TABLE 1. Summary of results

Mutations	Chandler		22L	
	Conversion[a]	Inhibition[b]	Conversion[a]	Inhibition[b]
SHa	−	+	−	2+
Hu	−	+	−	+
Q90R	+	NA	+	NA
Q97R	−	2+	−	2+
Q159R	+	NA	+	NA
Q167R	−	2+	−	+
Q171R	−	2+	−	2+
Q185R	+	NA	−	2+
Q185K	2+	NA	−	2+
Q185H	+	NA	+/−	+
Q185E	−	+	−	+/−
Q185L	+/−	+	+/−	+
Q211R	+	NA	+	NA
Q216R	−	+	−	2+
Q218R	−	2+	+	NA
Q218K	−	2+	−	−
Q218H	−	2+	+	NA
Q218E	−	+	+/−	−
Q218L	+/−	+	+	NA
Q222R	+	NA	+	NA

[a] PrP^{Sc} formation of each 3F4-positive construct was quantified by densitometric analysis. The percent conversion of Mo3F4 was assigned as 100%, and the relative scores compared with Mo3F4 are shown. 2+ indicates >200%; + indicates 50 to 200%; +/− indicates 10 to 50%; − indicates <10%. Each value represents the mean of two or three replicates.

[b] Relative inhibitory effect on PrP^{Sc} formation of conversion-defective mutated PrPs was assessed. 2+ indicates >80% inhibition of Mo3F4 PrP^{Sc} formation (in the DNA ratio of 1:1); + indicates 50 to 80% inhibition (1:1); +/− indicates <50% inhibition (1:1) and >50% inhibition (1:2); − indicates <50% inhibition (1:1 and 1:2). Each value represents the mean of two or three replicates. NA, not applicable.

of PrP^{Sc} formation by some of these PrP molecules, especially Q218K, is strain specific.

Q185R PrP converts to PrP^{Sc} in Ch-N2a58 but not 22L-N2a58 cells. To further analyze the strain-specific effect of PrP mutations on PrP^{Sc} formation in Ch-N2a58 and 22L-N2a58 cells, we generated nine 3F4-positive mutated PrPs with a single arginine substitution for each glutamine residue in the C-terminal half and examined their conversion efficiencies in the infected cells (see Fig. 6). The Q90R, Q159R, Q211R, and Q222R PrPs readily converted to PrP^{Sc} in Ch-N2a58 and 22L-N2a58 cells (Fig. 3A, upper panels), whereas the Q97R, Q167R, Q171R, and Q216R PrPs failed to convert in both cell lines (Fig. 3A, upper panels). These conversion-defective mutated PrPs potently inhibited the accumulation of wild-type Mo3F4-derived PrP^{Sc} in both cell lines (Fig. 3B, upper panels). Interestingly, Q185R PrP efficiently converted in Ch-N2a58 cells but not in 22L-N2a58 cells (Fig. 3A), where it actually had an inhibitory effect (Fig. 3B, upper panel, and Table 1).

Substitutions of various amino acid species at codons 185 and 218 differentially affect PrP^{Sc} formation between Ch- and 22L-N2a58 cells. To further examine the effect of amino acid substitutions at codons 185 and 218, where strain-specific effects were observed, we replaced each glutamine residue (Q) with various amino acid species, including basic amino acids (R, K, and H), an acidic amino acid (E), and a hydrophobic amino acid (L). As shown in Fig. 4A and Table 1, Q185K, Q185H, and Q185R PrP readily converted to PrP^{Sc} in Ch-N2a58 cells. Interestingly, the amount of Q185K-derived PrP^{Sc} accumulation in Ch-N2a58 cells was higher than that of Mo3F4-derived PrP^{Sc}, suggesting a more efficient conversion of these mutated PrPs in the cells. In contrast, in 22L-N2a58 cells, little PrP^{Sc} derived from Q185R, Q185K, and Q185H PrP accumulated. Q185E and Q185L PrP minimally converted to PrP^{Sc} in both Ch- and 22L-N2a58 cells.

The introduction of substitutions at codon 218, including basic amino acids (R, K, and H), resulted in the loss of conversion in Ch-N2a58 cells (Fig. 4B). Conversely, in 22L-N2a58 cells, Q218R, Q218H, and Q218L efficiently converted to PrP^{Sc}. Q218K PrP did not convert in either cell line (Fig. 4B) and failed to inhibit wild-type Mo3F4 PrP^{Sc} formation (Fig. 2C).

To determine whether different cellular localizations of the mutated PrPs might be the cause of the different conversion effects, we examined the mutated PrPs with indirect immunofluorescence using the 3F4 antibody. Mo3F4, Q185R, Q218R, and Q218K all localized to the cell surface of Ch-N2a58 and 22L-N2a58 cells (data not shown). In addition, phosphatidylinositol-specific phospholipase C treatment removed the mutated PrPs from the cell surface (data not shown). These results demonstrate that the localization of the mutated PrPs cannot account for their strain-specific properties.

Strain-specific properties of the PrP mutations are independent of antibody epitopes. To assess whether the 3F4 epitope can influence the strain-specific properties of the mutated PrPs, we replaced the 3F4 epitope with the L42 epitope (W144Y), because others have previously shown that MoL42 PrP, like Mo3F4 PrP, readily converted to PrP^{Sc} in ScN2a cells (55). Expression of the L42-positive PrPs, MoL42, Q185R, Q218H, Q218R, and Q218K PrPs, was confirmed by Western blotting using the L42 antibody (Fig. 5A, lower panels). The conversion efficiencies of these L42-positive mutated PrPs were similar to those of 3F4-positive mutated PrPs (Fig. 5A, upper panels). Moreover, as shown in Fig. 5B, Q218K PrP again showed strain-dependent effects on MoL42-derived PrP^{Sc}. The data shown in Fig. 5 indicate that changing from a 3F4 epitope to an L42 epitope in the mutant PrPs does not significantly affect their strain-specific effects on PrP^{Sc} formation.

22L and Chandler PrP^{Sc} have different conformations by IR spectra. To assess whether there are any detectable differences in structure between 22L and Chandler PrP^{Sc}, we performed IR spectroscopy. The amide I region (1,600 to 1,700 cm^{-1}) of protein IR spectra is sensitive to differences in protein secondary structure. Although it is difficult to make complete and unequivocal assignments of IR amide I bands, predominantly α-helical and β-sheet proteins have absorption maxima of 1,653 to 1,657 cm^{-1} and 1,615 to 1,640 cm^{-1}, respectively, in water-based (as opposed to D_2O-based) media (see Fig. 7). Unfolded or random-coil polypeptides tend to have absorbance maxima near 1,645 to 1,650 cm^{-1}, and turn structures tend to absorb between 1,660 and 1,680 cm^{-1}. Absorbance maxima are represented by negative deflections in the second-derivative spectra shown in Fig. 7. Previous studies have shown that the infrared spectrum of PrP^{Sc} of different hamster TSE strains can vary markedly despite being composed of PrP molecules of the same amino acid sequence (13, 52). Consistent with that theme, PK-treated PrP^{Sc} isolated from the brains of mice with 22L and Chandler scrapie differed in the IR spectral

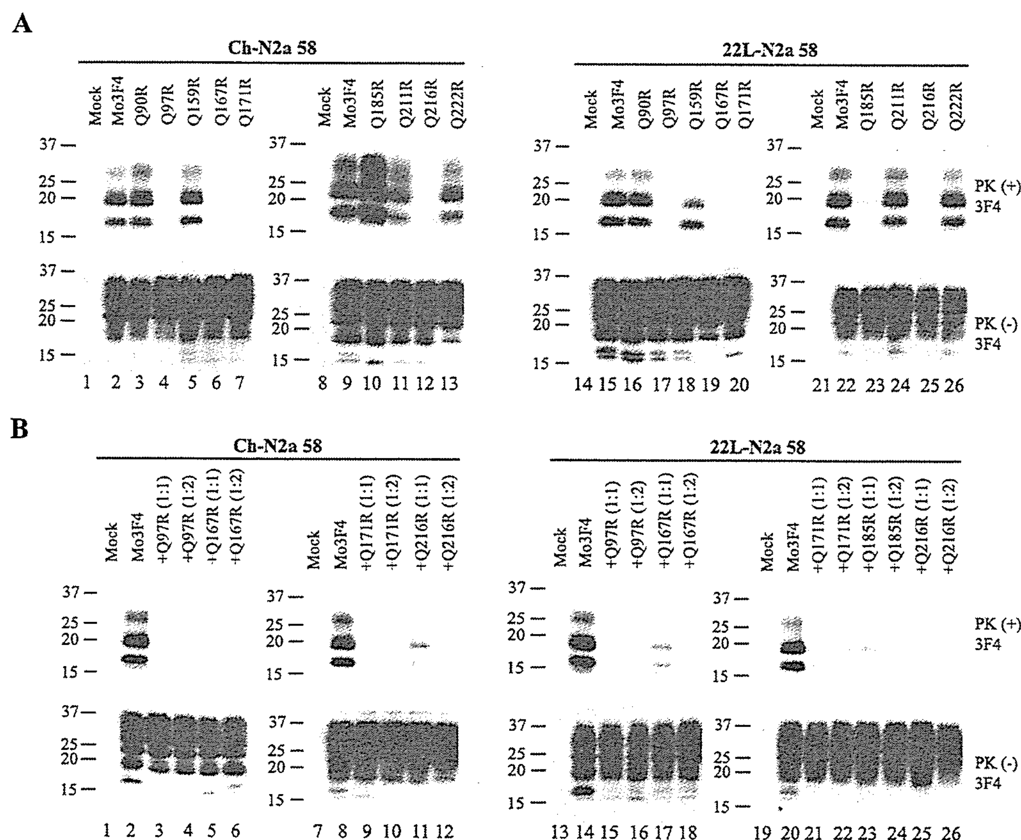

FIG. 3. Strain-specific effects of Q185R mutation on PrP^{Sc} formation. (A) Conversion to 3F4-positive PrP^{Sc} (upper panels) and expression of Mo3F4, Q90R, Q97R, Q159R, Q167R, Q171R, Q185R, Q211R, Q216R, and Q222R (lower panels) were measured by Western blotting using 3F4 antibody. The 3F4 epitope was present in all these constructs. (B) Inhibitory effects of constructs that did not convert were determined by cotransfection with Mo3F4 at a 1:1 or 1:2 DNA ratio. The blots were probed with 3F4 antibody.

region commonly ascribed to the β-sheet region (see Fig. 7). For comparison, PrP^{Sc} from another mouse scrapie strain, 87V, is also shown to have a distinct infrared spectrum in the β-sheet region. In contrast, hemoglobin, a highly α-helical protein, has very little absorbance in the β-sheet region. These results provide direct spectroscopic evidence for differences in conformation between 22L, Chandler, and 87V PrP^{Sc}.

DISCUSSION

In this study, we found evidence that TSE strain characteristics depend on their conformation. We showed that substitutions at codons 185 and 218 resulted in strain-specific PrP^{Sc} formation in cultured neuronal cells infected with two mouse-adapted scrapie prion strains, Chandler and 22L. While others previously demonstrated conformational differences between strains (13, 39, 44, 52), and some strain-specific differences in conformation have been observed in cell-free conversions (6), synthetic amyloid fibrils (25), and purified recombinant *Saccharomyces cerevisiae* Sup35 (31, 49), our results are the first to be obtained from a cell culture comparison of strain effects on the conversion of a panel of mutant PrP^c molecules.

The amino acid sequence of PrP can certainly influence the efficiency of transmission of the infectious agent to a new host species (45), but this "species barrier" cannot be explained on the basis of sequence heterogeneity alone. Our results demonstrate that TSE strains with the same sequence have various abilities to convert the PrP^c mutants at codons 185 and 218, implying a sequence-independent cause of strain specificity. Although the most efficient conversions are expected to occur between PrP^c and PrP^{Sc} with identical sequences, our Q185K mutation promoted PrP^{Sc} formation in Ch-N2a58 cells at a rate higher than that of the homologous wild-type PrP^c (Table 1), indicating that sequence homology between PrP^c and PrP^{Sc} does not necessarily guarantee the most efficient PrP^{Sc} formation.

The locations of residues 185 and 218 within the secondary structure of PrP may explain why mutations at these sites revealed strain-specific differences in conversion. The nuclear magnetic resonance structure of mouse PrP contains three α-helices comprised of residues 144 to 154, 175 to 193, and 200 to 219; two β-strands containing residues 128 to 131 and 161 to 164; and a disulfide bridge between C178 and C213, linking helices 2 and 3 (42). Amino acid 185 is in helix 2, and residue 218 is in the C-terminal portion of helix 3 (Fig. 6). Helices 2 and 3 and their disulfide bridge are crucial for PrP^{Sc} formation (22, 36), and many point mutations associated with familial human prion diseases are located within or adjacent to these

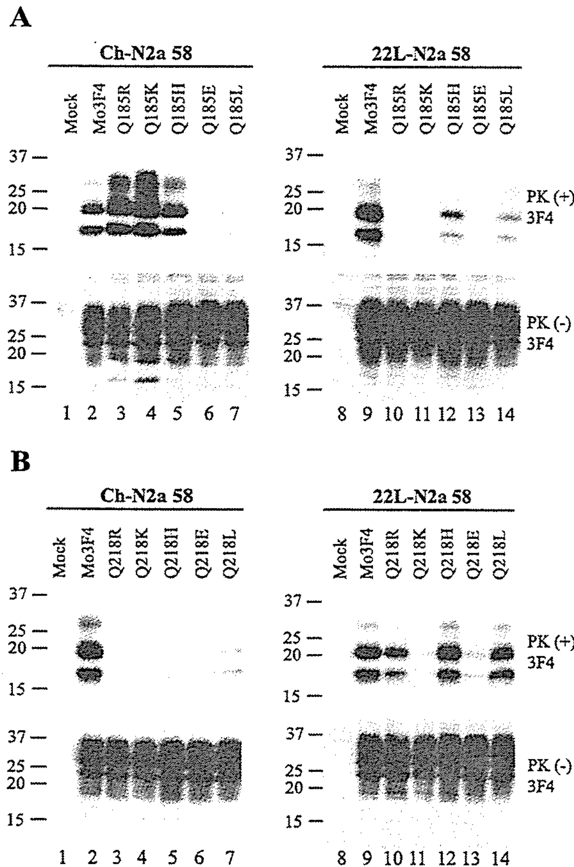

FIG. 4. Strain-specific PrP^{Sc} formation of Q185R, Q185K, Q218R, and Q218H mutated PrP. (A) Conversion to 3F4-positive PrP^{Sc} (upper panels) and expression of Mo3F4, Q185R, Q185K, Q185H, Q185E, and Q185L (lower panels) were measured by Western blot using 3F4 antibody. The 3F4 epitope was present in all these constructs. (B) Western blotting of Mo3F4, Q218R, Q218K, Q218H, Q218E, and Q218L was done as in A.

two helices (41). One such mutation, D178N, is seen in two clinicopathologically distinct diseases, fatal familial insomnia and Creutzfeldt-Jakob disease, the phenotype being determined by the methionine-valine polymorphism at codon 129 of the same mutant allele (21). The striking influence of codon 129 on the D178N mutation phenotype suggests that there may be a modifiable electrostatic interaction or hydrogen bonding between residues 129 and 178 in human PrP (1, 43). Of note, anti-PrP antibody binding studies have revealed that the main conformational differences between PrP^c and PrP^{Sc} actually lie toward the N-terminal region in residues 90 to 120, while the C-terminal regions, including helices 2 and 3, remain accessible to antibody in both forms of PrP, implying that their conformation is not significantly altered during conversion (40). This is also consistent with the maintenance of significant α-helical secondary structure content in PrP^{Sc} (13, 14, 38). In addition, a conformation-dependent immunoassay has localized the primary structural differences among PrP^{Sc} strains to their N termini (44). Such observations suggest that helices 2 and 3 may be involved in intra- or intermolecular interactions with the N-terminal domain during PrP^{Sc} formation and may influence the ultimate conformational change of the N terminus, perhaps through an altered β-sheet structure. In keeping with this, our IR spectra detected a difference in β-sheet structures between 22L-PrP^{Sc} and Chandler-PrP^{Sc} (Fig. 7). If these distinct N-terminal domains had differing interactions with helices 2 and 3, particularly around residues 185 and 218, then our mutations may have created structural changes that were compatible with only one of the strains. For example, the introduction of Q185R into helix 2 of mouse PrP^c may have interfered with the conformational change of its N-terminal domain into 22L-PrP^{Sc} via steric hindrance and/or electrostatic incompatibility while still allowing its conversion into Chandler-PrP^{Sc}. These strain-specific interactions between the N-terminal domain and helices 2 and 3 are likely quite localized, as mutations at other sites did not reveal any strain differences.

In addition to the location of the mutant residues, the nature of their amino acid change may have contributed to our ob-

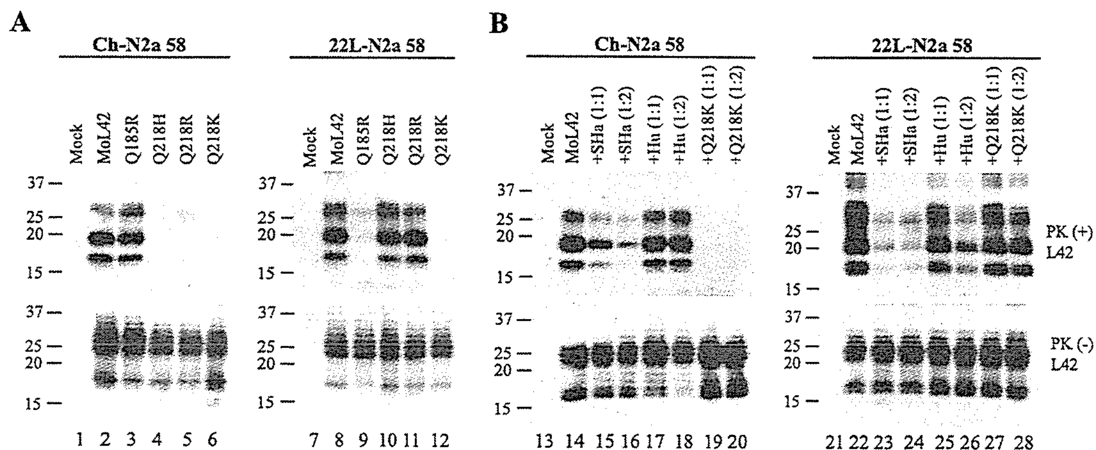

FIG. 5. Strain-specific effects of L42-positive mutated PrPs on PrP^{Sc} formation. (A) Conversion to L42-positive PrP^{Sc} (upper panels) and expression of MoL42, Q185R, Q218H, Q218R, and Q218K (lower panels) were measured by Western blot using L42 antibody. The L42 epitope was present in all these constructs. (B) Inhibitory effects of SHa, Hu, and Q218K were determined by cotransfection with MoL42 at a 1:1 or 1:2 DNA ratio. The blots were probed with the L42 antibody. Molecular mass markers are indicated in kilodaltons on the left side of each panel.

```
                                                           β1
              90        100        110        120       130       140
Mo3F4 PrP   GQPHGGGWGQGGGTHNQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMSRPMIHF
Mutations   *********R******R*******************************************
                     +      -

              α1                β2            α2
              150       160       170       180       190       200
Mo3F4 PrP   GNDWEDRYYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNITIKQHTVTTTTKGENFTET
Mutations   ******************R*******R***R*************R***************
                              +       -   -             C

              α3
              210       220       230
Mo3F4 PrP   DVKMMERVVEQMCVTQYQKESQAYYDGRRS
Mutations   **********R****R*R***R********
                      +    - L   +
```

FIG. 6. Amino acid sequence of Mo3F4 and the position of mutations. The amino acid residue number is based on Mo3F4 PrP. The secondary structures in mouse PrP^{C} are indicated in white boxes at the top. Boxed residues indicate the representative mutations tested; + indicates that conversion occurred in the two cells; − indicates that conversion did not occur in the two cells; "C" indicates Chandler-specific conversion; "L" indicates 22L-specific conversion.

Hemoglobin
87V
Chandler
22L
α-helix
β-sheet
1740 1700 1660 1620 1580
Wavenumber (cm^{-1})

FIG. 7. Comparison of 22L, Chandler, and 87V PrP^{Sc} by infrared spectroscopy. Second-derivative Fourier transform IR spectra are shown for PK-treated PrP^{Sc} samples isolated from the brains of mice affected by either 22L, Chandler, or 87V scrapie. Spectral differences, especially in the β-sheet region of the spectrum, provide evidence that PrP^{Sc} proteins associated with these murine-adapted scrapie strains have distinct conformations. For comparison, a highly α-helical protein, hemoglobin, gives strong absorbance (represented by a negative deflection) at ~1,657 cm^{-1}, with only minor absorbance in the β-sheet region. Similar results were obtained from at least two independent preparations of each strain of PrP^{Sc}.

servations. Similar mutations have been studied to a great extent in yeast, where the translation termination factor Sup35 can aggregate and self-propagate in a prion-like manner. The introduction of point mutations into Sup35 often prevents its aggregation and can block the phenotype of cells that contain aggregated Sup35 (the yeast prion state or [*PSI*]) in a dominant inhibitory fashion. Interestingly, random mutagenesis of Sup35 has revealed that most of these mutants have a glutamine or serine replaced with an arginine (18). Likewise, our PrP mutants, which contained an arginine instead of a glutamine, frequently did not convert and also inhibited the conversion of wild-type PrP. The large, charged arginine side chain most likely has a disruptive effect on the protein-protein interactions that are necessary for aggregation and/or conversion.

Another interesting relationship between our study and those of Sup35 is that in both settings, select mutants have shown strain-specific conversion or aggregation behaviors. When Sup35 aggregates, various levels of translation through premature stop codons can occur, which produces different [*PSI*] phenotypes (53, 58). [*PSI*] strains are heritable and have distinct biological properties that can be propagated in the same yeast genetic background (20). A few Sup35 mutants that displayed different levels of dominant inhibition of [*PSI*], depending on the variant to which they were exposed, have been discovered (19, 30), just as our codon 185 and 218 mutants showed different conversion rates depending on the PrP^{Sc} strain to which they were exposed. The analogous results suggest that various prion types may share similar strain determinants.

A second possibility that could account for the strain-specific properties of our PrP mutations is that the alterations affected interactions between PrP and a strain-specific agent or a host factor. The heterodimer model of the protein-only hypothesis

suggests that an as-yet-unidentified host factor, protein X, is responsible for the behavior of a number of dominant inhibitory forms of PrP^c (26, 51). Interestingly, codon 218 is one of the proposed binding sites for protein X; therefore, a mutation at this site should result in similar conversion rates in cells from the same line, which would have the same protein X. However, in our study, there was a dramatic difference in Q218R and Q218H mutant PrP^{Sc} formation in the same cell line infected with either the Chandler or the 22L strain. Moreover, we and others (55) have shown that PrP mutations with inhibitory effects on conversion are not restricted to the proposed protein X binding sites. There are several previous studies that demonstrated the importance of sulfated glycosaminoglycans (5, 12, 46, 48, 59) and the laminin receptor in PrP^{Sc} formation (33), and more recently, in vitro PrP^{Sc} formation experiments using brain homogenates revealed that host-encoded RNA molecules facilitated PrP^{Sc} formation (17). However, to fully explain how the same mutant PrP^c can convert differently when exposed to two PrP^{Sc} strains, any invoked factor must be associated with the strain itself. For example, the virus or virino hypothesis (15, 27, 34) proposes that agent-encoded nucleic acids are the true determinants of strain diversity. Unfortunately, evidence for such nucleic acids is lacking.

It should be noted that the strain-specific effects were not related to cloning artifacts or the influence of introduced epitopes. Our results were reproduced in other clones and mass cultures prior to cloning (data not shown). In addition, changing the epitope tag from 3F4 to L42 in the mutated PrPs did not affect the strain-specific effects on PrP^{Sc} formation (Fig. 5). This indicated that the properties observed were due only to the codon substitutions.

The best explanation for our data lies with the seeding model hypothesis, which proposes that mutated PrP^c, which is unable to convert, forms a heteropolymer with wild-type PrP^c and PrP^{Sc}, which prevents the conversion of both wild-type and mutated PrP^c. Cell-free conversion studies with purified mouse and hamster PrP isoforms have revealed that heterologous PrP^c, which itself cannot convert, can directly interfere with the conversion of homologous PrP^c into PrP^{Sc}. Furthermore, mouse PrP^c can form heteropolymers with hamster PrP^c and PrP^{Sc} and vice versa (24).

In conclusion, we have shown that mutations at codons 185 and 218 in mouse PrP^c reveal strain-specific effects on PrP^{Sc} formation in cell culture. The conversion differences and IR data suggest that distinct conformations underlie the characteristics of these strains, although the presence of an unidentified strain-specific cofactor cannot be excluded. Further study of these mutants may lead to a better understanding of the structure of PrP^{Sc} and the process by which it is formed. This, in turn, will help advance the knowledge of the molecular basis of TSE strains.

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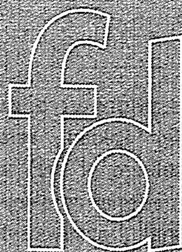

Recent developments in mucosal vaccines against prion diseases

Suehiro Sakaguchi† *and Takeshi Arakawa*

Bovine spongiform encephalopathy in cattle is highly suspected to be orally transmitted to humans through contaminated food, causing new variant Creutzfeldt–Jakob disease. However, no prophylactic procedures against these diseases, such as vaccines, in particular those stimulating mucosal protective immunity, have been established. The causative agents of these diseases, termed prions, consist of the host-encoded prion protein (PrP). Therefore, prions are immunologically tolerated, inducing no host antibody responses. This immune tolerance to PrP has hampered the development of vaccines against prions. We and others recently reported that the immune tolerance could be successfully broken and mucosal immunity could be stimulated by mucosal immunization of mice with PrP fused with bacterial enterotoxin or delivered using an attenuated *Salmonella* strain, eliciting significantly higher immunoglobulin A and G antibody responses against PrP. In this review, we will discuss these reports.

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The development of vaccines is one of the greatest medical and veterinary achievements in human history. Since the first experimental trial of smallpox vaccine by Edward Jenner in 1796, many vaccines have been developed and saved countless numbers of human lives worldwide, giving us evidence-based great reliance on vaccines. Vaccines currently licensed for use in humans and animals are parenterally injectable with a few exceptions, including oral polio vaccine, inactivated *Vibrio cholera* combined with cholera toxin B subunit and oral rotavirus vaccine. However, the recent accumulation of immunological knowledge, particularly regarding the mucosal immune system and its unique character, distinguishable from the systemic immune system, together with the development of recombinant DNA technology, opens up a new avenue for mucosal vaccines combating infectious diseases.

Mucosal vaccines have many advantages over parenteral immunization [1,2]. Mucosal vaccines are needle-free, noninvasive and painless. Mucosal vaccines may also be safer than conventional injected vaccines by reducing the risk of infection from blood-borne pathogens. Moreover, mucosal vaccines may be cost effective because their administration does not require highly trained personnel. In addition to these advantages, mucosal vaccines are effective in priming a full range of local, as well as systemic, immune responses by inducing not only secretory immunoglobulin (sIg)A at mucosal surfaces but also immunoglobulin (Ig)G in serum [3,4]. In addition, cell-mediated immunity can be induced by mucosal vaccines [3,4]. Hence, mucosal vaccines could be effective against infectious diseases caused by mucosally and nonmucosally invasive pathogenic organisms. Indeed, protective efficacy of mucosal vaccines to nonmucosal pathogens, such as arthropod vector-borne pathogens, has been demonstrated [5–7]. It is, therefore, justifiable that mucosal vaccines be evaluated for the next generation of vaccines.

Vaccines against prion protein (PrP), a major component of the causative agents of prion diseases termed prions, are urgently awaited. However, PrP is immunologically tolerated because PrP is a host-encoded protein. Recently, we succeeded in enhancing the mucosal immunogenicity of PrP by fusion with the B subunit of *Escherichia coli* heat-labile enterotoxin (LT) [8]. Other investigators

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also reported success in overcoming the immune tolerance using an attenuated *Salmonella* strain as a mucosal antigen-delivery vector for PrP and showed that immunized mice could survive significantly longer than nonimmunized control mice after oral prion challenge [9]. Here we will briefly review some aspects of prion diseases and introduce reports of mucosal vaccines against them.

Prion diseases

Etiologies of prion diseases

Transmissible spongiform encephalopathies or prion diseases, including Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker (GSS) syndrome, fatal familial insomnia and kuru in humans, and scrapie and bovine spongiform encephalopathy (BSE) in animals, are a group of devastating neurodegenerative disorders. The human prion diseases manifest sporadic, genetic and infectious disorders (TABLE 1) [10,11]. Most cases of human prion diseases, accounting for 85–90% of cases, are a sporadic type of CJD with unknown etiologies [12]. Approximately 10% of cases are an inherited type of disease, including familial CJD, GSS syndrome and fatal familial insomnia, all of which are associated with specific mutations of the *PrP* gene [12]. Only a small percentage of the cases are caused by an infectious event and most of them are iatrogenically transmitted, causing iatrogenic CJD via prion-contaminated intracerebral electroencephalogram electrodes, human growth hormone preparations, dura matter and corneal grafts [13-16]. It was also reported recently that blood transfusion could be a risk factor for prion transmission in humans, causing subsequent CJD in recipients [17,18]. Kuru is caused by ritualistic cannibalism among Papua New Guinea highland people [19]. Moreover, recent lines of evidence strongly suggest that BSE could be transmitted to humans via contaminated food, causing a new variant type of CJD (vCJD) in more than 150 people in England [20-22]. A substantial but much smaller number of vCJD cases were also reported in other countries, including France, Ireland, USA, Canada, Italy, Japan, The Netherlands, Portugal, Saudi Arabia and Spain [22].

Table 1. Human prion diseases.

Diseases	Etiology
CJD	
Sporadic	Unknown
Familial	Mutations in the *PrP* gene
Iatrogenic	Infection by medical practices
Variant	Infection from bovine spongiform encephalopathy (?)
Non-CJD disease	
Gerstmann–Sträussler–Scheinker syndrome	Mutations in the *PrP* gene
Fatal familial insomnia	Mutations in the *PrP* gene
Kuru	Infection by ritualistic cannibalism

CJD: Creutzfeldt–Jakob disease. PrP: Prion protein.

PrPs & the molecular nature of prions

According to the protein-only hypothesis, prions are postulated to be composed of the abnormally folded, relatively proteinase K-resistant, amyloidogenic isoform of PrP, termed PrP^{Sc} [10]. PrP^{Sc} is generated by conformational conversion of the normal cellular isoform of PrP, PrP^{C}, a glycosylphosphatidylinositol-anchored membrane glycoprotein abundantly expressed in neurons [10]. Prions (or PrP^{Sc}), having invaded the body interact with PrP^{C}, inducing changes in the protein conformation of the interacting PrP^{C} into that of PrP^{Sc}, resulting in the propagation of prions (FIGURE 1) [10]. The constitutive conversion of PrP^{C} into PrP^{Sc} also leads to the detrimental accumulation of PrP^{Sc} in the CNS.

We and others demonstrated previously that mice devoid of PrP^{C} are resistant to prion diseases, neither developing the diseases nor propagating prions, clearly indicating that the presence of PrP^{C} is essential for prion propagation and strongly supporting the protein-only hypothesis [23-26]. It was recently reported that β-sheet-rich amyloid fibrils formed by the N-terminally truncated recombinant mouse PrP alone was infectious, causing the disease in transgenic mice expressing the similarly truncated mouse PrP after intracerebral inoculation of the amyloid of the truncated PrP [27]. It was also recently demonstrated that prion infectivity could be increased in a cell-free conversion system, in which the protease-resistant PrP could be produced *in vitro* by incubating normal and infected hamster brain homogenates under certain specific conditions [28]. These results appear to be the conclusive evidence arguing for the protein-only hypothesis.

Prion transport to the CNS

For orally ingested prions to invade the body, they must cross the intestinal epithelium barrier. Heppner and colleagues showed that M cells in the follicle-associated epithelium overlying Peyer's patches might be a portal for prion entry into mucosal tissues by demonstrating that scrapie prions could cross the Caco-2 human epithelial cell monolayer through transcytotic transport by M cells [29]. On the other hand, Mishra and colleagues found that PrP^{Sc} formed a complex with the iron-binding protein ferritin and could transverse the epithelial layer of Caco-2 cells without M cells [30]. These results suggest that prions could also invade directly into mucosal tissues through transcytosis by the epithelial cells themselves. Among migratory bone marrow-derived dendritic cells (DCs) in mucosal tissues, some cells extend projections directly into the gut lumen and have the potential to sample antigens present in the lumen [31], suggesting the possibility that this type of DC transport prions directly into mucosal tissues.

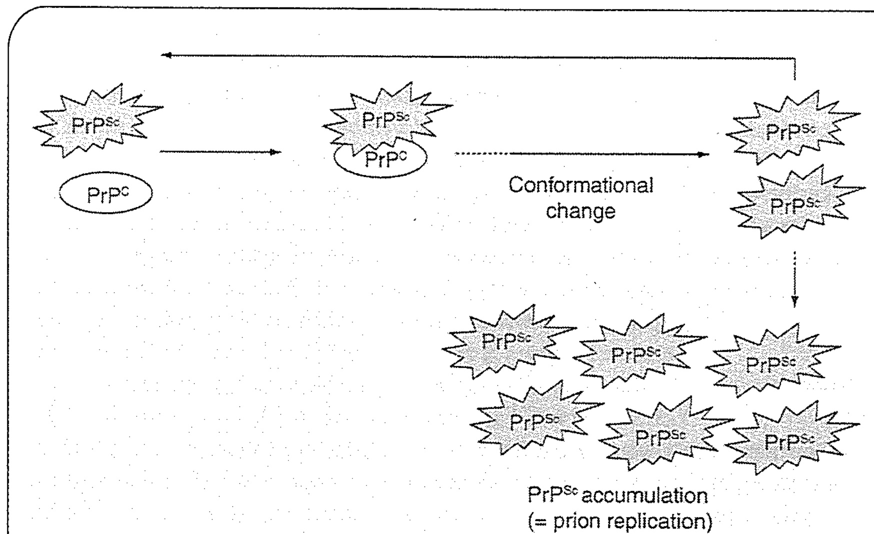

Figure 1. Mechanism for the propagation of prions or the accumulation of the abnormal isoform of PrP, namely PrP^{Sc}. Prions (or PrP^{Sc}) that have invaded the body interact with the normal isoform of PrP, PrP^C, and change the protein conformation of the interacting PrP^C into that of PrP^{Sc}, leading to the propagation of prions or the accumulation of PrP^{Sc} in the CNS.
PrP: Prion protein; PrP^C: Cellular isoform of prion protein; PrP^{Sc}: Prions.

It was recently reported that alymphoplasia (aly) mice, which are deficient in systemic lymph nodes and Peyer's patches due to a point mutation in the nuclear factor (NF)-κB-inducing kinase gene, were completely resistant to a scrapie prion when the prion was orally administered [32]. These results indicate that lymphoid tissues are important for orally ingested prions to transport from the alimentary tract to the CNS. In lymphoid tissues, PrP^{Sc} was shown to accumulate in follicular DCs (FDCs) of primary B-cell follicles and germinal centers [33,34]. Taken together, these results indicate that FDCs in lymphoid tissues might be important cells for orally ingested prions to invade the CNS. Interestingly, electron microscopic examination revealed that nervous fibers are in close proximity to FDCs in Peyer's patches [35]. It is, therefore, conceivable that prions in FDCs could be transmitted to the nervous system at such sites where FDCs and nervous fibers are closely encountered.

For directly invading prions, FDCs appear to be unnecessary for invasion of the CNS. Montrasio and colleagues demonstrated that depletion of mature FDCs by administration of soluble lymphotoxin β-receptor markedly inhibited prion replication in the spleen but the effects on the neuroinvasion of prions were very slight in mice intraperitoneally inoculated with a prion [36]. Consistently, *aly/aly* mice succumbed to the disease with a very slight delay of the incubation times, compared with control wild-type mice, after intraperitoneal inoculation of a prion [32]. Moreover, a drowsy (DY) prion of transmissible mink encephalopathy could be transported into the CNS without replication in spleen and lymph nodes when inoculated into the tongue, which is highly innervated by cranial nerves [37]. These results suggest that prions can also directly invade neuronal tissues without propagating in lymphoid tissues.

Immunological approaches against prion diseases

Attenuation of prions by antibodies against PrP

Given that PrP^{Sc} is thought to be a major component of prions, PrP is a plausible target molecule for the development of prion vaccines. Gabizon and colleagues reported previously that polyclonal antibodies against PrP, α-PrP27–30, could reduce the infectivity of hamster-adapted scrapie prions by a factor of 100 [38]. They dispersed the prion rods containing PrP^{Sc} into detergent–lipid–protein complexes, then mixed them with α-PrP27–30 and finally inoculated them into hamsters to evaluate the prophylactic effects of the antibodies. This was the first report of the immunological approaches to the prophylaxis of prion diseases. Much later on, Heppner and colleagues produced transgenic mice expressing a 6H4 mouse anti-PrP monoclonal antibody and intraperitoneally inoculated them with mouse-adapted scrapie Rocky Mountain Laboratory (RML) prions, showing that these transgenic mice were resistant to the disease [39]. White and colleagues further demonstrated that passive immunization with anti-PrP antibodies could prevent prion infection by demonstrating that intraperitoneal administration of two anti-PrP monoclonal antibodies, ICSM 18 and 35, could protect mice from the peripheral infection of RML prions [40]. However, at the same time, they showed that passive immunization with these prophylactic antibodies had no effects on prions directly infected into the brains of mice, probably owing to difficulties of the antibodies to cross the blood–brain barrier. Taken together, these results suggest that prophylactic anti-PrP antibodies are effective against the prion infection in the peripheral tissues but not in the CNS. It is, therefore, conceivable that prion vaccines have no therapeutic potential against prion diseases once prions have invaded the CNS.

Immune tolerance of PrP & prion vaccines

The successful passive immunization with anti-PrP antibodies highly encourages and promotes the studies of development of active vaccines against prions using PrP as an antigen. However, PrP is immunologically tolerant, having hampered the development of prion vaccines. Therefore, it is of great importance to break the tolerance to PrP for the development of vaccines against prion diseases.

In iatrogenic CJD or BSE cases, human or bovine PrP^{Sc} that has invaded the body as a prion interacts with endogenous human or bovine PrP^C and converts the interacting PrP^C into PrP^{Sc}, and this constitutive syngeneic conversion of PrP results in fatal progression of the diseases. Therefore, to prevent this type of transmission of prions, the syngeneic conversion of PrP should be efficiently blocked. In other words, to prevent

iatrogenic CJD or BSE it is necessary to elicit antibodies against the host PrP. By contrast, in vCJD, BSE prions that have invaded the body convert endogenous host human PrP^C to PrP^{Sc} upon the heterologous interaction between bovine PrP^{Sc} and human PrP^C and once host-derived PrP^{Sc} is generated, the conversion effectively takes place through the syngeneic interaction of host PrP^C and PrP^{Sc}. Therefore, to prevent this type of transmission of the diseases, it might be better to produce antibodies against PrPs of both species, thereby blocking not only heterologous but also syngeneic conversion of PrP.

Mucosal vaccine approaches against prion diseases

Mucosal vaccine advantages for prion diseases

The advent of vCJD owing to the entry of BSE-contaminated animal foodstuffs into the human food chain raised great public health concerns regarding the transmission of the animal prion diseases to humans. In North America, chronic wasting disease (CWD), another type of animal prion disease, is rapidly spreading within mule deer and elk populations, similarly causing concern about transmission of CWD to humans [41]. BSE and CWD, themselves, are also thought to be spread among animals through contaminated food. These days, BSE cases are dramatically declining, reducing the risk of transmission of BSE to humans. By contrast, the risk of iatrogenic infection in human populations through, for example, blood transfusion, contaminated surgical instruments and transplantation of infected tissues is now increasing. Thus, prion vaccines might be better to effectively block prions at both mucosal and nonmucosal entry sites.

Mucosal vaccines are able to elicit specific IgA and G antibody responses [1,2]. IgA is a key player in pathogen-specific mucosal immunity. It is, therefore, feasible that anti-PrP IgA antibodies block the entry of orally ingested prions into mucosal tissues. It is also feasible that IgG against PrP block the transmission of not only the orally ingested prions that have escaped from IgA protection but also the prions directly invading nervous tissues. Thus, mucosal vaccines may be useful to prevent both the mucosal and nonmucosal transmissions of prions because they can stimulate both mucosal and systemic protective immunity.

Adjuvant effects of bacterial toxins on mucosal immunogenicity of PrP

Bacterial toxins, such as the AB_5-type enterotoxin-like cholera toxin (CT) or LT of *E. coli* are the most powerful mucosal adjuvants [42,43]. They share 80% amino acid sequence identity. The A subunit possesses toxic ADP-ribosyltransferase activity and the B subunit forms a nontoxic pentamer with binding affinity for receptors located on the eukaryotic cell surface [44,45]. Many lines of evidence indicate that intranasal or oral delivery of recombinant proteins admixed with such toxin molecules elicit very strong humoral and cellular immune responses, often at comparable or even exceeding levels in comparison with parenteral vaccines [44,45]. Omitting such an adjuvant from vaccine formulations often nullifies the immune response. The precise mechanism underlying such effective immunomodulating activity of these molecules is not fully elucidated. However, the activity is well associated with its binding affinity for cell surface receptors, such as G_{M1}-ganglioside found on most nucleated cells, including DCs and direct uptake of the toxin molecules by DCs [46,47].

In spite of the attractive immune-enhancing effect of CT and LT, their toxicity or potential hazardous effects on olfactory nerves have raised safety concerns regarding the clinical use of these molecules [48]. However, site-directed mutagenesis at or near the enzymatic active site of the A subunit successfully generated a series of nontoxic LT or CT without significant loss of adjuvanticity [49], making it possible to use nontoxic derivatives of LT or CT as mucosal adjuvants [50]. Interestingly, DNA encoding the toxin or part of the toxin molecule administered, as plasmid DNA, has recently demonstrated their effectiveness as a genetic adjuvant [51,52], indicating that CT or LT should not be limited to use as protein adjuvants.

Bade and colleagues immunized Balb/c mice intranasally or intragastrically with recombinant mouse PrP90–231 together with CT as a mucosal adjuvant [53]. No antibody responses against PrP could be elicited by the intragastric administration of PrP90–231 [53]. By contrast, significantly higher IgG and IgA antibody responses could be observed in mice immunized intranasally with PrP90–231 and CT [53]. The authors also showed the protective effects of intranasal immunization with PrP90–231 on the infectivity of a 139A mouse prion by demonstrating that the immunized mice developed the disease significantly later than nonimmunized mice [53]. However, the protective effects were very marginal. The median survival times of the immunized mice were 266 days postinoculation (dpi), while those of nonimmunized mice were 257.5 dpi [53]. These results might indicate that bacterial toxins alone could not enhance the mucosal immunogenicity of PrP to levels high enough to elicit protective immunity against prions.

Mucosal immunogenicity of PrP fused with bacterial toxins

The B subunit of LT (LTB) or CT (CTB) is a highly efficient mucosal carrier molecule for chemically or genetically fused antigens, eliciting local, as well as systemic immunity, against them [54]. These molecules have also been found to be a useful vehicle for self-antigens of prophylactic vaccines against autoimmune diseases [55–57]. In general, the fusion of antigens with the B subunit greatly reduces the antigen dose required for T-cell activation by more than 10,000-fold compared with nonfused free antigen [58]. The efficient antigen carrier effect of the B subunit was not limited to *in vivo* use. Their DC-stimulating capacity, which is mediated by upregulation of major histocompatibility complex and secondary costimulatory molecules (i.e., CD80 and CD86), as well as the induction of cytokine or chemokine secretion, may provide a novel technology for *ex vivo* DC vaccines [59].

We investigated the effects of LTB fusion on the mucosal immunogenicity of PrP in mice [8]. The C-terminal residues 120–231 and 132–242 of mouse and bovine PrPs, respectively, were fused to the C-terminus of LTB with the hinge sequence Gly-Pro-Gly-Pro, resulting in respective fusion proteins

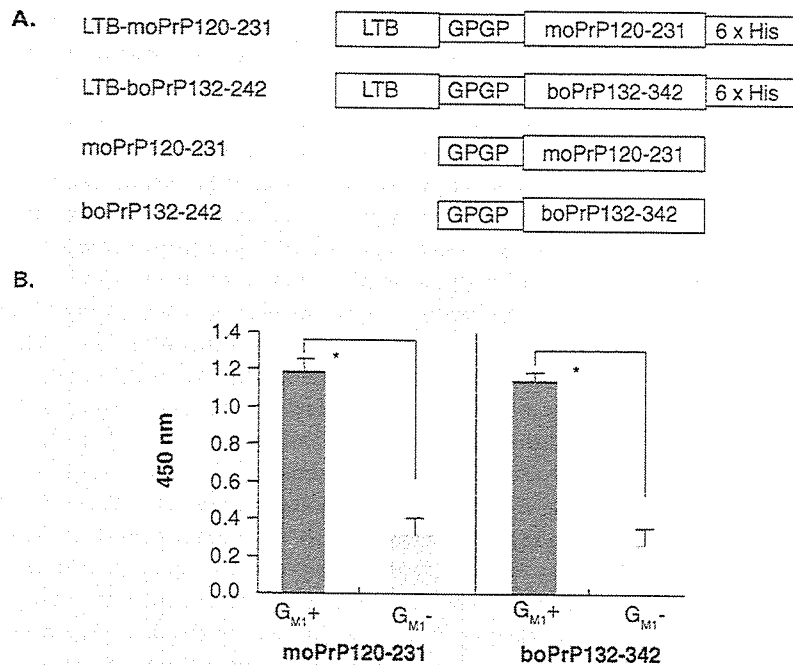

Figure 2. (A) Schematic structures of mouse (mo) and bovine (bo) PrPs fused with or without LTB. (B) Binding of LTB-moPrP120–231 and LTB-boPrP132–242 to G_{M1} ganglioside.
Wells coated with or without G_{M1} ganglioside were incubated with LTB-moPrP120–231 or LTB-boPrP132–242. Binding of the proteins to G_{M1} ganglioside was visualized by enzyme-linked immunosorbent assay using anti-LT mouse serum against recombinant mouse LT. The signals were expressed as colorimetric values measured at 405 nm, showing that LTB-moPrP120–231 and LTB-boPrP132–242 could similarly bind to G_{M1} ganglioside. Four independent data from each group were analyzed using the Mann–Whitney U-test. Data were represented by mean ± standard deviation.
*$p < 0.05$. LT: Heat-labile enterotoxin; LTB: B subunit of LT; PrP: Prion protein.
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termed LTB-moPrP120–231 and LTB-boPrP132–242 (FIGURE 2A). These recombinant fusion proteins were partially purified in a soluble pentameric form with high affinity to G_{M1}-ganglioside (FIGURE 2B).

We immunized Balb/c mice intranasally with LTB-moPrP120–231 fusion protein, as well as nonfusion moPrP120–231, in the presence of recombinant mutant non-toxic LT as an adjuvant [8]. In contrast to the results of Bade and colleagues [53], moPrP120–231 was not immunogenic in mice and no IgG antibody response against PrP could be detected (FIGURE 3A). However, LTB-moPrP120–231 fusion protein elicited significantly higher antibody responses in mice (FIGURE 3A), indicating that fusion with LTB could break the tolerance to PrP. However, efficacy of the tolerance breakdown for PrP was small, suggesting that fusion with LTB alone might not be enough to enhance the mucosal protective immunity against intraspecies transmission of prions.

We similarly immunized Balb/c and C57BL/6 mice with LTB-boPrP132–242 fusion protein, as well as nonfusion boPrP132–242. BoPrP132–242 itself elicited a moderate IgG antibody response in Balb/c mice but not in C57BL/6 mice (FIGURE 4). No specific IgA response could be detected in either mouse strain immunized with boPrP132–242 (FIGURE 4). By contrast, the mucosal immunogenicity of LTB-boPrP132–242 was markedly enhanced in both mouse strains, producing much higher titers of anti-boPrP IgG and A in serum, except for IgA in C57BL/6 mice (FIGURE 4). IgA was also abundantly secreted in the intestines of LTB-boPrP132–242-immunized Balb/c mice (TABLE 2). These results indicate that fusion with LTB could markedly augment the mucosal immunogenicity of bovine PrP. Of great note, antibodies raised against LTB-boPrP132–242 could react with bovine PrP residues 143–166, which corresponds to the antiprion epitope of mouse PrP residues 144–152 and 146–159. Therefore, these antibodies raised against LTB-boPrP132–242 could be effective against the heterologous interaction between BSE prions and human PrP. However, the efficiency of LTB-boPrP132–242 for breaking immune tolerance to PrP was very low, producing small amounts of antibodies cross-reactive with mouse PrP (FIGURE 3B). Thus, it is suggested that, in contrast to the possible effects on the heterologous interaction of PrP, LTB-boPrP132–242 might not be effective against the syngeneic interaction of host PrP^{C} and PrP^{Sc} that are produced in the host.

Mucosal immunogenicity of PrP delivered by an attenuated Salmonella vector

To enhance the mucosal immunogenicity of antigens, efficient mucosal antigen delivery systems have been developed using bacterial vectors, including live-attenuated pathogenic *Salmonella*, Bacillus Calmette–Guérin and *Bordetella*, as well as commensal lactobacilli or certain streptococci and staphylococci [2]. Virus vectors using vaccinia, poxviruses and adenoviruses have also been developed as mucosal antigen delivery systems [2].

Goñi and colleagues used an attenuated *Salmonella typhimurium* LVR01 LPS vaccine strain to mucosally deliver mouse PrP [9]. One two tandem copies of mouse full-length PrP were expressed as a fusion protein with nontoxic fragment C of tetanus toxin in the cells [9]. The authors orally imm unized these

Table 2. Anti-boPrP Immunoglobulin A in fecal extracts.

Immunogen	ng/ml
Unimmunized (n = 5)	<15
LTB-boPrP132–242	212.2 ± 159.8

bo: Bovine; LTB: B subunit of heat-labile enterotoxin; PrP: Prion protein.

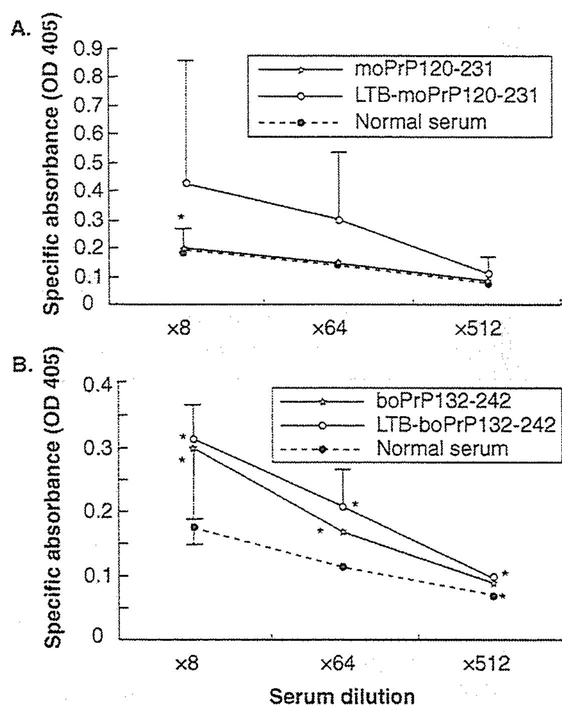

Figure 3. Anti-moPrP autoantibodies in Balb/c mice immunized with mo (A) and boPrPs (B) fused with or without LTB six times at 2-week intervals. Antisera were collected from four to five mice from each group and subjected to enzyme-linked immunosorbent assay against moPrP without a 6×His tag. Antibody titers were expressed by colorimetric values at 405 nm. Data were analyzed using the Mann–Whitney U-test. Data were represented by mean ± standard deviation.

*$p < 0.05$.

bo: Bovine; LTB: B subunit of heat-labile enterotoxin; mo: Mouse.

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viable cells into CD-1 mice and thereafter orally challenged them with a 139A mouse prion [9]. The immunized mice elicited significantly higher IgG and IgA antibody responses compared with control mice administered with *Salmonella* without PrP [9], indicating that this delivery system could be useful to disrupt immune tolerance to PrP. More importantly, approximately 30% of the mice immunized with the cells expressing either one or two copies of mouse PrP were alive without any clinical signs of prion diseases until at least 500 dpi [9]. By contrast, the remaining 70% of immunized mice developed the disease with very little or no delay in their survival times [9]. However, all control mice had died by up to 300 dpi [9]. These results indicate this delivery system for PrP is effective for stimulating protective immunity against prions but its effectiveness is very variable.

Nonmucosal vaccine approaches against prion diseases

Sigurdsson and colleagues reported that subcutaneous immunization of mice with recombinant mouse PrP could induce anti-PrP autoantibodies and slightly retarded onset of the disease after inoculation with a mouse-adapted 139A prion [60]. The immunized mice died at 189 ± 4 days while the control mice died at 173 ± 2 days after intraperitoneal inoculation with a tenfold dilution of the infected brain homogenate [60]. However, Polymenidou and colleagues described that recombinant mouse PrP failed to induce anti-PrP autoantibodies in mice [61]. We also failed to detect anti-PrP autoantibodies in mice intraperitoneally immunized with mouse recombinant PrP [62]. Moreover, we could not observe any prophylactic effects of the immunization against the Fukuoka-1 mouse prion [62]. However, very interestingly, we found that that heterologous bovine and sheep recombinant PrPs were highly immunogenic in mice, stimulating anti-PrP autoantibody responses [62]. More interestingly, mice intraperitoneally immunized with the heterologous recombinant PrPs exhibited a slightly but significantly extended survival after intraperitoneal infection with the mouse-adapted Fukuoka-1 prion [62]. Non-immunized mice developed the disease 291 ± 10 dpi and mice immunized with recombinant bovine PrP showed delayed onsets at 322 ± 15 dpi [62]. Recombinant sheep PrP showed variable effects against the prion in the immunized mice [62]. Approximately 70% of the immunized mice developed the disease with prolonged onsets [62]. These results might indicate that, rather than autologous PrP, heterologous recombinant PrPs are more potent stimulator of protective immunity against prions.

Other approaches to enhance immunogenicity of PrP

Pathogenic organism-derived pathogen-associated molecular patterns (PAMPs), including unmethylated CpG, are recognized by pattern recognition Toll-like receptors, stimulating strong innate and ultimately acquired immune responses [63]. Indeed, it was reported that CpG could break the immune tolerance to PrP in C57BL/6 mice when subcutaneously coadministered with PrP peptides [64]. However, it was shown that the repeatedly administrated CpG causes suppression of FDCs essentially involved in induction of the innate and acquired immune responses [65]. Gilch and colleagues reported successfully inducing anti-PrP autoantibodies by immunization of mice with mouse recombinant PrP [66]. They showed that dimeric but not monomeric recombinant mouse PrP could elicit autoantibodies that had the potential to cure the persistently infected mouse neuroblastoma N2a cells of prions [66]. It was also reported that fusion of mouse PrP with the heat-shock protein DnaK enhanced the immunogenicity of PrP in mice, inducing autoantibodies against PrP [67]. More recently, it was shown that PrP displayedon the surface of retrovirus particles could efficiently induce autoantibody responses in mice [68]. It is, therefore, very interesting to investigate whether or not these immunization approaches could be effective against prion transmission *in vivo*.

Perspectives on prevalence of prion diseases in humans

Polymorphism of methionine (M) or valine (V) at codon 129 of the *PrP* gene is known to be a major determinant of susceptibility to human prion diseases [69–72]. MM is the most susceptible, MV intermediate and VV is protective. All cases of vCJD so far reported to be infected from BSE are MM homozygous. No MV or VV cases were identified. However, we have to look carefully at whether or not the MV or VV cases could appear in the future.

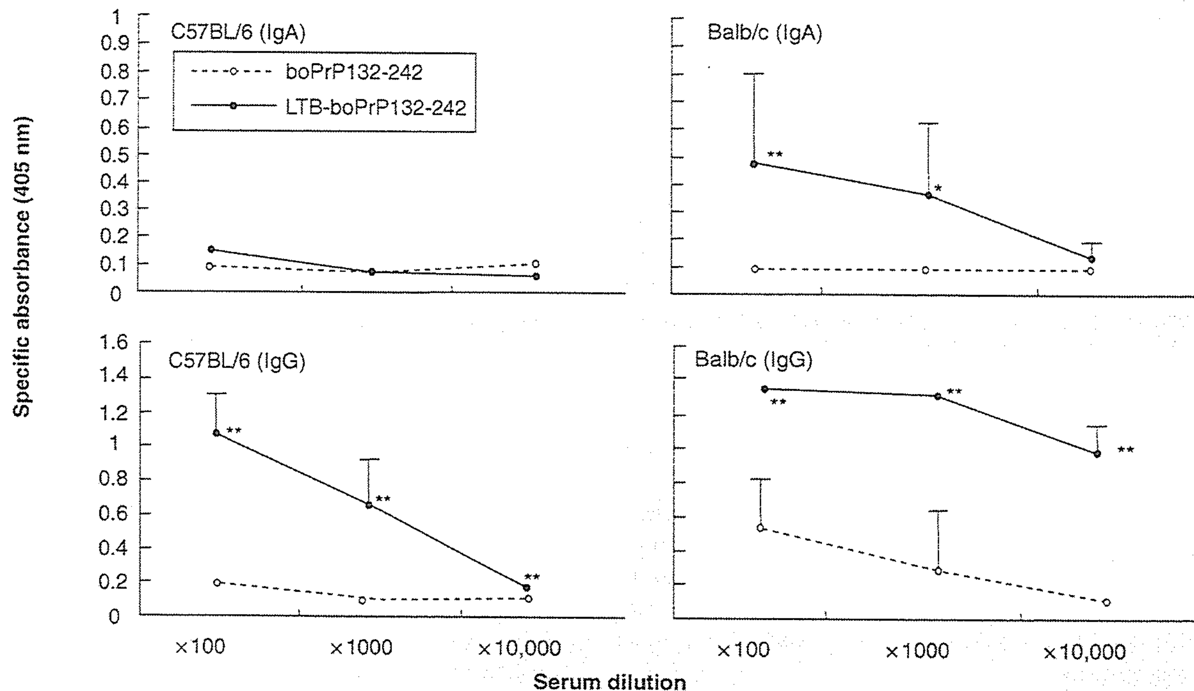

Figure 4. Specific IgA and G antibody titers in the serum of C57BL/6 and Balb/c mice intranasally immunized with LTB-boPrP132–242 or boPrP132–242 three times at 2-weekly intervals. Antisera were collected from five mice from each group and were subjected to enzyme-linked immunosorbent assay against 6×His-tagged boPrP. Antibody titers were expressed by colorimetric values at 405 nm. Data were analyzed using the Mann–Whitney U-test. Data were represented by mean ± standard deviation.
*$p < 0.05$.
**$p < 0.01$.
Reprinted in part from [8]. Copyright (2006), with permission from Elsevier. bo: Bovien; Ig: Immunoglobulin; LTB: B subunit of heat-labile enterotoxin.

Prions are more widely distributed in the body of vCJD patients than that in sporadic CJD cases [73], increasing the threat of iatrogenic secondary transmission of the disease through common medical practices. Indeed, iatrogenic transmission of vCJD through blood transfusion was reported in two cases and, surprisingly, one case was heterozygous at codon 129, raising concern about the spread of the disease within the human population [17,18]. These results indicate that individuals latently infected by vCJD prions could be sources for the iatrogenic transmission of vCJD within the human population in the future. Recent studies using transgenic mice expressing human PrP with the codon 129 MM, MV and VV genotypes showed that not only MM but also MV and VV transgenic mice could be infected by vCJD prions [74]. Therefore, development of prion vaccines, which can block such awful iatrogenic transmission of prions within human populations, is urgently awaited.

Expert commentary

At present, we do not have any available practical vaccines against prion diseases. Therefore, other possible prophylactic measures have been taken effectively. It is considered that BSE was spread rapidly within cattle being fed BSE-contaminated meat and bone meal (MBM). In the UK, the ruminant feed ban introduced in 1988, prohibited feeding cattle any bovine-derived MBM and has successfully reduced the number of new BSE cases. Therefore, feeding animals with MBM should only be carried out with a lot of care unless its safety has been guaranteed. Moreover, to prevent BSE prions from possibly entering the human food chain, tissues containing high prion infectivity, designated as specified risk materials (SRMs), are obliged to be removed in many countries, including all member states of the EU and Japan. SRMs are bovine heads (except for tongues and cheek meat, but including tonsils), spinal cords, distal ileum (two meters from connection to cecum) and vertebral column (excluding the transvers processes of the thoracic and lumbar vertebra, the wings of the sacrum and the vertebra of the tail).

We also have to be cautious about human-to-human transmission of vCJD since two cases have been reported through blood transfusion [17,18]. To reduce this kind of risk of infection, preclinical diagnosis of the diseases is critical. However, at present, identification of infected individuals is very difficult unless they have already developed specific symptoms. Soto and colleagues demonstrated that prions could be detected in the blood of presymptomatic hamsters after they had been experimentally infected with a scrapie prion, using protein misfolding cyclic amplification (PMCA) technology [75]. PMCA was

designed to amplify PrP^{Sc}, permitting easy detection of the amplified PrP^{Sc} by routine biochemical detection techniques, such as immunoblotting assay. The elucidation of whether PMCA is applicable to human samples is thus eagerly awaited. Moreover, development of disease treatment may also contribute to risk reduction for human-to-human transmission of prions.

Five-year view

The vaccines reported so far against prion diseases exhibit only marginal effects on prion infection in animal models. Their ineffectiveness is mostly attributable to the immunological tolerance of PrP. Thus, the issue of how tolerance to PrP can be broken down efficiently is a big question in this field.

We have shown that fusion with LTB markedly enhanced the mucosal immunogenicity of PrP, disrupting tolerance to PrP with low but significantly higher efficiency, thus stimulating antibody responses against host PrP [8]. Autoantibodies against PrP could be similarly induced using a *Salmonella* delivery system; 30% of the resulting immunized mice did not succumb to the disease [9]. These results indicate that development of more effective adjuvants or antigen delivery systems could allow more protective vaccines against prion diseases.

Molecular mimicry between microbial and host antigens, those sharing identical amino acid sequences or homologous but nonidentical amino acid sequences, is a well known hypothetical mechanism for triggering autoimmune diseases through the production of autoantibodies [76,77]. PrPs are highly conserved molecules among mammals with marked similarities in amino acid sequence. It is, therefore, conceivable that heterologous PrPs might mimic host PrP to overcome tolerance. Consistent with this concept, we showed that immunization of mice with bovine PrP could elicit antibodies capable of recognizing host mouse PrP [8]. Thus, molecular mimicry-based prion vaccines could be possible alternatives in the future.

The PrP^{Sc}-specific epitopes or conformation could be other potential targets for prion vaccines. Cashman and colleagues reported that the Tyr-Tyr-Arg epitopes are normally buried in PrP^{C} but become exposed outside of PrP^{Sc} owing to the structurally-altered conformation, giving rise to the interesting idea that these epitopes might be PrP^{Sc}-specific targets, stimulating antibody responses specific to PrP^{Sc} [78]. They confirmed this hypothesis by demonstrating that immunization of animals with the Tyr-Tyr-Arg peptide conjugated with keyhole limpet hemocyanin elicited antibodies specifically reactive with PrP^{Sc} not with PrP^{C} [78]. Moreover, in contrast to PrP^{C}, PrP^{Sc} has very high β-sheet-structure content. Therefore, prion vaccines that target a β-sheet structure might also be possible.

Gauczynski and colleagues recently reported interesting results showing that the 37-/67-kDa laminin receptor physically interacts with PrP^{C}, forming a receptor for prions and that antibodies against laminin receptor inhibited prions from adhering to the cell surface [79]. These results indicate the possibility that molecules other than PrP, such as the 37-/67-kDa laminin receptor, could be potential targets for prion vaccines in the future. Molecular mechanisms of prion infection, including exact identification of the prion receptor, how

Key issues

- Prions are mainly composed of the abnormally folded, amyloidogenic isoform of prion protein (PrP), PrP^{Sc}. Prions propagate through conformational conversion of PrP^{C}, the normal isoform of PrP. Having invaded the body, PrP^{Sc} interacts with endogenous PrP^{C} and then induces changes in the conformation of the interacting PrP^{C} into that of PrP^{Sc}, resulting in the multiplication of PrP^{Sc}. The syngeneic or heterologous conversion between PrP^{Sc} and PrP^{C} underlies the intraspecies or interspecies transmission of prions, such as human-to-human or cattle-to-human transmission, respectively.
- The highly suspected link between variant Creutzfeldt–Jakob disease (vCJD) and bovine spongiform encephalopathy (BSE) has raised concerns about a potential epidemic in the human population. Moreover, possible human-to-human transmission of vCJD through blood transfusion suggests that vCJD might be spread latently within the human population more widely than expected originally. However, no prophylactic measures against the disease have been developed.
- Passive immunization of mice with monoclonal antibodies against PrP, a major component of prions, successfully blocked infection with prions. This successful immunization encouraged and promoted developmental studies of vaccines against prion diseases. Compared with conventional vaccines, mucosal vaccines seem to be more suitable for preventing prion infection because BSE has been orally transmitted to humans through contaminated food.
- Host tolerance to PrP has hampered development of effective prion vaccines. Fusion with B subunit of heat-labile enterotoxin or delivery using attenuated *Salmonella* strains that enhanced mucosal immunogenicity of PrP in mice has been partly effective, breaking down tolerance to PrP and stimulating antibody responses against host PrP. Unfortunately, such effects were too weak to block prion transmission completely.
- Infection with vCJD prions of the transgenic mice expressing a different combination of a polymorphic amino acid (M or V) at codon 129 of the human *PrP* gene suggests that considerable numbers of individuals might be latently infected with vCJD. Therefore, in addition to the development of prion vaccines, a reliable assay for detection of such presymptomatic individuals is important to prevent further spread of prion diseases in the human population.

prions propagate in cells, how prions are released from cells and so on, are largely unknown. Further understanding of these mechanisms could also be very useful for the development of more effective prion vaccines.

PrP and laminin receptor are host molecules. No autoimmune-related abnormal symptoms could be reported in mice vaccinated with PrPs or passively immunized with anti-PrP antibodies [8,40,62] although immune responses against these molecules could have a possibility to cause adverse effects of autoimmunity. In the case of Alzheimer's disease vaccines using the Aβ-amyloid peptide derived from the host molecule amyloid precursor protein as an antigen, no adverse effects were similarly reported in immunized mice but severe encephalitis was observed in immunized people [80,81]. Moreover, anti-PrP antibodies markedly caused apoptosis of neurons when administrated directly into the hippocampus of mice probably through cross-linking PrP^C expressing on the cell surface [82]. Therefore, we must take these findings into serious consideration when prion vaccines are applied to human populations.

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Immunization with recombinant bovine but not mouse prion protein delays the onset of disease in mice inoculated with a mouse-adapted prion

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Abstract

Host tolerance to endogenous prion protein (PrP) has hampered the development of prion vaccines as PrP is a major component of prions. Indeed, we show that immunization of mice with mouse recombinant PrP elicited no prophylactic effect against a mouse-adapted prion. However, interestingly, mice immunized with recombinant bovine PrP developed the disease significantly later than non-immunized mice after inoculation of a mouse prion. Sheep recombinant PrP exhibited variable prophylactic effects. Mouse recombinant PrP stimulated only very weak antibody responses. In contrast, bovine recombinant PrP was higher immunogenic and produced variable amounts of anti-mouse PrP autoantibodies. Sheep recombinant PrP was also immunogenic but produced more variable amounts of anti-PrP autoantibodies. These results might open a new way for development of prion vaccines.
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1. Introduction

Transmissible spongiform encephalopathies or prion diseases, including Creutzfeldt–Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals, are a group of devastating neurodegenerative disorders transmitted by unconventional infectious agents, the so-called prions [1,2]. Many lines of recent evidence suggest that BSE prions could orally transmit to humans via contaminated food, causing new variant type CJD in young people [3–5]. It was also recently reported that blood transfusion could be a risk factor for prion transmission in humans, causing subsequent CJD in recipients [6,7]. However, no prophylactic measures against the transmission of prions have been developed.

Prions are thought to be mainly composed of the proteinase K (PK)-resistant, amyloidogenic isoform of prion protein, designated PrP^{Sc}, which is generated by conformational conversion of the normal cellular isoform of PrP (PrP^{C}) via unknown post-translational modifications [1,2]. PrP^{C} is

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a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein most abundantly expressed in neurons [1,2]. PrP is therefore a plausible target molecule for the development of prophylactic measures against prions. Gabizon et al. previously reported that polyclonal antibodies against PrP could reduce the infectivity of hamster-adapted prions by a factor of 100 [8]. Heppner et al. [9] recently showed that mice transgenically expressing anti-PrP monoclonal antibody, 6H4, were resistant to the disease after intraperitoneal inoculation of mouse-adapted scrapie RML prions. White et al. also demonstrated that two other anti-PrP monoclonal antibodies, ICSM 18 and 35 could prevent prion infection in mice by passive immunization [10]. This successful prevention of prion infection by anti-PrP antibodies indicates that active immunization or vaccination against PrP could be a promising prophylaxis against prion transmission.

In the present study, we immunized BALB/c mice with recombinant mouse, bovine, and sheep PrPs and thereafter intraperitoneally challenged these immunized mice with a mouse-adapted prion. Immunization with mouse recombinant PrP showed no prophylactic effect against the prion infection in mice. Instead, the immunization appeared to exacerbate the infection. In contrast, mice immunized with bovine recombinant PrP exhibited slightly but significantly prolonged incubation times, compared with those of non-immunized mice. The immunizing effects of sheep recombinant PrP on the infection were variable.

2. Materials and methods

2.1. Expression and purification of recombinant PrP immunogens

DNA fragments corresponding to the mouse PrP residues 23–231 (according to GenBank accession no. M13685), the sheep PrP residues 25–234 (GenBank accession no. U67922), and the bovine PrP residues 25–242 (GenBank accession no. AJ298878) were independently amplified by polymerase chain reaction (PCR) using appropriate primer pairs shown in Table 1. Following sequence confirmation of these PCR products, the fragments were digested with *Bam*HI and *Hin*dIII and inserted into a pQE30 vector (QIAGEN, Hilden, Germany). The pQE30 vector was developed to produce the proteins of interest with a N-terminal 6× His tag.

E. coli (M15) cells were freshly transformed by each plasmid, cultured in LB medium containing 1 mM isopropylthio-β-D-galactoside (IPTG), and collected by centrifugation. The collected cells were lysed using CelLytic B bacterial cell lysis/extraction reagent (Sigma–Aldrich Co., St. Louis, USA) in the presence of deoxyribonuclease I and the lysate was centrifuged at 25,000 × *g* for 10 min. The resulting pellet was suspended in Reagent containing 0.2 mg/ml lysozyme and incubated with occasional shaking at room temperature (RT) for 15 min. Volume of the suspension was then increased by addition of 1:10 diluted Reagent and centrifuged at 25,000 × *g* for 10 min. The resulting pellet was washed 3 times with the 1:10 diluted Reagent, suspended in a lysis buffer (8 M Urea, 10 mM Tris–HCl, 100 mM Na_2HPO_4, pH 8.0) and further purified using a Ni-NTA column (QIAGEN) as recommended in the manufacturer's protocol.

2.2. Purification of recombinant mouse PrP minus a 6× His tag

The DNA fragment corresponding to mouse PrP 23–231 was amplified by PCR using an appropriate pair of primers (Table 1). Following sequence confirmation, this fragment was digested with *Nde*I and *Bam*HI and inserted into a pET11a vector (Novagen, Inc., WI, USA). *E. coli* (BL21) cells were transformed by the resulting plasmid and cultured in LB medium containing 1 mM IPTG. The cells were collected by centrifugation and suspended in buffer (50 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, 1 mM PMSF, pH 8) containing 300 μg/ml lysozyme. After incubation for 20 min at RT, deoxycholic acid was added to the suspension for another 20 min and genomic DNA was digested with deoxyribonuclease I at RT for 30 min. The resulting extract was again centrifuged at 25,000 × *g* for 20 min and the pellet was solubilized in buffer (8 M urea, 50 mM Tris–HCl, 1 mM EDTA, pH 8). This extract was applied to a CM-sepharose column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and recombinant PrP was eluted using a linear NaCl gradient from 0 to 500 mM in the same buffer.

2.3. Immunization

Purified recombinant PrPs with a 6× His tag were dialyzed against PBS and 100 μg of each recombinant protein were intraperitoneally inoculated into a 4 week-old female

Table 1
The DNA sequences of primers used for constructs

Constructs	Forward primers	Reverse primers
6× His-tagged PrPs		
Mouse PrP23–231	gcggatccaaaaagcggccaaagcctggag	ccaagcttctatcagctggatcttctcccgtcgta
Bovine PrP25–242	gcggatccaagaagcgaccaaaacctggag	ccaagcttctatcaacttgcccctcgttggtaata
Sheep PrP25–234	gcggatccaagaagcgaccaaacctggcg	ccaagcttctatcaacttgcccccctttggtaata
Non-tagged PrP		
Mouse PrP23–231	ggatgccatatgaaaaagcggccaaag	gaggatcctattagctggatcttctccc

Underlined sequences indicate appropriate restriction enzyme sites described in Section 2.

BALB/c mouse (SLC Japan, Shizuoka, Japan) at 2-week intervals together with complete Freund's adjuvant (Difco Laboratories, Detroit, MI) for the first immunization and with incomplete Freund's adjuvant (Difco Laboratories) from the second immunization. Antisera were collected 1 week after the final immunization and stored at −20 °C until used. Mice were cared for in accordance with the Guidelines for Animal Experimentation of Nagasaki University.

2.4. Prion inoculation

Brains were removed from the diseased mice infected with the mouse-adapted Fukuoka-1 prion [11] and homogenized to 1% (w/v) in PBS. Aliquots (100 μl) of the homogenate were intraperitoneally inoculated into each mouse 1 week after receiving their fifth immunization with recombinant PrPs.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Each well of a 96-well immunoplate (Nunc) was coated with 500 ng of purified mouse recombinant PrP without a 6× His tag or other recombinant PrPs with a 6× His-tag by overnight incubation at 4 °C and then blocked with PBS containing 0.05% Tween-20 (T-PBS) and 25% Block Ace (Dainihonseiyaku Co., Tokyo, Japan) at 37 °C for 1 h. To detect specific IgG antibodies, serially 10-fold diluted antiserum was added to the wells for 1 h at 37 °C and unbound antibodies were removed by washing twice with T-PBS. Immune complexes were detected using secondary sheep anti-mouse IgG antibodies conjugated with HRP (Amersham Biosciences), 2 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), and 0.04% H_2O_2. Anti-PrP antibodies titers were determined using colorimetric values expressed at 405 nm.

For ELISA of mouse PrP peptides, moPrP90–109, moPrP131–154, and moPrP219–231, 1 μg of each peptide was coated on a 96-well immunoplate (Nunc) and similarly subjected to the procedures described above except for using 3,3′,5,5′-tetramentylbenzidine (Pierce, Rockford, IL) instead of 2 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and detecting signals at 450 nm instead of 405 nm. The peptides (>70% purity) were purchased from Sigma–Aldrich Japan K.K. (Hokkaido, Japan).

2.6. Constructions of expression vectors for mouse, sheep, and bovine PrP^C

The DNA fragment encoding full-length mouse PrP^C was amplified by PCR with a sense primer (5′-tcggatccagtcatc**atg**gcgaaccttggc-3′; the underlined sequence, a *Bam*HI site; the bold sequence, a start codon) and an antisense primer (5′-cctctagacc**tca**tcccacgatcaggaaga-3′; the underlined sequence, a *Xba*I site; the bold sequence, a stop codon) using a cloned mouse genomic DNA as a template. The DNA fragments for sheep and bovine PrP^C were similarly amplified with a sense primer (5′-tcggatccagtcatc**atg**gtgaaaagccac-3′; the underlined sequence, a *Bam*HI site; the bold sequence, a start codon) and an antisense primer (5′-cctctagacc**cta**tcctactatgagaaaaa-3′; the underlined sequence, a *Xba*I site; the bold sequence, a stop codon) using a cloned bovine PrP cDNA and a cloned sheep PrP genomic DNA as a template, respectively. After confirmation of the DNA sequences, each DNA fragment was digested by *Ban*HI and *Xba*I and introduced into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA).

2.7. Immunoblotting of eukaryotic PrP^C

African green monkey kidney COS-7 cells were transiently transfected by a pcDNA3.1 vector (Invitrogen) inserted with or without the DNA fragment encoding full-length mouse, sheep, and bovine PrP^C using lipofectamin 2000 (Invitrogen) and lysed in lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 300 mM NaCl, 100 mM Tris–HCl, pH 7.5) 3 days after transfection. Proteins were separated by 12% SDS-PAGE and electrically transferred onto an immobilon-P membrane (Millipore, MA, USA). The membrane was incubated with 1:400-diluted antiserum raised against recombinant PrPs in BALB/c mice and secondary sheep anti-mouse IgG antibodies conjugated with HRP (Amersham Biosciences). Immune complexes were visualized using the ECL system (Amersham Biosciences).

2.8. Flow cytometry

Cells were harvested with PBS containing 0.2% EDTA, suspended in BSS buffer (140 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 1 mM $CaCl_2$ pH 7.0), and incubated with 100-fold diluted antisera for 30 min on ice. The treated cells were then washed three times with BSS buffer, reacted with FITC-conjugated goat anti-mouse IgG (H+L) (Chemicon International, CA, USA), and analyzed by FACScan (Becton Dickinson, NJ, USA).

2.9. Statistical analysis

Logrank test was used for analysis of the incubation times between mice immunized with and without recombinant PrPs. Colorimetric data from ELISA were subjected to one way ANOVA followed by Tukey–Kramer multiple comparison test.

3. Results

3.1. Different effects of immunization with recombinant mouse, sheep, and bovine PrPs on mouse-adapted prion in mice

We intraperitoneally immunized BALB/c mice with purified recombinant mouse, sheep, and bovine PrPs (100 μg/mouse) five times at 2-week intervals and intraperitoneally inoculated a mouse-adapted Fukuoka-1 prion into