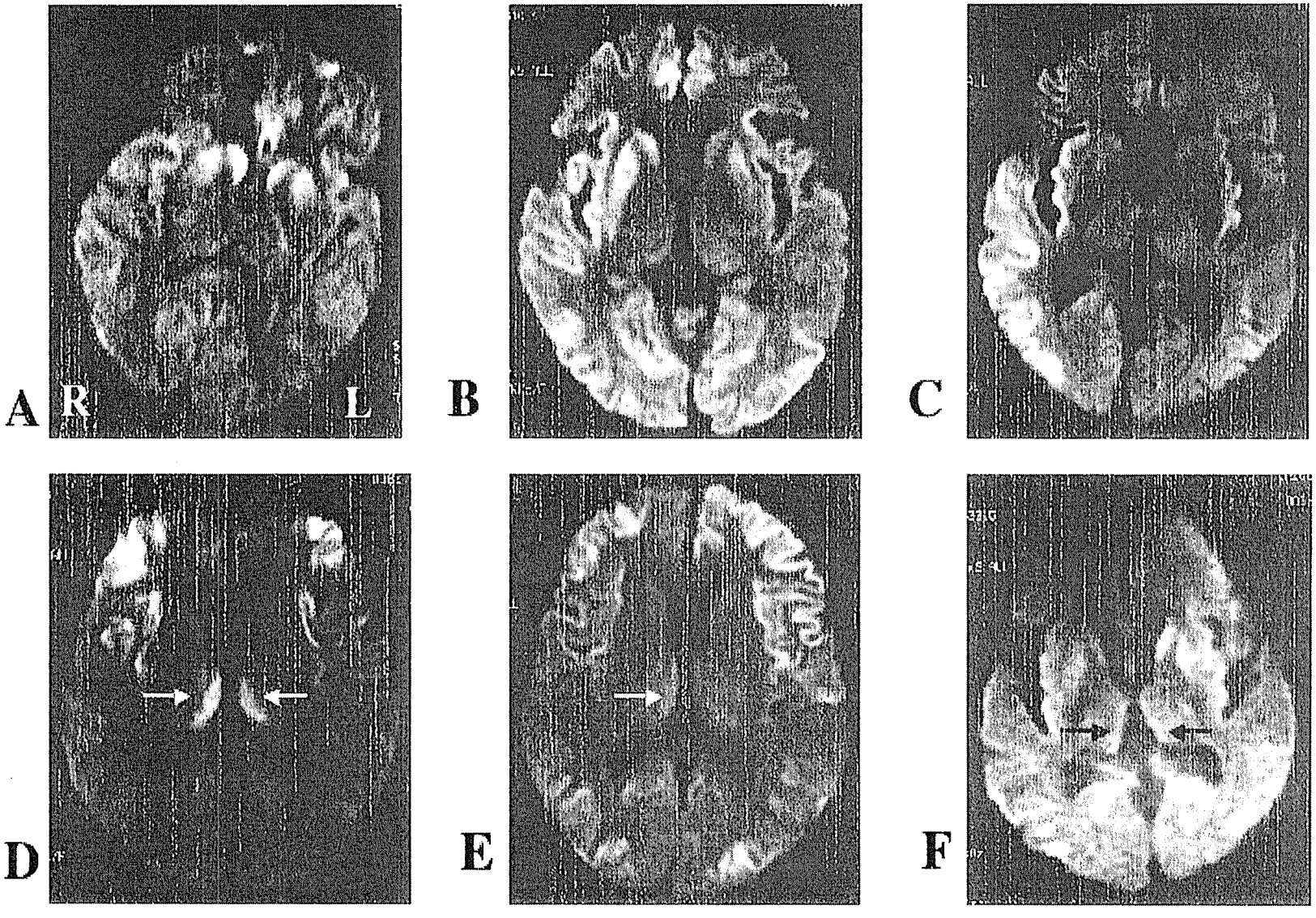


**Figure 3**

A

B

C

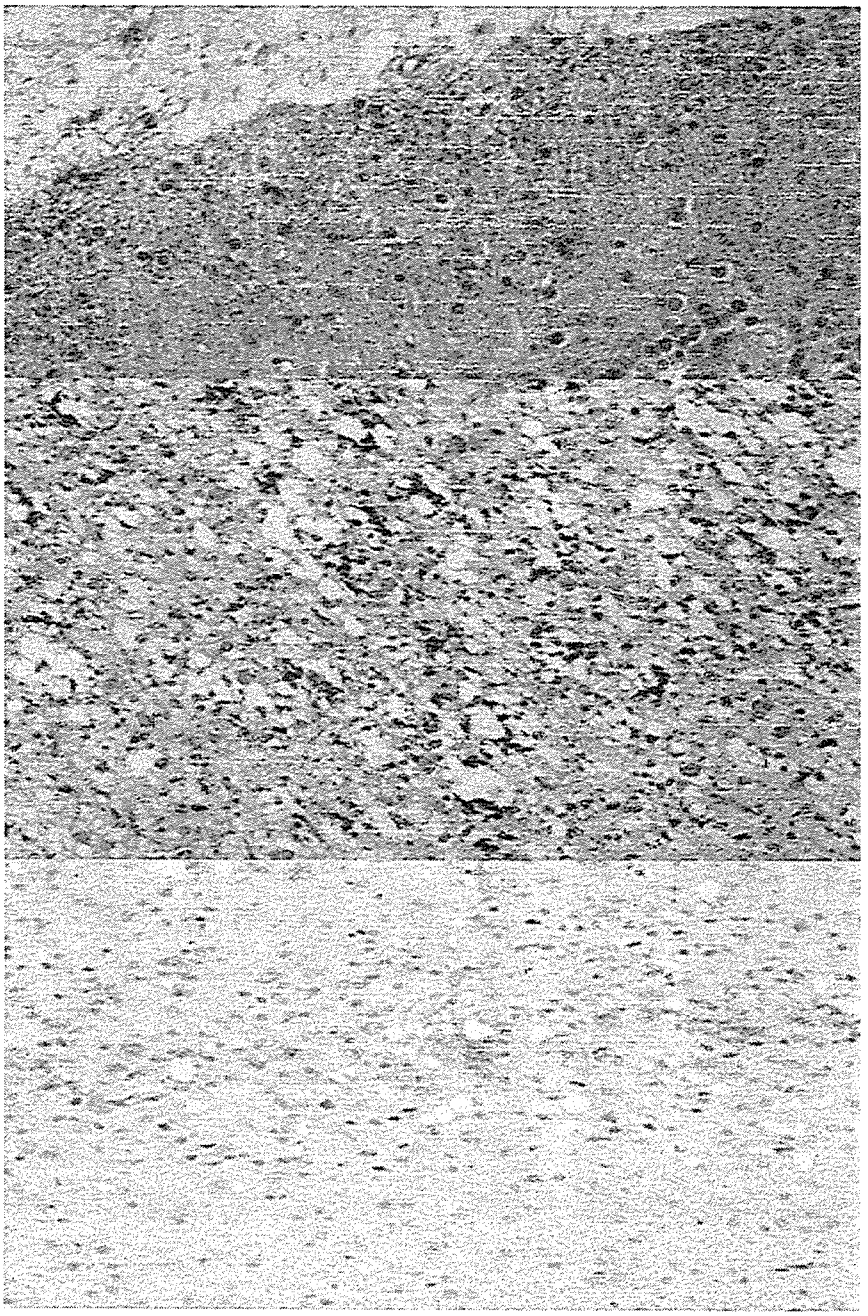

**Figure 4**

# 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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antibody, enterotoxin, mucosal vaccine, prion, prion diseases, prion protein, recombinant protein, *Salmonella*

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.

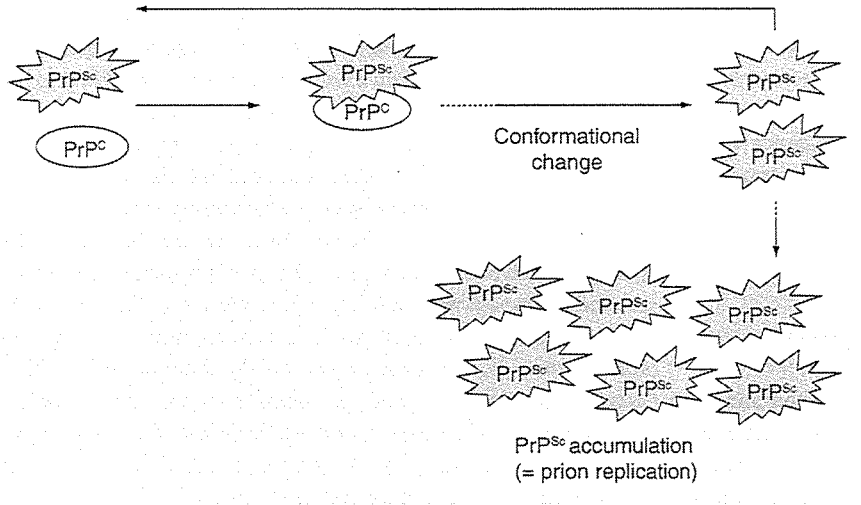


**Figure 1. Mechanism for the propagation of prions or the accumulation of the abnormal isoform of PrP, namely $PrP^{Sc}$.** Prions (or $PrP^{Sc}$) that have invaded the body interact with the normal isoform of PrP, $PrP^{C}$, and change the protein conformation of the interacting $PrP^{C}$ into that 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

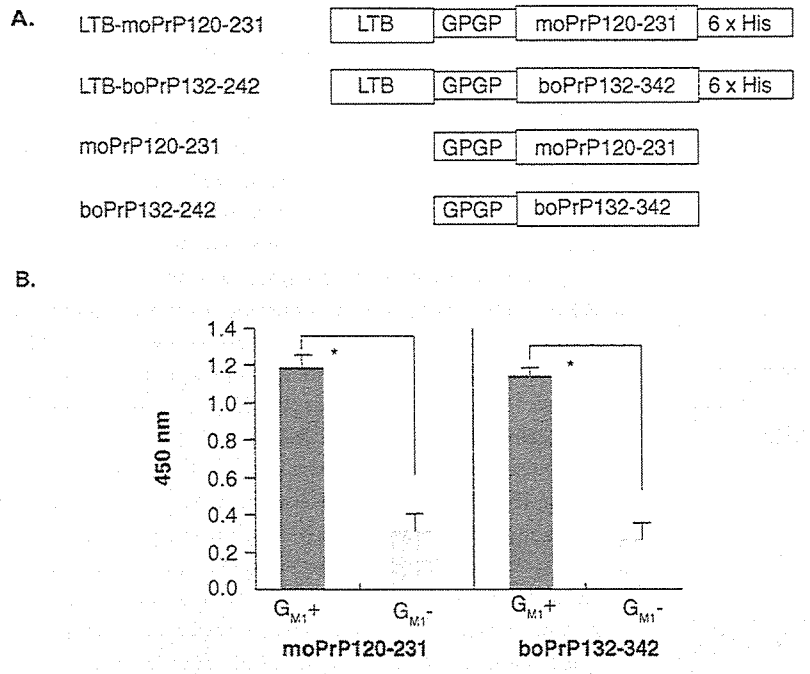


**Figure 2. (A) Schematic structures of mouse (mo) and bovine (bo) PrPs fused with or without LTB. (B) Binding of LTB-moPrP120–231 and LTB-boPrP132–242 to $G_{M1}$ ganglioside.**
Wells coated with or without $G_{M1}$ ganglioside were incubated with LTB-moPrP120–231 or LTB-boPrP132–242. Binding of the 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 nontoxic 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.

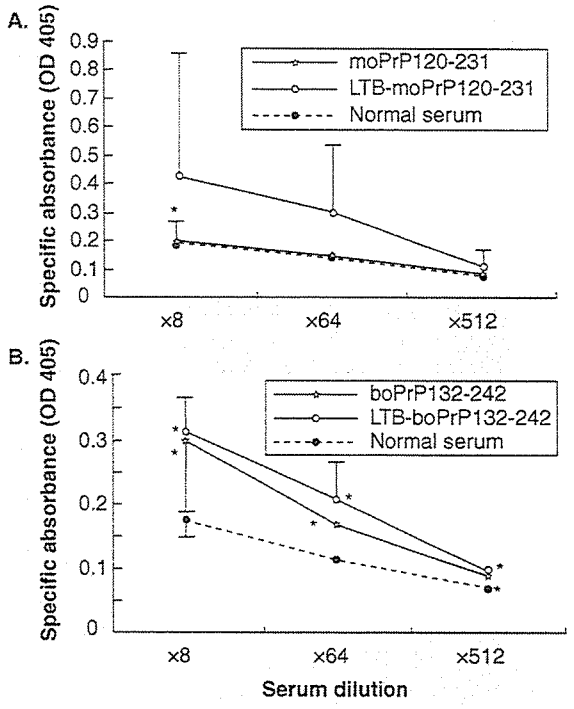


**Figure 3. Anti-moPrP autoantibodies in Balb/c mice immunized with mo (A) and boPrPs (B) fused with or without LTB six times at 2-week intervals.** Antisera were collected from four to five mice from each group and subjected to enzyme-linked immunosorbent assay against moPrP without a 6×His tag. Antibody 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.

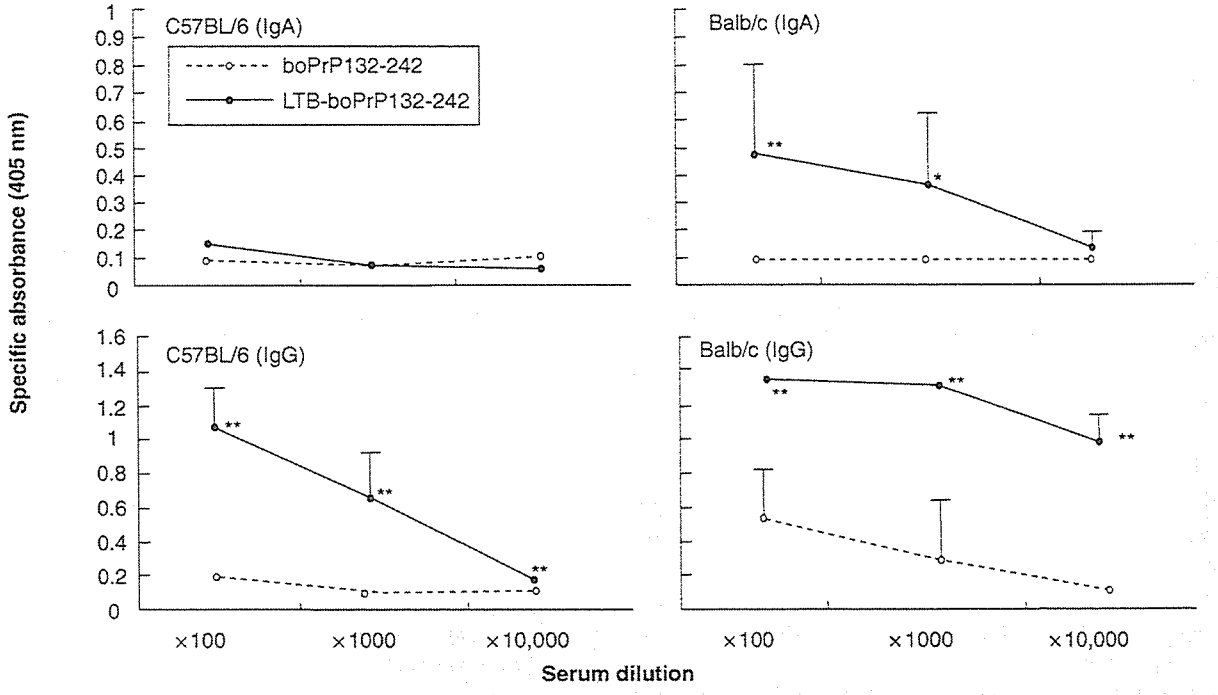


**Figure 4. Specific IgA and G antibody titers in the serum of C57BL/6 and Balb/c mice intranasally immunized with LTB-boPrP132–242 or boPrP132–242 three times at 2-weekly intervals.** Antisera were collected from five mice from each group and were subjected to enzyme-linked immunosorbent assay against 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.
*$p < 0.05$.
**$p < 0.01$.
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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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## Website


101 http://niah.naro.affrc.go.jp/disease/bse/count.html.


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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.


*Keywords:* Prion; Vaccine; Tolerance

## 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 | ccaagcttctatcaacttgccccccttggtaata |
| 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′-tcggatcc-agtcatc**atg**gcgaaccttggc-3′; the underlined sequence, a *Bam*HI site; the bold sequence, a start codon) and an antisense primer (5′-cctctagac**ctca**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′-cctctagac**cctca**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

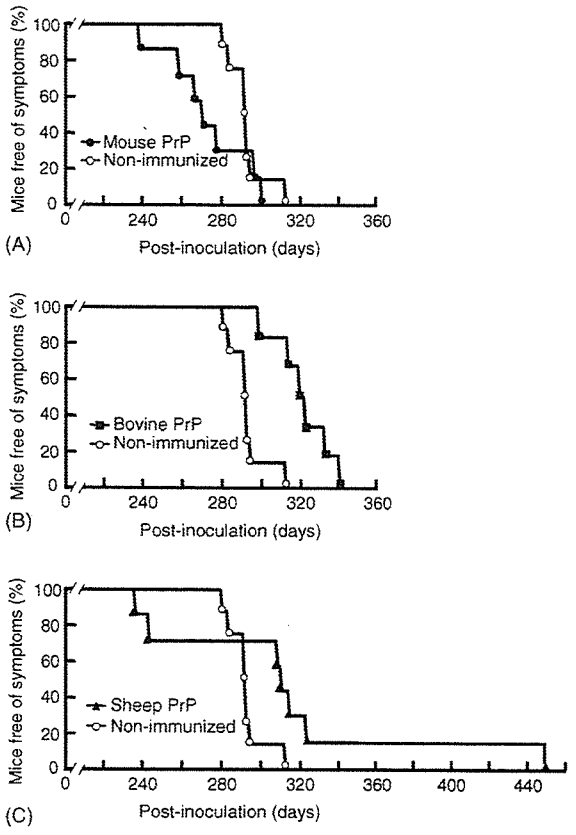


Fig. 1. Incubation times in mice immunized with mouse (A), bovine (B), and sheep (C) recombinant PrPs after intraperitoneal inoculation of a mouse-adapted Fukuoka-1 prion. (A) Incubation times in mice immunized with mouse recombinant PrP ($n=7$) and in non-immunized mice ($n=8$). No prophylactic effect from immunization with mouse recombinant PrP was detected. Instead, incubation times seemed to be shortened, compared with those of non-immunized mice. (B) Incubation times in mice immunized with bovine recombinant PrP ($n=6$) and in non-immunized mice ($n=8$). The immunized mice developed the disease with significantly delayed onset ($p=0.0008$, Logrank test). (C) Incubation times in mice immunized with sheep recombinant PrP ($n=7$) and in non-immunized mice ($n=8$). Except for two of the immunized mice, the other five mice showed extended incubation times compared to non-immunized mice.

the immunized mice 1 week after the final immunization. Non-immunized BALB/c mice developed the disease $291 \pm 10$ days post-inoculation (p.i.) (Fig. 1). Immunization with mouse recombinant PrP had no prophylactic effect against the disease. The immunized-mice succumbed to the disease at $269 \pm 22$ days p.i. (Fig. 1A). No significant difference in the incubation times could be detected between the mice immunized with and without mouse recombinant PrP ($p=0.22$, Logrank test), but incubation times of the immunized mice appeared to be shortened compared with those of the non-immunized mice. In contrast, mice immunized with recombinant bovine PrP showed significantly delayed onsets at $322 \pm 15$ days p.i., compared with non-immunized mice ($p=0.0008$, Logrank test, Fig. 1B). Immunization with recombinant sheep PrP showed variable effects against the prion. Five out of seven immunized mice developed the disease with prolonged onset (Fig. 1C). Two remaining mice became sick at 239 and 246 days p.i., as early as mice immunized with mouse recombinant PrP began to succumb (Fig. 1C). Accumulation of $PrP^{Sc}$ and pathological changes including vacuolation and gliosis were indistinguishable in the brains of terminally diseased mice (data not shown).

### *3.2. Bovine and sheep but not mouse recombinant PrP stimulates antibody responses against respective immunogens in mice*

To assess the immunogenicity of recombinant bovine, sheep, and mouse PrPs in mice, we investigated antibody responses in the immunized mice. Antisera were collected just before prion infection and each serum of the four to five immunized mice of each group was subjected to an ELISA to detect specific IgG antibodies against respective immunizing recombinant PrPs. In the mice immunized with mouse recombinant PrP, only slightly higher antibody binding expressed as optical density values at 405 nm ($OD_{405}$) were detected, compared with those of non-immunized mice (Fig. 2A). In contrast, much higher $OD_{405}$ values were observed in the mice immunized with recombinant bovine and sheep PrPs (Fig. 2A). We also performed Western blotting of COS-7 cells transiently expressing mouse, sheep, and bovine $PrP^C$ without a 6× His tag using the antisera. No mouse $PrP^C$ could be detected by the anti-mouse recombinant PrP sera on Western blotting (Fig. 2B). In contrast, all of the anti-sheep and -bovine recombinant PrP sera we used for Western blotting substantially detected sheep and bovine $PrP^C$ expressed in COS-7 cells, respectively (Fig. 2B). However, the signals were variable in intensity with each anti-sheep or -bovine recombinant PrP serum. Three out of four anti-bovine PrP sera showed relatively strong signals of bovine $PrP^C$ but the remaining one exhibited faint signals (Fig. 2B). In the case of anti-sheep PrP sera, one antiserum revealed relatively strong signals but the remaining ones exhibited weak signals (Fig. 2B). These results indicate that recombinant bovine and sheep but not mouse PrP were immunogenic but their immunogenicities were variable in BALB/c mice.

We further carried out fluorescence activated cell sorter (FACS) analyses and found that the antisera against bovine and sheep PrPs also contained various amounts of antibodies capable of reacting with respective native $PrP^C$ transiently expressed on COS-7 cells (Fig. 2C).

### *3.3. Anti-PrP autoantibodies are variably produced in mice immunized with recombinant bovine and sheep PrPs*

We investigated whether the antisera against recombinant bovine and sheep PrPs could crossreact with mouse PrP by carrying out ELISA. The immunizing recombinant PrPs contained a 6× His tag. Therefore, to eliminate immunoreactivity

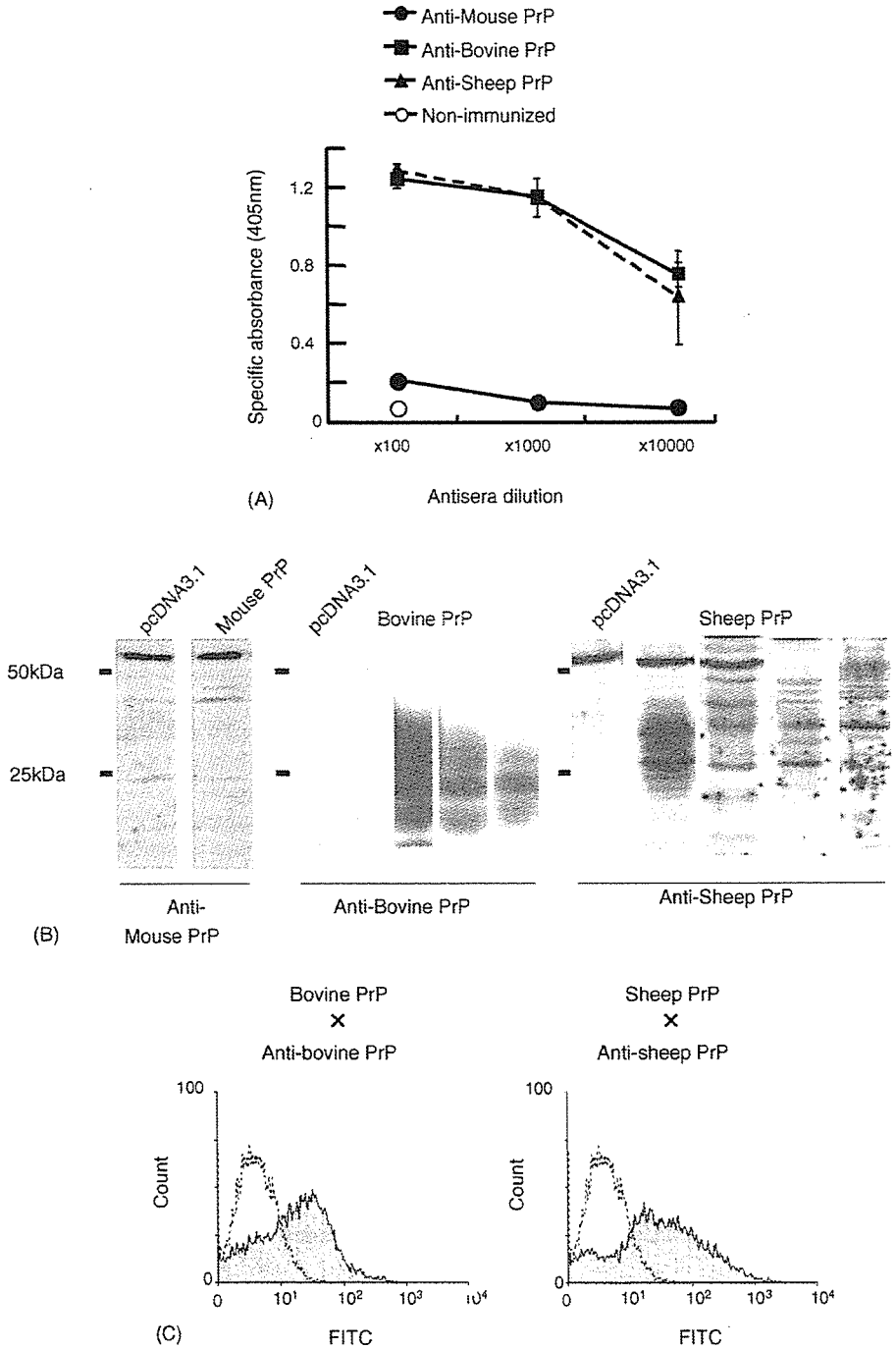


Fig. 2. Antibody responses in mice immunized with mouse, bovine, and sheep recombinant PrPs against the respective immunogens. (A) Each group of at least five mice was intraperitoneally immunized with the purified recombinant PrPs five times at 2-week intervals and anti-PrP IgG antibodies were detected in each serum of the immunized four to five mice of each group by an ELISA against the immunizing antigens. For anti-mouse PrP antibody detection, purified mouse recombinant PrP without a 6× His tag was used instead. (B) Antigenic specificities of each antiserum of the four immunized mice from each bovine and sheep recombinant PrP group were also examined by Western blotting of COS-7 cells transiently transfected with pcDNA3.1 vector alone or with pcDNA3.1 encoding each $PrP^C$. (C) FACS analysis of COS-7 cells transiently expressing each $PrP^C$. The cells transfected with the vector alone (unshaded) and the vector encoding bovine or sheep $PrP^C$ (shaded) were probed by each antiserum.

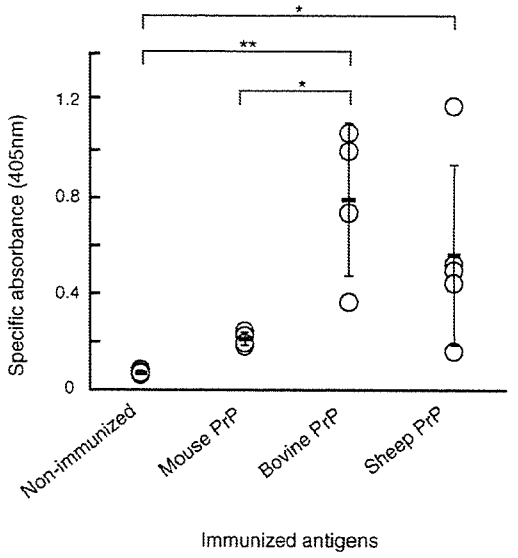


Fig. 3. Anti-PrP autoantibodies in mice immunized with recombinant mouse, bovine, and sheep PrPs. Each antiserum from four to five mice immunized with respective recombinant PrPs was diluted 1:100 and subjected to ELISA against purified mouse recombinant PrP without a 6× His tag. $^{*}p<0.05$; $^{**}p<0.01$.

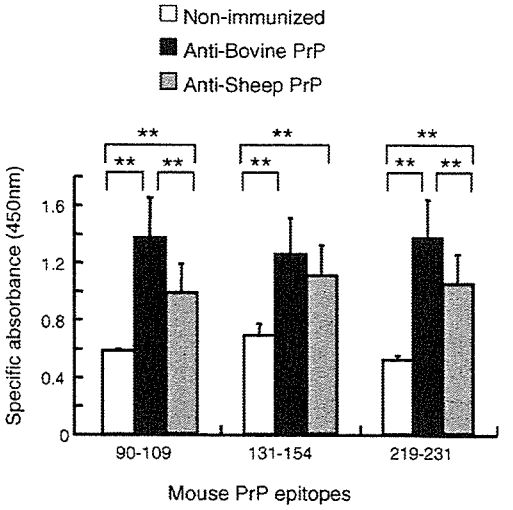


Fig. 4. Recognition of three different mouse PrP epitopes by antisera raised against bovine and sheep recombinant PrPs. The antisera used were collected from four to five mice of each immunization group. Mouse PrP peptides, moPrP90–109, moPrP131–154, and moPrP219–231, positively reacted with antisera raised against bovine and sheep recombinant PrPs on ELISA, compared with sera from non-immunized normal mice ($^{**}p<0.01$).

against the tag with antibodies that might be produced in the immunized mice, we used recombinant mouse PrP without the tag as an ELISA antigen. The antisera raised against mouse recombinant PrP showed only slightly higher $OD_{405}$ values depicting antibody responses at a 100-fold dilution, compared with those of non-immunized mice (Fig. 3). In contrast, a range of much stronger antibody responses showing as higher $OD_{405}$ values was detected with the antisera against bovine recombinant PrP (Fig. 3). The antisera against sheep recombinant PrP showed more variable titers of anti-PrP autoantibodies (Fig. 3). One mouse elicited the highest titer of anti-PrP autoantibodies among the immunized mice, but another mouse exhibited a very weak antibody response showing $OD_{405}$ values as low as those from mouse recombinant PrP-immunized mice (Fig. 3).

### *3.4. Anti-bovine and anti-sheep PrP antisera recognize prion epitopes*

Mouse PrP residues 91–110, 144–152, and 146–159 are the targets for protective monoclonal antibodies, ICSN 35, 6H4 and ICSN 18, respectively [9,10]. Thus, we investigated whether the antisera against bovine and sheep recombinant PrPs could recognize these epitopes. Two different mouse PrP peptides, moPrP90–109 and moPrP131–154, were synthesized and subjected to a more sensitive ELISA with each concentrated (20×) antiserum of the four to five immunized mice of each group because the conventional ELISA described above was less sensitive for detecting the specific signals. This sensitive ELISA resulted in higher backgrounds from non-immunized sera (Fig. 4). However, these two peptides were recognized with the anti-bovine and the anti-sheep PrP sera, showing higher $OD_{450}$ values compared to non-immunized sera (Fig. 4).

Mouse PrP residues 220–231 form target epitopes for PrP-specific Fab fragments, termed R1 and R2, both of which are capable of clearing $PrP^{Sc}$ from prion-infected N2a neuroblastoma cells [12]. We similarly performed the ELISA with a synthetic moPrP219–231 peptide (Fig. 3). Higher specific absorbance could be detected in the anti-bovine and anti-sheep sera, compared to non-immunized sera. However, we could not detect any therapeutic effects of these antisera using prion infected N2a cells (data not shown). This is probably due to very low titers of the antibodies against the peptide in these antisera, as the specific signals were undetectable by conventional ELISA.

## 4. Discussion

In the present study, we showed that BALB/c mice immunized with bovine recombinant PrP exhibited slightly but significantly extended survival after peripheral infection with the mouse-adapted Fukuoka-1 prion. In contrast, we could not detect any prophylactic effects against the prion in mice immunized with mouse recombinant PrP. Instead, the disease seemed to be accelerated in most of the immunized mice. Sheep recombinant PrP had variable effectiveness against the prion infection. Five out of seven immunized mice developed the disease later than non-immunized mice. However, the disease seemed to be exacerbated in two remaining mice with incubation times as short as those of mice immunized with mouse recombinant PrP. These results indicate that immu-

nization effects of recombinant bovine, sheep, and mouse PrPs on the prion infection were different in BALB/c mice.

We showed that heterologous bovine and sheep recombinant PrPs, but not mouse PrP, were immunogenic in mice, stimulating antibody responses against the respective immunizing antigens. Interestingly, we also showed that mice immunized with bovine and sheep recombinant PrPs variably produced a considerable amount of anti-PrP autoantibodies, and that these anti-PrP autoantibodies could react with the mouse PrP epitopes, moPrP90–109, moPrP131–154, and moPrP219–231. White et al. showed that passive immunization of mice with anti-PrP antibodies, which recognize the epitopes overlapping the two former epitopes, moPrP90–109 and moPrP131–154, efficiently blocked prion infection [10]. It has been also reported that titers of anti-PrP autoantibodies, which were induced by immunization with mouse recombinant PrP, were well correlated to the onset time of disease in mice inoculated with mouse 139A prion [13]. It is therefore likely that autoantibody-mediated humoral immunity could be associated with the attenuation of the Fukuoka-1 prion in mice immunized with bovine and sheep recombinant PrPs. However, at the present time, we do not know the exact mechanism of the protective effects of bovine and sheep recombinant PrP immunization on prion infection. It was reported that a cytotoxic T cell-like clone could be isolated by immunization of PrP-null mice with a PrP-derived peptide conjugated with keyhole limpet hemocyanin [14]. This might indicate an alternative possibility that cellular immunity may be involved in protection against prion infection.

The prophylactic effects of the immunization of mice with recombinant sheep PrP on the prion infection seemed variable, compared with those of recombinant bovine PrP. Western blotting of bovine $PrP^C$ with the anti-bovine PrP sera revealed that specific antibody responses were variable in the mice immunized with bovine recombinant PrP. Mice immunized with sheep recombinant PrP also showed variable antibody responses. The titers of anti-PrP autoantibodies were also various in amounts in the mice immunized with sheep or bovine recombinant PrP but seemed more variable in the mice with recombinant sheep PrP than in the mice with recombinant bovine PrP. One mouse immunized with recombinant sheep PrP elicited very weak autoantibody responses, showing $OD_{405}$ values as low as those of mice immunized with recombinant mouse PrP, while the other mouse produced anti-PrP autoantibodies higher than any mice immunized with recombinant PrP. Moreover, on Western blotting, specific antibody responses seemed weaker in the mice immunized with sheep PrP than in the mice immunized with bovine PrP. The titers of anti-mouse PrP autoantibodies also seemed lower and autoantibodies against moPrP90–109 and moPrP219–231 were significantly less produced in the mice with recombinant sheep PrP than in the mice with bovine recombinant PrP. It is therefore suggested that this more variable and lower amount of anti-PrP autoantibodies may reflect variable and less effective protection from the disease in mice immunized with recombinant sheep PrP, compared with that of the mice immunized with bovine recombinant PrP. However, unfortunately, because we did not individually identify the immunized mice, we could not directly compare the autoantibody titers to length of the incubation times in mice immunized with recombinant bovine and sheep PrPs in the present study. Thus, at this point, we are unable to directly answer the question why the immunization effects of recombinant sheep PrP on the prion infection were more variable than those of recombinant bovine PrP or why the two mice immunized with sheep recombinant PrP succumbed to the disease earlier than control non-immunized mice.

The disease also seemed to be exacerbated in the mice immunized with mouse recombinant PrP. It was reported that complement components C3 and C1q mediate the initial trapping of prions in lymphoreticular tissues [15,16]. Therefore, complement components, which might be upregulated by immunization, may be associated with the slight, but not significant, exacerbation of the disease. Alternatively, certain conditions induced in the peritoneal cavity by multiple immunizations could be considered to be involved in the disease exacerbation because the prion was inoculated into the same peritoneal cavity. However, these remain to be elucidated.

It was previously shown that recombinant mouse PrP was immunogenic eliciting anti-PrP autoantibodies in CD-1 mice and could slightly retard onset of the disease in immunized mice after inoculation with a mouse-adapted 139A prion [13]. However, we detected only a very weak antibody response in BALB/c mice immunized with mouse recombinant PrP and no such prophylactic effects of the immunization on the prion infection. Polymenidou et al. also reported that recombinant mouse PrP failed to induce anti-PrP autoantibodies in C57BL/6× 129Sv mice [17]. The different genetic background of mice used in each experiment may be responsible for the different antibody responses. Gilch et al. reported successfully inducing anti-PrP autoantibodies by immunization of mice with mouse recombinant PrP [18]. In this case, the recombinant PrP was inserted by a human or hamster-derived 3F4 epitope at the corresponding region, resulting in the recombinant PrP with two different amino acids from mouse PrP [18]. Thus, the recombinant PrP might acquire heterologous PrP-like immunogenicities in part and thereby induce anti-PrP autoantibodies in mice.

Molecular mimicry between microbial and host antigens is a well-known hypothetical mechanism for triggering autoimmune diseases via production of autoantibodies and/or autoreactive T cells [19,20]. This hypothesis postulates that shared identical amino acid sequences or homologous but non-identical amino acid sequences between microbial and host antigens could be essential for the initial processes of molecular mimicry [19,20]. PrPs are highly conserved molecules among mammals, sharing marked similarities in both amino acid sequence and tertiary structure [21–23]. Bovine and sheep recombinant PrPs contain 19 and 21 amino acids different from mouse recombinant PrP, respectively, indicating that the higher immunogenicity of bovine and