Vet Pathol 40:723–727 (2003)

Experimental Transmission of Abnormal Prion Protein (PrPsc) in the Small Intestinal Epithelial Cells of Neonatal Mice

M. Okamoto, H. Furuoka, M. Horiuchi, T. Noguchi, K. Hagiwara, Y. Muramatsu, K. Tomonaga, M. Tsuji, C. Ishihara, K. Ikuta, and H. Taniyama

Abstract. Using an immunohistochemical method, we attempted to detect the transmission of abnormal prion protein (PrPsc) to the enterocytes of the small intestine of neonatal mice by oral exposure with sheep brain affected by scrapie. Five 1-day-old neonatal mice were exposed by oral inoculation to the homogenized brain of a scrapie-affected sheep. In the small intestine of all mice 1 hour after inoculation, immunoreactivity with antinormal prion protein (PrPc) antibody was seen in the cytoplasm of villus enterocytes. This finding suggests transmission of abnormal PrPsc into the cytoplasm of enterocytes. In control mice treated with normal sheep brain, no PrPc signal was seen in enterocytes of the small intestine. Immunopositivity for neurofilament protein and glial fibrillary acidic protein was seen in the cytoplasm of enterocytes of mice inoculated with scrapie and normal sheep brain. This suggests that the enterocytes of neonatal mice can absorb PrPsc and other macromolecular proteins of the sheep brain affected by scrapie and may be more important than previously thought as a pathway for PrPsc transmission in neonatal animals.

Key words: Mice; PrPsc; absorption; enterocyte; immunohistochemistry.

Animal-transmissible spongiform encephalopathies or prion diseases, including ovine scrapie and bovine spongiform encephalopathy (BSE), are caused by acquisition of abnormal prion protein (PrPsc). Replication of the PrPsc depends critically on the host normal prion protein (PrPc), which develops into an abnormal, detergent-insoluble, proteinase-resistant isoform, PrPsc, in affected tissues such as the central nervous system (CNS) and lymphoid tissues.[3] However, how and when animals acquire PrPsc in the course of natural scrapie infection is not known. In experimental studies involving intragastric or oral inoculation of rodents with PrPsc from scrapie brain, PrPsc was detected in Payer's patches, gut-associated lymphoid tissues,[7,9,10] and ganglia of the enteric nervous systems before its detection in the CNS.[2,14] After the ingestion of PrPsc, uptake by the small intestine may follow either M-cell dependent or M-cell independent routes.[5] However, little is known about the transport mechanisms by which PrPsc reaches the germinal centers of lymphoid tissues from the gut lumen.

In neonatal animals, histologically, distended vacuoles

Fig. 1. Medulla oblongata; scrapie infected sheep, positive PrPc signal is seen in neuropil with spongiform change. ABC method, Mayer's hematoxylin counterstain. Bar = 20 mm.

Fig. 2. Small intestine; mouse, 1 hour after inoculation with homogenized sheep brain with scrapie. Positive signal for PrPc is present in the intralumeral sheep brain tissue (*arrows*) and in an enterocyte (*arrowhead*). ABC method, Mayer's hematoxylin counterstain. Bar = 30 mm.

Fig. 3. Small intestine; mouse, 1 hour after inoculation with homogenized sheep brain with scrapie. Positive signal for PrPc is present in the apical and basilar cytoplasm of villus enterocytes (*arrows*). ABC method, Mayer's hematoxylin counterstain. Bar = 20 mm.

Fig. 4. Small intestine; mouse, 1 hour after inoculation with homogenized sheep brain with scrapie. Immunostain omitting PrPc antibody shows no PrPc signal in enterocytes. ABC method, Mayer's hematoxylin counterstain. Bar = 20 mm.

containing colostral protein are frequently found in the cytoplasm of enterocytes lining villi of the small intestine, suggesting intestinal macromolecular uptake by pinocytosis during the first weeks after birth.[1,15] In this study, using immunohistochemical methods, we attempted to detect the entry of PrPsc into the enterocytes of the small intestine of neonatal mice following oral exposure to sheep brain containing abnormal prion of scrapie.

The first occurrence of ovine scrapie in Japan was reported in 1984.[13] Mice inoculated with fresh brain homogenates of an affected sheep showed the clinical signs and histopathologic lesions of scrapie after a long incubation period.[11]

Five 1-day-old neonatal mice were exposed by oral inoculation to sheep brain containing PrPsc. The sheep brain used for this study had been fixed in 10% formalin solution for 7 days and embedded in paraffin for 20 yrs.[13] Deparaffinized tissues (100 mg) of the medulla oblongata of the sheep were homogenized in 1 ml phosphate-buffered saline (PBS) with a polytron for 3 minutes, and 20 ml of homogenized brain was used for each peroral inoculation. For immunohistochemical controls, tissue sections were stained by procedures that omitted only the primary antibody. For negative controls, three neonatal mice were exposed by oral inoculation to normal sheep brain fixed in 10% formalin, embedded in paraffin, and extracted by identical procedures. All inoculated mice were sacrificed under anesthesia 1 hour after inoculation. Duodenum and jejunum were fixed in 4% paraformaldehyde in PBS for 24 hours and embedded in paraffin. Thin paraffin sections (4 mm) of the small intestine of mice

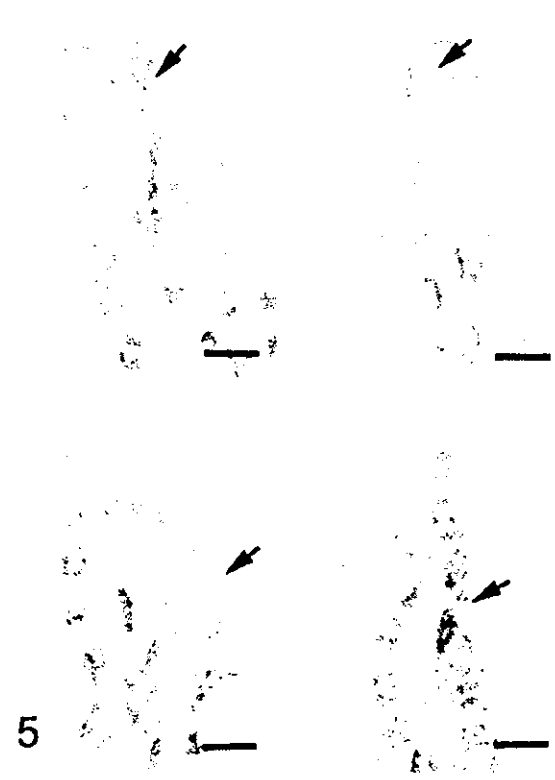

Fig. 5. Small intestine; mouse, 1 hour after inoculation with homogenized sheep brain with scrapie. There is PrPc signal mainly in the basilar cytoplasm of enterocytes at tip of the villus adjacent to the nucleus (arrows). ABC method, Mayer's hematoxylin counterstain. Bar = 10 mm.

and the medulla oblongata of the sheep brain affected by scrapie were deparaffinized and stained with hematoxylin and eosin (HE).

Histologic sections of sheep brain and mouse small intestine were stained immunohistochemically by the avidin-biotin-peroxidase complex (ABC) procedure (ABC-peroxidase staining kit (Elite; Vector Laboratories, Burlingame, CA). The specific antibody used in the present study was an affinity-purified polyclonal rabbit anti-PrPc antibody described in previous reports.[6,12] The synthetic peptide used as immunogen was B-103, corresponding to bovine PrP codons 103–121. This polyclonal antibody reacted strongly with PrPc-enriched fractions of brain tissues of cattle, sheep, and mouse on Western blots.[6]

Deparaffinized tissue sections for PrP immunostaining were autoclaved at 121 C for 30 minutes after immersion in 98% formic acid for 30 minutes and in 60 μg/ml proteinase K (Wako Pure Chemical Industries, Tokyo, Japan) in 0.1 mol/liter phosphate buffer saline for 5 minutes. Sections

→

Fig. 6. Small intestine; mouse, 1 hour after inoculation with homogenized sheep brain with scrapie. GFAP signal is seen in the apical cytoplasm of enterocytes (*arrows*). ABC method, Mayer's hematoxylin counterstain. Bar = 20 mm.

Fig. 7. Small intestine; mouse, 1 hour after inoculation with homogenized sheep brain with scrapie. NF signal is observed in the apical and basilar cytoplasm of enterocytes (*arrows*). ABC method, Mayer's hematoxylin counterstain. Bar = 20 mm.

Fig. 8. Small intestine; mouse, 1 hour after inoculation with homogenized brain of normal sheep (*). No PrPc signal is seen in the cytoplasm of enterocytes. Tissues were subjected to multiple pretreatments. ABC method, Mayer's hematoxylin counterstain. Bar = 50 mm.

were incubated with primary antibodies at room temperature for 30 minutes. After washing with distilled water, sections were incubated with 0.5% biotinylated goat antirabbit antibody for 30 minutes at room temperature and with ABC conjugated peroxidase for 30 minutes. Color was developed in a 0.05% 3,3′-diaminobenzidine solution. Tissue sections without pretreatment were incubated with rabbit antibody to bovine glial fibrillary acidic protein (GFAP) (Dako, Co., Carpinteria, CA) and a rabbit antibody cocktail to human neurofilament (NF) (Affinity Research Products Limited, Mamhead, UK); these proteins served as markers of nervous tissue.

In the brain of the sheep with scrapie, cytoplasmic vacuolation of neurons and spongiform change of the gray matter were identified in the HE sections of the medulla oblongata.[13] Immunoreactivity for PrPc was found in the neuropil of the medulla oblongate (Fig. 1).

In the small intestine of all five mice given scrapie-affected brain 1 hour prior to being killed, sheep brain tissue was present in the gut lumen. Immunopositive signals for PrPc were found in the intralumenal sheep brain tissue (Fig. 2) and the apical and basilar cytoplasm of villus and crypt enterocytes (Fig. 3) in the small intestine of all mice treated with scrapie brain. When PrPc antibody was omitted, no positive signals for PrPc were found in enterocytes (Fig. 4). PrPc-positive cells ranged from 1 to 2 per high-power field (Fig. 5). Positive signals for GFAP and NF were found in the medulla oblongata of the sheep brain with scrapie and in enterocytes of the small intestine of all mice inoculated with scrapie brain (Figs. 6 and 7).

In all mice given normal brain, no PrPc was seen in the intralumenal brain tissue or enterocytes of the small intestine (Fig. 8), but signal for GFAP- and NF-positive signals was found in the enterocytes.

For immunohistochemical detection of abnormal PrPsc in the brain, several pretreatments have been employed, including picric acid, formic acid, steam autoclaving, and microwave treatment, because epitope retrieval after formalin fixation and paraffin embedding of the brain tissue is very difficult.[4] Without pretreatment there is a high probability of false-negative immunostaining.[4] Therefore, single or multiple pretreatments are necessary for immunohistochemical detection of the PrPsc. In the present study, we used multiple pretreatments. Immunopositivity for PrPc was detected in the sheep brain with scrapie and in enterocytes of mice inoculated with sheep brain containing the abnormal scrapie prion. The immunohistochemical results suggest entry of PrPsc into the cytoplasm of enterocytes. However, absorption of sheep brain containing PrPsc occurred only in enterocytes in the duodenum and jejunum in this study. Intralumenal sheep brain was observed only as far distally as the jejunum. This material could not be observed in the ileum in mice at only 1 hour after ingestion. Observation of absorbed PrPsc in the ileum would require being killed later. We have demonstrated absorption of PrPsc in the jejunum and ileum of adult mice in another study (M. Okamoto, T. Noguchi, and H. Tamiyama, unpublished data). It is not clear from our studies whether different segments of the small intestine differ in their ability to absorb the macromolecular proteins of sheep brain.

In Germany, milk substitutes made from bone meal and fat of diseased animals are believed to play an important role in the transmission of BSE in cattle.[8] In the present study, immunohistochemistry suggests that PrPsc of sheep brain affected by scrapie enters neonatal murine enterocytes by pinocytosis. Thus, the results support the possibility that PrPsc included in milk substitutes may be absorbed by enterocytes of the small intestine of the neonate and may thus be an important pathway for neonatal PrPsc transmission in sheep, cattle, and mice. The mechanism of PrPsc transmission from enterocytes to the germinal centers of lymphoid tissues or peripheral nerve systems remains unclear.

Acknowledgement

The present study was supported in part by a grant from Gakujutsu-Frontier cooperative research in Rakuno Gakuen University.

References

1 Barker LK, van Dreumel AA, Palmer N: The alimentary system. *In*: Pathology of Domestic Animals, ed. Jubb KVF, Kennedy PC, and Palmer N, 4th ed., vol. 2, pp. 74–83, Academic Press, New York, 1993

2 Beekes M, McBride PA: Early accumulation of pathological PrP in the enteric nervous system and gut-associated lymphoid tissue of hamsters orally infected with scrapie. Neurosci Lett **278**:181–184, 2000

3 Bueler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C: Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature **356**:577–582, 1992

4 Everbroeck BV, Pals P, Martin JJ, Cras P: Antigen retrieval in prion protein immunohistochemistry. J Histochem Cytochem **47**:1465–1470, 1999

5 Ghosh S: Intestinal entry of prions. Z Gastroenterol **40**: 37–39, 2002

6 Horiuchi M, Yamazaki N, Ikeda T, Ishiguro N, Shinagawa M: A cellular form of prion protein (PrPc) exists in many non-neuronal tissues of sheep. J Gen Virol **76**: 2583–2587, 1995

7 Huang FP, Farquhar CF, Mabbott NA, Bruce ME, MacPherson GG: Migrating intestinal dendritic cells transport PrPSc from the gut. J Gen Virol **83**:267–271, 2002

8 Kamphues J, Zentek J, Oberthur RC, Flachowsky G, Coenen M: Animal-derived feeds as possible vectors for bovine spongiform encephalopathy (BSE) in Germany. 1. Comparative risk assessment for a single animal food of animal origin. Dtsch Tierarztl Wochenschr **108**:283–290, 2001

9 Maignien T, Lasmezas CI, Beringue V, Dormont D, Deslys JP: Pathogenesis of the oral route of infection of mice with scrapie and bovine spongiform encephalopathy agents. J Gen Virol **80**:3035–3042, 1999

10 McBride PA, Eikelenboom P, Kraal G, Fraser H, Bruce ME: PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. J Pathol **168**:413–418, 1992

11 Shinagawa M, Matsuda A, Sato G, Takeuchi M, Ichijo S, Ono T: Occurrence of ovine scrapie in Japan: clinical and histological findings in mice inoculated with brain homogenates of an affected sheep. Jpn J Vet Sci **46**:913–916, 1984
12 Takahashi H, Takahashi RH, Hasegawa H, Horiuchi M, Shinagawa M, Yokoyama T, Kimura K, Haritani M, Kurata T, Nagashima K: Characterization of antibodies raised against bovine-PrP peptides. J Neurovirol **5**:300–307, 1999
13 Taniyama H, Ichijo S, Ono T: The occurrence of scrapie of sheep in Japan. Jpn J Vet Sci **46**:741–744, 1984
14 Van Keulen LJ, Schreuder BE, Vromans ME, Langeveld JP, Smits MA: Scrapie-associated prion protein in the gastrointestinal tract of sheep with natural scrapie. J Comp Pathol **121**:55–63, 1999
15 Walker WA, Abel SN, Wu M, Bloch KJ: Intestinal uptake of macromolecules. V. Comparison of the vitro uptake by rat small intestine of antigen-antibody complexes prepared in antibody or antigen excess. J Immunol **117**: 1028–1032, 1976

Request reprints from Dr. H. Taniyama, Department of Veterinary Pathology, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyodai-Midorimachi, Ebetsu, Hokkaido 069-8501 (Japan). E-mail: taniyama@rakuno.ac.jp.

FULL PAPER *Virology*

Susceptibility of Transgenic Mice Expressing Chimeric Sheep, Bovine and Human PrP Genes to Sheep Scrapie

Altangerel GOMBOJAV[1,6], Ikuko SHIMAUCHI[1], Motohiro HORIUCHI[1], Naotaka ISHIGURO[1]*, Morikazu SHINAGAWA[1], Tetsuyuki KITAMOTO[2], Ichiro MIYOSHI[3], Shirou MOHRI[4] and Masuhiro TAKATA[5]

[1]*Laboratory of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080–8555,* [2]*Department of Neurological Sciences, Tohoku University Graduate School of Medicine,* [3]*Institute for Animal Experimentation, Tohoku University Graduate School of Medicine, Seiryou-cho, Aoba-ku, Sendai, 980–8575,* [4]*Laboratory of Biomedicine, Graduate School of Medical Science, Kyushu University, Maidashi 3–1–1 Higashi-ku, Fukuoka, 812–8582,* [5]*National Institute of Animal Health, Tsukuba, Ibaraki 305–0556, Japan and* [6]*School of Veterinary Medicine and Biotechnology, Mongolian State University of Agriculture, Ulaanbaatar 210153, Zaisan, Mongolia*

(Received 19 July 2002/Accepted 20 December 2002)

ABSTRACT. The use of Transgenic (Tg) mice expressing chimeric sheep/mouse (Sh/Mo) prion protein (PrP) and chimeric bovine/mouse (Bo/Mo) PrP genes was evaluated as a sheep scrapie model. We also investigated the potential for the transmission of sheep scrapie to a human/mouse (Hu/Mo) PrP Tg mouse line. The Sh/Mo PrP and Bo/Mo PrP Tg $Prnp^{+/+}$ or $Prnp^{0/0}$ mouse lines were inoculated intracerebrally with brain homogenates from three sheep with natural scrapie (KU, Y5 or S2). Incubation periods were slightly shorter in Sh/Mo PrP Tg $Prnp^{+/+}$, than in non-Tg mice inoculated with KU brain homogenate. In contrast, the incubation period was significantly prolonged ($p<0.05$) in Bo/Mo PrP Tg $Prnp^{+/+}$ mice inoculated with KU brain homogenate. The incubation period was significantly longer in all Tg $Prnp^{+/+}$ and $Prnp^{0/0}$, than in non-Tg mice ($p<0.01$) inoculated withY5 brain homogenate. None of the Tg $Prnp^{0/0}$ mice inoculated with S2 brain homogenate developed clinical signs and PrP^{Sc} was undetectable in their brains. These results suggested that expression of the Sh/Mo PrP or Bo/Mo PrP transgenes does not confer susceptibility to sheep prions upon mice, and thus none of the Tg mouse lines could be a suitable model of sheep scrapie. Hu/Mo PrP Tg $Prnp^{0/0}$ mice inoculated with natural and experimental scrapie or mouse prions did not develop clinical signs of scrapie and PrP^{Sc} was undetectable. These results suggested that neither sheep nor mouse strains of scrapie are highly transmissible to humans.

KEY WORDS: prion, PrP transgene, scrapie susceptibility, transgenic mouse.

J. Vet. Med. Sci. 65(3): 341–347, 2003

Transmissible spongiform encephalopathies (TSEs) including Creutzfelt-Jakob disease (CJD) in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in cattle are characterized by spongiosis, astrocytosis and the accumulation of an abnormal protease-resistant prion protein (PrP), termed PrP^{Sc} [14].

The transmission of prions between species is often stochastic, characterized by prolonged incubation periods or even the absence of clinical signs and symptoms during the first passage in a new host. The reduced susceptibility of one species to prions derived from another is due to the "species barrier" [12]. Studies using transgenic mice expressing PrP transgenes have conclusively shown that the PrP gene is the primary determinant that controls susceptibility to foreign prions [1, 3, 8, 15–18, 20, 21]. Moreover, the incubation period of TSEs in transgenic mice is inversely proportional to the level of PrP protein produced in the host brain [13, 22].

Sheep are natural hosts for scrapie, but many restrictions affect the use of these animals to study this disease. Such limitations include the high cost of large numbers of animals, space and facilities, biohazard problems and long incubation periods. Therefore, reliable and practical animal models of sheep scrapie are required to bioassay of sheep prions.

Tg mice expressing the sheep PrP gene would be useful tools with which to bioassay sheep scrapie [13]. A host factor, provisionally called "protein X", may be involved in species-specific prion propagation [20]. In the human/mouse Tg system, the "protein X" effect can be overcome using chimeric human and mouse PrP (MHu2M PrP) [14, 20].

We developed Tg mice expressing a chimeric PrP gene (Sh/Mo or Bo/Mo PrP) and assessed their susceptibility to sheep prions with a view to using these mice as a model of sheep scrapie. We also investigated the potential transmission of sheep scrapie to the Hu/Mo PrP Tg mouse line.

MATERIALS AND METHODS

Tg mice: Four lines of Tg mice, #4, #20, #50 and #61 expressing chimeric sheep and mouse PrP genes (Sh/Mo PrP Tg $Prnp^{+/+}$), and three lines of Tg mice, #10, #43 and #46, expressing chimeric bovine and mouse PrP genes (Bo/Mo PrP Tg $Prnp^{+/+}$), which were produced by crossbreeding Tg and C57BL/6 mice, were established by Kitamoto *et al.* at Tohoku University School of Medicine (unpublished data). Figure 1 shows the organization of both chimeric PrP genes. The above mice were crossbred with knockout mice

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

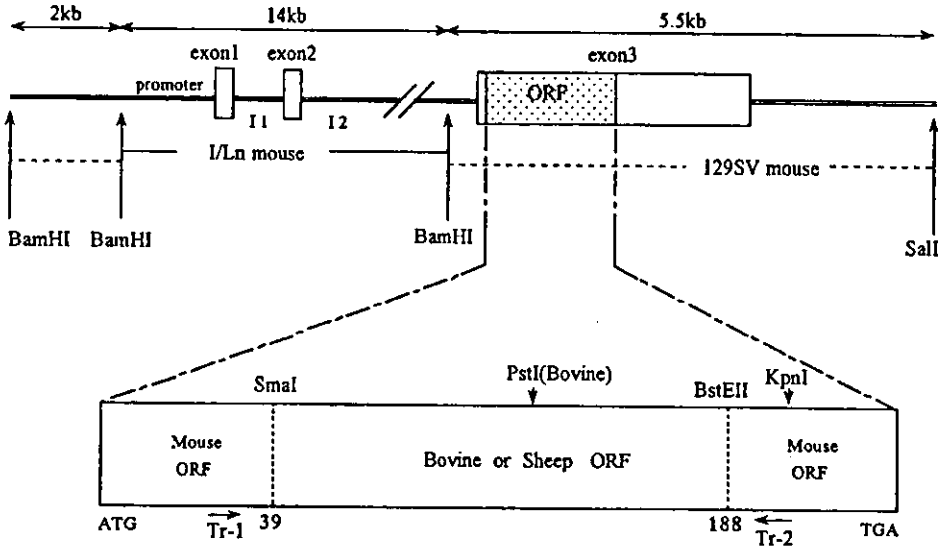

Fig. 1. Structure of chimeric PrP transgene construct. Recombinant mouse PrP gene was constructed from fragments derived from I/Ln (—) and 129SV (----) mice. Three exons indicate open boxes, and two introns indicate I1 and I2. Open reading frame (ORF) in transgenes indicated by dotted box was replaced with bovine or sheep ORF as described in Materials and Methods.

($Prnp^{-/-}$) [23], then screening these offspring for the transgene. We used Tg mice that were heterozygous for the transgene and non-transgenic littermates as controls.

Screening of transgene: Tail tissue from weaned pups was digested with 400 μl of proteinase K (150 $\mu g/ml$ in 50 mM Tris-HCl, 100 mM NaCl, 20 mM EDTA, 1% SDS, pH 8.0) at 37°C for 24 hr. Cellular DNA was extracted twice with buffered-phenol and once with chloroform:isoamyl alcohol (24:1 v/v) and precipitated with three volumes of ethanol. Mouse PrP residues 40 to 187 in the open reading frame (ORF; indicated by dotted box in Fig. 1) were replaced in the transgene with the corresponding regions of sheep PrP ORF (sheep substitutions extended from 34 to 190) or bovine PrP ORF (bovine substitutions extended from 34 to 198). The bovine ORF is a common type that contains six copies of an octarepeat sequence. The sheep ORF was derived from a sheep with the PrP amino acid polymorphisms, 136 Ala and 171 Gln. Figure 1 shows amplification of the coding region and digestion of the PrP gene. Endogenous mouse or chimeric PrP genes were differentiated by PCR using the primers, Tr-1 (5'-GCCCTCTTTGTGACTATGTGG) and Tr-2 (5'CCCCCT-TGGTGGTGGTGGTGAC). The products were digested with *Kpn* I, which cleaves the endogenous mouse PrP gene fragment at one site but not Sh/Mo PrP gene fragment, or with *Pst* I, which cleaves the Bo/Mo PrP gene fragment at one site but not the mouse PrP gene fragment.

Inoculation with sheep and mouse prions (PrP): Brains from sheep infected with natural scrapie (KU, PrP amino acid type PrP^{MARQ}/PrP^{TARQ}; Y5, PrP^{MARQ}/PrP^{MARQ} and S2, PrP^{MARQ}/PrP^{MARH}), experimental scrapie (VPH-G1, PrP^{MARQ}/PrP^{MARQ}) and the mouse adapted scrapie strain, Mo-Obihiro were sources of prions. Brain homogenates (10% w/v) in PBS were prepared by serial passage through 18-, 22- and 27-gauge needles. Mice were inoculated intracerebrally with 20 μl of 10% brain homogenate of KU, S2, VPH-G1, Mo-Obihiro and 1% brain homogenate of Y5 and S2. When death was clearly imminent, the inoculated mice were sacrificed under anesthesia. The brains were removed, cut into halves along the median line, and stored at –35°C before processing for of PrP^{Sc} detection.

Detection of PrP^{C} and PrP^{Sc}: Brain homogenate (10% w/v) from control mice was prepared in 20 mM Tris-HCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 6% Sarkosyl, 2 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5. The homogenate was stabilized for 30 min at 37°C and then separated by centrifugation at 17, 860 × g for 5 min at room temperature. The supernatant was decanted and centrifuged again at 173, 969 × g for 20 min at 20°C. The supernatant was precipitated with 10 volumes of methanol and boiled in a double-concentrated sample buffer (0.1 M Tris-HCl, 4% SDS, 2% 2-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue, pH 6.8) at 100°C for 10 min.

To detect PrP^{Sc}, brains of mice infected with scrapie were minced, suspended in 6.5 volumes of 50 mM Tris-HCl, 5 mM $MgCl_2$, 2% Triton X 100, pH 7.5 and digested with DNase I (65 μg/100 mg tissue) and collagenase (650 μg/100 mg tissue) at 37°C for 3~10 hr until tissue clumps disappeared. Sarkosyl was added to a final concentration of 6.25% and the mixture was equilibrated for 30 min at 37°C before centrifugation at 17, 860 x g for 5 min at room temperature. The supernatants were digested with proteinase K

Table 1. Reactivity of anti- PrP antibodies

Antibody	Animal	Immunogen (Synthetic Peptide)	Reactivity to PrP[a]			Dilution	Reference
			Bo	Sh	Mo		
B103	Rabbit	Bo PrP residues103–121	+	+	+	1:2000	[10]
BSPX54[b]	Mouse	Bo PrP residues146–159	+	+	–	1:5	[10]
H90	Rabbit	Ha PrP residues90–104	–	–	+	1:800	[19]

a) Reactivity to respective PrP at the indicated dilution.
b) Monoclonal antibody in culture medium.
Bo, bovine; Sh, sheep; Mo, mouse; Ha, hamster (Syrian hamster).

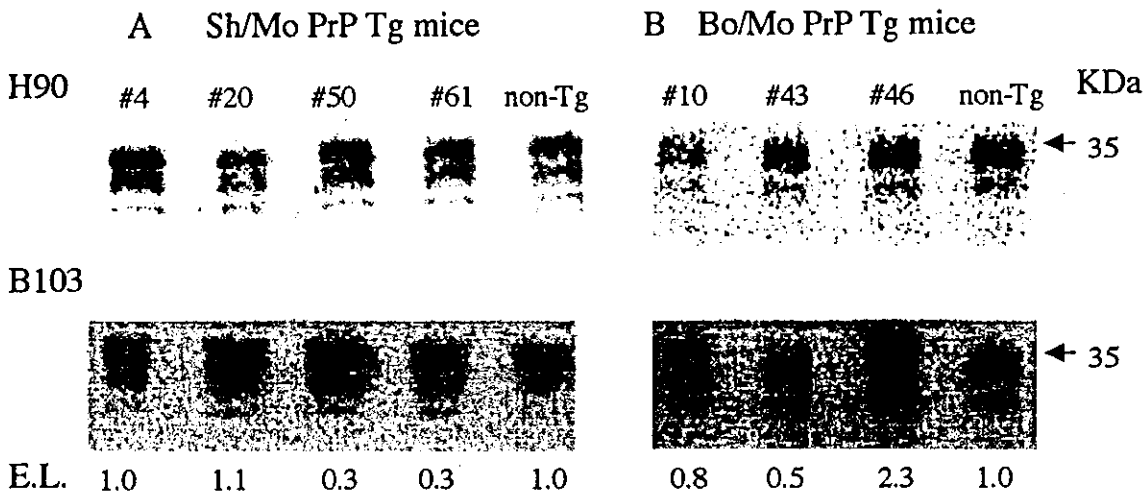

Fig. 2. Expression of PrP^C in Tg mice. Expression levels (E.L.) of PrP^C in Sh/Mo PrP Tg (A) and in Bo/Mo PrP Tg (B) mice brains estimated by relative expression of chimeric PrP gene to non-Tg PrP gene using antiserum H90 (upper panels) that reacts only with Mo PrP and antiserum B103 (lower panels) that reacts with Mo PrP, Sh PrP and Bo PrP. Membranes probed with H90 were reprobed with B103. Expression levels were estimated as described in the text. E.L.: estimated relative expression levels. Samples in A and B contained 0.5 and 0.4 mg tissue equivalent, respectively. Molecular weights of PrP^C (arrows) of 35 kDa were estimated using cytochrome *c* monomer and oligomers (Oriental Yeast, Tokyo, Japan).

(40 μg/100 mg tissue) for 2 hr at 37°C, then PMSF was added to a final concentration of 2 mM. Proteins were precipitated with nine volumes of methanol and collected by centrifugation at 17, 860 × g for 5 min at 4°C. The precipitates were dissolved in double-concentrated sample buffer at 100°C for 10 min.

Proteins were resolved by electrophoresis in 12% polyacrylamide gels containing 0.1% SDS and transferred to Hybond-PVDF membranes (Amersham, U.S.A.) using a wet blotter as described [6]. Non-specific binding was blocked using 5% skim milk, 0.2% Tween-20 in PBS on a rocking platform. PrP^{Sc}- or PrP^C- specific bands were visualized using the primary antibodies, B103, H90, or the monoclonal antibody (mAb) BSPX54 (Table 1), and the secondary antibody, horseradish peroxidase-conjugated anti-mouse or rabbit IgG (Amersham) in the ECL-Western blot detection system (Amersham). Bands were visualized and quantified using FUJIFILM LAS-1000 (FUJIFILM, Tokyo, Japan) and FUJIFILM Image Gauge (FUJIFILM, Tokyo, Japan), respectively.

RESULTS

Expression of PrP^C in Tg mice: Barriers between mouse and other species is thought to be influenced by the expression of transgene products and heterology between exogenous PrP^{Sc} and de novo PrP^C in mice. We estimated the expression of chimeric Sh/Mo PrP^C and Bo/Mo PrP^C in the brains of Tg mice by Western blotting using the antisera B103 that reacts to sheep and bovine PrP, and H90 that reacts with mouse, but not sheep and bovine PrP (Table 1 and Fig. 2). H90 antiserum detected authentic Mo PrP^C in the brains of all Tg mice (Fig. 2A and B). Authentic Mo PrP^C signals in the Tg mouse lines were quantified and normalized by comparison with the signals from non Tg mice as a standard. Signals from total PrP^C of the Tg mice in the

Table 2. Incubation periods for transmission of sheep scrapie to Tg $Prnp^{+/+}$ mice

Recipient Mouse Line	Prion strain[a]	Affected/ inoculated[b]	Incubation period (mean days ± SD)	p[c]	PrP^{Sc}[d]
Sh/Mo PrP Tg $Prnp^{+/+}$ mice					
# 50	KU	3/3	519 ± 31	0.1590	+
# 61	KU	3/3	505 ± 46	0.0837	+
Bo/Mo PrP Tg $Prnp^{+/+}$ mice					
# 10	KU	2/2	701 ± 0	0.0001	+
# 43	KU	3/3	598 ± 12	0.0116	+
# 46	KU	1/3	699	–	+
Non transgenic mice	KU	8/8	542 ± 34		+
Sh/Mo PrP Tg $Prnp^{+/+}$ mice					
# 4	Y5	8/8	512 ± 28	0.0019	+
# 20	Y5	10/10	555 ± 20	<0.001	+
# 50	Y5	3/3	493 ± 16	0.0064	+
# 61	Y5	5/5	510 ± 18	0.0003	+
Bo/Mo PrP Tg $Prnp^{+/+}$ mice					
# 10	Y5	5/5	657 ± 57	<0.001	+
# 43	Y5	6/6	598 ± 44	<0.001	+
Non transgenic mice	Y5	6/6	470 ± 5		+

a) KU, 10% brain homogenates of scrapie sheep KU; Y5, 1% brain homogenates of scrapie sheep Y5.
b) Numbers of affected/inoculated mice exclude mice dying after inoculation or developing clinical signs of scrapie.
c) P values determined by t-test.
d) All mice proteins examined by Western blotting.

same membranes reprobed with B103 antiserum were normalized by comparison with the signals from non-Tg mice in the same manner. Expression of transgene by each Tg mouse line was determined as follows. Normalized values of total PrP^C were divided by the respective normalized value of the authentic Mo PrP^C minus 1. The expression levels of the transgene in Sh/Mo PrP Tg mouse lines #4, #20, #50, and #61 were 1.0, 1.1, 0.3 and 0.3, respectively (Fig. 2A). Similarly, the levels in Bo/Mo PrP Tg mouse lines #10, #43, #46 were 0.8, 0.5, and 2.3, respectively (Fig. 2B).

Susceptibility of the Tg $PrP^{+/+}$ mice to sheep prion: Sh/Mo PrP Tg, Bo/Mo PrP Tg $Prnp^{+/+}$ and non-Tg littermate mice were inoculated with brain homogenates of sheep KU or Y5 naturally infected with scrapie as a source of sheep prion. The clinical course in affected Sh/Mo PrP and Bo/MoTg $Prnp^{+/+}$ and non-transgenic mice was characterized by progressive dysbasia, mild ataxia, whole-body tremors, ataxia, and paralysis followed by death. The incubation periods in the Sh/Mo PrP Tg $Prnp^{+/+}$ mice inoculated with KU brain homogenate seemed to be somewhat, but not significantly shorter than that in the non-Tg control. However, incubation periods were significantly prolonged in Sh/Mo PrP Tg $Prnp^{+/+}$ mice inoculated with the Y5 brain homogenate ($p<0.01$) and in Bo/MoTg $Prnp^{+/+}$ mice inoculated with KU and Y5 brain homogenates ($p<0.01$) (Table 2).

Detection of PrP^{Sc} in the brains of Tg mice: We analyzed the types of PrP^{Sc} accumulated in the brains of Sh/Mo PrP and Bo/Mo PrP Tg mice inoculated with scrapie KU homogenates using the mouse PrP specific antibodies H90 and the sheep/bovine PrP specific mAb BSPX54. Brains from the mice inoculated with sheep scrapie and the Obihiro strain [19] served as controls for Sh PrP^{Sc} and Mo PrP^{Sc} respectively (Fig. 3). Mo PrP^{Sc} was detected with H90 but not with mAb BSPX54, while Sh PrP^{Sc} was detected only with mAb BSPX54 (Fig. 3). Based on the antibody specificity, both Mo PrP^{Sc} and Sh/Mo PrP^{Sc} were detected in the brains of Sh/Mo PrP Tg mice #50 and #61 that developed symptoms of scrapie signs with KU (Fig. 3A). This indicates that the product of the transgene, Sh/Mo PrP^C, could be a substrate for the propagation of prions in Sh/Mo PrP Tg mice. However, less Sh/Mo PrP^{Sc} than Mo PrP^{Sc} apparently accumulated, indicating that authentic Mo PrP^C was dominant in Sh/Mo PrP Tg mice even though they were inoculated with sheep prions. Furthermore, Bo/Mo PrP^{Sc} in Bo/Mo PrP Tg mice inoculated with KU sheep prions was undetectable, even though Mo PrP^{Sc} was detected (Fig. 3B).

Susceptibility of the Tg $PrP^{0/0}$ mice to sheep prions: To eliminate the expression of de novo mouse PrP^C, Sh/Mo PrP Tg and Bo/Mo PrP Tg $Prnp^{0/0}$ mice were inoculated with brain homogenates containing the natural scrapie prions Y5 or S2. Incubation periods were significantly prolonged in

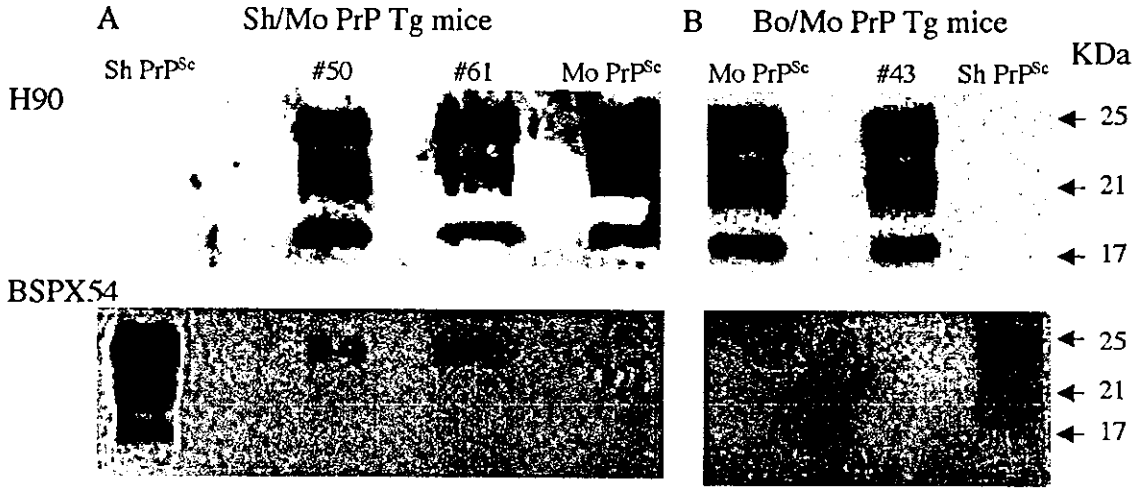

Fig. 3. Detection of PrP^{Sc} in Tg mice inoculated with sheep prions from KU. Types of PrP^{Sc} accumulated in the brain of Sh/Mo PrP Tg mice (A) and Bo/Mo PrPTg mice (B) inoculated with scrapie sheep KU brain homogenates were analyzed using mouse PrP specific antibodies H90 (upper panels) and sheep/bovine PrP specific mAb BSPX54 (lower panels). Sh PrP^{Sc}, PrP^{Sc} from sheep with scrapie; Mo PrP^{Sc}, PrP^{Sc} from ICR mice with scrapie. Molecular weights of PrP^{Sc} bands indicated by arrows estimated using cytochromec monomer and oligomers (Oriental Yeast, Tokyo, Japan).

Sh/Mo PrP Tg $Prnp^{0/0}$ mouse lines inoculated with sheep prion Y5 ($p<0.001$). However, Bo/Mo PrP Tg $Prnp^{0/0}$ mouse lines inoculated with Y5 did not develop clinical signs of scrapie and PrP^{Sc} was undetectable in their brains except for one mouse (#46) in which the incubation period was 644 days. Likewise, none of the mouse lines inoculated with sheep prion S2 developed clinical signs of scrapie and PrP^{Sc} was undetectable in their brains (Table 3).

Potential transmission of sheep scrapie to Hu/Mo PrP Tg $Prnp^{0/0}$ mice: Hu/Mo PrP Tg $Prnp^{0/0}$ mice were inoculated with three natural and experimental sheep scrapie sources, namely V5, S2, VPH-G1 and Mo-Obihiro mouse scrapie prions. None developed clinical signs of scrapie and PrP^{Sc} was undetectable in their brains. In another study, when Sporadic CJD isolate was inoculated into the same mouse line, the incubation period was 156 ± 14 days [11] (Table 3). These results indicated that sheep and mouse scrapie is less transmissible to humans.

DISCUSSION

We established Tg mice (Sh/Mo PrP Tg mice) expressing the Sh/Mo chimeric PrP gene, the organization of which is similar to that of the MHu2M PrP gene, and investigated their usefulness as a model of sheep scrapie. However, the incubation periods in Sh/Mo PrP Tg mice inoculated with sheep prions were not reduced (Tables 2 and 3). Moreover, almost all accumulated PrP^{Sc} in Sh/Mo PrP Tg mice infected with scrapie were from endogenous mouse PrP^{C} (Fig. 3A). These results suggest that Sh/Mo PrP transgene expression did not facilitate mouse susceptibility to sheep prions.

Bo/Mo PrP Tg mice are more resistant to inoculated sheep prions (Table 2). Moreover, PrP^{Sc} detected in the brains of affected Tg mice was derived only from authentic mice PrP^{C}. These results indicate that Bo/Mo PrP^{C} cannot act as a substrate for the propagation of sheep prions. During the course of this study, Scott *et al.* reported that mice deficient in the authentic PrP gene but expressing MBo2M bovine/mouse chimeric PrP^{C} (MBo2M PrP is similar to Bo/Mo PrP in this study) are completely resistant to BSE prions. In contrast, Tg mice deficient in authentic PrP but expressing bovine PrP^{C} are susceptible to BSE prions [17]. Our findings are consistent with these, since Bo/Mo PrP^{C} is less competent for the propagation of PrP^{Sc} and/or prions. Tg (BoPrP) mice expressing only bovine PrP are susceptible to BSE prion and tgOv or Tg (OvPrP4) mice expressing only sheep PrP are susceptible to sheep scrapie prions [4, 17, 22].

The susceptibility of Sh/Mo PrP Tg mice to sheep prions was not enhanced despite expression of the Sh/Mo PrP transgene perhaps because of the relatively low expression levels of Sh/Mo PrP^{C} in the Tg mice. The expression level of Syrian hamster (SHa) PrP or MH2M hamster/mouse chimeric PrP and tgOv lines expressing ovine PrP in Tg mice is inversely proportional to the incubation periods after inoculation with 263K hamster prions or with PG127 and LA404 sheep scrapie prions [13, 16, 22]. In fact, an expression level of hamster PrP^{C} that was over ten-fold higher than that of mouse PrP^{C} was required to completely overcome the species barrier effect [13]. However, the expression of Sh/Mo PrP^{C} was equal to or less than that of endogenous mouse PrP^{C}. Thus, the amount of Sh/Mo PrP^{C} expression may not have been high enough to confer susceptibility.

To eliminate the influence of mouse PrP^{C}, we repeated

Table 3. Incubation periods for transmission of sheep scrapie to Tg $Prnp^{0/0}$ mice

Recipient Mouse Line	Prion strain[a]	Affected/ inoculated[b]	Incubation period or Survival time	p[c]	PrP^{Sc} [d]
Sh/Mo PrP Tg $Prnp^{0/0}$ mice					
# 20	Y5	4/11	589 ± 88	<0.001	+
# 50	Y5	11/11	722 ± 41	<0.001	+
Bo/Mo PrP Tg $Prnp^{0/0}$ mice					
# 10	Y5	0/9	61~705[e]		-
# 43	Y5	0/6	29~705[e]		-
# 46	Y5	1/5	644		+
Non transgenic mice	Y5	11/11	431 ± 25		+
Sh/Mo PrP Tg $Prnp^{0/0}$ mice					
# 20	S2	0/8	162~724[e]		-
# 50	S2	0/8	386~726[e]		-
Bo/Mo PrP Tg $Prnp^{0/0}$ mice					
# 10	S2	0/11	169~717[e]		-
# 43	S2	0/10	539~724[e]		-
Non transgenic mice	S2	0/10	658~668[e]		-
Hu/Mo PrP Tg $Prnp^{0/0}$ mice					
	Y5	0/5	132~814[e]		-
	S2	0/4	71~862[e]		-
	VPH-G1	0/4	692~927[e]		-
	Mo- Obihiro	0/3	541~697[e]		-
	Sporadic CJD	11/11	156 ± 14[f]		+

a) S2, 10% and 1% brain homogenates of sheep S2 infected with scrapie; VPH-G1, 10% brain homogenates of sheep VPH-G1 infected with scrapie; Mo-Obihiro, 10% brain homogenates of mouse Mo-Obihiro scrapie; Sporadic CJD, 10% brain homogenates of codon 129 met/met patient via i.c.

b) Numbers of affected/inoculated mice exclude mice dying after inoculation or developing clinical signs of scrapie.

c) P values determined by t-test.

d) All mice proteins examined by Western blotting.

e) Cause of death was not due to scrapie but other causes (e.g. accident, old age).

f) Data from Mohri S *et al.*, 2000.

the same experiment using Sh/Mo and Bo/Mo PrP Tg $Prnp^{0/0}$ mice (Table 3). However, the incubation periods were not reduced. In other words, authentic mouse PrP^{C} inhibition of the conversion of PrP^{Sc} may be an insignificant factor.

Sh/Mo Tg mice may have failed to overcome the species barrier because chimeric PrP is not a suitable substrate for sheep prion propagation. If the interaction between Sh/Mo PrP^{C} and inoculated sheep prion (PrP^{Sc}) was favored over that between Mo PrP^{C} and Sh PrP^{Sc}, more Sh/Mo PrP^{Sc} would have accumulated in the brains of Sh/Mo PrP Tg mice. However, we found that PrP^{Sc} in the brains of Sh/Mo Tg mice mainly consisted of Mo PrP^{Sc} (Fig. 3A). The C-terminal part of PrP might also be involved in the process of PrP^{Sc} formation [9, 17]. If so, the C-terminal amino acids in the sheep PrP gene might have been substituted by mouse PrP amino acid residues. Codons 182–230 between sheep PrP and mouse PrP differ in only six amino acid residues [7], of which some are likely to be involved in the conversion from sheep PrP^{C} to PrP^{Sc}.

The lack of susceptibility of the transgenic and non-transgenic mice lines to inoculum S2 as compared with Y5 may be related to strain characteristics (Table 3). Several scrapie strains have different effects on experimental mice [4, 22]. The pathogenicity and incubation periods of prion strains S2 and Y5 may be different in infected mice. Furthermore, sheep scrapie S2 was only heterozygous at codon 171 Gln/Arg, therefore this genetic property may influence its pathogenicity. The structures of scrapie strains may also be different.

None of the human/mouse Tg lines of mice (Hu/Mo PrP Tg $Prnp^{0/0}$) inoculated with four natural and experimental types of scrapie developed clinical signs of scrapie and their brains were negative for PrP^{Sc} (Table 3). Incubation periods for scrapie isolates in laboratory primates were significantly

longer than those associated with Kuru and CJD isolates [5]. Moreover, a French investigation of scrapie and CJD found no epidemiological relationships between the two diseases [2]. Thus, these and the present findings support the contention that scrapie is minimally or not at all transmissible to humans.

ACKNOWLEDGEMENTS. This work was partly supported by a grant from the Ministry of Health and Welfare of Japan, from the Ministry of Agriculture, Forestry and Fisheries of Japan and a Grant-in-Aid for Science Research from the Ministry of Education, Science and Culture of Japan (12460130, 12575030, 10556069).

REFERENCES

1. Bueler, H., Aguzzi, A., Sailer, A., Greiner, R.A., Autenried, P., Aguet, M.and Weissmann, C. 1993. Mice devoid of PrP are resistant to scrapie. *Cell* **73**: 1339–1347.
2. Chatelain, J., Cathala, F., Brown, P., Raharison, S., Court, L. and Gajdusek, D.C. 1981. Epidemiologic comparisons between Creutzfeldt-Jakob disease and scrapie in France during the 12-year period 1968–1979. *J. Neurol. Sci* **51**: 329–337.
3. Collinge, J., Palmer, M.S., Sidle, K.C.L., Hill, A.F., Gowland, L., Meads, J., Asante, E., Bradley, R., Doey, L.J. and Lantos, P.L. 1995. Unaltered susceptibility to BSE in transgenic mice expressing human prion protein. *Nature (Lond.)* **378**: 21–28.
4. Crozet, C., Flamant, F., Bencsik, A., Aubert, D., Samarut, J. and Baron, T. 2001. Efficient transmission of two different sheep scrapie isolates in transgenic mice expressing the ovine PrP gene. *J. Virol.* **75**: 5328–5334.
5. Gajdusek, D.C. 1977. Unconventional viruses and the origin and disappearance of Kuru. *Science* **197**: 943–960.
6. Grathwohl, K. U., Horiuchi, M., Ishiguro, N. and Shinagawa, M. 1996. Improvement of PrPSc-detection in mouse spleen early at the preclinical stage of scrapie with collagenase-completed tissue homogenization and Sarkosyl-NaCl extraction of PrPSc. *Arch. Virol.* **141**: 1863–1874.
7. Harmeyer, S., Pfaff, E. and Groschup, H. M. 1998. Synthetic peptide vaccines yield monoclonal antibodies to cellular and pathological prion proteins of ruminants. *J. Gen. Virol.* **79**: 937–945.
8. Hill, A. F., Desbruslais, M., Joiner, S., Sidle, K. C. L., Gowland, I., Collinge, J., Doey, L. J. and Lantos, P.1997. The same prion strain causes vCJD and BSE. *Nature (Lond.)* **389**: 448–450.
9. Horiuchi, M. and Caughey, B. 1999. Specific binding of normal prion protein to the scrapie form via a localized domain initiates its conversion to the protease-resistant state. *EMBO. J* **18**: 3193–3203.
10. Horiuchi, M., Yamazaki, N., Ikeda, T., Ishiguro, N. and Shinagawa, M. 1995. A cellular form of prion protein (PrP^C) exists in many non-neuronal tissues of sheep. *J. Gen. Virol.* **76**: 2583–2587.
11. Mohri, S., Kitamoto, T., Miyoshi, I., Ishikawa, Y., Ohno, S. and Numano, J. 2000. Susceptibility for the transgenic mice expressing human/mouse chimeric prion gene to human prion (Part 3). pp. 88–93. J. Annual Report of the Slow Virus Infection Research Committee, the Ministry of Health and Welfare of Japan.
12. Pattison, I. H. 1965. Experiments with scrapie special reference to the nature of the agent and the pathology of the disease, pp. 249–257. *In*: Slow, Latent and Temperate Virus Infections. NINDB Monograph 2 (Gajdusek, D.C., Gibbs, C. J., Jr. and Alpers, M. P. eds.), U.S. Government Printing Office, Washington, D.C.
13. Prusiner, S. B., Scott, M., Foster, D., Pan, K. M., Groth, D., Mirenda, C., Torchia, M., Yang, S. L., Serban, D., Carlson, G. A., Hoppe, P. C., Westaway, D.and DeArmonds, S. J. 1990. Transgenic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* **63**: 673–686.
14. Prusiner, S. B. 1991. Molecular biology of prion diseases. *Science* **252**: 1515–1522.
15. Scott, M., Foster, D., Mirenda, C., Serban, D., Coufal, F., Walchli, M., Torchia, M., Groth, D., Carlson, G., DeArmond, S. J., Westaway, D. and Prusiner, S. B. 1989. Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell* **59**: 847–857.
16. Scott, M., Groth, D., Foster, D., Torchia, M., Yang, SL., DeArmond, S.J. and Prusiner, S. B. 1993. Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. *Cell* **73**: 979–988.
17. Scott, M. R., Safar, J., Telling, G., Nguyen, O., Groth, D., Torchia, M., Koehler, R., Tremblay, P., Walther, D., Cohen, F. E., DeArmond, S. J. and Prusiner, S. B. 1997. Identification of a prion protein epitope modulating transmission of bovine spongiform encephalopathy prions to transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* **94**: 14279–14284.
18. Scott, M.R., Will, R., Ironside, J., Nguyen, H.O.B., Tremblay, P., DeArmond, S.J. and Prusiner, S.B. 1999. Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. *Proc. Natl. Acad. Sci. U.S.A.* **96**: 15137–15142.
19. Shinagawa, M., Munekata, E., Doi, S., Takahashi, K., Goto, H. and Sato, G. 1986. Immunoreactivity of a synthetic pentadecapeptide corresponding to the N-terminal region of the scrapie prion protein. *J. Gen. Virol.* **67**: 1745–1750 .
20. Telling, G. C., Scott, M., Mastrianni, J., Gabizon, R., Torchia, M., Cohen, F. E., DeArmond, S. J. and Prusiner, S. B. 1995. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell* **83**: 79–90.
21. Telling, G. C., Scott, M., Hsiao, K. K., Foster, D., Yang, S. L., Torchia, M., Sidle, K. C., Collinge, J., DeArmond, S. J. and Prusiner, S. B. 1994. Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 9936–9940.
22. Vilotte, J.L., Soulier, S., Essalmani, R., Stinnakre, M.G., Vaiman, D., Lepourry, L., Silva, J.C.D., Besnard, N., Davson, M., Buschmann, A., Groschup, M., Petit, S., Madelaine, M.F., Rakatobe, S., Dur, A.L., Vilette, D. and Laude, H. 2001. Markedly increased susceptibility to natural sheep scrapie of transgenic mice expressing ovine PrP. *J. Virol.* **75**: 5977–5984.
23. Yokoyama, T., Kimura, M. K., Ushiki, Y., Yamada, S., Morooka, A., Nakashiba, T., Sassa, T. and Itohara, S. 2001. *In vivo* conversion of cellular prion protein to pathogenic isoforms, as monitored by conformation-specific antibodies. *J. Biol. Chem.* **276**: 11265–11271.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Virology 320 (2004) 40–51
VIROLOGY
www.elsevier.com/locate/yviro

Antigenic characterization of an abnormal isoform of prion protein using a new diverse panel of monoclonal antibodies

Chan-Lan Kim,[a] Atsushi Umetani,[b] Toshio Matsui,[b] Naotaka Ishiguro,[a] Morikazu Shinagawa,[a,1] and Motohiro Horiuchi[a,c,*]

[a] *Laboratory of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*
[b] *Obihiro Research Laboratory, Fujirebio Inc., Kawanishi-cho, Obihiro, Hokkaido 089-1111, Japan*
[c] *Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*

Received 14 July 2003; returned to author for revision 22 October 2003; accepted 22 October 2003

Abstract

We established a panel of monoclonal antibodies (mAbs) against prion protein (PrP) by immunizing PrP gene-ablated mice with the pathogenic isoform of prion protein (PrP^{Sc}) or recombinant prion protein (rPrP). The mAbs could be divided into at least 10 groups by fine epitope analyses using mutant rPrPs and pepspot analysis. Seven linear epitopes, lying within residues 56–90, 119–127, 137–143, 143–149, 147–151, 163–169, and 219–229, were defined by seven groups of mAbs, although the remaining three groups of mAbs recognized discontinuous epitopes. We attempted to examine whether any of these epitopes are located on the accessible surface of PrP^{Sc}. However, no mAbs reacted with protease-treated PrP^{Sc} purified from scrapie-affected mice, even when PrP^{Sc} was dispersed into a detergent–lipid protein complex, to reduce the size of PrP^{Sc} aggregates. In contrast, denaturation of PrP^{Sc} by guanidine hydrochloride efficiently exposed all of the epitopes. This suggests that any epitope recognized by this panel of mAbs is buried within the PrP^{Sc} aggregates. Alternatively, if the corresponding region(s) are on the surface of PrP^{Sc}, the region(s) may be folded into conformations to which the mAbs cannot bind. The reactivity of a panel of mAb also showed that the state of PrP^{Sc} aggregation influenced the denaturation process, and the sensitivity to denaturation appeared to vary between epitopes. Our results demonstrate that this new panel of well-characterized mAbs will be valuable for studying the biochemistry and biophysics of PrP molecules as well as for the immunodiagnosis of prion diseases.

Keywords: Scrapie; Prion; BSE; Monoclonal antibody; Epitope mapping

Introduction

Transmissible spongiform encephalopathies (TSEs), so-called prion diseases, are fatal neurodegenerative diseases including scrapie in sheep and goats, bovine spongiform encephalopathy, and Creutzfeldt–Jakob disease (CJD) in humans (Prusiner, 1991). The causative agent, prion, is thought to be composed solely, if not entirely, of a pathogenic isoform of the prion protein (PrP^{Sc}). PrP^{Sc} is generated from a host-encoded cellular prion protein (PrP^{C}) by certain post-translational modifications including a conformational transformation. Although the two PrP isoforms share the same primary structure (Hope et al., 1986), PrP^{Sc} is distinguished from PrP^{C} by biochemical and biophysical properties such as high β-sheet content (Pan et al., 1993; Safar et al., 1993), partial resistance to protease digestion, and insolubility in nonionic detergent (Meyer et al., 1986). The conformational transformation from PrP^{C} to PrP^{Sc} requires preexisting PrP^{Sc} as a template and is thought to be a central event in PrP^{Sc} formation and prion replication. However, the molecular mechanism of the conformational transformation remains unclear. To elucidate the mechanism, structural information from two PrP isoforms is needed. At this time, the NMR structure of a recombinant PrP resembling PrP^{C} has been determined (Riek et al., 1996), whereas the structure of PrP^{Sc} is not yet known.

* Corresponding author. Laboratory of Prion Diseases, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita, Sapporo 060-0818, Japan. Fax: +81-11-706-5293.
E-mail address: horiuchi@vetmed.hokudai.ac.jp (M. Horiuchi).
[1] Present address: Prio Disease Research Center, National Institute of Animal Health, Kannondai, Tsukuba, Ibaragi, 305–0856, Japan.

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2003.10.026

A diverse panel of anti-PrP mAbs is invaluable for analyzing the difference between PrP^C and PrP^{Sc}, as well as for the biochemical and structural analysis of PrP molecules. To date, many monoclonal antibodies (mAbs) against PrP have been generated by immunizing with PrP^{Sc} purified from scrapie-affected animals (Barry and Prusiner, 1986; Kascsak et al., 1987; Williamson et al., 1996), recombinant PrP (Zanusso et al., 1998), synthetic PrP peptides (Harmeyer et al., 1998; Horiuchi et al., 1995; O'Rourke et al., 1998), or a PrP expression plasmid (Krasemann et al., 1996). Most of the anti-PrP antibodies developed so far react with linear or discontinuous epitopes on PrP^C and recombinant PrP molecules, as well as with PrP^{Sc} treated with denaturant. These types of anti-PrP antibodies are now widely used for the immuno-detection of PrP^{Sc}. Because these antibodies themselves cannot distinguish the two PrP isoforms when the molecules are treated with denaturant, proteolytic removal of PrP^C before immuno-staining is essential for the detection of PrP^{Sc} by immunoblotting or enzyme-linked Immunosorbent assay (ELISA). One mAb, designated 15B3, generated by immunizing PrP gene-deficient mice with recombinant bovine PrP, was reported to recognize a PrP^{Sc}-specific conformational epitope consisting of two PrP molecules (Korth et al., 1997). However, further characterization of this mAb has not been published. Recent reports also describe mAbs that appear to recognize discontinuous epitopes on PrP^C but do not appear to react with PrP^{Sc} (Yokoyama et al., 2001).

Further PrP^{Sc}- and PrP^C-specific antibodies, as well as additional characterized antibodies to PrP molecules, will help to elucidate the structure–function relationships of PrP, structural differences between PrP^C and PrP^{Sc}, and mechanisms of conformational transformation. In addition, new antibodies showing higher reactivity and specificity than those currently available would help to improve the sensitivity of PrP^{Sc} detection. Although a large number of antibodies against PrP have been generated, it is possible that undefined epitopes still exist. In this study, we established a panel of mAbs that covers diverse epitopes on the mouse PrP molecules by immunizing $PrP^{-/-}$ mice with either recombinant mouse PrP or PrP^{Sc} purified from scrapie-affected mouse brains. The mAbs could be at least divided into 10 groups based on fine epitope mapping. Finally, we discuss how this panel of well-characterized mAbs will be useful for analyzing the molecular properties of PrP as well as for diagnostic purposes.

Results

Production of monoclonal antibodies against PrP molecules

For the immunization of PrP gene-ablated mice, we prepared rMoPrP as well as PrP^{Sc} from brains of scrapie-affected mice. Fig. 1 shows the purity of rMoPrP and PrP^{Sc}. rMoPrP23–231 expressed by pRSETB in *E. coli* formed inclusion bodies (Fig. 1A). The rMoPrP23–231 recovered from inclusion bodies was dissolved with 6 M GdnHCl, and further purified with Ni^{2+}-charged IMAC. After dialysis, rMoPrP possessing an intramolecular disulfide bond was

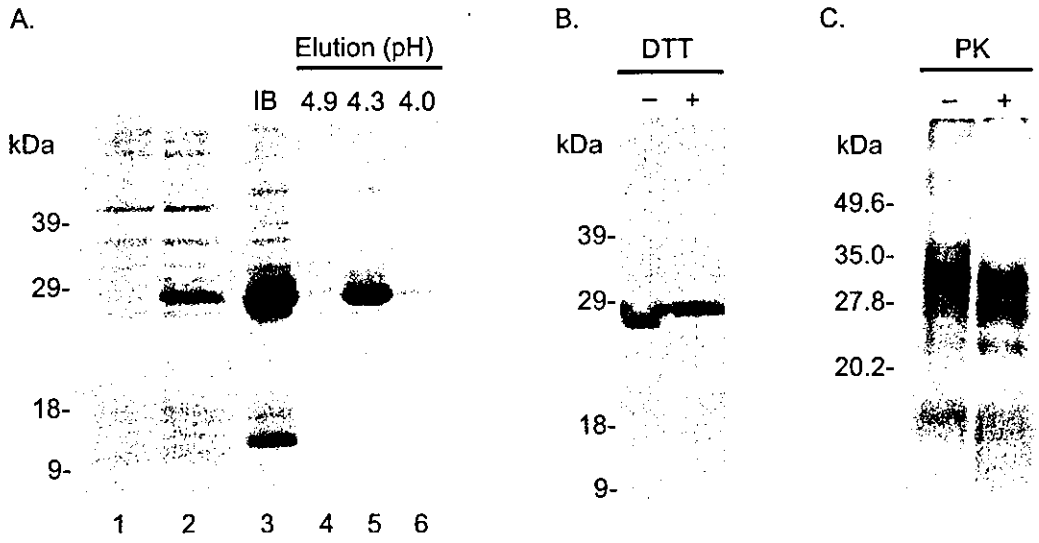

Fig. 1. Purity of rPrP and PrP^{Sc}. (A) Purification of rPrP from bacterial lysates. Whole lysates of *E. coli* transformed with pRSETB (lane 1) and pRSETB/MoPrP23–231 (lane 2) after induction with IPTG for 2 h. Inclusion bodies were prepared from whole bacterial lysates (lane 3) for partial purification of rPrP. Inclusion bodies were dissolved in 6 M GdnHCl and applied to Ni^{2+}-charged IMAC. The rPrP was eluted with a stepwise pH gradient (lanes 4–6), and rPrP eluted at pH 4.3 was dialyzed against acetate buffer to remove GdnHCl. (B) Formation of intramolecular disulfide bonds. After the dialysis, rPrP possessing an intramolecular disulfide bond was collected by reverse-phase HPLC. DTT + and – indicates purified rPrP dissolved with sample buffer with and without 100 mM DTT. (A and B) Coomassie blue staining. (C) Purity of PrP^{Sc} fraction from scrapie-affected mouse brains. The PrP^{Sc} fraction treated (+) and untreated (–) with PK was visualized by sliver staining and the purity was estimated by densitometric analysis by using Atto Densitograph (Atto Co. Ltd.). Molecular mass markers are in kilodaltons (kDa).

collected by reverse-phase HPLC (Fig. 1B). The $MoPrP^{Sc}$ prepared from brains of scrapie-affected mice was estimated to be >70% pure even without PK treatment (Fig. 1C), and a bioassay revealed that this fraction was estimated to contain more than10^{11} LD_{50}/mg from our dose–incubation time standard curve for Obihiro strain (data not shown). These PrP fractions were used for immunization of PrP gene-ablated mice, and splenocytes from immunized mice were fused with myeloma cells. Hybridomas positive for both rMoPrP and $MoPrP^{Sc}$, or those positive either for rMoPrP or $MoPrP^{Sc}$, were selected. Finally, we established 4, 10, and 15 hybridomas using rMoPrP23–231, rMoPrP89–231 or $MoPrP^{Sc}$, respectively, as the immunogen (Table 1).

Epitope analyses

To determine the epitopes recognized by the mAbs, we first examined their reactivity to various mutant rPrPs by ELISA. Based on their reactivity, the mAbs were divided into seven groups (Table 1). The mAbs in group I did not react with rMoPrP lacking the N-terminal region, indicating that their epitopes are located in residues 23–89. Group II mAbs reacted with all rMoPrPs except for rMoPrP155–231, indicating that they recognize the regions between residues 89 and 155. Group III consists of mAb 43C5, which reacted with all rMoPrPs, suggesting that its epitope is located between residues 155 and 167. The mAbs in group IV did not react with mutants lacking the C-terminus but reacted with other mutants, indicating that they recognize the epitopes on the C-terminal part of PrP molecule. The mAbs in group V reacted with rMoPrP155–231 but not with rMoPrP23–214. In addition, these mAbs did not react with point mutants rHaPrPC179A and rHaPrPC214A in which the cysteines at residues 179 and 214, respectively, were replaced with alanine. The mAbs reacted equally with rMoPrP23–231 and rHaPrP23–231 (data not shown), indicating amino acid differences between Mo and HaPrP did not influence the reactivity. These facts suggest that mAbs in groups V recognize discontinuous epitopes, including the region within residues 155–231, and that the epitopes are dependent on the intramolecular disulfide bond in the PrP molecule. The mAbs in group VI reacted with rMoPrP89–231, but neither reacted with rMoPrP155–231 nor point mutants of rHaPrP, suggesting that the epitope for these mAbs include the region within residues 89–231 and also are dependent on the intramolecular disulfide bond. The reactivity of mAb 72 (group VII) was difficult to figure out the epitope. It reacted with rMoPrP89–231 and 23–167, suggesting that the epitope is located within residues 89–167. However, the mAb did not react with rMoPrP23–214, rHaPrPC179A, or rHaPrPC214A, but reacted with rHaPrP23–231, again suggesting that the epitope depends on the presence of the intramolecular disulfide bond.

Further precise epitope mapping was carried out by pepspots analysis (Fig. 2). The reactivity of the group I mAbs revealed that they recognize a portion of octa-peptide repeat in N-terminus, although there is some difference in the amino acid sequence recognized by these mAbs. Based on the reactivity (Fig. 2A), the epitope for mAb 8 was WGQPHG at aa 56–61, 64–69, 72–77, and 80–85. mAbs 110 and 37 reacted with peptides 16–19 and 28–31, indicating that the recognized sequence is PHGGGWG at aa 59–65 and 83–89 (Fig. 2B). mAbs 40, 106, and 162 showed broad reactivity to peptides ranging from 13 to 33 (Fig. 2C). The reaction to peptides including 17 to 19 and 29 to 33 the most intense, suggesting that the major recognition sequence for these mAbs is PHGGGWGQ at aa 59–66 and 83–90, although the minimum required sequence appears to be WGQ. The group II mAb 132 reacted with peptides from 47 to 49, which share residues

Table 1
Grouping of mAbs based on the reactivity to rPrP deletion and point mutants

Group	mAb[a] (Isotype[b])	rPrP used as antigen[c]					
		rMoPrP23–167	rMoPrP23–214	rMoPrP89–231	rMoPrP155–231	rHaPrPC179A	rHaPrPC214A
I	8(2b), 37(2b), 40(2b), 106(2b), 110(2b), 162(2a)	+	+	–	–	+	+
II	13(2b), 32(2a), 118(2b), 132(G1), 149(2b), 31C6(G1)	+	+	+	–	+	+
III	43C5(G1)	+	+	+	+	+	+
IV	39(2b), 147(2b)	–	–	+	+	+	+
V	66(G1), 31B1(G1), 31B5(G1), 42B4(G1), 42D2(G1), 42D6(G1), 44A2(G1), 44A5(G1), 44B1(2a), 44B5(G1)	–	–	+	+	–	–
VI	23D9(G1), 42D3(G1), 44B2(G1)	–	–	+	–	–	–
VII	72(G1)	+	–	+	–	–	–

[a] MAbs named only with numbers were obtained by using $MoPrP^{Sc}$ as the immunogen, others named with a combination of numbers and letters were obtained using rMoPrP as the immunogen Among the latter, mAbs starting with 23 or 31, and 42, 43 or 44 were obtained by immunization with rMoPrP23–231 and rMoPrP89–231, respectively.
[b] G1, IgG1; 2a, IgG2a; 2b, IgG2b.
[c] Examined by ELISA.

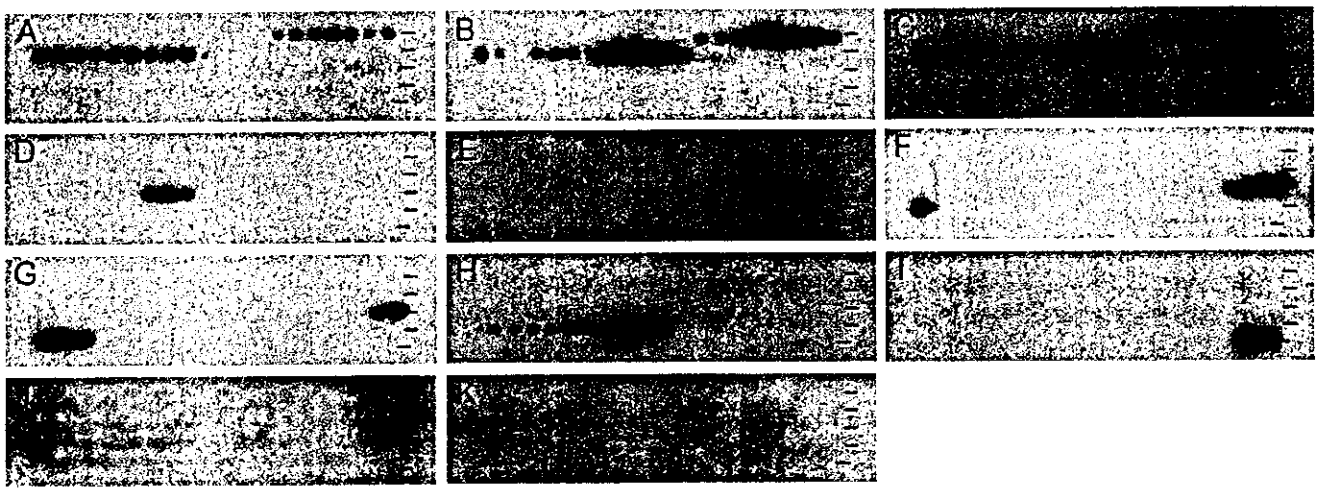

Fig. 2. Pepspot analysis. Bars on the right sides indicate lines of peptide spots. The first through fourth lines contain 20 spots per line, while fifth line contains 19 spots. (A) mAb 8 (group I); (B) mAb 110 (group I); (C) mAb 162 (group I); (D) mAb 132; (group IIa); (E) mAb 118 (group IIb); (F) mAb 31C6 (group IIc); (G) mAb 32 (group IId); (H) mAb 43C5 (group III); (I) mAb 147 (group IV); (J) mAb 72 (group VII); (K) mAb P1–284 (anti-parvovirus mAb, negative control).

119–127, AVVGGLGGY (Fig. 2D). mAbs 13 and 118 (group II) reacted with peptides from 55 to 58, which contain the fragment corresponding to residues 137–143, MIHFGND (Fig. 2E). The mAb 31C6 (group II) reacted with peptides from 58 to 61, which share in common residues 143–149, DWEDRYY (Fig. 2F). mAbs 32 and 149 (group II) reacted with five peptides from 59 to 63 that share a common sequence RYYRE, residues 147–151 (Fig. 2G). The mAb 43C5 (group III) reacted with 10 continuous spots but intense reactivity was observed to four peptides from 68 to 71, which contain the fragment corresponding to residues 163–169, RPVDQYS (Fig. 2H). This result is consistent with the epitope analysis of mutant recombinant PrPs. mAbs 39 and 147 (group IV) reacted with peptides 98 and 99, which correspond to the extreme C-terminus of PrP molecules, residues 219–229, KESQAYYDGRR (Fig. 2I). The mAbs in groups I–IV reacted with rHaPrPC179A and rHaPrPC214A in the ELISA (Table 1) and PrP^{Sc} in immunoblot analysis (Fig. 3), indicating that these mAbs recognize linear epitopes. mAb 72 appeared to recognize discontinuous epitope based on the reactivity to mutant rPrP (Table 1), although the mAb reacted with peptides 60 and 61, which share residues 143–153 (Fig. 2J).

Based on the fine epitope mapping by pepspot analysis, the mAbs in group II of Table 1 can be separated into four groups, designated IIa–IId. Together, the mAbs produced in this study can be divided into at least 10 groups; 7 that recognize linear epitopes, and 3 that recognize discontinuous epitopes (Table 2).

Species specificity of mAbs

We next examined the specificity of the mAbs by ELISA using species-specific versions of rMoPrP, rHaPrP, rShPrP, and rBoPrP. Most of the mAbs reacted with the rPrP from all four species (hamster, bovine, mouse and ovine), while the following mAbs showed obvious species-specific reactivity: mAbs 13 and 118 in group IIb, mAbs 39 and 147 in group IV, and mAb 66 reacted to Mo and HaPrP, while mAbs 42D2 and 44B5 showed intense reaction to Mo and HaPrP, moderate reaction to ShPrP but no reaction to BoPrP (data not shown).

Fig. 3 shows the reactivity to $MoPrP^{Sc}$, ovine PrP^{Sc} ($ShPrP^{Sc}$), and bovine PrP^{Sc} ($BoPrP^{Sc}$) in immunoblot analysis. mAbs 110, 132, 118, 31C6, 32, 43C5, and 147, which recognize linear epitopes, reacted with PrP^{Sc} prepared from brains of the disease-affected animals. Surprisingly, mAbs 44B1 and 72, which appeared to recognize discontinuous epitopes, showed an intense reaction to PrP^{Sc} in immunoblotting. The species-specific reactivities of mAbs 132, 118, 32, 43C5, 147, 44B1, and 72 are consistent with the results from ELISA; mAbs 118 and 147 only reacted with $MoPrP^{Sc}$ while other mAbs reacted with PrP^{Sc} from the other three species. In contrast to the results from ELISA, mAb 31C6 only reacted with $MoPrP^{Sc}$ and mAb 110 reacted with Mo and $ShPrP^{Sc}$ but not with $BoPrP^{Sc}$ in immunoblot analysis.

Reactivity of panel mAbs to purified PrP^{Sc}

To determine whether any of the antibody-reactive epitopes are exposed in the infectivity-associated PrP^{Sc}, we examined the reactivity of mAbs to purified $MoPrP^{Sc}$ by ELISA. A set of three PrP^{Sc} preparations, PK-untreated and non-denatured, PK-treated and non-denatured, and PK-treated and denatured, was used for each mAb (Fig. 4). All the mAbs reacted with PK-untreated non-denatured PrP^{Sc}, although the reactivity was lost when PrP^{Sc} was treated with PK. However, the mAbs regained reactivity when the PK-treated PrP^{Sc} was denatured with GdnHCl. These results suggest that the inability of the antibodies to react to PK-

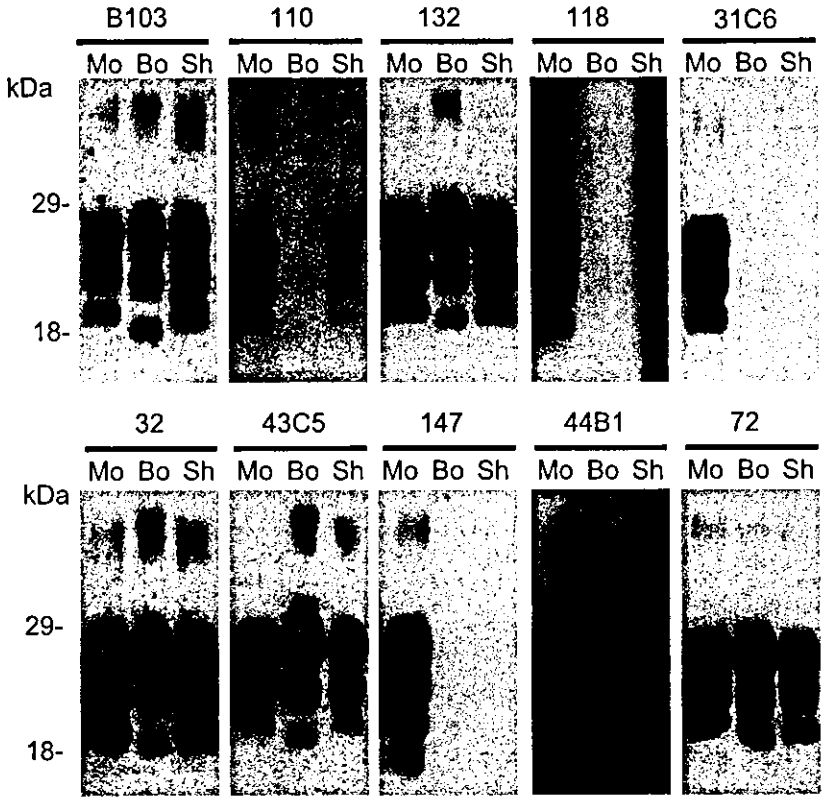

Fig. 3. Species specificity in Western blot analysis. Crude preparations of mouse (Mo), bovine (Bo), and sheep (Sh) PrP^{Sc} were prepared as described elsewhere (Grathwohl et al., 1997) and were separated by SDS-PAGE and transferred onto PVDF membranes. Brain tissue equivalents of 25 mg, 500, and 125 μg were loaded for Mo, Bo, and Sh PrP^{Sc}, respectively. The blots were stained with B-103 rabbit polyclonal antibodies (Horiuchi et al., 1995) or mAbs as indicated.

treated PrP^{Sc} is not due digestion of the PrP^{Sc}. Rather, it appears that the epitopes on PK-treated PrP^{Sc} are cryptic. A considerable amount of PK-sensitive PrP, including PrP^{C}, is co-purified during PrP^{Sc} purification in the absence of PK treatment (Caughey et al., 1995). Therefore, it is likely that reaction of the mAbs with PK-untreated non-denatured PrP^{Sc} accounts for the reaction to PK-sensitive PrP that exposes these epitopes on its accessible surface. Furthermore, the epitopes recognized by our panel of mAbs do not appear to be exposed on the surface of the PK-resistant core

Table 2
Characterization of mAbs against PrP

Group	mAb	Epitope[a]		Reactivity to PrP^{Sc} in ELISA[b]		
		position (amino acid)	L/DC	PK(−) GdnHCl(−)	PK(+) GdnHCl(−)	PK(+) GdnHCl(+)
I	8, 37, 40, 106, 110, 162	56–90	L	+	–	+
IIa	132	119–127	L	+	–	+
IIb	13, 118	137–143	L	+	–	+
IIc	31C6	143–149	L	+	–	+
IId	32, 149	147–151	L	+	–	+
III	43C5	163–169	L	+	–	+
IV	39, 147	219–229	L	+	–	+
V	66[c], 31B1, 31B5, 42B4, 42D2, 42D6, 44A2, 44A5, 44B1, 44B5[c]	155–231	DC	+(−)[c]	–	+(−)[c]
VI	23D9, 42D3, 44B2	89–231	DC	+	–	+
VII	72	89–231 (143–153)	DC	+	–	+

[a] L, linear epitope; DC, discontinuous epitope.
[b] Treatments of PrP^{Sc} are as described in Fig. 4.
[c] MAbs 66 and 44B5 reacted with rPrP but did not react with the three PrP^{Sc} preparations.

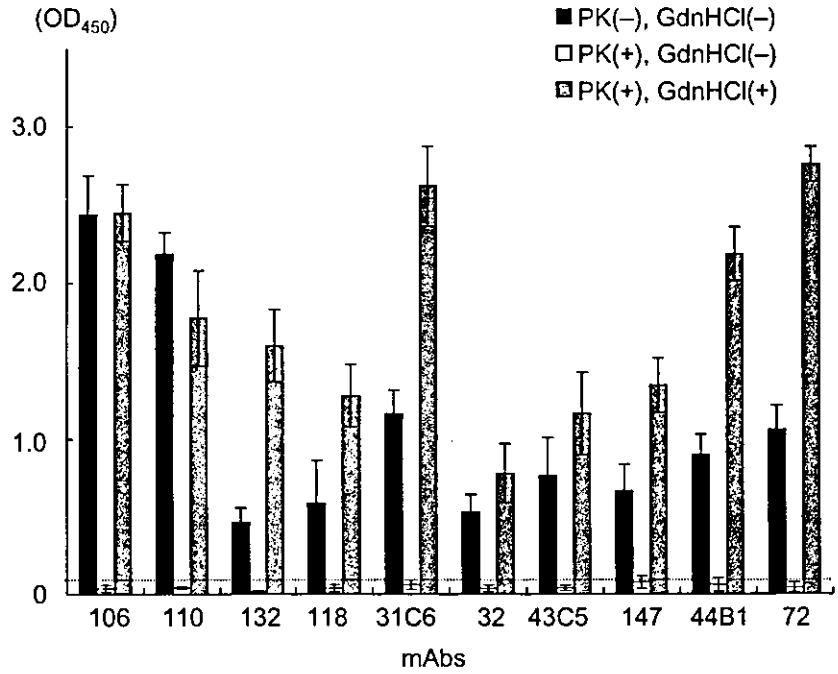

Fig. 4. Reactivity of mAbs to PrP^{Sc} fraction in ELISA. A set of three wells were used for each mAb: after the adsorption of purified PrP^{Sc} fraction, first well was neither treated with PK nor with GdnHCl [PK(−), GdnHCl(−)], second well was treated with 40 μg/ml PK for 60 min at 37 °C [PK(+), GdnHCl(−)], and third well was treated with PK and then further treated with 6M GdnHCl for 60 min to denature PrP^{Sc} [PK(+), GdnHCl(+)]. mAbs indicated in the figure were used as a representative for each group in Table 2. Dotted line indicates the cut-off value [An average OD_{450} value of negative control monoclonal antibody plus 5 times standard deviation ($n > 4$)].

of PrP^{Sc}. Instead, they become accessible to the mAbs after denaturation of PK-resistant core of PrP^{Sc}.

Reduction of PrP^{Sc} aggregate size does not expose cryptic epitopes

Purified PrP^{Sc} forms relatively large aggregates that can be precipitated by centrifugation at 10000 × *g*. We were concerned that the large aggregates themselves affect the antibody accessibility to PrP^{Sc}. To address this concern, we attempted detergent-lipid-protein complex (DLPC) treatment, which can reduce aggregate size without loss of infectivity (Gabizon et al., 1987). As shown in Fig. 5, nearly all of the purified PrP^{Sc} was present in the pellet after centrifugation at 10000 × *g* for 10 min, although more than the half of DLPC-treated PrP^{Sc} remained supernatant. Further centrifugation at 100000 × *g* of the soluble DLPC-treated PrP^{Sc} resulted in its precipitation. These results show that the DLPC treatment reduces the size of PrP^{Sc} aggregates without significant a loss of PK resistance. As described above (Fig. 4), mAbs did not react with PK-treated, DLPC-untreated PrP^{Sc} unless it was denatured (at 0 M GdnHCl in Fig. 6). Although all the mAbs except for mAbs 110, 132, and 32 faintly reacted with DLPC-treated PrP^{Sc} without denaturation (OD_{450} < 0.17 at 0 M GdnHCl), none of the mAbs showed a significant increase in reactivity to DLPC-treated PrP^{Sc} compared with DLPC-untreated PrP^{Sc}. These results suggest that the reduction of aggregate size by DLPC is not sufficient to expose cryptic epitopes on PrP^{Sc}.

Exposure of cryptic epitopes by denaturation

The reduction of PrP^{Sc} aggregate size by DLPC did not result in the efficient exposure of the hidden epitopes. In

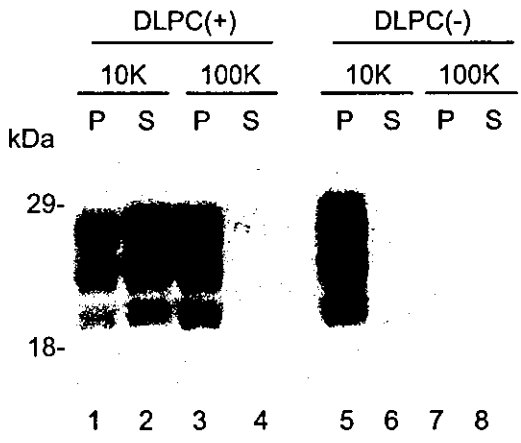

Fig. 5. Sedimentation analysis of DLPC-treated PrP^{Sc}. DLPC-treated (lanes 1–4) and untreated PrP^{Sc} (lanes 5–8) were digested by PK and then subjected to sedimentation analysis. Centrifugation at 10000 × *g* yielded pellet (lanes 1 and 5) and supernatant (lanes 2 and 6) fractions. The supernatants were further subjected to ultracentrifugation at 100000 × *g* to generate pellet (lanes 3 and 7) and supernatant (lanes 4 and 8). The PrP^{Sc} in each fraction was detected by immunoblot analysis.

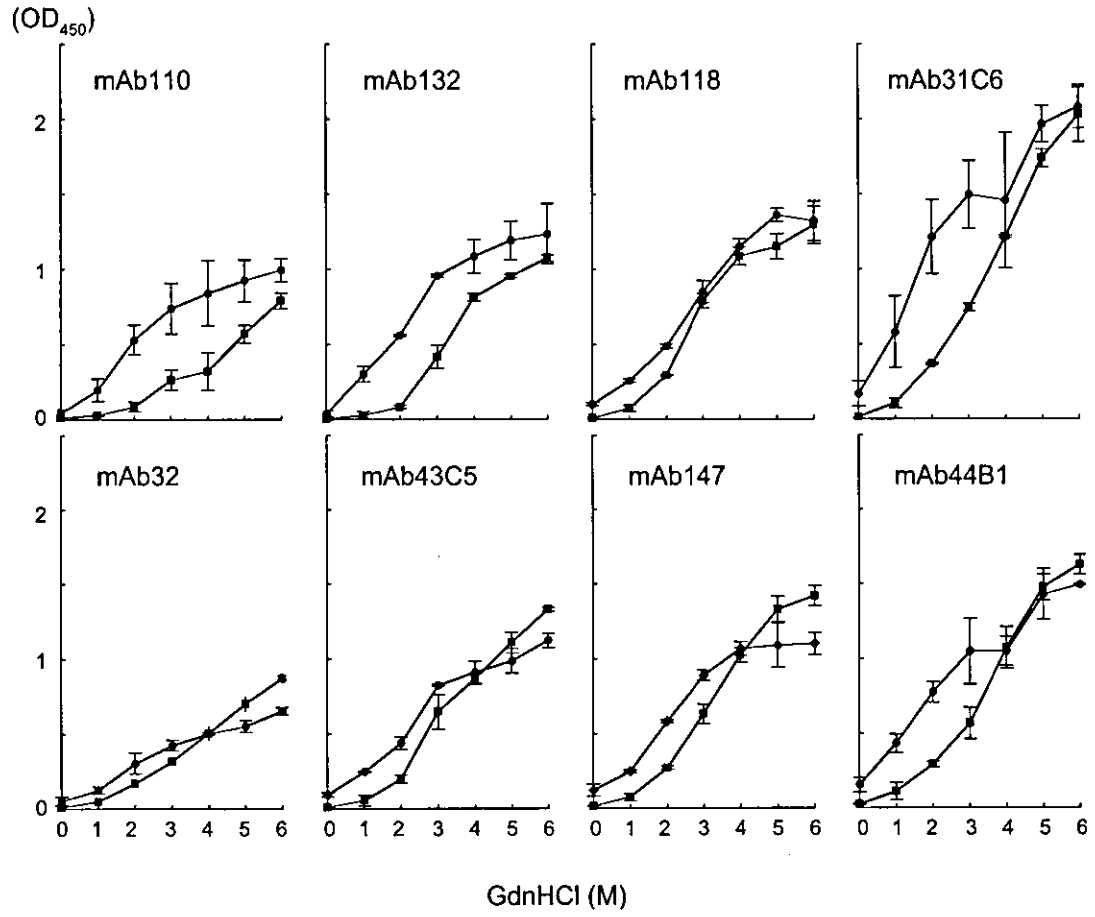

Fig. 6. Exposure of epitopes by GdnHCl treatment. DLPC-treated or untreated PrP^{Sc} was adsorbed to an ELISA plate, digested with PK, and then treated with the various concentrations of GdnHCl indicated at the bottom of the figure. Average and SD of three independent experiments were plotted. Circles indicate the DLPC-treated PrP^{Sc}, whereas squares indicate DLPC-untreated PrP^{Sc}.

contrast, Fig. 6 shows that treatment with of both PrP^{Sc} preparations to GdnHCl dramatically increased the reactivities to all mAbs. This indicates that the dissociation and denaturation of PrP^{Sc} aggregates resulted in pronounced exposure of epitopes. However, one striking difference in the reactivity of mAbs was observed especially at lower GdnHCl concentration: most of the mAbs displayed a higher reactivity to DLPC-treated PrP^{Sc} than to DLPC-untreated PrP^{Sc} at the lower GdnHCl concentrations. This suggests that DLPC-treated PrP^{Sc} may be more sensitive to denaturant than DLPC-untreated PrP^{Sc}.

PrP^{Sc} aggregates are thought to undergo partial denaturation in 2 M GdnHCl, and are believed to be almost completely denatured in 6 M GdnHCl. In parallel with denaturation of PrP^{Sc}, 2 M GdnHCl treatment does not completely abolish prion infectivity, although >4 M GdnHCl treatment drastically reduces the infectivity (Caughey et al., 1997; Prusiner et al., 1993). Table 3 shows the ratio of OD_{450} (in Fig. 6) at 2 and 6 M GdnHCl for both PrP^{Sc} preparations. In DLPC-untreated PrP^{Sc}, the ratios varied from 0.08 to 0.19, suggesting that less than 20% of the epitope for each mAb was exposed following 2 M GdnHCl treatment. In contrast, the ratios increased, varying from 0.37 to 0.58, following DLPC treatment, indicating that the reduction of PrP^{Sc} size influences the sensitivity to denaturation. The variation of the ratios may reflect the difference in the denaturation process for the specific epitopes. For example, epitopes for mAbs 118 and 43C5 appeared to be more resistant to denaturation by GdnHCl than other epitopes.

Table 3
Reactivity of mAbs to partially denatured PrP^{Sc}

mAb	DLPC-untreated		DLPC-treated	
	OD_{450} at 2M/6M GdnHCl	Ratio	OD_{450} at 2M/6M GdnHCl	Ratio
110	0.084/0.789	0.10	0.529/0.994	0.53
132	0.084/1.074	0.08	0.560/1.235	0.45
118	0.295/1.292	0.19	0.488/1.320	0.37
31C6	0.367/2.031	0.18	1.211/2.085	0.58
32	0.167/0.872	0.19	0.305/0.653	0.47
43C5	0.199/1.332	0.15	0.438/1.123	0.39
147	0.271/1.419	0.19	0.581/1.099	0.53
44B1	0.290/1.628	0.18	0.775/1.492	0.52

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-