Discussion

To generate a diverse panel of mAbs to PrP molecules, we established a variety of hybridomas by using rMoPrP and $MoPrP^{Sc}$ purified from scrapie-affected mice brain as immunogens. According to the extensive epitope analyses using rPrP and pepspot membrane, our mAb panel contained mAbs recognizing at least seven different linear epitopes and three discontinuous epitopes. Five of seven linear epitopes were located within the N-terminal half of the PK-resistant core of PrP^{Sc} (aa 119–127, 137–143, 143–149, 147–151, and 163–169). This region is thought to undergo a major conformational change from random coil or α-helix- to β-sheet-rich structure during the conversion of PrP^{C} to PrP^{Sc}. Among the antibodies, mAb 132, recognizing the epitope aa 119–127 (AVVGGLGGY), is of particular interest. This region is adjacent to the highly amyloidogenic sequence AVAAAAVA (aa 112–119) (Gasset et al., 1992) and the first short β-strand (aa 128–131). Studies have shown that this region plays an important role in the conversion of PrP^{C} to PrP^{Sc} (Holscher et al., 1998; Muramoto et al., 1996). In addition, this region is highly conserved between mammals and birds, suggesting the importance of this region in PrP^{C} biology (Wopfner et al., 1999). Thus, mAb 132 will facilitate studies of how this region is involved in the conversion process as well as how PrP^{C} functions. The epitope for mAb 43C5 (aa 163–169) on PrP^{C} is of also interest because this region, in conjunction with its C-terminal portion, is thought to be a binding domain for an unidentified factor tentatively named protein X, which is expected to act as a molecular chaperon during the conversion process (Kaneko et al., 1997). mAb 43C5 will also be a good tool for studying how this region is involved in the intermolecular interaction. In addition, the first α-helix on PrP^{C} may undergo α to β conformational change during the conversion process, although this has not been fully clarified (Zhang et al., 1995). The mAbs in groups IIb, IIc, and IId recognizing the first α-helix and its immediate N-terminal portion will therefore contribute to understanding of structural differences in this region.

Elucidation of the PrP^{Sc} structure is an important problem to understand the identity of prion. Although the model structure of PrP^{Sc} and its aggregates were recently proposed from electron crystallography (Wille et al., 2002), their atomic structures remain to be elucidated. Studies of antibody accessibility will help to clarify PrP^{Sc} structure (Kanyo et al., 1999). Our mAbs did not show intense reactivity to PK-treated PrP^{Sc}, which is associated with prion infectivity, although they strongly react with PrP^{Sc} after denaturation. This suggests none of the epitopes recognized by our mAb panel are accessible by mAbs on PrP^{Sc} aggregates. The epitope at the C-terminus of PrP^{Sc} is reported to be accessible to antibody (Peretz et al., 1997; Williamson et al., 1998). In that study, the authors used DLPC treatment, which can disperse the PrP^{Sc} aggregates into liposome and reduce particle size of PrP^{Sc} aggregates (Gabizon et al., 1987). Although we confirmed that DLPC treatment could reduce the PrP^{Sc} aggregate size, some mAbs including one recognizing the C-terminus showed a trace of reactivity even when we used DLPC-treated PrP^{Sc}. In contrast, denaturation of DLPC-treated PrP^{Sc} was required to expose the cryptic epitopes. This implies that DLPC treatment might not be sufficient to expose the cryptic epitope(s) on PrP^{Sc}. There are some differences in experimental conditions between our investigations and those of Peretz et al. that could explain the differences in our results. First, they used Sc237 hamster scrapie, while we used the Obihiro strain of mouse-adapted scrapie. Second, preparation of PrP^{Sc} for ELISA also varied. Peretz et al. treated PrP^{Sc} with PK and the resulting PrP^{27-30} was dispersed into liposomes. In contrast, we performed DLPC treatment first after which DLPC-treated PrP^{Sc} was digested with PK to eliminate PK-sensitive PrP, which is expected to possess some exposed epitopes. Third, they used streptavidin-coated plates to immobilize the PrP^{Sc} after biotinylation, while, in this study, we directly adsorbed PrP^{Sc} to the ELISA plate by possible hydrophobic interaction. Finally, the antibodies used in the two studies were different. Although pepspot analysis demonstrated that mAbs 39 and 147 recognize an extreme C-terminal part of PrP, we used purified IgG instead of a smaller single-strand Fab fragment. We do not know the reason for the difference in the mAb reactivity to the C-terminus in our results and those reported by Peretz et al., it is conceivable that these differences in the experimental conditions might influence the results.

Denaturation of PrP^{Sc} aggregates caused the exposure of cryptic epitopes (Serban et al., 1990; Williamson et al., 1996). Here we observed that the sensitivity to denaturant varies between the epitopes. In DLPC-treated PrP^{Sc}, the epitopes for mAb 118 and 43C5 appeared to be more resistant to denaturation as determined by the ratio of OD at 2 to 6 M GdnHCl treatment. This difference implies complexity in the inter- or intramolecular interactions involved in the formation of PrP^{Sc} aggregates. It is of particular interest to examine what kind of inter- or intramolecular interactions determine prion infectivity or if exposure of certain epitopes correlates to prion inactivation. Our data also showed that the DLPC-treated PrP^{Sc} is more sensitive to denaturant than DLPC-untreated PrP^{Sc}, indicating that prion inactivation methods are possibly influenced by the state of PrP^{Sc} aggregation and environment.

The epitopes for mAbs raised against rPrP seemed to be relatively restricted, and 9 of 14 mAbs recognized a discontinuous epitope within aa 155–231, indicating this epitope on rPrP was immunodominant in $PrP^{-/-}$ mice. In contrast, the epitopes for the mAbs raised against PrP^{Sc} broadly spanned the PrP molecules. Furthermore, 13 of 14 hybridomas from mice immunized with rPrP secreted IgG1, although hybridomas secreting IgG2b were predom-

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 **TA**CTGATCCACTGGCCTG-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 **TC**A 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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