, two mAbs, 110 and 44B1, obviously increased the total amount of PrP^C, while two other mAbs 31C6 and 72 did not influence the total amount of PrP^C. It is conceivable that binding of mAbs to specific epitopes of cell-surface PrP^C might result in downregulation of PrP^C synthesis; however, further experiments are required to resolve this.

It was recently reported that polyclonal antibodies against dimeric recombinant PrP inhibited PrP^{Sc} formation in the cell, while the corresponding Fab fragments had little effect on PrP^{Sc} formation (Gilch *et al.*, 2003). This suggests that antibody-mediated cross-linking of PrP^C on the cell surface is important for inhibition of PrP^{Sc} formation. Whether cross-linking of PrP^C by IgG is required for the retention of the mAb–PrP^C complex under our experimental conditions remains to be determined. Treatment of cells persistently

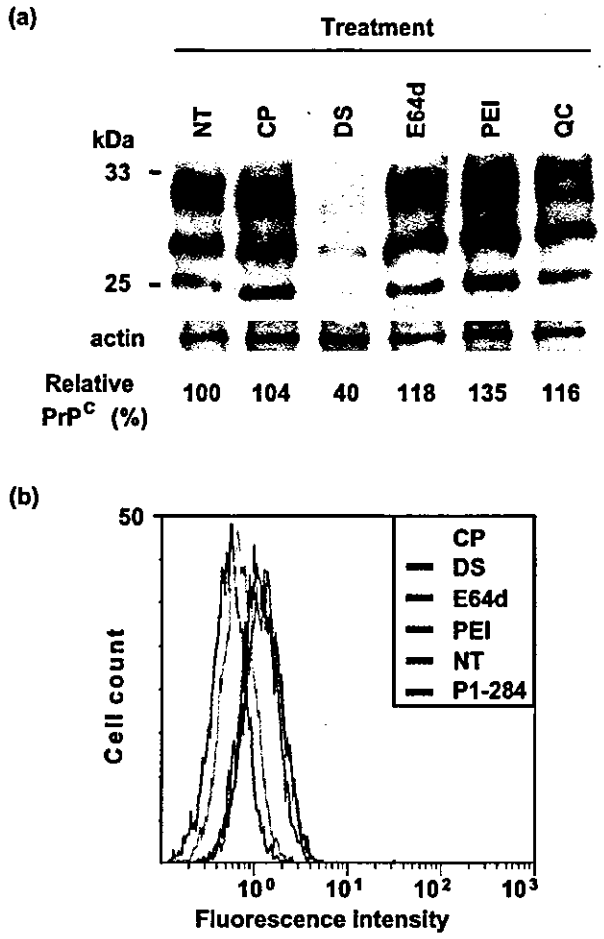

Fig. 6. Influence of chemical treatments on the expression of PrP^C. (a) Total amount of PrP^C. Neuro2a cells were treated for 12 h with various chemical compounds as indicated above the panel. Final concentrations were 3 μg chlorpromazine (CP) ml^{-1}, 25 μg DS500 (DS) ml^{-1}, 50 μM E-64d (E64d), 3 μg polyethyleneimine (PEI) ml^{-1} and 2 μM quinacrine (QC). Total PrP^C was detected in cell lysates by immunoblot analysis using mAb 31C6 (upper panel). The same blot was probed with anti-sarcomeric actin mAb to normalize for loading (lower panel). The intensity of the bands was quantified using an LAS-1000 lumino image analyser, and the relative amount of PrP^C compared with untreated control (NT) was calculated for each experiment. The data below the panel are means from three independent experiments. (b) Representative flow cytometric analysis of the cell-surface expression of PrP^C. Neuro2a cells were treated with compounds as described in (a), harvested, stained with mAb 110 followed by Alexa-488-conjugated secondary antibody and analysed by flow cytometry. The mean fluorescence intensity of the untreated control (NT) was assigned a value of 1 and the relative fluorescence intensities were calculated from the mean fluorescence intensity from each histogram. Quinarine was excluded from this experiment because of its autofluorescence. mAb P1-284 was used as a negative control for flow cytometric analysis.

Table 1. Effects of chemical treatment on cell-surface expression of PrP^C

Data represent means±SD (minimum of $n=3$) of relative fluorescence intensity compared with control (NT).

Treatment	mAb for detection		
	110	31C6	44B1
NT	1·00	1·00	1·00
Chlorpromazine	0·77±0·05	0·95±0·03	0·92±0·03
DS500	0·52±0·03*	0·55±0·10*	0·48±0·04*
E-64d	0·99±0·17	0·99±0·06	0·97±0·11
Polyethyleneimine	1·01±0·16	1·04±0·03	1·07±0·11

*Statistically significant differences ($P<0·05$). The conditions of the treatments are described in the legend to Fig. 6.

infected with prions using antibodies against the laminin receptor precursor/laminin receptor (LRP/LR) reduced PrP^{Sc} accumulation (Leucht *et al.*, 2003). Because binding of LRP/LR to PrP^C could be involved in PrP metabolism (Gauczynski *et al.*, 2001), it is conceivable that antibodies interfere with the interaction between PrP^C and a molecule(s) that participates in PrP^C internalization.

Many reagents, including small molecules, recombinant PrP and anti-PrP antibodies, have been identified as potential inhibitors of prion propagation. It is important to elucidate their mechanisms of action, not only for the establishment of therapeutics but also for an understanding of prion replication. In the present study, we have demonstrated that blocking of the internalization of PrP^C with anti-PrP mAbs prevents PrP^{Sc} accumulation. Although anti-PrP mAbs recognizing specific epitopes have recently been reported to induce neuronal death in the hippocampus and cerebellum (Solforosi *et al.*, 2004), we have not found an apparent adverse effect on the cell growth and clinical manifestation by intraventricular inoculation of the anti-PrP mAbs used in this study (data not shown). Further analyses using prion-infected animals are necessary for evaluation of anti-PrP antibodies as therapeutics for treating prion diseases.

After the submission of this paper, a paper was published by Perrier *et al.* (2004) in which it was described that recognition by mAb SAF34 of the octapeptide repeat region on the N-terminal part of human PrP inhibited PrP^{Sc} formation in prion-infected neuroblastoma cells.

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NOTE *Clinical Pathology*

Unique Amino Acid Polymorphisms of PrP Genes in Mongolian Sheep Breeds

Altangerel GOMBOJAV[1,3], Naotaka ISHIGURO[1]*, Motohiro HORIUCHI[1] and Morikazu SHINAGAWA[2]

[1]*Laboratory of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555,*
[2]*Prion Disease Research Center, National Institute of Animal Health, 5–1–3 Kannondai, Tsukuba, Ibaraki 305–0856, Japan and*
[3]*School of Veterinary Medicine and Biotechnology, Mongolian State University of Agriculture, Ulaanbaatar 210153, Zaisan, Mongolia*

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ABSTRACT. To characterize amino acid polymorphisms of sheep prion protein (PrP) gene, DNA from 740 sheep of nine breeds raised in Mongolia was isolated and analyzed. A total of 16 genotypes and seven allelic variants of the PrP gene at codons 112, 136, 154, and 171 were found. The MARQ/MARQ genotype associated with susceptibility to scrapie was found in 82.6% of the sheep while the MARR/MARR genotype associated with resistance to scrapie was found in 1.8% of the sheep. The polymorphisms of valine and serine at codon 127, and leucine and arginine at codon 189 were detected in eight Mongolian sheep breeds, suggesting that these polymorphisms are a common feature among Mongolian sheep breeds.
KEY WORDS: PrP genotype, scrapie susceptibility.

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Scrapie in sheep and goats is a fatal and infectious neurodegenerative disease that has been categorized as a transmissible spongiform encephalopathy (TSE) or prion disease also found in humans and other animals. Prion diseases are characterized by the accumulation in the tissues of the central nervous system of an "infectious" abnormal protease-resistant isoform (PrP^{Sc}) of cellular prion proteins (PrP^{C}) encoded by the PrP gene [12]. Polymorphisms of the PrP gene have been linked to host susceptibility and the incubation period of the disease [11]. PrP allelic variant valine/arginine/glutamine (VRQ) at codons 136, 154, and 171 is associated with high susceptibility to scrapie for sheep breeds. While the allele VRQ is rare in Suffolk sheep, the wild-type PrP allele alanine/arginine/glutamine (ARQ) is associated with susceptibility to scrapie. It has been widely considered that the PrP allelic variant alanine/arginine/arginine (ARR) at codons 136, 154, and 171 is associated with resistance to scrapie in several breeds [1–5, 7–10, 13]. Links between DNA polymorpisms and scrapie susceptibility have been identified in outbreaks of scrapie in various breeds or flocks in different countries where scrapie has been diagnosed [1, 7–8, 10, 13]. However, in central Asian countries where many sheep are raised, the polymorpism of PrP genes associated with scrapie have not yet been characterized. Therefore, we examined the PrP genotypes of 740 Mongolian sheep, including 271 sheep previously reported [6].

DNA samples were collected from several breeds from different prefectures in Mongolia. A total of 271 sheep came from the central region of Mongolia: 112 Khalkh sheep (native breed) from Tuv prefecture (designated I in Fig. 1.); 60 Khalkh sheep from Uvurkhangai prefecture (J); and 35 Yeroo sheep, 35 Orkhon sheep, and 29 Khangai sheep from Selenge prefecture (K) [6]. From the western region, a total of 345 sheep were used: 70 Khalkh sheep and 36 Sartuul sheep from Zavhan prefecture (A); 35 Khalkh sheep and 35 Govi-altai sheep from Govi-Altai prefecture (B); 71 Khalkh sheep and 33 Bayad sheep from Uvs prefecture (C); and 34 Khalkh sheep and 31 Darhad sheep from Huvsgul prefecture (D). For the eastern region, a total of 124 sheep were used: 32 Sumber Karakul sheep from Govi-Sumber prefecture (H); 32 Khalkh sheep from Dornogovi prefecture (E); 31 Khalkh sheep from Suhbaatar prefecture (F); and 29 Khalkh sheep from Hentii prefecture (G). The Khalkh sheep comprised about 90% of 13.8 million Mongolian sheep. Crossbreeding between the Khalkh sheep and local sheep including imported sheep started in the 1930s, and the crossbreeding has led to develop several local crossbreeds in Mongolia [6].

The entire 794-bp open reading frame (ORF) of the PrP gene was amplified by polymerase chain reaction (PCR) in 50 μl reactions, using PrP primers (SPrP-1, SPrP-2, SPrP-3, and SPrP-5) as described by Gombojav *et al.* [6]. To confirm amplification, a portion of each reaction product was electrophoresed on a 0.7% agarose gel containing ethidium bromide (0.5 $\mu g/ml$), and visualized under ultraviolet radiation. Then, the primers were removed using a Centricon 100 micro-concentrator (Amicon, Bedford, MA), and 1 to 5 μl of the concentrated PCR product was used for direct sequencing [6].

In this study, the relative genotype frequencies of four codons (112, 136, 154, and 171) of the PrP gene are newly reported in five sheep breeds (Sartuul, Govi-altai, Bayad, Darhad, and Sumber Karakul), in addition to four previously reported sheep breeds (Khalkh, Yeroo, Orkhon, and Khangai) [6]. Table 1 shows the genotype frequencies of 16 different PrP genotypes in the 740 sheep examined. The PrP genotypes MARQ/MARQ was found in all nine breeds.

Among all the breeds studied, the Khalkh sheep showed the greatest variation with 11 PrP genotypes composed from 6 alleles, while three to nine PrP genotypes were found in

* CORRESPONDENCE TO: ISHIGURO, N., Laboratory of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan.

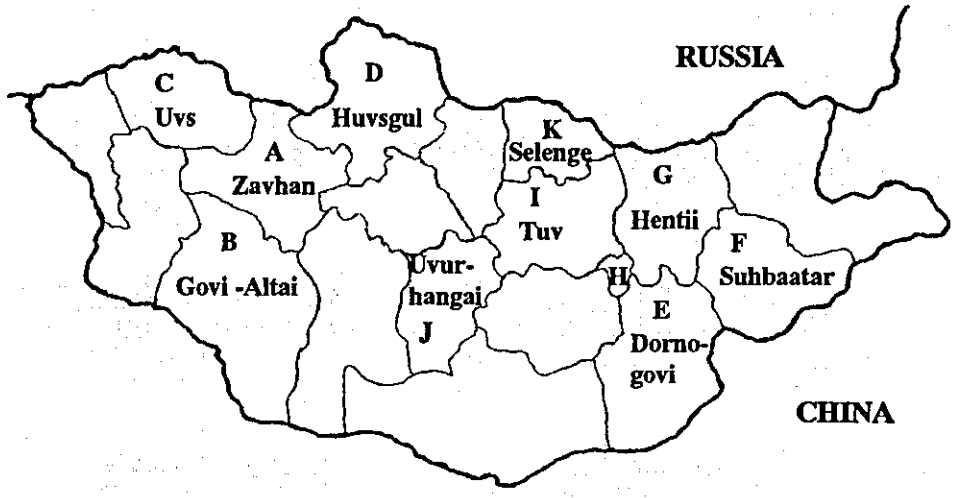

Fig. 1. Map of Mongolia. The blood samples were collected from different prefectures in Mongolia (A-K). H, Govi-Sumber prefecture.

Table 1. Frequency of PrP genotypes at codons 112, 136, 154 and 171 in Mongolian sheep breeds

Location[a]	A-G, I-J		A		B		C		D		H		K		K		K	
Breeds	Khalkh[b]		Sartuul		Govi-altai		Bayad		Darhad		Sumber		Yeroo[c]		Orkhon[c]		Khangai[c]	
PrP genotype											Karakul							
	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
MARQ/MARQ	317	66.8	29	80.6	27	77.1	29	87.9	21	67.7	27	84.3	17	48.5	13	37.0	15	51.7
MARQ/TARQ	68	14.4	3	8.3	2	5.7	3	9.1	2	6.5			4	11.4	7	20.0	2	6.9
MARQ/MARH	31	6.6	2	5.5	2	5.7	1	3.0	5	16.2	3	9.4			1	2.9		
MARQ/MARR	13	2.7			2	5.7							8	22.8	8	22.8	9	31.0
MARH/MARH	7	1.5	1	2.8					1	3.2	2	6.3			1	2.9		
TARQ/TARQ	12	2.5							1	3.2			1	2.9				
TARQ/MARH	8	1.7													1	2.9		
TARQ/MARR	4	0.8			1	2.9									1	2.9		
MARQ/MAHQ	6	1.3																
MARR/MARR	7	1.5			1	2.9			1	3.2			2	5.7			2	6.9
MARK/MARK	1	0.2	1	2.8														
MARQ/MVRQ													1	2.9	2	5.7		
MARR/MVRQ													1	2.9				
MARH/MAHQ													1	2.9				
MARR/MAHQ															1	2.9		
TARQ/MAHQ																	1	3.5
Total	474		36		35		33		31		32		35		35		29	

a) Locations are shown in Fig. 1.
b) A total of 474 samples include 172 samples previously reported by Gombojav *et al.* [6].
c) Data from Gombojav *et al.* [6].

the other sheep breeds. The great variations in PrP genotypes suggest that Khalkh sheep originated from a mixture of several breeds from surrounding countries, while lesser variations in the other sheep breeds suggest that these breeds originated from a mixture of fewer numbers of breeds within Mongolia.

The allelic variant VRQ at codons 136, 154, and 171 is rare in Suffolk sheep [4, 9, 13] and this tendency was also found in the Mongolian sheep breeds. Valine at codon 136 (136V) associated with high susceptibility to scrapie was detected from two sheep breeds, Yeroo and Orkhon, but not in the other sheep breeds of Mongolia. Yeroo and Orkhon sheep have been raised in Selenge prefecture, which is in close proximity to Russia. The sheep raised in the prefecture had been genetically developed by crossbreeding with Russian sheep. Therefore, it is thought that the polymorphism of valine at codon 136 may have been introduced through the Russian sheep breeds, although little information about the PrP polymorphism of Russian sheep.

We found valine (V; nucleotides GTC) and serine (S;

Table 2. Frequency of PrP genotypes at codons 127 and 189 in Mongolian sheep breeds

Location[a]	A-G, I-J		A		B		C		D		H		K		K		K	
Breeds	Khalkh[b]		Sartuul		Govi-altai		Bayad		Darhad		Sumber Karakul		Yeroo[c]		Orkhon[c]		Khangai[c]	
PrP genotype	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
GQ/GQ[d]	354	74.6	26	72.2	17	48.6	22	66.8	20	64.5	23	71.9	28	80.0	32	91.4	29	100
GQ/SQ	68	14.4	5	13.9	4	11.4	6	18.2	8	25.8	5	15.6	7	20.0	1	2.9		
SQ/SQ	5	1.1									1	3.1						
GQ/VQ	19	4.0					1	3.0			2	6.3						
GQ/GL	20	4.2	4	11.1	11	31.4	2	6.0	3	9.7	1	3.1			2	5.7		
GL/GL	1	0.2																
GQ/GR	2	0.4			1	2.9												
GL/SQ	5	1.1	1	2.8	2	5.7	2	6.0										
Total	474		36		35		33		31		32		35		35		29	

a) Locations are shown in Fig. 1.
b) A total of 474 samples include 172 samples previously reported by Gombojav *et al.* [6]
c) Data from Gombojav *et al.* [6].
d)Wild type of sheep PrP gene.

AGC) at codon 127, and leucine (L; CTA) and arginine (R; CGA) at codon 189 in eight Mongolian sheep breeds (Table 2). These amino acid polymorphisms of PrP gene were widely observed in Mongolian sheep breeds but have not been reported in other sheep from European countries, suggesting that these are unique to indigenous sheep breeds including Mongolian sheep. However, it remains to be determined whether these polymorphisms have any correlation with susceptibility to scrapie.

Among the different sheep breeds raised in Mongolia, 66.9% had the MARQ/MARQ genotype and 1.8% had the MARR/MARR genotype, which are linked to susceptibility and resistance to scrapie, respectively (Table 1). In the previous study [6], we examined the scrapie form of the prion protein (PrP^{Sc}) in brain tissues from 10 sheep with neurological symptoms, but no PrP^{Sc} was obtained by Western blot analysis. Therefore, although there have been no reports of scrapie in Mongolia, these results suggest that the majority of Mongolian sheep are genetically susceptible to scrapie. However, since PrP genes linked to scrapie resistance were observed in five of the nine breeds, individual sheep carrying the scrapie-resistant genes can be identified and used in breeding programs to develop scrapie-resistant populations.

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FULL PAPER *Virology*

Effect of Tissue Deterioration on Postmortem BSE Diagnosis by Immunobiochemical Detection of an Abnormal Isoform of Prion Protein

Hiroko HAYASHI[1], Masuhiro TAKATA[1], Yoshifumi IWAMARU[1], Yuko USHIKI[1,2], Kumiko M. KIMURA[1], Yuichi TAGAWA[1], Morikazu SHINAGAWA[1] and Takashi YOKOYAMA[1]*

[1]*Prion Disease Research Center, National Institute of Animal Health, 3–1–5 Kannondai, Tsukuba, Ibaraki 305–0856 and*
[2]*Nippi Research Institute of Biomatrix, Adachi, Tokyo 120–8601, Japan*

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ABSTRACT. Surveillance for bovine spongiform encephalopathy (BSE) in fallen stock in Japan is conducted with a commercial enzyme-linked immunosorbent assay (ELISA) for mass screening, with Western blotting (WB) and immunohistochemistry performed for confirmation of the ELISA. All tests are based on immunological detection of an abnormal isoform of the prion protein (PrP^{Sc}) in brain tissues, which have sometimes deteriorated by the time samples from fallen stock reach a diagnostic laboratory. To evaluate BSE surveillance procedures for fallen stock, we examined PrP^{Sc} detection from artificially deteriorated BSE-affected bovine brain tissues with a commercial ELISA kit and compared the results with those of WB. The optical density (OD) values of the ELISA decreased with advancing deterioration of the tissues, whereas no reduction in the signal for PrP^{Sc} was observed in WB, even when performed after 4 days of incubation at 37°C. The progressive decrease in the OD values in the ELISA appear to be caused by a partial loss of the N-terminal moiety of PrP^{Sc} due to digestion by endogeneous and/or contaminated microbial enzymes, and by the presence of ELISA inhibitors that are generated in deteriorated tissues. These results suggest that WB is the most reliable test for fallen stock, especially for cattle brains within decaying carcasses.

KEY WORDS: BSE, ELISA, PrP^{Sc}, Western blot.

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Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disease of cattle and belongs to a family of transmissible spongiform encephalopathies or prion diseases. A proteinaceous pathogen, termed prion, is regarded as the causative agent [12]. Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, and chronic wasting disease in cervids are also included in this category. Since BSE prion is thought to cause variant CJD in humans [2, 4], it has been highlighted as a public health problem.

Prion is considered to consist of an abnormal isoform of the prion protein (PrP^{Sc}) as the main component [8]. PrP^{Sc} is generated by post-translational modification of the host-encoded normal cellular prion protein (PrP), termed PrP^{C} [10]. PrP^{Sc} is known to have unusual resistance to various physiochemical treatments for inactivation [17]. Though PrP^{C} is easily digested by proteinase K (PK), PrP^{Sc} partially resists PK and is cleaved to a smaller fragment called PrP-core or PrP_{27-30} [1], which lacks an N-terminal subregion of 60 to 70 amino acids. Thus, PK digestion is used to distinguish PrP^{Sc} from PrP^{C} and is a key to the diagnosis of prion disease.

Currently, diagnosis of BSE is based on postmortem detection of PrP^{Sc} in brain tissue, by means of immunological techniques such as enzyme linked immunosorbent assay (ELISA), Western blotting (WB), and immunohistochemistry (IHC), together with histopathology [11]. Several commercial kits have been developed and applied for mass surveillance. With the institution of surveillance programs, BSE has been newly recognized in several countries. Although the occurrence of BSE has decreased in the UK, where it was originally reported [19], it has since spread to other countries.

In Japan, following the occurrence of BSE for the first time in 2001 [7], mass surveillance at abattoirs was started and the Platelia BSE kit (Bio-Rad, France) has introduced as the principal screening test. For confirmation, WB, histopathology and IHC are performed. With this active surveillance, six more BSE cases were detected by the end of June 2003, and these cases were all sampled at abattoirs. To understand the current status of BSE in Japan, examination of fallen stock has been strengthened, and all dead cattle over 24 months old are now required to be subjected to a BSE test. Livestock hygiene service offices that conduct the fallen stock examination are obliged to deal with considerable numbers of deteriorated samples from carcasses that have been left for long periods under poor storage conditions. It has been reported that PrP^{Sc} can be detected in autolyzed and/or deteriorated samples with WB and IHC [3, 5, 6, 13, 14], but there is no information about the effects of sample condition on PrP^{Sc} detection with the ELISA.

The Platelia BSE kit, which is an antigen-capture ELISA, uses two monoclonal antibodies (mAbs). The mAb used for antigen-capture in this kit recognizes an octarepeat sequence (personal communication with Mr. K. Sugimura from Nippon Bio-Rad Laboratories, Tokyo, Japan), which is located adjacent to (but mostly not included) in the PK-resistant PrPcore region, and therefore the epitope can be digested by PK or other enzymes. The epitope of the other mAb used

* CORRESPONDENCE TO: YOKOYAMA, T., Prion Disease Research Center, National Institute of Animal Health, 3–1–5 Kannondai, Tsukuba, Ibaraki 305–0856, Japan.

for detection is located within PrPcore. Because of this mAb combination, it is critical for this ELISA to control PK treatment precisely. PrP^{C} has to be digested away, whereas PrP^{Sc} has to be conserved with the epitope in the octarepeat region that is PK-sensitive. It is likely that endogenous or secondarily proliferated bacterial enzymes are active in postmortem tissues, which may induce conversion of PrP^{Sc} to PrPcore if the carcass is left for a long time before the brain is harvested, especially in warm summer conditions.

To evaluate current BSE surveillance procedures for fallen stock in Japan, we examined PrP^{Sc} detection in artificially deteriorated BSE brain samples by means of Platelia-BSE ELISA and WB.

MATERIALS AND METHODS

Preparation and treatment of brain tissue: BSE-affected brain samples were provided courtesy of the Veterinary Laboratories Agency, Weybridge, UK. Brain stem tissues of five BSE-positive cattle were pooled and then minced well to make PrP^{Sc} content uniform. Approximately 12 g from the pooled tissue was placed in a 50-m*l* plastic tube and incubated at 30°C for 4 days. Small aliquots were sampled every day (Experiment [Exp.] 1). In another experiment, minced tissue was divided into 5 small aliquots, followed by incubation at 37°C for 0 to 4 days, respectively (Exp. 2). After incubation, tissue samples were stored at −80°C until use.

ELISA: Twenty-percent brain homogenates were prepared in 5% glucose (Platelia BSE kit homogenization buffer) and subjected to ELISA and WB. The ELISA procedure followed the manufacturer's protocol. To more efficiently estimate the difference in PrP^{Sc} detection depending on the incubation time, samples were serially diluted and subjected to ELISA, and optical density (OD) values at 450/620 nm were compared.

Western Blotting: For WB, 250 μl of the homogenate was mixed with an equal volume of detergent buffer, containing 4% sulphobetaine 3–14 and 1% sarkosyl, and then incubated with 500 μg/m*l* collagenase, followed by incubation with 40 μg/m*l* PK at 37°C for 30 min. After PK digestion was terminated with 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF; Pefabloc SC, Roche Diagnostics GmbH, Manheim, Germany), the sample was mixed with 250 μl of an 2-butanol:methanol (5:1) mixture and then centrifuged at 20,000 × g for 10 min. The pellet was heated at 100°C for 5 min in sodium dodecyl sulfate (SDS)-containing sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and electrical blotting to poly-vinylidene-fluoride membrane. The blotted membrane was incubated with one of two anti-PrP mAbs, T2 or 1H7 that were used as a primary antibody at room temperature (RT) for 1 hr. These mAbs were generated with PrP-deficient mice, by immunization with mouse PrP121–231 or sheep PrP23–231 recombinant protein, respectively. T2 recognizes mouse PrP135–140, and 1H7 recognizes the octarepeat region, PHGGG/SWGQ. The membrane was then incubated with peroxidase-conjugated anti-mouse immunoglobulin (Jackson Immunoreseach Laboratories, Inc.,West Grove, PA, U.S.A.) at RT for 45 min. Signals were developed with a chemiluminescence substrate (SuperSignal, Pierce Biotechnology, Inc., Rockford, IL, U.S.A.).

RESULTS

Gross findings of deteriorated tissue samples: After the 4-day incubation, the color of the tissue had changed to yellow-brown or grayish-green and had also liquefied. The severity of the gross damage and effluvium increased with longer incubation times except in one sample. The color of the sample incubated for 2 days in Exp. 2 was dark grayish brown; more severe deterioration was seen than the samples incubated for 3 and 4 days.

Detection of PrP^{Sc} by ELISA: The ELISA OD values for all samples reached a plateau when samples were diluted at 1:4 or undiluted in Exp. 1. The plateau levels for the samples incubated for 2 days or more were lower than those for fresh and 1-day-incubated samples (Fig. 1A). Although the OD values fluctuated somewhat, a similar tendency was also observed in Exp. 2, with the exception of the 2-day-incubated sample (Fig. 1B). The color of all reaction solutions in the wells of the undiluted samples was dark yellow. One reason for the similar OD values of all undiluted samples and the samples diluted 1:4 seemed to be color quenching. For the unincubated sample in Exp. 1, the OD value was increased by dilution to 1:4 (Fig. 1A). This might have been due to generation of brown precipitates in the reaction solution of the undiluted samples, in additional to color quenching. Accordingly, the values for all undiluted samples diverged from the dose-dependent curve. Therefore, comparison of the OD values among the samples was carried out with the values for samples diluted 1:4 or 1:16, where dose-dependency was observed. The 2-day-incubated sample in Exp. 2 containing the above-mentioned severely deteriorated tissue, had a remarkably lower OD value than did the others at any dilution (Fig. 1B). This sample was omitted from the comparison in the time course of the deterioration.

As shown in Fig. 1A of Exp. 1, incubation at 30°C for 1 day caused no significant change in detection of PrP^{Sc} by ELISA. Incubation for one additional day induced a significant reduction in OD values, but no further reduction in OD values was observed with further incubation for up to 4 days. In Exp. 2, where incubation was carried out at 37°C, one-day incubation caused a significant decrease in the OD value. The OD values for the incubated samples diluted at 1:16 were similar, with the exception of the 2-day-incubated sample. This was consistent with the results of Exp. 1, in which the observed decrease in OD values occurred only during the early stage of deterioration. When deteriorated samples were diluted up to 1:16, the OD values remained above the cutoff value for a positive diagnosis (Figs. 1A and B), whereas at a dilution of 1:64 the values for the samples incubated at 30°C for 2 days or more and at 37°C for 1 day

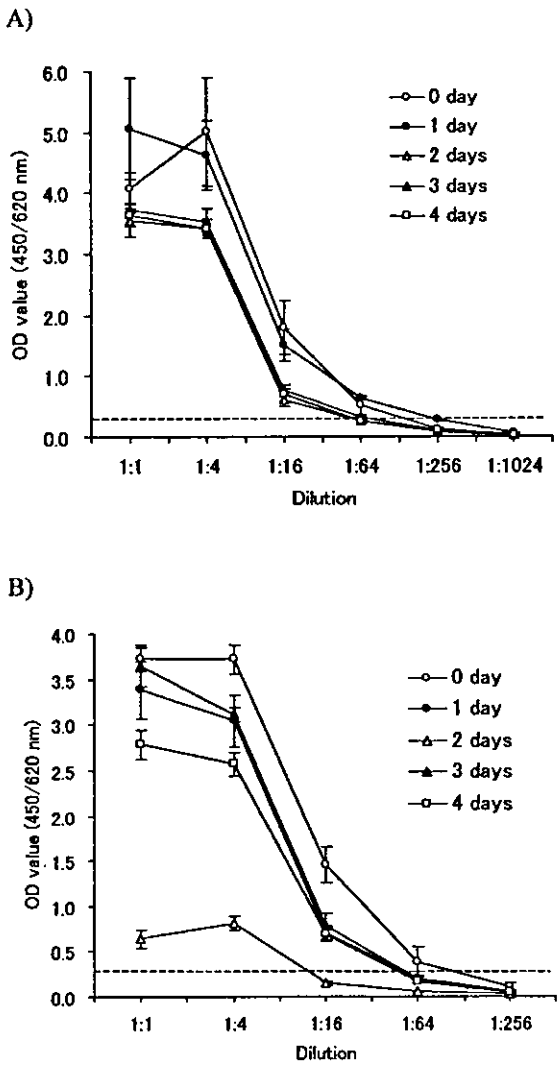

Fig. 1. ELISA results for deteriorated BSE samples. Samples were prepared from BSE-affected brain tissue incubated for 0 to 4 days, and diluted up to 1:1,024 with the dilution buffer included in the Platelia kit. Triplicate samples were assayed. The undiluted sample contained 33 mg of wet brain equivalent for examination. Broken lines indicate cutoff values. The values for the undiluted samples and samples diluted 1:4 are provided only for reference, because OD values at these dilutions diverged from the dose-dependent curve and therefore were not useful for comparison. (A) Exp. 1. Tissue was incubated at 30°C. The cutoff value was 0.23 (B) Exp. 2. Tissue was incubated at 37°C. The cutoff value was 0.25. The 2-day-incubated sample in Exp. 2, represented by white triangles, showed a markedly lower OD value than the others.

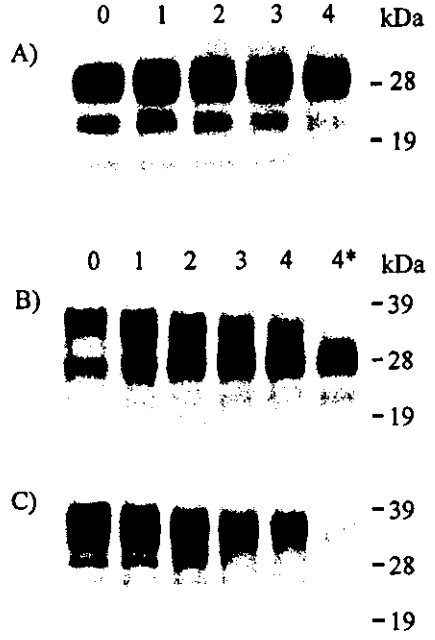

Fig. 2. Western blotting analysis of PrP^{Sc} from tissue incubated at 30°C in Exp. 1. Numbers at the top of each lane indicate days of incubation. (A) Samples treated with PK and detected with mAb T2. (B) Samples prepared without PK treatment and detected with T2. (C) Samples prepared without PK treatment and detected with mAb 1H7. Lane 4* in Figs. B and C represent PK-digested control of the 4-day-incubated sample. The equivalent of 500 μg of wet brain tissue was loaded in each lane. Molecular mass markers (kDa) are shown on the right.

or more, fell below the cutoff value (Fig. 1B). This suggests that the accuracy of diagnosis of BSE based on ELISA testing of deteriorated cattle brain tissues may be unreliable if the suspect tissue contains less than $1/64^{th}$ the amount of PrP^{Sc} particles that were present in the BSE brain tissues used here.

Detection of PrP^{Sc} by Western Blotting: As shown in Fig. 2A, PrP^{Sc} was consistently detected by mAb T2 in all samples after PK treatment. The intensity of the PrP^{Sc} signals in respective samples incubated for various time periods remained almost the same, i.e., sample deterioration did not affect the signal intensity. When PK treatment was omitted, the differential signal intensity of two major bands, of 32–36 and 27–30 kDa, shifted, depending on how long the tissue was incubated. The signal of the 32–36-kDa band, which represents full-length PrP, gradually diminished with increasing incubation time, whereas that of the 27–30-kDa band, representing PrPcore, became dominant after incubation for 3 days or more (Fig. 2B). Consistent with this shift, the PrP signal detected by mAb 1H7, which recognizes the octarepeat region, decreased depending on the incubation time of the tissue (Fig. 2C). In Exp. 2, one sample (incubated for 2 days) showed a remarkably lower OD value in the ELISA; nevertheless, the WB result showed that there was no less PrP^{Sc} in this sample than in the other samples, and that it still harbored the N-terminal sub-region (octare-