Prions: Pathogenesis and Reverse Genetics

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ABSTRACT: Spongiform encephalopathies are a group of infectious neurodegenerative diseases. The infectious agent that causes transmissible spongiform encephalopathies was termed *prion* by Stanley Prusiner. The prion hypothesis states that the partially protease-resistant and detergent-insoluble prion protein (PrP^{sc}) is identical with the infectious agent, and lacks any detectable nucleic acids. Since the latter discovery, transgenic mice have contributed many important insights into the field of prion biology. The prion protein (PrP^{c}) is encoded by the *Prnp* gene, and disruption of *Prnp* leads to resistance to infection by prions. Introduction of mutant PrP^{c} genes into PrP^{c}-deficient mice was used to investigate structure-activity relationships of the PrP^{c} gene with regard to scrapie susceptibility. Ectopic expression of PrP^{c} in PrP^{c} knockout mice proved a useful tool for the identification of host cells competent for prion replication. Finally, the availability of PrP^{c} knockout and transgenic mice overexpressing PrP^{c} allowed selective reconstitution experiments aimed at expressing PrP^{c} in neurografts or in specific populations of hemato- and lymphopoietic cells. The latter studies helped in elucidating some of the mechanisms of prion spread and disease pathogenesis.

RECENT HISTORY OF PRION DISEASES

The prototype of all prion diseases, scrapie in sheep and goats, has been known for more than two centuries. A recent form of animal prion disease designated bovine spongiform encephalopathy (BSE) has since its first recognition in 1986 developed into an epizootic, which fortunately has been receding since 1992.[1–3] The emergence of a new variant form of Creutzfeldt-Jakob disease (vCJD) in young people in the UK has raised the possibility that BSE has spread to humans by dietary exposure.[4,5] This scenario has recently been supported by experimental evidence claiming that the agent causing BSE is indistinguishable from the vCJD agent.[6–10]

THE NATURE OF THE INFECTIOUS AGENT

Prion diseases are caused by a novel type of pathogen, the "prion," which differs from known bacteria and viruses in major respects. For one, prions are extremely resistant to heat. Even prolonged heating to 100°C (212°F) and disinfection by most of the commonly used disinfectants does not lead to inactivation.[11] Prions are not

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easily biodegradable.[12] Purification of the infectious agent has led to the identification of a protein (denominated prion protein) which is intimately associated with infectivity. Although the exact physical nature of the transmissible agent is controversial, a very large body of experimental data supports the "protein only" hypothesis which postulates that the agent is devoid of nucleic acid and consists solely of an abnormal conformer of a cellular protein called PrP^c.[13,14] Accumulation of an abnormal isoform (PrP^{Sc} or PrP-res) of the host encoded prion protein (PrP^c or PrP-sen)[15–17] in the central nervous system (CNS) is a hallmark of prion diseases.

Because of its location at the outer surface of cells, anchored by phosphatidylinositol glycolipid,[18] PrP^c is a candidate for a signaling, cell adhesion or perhaps even for some transport functions. PrP^c is expressed on many cell types, including neurons,[19] astrocytes,[20] and lymphocytes[21] and appears to be developmentally regulated during mouse embryogenesis.[22] Although PrP^c is predominantly found in brain tissue, high levels are also present in heart, skeletal muscle, and kidney, whereas it is barely detectable in the liver.[23] Several candidate proteins that bind PrP^c have been reported. Among them is the amyloid precursor-like protein 1 (APLP1) which is a member of the amyloid precursor protein gene family. This opens up new questions in the light of its relevance to Alzheimer's disease.[24] Other possible PrP^c binding proteins are the human laminin receptor precursor, which serves as a cell surface receptor for infectious agents,[25,26] and an uncharacterized 66-kDa membrane protein.[27] Interaction of the PrP^c with Bcl-2, a protein that can rescue neurons, points towards a possible role of the prion protein in neuronal cell survival.[28] However, evidence that any of these interactions is physiologically significant is still missing.

PrP KNOCKOUT MICE AND THEIR PHENOTYPES

The "protein only" hypothesis states that PrP^c is a substrate for the PrP^{Sc}-mediated conversion of PrP^c into new PrP^{Sc} molecules. As a consequence of this hypothesis, an organism lacking PrP^c should be resistant to scrapie and unable to propagate the infectious agent. The mice generated by Büeler and colleagues[29] carry a targeted disruption of the *Prnp* gene. This was achieved by homologous recombination in embryonic stem cells. In the disrupted *Prnp* allele, 184 codons of the *Prnp* coding region (which consists of 254 codons) were replaced by a drug-resistance gene as selectable marker. A second line of PrP^c knockout mice was generated by Manson and co-workers[30] by inserting a selectable marker into a unique *Kpn*I site of the PrP^c open reading frame, thereby disrupting—but not deleting—the *Prnp* coding region. In a third PrP^c knockout line created by Sakaguchi and colleagues the whole PrP^c ORF and about 250 bp of the 5′ intron and 452 bp of 3′ untranslated sequences were replaced with a drug-resistance gene.[31] Both the Büeler and Sakaguchi mice were on a mixed genetic (129/Sv × C57BL) background whereas the Manson mice were bred on a pure 129/Ola background. According to the terminology which has become customary in the literature, and by which we abide in this manuscript, the Zurich mice have been designated $Prnp^{o/o}$ while the Edinburgh and the Japanese mice are termed $Prnp^{-/-}$.

Although it was proposed that PrP^c, which is an ubiquitously expressed neuronal protein, may have housekeeping function,[32] the homozygous PrP^c knockout mice

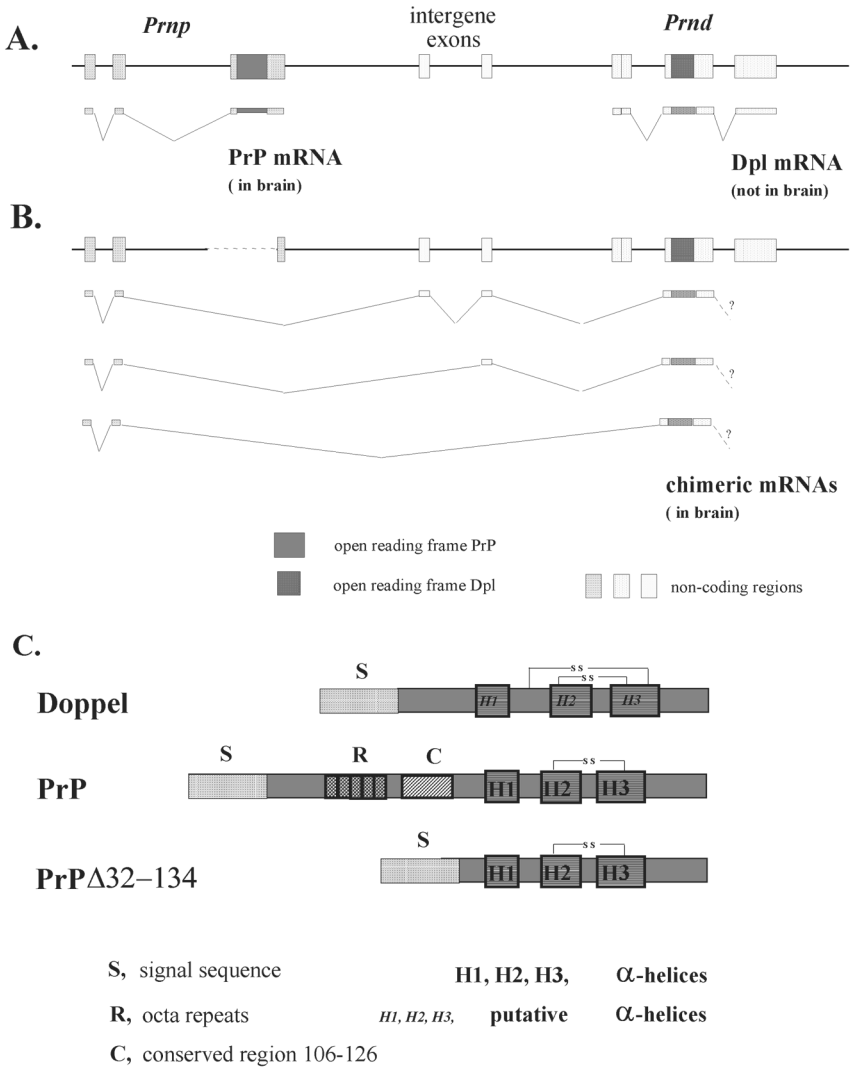

FIGURE 1. **(A)** The Prnp and Prnd loci and expression of the PrP^c and Dpl mRNAs and proteins that they encode. Coding and noncoding exons of *Prnp*, *Prnd*, and intergenic exons of unknown function. **(B)** Deletion of the exon of *Prnp* containing an open reading frame and its flanking regions results in the formation of several chimeric mRNAs that comprise the first two exons of *Prnp* , which are spliced directly or indirectly to the exon encoding Dpl. **(C)** Comparison of predicted domains of Dpl with full-length PrP^c and with PrP^c in which amino acid residues 32–134 have been deleted.

generated by Büeler[29] and Manson[30] were viable with no behavioral impairment and showed no overt phenotypic abnormalities, suggesting that PrP^c does not play a crucial role in development or function of the nervous system.[29,30] Although there was no overt phenotype, detailed analysis revealed electrophysiological defects such as weakened GABA-A receptor-mediated fast inhibition and impaired long-term potentiation in the hippocampus for these two lines of PrP^c knockout mice when compared to their corresponding wild-type counterparts, indicating that PrP^c might play a role in synaptic plasticity.[33] Tobler *et al.*[34] reported altered sleep patterns and rhythms of circadian activity in the Büeler and Manson mice.

Instead the PrP^c null mice derived by Sakaguchi *et al.*[31] developed severe progressive ataxia starting from 70 weeks of age. Analysis of the brains of affected animals revealed extensive loss of cerebellar Purkinje cells.[35] Because no such phenotype was observed in the other two lines of PrP^c knockout mice, it seems likely that this phenotype is not due to the lack of PrP^c but rather to the deletion of flanking sequences. Interestingly, a Purkinje cell-specific enhancer was proposed to be contained within the second intron of *Prnp*.[36] The report that expression of a *Prnp* transgene can rescue this phenotype argues against the hypothesis that the phenotype was caused by deletion of a regulatory element rather than of the *Prnp* reading frame.[37] Recently evidence has been forthcoming that the phenotype observed in the Sakaguchi mice is caused by upregulation of a second *Prnp*-like gene located 16 kb downstream of the *Prnp* gene (FIG. 1).[38] The exact mechanism of this process is still under discussion.[39]

NO SCRAPIE IN PRP NULL MICE

One of the milestones in scrapie research was the inoculation of PrP^c null mice with mouse-adapted scrapie strains. All three PrP^c null mouse lines were resistant to scrapie. The $Prnp^{o/o}$ mice generated by Büeler *et al.* inoculated with the RML isolate of mouse-adapted prions remained healthy for their whole life span and did not show any signs of scrapie typical neuropathology.[40] This observation was confirmed using different PrP^c null mice with different mouse-adapted scrapie inocula.[31,41] Mice hemizygous for the disrupted *Prnp* gene ($Prnp^{0/+}$) showed partial resistance to scrapie infection as manifested by prolonged incubation times of about 290 days as compared to about 160 days in the case of $Prnp^{+/+}$ mice. The incubation times until the first scrapie symptoms appear seem to correlate with levels of PrP^c in the host, whereas the severity of the disease in terms of neuropatholgical changes in the brain and levels of prion infectivity were not dependent on the PrP^c level.[41,42] The amount of PrP^c present in the brain seems to be the rate-limiting step in the development of the disease. Therefore therapeutic efforts aimed to reduce the amount of PrP^c may be effective.

STRUCTURE AND IMPLICATIONS ABOUT FUNCTION OF THE PRP GENE

When PrP^{Sc} undergoes limited proteolysis, the N terminus is cleaved off and a fragment termed $PrP^{27–30}$ is left. This portion of PrP^{Sc} is still infectious meaning that the last 60 amino-proximal residues of PrP^{Sc} are not required for infectivity.[16,43]

PrP^c lacking residues 23–88 can be converted into protease-resistant PrP^c in scrapie-infected neuroblastoma cells.[44] An important question arising from these experiments is the question whether N-terminally truncated PrP^c molecules can support prion replication in mice. Transgenic mice expressing N-terminal deletions of the prion protein on a PrP^c null background were established. These mutant PrP^c mice with amino-proximal deletions of residues 32–80 and 32–93 corresponding to truncations of 49 and 63 residues restore scrapie susceptibility, prion replication, and formation of truncated PrP^{Sc} in PrP^c-deficient mice.[45]

These experiments demonstrate that the octapeptide region encompassing residues 51–90 of murine PrP^c is dispensable for scrapie pathogenesis. This is remarkable in view of the fact that additional octapeptide repeats instead of the normal 5 segregate with affected individuals in families with inherited CJD,[46] and that expression of a mutant PrP^c with a pathological number of octarepeats induces a neurodegenerative disease in transgenic mice.[47]

PHENOTYPE IN MICE EXPRESSING TRUNCATED PrP^c

The three-dimensional structure determination using NMR revealed a highly flexible amino terminal tail that lacks ordered secondary structures extending from residue 23 to 121 within full-length mature PrP^c, whereas the carboxy terminal part of PrP^c consists of a stably folded globular domain.[48,49] The flexible tail, part of which is protease-sensitive in PrP^{Sc}, comprises the most conserved region of PrP^c across all species examined.[50] The possibility that the flexible tail may play a role in the conformational transition of PrP^c to PrP^{Sc} by initiating the structural rearrangements from α-helices to β-sheets was proposed.[48,51] To further analyze the importance of the flexible tail in regard to scrapie susceptibility, Shmerling and colleagues generated amino-proximal deletions of residues 32–121 and 32–134 and expressed them as transgenes in PrP^c-deficient mice. Mice overexpressing these transgenes developed severe ataxia and neuronal death limited to the granular layer of the cerebellum, as early as 1–3 months of age. No pathological phenotype was observed in transgenic mice with shorter deletions encompassing residues 32–80, 32–93 and 32–106. The selective degeneration of granule cells in the cerebellum argues against an unspecific toxic effect elicited by the truncated PrP^c. Another argument for a specific effect is the fact that neurons in the cortex and elsewhere express truncated PrP^c at similar levels but do not undergo cell death by apoptosis. If just one copy of a wild-type *Prnp* allele was introduced in these mice, the phenotype was abolished. These results are consistent with a model in which truncated PrP^c acts as dominant negative inhibitor of a functional homologue of PrP^c, with both competing for the same putative PrP^c ligand.[45]

A different spontaneous neurologic phenotype was reported in mice carrying PrP^c transgenes with internal deletions corresponding to either of the two carboxy-proximal α-helices. Two transgenic mouse lines generated on the $Prn^{po/o}$ background expressing mutant PrP^c with deletions of residues 23–88 and either residues 177–200 or 201–217 developed CNS dysfunction and neuropathological changes characteristic of a neuronal storage disease.[52] Since deletion of residues 23–88 alone did not lead to a spontaneous phenotype, it was concluded that ablation of either of

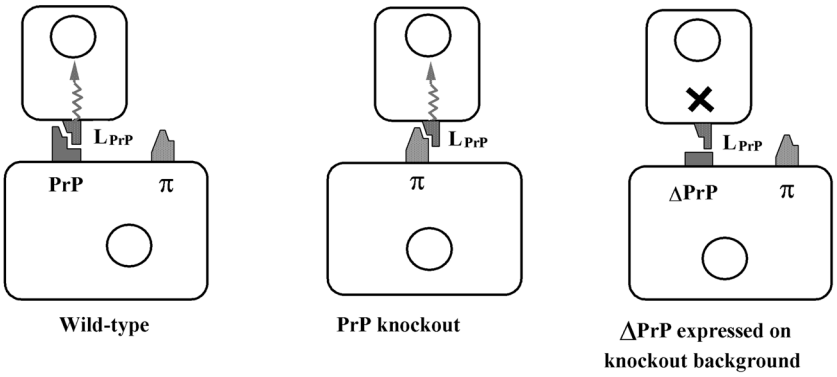

FIGURE 2. According to a hypothesis advanced by C. Weissmann, in wild-type mice PrP^c binds to its receptor (L) to elicit an essential signal. In the absence of PrP^c a PrP^c-like molecule (π) with a lower binding affinity can fulfill the same function. In PrP^c knockout mice with the truncated PrP^c, the truncated PrP^c can interact with the receptor, but without eliciting the signal which leads to the observed phenotype.

the two carboxy-terminal α-helices is sufficient to cause this novel CNS illness. Ultrastructural studies indicated extensive proliferation of the endoplasmic reticulum and revealed accumulation of mutant PrP^c within cytoplasmic inclusions in enlarged neurons. Since both Asn-linked glycosylation sites are located within residues 177–200, it is conceivable that aberrant glycosylation affects processing of the mutant PrP^c. However, it is unlikely that altered glycosylation of PrP^c is sufficient to account for neuronal storage disease, because transgenic mice expressing hamster PrP^c with point mutations that block Asn-linked glycosylation did not show this spontaneous disease phenotype.[53]

A completely new light is shed on all these studies by the discovery of a PrP^c-like gene named Dpl (Doppel, German for double) located 16 kb downstream of the murine *Prnp* gene (FIG. 1).[38] Dpl is overexpressed in certain strains of PrP^c knockout mice that develop neurological symptoms. The fact that the mice with a truncated *Prnp* transgene lacking the amino terminus which are devoid of the conserved 106–126 amino acid region develop granule cell degeneration which can be rescued by introduction of single intact PrP^c allele led to the hypothesis that PrP^c interacts with a ligand to produce an essential signal. In PrP^c knockout mice a PrP^c like molecule with a lower binding affinity could substitute the role of PrP^c. In the amino-terminated transgenic mice the truncated PrP^c could bind the ligand with high affinity without eliciting the survival signal. Dpl could act in a similar way and produce its effects through a competition with PrP^c for the PrP^c ligand thus blocking an important signal (FIG. 2).[38,39]

NEUROGRAFTS IN PRION RESEARCH

The fact that $Prnp^{o/o}$ mice show normal development and behavior,[29,30] has led to the hypothesis that scrapie pathology may come about because PrP^{Sc} deposition

is neurotoxic,[54] rather than by depletion of cellular PrP^c.[33] If the depletion of cellular PrP^c is really the reason for scrapie pathology, lack of PrP^c might result in embryonic or perinatal lethality, especially since PrP^c is encoded by a unique gene for which no related family members have been found. Until now there is no stringent mouse model where PrP^c can be depleted in an acute fashion. In this case the depletion of PrP^c may be much more deleterious than its lack throughout development, since the organism may then not have the time to enable compensatory mechanisms.

In order to study the question of neurotoxicity, we exposed brain tissue of *Prnp*$^{o/o}$ mice to a continuous source of PrP^{Sc}. This was achieved by grafting neural tissue overexpressing PrP^c into the brain of PrP^c-deficient mice using well-established protocols.[55–57] Following intracerebral inoculation with scrapie prions, neuroectodermal grafts accumulated high levels of PrP^{Sc} and infectivity, and developed severe histopathological changes characteristic for scrapie (FIG. 3). At later timepoints, substantial amounts of graft-derived PrP^{Sc} migrated into the host brain, and even in areas distant from the grafts, substantial amounts of infectivity were detected (FIG. 3).[36,58] Nonetheless, even 16 months after transplantation and infection with prions, no pathological changes were detected in the PrP^c-deficient tissue, not even in the immediate vicinity of the grafts or the PrP^c deposits. These results clearly suggest that PrP^{Sc} is inherently nontoxic, and PrP^{Sc} plaques found in spongiform encephalopathies may be an epiphenomenon rather than a cause of neuronal damage.[59] Maybe the PrP^{Sc}-containing plaques have to be formed and localized intracellularly in order to act neurotoxic. If this is the case, plaques that are localized extracellularly might not be toxic. This would explain the absence of pathological changes outside the PrP^c-containing grafts.

Because the host mice harboring a chronically scrapie-infected neural graft did not develop any sign of disease, they not only enabled us to study the effects of prions on the surrounding tissue but also were an ideal model to assess all changes occurring during the progression of scrapie disease in neuroectodermal tissue. The possibility of studying extremely late timepoints after infection with the scrapie agent was useful in order to observe phenomena that cannot be seen in PrP^c-containing mice, because these mice develop clinical symptoms eventually leading to death earlier. With increasing length of the incubation time, grafts underwent progressive astrogliosis and spongiosis which was accompanied by loss of neuronal processes within the grafts and subsequent destruction of the neuropil. The latest studied timepoint was 435 days after inoculation. Grafts showed an increase of cellular density probably due to astroglial proliferation and a complete loss of neurons. Intriguingly, with *in vivo* imaging with magnetic resonance imaging using gadolinium as a contrast-enhancing medium, a progressive disruption of the blood-brain barrier in scrapie-infected grafts was detected during the course of the disease.[60] These findings confirmed several predictions about the pathogenesis of spongiform encephalopathies, mainly that scrapie leads to selective neuronal loss while astrocytes and perhaps other neuroectodermal cells, while being affected by the disease, can survive and maintain their phenotypic characteristics for very long periods of time.

In other experimental models as experimental hamster scrapie, disruption of the blood-brain barrier was also visible,[61] yet no such observations were made in human spongiform encephalopathies. The localized blood-brain barrier disruption in chronically infected grafts might contribute to the spread of prions from grafts to the sur-

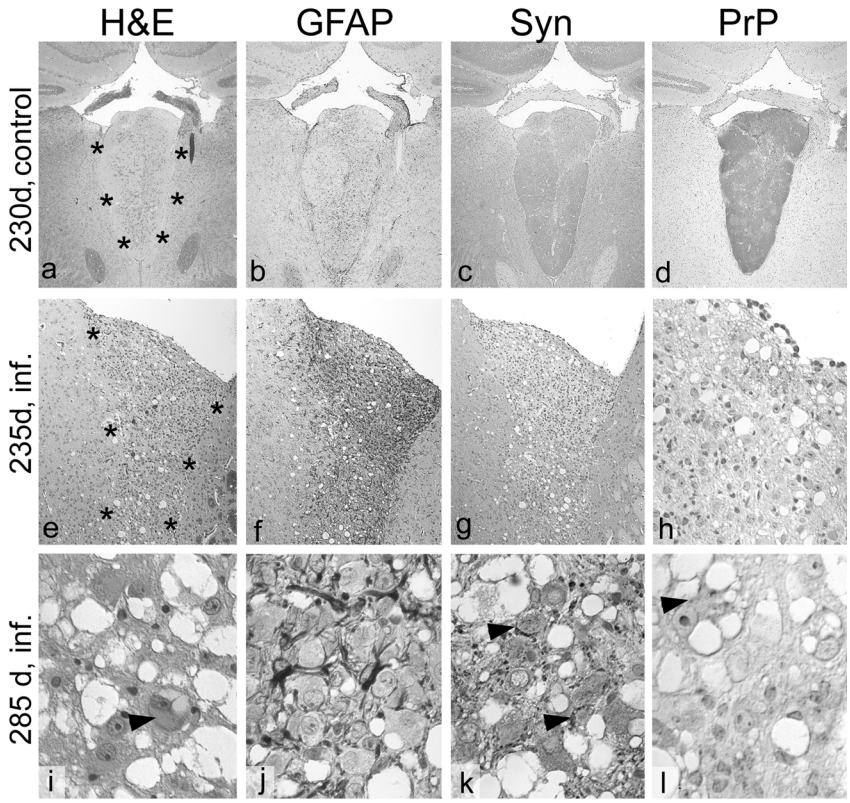

FIGURE 3. Noninfected and scrapie-infected neural grafts in brains of $Prnp^{o/o}$ mice. *Upper row* **(a, b, c, d)**: healthy control graft 230 days after mock inoculation. The graft is located in the third ventricle of the recipient mouse (a, see *asterisks*, hematoxylin-eosin), and shows no spongiform change, little gliosis (b, immunostain for GFAP) , and strong expression of synaptophysin (c) and of PrP^c (d). *Middle row* **(e, f, g, h)**: scrapie-infected graft 235 days after inoculation with increased cellularity (e), brisk gliosis (f), and a significant loss of synaptophysin (g) and PrP^c (h) staining intensity is shown. *Bottom row* **(i, j, k, l,)**: high magnification of a similar graft shows characteristic pathological changes in a chronically infected graft. (i) Appearance of large vacuoles and ballooned neurons (*arrow*). In the GFAP immunostain (j), astrocytes appear wrapped around densely packed neurons. Granular deposits and intracytoplasmic accumulation of synaptophysin (k) and $PrPrP^c$ PC immunoreactivity (l) in the cytoplasm of neurons.

rounding brain, as described previously.[58] It may also account for the accumulation pattern of protease-resistant PrP^c within the white matter and in brain areas surrounding the grafts. The accumulation of PrP^{Sc} in nonaffected neuropil surrounding the graft could also be explained through vasogenic diffusion from the affected graft towards the host brain.

SPREAD OF PRIONS IN THE CENTRAL NERVOUS SYSTEM

Scrapie can be transmitted by injecting scrapie-infected brain homogenate into suitable recipients by a number of different inoculation routes. Intracerebral inoculation is the most effective method for transmission of spongiform encephalopathies and may even facilitate circumvention of the species barrier. Other modes of transmission are oral uptake of the agent,[2,62,63] intravenous and intraperitoneal injection,[64] as well as conjunctival instillation,[65] implantation of corneal grafts,[66] and intraocular injection.[67] Intraocular injection is an elegant way of studying the neural spread of the agent, since the retina is a part of the CNS and intraocular injection does not produce direct physical trauma to the brain, which may disrupt the blood-brain barrier and impair other aspects of brain physiology. The assumption that spread of prions within the CNS occurs axonally rests mainly on the demonstration of diachronic spongiform changes along the retinal pathway following intraocular infection.[67]

It has been repeatedly shown that expression of PrP^c is required for prion replication[40,68] and for neurodegenerative changes to occur.[58] To investigate whether spread of prions within the CNS is dependent on PrP^c expression in the visual pathway, PrP^c-producing neural grafts were used as sensitive indicators of the presence of prion infectivity in the brain of an otherwise PrP^c-deficient host.

Following inoculation with prions into the eye of grafted *Prnp*$^{o/o}$ mice, none of the grafts showed signs of spongiosis, gliosis, synaptic loss, or PrP^{Sc} deposition. In one instance, the graft of an intraocularly inoculated mouse was assayed and found to be devoid of infectivity. Therefore, it was concluded that infectivity administered to the eye of PrP^c-deficient hosts cannot induce scrapie in a PrP^c-expressing brain graft.[69]

One problem that is encountered while conducting work with PrP^c-containing grafts in *Prnp*$^{o/o}$ mice is the fact that PrP^c-producing tissue might lead to an immune response to PrP^c [70] and possibly to neutralization of infectivity. Indeed, analysis of sera from grafted mice revealed significant anti-PrP^c antibody titers,[69] and it was shown that PrP^c presented by the intracerebral graft (rather than the inoculum or graft-borne PrP^c) was the offending antigen. In order to definitively rule out the possibility that prion transport was disabled by a neutralizing immune response, the experiments were repeated in mice tolerant to PrP^c, namely, the *Prnp*$^{o/o}$ mice transgenic for the PrP^c coding sequence under the control of the lck-promoter. These mice overexpress PrP^c on T lymphocytes, but were resistant to scrapie and did not replicate prions in brain, spleen, or thymus after intraperitoneal inoculation with scrapie prions.[71] Upon grafting with PrP^c-overexpressing neuroectoderm these mice do not develop antibodies to PrP^c presumably due to clonal deletion of PrP^c-immunoreactive lymphocytes. As before, intraocular inoculation with prions did not provoke scrapie in the graft, supporting the conclusion that lack of PrP^c, rather than immune response to PrP^c, prevented prion spread.[69] Therefore, PrP^c appears to be necessary for the spread of prions along the retinal projections and within the intact CNS.

These results indicate that intracerebral spread of prions is based on a PrP^c-paved chain of cells, perhaps because they are capable of supporting prion replication. When such a chain is interrupted by interposed cells that lack PrP^c, as in the case described here, no propagation of prions to the target tissue can occur. Perhaps prions require PrP^c for propagation across synapses: PrP^c is present in the synaptic

region,[72] and certain synaptic properties are altered in *Prnp*$^{o/o}$ mice.[33,73] Perhaps transport of prions within (or on the surface of) neuronal processes is PrP^{c}-dependent. These findings support the "domino-stone" model in which spreading of scrapie prions in the CNS occurs *per continuitatem* through conversion of PrP^{c} by adjacent PrP^{Sc}.[74]

SPREAD OF PRIONS FROM EXTRACEREBRAL SITES TO THE CNS

From an epidemiological point of view oral uptake of prions may be more relevant than intracerebral transmission, because it is thought to be responsible for the BSE epidemic and for transmission of BSE to a variety of species including humans.[8,9] Prions can find their way through the body to the brain of their host, yet histopathological changes have not been identified in organs other than the CNS. One of the main characteristics of prion diseases is the long incubation time. This incubation time can be explained by multiplication of prions in "reservoirs." A likely candidate that could constitute this reservoir is the lymphoreticular system (LRS). This is supported by the finding that prion replication in lymphoid organs always precedes prion replication in the CNS, even if infectivity is administered intracerebrally (FIG. 4).[75] Prions may multiply silently in "reservoirs" during the incubation phase of the disease. Infectivity can accumulate in all components of the LRS, including lymph nodes and intestinal Peyer's patches, where prions replicate almost immediately after oral administration of prions to mice.[76] Recently, it was shown that vCJD prions accumulate in the lymphoid tissue of tonsils in such large amounts that PrP^{Sc} can easily be detected with antibodies on histological sections.[77]

A wealth of early studies points to the importance of prion replication in lymphoid organs, yet little is known about which cells support prion propagation in the LRS. Whole-body ionizing radiation studies in mice[78] after intraperitoneal infection have suggested that the critical cells are long-lived. The follicular dendritic cell (FDC) would be a prime candidate, and indeed PrP^{Sc} accumulates in such cells of wild-type and nude mice (which have a selective T-cell defect).[79] In addition when mice with severe combined immunodeficiency (SCID) whose FDCs are thought to be functionally impaired are challenged with the scrapie agent intraperitoneally, they do not develop the disease nor is there any replication of prions in the spleen.[80] Upon reconstitution of SCID mice with wild-type spleen cells, susceptibility to scrapie is restored after peripheral infection.[81] These findings suggest that components of the immune system are required for efficient transfer of prions from the site of peripheral infection to the CNS.

To study the role of the immune system in more detail we used a panel of immune-deficient mice which were inoculated intraperitoneally with prions. We found that defects affecting T cells had no apparent effect, but that all mutations that disrupted the differentiation of B cells prevented the development of clinical scrapie.[82] From these results, one can conclude that B cells are important for the development of scrapie after peripheral infection. Do B cells physically transport prions all the way from the periphery to the CNS? This possibility seems very unlikely, since lymphocytes do not normally cross the blood-brain barrier unless they have a specific reason to do so (e.g., during an inflammatory reaction). Furthermore, up to 30% of B-cell-deficient mice contain prions in their brains despite no signs of clin-

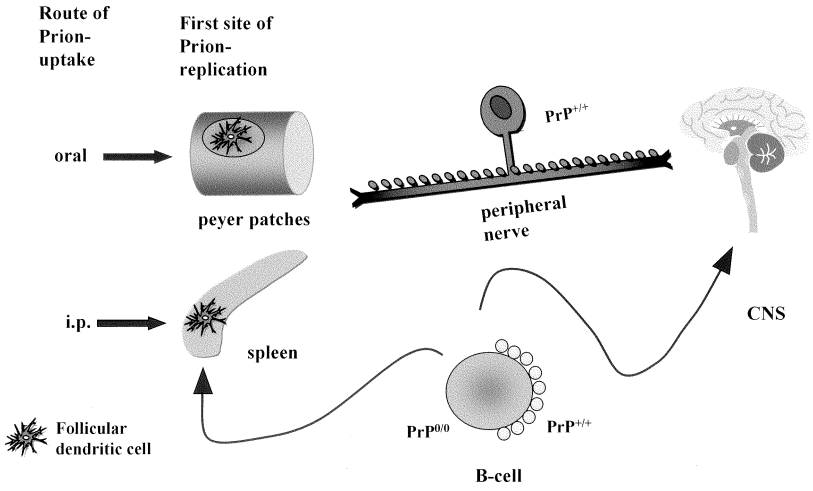

FIGURE 4. Neuroinvasion of prions. Depending on the route of administration of the infectious agent, the first site of replication is probably in the lymphoid tissue of the gut or in the spleen. From there neuroinvasion is most likely accomplished via the PNS. The presence of B cells is crucial for neuroinvasion. The precise mode of action of B cells is still unclear.

ical disease.[83] How is the spread of prions accomplished within the body? Perhaps, prions administered to peripheral sites are first brought to lymphatic organs by mobile immune cells such as B cells. Once infection has been established in the LRS, prions invade peripheral nerve endings.[84,85] How the neuroinvasion from cells belonging to the lymphoreticular system to peripheral nerves is accomplished, is still a matter of discussion (FIG. 4). Access to peripheral nerves may perhaps be facilitated if myelination of the nerves is reduced or absent.[86] Considering this, the mantle zone of the lymph follicles which is innervated by terminal unmyelinated nerve fibers could be the entry point of the scrapie agent into the PNS. It is also here that processes belonging to FDCs are in close contact with nerve fibers. Along the peripheral nerves the scrapie agent reaches the CNS where further spread occurs transsynaptically and along fiber tracts.[87,88] Is PrP^c also required for the spread of prions from peripheral sites to the CNS? Indirect evidence points in this direction: PrP^c-expressing neurografts in *Prnp*$^{o/o}$ mice did not develop scrapie histopathology after intraperitoneal or intravenous inoculation with prions and no infectivity was detectable in the spleen. Following reconstitution of the host lymphohemopoietic system with PrP^c-expressing cells, prion titers in the spleen were restored to wild-type levels but, surprisingly, PrP^c-expressing grafts failed to develop scrapie upon intraperitoneal or intravenous infection with prions (FIG. 5).[89] These findings suggest that transfer of infectivity from the spleen to the CNS is crucially dependent on the expression of PrP^c in a tissue compartment interposed between the LRS and the CNS that cannot be reconstituted by bone marrow transfer. Several studies suggest that this compartment may comprise part of the peripheral nervous system.[86,90]

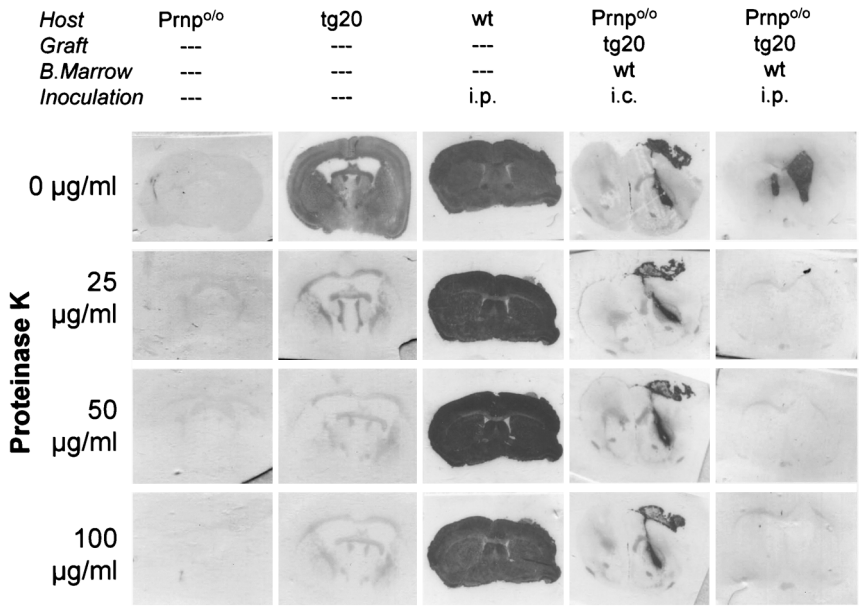

FIGURE 5. Accumulation of PrP^{Sc} in brain grafts. Histoblots showing immunoreactive PrP^{c} in brain sections natively (*first row*) and after digestion with increasing levels of proteinase K (*second through fourth row*). $Prnp^{o/o}$ mice (*first column*) show no immunoreactivity, while mock-inoculated *tg20* mice (which overexpress PrP^{c}) show proteinase K-sensitive PrP^{c} (*second column*), but no proteinase K-resistant PrP^{c}. Terminally sick scrapie-infected wild-type mice contain large amounts of both PrP^{c} and PrP^{Sc} (*third column*). *Prnp*$^{o/o}$ whose bone marrow has been reconstituted with wild-type FLCs accumulate PrP^{Sc} in their PrP^{c}-overexpressing grafts after *i.c.* (*fourth column*) but not after *i.p.* prion administration (*fifth column*).

The absolute necessity of PrP^{c} presence for the spread of scrapie from the periphery as well as within the CNS may offer a handle to interfere with this chain of events without resorting to ablation of a functional immune system such as in the case of SCID mice.

ROLE OF B LYMPHOCYTES IN NEUROINVASION

The replication of prions[40] and their transport from the periphery to the CNS[89] is dependent upon expression of PrP^{c}. With respect to the results described in the previous paragraph, we examined whether expression of PrP^{c} by B cells was necessary to support neuroinvasion. In order to study this matter we took mice with various immune defects and repopulated their LRS by adoptive transfer of hematopoietic stem cells which expressed or lacked expression of PrP^{c}.

Adoptive transfer of either *Prnp*$^{+/+}$ or *Prnp*$^{o/o}$ fetal liver cells (FLCs) induced formation of germinal centers in spleens of recipient mice and differentiation of FDCs as visualized by staining with antibody FDC-M1.[91] However, no FDCs were found in B- and T-cell-deficient mice reconstituted with FLCs from μ*MT* embryos (B-cell-deficient), consistent with the notion that B cells or products thereof are required for FDC maturation.

Mice reconstituted in the fashion explained above were challenged *i.p.* with scrapie prions. All mice that received FLCs of either genotype, *Prnp*$^{+/+}$ or *Prnp*$^{o/o}$, from immunocompetent donors, succumbed to scrapie after inoculation with a high dose of prions, and most mice after a low dose. Susceptibility to disease could not be restored upon transfer of FLCs from μ*MT* donors. Omission of the adoptive transfer procedure did not restore susceptibility to disease in any of the immune-deficient mice challenged with the low dose of prions. With the high dose inoculum, susceptibility to scrapie could be restored even in the absence of B cells and FDCs. However, reconstituted mice which received bone marrow from TCR$\alpha^{-/-}$ donors, which possess B cells and lack all T cells but those expressing TCR$\alpha^{-/-}$ receptors, regained susceptibility to scrapie, again confirming the dependency of infectibility upon the presence of B cells. Individual samples of brain and spleen from the scrapie-inoculated bone marrow chimeras were transmitted into highly susceptible indicator mice. We observed restoration of infectious titers and PrP^{Sc} deposition in spleens and brains of recipient mice either carrying *Prnp*$^{+/+}$ or *Prnp*$^{o/o}$ donor cells.[91]

B cells are clearly a cofactor in peripheral prion pathogenesis, but the identity of those cells in which prions actually replicate within lymphatic organs is uncertain. In a further step to clarify this issue, we investigated whether spleen PrP^{Sc} was associated with FDCs in repopulated mice. Double-color immunofluorescence confocal microscopy revealed deposits of PrP^{c}-immunoreactive material in germinal centers which appeared largely colocalized with the follicular dendritic network in spleens of reconstituted mice.

Taking together all the information we have gained with the above-described experiments, one can support the hypothesis that cells whose maturation depends on B cells are responsible for accumulation of prions in lymphoid tissue such as the spleen. FDCs, although their origin remains rather obscure, are a likely candidate for the site of prion replication, because their maturation correlates with the presence of B cells and their products. This hypothesis is not free of controversy; it is still possible that the follicular dendritic network serve merely as a reservoir for the accumulation of prions and that other B-cell-dependent processes are involved in the transport of the infectious agent. Prions may be transported on or within B cells directly as they cross peripheral lymphoid tissue to localize in autonomic nerve terminals. In recent studies where spleens were fractionated it was shown that prion infectivity is mainly associated with B and T lymphocytes and less with a stromal fraction containing FDCs.[92]

CONCLUSION

Peripheral pathogenesis of prion diseases is here defined as the process starting with the contact of the infectious agent with extracerebral sites, and eventually resulting in brain disease. This process occurs in distinct sequential phases. The first

and very early event in disease progression is certainly accumulation of prions in the LRS. This process is dependent upon components of the host immune system. Whether prions replicate or merely accumulate in the LRS is not known with certainty. FDCs play a major role in this process, but the details are still under discussion. In order to achieve efficient neuroinvasion either B cells per se or their products are essential. One B-cell-dependent event that is of relevance is the acquisition of a functional FDC network within the germinal centers of peripheral lymphoid tissue.

The second phase of neuroinvasion appears to encompass transfer of prions from lymphoid tissue to nerve endings of the peripheral nervous system. Because lymphoid organs are predominantly innervated by nerve fibers of the sympathetic nervous system, this part of the peripheral nervous system is a prime candidate. How neuroinvasion is accomplished and how the agent is transported within the peripheral nervous system, however, is still unclear. It is worthwhile noting that the innervation of lymphoid tissue is, at least in part, controlled by lymphocytes themselves, as both T and B cells secrete nerve growth factor and, vice versa, nerve terminals secrete a variety of factors that stimulate the immune system.[93] These factors may play a critical role in the neuroinvasion process and represent a critical site for modulation of disease progression. For example, drugs which act on lymphocytes or on the sympathetic innervation of lymphoid tissue, or those that prevent cytokine release or block neurotransmission, may have a strong influence in the immune modulation and might represent useful tools for studying the cellular and molecular basis of prion neuroinvasion.

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