Prions: Protein Aggregation and Infectious Diseases

ADRIANO AGUZZI AND ANNA MARIA CALELLA

Institute of Neuropathology, University Hospital of Zurich, Zurich, Switzerland

<table>
<thead>
<tr>
<th>I. Properties of Prions</th>
<th>1106</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Introduction</td>
<td>1106</td>
</tr>
<tr>
<td>B. Development of the prion concept</td>
<td>1107</td>
</tr>
<tr>
<td>C. Discovery of the prion protein</td>
<td>1108</td>
</tr>
<tr>
<td>D. PrP amyloid</td>
<td>1109</td>
</tr>
<tr>
<td>E. Formation of PrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
<td>1109</td>
</tr>
<tr>
<td>F. Prion diversity</td>
<td>1112</td>
</tr>
<tr>
<td>G. Prion-like properties of additional misfolded proteins</td>
<td>1114</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. Function of the Cellular Prion Protein</th>
<th>1116</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Teachings from Prnp-ablated mice</td>
<td>1116</td>
</tr>
<tr>
<td>B. PrP&lt;sup&gt;C&lt;/sup&gt; and its partners</td>
<td>1117</td>
</tr>
<tr>
<td>C. PrP&lt;sup&gt;C&lt;/sup&gt; double and its trouble</td>
<td>1117</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>III. Mechanisms of Prion Toxicity</th>
<th>1118</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Alteration of PrP&lt;sup&gt;C&lt;/sup&gt; mediated signaling</td>
<td>1119</td>
</tr>
<tr>
<td>B. PrP&lt;sup&gt;C&lt;/sup&gt; mislocalization</td>
<td>1119</td>
</tr>
<tr>
<td>C. PrP&lt;sup&gt;C&lt;/sup&gt;-derived oligomeric species</td>
<td>1121</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IV. Clinical and Pathological Aspects of Prion Diseases</th>
<th>1121</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Scrapie</td>
<td>1121</td>
</tr>
<tr>
<td>B. Bovine spongiform encephalopathy</td>
<td>1122</td>
</tr>
<tr>
<td>C. Chronic wasting disease of deer and elk</td>
<td>1122</td>
</tr>
<tr>
<td>D. Human prion diseases</td>
<td>1123</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>V. Peripheral Routes of Prion Infectivity</th>
<th>1126</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Pathway of orally administered prions</td>
<td>1126</td>
</tr>
<tr>
<td>B. Transepithelial enteric passage of prions</td>
<td>1127</td>
</tr>
<tr>
<td>C. Uptake of prion through the skin</td>
<td>1127</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VI. Immunological Aspects of Prion Disease</th>
<th>1127</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. The lymphoid system in prion disease</td>
<td>1127</td>
</tr>
<tr>
<td>B. Tropism of prions for inflammatory foci</td>
<td>1130</td>
</tr>
<tr>
<td>C. Neuroinflammation in prion pathogenesis</td>
<td>1131</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VII. Spread of Prions Along Peripheral and Central Nervous System Pathways</th>
<th>1131</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII. Towards Antiprion Therapeutics</td>
<td>1134</td>
</tr>
<tr>
<td>A. Innate immunity and antiprion defense</td>
<td>1135</td>
</tr>
<tr>
<td>B. Adaptive immunity and preexposure prophylaxis against prions</td>
<td>1137</td>
</tr>
<tr>
<td>C. Soluble prion antagonists</td>
<td>1138</td>
</tr>
</tbody>
</table>

| IX. Progress in the Diagnostics of Prion Diseases                       | 1138 |
| X. Concluding Remarks                                                   | 1139 |

Aguzzi A, Calella AM. Prions: Protein Aggregation and Infectious Diseases. Physiol Rev 89: 1105–1152, 2009; doi:10.1152/physrev.00006.2009.—Transmissible spongiform encephalopathies (TSEs) are inevitably lethal neurodegenerative diseases that affect humans and a large variety of animals. The infectious agent responsible for TSEs is the prion, an abnormally folded and aggregated protein that propagates itself by imposing its conformation onto the cellular prion protein (PrP<sup>C</sup>) of the host. PrP<sup>C</sup> is necessary for prion replication and for prion-induced neurodegeneration, yet the proximal causes of neuronal injury and death are still poorly understood. Prion toxicity may arise from the interference with the normal function of PrP<sup>C</sup>, and therefore, understanding the physiological role of PrP<sup>C</sup> may help to clarify the mechanism underlying prion diseases. Here we discuss the evolution of the prion concept and how prion-like mechanisms may apply to other protein aggregation diseases. We describe the clinical and the pathological features of the prion diseases in human and animals, the events occurring during neuroinvasion, and the possible scenarios underlying brain damage. Finally, we discuss potential antiprion therapies and current developments in the realm of prion diagnostics.
I. PROPERTIES OF PRIONS

A. Introduction

Prion diseases, also termed transmissible spongiform encephalopathies (TSEs), are inevitably fatal neurodegenerative conditions that affect humans and a wide variety of animals. Prion diseases may present with certain morphological and pathophysiological features that parallel other progressive encephalopathies, such as Alzheimer’s and Parkinson’s disease (12). However, prion diseases were considered unique in that they are transmissible. As it happens, the latter assertion may not be entirely correct. It has been reported that aggregates of amyloid-β (Aβ) peptide associated with Alzheimer’s disease behave like an infectious agent when injected into the brain of a mouse model of Alzheimer’s disease, showing a pattern of Aβ deposition that depends on both the host and the agent (345). These findings suggest that what was considered to be a unique feature of prion disease may be a more general property of amyloids.

The agent that causes TSEs was termed “prion” by Stanley B. Prusiner and is defined as “a small proteinaceous infectious particle that is resistant to inactivation by most procedures that modify nucleic acids” (403). Prions certainly differ from all other known infectious pathogens in several respects. First, prions do not appear to contain an informational nucleic acid genome longer than 50 bases that encodes for their progeny (423). Second, the only known component of the prion is a modified form of the cellular prion protein, PrP^C, which is encoded by the gene Prnp. PrP^C is a cell surface glycoprotein (481) of unknown function that has been identified in all mammals and birds examined to date, as well as in Xenopus laevis (482) and fish (425). Third, the central event in prion pathogenesis is the conformational conversion of PrP^C into PrP^Sc, an insoluble and partially protease-resistant isoform that propagates itself by imposing its abnormal conformation onto PrP^C molecules.

Numerous experiments have provided evidence that PrP^C is a key player in prion replication as well as in prion-induced neurodegeneration. Mice lacking the prion gene are resistant to the disease (84), and PrP^C expression is required for neurodegeneration in host neurons, because the presence of PrP^Sc alone does not cause disease. For example, when neurografts propagating PrP^Sc were implanted into Prnp knockout mice, no pathological changes were seen in PrP-deficient tissue, even in the immediate vicinity of the grafts (64). Additionally, transgenic mice expressing only a secreted form of PrP^C lacking a glycosylphosphatidylinositol (GPI) anchor do not develop clinical signs of prion disease, although prion inoculation induces PrP^Sc formation and aggregation of amyloid plaques (108). These findings suggest that expression of membrane-anchored PrP^C is necessary to initiate disease. Finally, neuron-specific ablation of PrP^C in transgenic mice (321) or RNAi knockdown of PrP^C expression in mice with established prion disease (519) rescues early neuronal dysfunction and prolongs the survival of mice despite the accumulation of extraneuronal PrP^Sc.

Attempts to identify posttranslational modifications that distinguish PrP^Sc from PrP^C have been mostly unsuccessful (480); however, increasing evidence indicates that they may exist (89, 122). Almost 45% of the PrP^C protein is α-helical with two very short stretches of β-sheet (421). Conversion to PrP^Sc results in a protein comprised of ~30% α-helix and 45% β-sheet. The mechanism by which PrP^C is converted into PrP^Sc is unknown, but PrP^C appears to bind to PrP^Sc, perhaps in combination with ancillary proteins, to form an intermediate complex during the formation of nascent PrP^Sc (343). Transgenic mouse studies have provided genetic evidence that incoming prions in the inoculum interact preferentially with homo- and heterotypic PrP^C during the propagation of prions (412, 447).

Prion disease is characterized by widespread neurodegeneration; therefore, affected individuals exhibit clinical symptoms of both cognitive and motor dysfunction. In addition, the disease is characterized by the propagation of infectious prions and, in many instances, the formation of amyloid plaques. The human prion diseases include Kuru, Creutzfeldt-Jakob disease (CJD), variant CJD (vCJD), Gerstmann-Sträussler-Scheinker disease (GSS) and fatal familial insomnia (FFI) (Table 1). The most common prion diseases of animals are scrapie, which affects sheep and goats, bovine spongiform encephalopathy (BSE) or “mad cow” disease, and chronic wasting disease (CWD), which affects deer and elk. Kuru was the first of the human prion diseases to be transmitted to experimental animals, and Gajdusek discovered that Kuru spread among the Fore people of Papua New Guinea through ritual cannibalism (172). The experimental and presumed human-to-human transmission of Kuru led to the belief that prion diseases are infectious disorders caused by unusual viruses similar to those causing scrapie in sheep and goats. Yet autosomal dominant inheritance of CJD was first reported almost 90 years ago (260, 342),

<table>
<thead>
<tr>
<th>Disease</th>
<th>Etiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuru</td>
<td>Infection</td>
</tr>
<tr>
<td>Creutzfeldt-Jakob disease</td>
<td></td>
</tr>
<tr>
<td>Iatrogen</td>
<td></td>
</tr>
<tr>
<td>Sporadic</td>
<td></td>
</tr>
<tr>
<td>Familial</td>
<td></td>
</tr>
<tr>
<td>Variant</td>
<td></td>
</tr>
<tr>
<td>Gerstmann-Sträussler-Scheinker disease</td>
<td>PRNP mutation</td>
</tr>
<tr>
<td>Fatal familial insomnia</td>
<td>PRNP mutation</td>
</tr>
</tbody>
</table>

BSE, bovine spongiform encephalopathy.
suggesting that CJD was also a genetic disease. The significance of familial CJD (fCJD) remained largely unappreciated until mutations were discovered in the protein coding region of the PRNP gene on the short arm of chromosome 20 (478). The earlier finding that brain extracts from patients who had died of familial prion diseases inoculated into experimental animals often transmit disease posed a conundrum that was resolved with the genetic linkage of these diseases to mutations of the PRNP gene that encodes PrP<sup>C</sup> (496). To date, all known cases of familial prion disease cosegregate with PRNP mutations.

The most common form of prion disease in humans is "sporadic" CJD (sCJD) whose cause is unknown. Indeed, many attempts to show that the sporadic prion diseases are caused by infection have been unsuccessful (71, 125, 324). The discovery that inherited prion diseases are caused by germ-line mutations in the PRNP gene raised the possibility that sporadic forms of these diseases might result from somatic mutations (402). Alternatively, since PrP<sup>Sc</sup> is derived from PrP<sup>C</sup> by a posttranslational process (58), sporadic prion diseases may result from the spontaneous conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup>.

The incidence of sCJD is low in all ethnicities and has been often said to affect one person in one million annually. However, in countries with active surveillance programs, the reported CJD incidence is often higher (13), and in Switzerland it has reached 3.0/10<sup>6</sup> per year (187, 429), suggesting that many cases may go undetected. Less than 1% of CJD cases arise from exposure to infected material, and most of these seem to be iatrogenic. Between 5 and 15% of prion disease cases are inherited, whereas the remaining cases are sporadic. Kuru was once the most common cause of death among New Guinea women in the Fore region of the Highlands (171, 173), but the incidence has since dissipated with the cessation of ritualistic cannibalism (341).

In the past 20 years, more than 280,000 cattle suffering from BSE has provoked a major worldwide food crisis with thus far incomparable economic consequences for the European Union and other countries. In addition, transmission of BSE to humans is believed to have caused ≥200 cases of variant CJD (vCJD). Direct experimental proof that vCJD is caused by the transmission of BSE prions to humans has not been demonstrated. However, epidemiological, biochemical, and neuropathological evidence, as well as transmission studies, strongly suggest that BSE has been transmitted to humans resulting in vCJD (106, 231, 523).

In recent years, the United States has witnessed an enigmatic rise of CWD cases affecting elk and deer, and there has been a recrudescence of scrapie outbreaks among European sheep flocks (e.g., Sweden, Austria, and Sardinia). This resurgence of new cases might be linked to an increased sensitivity and frequency of the currently executed testing procedures. In any case, these findings have exposed our deficit in knowledge about prion epidemiology and possible transmission routes of prion diseases in humans and animals.

B. Development of the Prion Concept

Scrapie was originally hypothesized to be a muscle disease caused by parasites (310, 311). The transmissibility of scrapie was demonstrated in an early, seminal study (128) and led to the hypothesis that scrapie is caused by a "filterable" virus (527). The findings of Tikvah Alper and colleagues (23, 24) that scrapie infectivity was resistant to inactivation by ultraviolet (UV) and ionizing irradiation resulted in a myriad of hypotheses on the chemical nature of the scrapie agent, including the clairvoyant conjecture by Griffith (197) that it may consist of a self-replicating protein.

The experimental transmission of scrapie from sheep to mice (105) resulted in a much more convenient laboratory model, which yielded considerable information on the unusual infectious pathogen that causes scrapie (23–25, 182, 350, 383). Yet progress was, and in many instances continues to be, slow because quantification of infectivity in a single sample required holding many mice for at least 1 yr before accurate scoring could be accomplished (105).

After scrapie incubation times were reported to be ~50% shorter in Syrian hamsters than in mice (329), studies were undertaken to determine whether the incubation times in hamsters could be related to the titer of the inoculated sample. There was an inverse correlation between the length of the incubation time and the logarithm of the dose of inoculated prions, which allowed for a more rapid and economical (albeit considerably less precise) bioassay (406, 408).

The development of a quantitative, highly sensitive cell-based bioassay by Kloxin and Weissmann was crucial for many practical purposes (270). In the scrapie cell assay (SCA), susceptible neuroblastoma cells (N2a) are exposed to prion-containing samples for 3 days, grown to confluence, passaged several times, and then the proportion of PrP<sup>Sc</sup>-containing cells is determined. In a log-log plot, the dose response is linear over two logs of prion concentrations. The SCA is about as sensitive as the mouse bioassay, 10 times faster, >2 orders of magnitude less expensive, and suitable for automation. SCA performed in a more time-consuming end point titration format (SCEPA) extends the sensitivity and shows that infectivity titers measured in tissue culture and in the mouse are similar.

Once an effective protocol was developed for the preparation of partially purified fractions of scrapie agent from hamster brain, it became possible to demonstrate...
that the procedures that modify or hydrolyze proteins produce a diminution in scrapie infectivity (405, 406). At the same time, tests done in search of a scrapie-specific nucleic acid were unable to demonstrate any dependence of infectivity on a polynucleotide (403, 424), in agreement with earlier studies reporting the extreme resistance of infectivity to UV irradiation at 254 nm (23). On the basis of these findings, it seemed unlikely that the infectious pathogen capable of transmitting scrapie was a virus or a viroid. For this reason, the term prion was introduced by Prusiner to distinguish the proteinaceous infectious particles that cause scrapie, CJD, GSS, and Kuru from both viroids and viruses (403).

A wealth of evidence has accumulated over the past decade supporting the protein-only hypothesis, which proposes that the main or perhaps only constituent of the infectious agent is the mammalian prion PrP\textsuperscript{Sc}. Final proof of this hypothesis could be achieved if pure PrP\textsuperscript{C} or, better yet, recombinant PrP produced in \textit{E. coli}, could be converted into a form that elicits prion disease. A first, seminal step in this direction was the demonstration that radioactive PrP\textsuperscript{C} incubated with unlabeled PrP\textsuperscript{Sc} in a cell-free system generated radioactive PrP\textsuperscript{Sc}, as characterized by its physical properties (97). However, an increase of infectivity was not demonstrated in these experiments (510). Another important advance was provided by the invention of protein misfolding cyclic amplification (PMCA), a technique by which PrP\textsuperscript{Sc} is amplified by cycles of sonication followed by incubation with brain homogenate (433, 434). Soto and co-workers (94) showed that amplification of PrP\textsuperscript{Sc} was accompanied by an amplification of infectivity. The use of purified PrP\textsuperscript{C} instead of brain homogenate as a substrate for the in vitro PrP\textsuperscript{Sc} generation decreased the efficiency of amplification, suggesting that additional cofactors may facilitate the conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc} (134). In addition, initial attempts to use recombinant forms of PrP as a substrate in PMCA have failed. Caughey and co-workers (30) have since succeeded in carrying out PMCA using bacterially expressed hamster PrP as a substrate. While this represents a major advance in many ways, the sensitivity is not quite as high as with brain homogenate (5, 30).

In an intriguing study, Supattapone and co-workers (135) identified the minimal components (PrP\textsuperscript{C}, copurified lipids, and single-stranded polyanionic molecules) required for the amplification of proteinase K-resistant PrP, and they convincingly showed that prion infectivity can be generated de novo in brain homogenates derived from healthy hamsters using PMCA. Inoculation of healthy hamsters with de novo formed prions caused prion disease (135), providing strong support for the prion hypothesis.

Additional support for the prion hypothesis comes from the demonstration that infectious material can be generated in mice by altering the sequence of the prion protein (461). Moderate overexpression in transgenic mice of a mouse PrP with two point mutations (170N, 174T) that subtly affect the structure of its globular domain causes a fully penetrant lethal spongiform encephalopathy with cerebral PrP plaques. This genetic disease was reproduced with 100% attack rate by intracerebral inoculation of brain homogenate to mice overexpressing wild-type PrP (154), and from the latter to wild-type mice, but not to PrP-deficient mice. This work provides an important step forward in elucidating the role of a specific domain of the prion protein in the generation of infectivity (471).

C. Discovery of the Prion Protein

The dependence of prion infectivity on a protein intensified the search for a scrapie-specific protein. Although the insolubility of scrapie infectivity made purification problematic, this property, along with its relative resistance to degradation by proteases, was used by Prusiner to facilitate purification (409, 411). Radiiodination of partially purified brain fractions revealed a protein unique to preparations from scrapie-infected hamsters (55, 404), and in subcellular fractions of hamster brain enriched for scrapie infectivity, a protease-resistant polypeptide of 27–30 kDa (designated PrP\textsuperscript{27–30}) was identified (55, 337).

Purification of PrP\textsuperscript{27–30} to near-homogeneity allowed determination of its NH\textsubscript{2}-terminal amino acid sequence, which permitted the synthesis of an isocoding mixture of oligonucleotides that was subsequently used to identify incomplete PrP cDNA clones from hamster (370) and mouse (107). cDNA clones encoding the entire open reading frames (ORFs) of Syrian hamster and mouse PrP were subsequently isolated (30, 305). The above experiments established that PrP is encoded by a chromosomal gene, and not by a nucleic acid in the infectious scrapie prion particle (39, 370). Levels of PrP mRNA remain unchanged throughout the course of scrapie infection, an unexpected observation that led to the identification of the normal \textit{Prnp} gene product, a protein of 33–35 kDa designated PrP\textsuperscript{C} (39, 370). PrP\textsuperscript{C} is protease sensitive and soluble in nonionizing detergents, whereas PrP\textsuperscript{27–30} is the protease-resistant core of a 33- to 35-kDa disease-specific protein, designated PrP\textsuperscript{Sc}, which is insoluble in detergents (346).

The entire ORF of all known mammalian and avian \textit{Prnp} genes is contained within a single exon (39, 170, 230, 515). This feature of the \textit{Prnp} gene eliminates the possibility that PrP\textsuperscript{Sc} arises from alternative RNA splicing (39, 516), although mechanisms such as RNA editing, protein splicing, and alternative initiation of translation remain a possibility (54, 241, 246). The \textit{Prnp} gene is located in the short arm of human chromosome 20 and in a homologous
region in mouse chromosome 2 (479). Hybridization studies demonstrated <0.002 Prnp gene sequences per ID₅₀ unit in purified prion fractions, strongly suggesting that a nucleic acid encoding PrPSc cannot constitute a component of the infectious prion particle (370). This is a major feature that distinguishes prions from viruses, including retroviruses which carry cellular oncogenes and "satellite viruses" bearing coat proteins from viruses that had previously infected the host.

D. PrP Amyloid

The discovery of PrP²⁷⁻³⁰ in fractions enriched for scrapie infectivity was accompanied by the identification of rod-shaped particles in the same fractions (404, 410). The fine structure of these particles, which had been originally described by Mertz as “scrapie-associated fibrils” (344), failed to reveal any regular substructure characteristic of most viruses (526). Conversely, prion rods are indistinguishable from many purified amyloids (114). This analogy was extended when the prion rods were found to display the tinctorial properties of amyloids (410). Small amyloidotropic dyes, such as derivatives of Congo red and thioflavins, bind with various degrees of selectivity to protein aggregates having an extensive cross β-pleated sheet conformation and sufficient structural regularity and give rise to an enhanced fluorescence (thioflavins) or apple-green birefringence under cross-polarized light (Congo red) (47, 138). However, these dyes are not suitable for recognizing prefibrillary species and amyloid deposits of diverse morphological origin. Advancement in this direction has been provided by luminescent conjugated polymers (LCPs), a recently developed, novel class of amyloidotropic dyes (216, 366, 367).

The amyloid plaques in the brains of humans and other animals with prion disease contain PrP, as determined by immunoreactivity and amino acid sequencing (45, 132, 265, 426, 488). Solubilization of PrP²⁷⁻³⁰ into liposomes with retention of infectivity (169) suggests that large PrP polymers are not required for infectivity, although it is certainly possible that PrPSc oligomers may constitute nucleation centers pivotal to prion replication (238, 288). To systematically evaluate the relationship between infectivity, converting activity, and the size of various PrPSc-containing aggregates, Caughey and co-workers (464) partially disaggregated PrPSc. The resulting species were fractionated by size and analyzed by light scattering and non-denaturing gel electrophoresis. Intracerebral inoculation of the different fractions into hamsters revealed that with PrP content, infectivity peaked markedly with 17–27 nm (300–600 kDa) particles. These results suggest that nonfibrillar particles, which have masses equivalent to 14–28 PrP molecules, are the most efficient initiators of TSE disease (464).

E. Formation of PrPSc

It remains to be established whether any form of PrPSc can act as a substrate for PrPSc formation, or whether a restricted subset of PrP molecules are precursors for PrPSc (511). It is also unknown whether reflective transition states between the two exist. Several experimental results argue that PrP molecules destined to become PrPSc exit to the cell surface prior to their conversion into PrPSc (59, 99, 494). Similar to other GPI-anchored proteins, PrPSc appears to localize in cholesterol-rich, nonacidic, detergent-insoluble membranes known as rafts (26, 195, 253, 456, 495). Within the raft compartment, GPI-anchored PrPSc is apparently either converted into PrPSc or partially degraded (343, 495). Chemical and enzymatic treatment of purified PrP²⁷⁻³⁰ leads to the release of glycolipid components, suggesting that PrPSc could be tethered to the membrane by a GPI anchor (481).

The role of the GPI membrane anchor in the formation of PrPSc in vivo has been addressed by Chesebro et al. (108), who have established a transgenic mouse model expressing anchorless, and hence secreted, PrP. When these transgenic mice were subsequently infected with protease-resistant PrPSc, they developed significant amyloid plaque pathology in the brain, but clinical manifesta-
tions were minimal. In contrast, combined expression of anchorless and wild-type PrP resulted in accelerated clinical scrapie (108).

1. Structures of purified PrP<sup>C</sup> and PrP<sup>Sc</sup>

Early low-resolution structural studies indicated that PrP<sup>C</sup> had a high α-helix content (~40% of the protein) and relatively little β-sheet (3% of the protein) (379). These findings were further refined by Wüthrich and colleagues who determined the fine structure of PrP<sup>C</sup> by nuclear magnetic resonance spectroscopy (Fig. 1A) (229, 421), and later also by crystallographic studies (271). The NH<sub>2</sub>-proximal half of the molecule is not structured at all, whereas the COOH-proximal half is arranged in three α-helices corresponding, for the human PrP<sup>C</sup>, to the residues 144–154, 173–194, and 200–228, interspersed with an antiparallel β-pleated sheet formed by β-strands at residues 128–131 and 161–164. A single disulfide bond is found between cysteine residues 179 and 214 (421, 422, 531). It is unlikely that the NH<sub>2</sub> terminus is randomly coiled in vivo, since functional studies in transgenic mice imply that the domain comprising amino acids 32–121 carries out important physiological functions (453). It is possible that the flexible tail of PrP<sup>C</sup> acquires a defined structure when PrP<sup>C</sup> is present within membrane rafts (362).

In contrast to PrP<sup>C</sup>, the β-sheet content of PrP<sup>Sc</sup> comprises ~40% of the protein, whereas α-helices comprise 30% of the protein as measured by Fourier-transform infrared (379) and CD spectroscopy (Fig. 1B) (436). No high-resolution structure is available for PrP<sup>Sc</sup>, although interesting models have been conjectured on the basis of electron crystallography studies (524). Prion rods of the NH<sub>2</sub>-terminally truncated form of PrP<sup>Sc</sup> derived by limited proteolysis, PrP<sup>27–30</sup>, exhibit green-gold birefringence after staining with Congo red, indicating that this isoform has a high β-sheet content (410). Indeed, PrP<sup>27–30</sup> poly-

**Fig. 1.** Structural features of PrP<sup>C</sup> and PrP<sup>Sc</sup>. A: NMR structure of the mouse prion protein domain (121–231). The ribbon diagram indicates the positions of the three helices (yellow) and the antiparallel two-stranded β-sheet (cyan). The connecting loops are displayed in green if their structure is well defined and in magenta otherwise. The disulfide bond between Cys-179 and Cys-214 is shown in white. The NH<sub>2</sub>-terminal segment of residues 121–124 and the COOH-terminal segment 220–231 are disordered and not displayed. (Figure kindly provided by S. Hornemann.) B: Fourier transform infrared spectroscopy of prion proteins. The amide I band (1,700–1,600 cm<sup>–1</sup>) of transmission FTIR spectra of PrP<sup>C</sup> (black line), PrP<sup>Sc</sup> (gray line), and PrP<sup>27–30</sup> (dotted line). These proteins were suspended in a buffer in D<sub>2</sub>O containing 0.15 M sodium chloride/10 mM sodium phosphate, pD 7.5 (uncorrected)/0.12% ZW. The spectra are scaled independently to be full scale on the ordinate axis (absorbance). [From Pan et al. (379).] C: electron micrographs of negatively stained and immunogold-labeled prion proteins. Each panel shows in following order PrP<sup>C</sup>, PrP<sup>Sc</sup>, and Prion rods, composed of PrP<sup>27–30</sup>, that were negatively stained with uranyl acetate. Bar, 100 nm. [From Pan et al. (379).]
merized into rod-shaped particles with an ultrastructural appearance of amyloid (Fig. 1C) containing ~50% β-sheet and 20% α-helix content (103, 176, 435). In contrast to PrP27–30, neither purified PrP<sup>C</sup> nor PrP<sup>Sc</sup> forms aggregates detectable by electron microscopy (338, 379).

2. Models describing the replication of prions

Transgenic mouse studies have provided genetic (412) and biochemical (343) evidence that the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> occurs through the formation of a PrP<sup>C</sup>/PrP<sup>Sc</sup> complex. However, such a complex has never been isolated to purity. Consequently, it is unclear whether PrP<sup>C</sup> binds to one or more additional macromolecules during the process of PrP<sup>C</sup> to PrP<sup>Sc</sup> conversion. The formation of a PrP<sup>C</sup>/PrP<sup>Sc</sup> complex may be facilitated when the amino acid sequences of the two PrP isoforms are identical. For instance, hamster PrP differs from mouse PrP at 16 of 254 positions, perhaps explaining the strong species barrier between mice and hamsters. Differences in amino acid sequence delay the onset of prion disease by prolonging the incubation time (446). As the sequence specificity of PrP<sup>Sc</sup> formation is more important for the conversion to protease resistance than for the initial binding steps, additional intermolecular interactions between PrP<sup>C</sup> and PrP<sup>Sc</sup> may be required to complete the process of conversion to the protease-resistant state (228).

The level of PrP<sup>C</sup> expression appears to be directly proportional to the rate of PrP<sup>Sc</sup> formation and, thus, inversely related to the length of the incubation time. Prnp<sup>o/o</sup> mice are resistant to prions and do not propagate scrapie infectivity (84, 407, 439). Mice hemizygous (Prnp<sup>+/+</sup>) for ablation of the Prnp gene have prolonged incubation times when inoculated with mouse prions (83, 326).

According to the “protein only” hypothesis, there are two models to explain the conformational conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup>. The “template-directed refolding” hypothesis predicates an instructionist role for PrP<sup>Sc</sup> on PrP<sup>C</sup> (Fig. 2A). Alternatively, the seeded nucleation hypothesis suggests that PrP<sup>Sc</sup> exists in equilibrium with PrP<sup>C</sup>. In a nondisease state, such an equilibrium would be heavily shifted toward the PrP<sup>C</sup> conformation, such that only minute amounts of PrP<sup>Sc</sup> would coexist with PrP<sup>C</sup>. If this were the case, PrP<sup>Sc</sup> could not possibly represent the infectious agent, since it would be ubiquitous. According to this “seeded nucleation” hypothesis (238), the infectious agent would consist of a highly ordered aggregate of PrP<sup>Sc</sup> molecules. The aggregated state would be an intrinsic property of infectivity. Monomeric PrP<sup>Sc</sup> would be harmless, but it might be prone to incorporation into nascent PrP<sup>Sc</sup> aggregates (Fig. 2B) (19).

The recently developed PMCA (92, 93, 196, 432, 434) is designed to mimic PrP<sup>Sc</sup> autocatalytic replication (Fig. 3). In the PMCA, PrP<sup>Sc</sup> is amplified in a cyclic manner by incubating small amounts of PrP<sup>Sc</sup>-containing brain homogenate with uninfected, PrP<sup>C</sup>-containing brain homogenate, resulting in the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup>. The latter forms aggregates that are sonicated to generate multiple smaller units functioning as a seed for the continued formation of new PrP<sup>Sc</sup> aggregates.

AGGREGATION AND INFECTIOUS DISEASE

Fig. 2. Models for the conformational conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup>. A: the “refolding” or template assistance model postulates an interaction between exogenously introduced PrP<sup>Sc</sup> and endogenous PrP<sup>C</sup>, which is induced to transform itself into further PrP<sup>Sc</sup>. A high-energy barrier may prevent spontaneous conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup>. B: the “seeding” or nucleation-polymerization model proposes that PrP<sup>C</sup> and PrP<sup>Sc</sup> are in a reversible thermodynamic equilibrium. Only if several monomeric PrP<sup>Sc</sup> molecules are mounted into a highly ordered seed, can further monomeric PrP<sup>Sc</sup> be recruited and eventually aggregate to amyloid. Fragmentation of PrP<sup>Sc</sup> aggregates increases the number of nuclei, which can recruit further PrP<sup>Sc</sup> and thus results in apparent replication of the agent.
An important part of the prion replication process is the propagation of prions through the fragmentation of existing fibrils. This mechanism has been experimentally verified for yeast prions (62, 491). There are indications that similar processes govern the growth of mammalian prions (94, 295) as well as non-prion-related amyloid fibrils (139).

Analysis of yeast prions has shown that the mechanical frangibility of formed prion fibrils plays a key role in fibril growth, complexity, and diversity (492). The fragility of yeast prions varies substantially between different classes of fibrils, but is relatively homogeneous within a single class (237, 492). Yeast prion classes with an aggressive tendency to multiply in vivo have been found to be composed of amyloid fibrils with the highest propensity to fragment. It is interesting to note that in terms of their mechanical rigidity, amyloid fibrils can be very heterogeneous (272), implying a substantial potential for intrinsic variability in their breakage rates. Although prion propagation in mammalian tissues is likely to be inherently more convoluted, recent observations indicate that the propagation of mammalian prion strains may be kinetically similar to fungal prions. Indeed, shorter fibrils, which may result from breakage of frangible structures, are more infectious than longer fibrils (464), an observation consistent with the fact that a larger number of free ends in a short fibril population leads to more rapid conversion of soluble cellular prion protein into misfolded fibrillar form and eventually overwhelming cellular clearance mechanisms.

In agreement with this idea, an inverse correlation was recently found between the stability of prion aggregates and incubation times in vivo (296), a finding that is closely analogous to results obtained in yeast prions (499). Furthermore, in in vitro growth assays of many amyloid fibril systems, it has been noted that agitation significantly enhances the overall conversion rate of proteins into fibrillar forms (255, 371, 467), indicating that fibril breakage is an essential factor determining the rate of amyloid formation. In living systems, the rate constants for prion replication are clearly influenced not only by the intrinsic strength of PrPSc aggregates, but also by other cellular components. Molecular chaperones in yeast, for instance, have been identified as important players in the rate of prion replication (454).

F. Prion Diversity

One of the most puzzling phenomena in prion biology is the existence of prion strains. Prion strains are defined as infectious isolates that, when transmitted to identical
hosts, exhibit distinct prion-disease phenotypes. The phenotypic traits may include distinct patterns of protein aggregate deposition, incubation times, histopathological lesion profiles, and specific neuronal target areas. Most of these traits are relatively stable across serial passages. Often new distinct strains can be observed upon transmission of prions across an interspecies barrier or into animals of the same species expressing different polymorphisms of the prion gene, a phenomenon sometimes referred to as a “strain mutation” (76, 504).

The diversity of scrapie prions was initially noted in goats inoculated with “hyper” and “drowsy” isolates (386). Scrapie isolates or “strains” from goats with a drowsy syndrome transmitted a similar syndrome to inoculated recipients, whereas those from goats with a “hyper” or ataxic syndrome transmitted an ataxic form of scrapie to recipient goats. Subsequent studies in mice have also demonstrated the existence of diverse scrapie strains that produce characteristic phenotypes in inoculated recipients (78, 136, 137) with respect to incubation times, the distribution of central nervous system (CNS), and the formation of amyloid plaques, although the clinical signs of scrapie tend to be similar.

The identification of strains through the inoculation of mice is time-consuming, cumbersome, and prohibitively expensive. Moreover, the reliability of the resulting classification is based on mere correlative evidence. However, over time, a number of biochemical correlates for prion strains have been identified (Table 2) (9), and it is expected that these criteria will facilitate the study and the diagnosis of prion strains.

1. Prion strains and variations in patterns of disease

Although defining of prion strains according to biochemical characteristics is useful in terms of nomenclature and classification of prion diseases, it certainly does not explain why strains display different organ tropisms. For example, some prion strains preferentially propagate in the CNS and exhibit low abundance in secondary lymphoid organs. This neurotropic pattern of tissue distribution is observed in “classical” BSE in bovines. In other strains, PrP \textsuperscript{Sc} and/or prion infectivity are detected not only in the CNS but also, to a large extent, in secondary lymphoid organs. These lymphotropic prion strains include vCJD, CWD (460), and many scrapie strains (20). However, these differences are not absolute. For example, in patients with sCJD, it was originally believed that prions were present in the CNS and other peripheral tissues, but not in secondary lymphoid organs (74). However, it is now acknowledged that PrP \textsuperscript{Sc} can also be found in the spleen and skeletal muscle of patients with sCJD (183, 388).

What defines the organ tropism of a given prion strain is still a completely open question. One might speculate that tropism is defined by the tertiary or (more probably) by the supramolecular structure of the prion agent, which influences its ability to bind to or interact with specific molecules (for example, receptors), and as a result affects specific cell types (9). The phenomenon of cellular prion tropism has been elegantly demonstrated by Charles Weissmann’s group in an array of cell lines which replicate different prion strains to varying degrees (319). The differential cell tropism of the various prion strains suggests the requirement for cell-specific cofactors, be they chaperones, specific uptake receptors, RNA species, a particular lipid environment, a specific posttranslationally modified PrP molecule, or a particular prion replicating subcellular environment. This hypothesis relies on the assumption that cofactors supporting the conversion of distinct prion strains exist in defined cell groups (16).

2. Molecular basis of prion strains

The mechanism by which isolate-specific information is carried by prions remains enigmatic. A dwindling num-

### Table 2. Synopsis of currently used prion strain differentiation assays

<table>
<thead>
<tr>
<th>Assay Principle</th>
<th>Test Substrate</th>
<th>Speed</th>
<th>Cost</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation period in indicator mice</td>
<td>Mice</td>
<td>Years</td>
<td>+++</td>
<td>164</td>
</tr>
<tr>
<td>Histological lesion profile</td>
<td>Mice</td>
<td>Years</td>
<td>+++</td>
<td>79, 163</td>
</tr>
<tr>
<td>Histoblots</td>
<td>Immunohistology</td>
<td>Days</td>
<td>+</td>
<td>493</td>
</tr>
<tr>
<td>Conformation-dependent immunoassay</td>
<td>ELISA</td>
<td>Days</td>
<td>+</td>
<td>437</td>
</tr>
<tr>
<td>Conformational stability assay</td>
<td>Western blot</td>
<td>Days</td>
<td>+</td>
<td>300</td>
</tr>
<tr>
<td>Conformation assay</td>
<td>Infrared spectroscopy</td>
<td>Days</td>
<td>+</td>
<td>101</td>
</tr>
<tr>
<td>PK cleavage site</td>
<td>Western blot</td>
<td>Days</td>
<td>+</td>
<td>51</td>
</tr>
<tr>
<td>Detection with NH\textsubscript{2}-terminal antibodies</td>
<td>Western blot</td>
<td>Days</td>
<td>+</td>
<td>396</td>
</tr>
<tr>
<td>Glycosylation profile</td>
<td>Western blot</td>
<td>Days</td>
<td>+</td>
<td>220</td>
</tr>
<tr>
<td>Amyloid detection by thioflavin and Congo red stains</td>
<td>Histochecmistry</td>
<td>Hours</td>
<td>+</td>
<td>460</td>
</tr>
<tr>
<td>Luminescent-conjugated polymers</td>
<td>Histochecmistry</td>
<td>Hours</td>
<td>+</td>
<td>462</td>
</tr>
<tr>
<td>Cell panel assay</td>
<td>Cell culture</td>
<td>Weeks</td>
<td>+</td>
<td>319</td>
</tr>
</tbody>
</table>

Most assays have high discriminatory power between few specific strains (e.g., glycosylation profiles for bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease) but may perform poorly with other strains. +, Low; +++, high; ++++, extremely high. [Adapted from Aguzzi (9).]
ber of investigators still contend that scrapie is caused by a viruslike particle containing a scrapie-specific nucleic acid that encodes the information expressed by each isolate (78). But to date, no such polynucleotide has been identified, despite the use of a wide variety of techniques, including the measurement of nucleic acids in purified preparations. An alternative hypothesis is that PrPSc alone is capable of transmitting the disease and that the characteristics of PrPSc are modified by a cellular nucleic acid (512). This accessory cellular polynucleotide is postulated to induce its own synthesis upon transmission from one host to another, but there is currently no experimental evidence to support its existence.

In striking contrast, mice expressing Prnp transgenes have demonstrated that the level of PrP expression is inversely related to the incubation time (412). Taken together, these observations indicate that PrPSc itself is the molecule in which prion "strain"-specific information is encrypted. Preliminary immunochemical evidence suggests that PrPSc of different strains expose different epitopes (438) and display different degrees of stability to chaotropic salts (390). Infrared spectroscopy measurements have shown that types of PrPSc associated with distinct hamster TSE strains can possess different conformations, even though they are derived from PrP with the same amino acid sequence (101). This could be interpreted as indirect evidence for conformational differences or could indicate that different PrPSc strains associate with different molecules of proteinaceous or other nature.

Both PrPC and PrPSc exist in three main glycosylation states: unglycosylated, monoglycosylated, and diglycosylated. The relative ratios of these three forms of PrPSc differ in various prion strains. In some cases, such differences are remarkably robust and are widely used for prion strain typing. On the basis of the fragment size and the relative abundance of individual bands, three distinct patterns (PrPSc types 1–3) have been identified for sporadic and iatrogenic CJD cases (118, 381). In contrast, all cases of vCJD and of BSE displayed a novel pattern, designated as type 4 pattern.

The "seeded nucleation" theory (238) described for PrP may also apply to amyloid formation in other protein misfolding disorders (PMDs). In fact, protein conformational changes associated with the pathogenesis of most PMDs result in the formation of abnormal proteins that are rich in β-sheet structure, partially resistant to proteolysis, and have a high tendency to form larger-order aggregates, similar to PrPSc. Amyloid formation depends on the slow interaction of misfolded protein monomers to form oligomeric nuclei, around which a faster phase of elongation takes place. The ability of oligomeric species to seed their own growth is analogous to the self-propagating activity of prions.

These theoretical considerations are supported by extensive experimental data (474). In addition, it has been
reported that polyglutamine aggregates (420) and tau aggregates (167) can be internalized by mammalian cells in culture. These internalized fibrillar aggregates may then selectively recruit soluble cytoplasmic proteins and stimulate further misfolding of otherwise stable intracellular proteins. From a molecular standpoint, all PMDs can be considered “infectious” in the sense that misfolded and aggregated conformers act as templates for the recruitment of native proteins. However, in no PMDs other than PrPSc pathologies has bona fide “microbiological” infectivity been detected, and none of the non-PrP aggregation diseases has ever given rise to macroepidemics such as Kuru and BSE.

Some experimental amyloidoses in mammals have shown evidence of being transmissible in the sense that amyloid taken from an amyloidotic individual can sometimes greatly accelerate amyloidosis in another individual. However, it is important to note that such “transmissions” have only been shown under experimental conditions in which the recipient animal is highly primed for susceptibility to amyloidosis and would be expected to spontaneously develop amyloidosis over time. Typical examples are AA amyloidosis and Alzheimer’s disease (AD). Most cases of AA amyloidosis in mammals appear to occur spontaneously due to chronic inflammation or genetic peculiarities that elevate blood levels of serum amyloid A (SAA) and predispose the organism to AA amyloid formation. AA amyloid is characterized by the accumulation of aggregated SAA NH2-terminal-derived peptides in organs such as the kidney, liver, and spleen. Inoculation of unaffected mice with tissue extracts from amyloidotic mice can rapidly induce amyloidosis, as long as the recipients are “primed” for amyloidosis with proinflammatory treatments such as silver nitrate injections (261, 308).

Given the “infectious” characteristics of AA amyloidosis, transmission between captive cheetahs may explain the high incidence of AA amyloidosis observed in this species. Acceleration of amyloidosis in mice upon oral administration of AA amyloid suggests a plausible fecal-oral route for the natural transmission of AA amyloidosis in cheetahs. Indeed, amyloidotic cheetahs shed AA amyloid in the feces, and this fecal amyloid can accelerate AA amyloidosis when injected intravenously into silver nitrate-primed mice (533).

However, unlike wild cheetahs, captive cheetahs exhibit chronic inflammation and high blood SAA levels (359). These factors could make them susceptible to spontaneous amyloidosis, as it is the case with silver nitrate-treated mice. Therefore, the high incidence of amyloidosis in captive cheetahs might also be due to spontaneous disease, rather than infection with amyloid from other animals. This raises the important question of whether, in the case of non-PrPSc amyloids, inoculation with the amyloid actually initiates the disease, as is clearly the case with legitimate TSE prions, or merely enhances an already ongoing pathogenic process (96).

Several attempts have been made to transmit AD to experimental animals, which have yielded intriguing, yet conflicting, results. An early report of buffy coat-borne AD transmission to hamsters (327) initially caused great consternation, but was later found to be irreproducible. Marmosets injected with brain homogenates from individuals with AD developed scattered deposits of the amyloid-β protein (Aβ) in the brain parenchyma and cerebral vasculature 6–7 yr after inoculation (35). However, extensive studies by Gajdusek and colleagues (74) have failed to transmit AD and other dementias to primates.

Jucker and co-workers (345) showed that the injection of the Aβ peptide derived from human AD brains to transgenic mice overexpressing the Aβ precursor protein APP induced cerebral β-amyloidosis. The pathology in the APP transgenic mice was determined by both the host and the inoculum (345). The authors postulated the existence of Aβ strains that can initiate and accelerate aggregation and Aβ pathology. It remains to be seen whether different Aβ strains with distinct biochemical or neuropathological characteristics occur in humans. Once again, as was the case with the AA inoculation studies mentioned above, it is unclear whether inoculation with Aβ amyloid causes amyloidosis or simply accelerates an ongoing pathogenic process, since transgenic recipients ultimately develop amyloid pathology in the brain, even without inoculation with Aβ amyloid extracts.

Another major unresolved issue is the extent to which the induced amyloidotic lesions can spread from localized sites of seeding by the inoculum to other sites within the host. Surprisingly, a recent study reported that healthy fetal tissue grafted into the brains of Parkinson’s disease patients acquired cytoplasmic α-synuclein-rich Lewy bodies, suggesting that misfolded α-synuclein spreads to healthy cells and acts as a template for the conversion of native α-helices to pathogenic β-sheets (299).

Type 2 diabetes is yet another disease whose pathogenesis may involve ordered protein aggregation. Evidence for this was discovered early on (373) but was largely forgotten for almost a century. It is now evident that aggregation of islet amyloid polypeptide (IAPP) is an exceedingly frequent feature of type 2 diabetes. IAPP amyloid damages the insulin-producing β-cells within pancreatic islets and may crucially contribute to the pathogenesis of diabetes (233). It is unknown, however, whether IAPP deposition simply accrues linearly with IAPP production, or whether it spreads in a prion-like fashion from one pancreatic islet to the next.

In summary, the role of protein misfolding and aggregation in various human diseases has been clearly established in the past decade, yet an important challenge for the coming years will be to determine whether the
prion mechanism of disease transmission might be operating in other human diseases, some of which are highly prevalent.

II. FUNCTION OF THE CELLULAR PRION PROTEIN

PrP\textsuperscript{C}, following the cleavage of a 22-amino acid (aa) signal peptide, is exported to the cell surface as an N-glycosylated, GPI-anchored protein of 208–209 aa. PrP\textsuperscript{C} contains a long NH\textsubscript{2}-proximal flexible random-coil sequence, which is followed by a globular COOH-proximal domain. The structurally less-defined NH\textsubscript{2} proximal region consists of residues 23–124 and contains a stretch of several octapeptide repeats (OR), flanked by two positively charged clusters, CC1 (aa 23–27) and CC2 (aa 95–110). These domains are linked by a hydrophobic stretch of amino acids known as the HC region (aa 111–134) (Fig. 5).

The structure of the globular half of human PrP\textsuperscript{C} is identical to many other mammals, as expected from the high degree of sequence identity (309). Interestingly, despite the low sequence identity between PrP\textsuperscript{C} in chicken, turtle, or frog, and the mammalian isoforms, the major structural features of PrP\textsuperscript{C} are remarkably preserved in nonmammalian species (88).

Full-length PrP\textsuperscript{C} is found in non-, mono-, or diglycosylated forms, corresponding to the variable occupancy of glycosyl groups on residues Asn-181 and Asn-197 in human and Asn-180 and Asn-196 in mice (201). A rather large variety of N-glycans are found attached to both full-length and truncated PrP\textsuperscript{C} (380, 428), which may be differentially distributed in various areas of the central nervous system (49, 133). The physiological significance of PrP\textsuperscript{C} glycosylation is unknown.

Despite advances in the characterization of PrP\textsuperscript{C} structure, the physiological function of PrP\textsuperscript{C} is still unclear. An unrealistic plethora of possible functions have been ascribed to PrP. Therefore, there is a lingering suspicion that each of these reports represents a specific aspect of a more complex reality whose full picture is still not entirely understood. Importantly, it is not yet clear whether the physiological function of PrP\textsuperscript{C} is actually linked to toxicity associated with prion diseases.

A. Teachings From Prnp-Ablated Mice

Obviously, the study of PrP knockout mice has thus far failed to elucidate the molecular function of the protein. This is particularly sobering when one considers the fact that independent lines of mice lacking PrP\textsuperscript{C} have been generated by homologous recombination in embryonic stem cells in many laboratories. Mice with disruptive gene modifications restricted to the open reading frame are known as Prnp\textsuperscript{o/o} (Zurich I) (85) and Prnp\textsuperscript{−/−} (Edinburgh) (325). These mice were found to develop normally, although a number of subtle abnormalities have been described (120, 368, 498) whose molecular basis remains undefined. Among other functions, it has been suggested that PrP\textsuperscript{C} may have a role in the synaptic machinery of the olfactory bulb (293). However, the reported involvement of PrP\textsuperscript{C} in synaptic hippocampal activity has been the subject of intense controversy (301).

The only molecularly well-defined phenotype of Prnp\textsuperscript{o/o} mice is their resistance to prion inoculation (84). However, it seems rather unlikely that a protein as highly conserved among species as PrP\textsuperscript{C} has evolved purely for the purpose of bestowing susceptibility to prion diseases upon organisms. If the function of PrP\textsuperscript{C} were completely unrelated to prion disease pathogenesis, PrP\textsuperscript{C} would be just one of many proteins whose function awaits clarification. Otherwise, the function of PrP\textsuperscript{C} may reflect, in a subtle way, prion-induced damage. However, Prnp ablation does not elicit disease, even when induced postnatally (322). Therefore, prion pathology is unlikely to occur due to a simple loss of PrP\textsuperscript{C} function. If PrP\textsuperscript{C} possessed enzymatic activity or transduced a signal (similarly to many other GPI-linked proteins), one could hypothesize that conversion to PrP\textsuperscript{Sc} alters PrP\textsuperscript{C} substrate specificity or signal transduction strength, conferring as a result a toxic dominant function. Therefore, understanding the function of PrP\textsuperscript{C} may be instrumental to deciphering prion pathol-

![FIG. 5. Outline of the primary structure of the cellular prion protein including posttranslational modifications. A secretory signal peptide resides at the extreme NH\textsubscript{2} terminus. CC\textsubscript{1} and CC\textsubscript{2} define the charged clusters. OR indicates the octapeptide repeat, and four are present. HC defines the hydrophobic core. MA denotes the membrane anchor region. S-S indicates the single disulfide bridge, and the glycosylation sites are designated as CHO. The numbers describe the position of the respective amino acids.](http://www.prv.org)
ogy, and perhaps even to devising therapeutic approaches.

Glockshuber et al. (190) noted that PrPC had similarities to membrane-anchored signal peptidases, but their observation has not been substantiated by functional data. PrPC can bind copper (67, 505), but reports of increased copper content in neurons lacking PrPC have been called into question by contradictory findings (506). The idea that PrPC might be a superoxide dismutase (68, 69) was perceived as particularly attractive in view of its multiple copper-binding sites. However, PrPC does not provide any measurable contribution to dismutase activity in vivo (234, 506), although it has been suggested that NH$_2$-proximally-truncated PrPC may suppress endogenous dismutase activity (441).

**B. PrPC and Its Partners**

Perhaps PrPC and PrPSc do not possess any intrinsic biological activity of their own, but modify the function of other proteins. GPI-anchored proteins must interact with transmembrane adaptors on the same cell or an adjacent cell to influence intracellular signal transduction pathway. This supposition has led to the search for PrP-interacting partners using methods such as yeast-two hybrid, cross-linking experiments, and co-immunoprecipitations. All known PrPC interaction partners have been summarized in a recent review (10). Putative PrPC interacting proteins include membrane proteins (receptors, enzymes, caveolin-1, Na$^+$-K$^+$-ATPase, and a potassium channel), cytoplasmic proteins (components of the cytoskeleton, heat-shock proteins, and adaptor proteins involved in signaling), and even nuclear proteins. Recently, transgenic mice expressing functional tagged prion protein have been used to characterize the PrPC interactome (431). However, many studies used to identify such interactors are fraught with methodological issues. PrPC is exposed to the extracellular space, and therefore, it is questionable whether conventional yeast two-hybrid screens, which artificially expose PrPC to the cytosolic compartment, represent an appropriate method to investigate PrPC interaction partners. The same concerns may apply to chemical cross-linking experiments. Cross-linking fuses together any proteins that reside in the same microenvironment, even if they do not necessarily interact with each other. Furthermore, the choice of detergent conditions in coimmunoprecipitation experiments is also crucial, since it should allow for weak and transient protein-protein interactions, but should destroy any potential artificial or postlysis nonspecific interactions. Ultimately, it would be desirable to validate reported interactions by physiological read-outs, yet this lofty goal has not been attained for most of the candidates described thus far.

**C. PrP’s Double and Its Trouble**

After the original report by the Weissmann laboratory (85), several independent Prnp knockout mouse lines were generated, including Prnp$^{-/-}$ (Nagasaki), Rcm0, and Prnp$^{-/-}$ (Zurich II) (356, 427, 440). These endeavors have led to very surprising insights. All of the latter mice, but none of the Zurich I mice, developed ataxia and Purkinje cell loss later in life. The discrepancy among the different lines of PrP knockout mice was not resolved until a novel gene (Prnd), encoding a protein called Doppel (Dpl), was discovered. Prnd is localized 16 kb downstream of Prnp. In all three lines of PrPC-deficient mice developing ataxia and Purkinje cell loss, a splice acceptor site in the third exon of Prnp was deleted. This placed Prnd under transcriptional control of the Prnp promoter, resulting in the formation of chimeric transcripts and overexpression of Dpl in the brain (513).

The Prnd gene is evolutionarily conserved from humans to sheep and cattle and shares roughly 25% homology with the COOH-proximal two-thirds of PrPC, but it lacks the PrPC octapeptide repeats and hydrophobic region. Structural studies indicate that Dpl contains three $\alpha$-helices, similar to PrPC, and two disulfide bridges between the second and third helix (307, 465). Dpl mRNA is expressed at high levels in testis and heart, moderately in spleen and some other organs and, notably, at very low levels in brain of adult wild-type mice. However, elevated Prnd mRNA transcripts have been detected during embryogenesis and in the brains of newborn mice, thus arguing for a possible function of Dpl in brain development (298). To probe the function of Dpl, the Prnd gene was inactivated in embryonic stem cells (43). Similarly to mice lacking PrPC, mice devoid of Dpl survive to adulthood and do not show obvious phenotypical alterations, suggesting that Dpl is dispensable for embryogenesis and postnatal development (44). The similarities between PrPC and Dpl in primary amino acid sequence, in structure, and in subcellular localization suggest related biological functions (42). Therefore, a possible role of PrPC and Dpl during development may be masked by functional redundancy. To address this possibility, Prnp/Prnd double knockouts were generated using transallelic targeted meiotic recombination (177). Offspring carrying the double deletion exhibited no abnormalities, except for male sterility, and they did not develop ataxia, implicating a causal role for Dpl overexpression in the Zurich II phenotype. Furthermore, Dpl did not appear to compensate for the loss of any unrecognized function of PrP.

Why the overexpression of Dpl is deleterious is still unclear. Neurodegeneration is not uniquely associated with overexpression of Dpl. Transgenic mice overexpressing partially deleted Prnp variants provoke spontaneous neurodegenerative disease, and some domains are also essential for restoring prion susceptibility (Table 3).
Deletions of amino acids 32–121 or 32–134 (collectively termed ΔPrP) confer strong neurotoxicity to PrPSc in vivo, which indicates that ΔPrP is a functional antagonist of PrPC (414, 453). Furthermore, a small deletion within the hydrophobic stretch (amino acids 94–134) is sufficient to produce a highly neurotoxic molecule (40). This pathology can be abrogated by the reintroduction of wild-type, full-length PrPC. Because the lack of PrPC itself does not induce an obvious phenotype, the latter pathways imply the existence of pathways in which PrPC is functionally active. Hence, mice expressing PrP variants may allow for the identification of functionally relevant domains within PrPC (10).

### III. MECHANISMS OF PRION TOXICITY

Despite considerable knowledge about the characteristics of the TSE infectious agent, its mechanism of replication, and the association of PrPSc with neurodegeneration, the molecular pathways leading to cerebral damage are, for the most part, unknown. Depletion of PrPC is an unlikely cause, in view of the finding that abrogation of PrP does not cause scrapie-like neuropathological changes (85), even when elicited postnatally (322). Moreover, it was observed that depletion of PrPC in mice with established prion infection reversed early spongiform degeneration and prevented neuronal loss and progression to clinical disease (321, 323). Importantly, this occurred despite the accumulation of extraneuronal PrPSc to levels similar to terminally ill, wild-type infected animals. These data suggest that PrPSc might not be directly responsible for neurodegeneration; it is more likely that the toxicity of PrPSc depends on some PrPC-dependent process culminating in neuronal dysfunction and death (63, 64).

The first experimental evidence in support of this hypothesis was provided when neural tissue overexpressing PrPC was grafted into the brains of PrP-deficient mice (153). Following scrapie inoculation, these mice remained free of symptoms for at least 70 wk, exceeding the survival time of scrapie-infected donor mice by at least sevenfold (64). Therefore, the presence of a continuous source of PrPSc does not exert any clinically detectable adverse effects on a mouse devoid of PrPC. On the other hand, the grafted tissue developed characteristic histopathological features of scrapie after inoculation. The course of the disease in the graft was very similar to that observed in the brain of scrapie-inoculated wild-type mice (63). Since the grafts had extensive contact with the recipient brain, prions could navigate between the two compartments, as shown by the fact that inoculation of wild-type animals engrafted with PrP-expressing neuroectodermal tissue resulted in scrapie pathology in both graft and host tissue. Nonetheless, histopathological changes never extended into host tissue, even at the latest stages (>450 days), although PrPSc was detected in both grafts and recipient brain, and immunohistochemistry revealed PrP deposits in the hippocampus, and occasionally in the

---

**TABLE 3. Summary of transgenic mutant for PrP**

<table>
<thead>
<tr>
<th>PrP Deletion Mutants</th>
<th>Phenotype</th>
<th>Rescue by PrP (if applicable)</th>
<th>Prion Propagation</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrP Δ1-22 231#</td>
<td>Cerebellar disorder</td>
<td>No</td>
<td>No</td>
<td>314</td>
</tr>
<tr>
<td>PrP Δ23-88</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>360, 361</td>
</tr>
<tr>
<td>PrP Δ23-88 C178A</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>360, 361</td>
</tr>
<tr>
<td>PrP Δ23-88 Δ95-107</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>360, 361</td>
</tr>
<tr>
<td>PrP Δ23-88 Δ108-121</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>360, 361</td>
</tr>
<tr>
<td>PrP Δ23-88 Δ122-140</td>
<td>Short-lived protein</td>
<td>No</td>
<td>No</td>
<td>360</td>
</tr>
<tr>
<td>PrP Δ23-88 Δ141-170</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>360, 483</td>
</tr>
<tr>
<td>PrP Δ23-88 Δ141-221</td>
<td>Cerebellar disorder</td>
<td>No</td>
<td>No</td>
<td>484</td>
</tr>
<tr>
<td>PrP Δ23-88 Δ177-200</td>
<td>Storage disease</td>
<td>No</td>
<td>No</td>
<td>360</td>
</tr>
<tr>
<td>PrP Δ23-88 Δ201-217</td>
<td>Storage disease</td>
<td>No</td>
<td>No</td>
<td>360</td>
</tr>
<tr>
<td>PrP Δ32-80</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>153</td>
</tr>
<tr>
<td>PrP Δ32-93</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>154, 453</td>
</tr>
<tr>
<td>PrP Δ32-106</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>453*</td>
</tr>
<tr>
<td>PrP Δ32-121</td>
<td>Cerebellar disorder</td>
<td>Yes</td>
<td>No</td>
<td>453*</td>
</tr>
<tr>
<td>PrP Δ32-134</td>
<td>Cerebellar disorder</td>
<td>Yes</td>
<td>No</td>
<td>453*</td>
</tr>
<tr>
<td>PrP Δ104-134</td>
<td>Cerebellar disorder</td>
<td>Yes</td>
<td>Not done</td>
<td>40</td>
</tr>
<tr>
<td>PrP Δ104-114</td>
<td>No</td>
<td>Not done</td>
<td>No</td>
<td>204</td>
</tr>
<tr>
<td>PrP Δ105-125</td>
<td>Cerebellar disorder</td>
<td>Yes</td>
<td>Not done</td>
<td>297</td>
</tr>
<tr>
<td>PrP Δ114-121</td>
<td>No</td>
<td>Not done</td>
<td>No</td>
<td>40, 225</td>
</tr>
<tr>
<td>PrP 231#</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>108</td>
</tr>
<tr>
<td>PrP 144#</td>
<td>Short-lived protein</td>
<td>Yes</td>
<td>No</td>
<td>360</td>
</tr>
<tr>
<td>PrP PG 14</td>
<td>Cerebellar disorder</td>
<td>No</td>
<td>Yes</td>
<td>109</td>
</tr>
</tbody>
</table>

The left column denotes the individual mutants. The column named “phenotype” indicates presence or absence of phenotypic abnormalities in transgenic mice when expressed on a PrP-deficient genetic background; when the phenotype is rescued by endogenous PrP is indicated in the next column. The column named “prion propagation” describes the susceptibility of transgenic mice or transfected cell lines to prions. #Stop codon. *E. Flechsig, I. Hegyi, A. Aguzzi, and C. Weissman, unpublished results.
A. Alteration of PrP<sup>C</sup> Mediated Signaling

Although it is unlikely that the toxic activity of PrP<sup>Sc</sup> results primarily from loss of PrP<sup>C</sup> function, neurons derived from Prnp-deficient mice were originally reported to be more susceptible to the induction of apoptosis by serum deprivation. This effect could be rescued by expression of either B-cell lymphoma protein 2 (BCL2) or PrP<sup>C</sup> (286). Furthermore, it was shown that PrP<sup>C</sup> exerts its cytoprotective function by decreasing the rate of apoptosis induced by particular apoptotic stimuli such as Bax overexpression or tumor necrosis factor (TNF)-α. Bax overexpression normally induces apoptosis in human neuronal cells, but coexpression of Bax with wild-type PrP<sup>C</sup>, but not of PrP lacking the octarepeats, could reverse this effect (60). Therefore, PrP<sup>C</sup> can be neuroprotective against various insults, suggesting that its conversion to PrP<sup>Sc</sup> could abrogate this function and induce neurodegeneration.

A second possibility is that the binding of PrP<sup>Sc</sup> to PrP<sup>C</sup> triggers a signal transduction pathway leading to neuronal damage. This hypothesis is supported by a report that injection of antibodies that recognize and cross-link PrP<sup>C</sup> in the mouse hippocampus results in rapid and extensive neuronal apoptosis (469), although in the intervening 5 years since this publication, no further insights have come forth to explain this phenomenon.

B. PrP<sup>C</sup> Mislocalization

Abnormal topology or altered trafficking of PrP<sup>C</sup> has also been proposed to underlie PrP-related neuronal toxicity. After processing in the endoplasmic reticulum (ER) and Golgi apparatus, where it is glycosylated, mature PrP<sup>C</sup> is attached to the cell surface by its GPI anchor at its COOH terminus. PrP<sup>C</sup> can adopt at least two transmembrane topologies in the ER, CtmPrP and NtmPrP, which have either their COOH or NH<sub>2</sub> terminus in the ER lumen, respectively. It has been suggested that CtmPrP and NtmPrP normally comprise only a small portion of cellular PrP<sup>C</sup>, and an excess of CtmPrP induces neurodegeneration (204, 205). Some PrP mutations favor CtmPrP formation and are associated with a disease state characterized by low levels of PrP<sup>Sc</sup> accumulation. Thus the propensity for the formation of the CtmPrP and NtmPrP intermediates might lead to neurotoxicity in the absence of PrP<sup>Sc</sup> formation (Fig. 6).

Another suggested mechanism of PrP<sup>C</sup>-mediated neurotoxicity is aberrant PrP<sup>C</sup> trafficking resulting in the toxic accumulation of PrP<sup>C</sup> in the cytoplasm (Fig. 6). Here, it is important to consider the mechanisms controlling protein synthesis and degradation. The fidelity of protein synthesis is ensured by quality control within the ER; misfolded proteins undergo retrograde transport to the cytosol, become polyubiquitinated, and are degraded by the proteasome through a process called ER-associated degradation (ERAD). Both wild-type and misfolded forms of PrP<sup>C</sup> undergo ERAD (313, 529). It has been shown in vitro that the transfer of newly synthesized PrP<sup>C</sup> to the cytoplasm by ERAD, and its cytosolic accumulation through the pharmacological inhibition of the proteasome, leads to PrP<sup>Sc</sup>-like formation and neurotoxicity (312). Furthermore, the artificial expression of PrP in the cytosol in transgenic animals expressing a construct lacking ER-targeting and GPI-anchoring signals causes severe neurodegeneration (314).

Recently, Rane et al. (417) have described a possible mechanism to explain the accumulation of cy(PrP) during TSE neurodegeneration. Chronic ER stress, produced by PrP<sup>Sc</sup> accumulation, leads to persistent activation of a "preemptive" quality control system (pQC) that aborts ER translocation of PrP, thereby facilitating proteasome-mediated degradation of PrP in the cytosol. This pathway constitutes a defense mechanism to prevent nascent protein entry into the ER lumen during conditions of com-
promised ER function. However, under chronic ER stress conditions, the proteasome may become overwhelmed, resulting in PrP accumulation in the cytosol. According to the study of Rane et al. (417), even a modest increase in PrP routing to pQC for prolonged periods of time causes clinical and histological neurodegenerative changes reminiscent, in some aspects, of those observed in prion diseases.

Although ER stress in prion diseases is well documented (217), the relevance or even the existence of cy(PrP) continues to be rather controversial (142, 152). Furthermore, the transgenic mice with increased PrP translocation to the pQC pathway reported by Rane et al. (417) showed a relatively mild neurodegenerative phenotype that resembles only a subset of TSE pathology, and other cellular pathways may also be contributing to prion-induced neurodegeneration (472). A role for dysfunction of the ubiquitin-proteasome system (UPS) in the pathogenesis of prion disease was also suggested: neuronal propagation of prions in the presence of mild proteasome impairment triggered a neurotoxic mechanism involving the intracellular formation of cytosolic PrPSc aggregates that, in turn, activated caspase-dependent neuronal apoptosis. A similar effect was also seen in vivo in brains of prion-infected mice (285). In a follow-up study, the same group reported that disease-associated prion protein specifically inhibited the proteolytic H9252-subunits of the 26S proteasome. Upon challenge with recombinant prion and other amyloidogenic proteins, only the prion protein in a nonnative β-sheet conformation inhibited the 26S proteasome at stoichiometric concentrations. Furthermore, there...
appears to be a direct relationship between prion neuropathology and impairment of the UPS in prion-infected UPS-reporter mice (284). Together, these data suggest a possible mechanism of intracellular neurotoxicity mediated by misfolded prion protein.

C. PrP-Derived Oligomeric Species

The presence of highly organized and stable fibrillar deposits in the brain of patients and animals suffering from prion diseases initially led to the reasonable conjecture that this material is a causative agent of neurodegeneration. However, the most highly infective form of the mammalian prion protein is an oligomeric species of ~20 molecules, and such small aggregates are the most effective initiators of transmissible spongiform encephalopathies (464).

More recent findings from other disorders associated with protein misfolding and aggregation, such as AD and Parkinson’s disease, have raised the possibility that precursors to amyloid fibrils, particularly including low-molecular-weight oligomers and/or structured protofibrils, may represent the most toxic pathogenic species. In this respect, it is certainly plausible to speculate that the mechanism leading to neurotoxicity in prion disease could be similar to that of other disorders associated with protein misfolding.

In AD, the severity of cognitive impairment correlates with the levels of low-molecular-weight species of Aβ, rather than with the amyloid burden (202, 451). In addition, transgenic mice (290, 352), as well as Drosophila expressing Aβ1–42 and Aβ1–40 (127, 236), show deficits in synaptic plasticity before significant accumulation of amyloid. The existence of a toxic oligomeric intermediate is further supported by evidence that the aggressive “arctic” (E693G) mutation of the amyloid β-precursor protein, associated with heritable early-onset manifestation of AD, has been found in vitro to enhance protofibril, but not fibril, formation (364).

The toxicity of early aggregates has also been shown for Parkinson’s disease. In patients harboring mutations associated with juvenile Parkinson’s disease or early-onset forms of Parkinsonism, the loss of nigral and locus coeruleus neurons occurs in the absence of Lewy bodies (intracellular fibrillar deposits) (262). In transgenic fly or rat models of Parkinson’s disease, neuronal degeneration is not associated with the formation of detectable intracellular deposits (32, 304). Furthermore, transgenic mice exhibit substantial motor deficiencies and loss of dopaminergic neurons in the presence of nonfibrillar α-synuclein deposits in various regions of the brain (330).

Toxicity of smaller aggregates appears not to be restricted to the brain. For example, transthyretin (TTR) is an amyloidogenic protein implicated in systemic senile amyloidosis (517) and familial amyloidotic polyneuropathy (124). The primary toxic species in TTR-associated diseases appear to be low-molecular-weight oligomers that do not stain with Congo red (470, 477).

The toxicity of aggregated “preamyloid” proteins appears to be a fairly general phenomenon. For example, the prefibrillar forms of the non-disease-related HypF-N from E. coli, the SH3 domain from bovine phosphatidylinositol 3-kinase, lysozyme from horse, and apomyoglobin from sperm whale are all highly toxic to cultured fibroblasts and neurons, whereas the monomeric native states and the amyloid-like fibrils (formed in vitro) displayed very little, if any, toxicity (81, 320, 466). The finding that polyclonal antisera can bind to protofibrillar species from different sources, but not to their corresponding monomeric or fibrillar states, suggests that soluble amyloid oligomers have some important common structural elements (252).

With the assumption that oligomeric intermediates of a particular structure are common initiators of neurotoxicity observed in prion disease and in other protein misfolding disorders, the crucial question remains why these protein species are toxic to cells. The conversion of a protein from its native state into an oligomeric form invariably generates a wide distribution of misfolded species which exposes an array of groups that are normally masked in globular proteins. The improper interactions between oligomers and cellular components, such as membranes, small metabolites, proteins, or other macromolecules, may conceivably lead to the malfunctioning of crucial aspects of the cellular machinery. Depending on the cell type involved, the result could be impairment of axonal transport, oxidative stress, sequestration of essential proteins, or a combination of disparate factors, ultimately leading to apoptosis or other forms of cell death. Small aggregates have a higher proportion of residues on their surfaces than larger aggregates, including mature amyloid fibrils, and therefore may be likely, in general, to have a higher relative toxicity (110).

Elucidation of the mechanism of tissue damage by amyloid fibril proteins is undoubtedly an important issue in the development of therapeutic approaches, although the optimum strategy may be to prevent aggregation or even production of the amyloidogenic protein before it can generate any potentially damaging intermediates.

IV. CLINICAL AND PATHOLOGICAL ASPECTS OF PRION DISEASES

A. Scrapie

Even though scrapie was recognized as a distinct disorder of sheep with respect to its clinical manifestations as early as 1738, the disease remained an enigma,
even with respect to its pathology, for more than two centuries (382). Some veterinarians thought that scrapie was a disease of muscle caused by parasites, whilst others thought that it was a dystrophic process (311). An investigation into the etiology of scrapie followed the vaccination of sheep for louping-ill virus with formalin-treated extracts of ovine lymphoid tissue unknowingly contaminated with scrapie prions (194). Two years later, more than 1,500 sheep developed scrapie from this vaccine.

In 1998, aberrant cases of sheep scrapie were described in Norway, and the strain was newly classified as Nor98 (46). Active European Union surveillance later revealed additional cases of atypical scrapie in several other countries (87, 147); in fact, atypical scrapie appears to be the rule rather than the exception in some geographical areas.

Scrapie of sheep and goats seems to be readily communicable within flocks, but the mechanism of the natural spread of scrapie among sheep is puzzling. The finding that chronic inflammation can alter the tropism of prion infectivity to organs hitherto believed prion free, like mammary glands, raised concerns about scrapie transmission through milk (210, 300).

Polymorphisms at codons 136 and 171 of the Prnp gene in sheep that produce amino acid substitutions have been studied with respect to the occurrence of scrapie in sheep (113).

B. Bovine Spongiform Encephalopathy

In 1986, an epidemic of a previously unknown disease appeared in cattle in Great Britain (514): bovine spongiform encephalopathy (BSE) or “mad cow” disease. BSE was found to be a prion disease upon the discovery of protease-resistant PrP in the brains of ill cattle (226). On the basis mainly of epidemiological evidence, it has been proposed that BSE represents a massive common source epidemic that has caused more than 190,000 cases to date (http://www.oie.int/) (Fig. 7A). In Britain, cattle, particularly dairy cows, were routinely fed meat and bone meal as a nutritional supplement (521). Since 1988, the practice of using dietary protein supplements for domestic animals derived from rendered sheep or cattle offal has been forbidden in the United Kingdom.

In 1992, the BSE epidemic reached a peak, with over 35,000 cattle afflicted. In 1993, fewer than 32,000 cattle were diagnosed with BSE, and in 1994, the number was ~22,000. By 2003, BSE had become a rare disease in British and European cattle, but regrettably (and quite inexplicably) it still has not completely disappeared.

Brain extracts from BSE cattle have transmitted disease to mice, cattle, sheep, and pigs after intracerebral inoculation (75, 130, 131, 165). Of particular importance to the BSE epidemic is the transmission of BSE to the non-human primate marmoset after intracerebral inoculation followed by a prolonged incubation period (34).

C. Chronic Wasting Disease of Deer and Elk

Chronic wasting disease of deer and elk (CWD) was initially reported in 1980 as a TSE in captive research deer in Colorado and Wyoming. The disease origin still remains completely obscure (525). Since 1980, cases of CWD in free-ranging mule deer (Odocoileus hemionus), whitetailed deer (O. virginanus), and Rocky Mountain elk (Cervus elaphus nelsoni) have been detected in the same region of Colorado and Wyoming. Recently, increased surveillance efforts across the United States and Canada have startlingly revealed CWD in adjacent states (Nebraska, New Mexico, and Utah) but also distant (Wisconsin, Illinois, Canada) from this original endemic region. Thus far, CWD is only known to occur in North America and in South Korea. That said, international testing for CWD has been minimal with the exception of a CWD surveillance program in Germany (458).

CWD is believed to be horizontally transmitted among cervids with high efficiency (348). Exposure through grazing in areas contaminated by prion-infected secretions, excretions (saliva, urine, feces), tissues (placenta), or decomposed carcasses is most likely the major source of spreading. Insightful experimental studies have recently revealed two key findings: 1) saliva from CWD-infected deer can transmit disease (332), and 2) CWD-infected carcasses allowed to decay naturally in confined pastures can lead to CWD infections in captive deer (349). Additionally, abundant CWD prion accumulation within lymphoid tissues may also lead to CWD prion buildup in nonlymphoid organs with lymphoid follicles, as was recently demonstrated in kidney, potentially shifting shedding routes (200). It is unknown whether other types of inflammation, such as the granulomatous inflammation in the intestine seen in Johne’s disease (Mycobacterium avium subsp. paratuberculosis); affects ruminants, including deer and elk), or parasitic inflammation could lead to or perhaps increase prion excretion by fecal routes.

The potential for CWD transmission to other species is clearly an area of great concern. Wild predators and scavengers are presumably feeding on CWD-infected carcasses. Skeletal muscle has been shown to harbor CWD prion infectivity (29), indicating that other species are almost certainly being exposed to CWD through feeding. However, CWD has not been successfully transmitted by oral inoculation to species outside of the cervid family, suggesting a strong species barrier for heterologous PrP conversion (458). Human susceptibility to CWD is still unclear, although we can be somewhat reassured in that there have been no large-scale outbreaks of human TSE...
cases in Colorado and Wyoming, where CWD has existed for decades (334), and there is no doubt that people have been exposed to CWD through venison consumption, particularly in light of recent data showing CWD prion accumulation in muscle.

Other indirect studies of human susceptibility to CWD also suggest that the risk is low. In biochemical conversion studies, the efficiency of CWD in converting recombinant human PrP into amyloid fibrils was low, yet similar to that of both BSE and scrapie (419). Accordingly, it has also been reported that human PrP-overexpressing mice were not susceptible to nine CWD isolates from mule deer, white-tailed deer, and elk (490), whereas the elk PrP expressing mice developed disease after only 118–142 days postinoculation (275). However, mice have a limited life span, and further passages may be necessary to detect low levels of prion infectivity that may be present subclinically.

**D. Human Prion Diseases**

Rapidly progressive dementia, myoclonus, visual or cerebellar impairment, pyramidal/extrapyramidal signs, and akinetic mutism clinically characterize fatal neurological diseases caused by prions. The human prion diseases are classified as infectious, inherited or sporadic disorders, depending on the clinical, genetic, and neuropathological findings.

Prion disease, not only in inherited forms but also in iatrogenic and sporadic forms, is influenced by an amino...
acid polymorphism resulting in methionine (M)→valine (V) substitution at PrP codon 129 (375). The finding that homozygosity at codon 129 predisposes individuals to CJD supports the theory that protective polymorphisms in the human prion gene have been heavily selected for, possibly due to evolutionary pressure from cannibalism-propagated prions (341). However, several publications have pointed out that these conclusions may be incorrect (449, 468). It was recently described that incubation periods of Kuru could be as long as 56 years. PRNP analysis showed that most of those patients with Kuru were heterozygous at polymorphic codon 129, a genotype associated with extended incubation periods and resistance to prion disease (119).

A second polymorphism resulting in glutamate (E)→lysine (K) substitution at codon 219 has been identified in the Japanese population, and it has also been reported to have an effect on the susceptibility of individuals to prion disease (264). Recently, additional candidate loci have been identified by a genome-wide association study of the risk of vCJD (340) which justifies functional analyses of these biological pathways in prion disease.

1. Iatrogenic prion diseases

Iatrogenic CJD is accidentally transmitted during the course of medical or surgical procedures. The first documented case of iatrogenic prion transmission occurred in 1974 and was caused by corneal transplantation of a graft derived from a patient suffering from sCJD (143). Iatrogenic CJD has also been transmitted by neurosurgery using contaminated EEG electrode implantations (50) and surgical operations using contaminated instruments or apparatuses (129, 331). Moreover, the transmission of CJD from contaminated human growth hormone (HGH) preparations derived from human pituitaries was discovered when fatal cerebellar disorders with dementia occurred in >80 patients ranging in age from 10 to over 60 yr (73, 82, 160). Five cases of CJD have occurred in women receiving human pituitary gonadotropin (203). Although one case of spontaneous CJD in a 20-yr-old woman has been reported (377), CJD in people under 40 yr of age is extremely rare.

Molecular genetic studies have revealed that most patients developing iatrogenic CJD after receiving pituitary-derived HGH are homozygous for either Met or Val at codon 129 of the PRNP gene (72, 117).

2. Inherited prion diseases

Familial TSEs are associated with an autosomal dominant PRNP gene alteration. There is variability in the clinical and pathological findings, the age of onset, and the duration depending on the particular PRNP mutation involved (Table 4). Familial TSEs account for 10–20% of all TSE cases in humans and include fCJD, GSS, and FFI. Clinically, fCJD is characterized by rapidly progressive dementia, with myoclonus and pseudoperiodic discharges on electroencephalogram; GSS by slow progression of ataxia followed by later onset dementia, and FFI from refractory insomnia, hallucinations, dysautonomia, and motor signs. The clinical categories may be seen as phenotypic extremes of a continuum, because in reality the syndromes overlap considerably (339).

fCJD have been associated with point mutations affecting the region between the second and the third helix of the COOH terminus, insertions in the octarepeat region in the NH2 terminus, and even a premature termination codon at position 145. The inheritance is, in all cases, autosomal dominant, often with very high penetrance. In

<table>
<thead>
<tr>
<th>Presentation</th>
<th>Mutation in PRNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatal familial insomnia phenotype</td>
<td>D178N-120M</td>
</tr>
<tr>
<td>Vascular amyloid deposition</td>
<td>Y145STOP</td>
</tr>
<tr>
<td>Proven but unclassified prion disease</td>
<td>H187R, 216 bpi</td>
</tr>
<tr>
<td>Polymorphisms, established influence on phenotype</td>
<td>M120V</td>
</tr>
<tr>
<td>Polymorphisms, suggested influence on phenotype</td>
<td>N171S, E219K</td>
</tr>
</tbody>
</table>

The wild-type human PRNP gene contains 5 octarepeats [P(Q/H)GGG(G/–) WGG] from codons 51–91. Deletion of a single octarepeat at codon 81 or 82 is not associated with prion disease (289, 413, 503); whether this deletion alters the phenotypic characteristics of a prion disease is unknown. There are common polymorphisms at codons 117 (Ala→Ala) and 129 (Met→Val); homozygosity for Met or Val at codon 129 seems to increase susceptibility to sporadic CJD (378). Octarepeat insertions of 16, 32, 40, 48, 56, 64, and 72 amino acids at codons 67, 75, or 83 are designated as bpi (base pair insertion); bpd indicates base pair deletion. Point mutations are designated by the wild-type amino acid preceding the codon number and the mutant residue follows, e.g., P102L. These point mutations segregate with the inherited prion diseases, and significant genetic linkage has been demonstrated where sufficient specimens from family members are available. The single letter code for amino acids is as follows: A, Ala; D, Asp; E, Glu; F, Phe; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

Physiol Rev • VOL 89 • OCTOBER 2009 • www.prv.org
all fCJD, the clinicopathological disease phenotype varies depending on the actual mutation, as well as on polymorphisms at codon 129, and most likely on a plethora of yet unidentified modifiers and cofactors (280).

The first reports of GSS originate from 1928 and 1936 in an Austrian family (179, 199). GSS syndrome is a rare, inherited autosomal dominant disease that is associated with mutations in the PRNP gene (the most common are at codons 102 and 198). GSS syndrome is characterized by chronic progressive ataxia, terminal dementia, a long clinical duration (2–10 yr), and multicentric amyloid plaques that can be visualized by antibodies directed against the prion protein.

FFI is characterized by sleep disturbances as well as vegetative and focal neurological signs as a result of thalamic lesions. The clinical phenotype depends on the D178N point mutation of the PRNP gene coupled with a methionine at codon 129 (175).

3. Sporadic Creutzfeldt-Jakob disease

Approximately 85% of all human prion diseases are sporadic forms of CJD. For sCJD, there is no association with a mutant PRNP allele, nor is there any epidemiological evidence for exposure to a TSE agent through contact with people or animals infected with TSEs.

sCJD cases are currently subclassified according to the methionine/valine polymorphism at codon 129 of the PRNP gene and the size and glycoform ratio of protease-resistant prion protein identified on western blot (type 1 or type 2) (174). Heterozygosity (Met/Val) at PrP codon 129 appears to be associated with a lower risk (378) and/or prolonged incubation time (119, 387).

The lack of routine laboratory testing for preclinical diagnosis makes the search for agent sources and other risk factors extremely difficult. At present, the means of acquisition of a TSE agent in these patients remains a mystery. So far, there is no evidence for spontaneous PrPSc formation in any animal or human TSE. In humans, the peak age incidence of sporadic CJD is 55–60 years. However, if spontaneous misfolding were the primary event, one might expect a continuously increasing incidence with age because more time would allow more opportunity for rare misfolding events.

4. Variant Creutzfeldt-Jakob disease

The most recently identified form of CJD in humans, new variant CJD (vCJD), was first described in 1996 and has been linked to BSE (522).

At the time of this writing, vCJD has killed ~200 individual victims worldwide (http://www.cjd.ed.ac.uk/). Most of the affected individuals lived in the United Kingdom and France. Fortunately, in the United Kingdom, the incidence appears to be decreasing since 2001 to five diagnosed cases yearly in 2006 and 2007 and one case in 2008. In contrast, in France, the number of probable and definite cases of vCJD increased from zero to three diagnosed cases per year in 1996–2004 to six per year in 2005 and 2006. In 2007, the number of cases was back to three again, and no cases were reported in 2008 (http://www.invs.sante.fr/publications/mcj/donnees_mcj.html) (Fig. 7B).

vCJD represents a distinct clinicopathological entity that is characterized at onset by psychiatric abnormalities, sensory symptoms, and ataxia and eventually leads to dementia along with other features usually observed in sporadic CJD. What distinguishes vCJD from sporadic cases is that the age of patients is abnormally low (vCJD: 19–39 yr; sporadic CJD: 55–70 yr) and duration of the illness is rather long (vCJD: 7.5–22 mo; sporadic CJD: 2.5–6.5 mo). Moreover, vCJD displays a distinct pathology within the brain characterized by abundant “florid plaques,” decorated by a daisylike pattern of vacuolation. Florid plaques are spherical conglomerates of fibrillary birefringent material that stains positive with “amyloidotropic” dyes such as Congo red.

What is the evidence that the agent causing vCJD may be identical to that of BSE when transmitted to humans? None of the arguments to date are conclusive. Considerable effort has been devoted to characterizing the “strain properties” of the agent that affects cows and humans. Since the molecular substrate underlying the phenomenon of prion strains is not known, strain-typing of prions must rely on surrogate markers.

Two such markers have been particularly useful. One is the distribution of neuronal vacuoles in the brains of affected animals. BSE prions (extracted from the brains of affected cows) and vCJD prions derived from the brains of British patients produce the same lesion profile when transmitted to panels of susceptible mice (3, 22, 80). The second marker for strain typing of prions comes from analyzing the biochemical properties of disease-associated PrP recovered from the brains of cattle and humans. These traits were again found to be indistinguishable between BSE and human vCJD prions (118, 219). A third line of evidence linking BSE to human vCJD comes from the epidemiology of the disease. To date, the majority of vCJD cases have arisen in the United Kingdom, with relatively few cases in other countries including Ireland, France (106), and Italy. With the assumption that the quality of the epidemiological surveillance is similar in these countries and in the rest of Europe (which has not reported cases of vCJD), the unavoidable conclusion is that the incidence of vCJD correlates with the prevalence of BSE.

Convincing as all of these arguments may seem, each is phenomenological rather than causal. Given that a large fraction of the European population may have been exposed to BSE prions, yet only a minute fraction actually developed vCJD, there can be hardly any doubt that additional genetic modifiers exist, other than PRNP polymorphisms. So what will the numbers of vCJD victims be.
in the future? Although many mathematical models have been generated (180, 181), the number of cases is still too small to predict future developments with any certainty. A 30-yr mean incubation time of BSE/vCJD in humans is not entirely implausible, and therefore, some authors have predicted a multiphasic BSE endemic with a second increase in the incidence of vCJD affecting people heterozygous at codon 129 (119). Others, these authors included, regard the incidence of vCJD as subsiding (28). It is important to note, however, that the above considerations apply primarily to the epidemiology of primary transmission from cows to humans. Although by now a pool of preclinically infected humans may have been built, human-to-human transmission may present with characteristics very different from those of primary cow-to-human transmission, including enhanced virulence, shortened incubation times, disregard of allelic PRNP polymorphisms (129MM, MV or VV), and heterodox modes of infection including blood-borne transmission. If we account for the time it will take to eradicate these secondary transmissions in the population, vCJD is not likely to disappear entirely in the coming four decades. Four cases (three definite and one presumed) of vCJD transmission by blood transfusions have been reported in the United Kingdom (303, 387, 528) (see also http://www.cjd.ed.ac.uk/TMER/TMER.htm). Most worringly, the Health Protection Agency of the United Kingdom has reported a case of a hemophiliac patient who has most likely acquired vCJD prions through factor VIII preparation derived from plasma donated by a “preclinical” vCJD patient (http://www.hpa.org.uk).

The fact that preclinical infected individuals can transmit vCJD underscores the important medical need for sensitive diagnostic tools, which could be used for screening blood units prior to transfusion.

V. PERIPHERAL ROUTES OF PRION INFECTIVITY

The fastest and most efficient method of inducing spongiform encephalopathy in the laboratory is intracerebral inoculation with brain homogenate. Inoculation of 10^6 ID_{50} infectious units (defined as the amount of infectivity that will induce TSE with 50% likelihood in a given host) will yield disease in approximately half a year; a remarkably strict inverse relationship can be observed between the logarithm of the inoculated dose and the incubation time (406).

However, the above situation does not correspond to what typically happens in the field. There, acquisition of prion infectivity through any of several peripheral routes is the rule. Prion infections can be induced by oral challenge (159) and occur naturally as a result of food-borne contamination, as has been shown for Kuru, transmissible mink encephalopathy, BSE, and vCJD (27, 119, 222, 328).

However, prion diseases can also be initiated by intravenous and intraperitoneal injection (258) as well as from the eye by conjunctival instillation (445), corneal grafts (143), and intraocular injection (161). Two routes of infection have suggested for a long time that immune cells might be of importance for this phase of prion pathogenesis: oral challenge and administration by scarification (7). Therefore, these routes will be discussed in some detail in the following paragraphs.

A. Pathway of Orally Administered Prions

After oral infection, an early increase in prion infectivity is observed in the distal ileum. Within 2 wk of prion ingestion, prions appear to enter peripheral nerves and proceed by invasion of the dorsal motor nucleus of the vagus in the brain, as has been shown in mouse and hamster scrapie studies (336). Gastrointestinal infections caused by viruses, bacteria, and parasites, as well as idiopathic inflammatory diseases, could alter the dynamics of prion entry and systemic spread. It has been recently reported that moderate colitis, caused by an attenuated Salmonella strain, more than doubles the susceptibility of mice to oral prion infection and modestly accelerates the development of disease after prion challenge (459).

How do prions reach the peripheral nerves after having entered the gastrointestinal tract? Following exposure, many acquired TSE agents accumulate in lymphoid tissue, in the case of oral exposure, the gut-associated lymphatic tissue (GALT), which includes Peyer’s patches (PPs) and membranous epithelial cells (M cells), and mesenteric lymph nodes. Following experimental intra-gastric or oral exposure of rodents to scrapie, or non-human primates and sheep to BSE, protease-resistant prion protein accumulates rapidly in the GALT, PPs, and ganglia of the enteric nervous system (41, 57, 156, 259), long before they are detected in the CNS.

Recruitment of activated B lymphocytes to PPs requires αββ integrin as an essential homing receptor; PPs of mice that lack β7 integrin are normal in number, but are atrophic and almost entirely devoid of B cells (507). Therefore, it seemed interesting to investigate the susceptibility to orally administered prions of β7-deficient mice. Surprisingly, minimal infectious dose and disease incubation after oral exposure to logarithmic dilutions of prion inoculum were similar in β7-deficient and wild-type mice (399). Despite their atrophy, PPs of both β7-knockout and wild-type mice contained 3–4 log ID_{50}/g prion infectivity at 125 or more days after challenge.

Why does reduced mucosal lymphocyte trafficking not impair, as expected, the susceptibility to orally initiated prion disease? One possible reason may relate to the fact that, despite marked reduction of B cells, M cells...
were still present in βT–/– mice. In contrast, mice deficient in both TNF and lympho toxin (LT)-α (TNF-α–/– × LTα–/–) or in lymphocytes (RAG-1–/–, μMT), in which Peyer’s patches are reduced in number, were highly resistant to oral challenge, and their intestines were virtually devoid of prion infectivity at all times after challenge. Therefore, lymphoreticular requirements for enteric and for intraperitoneal uptake of prions differ from each other in that susceptibility to prion infection following oral challenge correlates with the number of Peyer’s patches, but is independent of the number of intestinal mucosa-associated lymphocytes (189, 399).

B. Transepithelial Enteric Passage of Prions

The requirements for transepithelial passage of prions are of obvious interest to prion pathogenesis. M cells are key sites of antigen sampling for the mucosal-associated lymphoid system (MALT) and have been recognized as major ports of entry for enteric pathogens in the gut via transepithelial transport (363).

Interestingly, maturation of M cells is dependent on signals transmitted by intraepithelial B lymphocytes. Efficient in vitro systems have been developed, in which epithelial cells can be instructed to undergo differentiation to cells that resemble M cells, as judged by morphological and functional-physiological criteria (254). This led to the proposal that M cells could be a site of prion entry, a hypothesis that has been substantiated in coculture models (212). Although in vivo confirmation is required, M cells are plausible sites for the transepithelial transport of scrapie across the intestinal epithelium. However, the transport across the intestinal epithelium might not rely entirely on M-cell-mediated transcytosis: several reports have also indicated that prion transport may occur through enterocytes (372) and could be mediated by a ferritin-dependent mechanism (351) or via laminin-receptor binding and endocytosis (357).

Dendritic cells (DCs), localized within the PPs beneath the M-cell intraepithelial pocket, are ideally situated to acquire antigens that have been transcytosed from the gut lumen and deliver them to peripheral nerves in lymphoid organs, thereby facilitating the process of neuroinvasion (232, 317). The contribution of CD11c+ DCs has been investigated in vivo using CD11c-diphtheria toxin receptor-transgenic mice in which CD11c+ DCs can be specifically and transiently depleted (418). With the use of two distinct scrapie agent strains (ME7 and 139A), depletion of CD11c+ DCs in the GALT and spleen before oral exposure blocked early prion accumulation in these tissues. These data suggest that migratory CD11c+ DCs play a role in the translocation of the scrapie agent from the gut lumen to the GALT, from which neuroinvasion proper may then ensue.

C. Uptake of Prion Through the Skin

Even less well understood, yet possibly much more efficient than oral administration of prions, is challenge by scarification. Removal of the most superficial layers of the skin, and subsequent administration of prions, has been known for long time to be a highly efficacious method of inducing prion disease (90, 497). It is conceivable that dendritic cells in the skin may become loaded with the infectious agent by this method, and in fact, dendritic cells have been implicated as potential vectors of prions in oral (232) and in hematogeneous spread (31) of the agent.

Following inoculation with scrapie by skin scarification, replication in the spleen and subsequent neuroinvasion is critically dependent on mature follicular dendritic cells (FDCs) (353). However, which lymphoid tissues are crucial for TSE pathogenesis following inoculation via the skin was not known until mice were created that lacked the draining inguinal lymph node (ILN), but had functional FDCs in remaining lymphoid tissues such as the spleen. In these mice, inoculated with the scrapie agent by skin scarification, the disease susceptibility was dramatically reduced demonstrating that, following inoculation by skin scarification, scrapie agent accumulation in FDCs in the draining lymph node is critical for the efficient transmission of disease to the brain (188).

It is equally possible (and maybe more probable), however, that scarification induces direct neural entry of prions into skin nerve terminals. This latter hypothesis has not yet been experimentally tested, but it would help explain the remarkable speed with which CNS pathogenesis ensues following inoculation by this route: dermal inoculation of killed mice yields typical latency periods of the disease that are similar to those obtained by intracerebral inoculation.

Rapid neuroinvasion was reported following intralingual inoculation and may also exploit a direct intraneural pathway (38).

VI. IMMUNOLOGICAL ASPECTS OF PRION DISEASE

In a discussion of the immunological aspects of prion diseases, it is important to distinguish between an early extraneural phase and a late CNS phase of pathogenesis. There is no doubt that components of the immune system participate in pathogenesis in both compartments, but their respective functional implications are strikingly different.

A. The Lymphoid System in Prion Disease

More than 40 years ago it was discovered using bioassays that lymphoreticular organs contain prion infectiv-
ity (162, 385). For scrapie in sheep and goats, infectivity was assessed in a broad range of tissues using experimentally inoculated goats as donors and uninfected goats as recipients (384), and both spleen and lymph nodes were shown to transmit scrapie.

In the decades following these discoveries, our understanding of the extraneural immune phase advanced considerably, and an impressive collection of data has accrued that provides clues about peripheral prion pathogenesis. The normal prion protein was found to be consistently expressed, albeit at moderate levels, in circulating lymphocytes (91). Subsequently it was clearly shown in a wealth of experimental paradigms that innate or acquired deficiency of lymphocytes would impair peripheral prion pathogenesis, whereas no aspects of pathogenesis were affected by the presence or absence of lymphocytes upon direct transmission of prions to the CNS (263, 291). Klein and colleagues (4, 267) pinpointed the lymphocyte requirement to B cells. Interestingly, it was shown that only B cells within the secondary lymphoid organs accumulated prions in a PrPC-dependent fashion, while circulating lymphocytes contained no detectable infectivity (415). A series of bone marrow transfer experiments demonstrated that peripheral prion pathogenesis required the physical presence of B cells, but the expression of the cellular prion protein by these cells is dispensable for pathogenesis upon intraperitoneal infection in the mouse scrapie model (11). Adoptive transfer of Prnp<sup>+/+</sup> bone marrow into Prnp<sup>–/–</sup>-recipient mice did not suffice to restore infectibility of Prnp-expressing brain grafts, indicating that neuroinvasion was still defective (53), yet intraperitoneal infection occurred efficiently even in B-cell-deficient hosts that had been engrafted with B cells from Prnp knockout mice (268, 354).

Although the requirement for B cells appears to be very stringent in most instances investigated, it has emerged that not all strains of prions induce identical patterns of peripheral pathogenesis, even when propagated in the same, isogenic strain of host organism (452). Another interesting discrepancy that remains to be addressed concerns the actual nature of the cells that replicate and accumulate prions in lymphoid organs. In the RML paradigm, four series of rigorously controlled experiments over 5 years (18, 243, 269, 400) unambiguously reproduced the original observation by Blättler et al. (53) that transfer of Prnp<sup>+/+</sup> bone marrow cells (or fetal liver cells) to Prnp<sup>–/–</sup> mice restored accumulation and replication of prions in spleen. In contrast, Brown et al. (70) reported a diametrically opposite outcome of similar experiments when mice were inoculated with prions of the ME7 strain. This discrepancy may identify yet another significant difference in the cellular tropism of different prion strains.

It is important to note that the results of the Blättler study do not necessarily indicate that lymphocytes are the primary splenic repository of prions: in fact, other experiments suggest that this is quite unlikely. Instead, bone marrow transplantation may 1) transfer an ill-defined population with the capability to replenish splenic stroma and to replicate prions, or less probably, 2) donor-derived PrPC expressing hematopoietic cells may confer prion replication capability to recipient stroma by virtue of "GPI painting," i.e., the posttranslational cell-to-cell transfer of glycosphingositol linked extracellular membrane proteins (277). Some evidence might be accrued for either possibility: stromal splenic follicular dendritic cells have been described by some authors to possibly derive from hematopoietic precursors, particularly when donors and recipients were young (250, 486). Conversely, instances have been described in which transfer of GPI-linked proteins occurs in vivo with surprisingly high efficiency (278). GPI painting has been described specifically for the cellular prion protein (302).

While it has been known for a long time that specific strains of prions may preferentially affect specific subsets of neurons, the Blättler/Brown paradox may uncover an analogous phenomenon in peripheral prion pathogenesis. The latter question may be much more important than it appears at face value, since the molecular and cellular basis of peripheral tropism of prion strains is likely to be directly linked to the potential danger of BSE in sheep (77, 185, 249), as well the potential presence of vCJD prions in human blood (6).

1. Follicular dendritic cells and prion replication

As mentioned above, prion infectivity rises very rapidly (in a matter of days) in the spleens of intraperitoneally infected mice. Although B lymphocytes are crucial for neuroinvasion, all evidence indicates that most splenic prion infectivity resides in a "stromal" fraction. Instead, the apparent requirement for B lymphocytes in peripheral pathogenesis is more likely to be derived, at least in part, from indirect effects, including the provision of chemokines or cytokines to cells that efficiently replicate prions in peripheral regions of the host body (14).

Any potential profiteer from these B-lymphocyte-derived signals would be localized in close proximity to the B lymphocytes, be of stromal origin, and should also display PrPC on its cell surface. FDCs fulfill each of these criteria. FDCs support the formation and maintenance of the lymphoid microarchitecture by expressing homoeostatic chemokines and have a role in antigen trapping and capturing of immune complexes by Fcγ receptors. Identification of FDC-specific genes is extremely important and useful, to assess the contribution of FDCs to prion pathogenesis. Recently, the antigen FDC-M1 has been identified as Milk fat globule EGF factor 8 (Mfge8) (282).

Gene deletion experiments in mice have shown that signaling by both TNF and lymphotaxins is required for
generation and maintenance of FDCs (145, 276). LTs belong to a family of structurally related cytokines, the TNF superfamily (306), and are expressed primarily by B lymphocytes and activated T lymphocytes. LTs and LTβ are proinflammatory cytokines; ectopic expression of LTs in the liver, kidney, or pancreas leads to the generation of follicular structures resembling tertiary follicles (210, 283), which are very similar to activated lymphoid follicles found in the spleen or lymph nodes.

Membrane-bound LT-α/β (LT-α1/β2 or LT-α2/β1) heterotrimers signal through the LT-β receptor (LT-βR) (508), thereby activating a signaling pathway required for the development and maintenance of secondary lymphoid organs (318, 333).

A series of studies has been performed to define the effect of selective ablation of functional FDCs on the pathogenesis of scrapie in mice.

FDCs accumulate PrPSc following scrapie infection (263), and prion replication in the spleen was reported to depend on PrPSc-expressing FDCs, at least in the ME7 prion strain (70).

Inhibiting the LTβR pathway in mice and nonhuman primates, by treatment with LTβR-Ig, results in the disappearance or dedifferentiation of mature, functional FDCs (191, 192). In addition, treatment with LTβR-Ig was found to impair peripheral prion pathogenesis (316, 355). Treatment of mice with LTβR-Ig for 1 wk before exposure to a relatively low dose (intraperitoneally) of prions, followed by further treatment for 2 wk postinoculation, resulted in the complete protection of the mice from disease (15). This suggests that B cell-deficient μMT mice (266) may be resistant to intraperitoneal prion inoculation (267) because of impaired FDC maturation (268, 355).

However, additional experimentation appears to indicate that PrPSc-expressing hematopoietic cells are required in addition to FDCs for efficient lymphoreticular prion propagation (53, 243). This apparent discrepancy called for additional studies of the molecular requirements for prion replication competence in lymphoid stroma. Therefore, peripheral prion pathogenesis was studied in mice lacking TNF-α, LTα/β, or their receptors. After intracerebral inoculation, all treated mice developed clinical symptoms of scrapie with incubation times, attack rates, and histopathological characteristics similar to those of wild-type mice, indicating that TNF/LT signaling is not relevant to cerebral prion pathogenesis. Upon intraperitoneal prion challenge, mice defective in LT signaling (LTα−/−, LTβ−/−, LTβR−/−, or LTα−/−TNF-α−/−) proved virtually noninfectible with ≤5 log LD50 scrapie infectivity, and establishment of subclinical disease (166) was prevented. In contrast, TNFR1−/− mice were almost fully susceptible to all inoculum sizes, and TNF-α−/− mice showed dose-dependent susceptibility. TNFR2−/− mice had intact FDCs and germinal centers and were fully susceptible to scrapie. Unexpectedly, all examined lymph nodes of TNFR1−/− and TNF-α−/− mice had consistently high infectivity titers (400). Even inguinal lymph nodes, which are distant from the injection site and do not drain the peritoneum, contained infectivity titers equal to all other lymph nodes. Therefore, TNF deficiency prevents lymphoreticular prion accumulation in spleen but not in lymph nodes.

Why is susceptibility to peripheral prion challenge preserved in the absence of TNFR1 or TNF-α−/−, while deletion of LT signaling components confers high resistance to peripheral prion infection? After all, each of these defects (except TNFR2−/−) abolishes FDCs. For one thing, prion pathogenesis in the lymphoreticular system appears to be compartmentalized, with lymph nodes (rather than spleen) being important reservoirs of prion infectivity during disease. Second, prion replication appears to take place in lymph nodes even in the absence of mature FDCs; therefore, poorly defined immune cells or possibly stromal precursor cells (for example, for FDCs) might be capable of replicating the infectious agent.

In an unexpected turn of events, it was found that lymphotoxin-dependent prion replication can occur in inflammatory stromal cells that are distinct from FDCs (208). Granulomas, a frequent feature of chronic inflammation, express PrPSc and the LTβR, even though they lacked FDCs and did not display lymphoneogenesis. After intraperitoneal prion inoculation, granulomas accumulated prion infectivity. Bone marrow transfers between Prnp+/+ and Prnp−/− mice and administration of lymphotoxin signaling antagonists indicated that prion replication required radiocresistant PrPSc-expressing cells and LTβR signaling. The finding that a cell type other than mature FDCs is involved in prion replication and accumulation within lymph nodes may be relevant to the development of postexposure prophylaxis strategies.

2. A role for the complement cascade in prion accumulation

As FDCs interact with opsonized antigens through the CD21/CD35 complement receptors, a pertinent question is whether there is a role for the complement cascade in the pathology of prion diseases. Indeed, depletion of either one of the early complement factors (C1q, Bf/C2 and C3) or the complement receptor (CD21/CD35) significantly delays the onset of disease symptoms in mice injected with limiting doses of the scrapie strains RML and ME7 (269, 315). The relative importance of CD21/35 on hemopoietic and stromal cell types has been assessed through reciprocal reconstitution experiments by bone marrow transplantation. CD21/35 expression exclusively on FDCs in white pulp follicles resulted in prion titers, PrPSc retention, and disease kinetics and severity similar to those of wild-type mice. CD21/35 expression on hemopoietic cells also significantly affected these parameters,
but far less than FDC expression of CD21/35. Therefore, complement-mediated antigen trapping on FDCs is an important mechanism for lymphoid prion accumulation (530). The role of the classical complement cascade during peripheral prion infection certainly warrants further investigations, since complement is clearly an important determinant of pathogenesis yet its mechanics are not yet fully understood (14).

**B. Tropism of Prions for Inflammatory Foci**

Since proinflammatory cytokines and immune cells are involved in lymphoid prion replication, it is likely that chronic inflammatory conditions in nonlymphoid organs could affect the dynamics of prion distribution. Indeed, inclusion body myositis, an inflammatory disease of muscle, was reported to lead to the presence of large PrPSc deposits in muscle (281). Many chronic inflammatory conditions, some of which are very common and include rheumatoid arthritis, type 1 diabetes, Crohn’s disease, Hashimoto’s disease and chronic obstructive pulmonary disease, result in organized inflammatory foci of B lymphocytes, FDCs, DCs, macrophages, and other immune cells associated with germinal centers (141, 224, 244, 489). In addition, tertiary follicles can be induced, and are surprisingly prevalent, in nonlymphoid organs by naturally occurring infections in ruminants (141, 502).

Lymphoid follicles with prominent FDC networks can replicate prions in mice (210, 448), sheep (300), and deer (200) (Fig. 8). In fact, in mice suffering from nephritis, hepatitis, or pancreatitis, prion accumulation occurs in otherwise prion-free organs (210). The presence of inflammatory foci consistently correlated with an upregulation of LTs and the ectopic induction of PrPSc-expressing FDCs. Inflamed organs of mice lacking LTα or LTβR did not accumulate either PrPSc or infectivity following prion inoculation. These data have raised concerns that analogous phenomena might occur in farm animals, since these are commonly in contact with inflammogenic pathogens. Indeed, PrPSc has been observed in the inflamed mammary glands of sheep with mastitis and which are also infected with scrapie (300). These observations indicate that inflammatory conditions induce accumulation and replication of prions in organs previously considered to be free from prion infection.

In addition, inflammatory conditions could result in the shedding of the prion agent by excretory organs,
including the kidney. In fact, in various transgenic and spontaneous mouse models of nephritis, prion infectivity was observed in the urine with both subclinical and terminal scrapie (448). Recently, consistent levels of infectivity have been detected in colostrum and milk from sheep incubating natural scrapie, several months prior to clinical onset. The presence of ectopic lymphoid follicles in ewes with lymphoproliferative mastitis could increase prion shedding in milk, although the prion infectivity was revealed in sheep with healthy mammary glands as well as those with chronic lymphoproliferative mastitis (287). If confirmed, these findings could have an impact on the risk assessment of dairy foodstuffs (for example, milk) and could lead to a readjustment of current rules. It is possible that the horizontal spread of prions between hosts is mediated by secreted body fluids (for example, urine and milk) that are derived from potentially infectious secretory organs (for example, kidney and mammary glands).

However, chronic inflammation more commonly gives rise to granulomas whose cellular composition is different from that of lymphoid follicles. Granulomas occur, for example, in Crohn’s disease, sarcoidosis (235), and most mycobacterial (66, 146, 430), fungal, and parasitic infections, as well as in foreign body reactions. A recent study demonstrates that experimentally induced granulomas in mice enable prion replication after intraperitoneal inoculation (208). Because granulomas can occur at any site of the body, these results suggest that prions may colonize a broader spectrum of organs than previously anticipated. With respect to the relevance of the above findings for public health, it will be interesting to test whether prion replication in granulomas occurs in farm animals and in humans.

C. Neuroinflammation in Prion Pathogenesis

In the CNS, prion pathology involves large-scale activation and proliferation of microglia (215), the CNS equivalent of macrophages. The use of a prion organotypic slice culture assay (POSCA) (148) allowed pharmacogenetic ablation of microglia (Fig. 9A). In microglia-depleted slices, a 15-fold increase in prion titers and PrPSc concentrations was observed. This suggests that the extensive microglia activation accompanying prion diseases represents an efficacious defensive reaction (Fig. 9, B–E) (149).

In addition, it has been reported that T cells also infiltrate the CNS to some extent (52), which raises the possibility of atypical CNS inflammation, since the expression of both proinflammatory and effector cytokines are upregulated in prion infection (392). More importantly, intracerebral administration of prions has been shown to induce TSE in mice that have various immune deficiencies, including lack of B cells, T cells, interferon receptors, TNF, LTs or their receptors (267), receptors for IgG (FcγRs), complement factors or their receptors (269), Toll-like receptors (398), or chemokine receptors (397).

In all of these models, pathogenesis after intracerebral prion delivery proceeded with the same kinetics as in wild-type immunocompetent mice, and in terminally sick mice, intracerebral prion concentrations were unchanged (20). This evidence indicates that, after prions are present in the CNS, the disease course cannot be modified by manipulating immunological parameters.

Furthermore, several studies have addressed the impact of the nuclear factor-κB (NFκB) pathway in prion pathogenesis. NFκB is involved in a variety of physiological and pathological responses and plays an essential role in immune responses involving induction of inflammation, proliferation, and the regulation of apoptosis (56, 251). In acute and chronic neurodegenerative conditions such as AD, NFκB is activated in neurons and glia, and it has been proposed that NFκB might promote survival of neurons (245). Alternatively, NFκB may contribute to neuronal degeneration through the production and release of inflammatory cytokines or of reactive oxygen molecules by microglia and astrocytes. In prion diseases, NFκB activity was shown to be enhanced in parallel with the first neuronal pathological changes (256). Furthermore, the cytotoxic synthetic peptide PrP106–126 activates NFκB in microglia in vitro (33). Notably, it has been reported that NFκB activity leads to mitochondrial apoptosis after prion infection (61). Despite these findings, it remains unclear whether NFκB activation is protective or pathogenic in prion disease. However, when the impact of NFκB signaling on prion disease was investigated in mouse models with a CNS-restricted elimination of IKKβ and in mice containing a nonphosphorylatable IKK subunit, no impairment of prion pathogenesis in the brain was observed (242).

VII. SPREAD OF PRIONS ALONG PERIPHERAL AND CENTRAL NERVOUS SYSTEM PATHWAYS

The process of prion infectivity transfer from the spleen to the CNS is termed “neuroinvasion.” In the last several years, a biphasic model has emerged to explain the process of prion. The first phase is characterized by widespread colonization of lymphoreticular organs, the details of which have been described in the previous section. The second phase has long been suspected to involve peripheral nerves. The innervation pattern of lymphoid organs is primarily sympathetic (150), and many studies suggest that is the way how the prion agent travels from lymphoid organs to the CNS (111, 115, 335). Not surprisingly, the sympathetic nervous system is affected in vCJD patients (198). The detection of PrPSc in the
FIG. 9. Role of microglia in prion disease. A: schematic representation for the conditional ablation of microglia in vitro and in vivo by using the cytotoxic prodrug ganciclovir (GCV) in CD11b-HSVTK mice (213). B: histoblot analysis of PrPSc deposition in POSCA slices after 35 days in culture shows that PrPSc accumulates predominantly in the molecular layer. Organotypic brain slices were prepared from 10-day-old tga20TK (mice overexpressing PrP and carrying the CD11b-HSVTK transgene) and were inoculated with 100 μg RML (R) or uninfected homogenate (Ø). PrPSc was detected with anti-PrP antibody (POM1). C: accumulation of PrPSc in slices was also shown by Western blot; tga20TK and prnp-o/o slices were cultured for 35 days, optionally digested with PK (μg/ml), and probed with antibody POM1. D: microglia depletion in organotypic slices increases the amount of PK-resistant PrP. Organotypic slices from mice overexpressing PrP with the CD11b-HSVTK transgene (tga20TK+) or without (tga20TK−) were treated with GCV and inoculated with RML. Samples were PK-digested and PrP detected with POM1. E: microglia depletion in organotypic slices increases the infectivity as evaluated by SCEPA of homogenates of tga20TK+ or prnp-o/oTK+ slices treated with GCV. Three independent biological replicates of tga20TK+ or prnp-o/oTK+ slices or RML were analyzed in 10-fold dilution steps using 6–12 replica N2a-containing wells per dilution. Data are indicated as the number of infectious tissue culture units per gram of slice culture protein and are the averages of biological replicas ± SD. Slices from the same animal (pairs of GCV and −GCV samples) are represented by the same color. Sc, positive-control homogenate from brain of a mouse with scrapie; Inoc, inoculum. Left lane on all blots, molecular weight marker spiked with recombinant PrP3', yielding a PrP signal at 23 kDa with a cleavage product at 15 kDa. [B–E are adapted from Falsig et al. (149).]
spleens of sCJD patients (183) indicates that the interface between cells of the immune and peripheral nervous systems might also be of relevance in sporadic prion diseases.

Sympathectomy appears to delay the transport of prions from lymphatic organs to the thoracic spinal cord, which is the entry site of sympathetic nerves to the CNS. Denervation by injection of the drug 6-hydroxydopamine, as well as “immunosympathectomy” by injection of antibodies against nerve growth factor (NGF) leads to a rather dramatic decrease in the density of sympathetic innervation of lymphoid organs, and significantly delayed the development of scrapie (186). No alteration in lymphocyte subpopulations was detected in spleens at any time point investigated. In particular, no significant differences in the content of FDCs were detected between treated and untreated mice, which negate the possibility that the observed protection may be due to modulation of FDC microanatomy. Transgenic mice overexpressing NGF under control of the K14 promoter, whose spleens are hyperinnervated, developed scrapie significantly earlier than nontransgenic control mice.

The distance between FDCs and splenic nerves influences the rate of neuroinvagination (397). FDC positioning was manipulated by ablation of the CXCXR5 chemokine receptor, directing lymphocytes towards specific microcompartments (155). As such, the distance between germinal center-associated FDCs and nerve endings was reduced. This process resulted in an increased rate of prion entry into the CNS in CXCR5-deficient mice, probably owing to the repositioning of FDCs in juxtaposition with highly innervated, splenic arterioles.

The cellular and molecular prerequisites for prion trafficking within the lymphoreticular system are not fully understood. Genetic or pharmacological ablation of germinal center B cells, located in close vicinity to FDCs, has shown no significant effects on splenic prion replication efficacy, PrPSc distribution pattern, or latency to terminal disease (206), although in a previous report, the ablation of CD40L accelerated prion disease (86).

The mechanism by which prions reach neurons and take advantage of neuronal tracks to propagate from the periphery toward the CNS remains to be determined. Three different hypothesis could explain how prions enter neurons: 1) cell-cell contact, 2) vesicle transport, or 3) “free-floating” prion infectivity.

Convincing evidence for contact-mediated transmission of prion infectivity was presented in a study by Flechsig et al. (153a), where the inherent capacity of prions to strongly adhere to steel wires was used to study contact-mediated prion infection. Neuroblastoma cells adhering to such prion-coated steel wires become infected in tissue culture. Additionally, subsequent use of the identical steel wire mediates prion transmission efficiently to several PrPSc-overexpressing indicator mice, in a short contact period of 30 min. Remarkably, this occurs without any reduction in prion infectivity. These data strongly suggest that contact-mediated transmission rather than an exchange of the infectious agent likely takes place. Consistent with these findings, cell-mediated infection was shown in a tissue-culture model (247). This study took advantage of genetically marked target cells in combination with scrapie mouse brain (SMB) cells chronically propagating PrPSc (112). Several lines of evidence in this study, in particular efficient transmission after aldehyde fixation of the SMB cells, suggest that direct contact is responsible for the cell-to-cell transmission.

A second possibility is neuronal prion transfer by vesicle-associated infectivity. Exosome-bound prion infectivity has been reported in two cell lines: Rov, a rabbit epithelial cell line, and Mov, a neuroglial cell line, which both express ovine PrP (151). This report of exosomes containing prions in vitro is in agreement with a study that suggests that retroviral infection robustly enhances the release of prion infectivity in cell culture (294). For example, prion infectivity within exosomes, which in turn may be released by prion-infected FDCs, may encounter peripheral nerves and contribute to neuroinvasion (15, 397).

The third potential source of prion infectivity may be cell-free, free-floating oligomeric or protofibrillar infectious particles (207). Several cell lines can be infected with brain homogenates or brain fractions treated in a way that renders the existence of intact cells or exosomes highly unlikely. However, this most likely does not exclude the possibility of prion infectivity encapsulated in micelles. One recent study that favors the latter hypothesis investigated the relationship between infectivity, converting activity, and the size of various PrPSc-containing aggregates (464) and concluded that nonfibrillar particles, with masses equivalent to 14–28 PrP molecules, are the most efficient initiators of prion disease.

All of these mechanisms shown in vitro potentially play a role in vivo. However, there is currently no convincing evidence that implies an exclusive role of any of the three possibilities in prion uptake by peripheral nerves in vivo. It remains plausible, therefore, that more than one of these pathways contributes to efficient prion uptake by peripheral nerves simultaneously.

Although PrPC expression is known to modulate intraneural transport, it is unclear how prions are actually transported within peripheral nerves (184). Axonal and nonaxonal transport mechanisms may be involved, and nonneuronal cells (such as Schwann cells) may also play a role. Within the framework of the protein-only hypothesis, one may hypothesize a “domino” mechanism, by which infiltrating PrPSc converts resident PrPC on the axolemmal surface, sequentially propagating the infection. While speculative, this model is attractive, since it may accommodate the finding that the velocity of neural
prion spread is extremely slow (257) and may not follow the canonical mechanisms of fast axonal transport. The second hypothesis is that prion transport occurs within the axons of peripheral nerves similar to normal cargo proteins. This is known as the “streetcar” hypothesis (17). In the streetcar hypothesis, PrPSc is taken up at nerve endings and then transported retrogradely, presumably by a microtubule-based mechanism, to the CNS.

Despite evidence that neurons are the key mediators of prion propagation toward the CNS, low-efficiency alternatives such as transport via blood cannot be excluded completely: prion infectivity could directly invade the brain via the blood-brain barrier (BBB) (36). The cellular and molecular preconditions for such a process remain elusive. However, invasion of prion infectivity through the BBB could depend on the prion strain and the dose of administered prions (207).

The existence of multiple prion strains is likely to make neuronal transport even more complex. It is conceivable that distinct strains use different mechanisms for uptake and transport (37). This may affect not only the mechanisms of prion uptake by different neuronal cell populations, and cell-to-cell transmissions, but may also alter the route of neuroinvasion, type of transport, lesion profile, or the incubation time of an infected host (8).

The spread of prions within the central nervous system has been studied by ocular administration of prions. The retina is a part of the CNS, and intraocular injection does not induce direct physical trauma to the brain, which may disrupt the BBB and impair other aspects of brain physiology.

Evidence that spread of prions occurs axonally relies mainly on the demonstration of diachronic spongiform changes along the retinal pathway after intraocular infection (161). Reports of iatrogenic prion transmissions in humans via cornea transplants are consistent with the idea that prions spread axonally, implying that prions propagate retrogradely toward the cornea and anterograde from the cornea to the brain (143).

To investigate whether the spread of prions within the CNS is dependent on PrPSc expression in the visual pathway, PrP-producing neural grafts have been used as sensitive indicators of the presence of prion infectivity in the brain of Prnp−/− hosts. After prion inoculation in the eyes of grafted Prnp−/− mice, none of the grafts showed signs of scrapie. It was concluded, therefore, that infectivity administered to the eye of PrP-deficient hosts cannot induce scrapie in a PrP-expressing brain graft (65).

Engraftment of Prnp−/− mice with PrPSc-producing tissue might lead to an immune response to PrP which neutralizes PrPSc infectivity (214). To definitively rule out the possibility that prion transport was disabled by a neutralizing immune response, Prnp−/− mice were rendered tolerant by expressing PrPC under the control of the lck promoter. These mice overexpress PrP on T-lymphocytes but are resistant to scrapie and do not replicate prions in brain, spleen, and thymus after intraperitoneal inoculation with scrapie prions (416). Engraftment of these mice with PrP-overexpressing neuroectoderm did not lead to the development of antibodies to PrP after intracerebral or intraocular inoculation, presumably due to clonal deletion of PrP-immunoreactive T-lymphocytes. As before, intraocular inoculation with prions did not provoke scrapie in the graft, supporting the conclusion that lack of PrPSc, rather than immune response to PrP, prevented prion spread (65). Therefore, PrPSc appears to be necessary for the spread of prions along the retinal projections and within the CNS.

These results indicate that intracerebral spread of prions is based on a PrPSc-paved chain of cells, perhaps because they are capable of supporting prion replication. When such a chain is interrupted by interposed cells that lack PrPSc, as in the case described here, no propagation of prions to the target tissue can occur. Perhaps prions require PrPSc for propagation across synapses. PrPSc is present in the synaptic region (157) and certain synaptic properties are altered in Prnp−/− mice (120, 520).

Although a wealth of data has been acquired about the transport of PrPSc from the periphery into the brain in recent years, the exact molecular mechanisms of neuronal prion transport are still unclear. It also is becoming clear that investigation of the precise molecular transport mechanisms within neurons is much more difficult than previously thought. It is not trivial to test the involvement of retrograde neuronal transport in prion pathogenesis because a complete block of this transport system is incompatible with long-term survival. Furthermore, mouse models with partially impaired transport mechanisms do not display altered prion pathogenesis, yet such negative results by no means disprove a role for axonal transport.

One possible method of circumventing problems associated with in vivo studies may be to establish cell culture model systems of transport in vitro and ex vivo. Such systems may pave the way to a more precise molecular understanding of prion transport. Another advantage of in vitro systems may be the ability to pharmacologically manipulate specific neuronal transport pathways, which may be difficult to perform in vivo (207).

VIII. TOWARDS ANTIPRION THERAPEUTICS

The current therapeutic landscape for prion diseases gives little reason to rejoice. An impressive wealth of molecules has been considered as potential antiprion lead compounds. However, none of these therapeutic leads has yet proven their usefulness in clinical settings, and some have conspicuously failed. A startling variety of substances appears to possess prion-curing properties. A nonexhaustive list includes compounds as diverse as
Congo red (98), amphotericin B, anthracyclins (487), sulfated polyanions (100), porphyrins (104, 401), branched polyamines (485), “β-sheet breakers” (475), the spice curcumin (102), and the single-stranded phosphorothioate analogs of natural nucleic acids (273).

In drug discovery generally, and in TSE therapeutics specifically, promising compounds are often initially screened using relatively high-throughput in vitro tests, prior to testing of the best candidates in vivo. However, relatively few of the antiprion compounds that are active in vitro have shown any efficacy in vivo. Additional hurdles may lie in the identification of drugs that have sufficient in vivo efficacies, bioavailabilities, pharmacokinetics, and safety profiles to allow for applicability in the clinical phase of disease. Alternatively, one might conclude (and we lean towards this conclusion) that the initial cell culture screens are not very good at predicting in vivo antiprion activity.

In our view, it is premature to treat patients with alleged antiprion drugs on the sole basis of antiprion efficacy in neuroblastoma cells. This shortcut was taken in the case of quinacrine, which cures scrapie-infected cultured cells with impressive efficacy (140), yet appears to be utterly ineffective in scrapie-infected mice (121) and in CJD patients (123), besides being severely hepatotoxic (444). On the other hand, it is only fair to acknowledge that these are daunting tasks for any drug discovery project, even with great lead compounds in hand, and the lack of success thus far in identifying drugs that impact the course of prion disease in the clinical phase does not diminish the importance of prion drug discovery efforts that have been undertaken to date.

For therapeutic or prophylactic purposes, it is possible to exploit the roles of the lymphoid system and immune cells, including monocytes, macrophages, and dendritic cells, in prion replication sites; stimulation of the innate immune system; enhancement of elimination of PrPSc using PrP-specific antibodies; or binding of available PrPc or PrPSc so that they are unavailable for conversion (Fig. 10). All of these approaches, which include both suppression and stimulation of the immune system, are now being tested in suitable in vivo systems using mice experimentally infected with mouse-adapted scrapie. However, because the lymphoid system has been found to be involved in almost all forms of TSE, it is reasonable to presume that mouse-adapted scrapie provides a realistic generic model for TSE therapy.

### A. Innate Immunity and Antiprion Defense

Antiprion treatment strategies focusing on immunosuppression consist of conditional dedifferentiation of FDCs with LTβR-Ig, suppression of germinal centers, and disruption of lymphoid microarchitecture. LTβR-Ig treatment causes germinal center disruption due to the breakdown of FDC networks (Fig. 10A). The data from studies using LTβR-Ig indicate that postexposure treatment of humans with LTβR-Ig could only be considered for prophylaxis at well-defined, early time points in cases of known prion exposure. These cases might include recipients of blood transfusions from patients with CJD and researchers, pathologists, neurologists, neurosurgeons, and technicians after accidental CJD injection. In these situations, we think that a case could be made for experimental use of postexposure prophylaxis with LTβR-Ig, although it is not known how soon after exposure LTβR-Ig would need to be administered. It should be considered that LTβR-Ig will not offer any relief to patients with overt CJD, for whom neural entry has already taken place.

Theoretically, LTβR-Ig treatment might have unwanted effects on immune function. However, manipulation of the LT pathway has been carried out in macaques without apparent side effects. Primary antibody responses to keyhole limpet hemocyanin were found to be unaltered during a 20-day period (192). Moreover, LT-fusion proteins are already entering clinical trials as a treatment for rheumatoid arthritis. Hopefully, the results of such studies will become available in the foreseeable future (20).

The ordered aggregate state of PrPSc might render it recognizable as a pathogen-associated molecular pattern; therefore, the members of the Toll-like receptor (TLR) family might be involved in the recognition and subsequent degradation of prions.

However, the kinetics of prion pathogenesis in MyD88 (myeloid differentiation primary-response protein 88)-deficient mice inoculated with prions (by the intraperitoneal route) are identical to the kinetics observed in the wild-type control mice (398), suggesting that signaling by TLR1, -2, -6, and -9 (mediated by the adapter protein MyD88; Ref. 1) are probably not involved in prion recognition and signaling. Nevertheless, targeting TLR9 therapeutically might have other beneficial effects. TLR9 recognizes DNA sequences that are overrepresented in bacterial DNA. For example, unmethylated cytosine phosphate guanosine (CpG) motifs present in bacterial DNA can stimulate mouse and human immune responses through TLR9 (211) (Fig. 10B). Repetitive CpG motifs in synthetic oligodeoxynucleotides (CpG-ODN) stimulate bacterial unmethylated nucleic acid sequences, and thereby stimulate the innate immune system through TLR9 expressed on various immune cells, including monocytes, macrophages, and dendritic cells.

CpG-ODN treatment has been discussed as a possible therapy to delay prion disease, primarily based on promi...
ising results in mouse scrapie (450). The delay in the development of prion disease in this model could be due to the destruction of the primary site of peripheral prion amplification, the lymphoid follicles (209). Alternatively, the massive expansion of macrophage and dendritic cells that is evident following repetitive CpG-ODN treatment might lead to enhanced PrPSc degradation or prion sequestration.

Macrophages might conceivably function as prion transporters when exposed to high prion titers; on the other hand, they might also inhibit prion infectivity when confronted with manageable prion titers (48). However,

FIG. 10. Immunotherapeutic strategies for prion disease. 

A: treatment with the lymphotoxin-β receptor fusion protein (LTβR)-Ig breaks up the FDC networks and disrupts lymphoid follicles. The best protection is achieved when the fusion protein is administered immediately after exposure to prions. B: ablation of mature follicular dendritic cells (FDCs) delays the development of prion disease in mice. However, treatment with multiple doses of CpG-containing oligodeoxynucleotides (CpG ODNs) produces severe unwanted side effects, including immunosuppression, liver necrosis, and thrombocytopenia. C: vaccination against a self-protein is difficult because of immune tolerance, and it has the potential to induce autoimmune disease. Transgenic expression of an immunoglobulin µ chain containing the epitope-interacting region of 6H4, a high-affinity anti-PrP monoclonal antibody, associated with endogenous κ and λ chains, leads to high anti-PrPSc titers in Prnp<sup>−/−</sup> and Prnp<sup><sub><sub>o/o</sub></sub></sup> mice. It suffices to block prion pathogenesis upon intraperitoneal prion inoculation. After active immunization with full-length PrP in attenuated Salmonella vector, the mice develop high PrP specific antibody titers. D: treatment with dimeric full-length PrP fused to the Fc portion of human IgG1 (PrP-Fc<sub>2</sub>) delays the development of prion disease in mice, most probably owing to its interaction with the disease-associated PrP (PrP<sup>Sc</sup>). LT<sub>α,β2</sub>, LT heterotrimer; TLR, Toll-like receptor.
despite these promising results, CpG-ODNs are not likely to be a viable antiprion therapy owing to the severe toxic side effects associated with repeated administration. One might argue that severe side effects could be tolerable in the case of a disease that is invariably fatal. However, if the antiprion action of CpG ODNs is mediated by indiscriminate immune suppression, then there are alternative ways to achieve this goal that would at least not lead to hepatotoxicity.

An interesting alternative approach might include phagocyte activation and targeted prion degradation without the reported adverse side effects associated with CpG-ODNs (20).

B. Adaptive Immunity and Preexposure
Prophylaxis Against Prions

For many conventional viral agents, vaccination is the most effective method of infection control. But is it at all possible to induce protective immunity in vivo against prions? Prions are extremely sturdy, and their resistance to sterilization is proverbial. Preincubation of PrPSc inoculum with anti-PrP antisera was reported to reduce the prion titer of infectious hamster brain homogenates by up to 2 log units (168), and an anti-PrP antibody was found to inhibit formation of PrPSc in a cell-free system (227). Also, antibodies (269) and F(ab) fragments raised against certain domains of PrP (144, 389) can suppress prion replication in cultured cells. However, it is difficult to induce humoral immune responses against PrPC and PrPSc. This is most likely due to tolerance of the mammalian immune system to PrPSc, which is an endogenous protein expressed rather ubiquitously. Ablation of the Prnp gene (85) renders mice highly susceptible to immunization with prions (65). Many of the best available monoclonal antibodies to the prion protein have been generated in Prnp−/− mice.

However, Prnp−/− mice are unsuitable for testing vaccination regimens, since they do not support prion pathogenesis (84). Second, it was generally thought (with good reason) that antibodies specific for PrPSc if they could be elicited at all, might lead to severe systemic immunemediated diseases, because PrP is expressed by many cell types. Third, PrP-specific antibodies would be unlikely to cross the BBB in therapeutic concentrations.

To test these various possibilities and circumvent the problem of PrP tolerance, genes encoding high-affinity anti-PrP antibodies (originally generated in Prnp−/− mice) were used to reprogram B-cell responses of prion-susceptible mice that express PrPSc. In 2001, it was reported that mice transgenic for the μ-chain of a PrP-specific antibody were protected against prion disease after exposure by intraperitoneal inoculation (214) (Fig. 10C). A blatant autoimmune disease as a consequence of antiprion immunization was not observed, unless transgenic PrPSc was artificially expressed at nonphysiological, extremely high levels.

The strategy outlined above delivers proof-of-principle that a protective humoral response against prions can be mounted by the mammalian immune system and suggests that B cells are not intrinsically tolerant to PrPSc. The challenges to practical antiprion immunization, however, are enormous. While providing an encouraging proof of principle, transgenic immunization cannot easily be reduced to practice. Furthermore, no protection was observed if treatment was started after the onset of clinical symptoms, suggesting that passive immunization might be a good candidate for prophylaxis rather than therapy of TSEs. It was then found, in a follow-up study, that passive transfer of anti-PrP monoclonal antibodies can delay the onset of scrapie in prion-infected mice (518). Moreover, intracranial delivery of PrPSc-specific antibodies (those having the epitope between aa 95–105 of the PrP sequence) has been shown to result in neuronal apoptosis in the cerebellum and hippocampus, most likely through clustering of PrPSc, which is thought to trigger an abnormal signaling pathway (469). These results are alarming and certainly reinforce the concept that adequate in vivo safety studies must be carried out before prion immunophrophylaxis trials take place in humans. Active immunization, like in most antiviral vaccines, may be more effective, but is rendered exceedingly difficult by the stringent tolerance to PrPSc (476). The laboratory of Thomas Wisnieswki was able to induce active immunization with recombinant prion protein and a delay in the onset of prion disease in wild-type mice was observed, although the therapeutic effect was relatively modest and, eventually, all the mice succumbed to the disease (463). This limited therapeutic effect may be explained by the observation that antibodies generated against prokaryotic PrP often do not have a high affinity towards PrPSc (394).

The possibility of producing protective immunity against prions has captured the imagination of a considerable number of scientists, and additional reports have surfaced describing original methods of circumventing PrP tolerance and inducing an immune response in animals that express the normal prion protein, including mixing of the immunogenic moiety with bacterial chaperons (274). In a different study, tolerance was overcome by oral inoculation of the PrP protein expressed in an attenuated Salmonella vector. This mucosal vaccine induced gut anti-PrP immunoglobulins IgA and systemic anti-PrP IgG in 100% of mice with a high mucosal anti-PrP IgA titer and a high systemic IgG titer. Upon oral challenge with PrPSc scrapie strain 139A, mice remained without symptoms of PrP infection at 400 days (193) (Fig. 10C). At present, it is unclear whether vaccination approaches will ultimately be translated to clinical practice. The predic-
tive value of the different in vitro and in vivo experiments is limited, and further investigations will be required.

C. Soluble Prion Antagonists

In several paradigms, expression of two PrP moieties with subtle differences antagonizes prion replication. The molecular basis for these effects is unknown. Perhaps the subtly modified PrP moieties acts as a decoy by binding incoming PrPSc (or protein X) and sequestering it into a complex incapable of further replication.

To test the latter hypothesis, a transgenic mouse was developed that expresses soluble full-length mouse PrP rendered dimeric by fusion with the Fc portion of human IgG1 (known as PrP-Fc2) (Fig. 10D). After prion inoculation, these mice were surprisingly resistant to prion disease (343). The PrP-Fc2 was not converted to a prion-disease-causing isoform. Moreover, when the transgenic mice expressing PrP-Fc2 were back-crossed with wild-type mice and then inoculated with prions, they showed marked retardation in the development of prion disease, which was equivalent to a 10^3-fold reduction in titer of the prion-infected inoculum. This antiprion effect occurred in two different lines of PrP-Fc2-expressing transgenic mice after either intracerebral or intraperitoneal infection with scrapie. Therefore, it seems that PrP-Fc2 effectively antagonizes prion accumulation in the spleen and brain. Because PrP-Fc2 cannot be converted to the protease-resistant, disease-causing isoform, it might be effectively functioning as a “sink” for PrPSc, by binding PrPSc and preventing the binding and conversion of PrP (20).

Delivery of the soluble prion antagonist PrP-Fc2 to the brains of mice by lentiviral gene transfer impaired replication of disease-associated PrPSc and delayed disease progression (178). These results suggest that somatic gene transfer of prion antagonists may be effective for postexposure prophylaxis of prion diseases. In addition, it remains to be established whether the current form of PrP-Fc2 has the strongest antiprion properties. For instance, the introduction of dominant-negative mutations analogous to those described for Prnp (374, 391) might considerably augment its efficacy. However, further research is needed to establish whether it may be effective as a biopharmaceutical.

IX. PROGRESS IN THE DIAGNOSTICS OF PRION DISEASES

As with any other disease, early diagnosis would significantly advance the chances of success of any possible intervention approaches. Unfortunately, prion diagnostics continue to be rather primitive. Presymptomatic diagnosis is virtually impossible, and the earliest possible diagnosis is based on clinical signs and symptoms. Hence, prion infection is typically diagnosed after the disease has already progressed considerably.

A significant advance in prion diagnostics was accomplished in 1997 by the discovery that protease-resistant PrPSc can be detected in tonsillar tissue of vCJD patients (221). It was hence proposed that tonsil biopsy may be the method of choice for diagnosis of vCJD (218). Furthermore, there have been reports of individual cases showing detectable amounts of PrPSc at preclinical stages of the disease in the tonsil (443) as well as in the appendix (223), indicating that lymphoid tissue biopsy may represent a potential test for asymptomatic individuals. These observations triggered large screenings of human populations for subclinical vCJD prevalence, using appendectomy and tonsillectomy specimens (187). PrPSc-positive lymphoid tissues were long considered to be a vCJD-specific feature that would not apply to any other forms of human prion diseases (218). However, a successive survey of peripheral tissues of patients with sporadic CJD has led to the identification of PrPSc in as many as one-third of skeletal muscle and spleen samples (183). In addition, PrPSc was found in the olfactory epithelium of patients suffering from sCJD (532). These unexpected findings raise the hope that minimally invasive diagnostic procedures may take the place of brain biopsy in intravital CJD diagnostics.

For almost three decades, all gold standard methods for the molecular diagnosis of prion diseases have relied on the use of proteinase K (PK) to differentiate PrPSc and PrP. Recently, the complementary use of the protease thermolysin has been introduced. This protease digests PrP but, unlike PK, leaves PrPSc intact without truncation of the NH2 terminus (126, 376).

The development of highly sensitive assays for biochemical detection of PrPSc in tissues and body fluids is a top priority. One way to achieve this goal is to develop high-affinity immunoreagents that recognize PrPSc. Examples include the “POM” series of antibodies that recognize various well-defined conformational epitopes in the structured COOH-terminal region of PrP and linear epitopes in the unstructured NH2-terminal region (395). Because of the particular nature of the epitopes to which they are directed, some of these antibodies have affinities for the prion protein in the femtomolar range. Antibodies that specifically bind PrPSc without binding PrP have also been reported (279, 358), yet their affinity seems to be limited and their diagnostic value has awaited confirmation for more than one decade to no avail.

Searching for PrPSc binding reagents, Lauede et al. (292) discovered PrP-derived peptides that bound PrPSc. When coupled with a sandwich ELISA for detection, these peptide binding reagents create a sensitive assay that can detect PrPSc nanoliter amounts of 10% (wt/vol) vCJD brain homog-
enate diluted in plasma, thereby yielding a powerful and scalable assay that is very well suitable to blood testing.

PMCA is also a promising method for the sensitive detection of the pathological prion protein (434, 473). It was recently shown to increase sensitivity 6,600-fold over standard detection methods (95). Amplifiable PrPSc was detected in the blood of scrapie-infected hamsters by PMCA during most of the presymptomatic phase of disease (432).

LCPs were recently developed as a novel class of amyloidotropic dyes (216, 366). These dyes contain a swiveling thiophene backbone, and the optical processes, e.g., the fluorescence from the dye, are highly sensitive to the geometry of the thiophene backbone. Hence, upon interaction with protein aggregates with a distinct morphology, the rotational freedom of the LCP backbone is restricted in a specific way and a unique optical fingerprint is obtained for a given protein conformation. Instead of measuring simply the total amount of aggregated protein, heterogeneous populations of specific protein aggregates can be differentiated by LCP staining. In a transgenic mouse model of AD pathology, LCPs identified a striking heterogeneity in amyloid plaques (365). LCPs also specifically bind prion deposits, even those which were negative for binding to other amyloidotropic dyes (Congo red and ThT), and different prion strains could be discriminated via individual staining patterns of LCPs with distinct ionic side chains (462).

Another exciting prospect may be the combination of PMCA with LCPs for PrPSc detection. The unique photophysical properties of polythiophenes may pave the way for a “real-time PMCA,” where the buildup, lifetime, and conformational properties of prion aggregates may be visualized kinetically (9).

The use of surrogate biomarkers represents a diagnostic strategy fundamentally different from those delineated above. Since they typically identify secondary host reactions, surrogate biomarkers of prion infection cannot aspire to match the specificity of PrPSc detection. On the other hand, surrogate biomarkers may be useful for identifying subjects at risk and specifying acceptance or deferral of blood donations. In such cases, high sensitivity (i.e., the identification of all suspect individuals) is more important than absolute diagnostic specificity, as the latter can be supplied by confirmatory assays. Ideally, these surrogate markers should be detectable at preclinical stages of disease and differentially expressed in easily accessible body fluids such as blood or urine.

S-100, neuron-specific enolase, and 14-3-3 protein have been reported to be elevated in cerebrospinal fluid (CSF) of sCJD patients (240, 442). These proteins may represent consequences of CNS damage and neuronal death. The cysteine proteinase inhibitor cystatin C was also reported to be elevated in CSF of sCJD patients (393). Recently, urinary α1-antichymotrypsin has also been identified as a novel biomarker of prion infection (347).

The gold standard of prion diagnostics is the ability to detect prion infectivity itself. Until recently, the animal bioassay was the only method available to screen for prion infectivity, for example, by using transgenic mice overexpressing PrPSc (tga20) (154). Recently, the bank vole (Clethrionomys glareolus) has been suggested as a new and efficient model for bioassay of TSEs from different species, including human, sheep, goat, mouse, and hamster (2, 369). Additionally, the establishment of a human immune system in the mouse could be an efficient tool to test the potential human pathogenicity of various prion strains in vivo (14).

However, these bioassays take 6–7 mo to complete and are very costly. More recently, the development of highly susceptible, cloned neural cell lines (PK1 N2a) have provided an assay that improves the precision, cost, and time required to do prion detection bioassays, and might lend itself to high-throughput automation (270). Although such assays have the potential to advance methodologies aimed at the diagnostic assessment of whether the prion agent is present, it should be noted that these cell lines are currently reported to be permissive only to murine prions. Future advances will hopefully provide a means by which the full range of prion strains and species can be assayed.

X. CONCLUDING REMARKS

The study of prions has taken several unexpected turns over the years. The development and appropriate use of tools and technologies has enabled us to answer some long-standing key questions; however, many questions are still left unanswered. The physiological function of PrPSc remains unknown. Although the Prnp gene encoding PrPSc was identified in 1985 (39, 370) and the Prnp knockout mice were described in 1992 (85), the function of the protein is shrouded in mystery. Elucidation of the physiological function of PrPSc has the potential to help researchers understand the mechanisms involved in prion-induced pathogenesis.

The molecular mechanisms of prion replication are not completely defined, and the possibility that other proteins assist the process cannot be excluded. We still do not understand how strain information is maintained and transmitted, and we have no notion of the mechanisms that define the tropisms of prion strains. Finally, we do not understand how neurotoxicity is induced by the prion agent, and why it is less toxic to cells of the immune system, where it also undergoes live replication. The ability to answer these questions in the future will rely mainly
on the quality of the tools and technologies available to the prion field. These unresolved questions provide an opportunity for additional research which may have a significant impact on further protein misfolding disorders.

ACKNOWLEDGMENTS

This article honors the present and past members of the Aguzzi laboratory, whose enthusiastic commitment led to much of the work described here. Special thanks to Petra Schwarz for maintaining our prion-infected mouse colony in impeccable shape and to Rita Moos for excellent technical assistance. Mathias Heienwälter, Christina Sigurdson, Jeppe Falsig, and Mario Nuvolone provided crucial help with the section on “Immunological aspects of Prion disease” and Simone Homemann with the section on “Structures of purified PrPSc and PrPSc.” Certain parts of the history of prion research were adapted from a previously published book chapter [A. Aguzzi. In: Topley & Wilson’s Microbiology and Microbial Infections (10th ed.), 2005] whose previous edition had been published by Dr. Stanley B. Prusiner. We are grateful to Dr. Prusiner for giving us permission to utilize some of his material.

Address for reprint requests and other correspondence: A. Aguzzi, Institute of Neuropathology, University Hospital Zurich, Schmelzbergstrasse 12, CH-8091 Zürich, Switzerland (e-mail: adriano.aguzzi@usz.ch).

GRANTS

A. Aguzzi is supported by grants from the University of Zurich; the Swiss Federal Offices of Education, Science, and Health; the Swiss National Foundation; the National Center for Competence in Research on neural plasticity and repair; the United Kingdom Department for Environment, Food, and Rural Affairs; and the Stammbach Foundation. A. M. Calella was supported by the Roche Research Foundation and an EMBO fellowship.

REFERENCES


84. Caughey WS. Secondary structure analysis of the scrapie-associated protein PrPC


AGGREGATION AND INFECTIOUS DISEASE


AGGREGATION AND INFECTIOUS DISEASE


Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice. J Gen Virol 77:1505–1509, 1996.


