the Purkinje cell degeneration. Interestingly, we showed that PrPΔ23-88 has no such trans-neuroprotective activity, unable to antagonize against PrPLP/Dpl (39). It is therefore suggested that N-terminal residues 23-88 are involved in the trans-neuroprotection of PrP^C against PrPLP/Dpl and probably the truncated PrPs as well.

Antagonistic signals of PrP and PrPLP/Dpl in neuronal cell death

Kuwahara, *et al.* previously reported that hippocampal neuronal cells from PrP-/- mice easily undergo apoptosis after withdrawal of serum, which can be prevented by either re-introduction of PrP^C or by expressing the anti-apoptotic protein, Bcl-2 (46). Bounhar, *et al.* also showed that PrP^C protected human primary neurons from the apoptosis induced by the pro-apoptotic protein Bax (47). It is therefore suggested that PrP^C might be an anti-apoptotic protein while PrPLP/Dpl might be pro-apoptotic.

It was shown that the primary cultured cerebellar neurons from non-ataxic Npu PrP-/- mice were more sensitive to oxidative stress than those from PrP+/+ mice (19), indicating that PrP^C might be involved in mitigation of oxidative stress. Interestingly, Wong, *et al.* showed that oxidative stress was much more elevated in the brains of ataxic Rcm0 PrP-/- mice than in those of non-ataxic Npu PrP-/- mice (48). It is therefore suggested that, in contrast to PrP^C, PrPLP/Dpl aggravates oxidative stress. Oxidative stress is associated with overproduction of radical oxygens, such as superoxide and nitric oxide (NO). Interestingly, it was shown that enzymatic activity of superoxide dismutase, a superoxide-detoxifying enzyme, was significantly decreased in PrP-/- mice (18). Moreover, it was reported that recombinant PrPLP/Dpl was toxic to the cultured cerebellar neurons of non-ataxic Zrch I PrP-/- mice and that this PrPLP/Dpl-neurotoxicity could be prevented by L-*N*-acetyl methyl ester, a pharmacological inhibitor of NO synthases (49). It is therefore possible that PrPLP/Dpl might aggravate oxidative stress by overproducing NO and superoxide, thereby causing Purkinje cell death, and that PrP^C could detoxify it, preventing PrPLP/Dpl-induced neurodegeneration.

It was previously shown that, in Zrch I PrP-/- mice, Ca^{2+}-activated K^+ currents in hippocampal pyramidal neurons were abnormal and intracellular Ca^{2+} contents in cerebellar granule cells were altered (50). Moreover, we showed that the T- and L-type Ca^{2+}-antagonist, flunarizine, significantly reduced the PrPLP/Dpl-aggravated ischemic neuronal apoptosis in Ngsk PrP-/- mice (40). Since excess of intracellular Ca^{2+} has been shown to be toxic to neurons (51), it is alternatively possible that PrP^C could reduce the intracellular Ca^{2+} load in neurons, thereby being neuroprotective, and that, in contrast, PrPLP/Dpl increases the intracellular Ca^{2+} load in neurons, thereby enhancing the susceptibility of neurons to apoptosis.

A proposed model of antagonistic interaction between PrP and PrPLP/Dpl

Weissmann and colleagues have proposed an interesting hypothesis, in which two conjectural molecules, protein π, which is another PrP-like protein, and a cognate ligand for PrP^C, are introduced (42, 52). In normal mice, PrP^C binds its cognate ligand to elicit a neuroprotective signal, thereby Purkinje cells are able to survive. But, in ataxic lines of PrP-/- mice, PrPLP/Dpl or the neurotoxic truncated PrPs compete with PrP^C for the ligand and blocks the signal, resulting in Purkinje cell degeneration. In non-ataxic lines of PrP-/- mice, instead of PrP^C, the protein π could elicit the same neuroprotective signal via binding to the ligand, thereby causing no Purkinje cell degeneration. However, this hypothesis can be verified only when the putative molecules are identified.

FINDINGS IN PRP-NULL MICE AND PATHOGENESIS OF PRION DISEASES

The molecular pathogenesis of prion diseases remains elusive. Since PrP-/- mice were resistant to these diseases (11-14), it is considered that the conformational conversion of PrP^C to PrP^{Sc} plays an essential role in the pathogenesis of the diseases. However, the exact nature of this role has not been fully understood. PrP^{Sc} is markedly accumulated in infected brains due to constitutive conversion. Forloni, *et al.* showed that an amyloidogenic PrP peptide (PrP106-126) was highly toxic to primary cultured neurons (53). It is therefore conceivable that PrP^{Sc} might itself be neurotoxic. In contrast, Yokoyama, *et al.* showed that the PrP^C-specific immunoreactivity was decreased in the brain regions where PrP^{Sc} had accumulated in experimentally infected mice (54), indicating that PrP^C is reduced in the infected neurons due to conversion. It is therefore alternatively possible that PrP^C might be functionally disturbed due to its marked reduction, thereby resulting in the nerodegeneration. Or, both the accumu-

lation of PrP^{Sc} and the functional loss of PrP^{C} might be required for the neuronal cell death.

PrP-/- mice without ectopic expression of PrPLP/Dpl exhibited neurological abnormalities, including impairment of LTP, alteration in sleep and circadian rhythm, as well as demyelination in the spinal cord and peripheral nervous system (28, 31, 32). LTP is a form of synaptic plasticity that is thought to underlie memory formation. Memory loss or dementia is a common symptom in prion diseases (55). Alteration in sleep and circadian rhythms is also a symptom characteristic for the inherited human prion disease, fetal familial insomnia (55). Moreover, demyelinating peripheral neuropathy has been reported in some cases of inherited prion disease (56, 57). Taken together, these results strongly support that the functional loss of PrP^{C} is involved in these pathogenic changes in the diseases. However, no neuronal cell degeneration could be detected in mice devoid of PrP^{C} alone, indicating that loss of PrP^{C} alone might not be enough to induce neuronal cell death or that other neurotoxic mechanisms might associate with neuronal cell death in prion diseases.

Interestingly, the ectopic expression of PrPLP/Dpl, PrPΔ32-121, or PrPΔ32-134 in the absence of PrP^{C} caused degeneration of Purkinje cells and granule cells (6, 38, 42). Purkinje cells and granule cells are markedly degenerative in human prion diseases (55). It is therefore conceivable that PrPLP/Dpl and the truncated PrPs might be involved in the pathogenesis of the diseases. However, PrPLP/Dpl itself is unlikely to be involved in the pathogenesis of prion diseases because PrPLP/Dpl could not be detected in the brains affected by experimental prion diseases (6, 8). Instead, PrP^{Sc} is markedly accumulated and its fragmented C-terminal products are often observed in the affected brains (58). It is therefore very interesting to speculate that PrP^{Sc} or its fragmented products possess a neurotoxic potential equivalent to that of PrPLP/Dpl, PrPΔ32-121, or PrPΔ32-134. Thus, elucidation of the molecular mechanism for the Purkinje cell degeneration in ataxic lines of PrP-/- mice might be useful for understanding of the molecular pathogenesis of prion diseases.

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Recent developments in mucosal vaccines against prion diseases

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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.

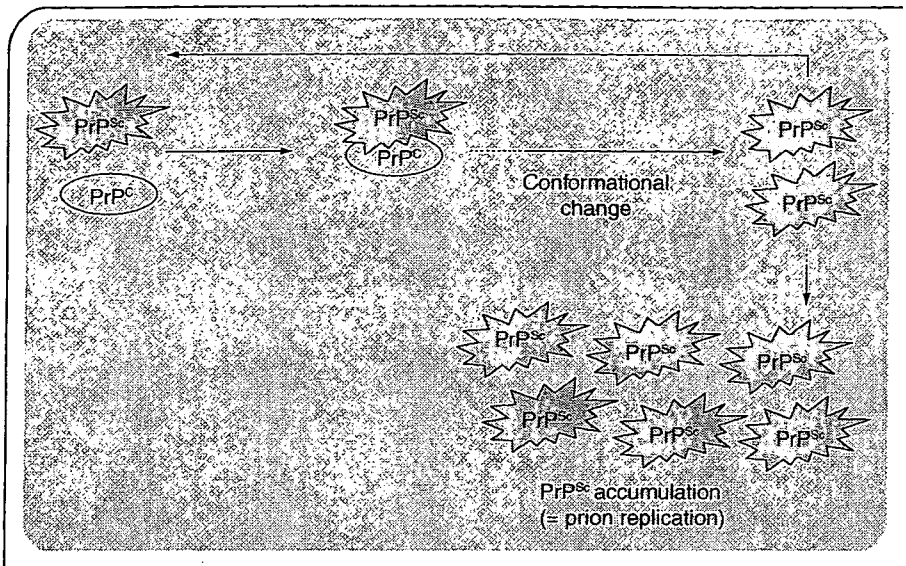

Figure 1. Mechanism for the propagation of prions or the accumulation of the abnormal isoform of PrP, namely PrP^{Sc}. Prions (or PrP^{Sc}) that have invaded the body interact with the normal isoform of PrP, PrP^{C}, and change the protein conformation of the interacting PrP^{C} into that of PrP^{Sc},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

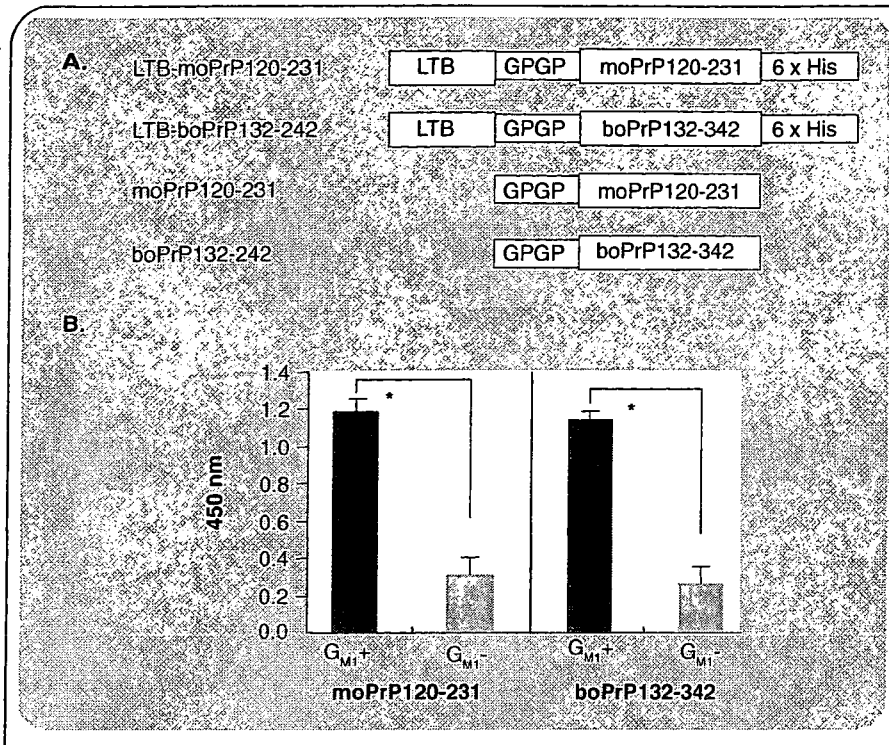

Figure 2. (A) Schematic structures of mouse (mo) and bovine (bo) PrPs fused with or without LTB. (B) Binding of LTB-moPrP120–231 and LTB-boPrP132–242 to G_{M1} ganglioside.
Wells coated with or without G_{M1} ganglioside were incubated with LTB-moPrP120–231 or LTB-boPrP132–242. Binding of the proteins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

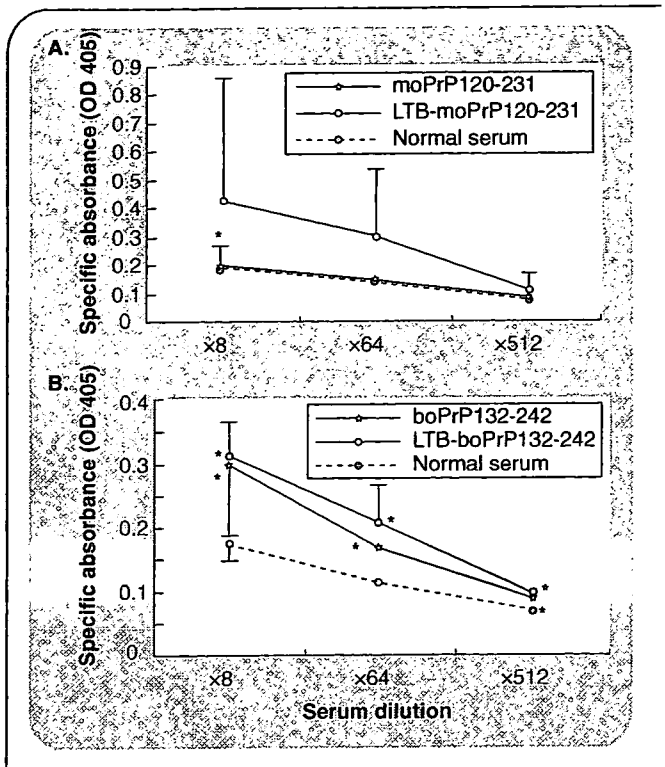

Figure 3. Anti-moPrP autoantibodies in Balb/c mice immunized with mo (A) and boPrPs (B) fused with or without LTB six times at 2-week intervals. Antisera were collected from four to five mice from each group and subjected to enzyme-linked immunosorbent assay against moPrP without a 6xHis tag. Antibody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

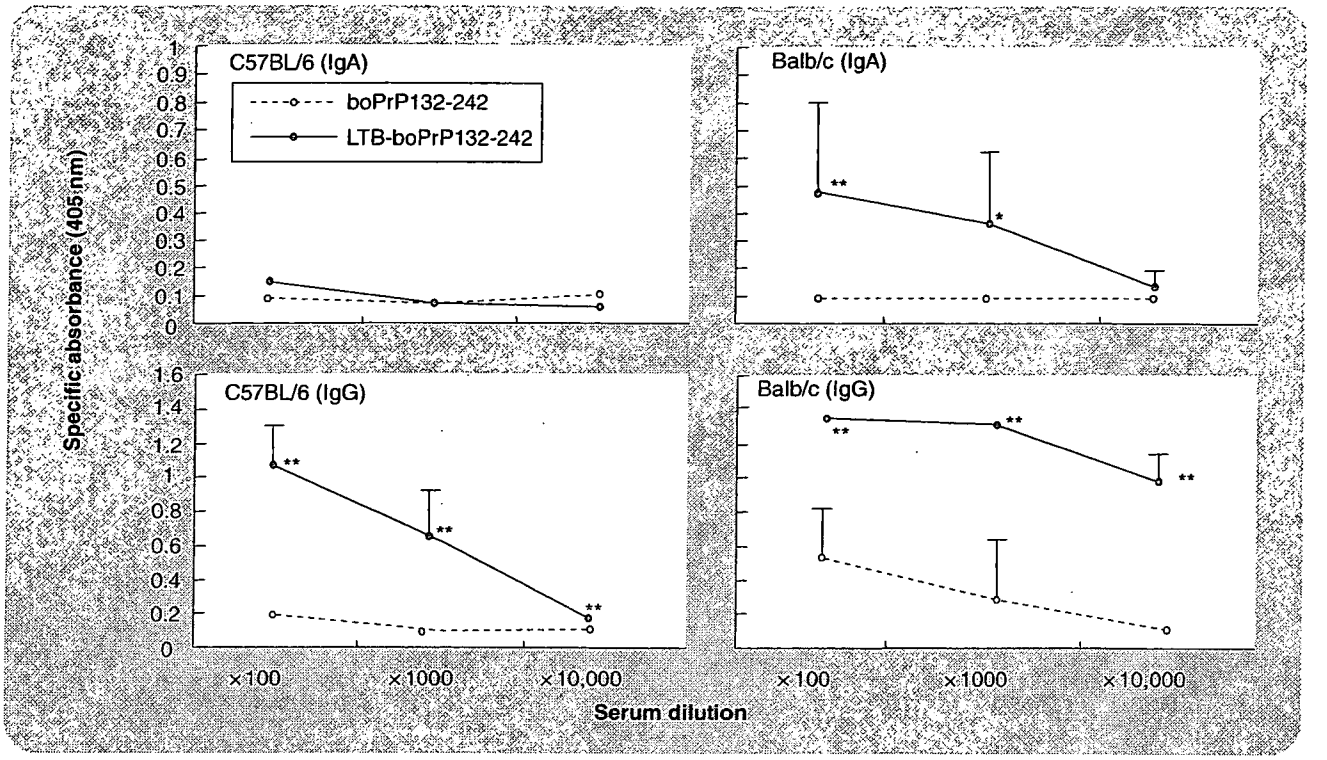

Figure 4. Specific IgA and G antibody titers in the serum of C57BL/6 and Balb/c mice intranasally immunized with LTB-boPrP132–242 or boPrP132–242 three times at 2-weekly intervals. Antisera were collected from five mice from each group and were subjected to enzyme-linked immunosorbent assay against 6×His-tagged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

Acknowledgements

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Neurobiology

Doppel Induces Degeneration of Cerebellar Purkinje Cells Independently of Bax

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Doppel (Dpl) is a prion protein paralog that causes neurodegeneration when expressed ectopically in the brain. To investigate the cellular mechanism underlying this effect, we analyzed Dpl-expressing transgenic mice in which the gene for the proapoptotic protein Bax had been deleted. We found that Bax deletion does not alter either clinical symptoms or Purkinje cell degeneration in Dpl transgenic mice. In addition, we observed that degenerating Purkinje cells in these animals do not display DNA fragmentation or caspase-3 activation. Our results suggest that non-Bax-dependent pathways mediate the toxic effects of Dpl in Purkinje cells, highlighting a possible role for nonapoptotic mechanisms in the death of these neurons. *(Am J Pathol 2007, 171:599–607; DOI: 10.2353/ajpath.2007.070262)*

Transmissible spongiform encephalopathies are fatal neurodegenerative disorders of humans and animals caused by infectious proteins called prions.[1,2] Mammalian prions are composed of PrP^{Sc}, a conformationally altered isoform of a normal cellular glycoprotein called PrP^{C}. Prions propagate by the templated autocatalytic conversion of PrP^{C} into PrP^{Sc}. Although a great deal is known about the role of PrP^{Sc} in the infectious transmission of prion diseases, much less is understood about PrP^{C}.[3] The PrP^{C} molecule of ~250 amino acids consists of a flexible, N-terminal tail and a structured C-terminal half composed of three α-helices and two β-strands flanking the first helix.[4] The N-terminal tail contains a series of five octapeptide repeats and a highly conserved hydrophobic segment that serves as a transmembrane anchor under some circumstances.[5,6] The C terminus of PrP^{C} is attached to the cell membrane via a glycosylphosphatidylinositol anchor.[7,8]

Analysis of the region surrounding the PrP gene (*Prn-p*) uncovered a second gene (*Prn-d*) that encodes a PrP-like protein called Doppel (Dpl, German for "double").[9] Dpl is a protein of 179 amino acids that is homologous to the structured C-terminal half of PrP and lacks the flexible N-terminal tail. Dpl is normally expressed primarily in testis, where it seems to play a role in spermatogenesis.[10,11] However, several lines of PrP knockout ($Prn\text{-}p^{o/o}$) mice display ectopic expression of Dpl in the central nervous system (CNS) because of aberrant mRNA splicing between the adjacent *Prn-p* and *Prn-d* genes.[9,12–14] Surprisingly, these mice, but not those lines of $Prn\text{-}p^{o/o}$ mice without up-regulation of Dpl, develop a severe neurodegenerative disorder characterized by ataxia and loss of cerebellar Purkinje cells. Interestingly, this phenotype is completely suppressed by the presence of the *Prn-p* gene. Subsequent studies of Dpl transgenic mice and $Prn\text{-}d^{o/o}$ mice confirmed that expression of Dpl in the CNS is sufficient to produce neurodegeneration and that the neurotoxic effect of Dpl is antagonized in a dose-dependent fashion by coexpression of PrP.[12,15–18] Dpl causes loss of both Purkinje and granule cells in the cerebellum, depending on the cell type in which the protein is expressed.[15,16] Dpl also produces a leukoencephalopathy characterized by axon loss and myelin degeneration.[19]

Although it is not essential for propagation of prions,[20,21] Dpl is likely to provide important clues to the normal physiological function of PrP^{C}. Interestingly, transgenic mice expressing PrP forms deleted for portions of the N-terminal tail (Δ32–121, Δ32–134, Δ94–134, and Δ105–125) display neurodegenerative phenotypes that are suppressible by coexpression of wild-type PrP, as is the case for mice expressing Dpl in the CNS.[22–24] These observations suggest that Dpl and the deleted forms of

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PrP act via a similar neurotoxic mechanism and that the N-terminal tail of PrP (in particular, residues 105 to 125) is capable of suppressing a potent neurotoxic activity that resides in the C-terminal half of both PrP^C and Dpl.

What are the neurotoxic pathways activated by Dpl? To begin to address this question, we have analyzed how the phenotype of Tg(Dpl) mice is affected by deleting the gene that encodes Bax. Bax is a proapoptotic member of the Bcl-2 family that plays a major role in regulating cell death in the CNS, both during development and following injury.[25,26] Bax is a cytoplasmic protein that translocates to mitochondria in response to extrinsic or intrinsic signals, thereby causing release of cytochrome *c* and subsequent activation of caspases.[27,28] In this study, we find that Bax deletion does not alter clinical symptoms or Purkinje cell degeneration in Tg(Dpl) mice, suggesting that the neurotoxic effects of Dpl occur independently of Bax, possibly via nonapoptotic processes.

Materials and Methods

Mice

Tg(N-Dpl) mice (line 32)[29] and $Bax^{0/0}$ mice[30] have been previously described. *Prn-p*$^{0/0}$ mice,[31] which do not spontaneously develop ataxia, were obtained from Charles Weissmann (The Scripps Research Institute, Jupiter, FL) and Tg(F35) mice[22] from Adriano Aguzzi (University of Zurich, Zurich, Switzerland). All of these mice were maintained on a C57BL/6J × CBA/J hybrid background. *Lurcher* mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

To generate mice for this study, we first crossed $Dpl^{+/0}$ *Prn-p*$^{+/0}$ $Bax^{+/+}$ males with *Prn-p*$^{+/+}$ $Bax^{0/0}$ females and recovered $Dpl^{+/0}$ *Prn-p*$^{+/0}$ $Bax^{+/0}$ offspring. Males of the latter genotype were then mated to *Prn-p*$^{0/0}$ $Bax^{0/0}$ females to produce littermate offspring in groups 1 to 4 (see Results). *Prn-p*$^{0/0}$ $Bax^{0/0}$ mice were produced by mating *Prn-p*$^{+/+}$ $Bax^{0/0}$ females to *Prn-p*$^{0/0}$ $Bax^{+/+}$ males and then intercrossing the resulting *Prn-p*$^{+/0}$ $Bax^{+/0}$ offspring. *Prn-p*$^{0/0}$ $Bax^{+/0}$ mice were generated by crossing *Prn-p*$^{0/0}$ $Bax^{0/0}$ females to *Prn-p*$^{0/0}$ $Bax^{+/+}$ males.

Mice were genotyped by polymerase chain reaction analysis of tail DNA prepared using the Puregene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN). Primer pairs for *Prn-p*,[32] Dpl,[29] and Bax[30] have been previously been reported. Ataxia was assessed using a set of objective criteria as described previously.[32]

Histology

Mice were anesthetized and perfused transcardially with 40 ml of 0.9% (w/v) NaCl and then with 40 ml of 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.35. Brains were removed and postfixed in 4% paraformaldehyde for 1 hour and then transferred to PBS for 24 hours at 4°C. After freezing, sagittal sections (14-μm thickness) were cut on a cryostat starting from the midline of each bisected brain. Sections were floated in PBS and stored at 4°C before staining.

Staining was performed on free-floating cryostat sections. Sections were permeabilized in PBS containing 0.1% Triton X-100 for 15 to 30 minutes, followed by blocking with 1% bovine serum albumin-PBS for 1 hour, all at room temperature. Sections were then incubated overnight at 4°C with antibodies directed against the following antigens: calbindin (1:1000 dilution; Sigma Chemical Co., St. Louis, MO); glial fibrillary acidic protein (1:1000 dilution; Dako, Carpinteria, CA); activated caspase-3 (1:1000 dilution; Cell Signaling Technology, Danvers, MA). Antibodies were diluted in PBS containing 0.1% bovine serum albumin and 0.1% Triton X-100. Sections were washed in PBS and then incubated with Alexa Fluor 488- or Alexa Fluor 594-labeled anti-IgG antibody (1:100 dilution; Invitrogen, Carlsbad, CA) for 45 minutes at room temperature. After further washing in PBS, sections were mounted on slides and imaged with a Zeiss LSM 510 confocal fluorescence microscope. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) was performed on calbindin-stained cryostat sections using the In Situ Cell Death Detection Kit according to the manufacturer's directions (Roche Diagnostics, Indianapolis, IN).

Purkinje Cell Counts

Counts were performed in cerebellar lobule IV, which displayed consistent Purkinje cell loss in all sections analyzed. Serial cryostat sections (14-μm thickness) were cut from each half brain beginning at the midline. The first section to show all of the cerebellar lobules was identified, and then every third consecutive section after that one was collected for a total of six sections. Sections were stained for calbindin as described above, and the total number of Purkinje cells residing in lobule IV of all six sections was counted. The position of the first section used for counting varied by <28 μm (plus or minus one section thickness). The six sections used for counting spanned a total thickness of 224 μm in each half cerebellum.

Western Blotting

Brain homogenates 10% (w/v) in PBS were centrifuged at 1000 × *g* for 10 minutes, and the supernatant was collected. Total protein concentration was determined using BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Two hundred micrograms of protein was analyzed in each lane of 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gels. After transfer, the blot was blocked in 5% nonfat dried milk-PBS and then probed with primary antibodies to PrP (8H4[33]), Dpl,[34] Bax (Santa Cruz Biotechnology, Santa Cruz, CA), Bak (Sigma Chemical Co.), or β-actin (Chemicon, Temecula, CA). Blots were developed using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Table 1. Bax Deletion Does Not Affect the Clinical Phenotype of Tg(Dpl) Mice

Genotype	Symptom onset	Death
Group 1 ($Dpl^{+/0}$ $Prn\text{-}p^{0/0}$ $Bax^{+/0}$)	56 ± 5 (46)	157 ± 35 (9)
Group 2 ($Dpl^{+/0}$ $Prn\text{-}p^{0/0}$ $Bax^{0/0}$)	55 ± 6 (31)	154 ± 30 (11)
Group 3 ($Dpl^{+/0}$ $Prn\text{-}p^{+/0}$ $Bax^{+/0}$)	266 ± 40 (6)	>365 (12)
Group 4 ($Dpl^{+/0}$ $Prn\text{-}p^{+/0}$ $Bax^{0/0}$)	261 ± 52 (5)	>365 (10)

Ages at symptom onset and death are given as mean number of days ± SD, with the number of animals in each group in parentheses. Mice in groups 3 and 4 were still alive at the time of writing.

Results

Bax Deletion Does Not Affect the Clinical Phenotype of Tg(Dpl) Mice

The Tg(Dpl) mice used in this study (N-Dpl, line 32) express Dpl in the CNS driven by the neural-specific enolase (NSE) promoter.[29] On the $Prn\text{-}p^{0/0}$ background, these mice develop ataxia at ~60 days of age, accompanied by massive degeneration of cerebellar Purkinje cells. This phenotype is rescued in a dose-dependent fashion by coexpression of wild-type PrP encoded by the endogenous *Prn-p* allele.

To determine whether Bax inactivation affects the neurodegenerative phenotype of Tg(Dpl) mice, we generated four groups of mice with the following genotypes, as described in Materials and Methods: $Dpl^{+/0}$ $Prn\text{-}p^{0/0}$ $Bax^{+/0}$ (group 1); $Dpl^{+/0}$ $Prn\text{-}p^{0/0}$ $Bax^{0/0}$ (group 2); $Dpl^{+/0}$ $Prn\text{-}p^{+/0}$ $Bax^{+/0}$ (group 3); and $Dpl^{+/0}$ $Prn\text{-}p^{+/0}$ $Bax^{0/0}$ (group 4). We did not observe any clinical or histological differences between mice from groups 1 and 3 (which are $Bax^{+/0}$) and mice with the same genotypes but carrying two Bax alleles ($Bax^{+/+}$) (data not shown). Thus, one intact Bax allele is sufficient for production of the Tg(Dpl) phenotype. In the experiments described below, we therefore used $Bax^{+/0}$ mice (groups 1 and 3) as littermate controls to compare with $Bax^{0/0}$ mice (groups 2 and 4).

Group 1 mice, which carry a single *Bax* gene, developed ataxia beginning at 56 ± 5 days of age and became terminally ill at 157 ± 35 days (Table 1), similar to the values reported previously.[29] Group 2 mice, which lack *Bax* expression, showed almost identical times of symptom onset and terminal illness (55 ± 6 and 154 ± 30 days, respectively). Introduction of a single endogenous *Prn-p* allele significantly delayed the onset of ataxia and development of terminal illness to ~260 and >365 days, respectively, regardless of the presence of a copy of the *Bax* gene. Thus, deletion of the *Bax* gene had no significant effect on the clinical phenotype of Tg(Dpl) mice.

Bax Deletion Does Not Prevent Purkinje Cell Degeneration in Tg(Dpl) Mice

To determine whether Bax deletion had any effect on the neuropathology of Tg(Dpl) mice, we stained brain sections with an antibody to calbindin to visualize Purkinje cells, the major neuronal type that undergoes degeneration in these animals. Mice were analyzed during the presymptomatic, symptomatic, and terminal phases of illness (50, 100, and 130 days of age, respectively). In group 1 animals, Purkinje cell numbers decreased progressively from 50 to 130 days (Figure 1, A, E, and I). Calbindin-positive deposits in the granule cell layer and underlying white matter could also be observed (Figure 1, E and I). These most likely represent swellings or torpedoes in the axons of degenerating Purkinje cells, a feature common in other cerebellar mutant mice with Purkinje cell loss.[35] There was also a decrease in the staining of Purkinje cell dendrites in the molecular layer as the illness progressed (Figure 1, A, E, and I). Deletion of Bax (group 2) did not have any observably effect on the development of these pathological features (Figure 1, B, F, and J). The presence of a single *Prn-p* allele suppressed Purkinje cell degeneration, regardless of whether or not Bax was present (Figure 1, C, D, G, H, K, and L). We did not observe any loss of granule neurons in Tg(Dpl) mice from any of the four experimental groups (data not shown).

We observed that loss of Purkinje cells in Tg(Dpl) mice was not uniform in all parts of the cerebellum. Purkinje cell loss was most severe in lobules III to V, with lobules VI to VIII showing moderate loss, and lobules IX and X remaining relatively unaffected (Figure 2). This gradient of Purkinje cell degeneration was apparent at all stages of the disease but was most obvious during the middle of the clinical phase (100 days of age). A similar sparing of Purkinje cells in lobules IX and X has been observed in other mice expressing Dpl in the CNS.[12,16] Even within a single lobule, Purkinje cell loss was often patchy, with some areas being completely devoid of Purkinje cells, other areas showing calbindin-positive torpedoes of degenerating Purkinje cell axons, and still other areas displaying a relatively intact Purkinje cell layer. Areas with fewer Purkinje cells showed diminished calbindin staining of the molecular layer, due to the absence of Purkinje cell dendrites (Figure 2, A and B, arrows). Nonuniform loss of Purkinje cells in a parasagittally oriented stripe-like pattern has been observed in several cerebellar mouse mutants and is thought to reflect differential sensitivity of cells residing in different anatomical compartments.[36]

To quantitate Purkinje cell loss, the number of Purkinje cells in lobule IV of the cerebellum was counted in six regularly spaced serial sections from three mice of each genotype (Figure 3). Group 1 mice ($Bax^{+/0}$) showed a progressive loss of Purkinje cells beginning as early as 50 days of age (before development of symptoms), with about 50% of the cells being lost by 130 days (in the terminal phase). Purkinje cell loss in group 2 mice ($Bax^{0/0}$) was almost the same as in group 1 mice at 50, 100, and 130 days of age. The presence of a single *Prn-p* allele (groups 3 and 4) effectively preserved Purkinje cells, so that at 130 days their numbers were almost the same as those in mice lacking the Dpl transgene (Figure 3, bars labeled $Prn\text{-}p^{+/0}$ $Bax^{+/0}$ and $Prn\text{-}p^{+/0}$ $Bax^{0/0}$). We noted that Purkinje cell number was consistently 5 to 10% higher in $Bax^{0/0}$ mice compared with $Bax^{+/0}$ mice. This difference was observable in $Dpl^{+/0}$ mice whether or not the *Prn-p* gene was present (compare group 1 versus group 2, group 3 versus group 4), and was also seen in the absence of the Dpl transgene (compare $Prn\text{-}p^{+/0}$

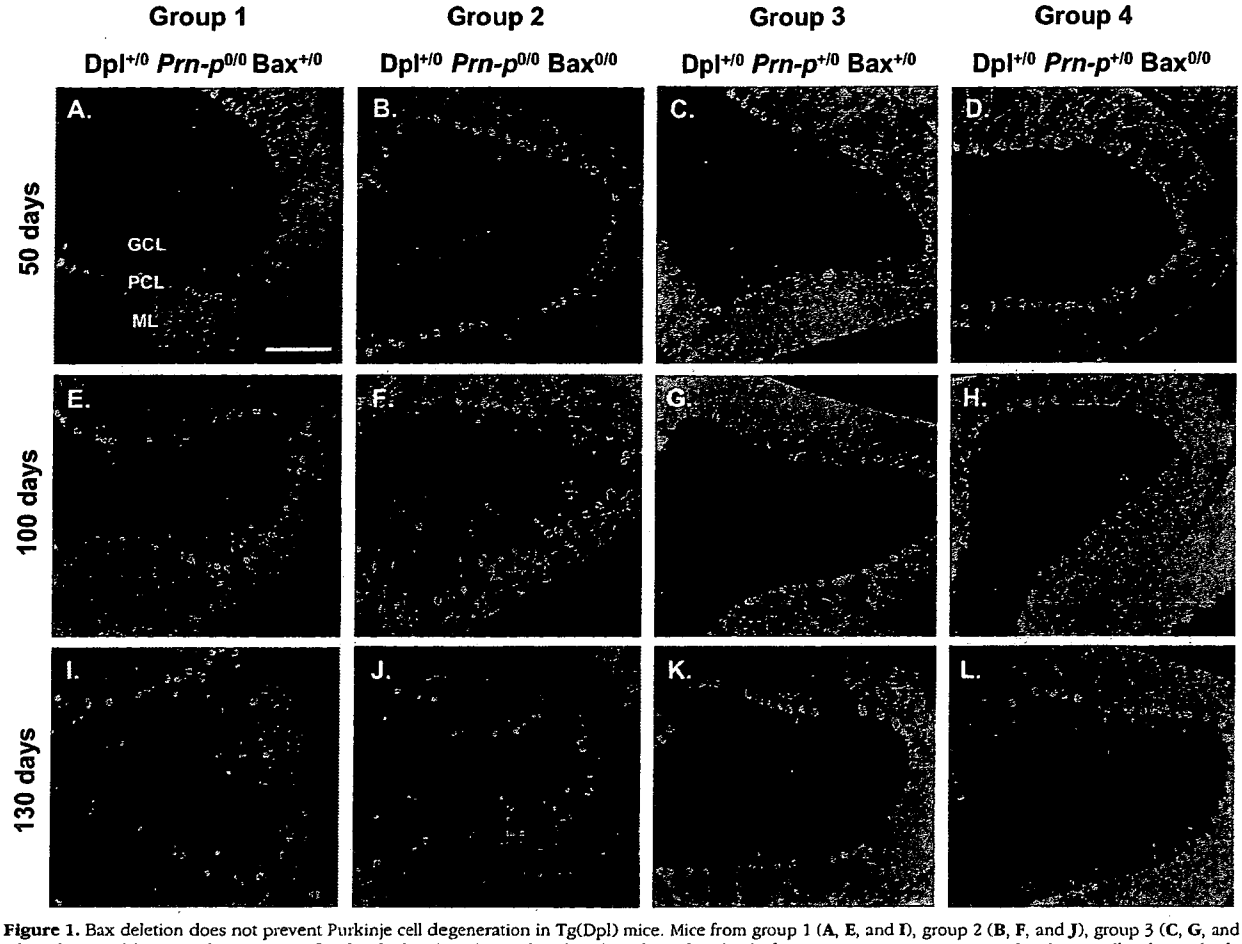

Figure 1. Bax deletion does not prevent Purkinje cell degeneration in Tg(Dpl) mice. Mice from group 1 (**A**, **E**, and **I**), group 2 (**B**, **F**, and **J**), group 3 (**C**, **G**, and **K**), and group 4 (**D**, **H**, and **L**) were sacrificed at 50 days (**A–D**), 100 days (**E–H**), and 130 days (**I–L**) of age. Brain sections were stained with anti-calbindin antibody to visualize cerebellar Purkinje cells. A representative area from lobule IV is shown for each brain. Progressive loss of Purkinje cells occurs between 50 and 130 days of age in Dpl$^{+/0}$ *Prn-p*$^{0/0}$ mice and is not affected by Bax deletion (compare groups 1 and 2). GCL, granule cell layer; PCL, Purkinje cell layer; ML, molecular layer. Scale bar = 100 μm.

Bax$^{+/0}$ versus *Prn-p*$^{+/0}$ Bax$^{0/0}$ at 130 days). This difference is probably due to a rescuing effect of Bax deletion on developmental death of Purkinje cells, a phenomenon described previously.[37] Taken together, the quantitative results confirm the lack of effect of Bax deletion on Dpl-induced Purkinje cell loss.

Purkinje cell degeneration in Tg(Dpl) mice is accompanied by a marked astrocytic reaction in the molecular and granule cell layers of the cerebellum, including prominent hypertrophy of radial Bergmann glial fibers, as revealed by staining for glial fibrillary acidic protein (Figure 4A). This pathology is not altered by deletion of Bax (Figure 4B). As expected, introduction of a *Prn-p* allele prevented the astrocytic reaction (Figure 4, C and D).

Purkinje Cell Death in Tg(Dpl) Mice Occurs Independently of Detectable DNA Cleavage and Caspase-3 Activation

Bax-mediated apoptosis is typically accompanied by internucleosomal cleavage of DNA and activation of caspase-3.[27,28] To test for these changes in the cerebella of Tg(Dpl) mice, we stained brain sections using the TUNEL method and using an antibody that selectively recognizes the cleaved form of caspase-3. We failed to observe any Purkinje or granule cells that were positive for TUNEL or activated caspase-3 in cerebellar lobule IV of Tg(Dpl) mice from groups 1 or 2 at 100 days of age (Figure 5, A, B, E, and F). Purkinje cells are undergoing extensive degeneration in lobule IV at this stage (Figure 1). To be sure that we had not missed positively stained cells at other ages or in other lobules, we analyzed the entire cerebellar cortex (lobules II to X) of groups 1 and 2 mice at 30, 100 and 180 days of age, corresponding to the presymptomatic, symptomatic, and terminal phases of illness. We again failed to detect any TUNEL-positive or activated caspase-3-positive cells (data not shown). Because the loss of Purkinje cells occurs in a gradient from lobules III to X (see above), it is likely that our analysis would have captured cells in several different stages of degeneration. As controls for the staining procedures, we analyzed cerebellar sections from *Lurcher* mice, which display apoptosis of Purkinje cells due to a mutation in