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An overview of transmissible spongiform encephalopathies

K. Takemura,[1] M. Kahdre,[2] D. Joseph,[1] A. Yousef[2] and S. Sreevatsan[1]*

[1]Food Animal Health Research Program, Department of Veterinary Preventive Medicine, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, OH 44691 and [2]Department of Food Science and Technology, Ohio State University, Columbus, OH 43210, USA

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Abstract

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders of humans and animals associated with an accumulation of abnormal isoforms of prion protein (PrP) in nerve cells. The pathogenesis of TSEs involves conformational conversions of normal cellular PrP (PrP^{c}) to abnormal isoforms of PrP (PrP^{Sc}). While the protein-only hypothesis has been widely accepted as a causal mechanism of prion diseases, evidence from more recent research suggests a possible involvement of other cellular component(s) or as yet undefined infectious agent(s) in PrP pathogenesis. Although the underlying mechanisms of PrP strain variation and the determinants of interspecies transmissibility have not been fully elucidated, biochemical and molecular findings indicate that bovine spongiform encephalopathy in cattle and new-variant Creutzfeldt–Jakob disease in humans are caused by indistinguishable etiological agent(s). Cumulative evidence suggests that there may be risks of humans acquiring TSEs via a variety of exposures to infected material. The development of highly precise ligands is warranted to detect and differentiate strains, allelic variants and infectious isoforms of these PrPs. This article describes the general features of TSEs and PrP, the current understanding of their pathogenesis, recent advances in prion disease diagnostics, and PrP inactivation.

Keywords: prion; transmissible spongiform encephalopathies; bovine spongiform encephalopathy; scrapie; Creutzfeldt–Jakob disease; diagnostics

Prion diseases: an overview

Prion diseases are a group of fatal neurodegenerative disorders characterized by loss of motor control, dementia, paralysis, wasting and eventual death. Prion diseases are generally referred to as spongiform encephalopathies (SE) because of the histological appearance of large vacuoles in the cortex and cerebellum. Prion diseases affect both animals and humans, causing sponge-like degeneration of brain tissue associated with the accumulation of an abnormal isoform of the host prion protein (PrP) in nerve cells. SEs that can be transmitted from one host to another are called transmissible SEs (TSEs). Despite the communicable nature of the disease, it remains contentious whether an infectious agent, an allelic variation in the host prion gene or a combination of both causes the conversion of normal PrP to an abnormal isoform, which accumulates in the host. Thus, one feature of prion diseases is that they are both infectious and hereditary. Any explanation for the cause of a prion disease therefore must account for inherited and transmitted variants of the disease.

Research on prion disease has recently accelerated for several reasons. First, mounting experimental evidence has generated great interest in what appears to be a totally new mechanism of disease. Secondly, the demon-

*Corresponding author. E-mail: sreevatsan.1@osu.edu

stration that PrPs are responsible for bovine spongiform encephalopathy (BSE), which affected more than 180 000 cattle in Great Britain alone, has lent new urgency to the elucidation of the mechanisms of prion diseases and the search for modalities for their cure. The development of highly sensitive methods to detect prion diseases is especially warranted, since consumption of BSE-affected beef products has been suggested to be causally associated with the new variant of Creutzfeldt–Jakob disease in humans.

TSEs have been reported in both humans and animals. Specific examples in humans include Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler–Scheinker syndrome (GSS), familial fatal insomnia (FFI) and kuru. TSEs described in animals include scrapie of sheep and goats, bovine spongiform encephalopathy (BSE) of cattle, chronic wasting disease (CWD) of deer and elks, feline spongiform encephalopathy of cats (Wyatt *et al.*, 1991; Sigurdson and Miller, 2003) and transmissible encephalopathy of farmed mink (Marsh and Hadlow, 1992; Sigurdson and Miller, 2003). In Europe, TSEs have also been reported in captive wild ruminants in the bovid family and monkeys (Sigurdson and Miller, 2003).

Human prion diseases

Creutzfeldt-Jacob disease

Based on the epidemiological and clinicopathological characteristics, CJD is further classified as sporadic, familial, iatrogenic and variant. Sporadic CJD (sCJD) occurs with an annual incidence of approximately one case in every 10^6 humans. The disease mostly affects people in middle and old age, with a peak onset between age 60 and 65 years (Brown *et al.*, 1994; Will *et al.*, 1998). The microscopic anatomy of affected brains demonstrates widespread spongiform vacuolation and the accumulation of only a few or no amyloid plaques. The cerebral cortex, basal ganglia and caudate nuclei, and the cerebellum are frequently affected. Early symptoms of sCJD may include mental deterioration or emotional disturbance along with physical incoordination. The clinical symptoms progress over weeks and death may occur within several months. In one study, among identified CJD cases, 87% were sporadic, 8% genetic and 5% iatrogenic (Will *et al.*, 1998).

In March 1996, an unusual clinical and pathological phenotype of CJD was first reported and named new variant Creutzfeldt–Jakob disease (Will *et al.*, 1996). These cases of new variant CJD (nvCJD or vCJD) had an average age of 29 years at death and absence of electroencephalogram features typical of sCJD. As of 2 June 2004, a total of 156 cases of vCJD have been reported in the world (146 in the UK, 6 in France and one each in Canada, Ireland, Italy and the USA) (UK Department of Health, Monthly CJD Statistical Figures. http://www.doh.gov.uk/cjd/cjd_stat.htm). The CJD Surveillance Unit at Edinburgh University and the Spongiform Encephalopathy Advisory Committee of the British government concluded that the most likely cause was ingestion of infected beef and beef products. The conclusions were based on reports that demonstrated experimental reproduction of clinically and pathologically similar CJD in macaques by intracerebral injection of brain homogenate from BSE-affected cows (Lasmezas *et al.*, 1996). In addition, the biochemical properties shared between PrP^{Sc} associated with the human vCJD cases and BSE supported the contention that BSE had been transmitted to humans. It is likely that a large proportion of the UK population has been exposed to the infectious agent. The incubation period for vCJD is about 10 years. The size of the vCJD epidemic will depend on infectious dose exposure via the gastric route. The importance of studying the origin and nature of strain differences is emphasized by this zoonotic transmission.

A number of observations suggest that vCJD represents a novel disease distinct from sporadic CJD. First, vCJD has a distinct pathology characterized by the presence of abundant large plaques surrounded by a halo of spongiform change (Ironside, 1998). Secondly, vCJD is associated with a significantly younger age of onset than sporadic CJD. To date, there is no explanation for the predilection of vCJD mostly for young adults or, conversely, why older individuals are less liable to develop the clinical and pathological changes that are characteristic of the disease. Early in the illness, patients usually experience psychiatric symptoms, which most commonly take the form of depression; less often they may appear as a schizophrenia-like psychosis. Unusual sensory symptoms, such as stickiness of the skin, have been experienced by half of the cases early in the illness. Neurological signs, including unsteadiness, difficulty in walking and involuntary movements, develop as the illness progresses and, by the time of death, patients become completely immobile and mute.

Other human TSEs

GSS syndrome is characterized by early onset, between the ages of 35 and 55, prominent cerebellar signs including varying levels of ataxia, dementia and dysarthria, and visual disturbances. The progression of GSS is rather slow compared with sCJD and often takes several years. FFI is an inherited prion disease with lesion formation dominantly in the thalamus. Because the thalamus is responsible for the regulation of sleep, FFI is typically characterized by progressive insomnia. Kuru was an epidemic among the Fore tribe in the Eastern Highlands of Papua New Guinea. Over 3000 fatalities have been attributed to the cannibalistic mourning practices of

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affected individuals (Gajdusek and Zigas, 1957). Kuru is manifested as a fatal cerebellar ataxia; however, in contrast to sCJD, other neurological signs, such as myoclonus, do not occur and dementia is absent in most cases.

Genetic predisposition to prion disease in humans

The human PrP gene, *PRNP*, is located on the short arm of chromosome 20. Over 20 mutations associated with increased susceptibility to prion disease have been described (Wadsworth *et al.*, 2003). The insertion mutations are in the N-terminus octapeptide repeat region between codons 51 and 91, while most of the point mutations are found in the globular C-terminal domain. Selection and maintenance of protective alleles of *PRNP* during the course of evolution of modern humans have been demonstrated recently (Mead *et al.*, 2003).

Homozygosity at codon 129 of the *PRNP*, encoding either methionine (M) or valine (V), is strongly associated with increased susceptibility to sporadic and iatrogenic CJD (Collinge *et al.*, 1991; Palmer *et al.*, 1991). All known vCJD cases to date are methionine homozygous at codon 129 (Collinge *et al.*, 1996a). Heterozygosity at codon 129 appears to offer relative resistance to prion disease. The character and frequency of polymorphisms in codon 129 seems to differ between iatrogenic CJD and sCJD. In iatrogenic CJD cases resulting from infection with PrP-contaminated human growth hormone in France and the UK, 129VV homozygosity was more frequently observed (29%) than in sCJD patients (16%) (Brandel *et al.*, 2003). Furthermore, among 27 patients with CJD transmitted via human growth hormone in the UK, 14 (52%) had the VV allele, while only one was found to be homozygous for MM at codon 129. These observations support the contention that the strain of infectious agent as well as the genotype of the host *PRNP* may affect the outcome of prion diseases (Brandel *et al.*, 2003). Among 56 polymorphic sites identified within 25 kb of the *PRNP* open reading frame, a C → T polymorphism at nucleotide 1368 located upstream of the *PRNP* exon 1 was found to be strongly associated with sCJD but not vCJD or iatrogenic CJD (Mead *et al.*, 2001). The substitution of lysine (K) for glutamate (E) at codon 200 of the *PRNP* has been reported in over 70% of familial CJD cases worldwide. The E200K variant of familial CJD is most prevalent among populations of Libyan and Tunisian Jews (Lee *et al.*, 1999).

Fatal familial insomnia is associated with the substitution of asparagine (D) for aspartate (D) at codon 178 of *PRNP* (Medori *et al.*, 1992). CJD and FFI are linked to the same D178N mutation; however, the illness is also determined by the polymorphism at codon 129. The presence of 129M coupled with 178N was prevalent in FFI patients, whereas patients with familial CJD had 129V coupled with the 178N allele (Goldfarb *et al.*, 1992).

A proline (P) to leucine (L) mutation in the *PRNP* gene at codon 102 was associated with GSS syndrome patients (Goldgaber *et al.*, 1989). GSS patients carrying the L102 genotype and 129MM developed rapid dementia and severe cortical damage that was clinically and pathologically indistinguishable from CJD (Hainfellner *et al.*, 1995). An alanine (A) to valine (V) substitution at amino acid residue 117 was found in a French-Alsatian kindred with GSS disease over three generations (Heldt *et al.*, 1998). A GSS phenotype with an unusual sporadic CJD case was reported. This patient was heterozygous at codon 129 and did not have pathogenic substitutions at codons 102, 200 and 178, or any of the previously described pathogenic mutations at codons 102, 105 and 117 associated with GSS (Liberski *et al.*, 1998).

It has been suggested that these amino acid changes may reduce the thermodynamic stability of PrP, thus facilitating the pathogenic process. However, some studies involving the nuclear magnetic resonance spectroscopic structure of murine PrP121–231 (Riek *et al.*, 1998) and the thermodynamics of human PrP90–231 (Swietnicki *et al.*, 1998) have questioned the contribution of mutation-induced thermodynamic destabilization to the pathogenic conversion of cellular PrP.

Prion diseases in animals

Scrapie

Scrapie is a fatal, neurodegenerative transmissible prion disease in sheep and goats. Since the first case of sheep scrapie was described in UK around 1732 (Parry, 1983), it has been endemic throughout the world, except in Australia and New Zealand. Australia (in 1952) and New Zealand (in 1952–1954 and 1976–1977) imported Suffolk sheep that developed scrapie later. However, in both countries, eradication was successful by slaughtering all affected and in-contact animals. Scrapie is thought to be transmitted from the ewe to offspring and to other lambs through contact with the placenta and placental fluids. Experimental transmission of scrapie in sheep and goats was demonstrated by oral administration of fetal membranes from scrapie-affected sheep (Pattison *et al.*, 1972). Clinical signs of scrapie vary widely among individual animals and develop very slowly. Scrapie-affected animals usually demonstrate behavioural changes, including tremors, pruritus and incoordination, subsequently progressing to death (Dickinson, 1976). The clinicopathological observations following scrapie exposure appear to be controlled by the infecting scrapie strain and the host PrP genotype (Goldmann *et al.*, 1994). The extent of strain variation in naturally occurring scrapie is unknown. In genetically defined mouse models, at least, 20 strains of scrapie associated

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with varying degrees of neuropathological and infectious properties have been described. A new distinct scrapie strain (221C) was recently identified in ten naturally occurring cases in the UK (Bruce *et al.*, 2002). In the mouse model, the strain 221C produced distinct outcomes in terms of the incidence of disease, the incubation period and neuropathology when compared with other known sheep scrapie strains. The small sample size used in this study precludes conclusions regarding whether the strain 221C was an emerging scrapie strain or an existing one that had not been isolated in the past. Large epidemiological studies that prospectively sample natural scrapie cases will be necessary to fully define strain variation in scrapie and other prion diseases. Knowledge of strain variation will also greatly aid in the understanding of interspecies transmission of TSEs.

Sheep present considerable diversity in the prion gene that affects natural and experimental scrapie transmission. The amino acids encoded at residues 136 [valine (V) or alanine A)], 154 [histidine (H) or arginine (R)] and 171 [glutamine (Q), arginine (R), lysine (K), or histidine (H)] of the prion gene are strongly associated with individual susceptibility to scrapie in sheep. Thus, the PrP genotype in sheep is usually expressed as V136 R154 Q171 or simply VRQ. Sheep carry two alleles of the PrP gene; therefore the genotype could be such as VRQ/ARR. Among the several possible combinations of amino acids at these loci, five allelic variants, VRQ, ARR, ARQ, ARH and AHQ, have been commonly identified worldwide. In common sheep breeds, VRQ/VRQ homozygotes were found to be most susceptible to developing scrapie (Goldmann *et al.*, 1991; Hunter *et al.*, 1993, 1996). In Suffolk sheep, the V136 is rarely reported and the scrapie susceptibility seems to be primary associated with Q171 homozygosity in that breed (Hunter *et al.*, 1997b). Analysis of the PrP genotypes among scrapie-infected and healthy sheep (Belt *et al.*, 1995) showed that the VRQ variant was most frequently identified in scrapie animals, whereas the ARR variant was almost exclusively present in the healthy animals. Two other variants, ARQ and ARH, were equally distributed in both affected and healthy sheep. Animals carrying ARQ/ARR genotype appear less likely to develop natural scrapie (Belt *et al.*, 1995; Hunter *et al.*, 1996) although these genotypes are susceptible to experimental challenge. ARR/ARR homozygotes are most resistant to scrapie infection, and there has been only one case of scrapie with this genotype reported to date (Ikeda *et al.*, 1995).

While there is a strong association between certain genotypes and susceptibility, scrapie is considered an infectious disease (Hunter *et al.*, 1997a; Houston *et al.*, 2002). Scrapie-susceptible genotypes have been identified in Cheviot and Suffolk sheep in Australia and New Zealand, countries that are considered to be scrapie-free. Genotyping studies of Merinos and Poll Dorset sheep also reported that susceptible PrP alleles exist in Australia and New Zealand (Hunter and Cairns, 1998). A study on Romney Marsh sheep, a common breed in New Zealand, showed that these animals also possessed scrapie-susceptible PrP alleles (Bossers *et al.*, 1999). In addition, Suffolk sheep imported from New Zealand into a scrapie-infected flock in the USA eventually developed the disease (Hourrigan, 1979). When the sheep from New Zealand carrying various PrP genotypes, similar to those reported in the UK, were subcutaneously challenged with a sheep-passaged scrapie, all the sheep carrying the VRQ allele developed typical clinical signs of scrapie with neurodegenerative changes. PrP^{Sc} was detected histopathologically in the brains and lymphoid tissues (Houston *et al.*, 2002). Taken together, these studies support the notion that scrapie is not a spontaneous genetic disorder but requires exogenous exposure to an infectious agent. These findings also suggest that the infectious agent(s) is absent in Australia and New Zealand.

The scrapie eradication program implemented by United States Department of Agriculture (USDA) is based on decreasing the genetic susceptibility of sheep stocks to scrapie and destroying the infectious agents. The program includes the identification of infected/suspected animals and active slaughter surveillance, the effective tracing of infected/suspected animals and indemnity that will allow producers to stay in business. Animal and Plant Health Inspection Service–Veterinary Services of USDA and other approved laboratories, such as GenMark (DeForest, WI, USA), GeneCheck (Fort Collins, CO, USA) and GeneSeek (Lincoln, NE, USA) offer sheep genotyping services that are critical to this eradication scheme and, for producers, an important breeding tool to increase resistance to scrapie in their flocks. While scrapie has existed for several centuries in domestic sheep flocks, there is no evidence of an association between sheep scrapie and human CJD (Brown *et al.*, 1987).

Bovine spongiform encephalopathy

Bovine spongiform encephalopathy is a TSE of cows. Since the first case of BSE was reported in the UK in 1986, over 180 000 cases of BSE have been confirmed in that country. Mathematical models suggest that 1–3 million cattle may have been infected with the BSE agent (Anderson *et al.*, 1996; Donnelly *et al.*, 2002). Conventional wisdom is that the recycling of ruminant tissues contaminated with BSE agent to cattle as feed supplement has been the cause of the BSE epidemic. The suspected feed supplement was meat-and-bone meal (Wilesmith *et al.*, 1988, 1991). In the UK, meat-and-bone meal was routinely fed to calves, which are considered to be more susceptible to the BSE agent (Anderson *et al.*, 1996). Experimental transmission of

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BSE has been successfully established in calves via an oral challenge (Wells *et al.*, 1994) and in sheep and goats via intracerebral or oral routes (Foster *et al.*, 1993). Establishment of experimental BSE infection in cattle, early in the course of the disease, was detected using a mouse bioassay. Inbred mice were intracerebrally inoculated with homogenates of the distal ileum derived from the orally challenged calves (Wells *et al.*, 1994).

Since the neuropathology of BSE in cattle is similar to that seen in scrapie, the origin of BSE has been suggested to be a scrapie-like agent from sheep or cattle (Wilesmith *et al.*, 1988, 1991). A major argument against this hypothesis is that the BSE agent is biologically and molecularly different from any of the scrapie-causing strains in sheep that have been described so far. Traditionally, strain typing of TSE agents has involved the characterization of clinical and pathological phenotype following transmission into inbred strains of mice. However, this method is time-consuming, multiple passages are required to stabilize and define strains, and not all TSE sources are transmissible to mice (e.g. CWD). While antibody-based glycosylation profiles of PrP^{Sc} following proteinase K digestion are also used for differentiation of BSE from scrapie or classical CJD, the method has not been standardized, resulting in inter-laboratory variations. Glycosylation profiles of PrP^{Sc} are only an indirect measure of strain characteristics (such as the density and distribution of PrP^{Sc} in the brain) and not conformation, which is a molecular determinant of strain properties. Regardless, using the currently available detection systems, naturally occurring scrapie strains identical to BSE in cattle have not been identified to date. Sheep in the UK also received meat-and-bone meal in their feed, with no evidence of a similar TSE epidemic in the sheep population. It is now known that sheep can be experimentally infected with BSE by the oral route with brain homogenate from BSE-affected cows (Foster *et al.*, 1993). An age cohort analysis conducted in the UK estimated that at the peak of the BSE epidemic in 1990 the number of cases of BSE-infected sheep would have ranged from fewer than 10 to about 1500, with a prediction of less than 20 clinical cases in 2001 (Kao *et al.*, 2002). It is speculated that, if BSE infection occurred in sheep naturally, they would possess a greater risk to public health than would cattle, resulting in an estimated 150 000 vCJD mortalities (Ferguson *et al.*, 2002). However, the possibility of emergence of a new strain, with enhanced infectious properties, during the process of interspecies (sheep to cow) transmission cannot be ruled out.

Chronic wasting disease

CWD is a fatal, neurodegenerative disorder affecting free-ranging and wild ruminant species (Williams and Miller, 2002). Although the illness was first identified as a clinical syndrome among captive mule deer (*Odocoileus hemionus hemionus*) in Colorado in the 1960s, CWD had not been definitively identified as a TSE until 1978 (Williams and Young, 1980). Clinical signs of CWD include behavioural changes, progressive weight loss and eventual death. The incubation period of CWD, as determined by experimental intracerebral inoculation of infected brain homogenate into deer, is between 17 and 21 months (Williams and Young, 1992). More recent oral exposure experiments in mule deer estimated the incubation period to be about 15 – 25 months (Sigurdson and Miller, 2003). Studies on mule deer and black-tailed deer (*Odocoileus hemionus columbianus*) in Colorado and Wyoming have identified neuropathology consistent with widespread spongiform damage in the central nervous system of affected animals (Williams and Young, 1980). Similar microscopic neuropathology has been described in Rocky Mountain elks (*Cervus elaphus nelsoni*) maintained in captivity in the same states (Williams and Young, 1982). Between 1981 and 1995, spongiform encephalopathy with clinicopathology consistent with CWD was identified in free-ranging mule deer, Rocky Mountain elks and white-tailed deer (*Odocoileus virginianus*) in north-central Colorado, and PrP^{Sc} was detected in the brain of affected animals. The histopathological and immunohistochemical appearance of tissues from these animals was identical to that of tissues from captive cervids with CWD (Spraker *et al.*, 1997). In northeastern Colorado and southeastern Wyoming, CWD was identified in a total of 513 free-ranging deer and Rocky Mountain elks by immunohistochemical of obexx sections. The prevalence was estimated at 4.9% in mule deer, 2.1% in white-tailed deer and 0.5% in elks in these endemic areas (Miller *et al.*, 2000). In Wisconsin, the prevalence of CWD was estimated as 3% based on immunohistochemical staining of 476 obexx sections derived from white-tailed Deer (Joly *et al.*, 2003). To date, CWD has been found in Colorado, Illinois, Kansas, Minnesota, Montana, Nebraska, New Mexico, Oklahoma, South Dakota, Utah, Wisconsin, Wyoming and parts of Canada. Outside of North America, a 7-year-old male elk exported from Canada to Korea developed a wasting disease that was confirmed as CDW (Sohn *et al.*, 2002). Although the mode of transmission of this infection among individual animals is unknown, horizontal transmission by ingesting materials contaminated with CWD-causing agent has been suggested (Miller *et al.*, 1998, 2000). Under experimental conditions, brain homogenate from mule deer with naturally occurring CWD was orally inoculated into mule deer (*Odocoileus hemionus*) fawns. None of the challenged animals showed clinical signs over the experimental period of 80 days. However, PrP^{Sc} was detected in retropharyngeal lymph node, tonsil, Peyer's patch and ileocecal lymph node 6 weeks after the inoculation. The infection was confirmed in all inoculated animals (Sigurdson *et al.*,

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1999). The presence of PrP^{Sc} in various lymphoid tissues including tonsil, Peyer's patches and mesenteric lymph nodes (Sigurdson *et al.*, 1999; Spraker *et al.*, 2002) led to the speculation that PrP^{Sc} could be shed through the digestive tract of infected animals and contaminate surrounding environments. In fact, contaminated pastures and premises seemed to serve as sources of infection in some CWD epidemics (Miller *et al.*, 1998).

There are several known polymorphisms in the PrP gene of Rocky Mountain elks, mule deer and white-tailed deer at codons 96, 132, 138 and 226. These alleles are G96 M/L132 S138 E226 in elks, G96 M132 S/N138 E226 in mule and white-tailed deer, and S96 M132 S138 E226 in while-tailed deer alone (Raymond *et al.*, 2000). The M132L substitution found in elks corresponds to the M129V polymorphism in humans. As discussed previously, M129M homozygosity is associated with increased susceptibility to CJD. Similarly, the M132M-homozygous genotype appears to be significantly over-represented in CWD-affected elks compared with normal free-ranging animals (O'Rourke *et al.*, 1999).

Biochemistry of prion proteins

Cellular prion protein (PrP^{c}) is a cell-surface-associated sialoglycoprotein with a molecular weight of approximately 27–35 kDa (Prusiner, 1998). A single exon encodes the prion protein, which consists of 254 amino acids in humans. The N terminus is cleaved at amino acid 23, generating the fragment 1–22, a signal peptide for prion trafficking, and the C terminus is cleaved at residue 231 for the glycosyl phosphatidyl inositol (GPI) moiety to anchor the protein on the cell surface (Stahl *et al.*, 1987). Human PrP contains an octarepeat domain consisting of four sequential repeats of the sequence Pro-His-Gly-Gly-Gly-Trp-Gly-Gln in the N terminus from amino acid residue 60 to residue 91, which serves as the major copper (II)-binding site (Hornshaw *et al.*, 1995; Brown *et al.*, 1997). PrP^{c} is synthesized in the rough endoplasmic reticulum and is transferred cotranslationally into the endoplasmic reticulum (ER). During the biosynthesis of PrP^{c}, the N terminal signal peptide is cleaved, oligosaccharide chains are added, a single disulfide bond is formed, and the C-terminal peptide is cleaved and modified for the attachment of a GPI moiety (Stahl *et al.*, 1987; Caughey *et al.*, 1989). Glycosylation occurs at amino acid residues N181 and N197 (Kretzschmar *et al.*, 1986), generating unglysosylated, monoglycosylated and diglycosylated PrP isoforms. The GPI moiety, in addition to the mannose, contains ethanolamine and sialic acid (Stahl *et al.*, 1992). The addition of sialic acid to mammalian peptide anchors is relatively unusual. PrP^{c} is translocated through Golgi and anchored to the cell surface via a GPI moiety (Stahl *et al.*, 1987; Caughey *et al.*, 1989). On the cell surface, PrP^{c} is segregated into cholesterol and sphingomyelin-rich rafts in the plasma membrane (Stahl *et al.*, 1987; Vey *et al.*, 1996; Naslavsky *et al.*, 1997). From the cell surface, the PrP^{c} is transported retrogradely into the endocytic compartments, from which most of the protein is recycled and a small percentage undergoes proteolysis (Yedidia *et al.*, 2001).

The structure of mouse (Riek *et al.*, 1997) and human (Zahn *et al.*, 2000) cellular prion protein consists of three α-helices [amino acids 144–154 (helix 1), 173–194 (helix 2) and 200–228 (helix 3) in the human] and antiparallel β-sheet (amino acids 128–131 and 161–164 in the human). The C-terminus of full-range prion protein possesses a globular structure while the N-terminus part is more flexible and lacks defined secondary structures under the experimental conditions (Donne *et al.*, 1997; Riek *et al.*, 1997). The disulfide bond forms between the cysteine residues at 179 and 214 (Turk *et al.*, 1988) that connect helix 2 and helix 3. The region between the β-sheet and helix 2 (amino acids 166–171) is of particular interest because structural differences among species may lie in the region (Zahn *et al.*, 2000).

Although PrP^{Sc} possesses an amino acid sequence identical to that of PrP^{c}, the biophysical features, such as detergent solubility, secondary and tertiary structures and stability against enzymatic digestion, are dramatically different from those of PrP^{c} (Prusiner, 1982). Biochemically, PrP^{c} is soluble in detergents and sensitive to proteinase K digestion. Under physiological conditions, PrP^{Sc} is highly insoluble in common detergents (Bolton *et al.*, 1982) and tends to aggregate (Pan *et al.*, 1993). Proteinase K digestion of PrP^{Sc} leads to the generation of protease-resistant prion protein fragments with molecular weights of 27–30 kDa (PrP27–30) that are the N-terminus truncated PrP^{Sc} isoforms which maintain full infectivity (Bolton *et al.*, 1982; Prusiner *et al.*, 1984; Basler *et al.*, 1986). Current diagnostics of TSE are mostly based on the detection of PrP27–30 following proteinase K digestion. PrP^{c} consists of 42% α-helix structure and only 3% β-sheet, whereas PrP^{Sc} contains 30% α-helix and 43% β-sheet. The N-terminally truncated PrP27–30 has a higher content of β-sheets (54%) and fewer α-helices (21%) (Pan *et al.*, 1993).

The exact function of PrP^{c} is unknown; however, some evidence suggests that it functions as a major copper-binding protein (Hornshaw *et al.*, 1995; Brown *et al.*, 1997) or as a copper delivery protein (Kretzschmar *et al.*, 2000). Experimental evidence suggests that PrP^{c} may be involved in signal transduction. PrP^{c} has been shown to interact with a tyrosine kinase (Fyn) (Mouillet-Richard *et al.*, 2000), casein kinase 2 (Meggio *et al.*, 2000) and growth factor receptor-bound protein-2 (Spielhaupter and Schatzl, 2001). In mice, intracerebral injection of monoclonal antibodies (mAbs) directed against PrP^{c} resulted in apoptotic neuronal cell death (Solforosi *et al.*, 2004). This led to the speculation that the mAb efficiently dimerized PrP^{c}, thus initiating an apoptotic cascade in neurons, possibly via docking with other

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molecule(s). The apoptotic cell death was observed for the mAb that recognized epitopes within amino acid residues 95–105, but not 133–157, even at a high concentration (1 mg/ml) of the mAb. This model seems to be promising for further study of PrP^{c} function and the neurotoxicity of PrP.

Biochemical evidence of vCJD–BSE and BSE–scrapie links

The hypothesis that vCJD and BSE are linked was first suggested on the basis of epidemiological observations of the time and place where these diseases were prevalent. The hypothesis was later supported by experimental transmission studies using other animals, including transgenic mice. Biochemical and molecular typing of PrP^{Sc} strain characteristics further strengthened the contention that the two prion diseases were caused by the same agent. PrP^{Sc} obtained from vCJD-affected brain had a glycosylation profile distinct from those of other forms of CJD but the profile was identical to that seen in BSE transmitted to mice (Collinge *et al.*, 1996b). Western blotting following proteinase K digestion demonstrated that banding patterns of PrP^{Sc} from different prion diseases could be classified into four types based on molecular weights and glycoform ratio (Collinge *et al.*, 1996b). Types 1 and 2 were most frequently seen in sCJD (Parchi *et al.*, 1996) and in some cases of iatrogenic CJD, while type 3 was reported in prion diseases acquired by peripheral prion exposure. The banding pattern of vCJD, designated type 4, differed from types 1 and 2 in molecular weights and from type 3, in glycoform ratio (Collinge *et al.*, 1996b). CJD-derived PrP^{Sc} inoculated into transgenic mice that exclusively expressed human PrP demonstrated PrP^{Sc} banding patterns typically seen in CJD; however, the transgenic mice inoculated with BSE revealed the PrP^{Sc} pattern seen in vCJD cases. Furthermore, BSE-inoculated macaques and naturally acquired BSE in domestic cats demonstrated PrP^{Sc} banding patterns indistinguishable from those of BSE-inoculated mice and vCJD (Collinge *et al.*, 1996b).

When brain homogenates from BSE, feline spongiform encephalopathy and vCJD were inoculated into mice, patterns of incubation periods and vacuolar degeneration in the brain were uniform for the inoculated mice; however, these biological characteristics were different from those in the scrapie-inoculated mice (Bruce *et al.*, 1997). These studies provide experimental evidence that vCJD in humans may be due to the same etiological agent as BSE. More recent findings in transgenic mice that express human prion protein genotype 129MM were rather surprising. Among BSE-inoculated transgenic mice, some produced vCJD-like PrP^{Sc} glycosylation profile type 4, but others produced a type 2 PrP^{Sc} fingerprint that is typically seen in sporadic CJD (Asante *et al.*, 2002). The experiment using transgenic mice 129MM is considered to be more relevant to BSE transmission to humans, because all of the known cases of human vCJD to date have had the 129MM genotype. Transgenic mice with the 129MM genotype also seem to be more susceptible to developing subclinical BSE. Consequently, it was speculated that some sporadic CJD cases might have arisen as a result of BSE exposure. However, such a hypothesis needs to be carefully examined as there still are no definitive tools to differentiate PrP strains (or conformations) corresponding to a particular prion disorder in a particular species. Taken together, the data from experimental animal models provide unequivocal evidence that vCJD and BSE are caused by the same etiological agent(s). Nevertheless, arguments that these mice were not syngeneic and that the findings may reflect the differences in the genetic background of the mice have been raised. This is further complicated by the notion that a fairly well outbred human population may not behave in the same way as 129MM transgenic mice when exposed to the BSE agent.

It is still unknown whether BSE-causing agent(s) are present in natural sheep populations and, if so, whether sheep-adapted BSE strains differs from PrP^{Sc} strains found in BSE-affected cattle. Various attempts have been made to profile PrP^{Sc} from sheep infected with natural scrapie, sheep-passaged experimental scrapie, sheep-adapted BSE and cows naturally infected with BSE. Western blotting analysis of brain samples from BSE-infected and scrapie-infected sheep and of PrP^{Sc} extracted from sheep infected with the experimental scrapie strain CH1641 demonstrated a glycoform banding pattern indistinguishable from that of BSE-infected sheep (Hope *et al.*, 1999). CH1641-infected sheep had various PrP genotypes, and no correlation was found between sheep PrP allotypes and PrP^{Sc} banding profiles. Another study using two mAbs to differentiate PrP^{Sc} banding patterns provided further distinction among different PrP^{Sc} fingerprints (Stack *et al.*, 2002). Western blotting with mAb 6H4 generated distinct glycoform profiles between the sheep with experimental BSE infection and natural cases of scrapie. Furthermore, when mAb P4 was used in Western blotting, strong signals were obtained from the natural scrapie samples, although weak signals from sheep samples infected with BSE agent and scrapie strain CH1641 and no signals from BSE-infected cattle samples were observed (Stack *et al.*, 2002). However, a more recent experimental infection study in transgenic mice expressing sheep PrP clearly differentiated PrP^{Sc} from CH1641 and that from sheep-adapted BSE by comparing percentages of mono- and diglycosylated PrP^{Sc} detected by western blotting (Baron *et al.*, 2004). Using two different anti-PrP mAbs in a western blot to detect PrP^{Sc}, an antibody-binding ratio of 1.5 was suggested as a practical indicator for possible BSE infection in sheep (Thuring *et al.*, 2004).

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Whether strain characteristics and/or conformations will change and new barriers will be created when there is interspecies transmission under natural conditions is still unknown. Thus, there is a critical need to develop tools to define the presence and distribution of strains (or conformations) of PrP^{Sc} in animal populations that can potentially spill over to the human food chain.

Pathogenesis of prion diseases

The protein-only hypothesis states that (i) PrP^{Sc} is the etiological agent of TSEs and (ii) PrP^{Sc} replicates itself by converting normal cellular prion proteins to a misfolded pathogenic conformation.

Studies with yeast models of prion disease have uncovered much critical information that supports the protein-only hypothesis in TSE pathogenesis. In *Saccharomyces cerevisiae*, two non-Mendelian inheritance elements, [*PSI*+], which is associated with partially defective nonsense suppression, and [*URE*3], which alters nitrogen metabolism, have been studied extensively as surrogates in prion research (for review, see Wickner *et al.*, 2000a; Chernoff, 2001). The proteins Sup35p, from the [*PSI*+] phenotype (Derkatch *et al.*, 1996), and Ure2p, from the [*URE*3] phenotype (Wickner, 1994), contain the N-terminal glutamine- and asparagine-rich domains, termed prion-determining domains (PrD), that are associated with PrP-like behaviors of these yeast proteins. The altered amyloidogenic or prion-like state of Sup35p and Ure2p is inactive and converts the corresponding normal protein into the same altered form, resulting in the formation of protein aggregation *de novo* (for review, see Wickner *et al.*, 2000b).

In yeast, an intermediate amount of the molecular chaperone Hsp104 has been known to be essential for the propagation of the prion-like isoform of Sup35p (Chernoff *et al.*, 1995). Hsp104 was shown to enhance the resolubilization of aggregated, heat-denatured proteins (Parsell *et al.*, 1994). Overproduction of Hsp104 led to the loss of [*PSI*+] forms, which was interpreted as a cure (Chernoff *et al.*, 1995). Recently, possible mechanisms of Hsp104 to control the formation, replication and cure of [*PSI*+] were reported, by measuring the effects of different concentrations of Hsp104 on various conformational states of PrD of Sup35p (Shorter and Lindquist, 2004). At low concentrations (such as a Sup35p-PrD:Hsp104 ratio of 250:1), Hsp104 significantly accelerated the formation of amyloid fibers of Sup35p-PrD. Electron microscopy revealed that the amyloid fibers were indistinguishable from spontaneously formed fibers. After the Hsp104 had been removed from the fibers, the fibers seeded the polymerization of Sup35p-PrD as well as that of the spontaneous fibers, suggesting that Hsp104 catalysed the acquisition of a self-replicating prion conformation (Shorter and Lindquist, 2004). At high concentrations (such as a Sup35p-PrD to Hsp104 ratio of 15:1), Lindquist and colleagues discovered three activities of Hsp104-promoted prion-curing, including the passive inhibition of Sup35p-PrD oligomer maturation, the elimination of amyloidogenic oligomer maturation, and the disassembly of amyloid fibers into non-amyloidogenic species (Shorter and Lindquist, 2004). In the same study, using an anti-oligomer antibody it was also demonstrated that an amyloidogenic oligomer was an obligate intermediate to nucleate formation of Sup35p-PrD amyloid fibers *de novo* (Shorter and Lindquist, 2004).

While yeast prion models provide an excellent example of protein–protein interactions in the template-like reproduction of alternative conformations, these investigations may not be directly relevant to mammalian prions, as mammalian PrP is a membrane-associated protein whereas the yeast prions are cytoplasmic. In the mammalian cellular environment, translated PrP is directed into the ER similarly to other membrane-associated and secreted proteins. In the ER, misfolded proteins, including PrPs, are transported back into the cytosol through the retrotranslocation pathway to be degraded by the proteasome (Tsai *et al.*, 2002). Proteasome inhibitor-induced cytosolic accumulation of PrP has been shown to be extremely neurotoxic in cultured cells and transgenic mice (Ma *et al.*, 2002). The inhibition of proteasome also produced amorphous aggregates and a proteinase K-resistant PrP^{Sc}-like conformation (Ma and Lindquist, 2002). However, conflicting results were found recently in cultured cells (Drisaldi *et al.*, 2003). In this study, cytosolic PrP that accumulated due to proteasome inhibitors was unglycosylated and retained a signal peptide, indicating that the PrP neither entered the ER nor underwent retrotranslocation prior to proteasome degradation. Furthermore, it was noted that the effects seen with proteasome inhibitors might have been artifacts due to increased PrP mRNA and protein translation rate. It was speculated that the accumulation of misfolded PrP in the lumen of ER was a more likely event in prion diseases (Drisaldi *et al.*, 2003).

One universal property of PrP^{Sc} is resistance to thermal and enzymatic degradation. Protease-resistant PrP successfully confers its characteristics on PrP^{c} in a cell-free environment (Kocisko *et al.*, 1994). However, a chimeric hamster–mouse PrP^{c} converted to PrP^{Sc} *in vitro* failed to reproduce infectivity in a bioassay (Hill *et al.*, 1999a). Protease resistance may not be necessary for neurotoxicity. Genetically modified PrP^{c} developed spontaneous neurodegeneration similar to that seen in TSEs without generating protease-resistant PrP^{Sc}. Although transgenic mice expressing secondary structure-deleted PrP^{c} (Muramoto *et al.*, 1997) and a transmembrane form of PrP^{c} (Hegde *et al.*, 1998) developed fatal neurodegenerative changes in the central nervous system, PrP^{Sc} was not found in the affected mice. In another study, transgenic mice that carried PrP^{c} containing a nine-octapeptide

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insertion (Chiesa *et al.*, 1998) spontaneously developed a fatal neurodegenerative illness with aggregations of weakly proteinase-resistant PrP (Chiesa *et al.*, 2003). However, intracerebral inoculation of the brain homogenates from the affected transgenic mice failed to transmit the disease in recipient transgenic mice, indicating the mutant PrP was neurotoxic and proteinase resistant but not infectious.

Until recently, one major missing piece of evidence in support of the protein-only hypothesis was that recombinant prion protein that is misfolded *in vitro* is infectious by itself (Aguzzi and Heikenwalder, 2003). A mouse PrP peptide consisting of amino acids 89–143 with a substitution of leucine (L) for proline (P) at codon 102 was synthesized and chemically converted to a β-sheet-rich conformation (Kaneko *et al.*, 2000). The β-sheet rich peptide was inoculated into the brain of transgenic mice expressing mutated PrP carrying a P101L mutation, which corresponds to the P102L substitution seen in GSS-affected humans (Kaneko *et al.*, 2000). The β-rich isoform of the peptide, but not the non-β-rich conformer, generated neuropathological changes similar to those in human GSS. However, these experiments were inconclusive, because (i) the transgenic mice spontaneously developed prion disease and (ii) the transgenic mice inoculated with brain extracts from asymptomatic transgenic mice also developed prion disease. In a subsequent study (Tremblay *et al.*, 2004) using a conformation-dependent immunoassay (CDI), it was demonstrated that the peptide-initiated neuropathology could be transmitted to other transgenic mice by intracerebral inoculation of brain extracts from mice with peptide-initiated neuropathological lesions. The CDI approach clearly demonstrated that PrP^{Sc} in transgenic mice with spontaneous GSS was different from that found in transgenic mice inoculated with sheep scrapie or BSE. The spontaneous GSS PrP^{Sc} was, however, indistinguishable from that seen in transgenic mice with peptide-induced GSS. Following proteinase K digestion at 4°C, a molecular signature of PrP^{Sc} for both spontaneous and peptide-induced GSS in the transgenic mice was generated (Tremblay *et al.*, 2004). Based on these findings, two possible misfolding pathways for mutant PrP were suggested: one that led to the accumulation of misfolded PrP and caused neurodegeneration, and another that facilitated the formation of misfolded PrP that were infectious. A breakthrough in support of the protein-only hypothesis comes from an elegantly designed experiment using a recombinantly expressed and *in vitro* polymerized β-sheet rich (synthetic) mouse, PrP 89–230, which showed that PrP was necessary and sufficient for infectivity and development of spongiform neuropathology (Legname *et al.*, 2004). In sum, these studies establish the role of PrP^{Sc} alone in the development of spongiform encephalopathy.

In order to fulfill all Koch's molecular postulates, it is also necessary to demonstrate the ability of PrP^{Sc} to chemically revert its structure and thus abolish the infectivity followed by reconstitution of the original set of properties. That there may be other cellular components or as-yet undefined infectious agents that are involved in the pathogenesis of TSE will be completely ruled out when this gap in our knowledge is closed. To this end, several investigations directed at understanding intermediary factors and processes involved in PrP conformation changes have been undertaken.

It has been demonstrated that the conversion of PrP^{c} to protease-resistant forms similar to PrP^{Sc} occurs in a cell-free system (Kocisko *et al.*, 1994), and the presence of cell lysate facilitates PrP^{Sc} conversion of PrP^{c} (Saborio *et al.*, 1999). Furthermore, the *in vitro* conversion was not observed without the cell lysate in the same system, suggesting that cellular components other than prion proteins might be involved in the conversion process. The concept of additional factors involved in prion replication was suggested nearly a decade ago to explain the PrP^{Sc} transmission in transgenic mice (Telling *et al.*, 1994). Transgenic mice expressing both mouse and human PrP were resistant to human PrP^{Sc} infection, whereas the transgenic mice expressing only human PrP were susceptible to the human PrP^{Sc}. Prusiner and colleagues postulated that a species-specific cofactor, designated 'protein X', plays a critical role in the conversion of PrP conformation, and that the mouse protein X binds to mouse PrP^{c} with higher affinity than it does to human PrP^{c}. A follow-up study with transgenic mice expressing human–mouse chimeric PrP^{c} further supported the involvement of the provisional protein X in the PrP^{Sc} conversion. Genetic evidence suggested that the locus of protein X was distinct from the PrP gene, and the protein X bound to PrP^{c} through residues near the C-terminus (Telling *et al.*, 1995). It was then hypothesized that protein X may act as a molecular chaperone during the structural transition from PrP^{c} to PrP^{Sc} (Prusiner, 1998). However, such a protein has not been discovered yet.

The cofactor involved in the PrP^{Sc} conversion, if such a molecule exists, could be a protein or other cellular component, including nucleic acid. Because prion protein is a basic protein, similar to other positively charged proteins, such as histones, it can bind to negatively charged nucleic acids and form nucleoprotein complexes. In recent years, nucleic acids have been shown to bind to PrP, with catalytic and chaperone-like activities involved in the structural conversion. Recombinant murine PrP^{c} polymerized in the presence of DNA and became proteinase K-resistant (Nandi and Leclerc, 1999). Another study demonstrated that DNA molecules bound to recombinant murine PrP converted the α-helical structure to the β-sheets seen in pathogenic isoforms of PrP (Cordeiro *et al.*, 2001). Recently it was shown that an anti-DNA antibody, OCD4, and a DNA-binding protein, g5p, selectively precipitated PrP^{Sc}, but not PrP^{c}, from TSE-affected brain samples from both humans and

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animals (Zou *et al.*, 2004). The binding of OCD4 to PrP^{Sc} was diminished by addition of exogenous DNA, but not affected by nuclease or proteinase K digestion of the brain homogenate. These findings suggest that OCD4 binds to DNA or DNA-related molecules associated with PrP^{Sc}, and that the nucleic acid material is protected from enzymatic digestion (Zou *et al.*, 2004).

The postulated cellular component involved in PrP^{Sc} conversion could be RNA. Single-stranded RNA molecules that specifically bound to recombinant hamster PrP^{c} were selected from a synthesized random nucleic acid library using systematic evolution of ligands by nexponential enrichment technique (Weiss *et al.*, 1997). The selected RNA molecules did not bind to PrP^{Sc}. Another study also demonstrated the binding of single-stranded RNA molecules to recombinant human PrP^{c} and proteinase K-treated human PrP^{Sc} (Zeiler *et al.*, 2003). The study suggested that PrP possessed two RNA binding sites. One was non-specific and was found in the N-terminus between amino acid residues 23 and 90 and the other was a specific site located in the core structure of prion protein (Zeiler *et al.*, 2003).

Recently, it was demonstrated that *in vitro* conversion of PrP^{c} to PrP^{Sc} required single-stranded RNA but not double-stranded RNA, RNA–DNA hybrids or DNA molecules (Deleault *et al.*, 2003). PrP^{Sc} was amplified six-fold following an overnight incubation of normal hamster brain homogenate with prion-infected hamster brain. In the presence of total brain RNA obtained from mouse or hamster, PrP^{Sc} amplification increased up to 24-fold. The size of the RNA species necessary to amplify PrP^{Sc} was estimated to be over 300 nucleotides. Interestingly, addition of RNA preparations from *Escherichia coli, Saccharomyces, Caenorhabditis* and *Drosophila* did not stimulate *in vitro* PrP^{Sc} amplification. Total RNA extracted from hamster liver also increased PrP^{Sc} conversion, indicating that RNA-mediated PrP conversion was species-specific but not tissue-specific. The stimulatory activity of RNA was destroyed by glyoxylation but not by deproteination, heating to 60°C or exposure to 50% formamide. These findings may support the hypothesis that specific RNA molecules are cellular cofactors to convert PrP^{c} to PrP^{Sc}. However, Deleault's study failed to convert full-length recombinant PrP to PrP^{Sc}.

In vitro cultivation systems for PrP

The successful adaptation of prions to several cell lines has made possible *in vitro* research into the pathobiology of TSEs. These systems have been efficiently applied in the study of PrP^{Sc} pathogenesis and propagation, efficacy testing for novel therapeutic agents, and potential use as diagnostic tools (for review, see Solassol *et al.*, 2003). The most commonly used cell is the mouse neuroblastoma N2a cell line, which is chronically infected with mouse-adapted scrapie; its infectivity has been demonstrated in a bioassay (Race *et al.*, 1987; Butler *et al.*, 1988). However, generally only a small fraction of N2a cells becomes infected, resulting in low PrP^{Sc} yield (Race and Chesebro, 1987).

The mouse hypothalamic cell line GT1 is derived from highly differentiated gonadotropin-releasing hormone neurons immortalized by genetically targeted tumorigenesis in transgenic mice (Mellon *et al.*, 1990). GT1-trk cells are subclones of GT1 cells transfected with the *trkA* gene, encoding the nerve growth factor (NGF) receptor (Zhou *et al.*, 1994). When GT1-trk cells were infected with mouse-adapted scrapie, the cells exhibited morphological alterations of neurodegeneration and vacuolation, and apoptotic features, including clumping of dense chromatin and nuclear pyknosis (Schatzl *et al.*, 1997). Intense accumulation of PrP^{Sc} was observed in endosome-related structures and secondary lysosomes. With NGF treatment, the infected GT1-trk cells gained viability, morphological changes occurring on cell infection were reduced, and the DNA fragmentation that was a hallmark of apoptosis was diminished (Schatzl *et al.*, 1997). Since such cytopathic changes and DNA fragmentation were not detected in scrapie-infected N2a cells, the GT1 cell line may be a suitable system for remodeling prion neuropathology.

The SMB cell line was originally established from cultures derived from the brain of a mouse clinically affected by the Chandler scrapie isolate (Clarke and Haig, 1970). SMB cells have been shown to be curable with pentosan sulfate, and can be re-infected with a different strain of scrapie (Birkett *et al.*, 2001).

It was reported that hamster-adapted scrapie did not infect N2a cells of mouse origin (Race *et al.*, 1987). The commonly used cell lines described above are homologous systems that support the propagation of TSE agents only if they are experimentally adapted in rodents. Infection of the rat pheochromocytoma cell line PC12 with a mouse-adapted scrapie demonstrated that species barriers could be overcome in a cell culture systems (Rubenstein *et al.*, 1984), making them amenable to the study of interspecies transmission and strain characteristics. A heterologous cultivation system, Rov, was developed using a rabbit kidney epithelial cell line expressing a tetracycline-regulatable sheep PrP gene (Vilette *et al.*, 2001). The transfected sheep PrP gene carried the VRQ allele that was associated with high susceptibility to developing scrapie (Hunter *et al.*, 1993, 1996). After induction, Rov cells expressed high levels of PrP on the outer membrane. The Rov cells were infected with brain homogenate prepared from VQR-homozygous sheep with natural scrapie. After infection, PrP^{Sc} was readily detected by western blotting; however, the glycosylation profile of Rov system-derived PrP^{Sc} was distinct from that of the scrapie brain inocula, indicating that the PrP^{Sc} was produced *de novo* in the Rov cells (Vilette *et al.*, 2001). Scrapie-infected Rov cells successfully transmitted the infection to uninfected fresh Rov

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cells and to transgenic mice. The findings suggest that the expression of homologous PrP may allow natural TSE agents to cross species barriers and propagate their infectivity in heterologous cell systems.

A heterologous cultivation system, Mov, with mouse glial cell clones derived from dorsal root ganglia that express sheep PrP with the VRQ allele and simian virus 40 T antigen, has been developed recently (Archer *et al.*, 2004). Two Mov phenotypes, MovS2 and MovS6, exhibited Schwann cell-like morphology and were further characterized. The MovS cells could be infected with transgenic mouse-adapted scrapie. PrP^{Sc} was detected within three subpassages after the infection, and the MovS cells propagated PrP^{Sc} over 100 subpassages. The MovS cells could also be infected *in vivo* by grafting into the brains of scrapie-infected transgenic mice. The PrP^{Sc}-propagating MovS cells transmitted the infection to transgenic mice expressing sheep PrP. These findings suggested that TSE agents could infect non-neuronal cultured cells derived from the peripheral nervous system. In the same study, PrP^{Sc} accumulation was detected in neurons, glial cells and Schwann cells from dorsal root ganglia of sheep with natural infections of scrapie and transgenic mice inoculated with scrapie intraperitoneally (Archer *et al.*, 2004). Experimental oral dosing and peripheral inoculation of TSE agent(s) have shown transmission of the diseases, and in naturally occurring TSEs these modes of transmission probably occur (for a review, see Weissmann *et al.*, 2002). However, the precise mechanisms of the peripheral nervous system involvement in the pathogenesis of TSE remain to be elucidated. The Mos system seems to have potential for use in the study of the peripheral pathobiology of TSEs.

In sum, *in vitro* cultivation systems open new avenues for prion diagnostics and the elucidation of prion biology. The studies have also laid the foundation for the analysis of factors involved in the emergence of new strains and the abrogation of species barriers. Developing such *in vitro* cultivation systems for human and animal TSEs seems to be a promising strategy to accelerate understanding of the mechanisms of TSE pathogenesis at the molecular and cellular levels.

Prion diagnostics

Diagnosis of prion diseases has been traditionally made based on clinical observations in the late stages of the illness. Definitive diagnosis still largely depends upon post-mortem histopathology and immunohistochemical staining of brain samples from the affected patients or animals. The infectivity of TSE has been exclusively measured by animal bioassays. Despite a few drawbacks, such as the species barrier and considerably long incubation times, animal bioassays remain the most sensitive tool for detection of infectious agent(s). Sheep and goats have been used in scrapie research; however, the development of a murine scrapie model (Chandler, 1961) significantly reduced the time required to observe the disease in this assay. The incubation time for animal bioassay was further reduced in Syrian hamster models (Marsh and Kimberlin, 1975). The development of PrP knockout (Bueler *et al.*, 1992; Manson *et al.*, 1994), mutant (Manson *et al.*, 2000) and transgenic mice (Weissmann and Flechsig, 2003) has remarkably accelerated the elucidation of the biochemical and molecular mechanisms of prion disease as well as PrP^{c} functions. Transgenic mice, especially transgenic mice expressing human *PRNP* for human TSE research, have been used extensively to study TSEs of most species (Telling, 2000).

Ante-mortem diagnosis

The pathogenic form of prion protein is considered to replicate or accumulate in lymphoreticular systems before neuroinvasion. Ante-mortem immunological and immunohistochemical examination of lymphoreticular tissues, such as tonsils, has shown promise in the diagnosis of vCJD and CWD. Tonsil biopsies from mule deer orally inoculated with a brain homogenate from CWD-infected deer were PrP^{Sc}-positive as early as 42 days after the inoculation (Sigurdson *et al.*, 1999). Among human tonsillar biopsies, those from patients that were eventually diagnosed with vCJD were positive for PrP^{Sc} with the type 4 glycoform profile. Tonsil biopsies from patients subsequently confirmed with other diagnoses were negative for PrP^{Sc} (Hill *et al.*, 1999b). Tissue samples from neuropathologically confirmed vCJD patients were analysed by western blotting with enhanced chemiluminescence to detect PrP^{Sc}. PrP^{Sc} was found in tonsil, spleen, and lymph node with the highest PrP^{Sc} concentrations consistently seen in tonsil samples (Wadsworth *et al.*, 2001). The study suggested tonsil biopsy as a diagnostic tool to detect preclinical vCJD infection.

An immunohistochemistry-based test to detect the presence of PrP^{Sc} in the lymphoid tissue of the third eyelid (nictitating membrane) of sheep is currently applied in the scrapie eradication program (O'Rourke *et al.*, 2000). Based on well-designed prospective studies performed with sheep flocks with or without a history of exposure to scrapie, the third eyelid test had sensitivity of 87.8% and specificity of 99.2% (O'Rourke *et al.*, 2000). Although scrapie-infected sheep typically show clinical signs at 35–45 months of age (Wineland *et al.*, 1998), infectivity could be detected in lymphoid tissues of affected animals as early as 10–14 months (Hadlow *et al.*, 1982). Thus, the third eyelid test could identify affected animals in the preclinical stages; however, a negative result may not indicate the absence of scrapie. Although the nictitating membrane can be obtained from live animals under local anesthesia, such tissue col-

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lection is more labor-intensive than withdrawal of peripheral blood.

Low amounts of PrP^{Sc} in blood and the insoluble nature of PrP^{Sc} under physiological conditions present challenges for the detection of PrP in peripheral blood. An immunocompetitive capillary electrophoresis (ICCE) assay with fluorescence detection technology to detect PrP^{Sc} in blood was evaluated recently (Schmerr *et al.*, 1999). The ICCE assay involves two contiguous fluorescence measurements: the first signal is the antibody binding to the fluorescent-labeled peptide raised against the carboxyl terminal region of PrP, and the second one is generated by the displacement of the peptide by the abnormal PrP in samples. Abnormal PrP could be detected in blood from scrapie-infected sheep and elks and CWD-infected elks, but not from normal animals. However, when ICCE was performed by another research group, it was unable to distinguish between PrP^{Sc} in blood from healthy and CJD-affected humans and chimpanzees (Cervenakova *et al.*, 2003).

A novel *in vitro* technique developed by Saborio and colleagues may improve analytical sensitivity in detecting PrP^{Sc} even when it is present in limited quantities in clinical samples (Saborio *et al.*, 2001). The method of protein misfolding cyclic amplification (PMCA) is conceptually an analog of the polymerase chain reaction (PCR) and converts PrP from the cellular form to a protease-resistant form by means of sonication. Sonication disrupts aggregates formed upon incubation of an excess amount of PrP^{c} with a minute quantity of PrP^{Sc} template. Subsequently, the disrupted pieces of the aggregates serve as templates to continue the conversion of PrP^{c} to PrP^{Sc}. The study demonstrated that there was 15-fold greater sensitivity in the low-end detection limit of PrP^{Sc} in hamster brain homogenates after ten cycles of PMCA (Saborio *et al.*, 2001). In conjunction with existing PrP detection methods, the PMCA technique appears to have significant potential in prion disease diagnosis (Soto *et al.*, 2002).

Most conventional infectious agents elicit a host immune response; therefore, many diagnostic assays take advantage of the specific antibodies against pathogens for their detection. In prion disease, because the etiological agent is a host-encoded protein that differs only in its conformation, host immune responses may be limited to conformational epitopes. In addition, the accessibility of antibodies to these epitopes is also restricted because of large glycan moieties that mask the protein and the aggregative nature of PrP^{Sc}. Alternatively, the macrophages or dendritic cells may process both conformations of proteins into similar epitopes for B-cell presentation, leading to indistinguishable responses. Several attempts have been made to generate immunological reagents that detect conformational epitopes of PrPs and differentiate PrP^{Sc} from PrP^{c}.

In 1997, a mAb that precipitated bovine, murine and human PrP^{Sc}, but not PrP^{C}, was reported (Korth *et al.*, 1997). Recently, mAbs against the tyrosine-tyrosine-arginine motif of PrP that was hidden in PrP^{c} but became solvent accessible upon its conformational changes to a β-sheet-rich structure were generated in mice, goats and rabbits (Paramithiotis *et al.*, 2003). The antibodies directed against the Tyr-Tyr-Arg motif not only differentiate PrP^{Sc} from PrP^{c} but also recognize misfolded protease-sensitive PrP, which is considered to be a transient intermediate form between normal and pathogenic PrPs.

The conformation-dependent immunoassay (CDI) is a newly developed, highly sensitive PrP^{Sc} detection method utilizing a recombinant antibody fragment (recFab) directed against amino acid residues 132–156 of bovine PrP, which is hidden for PrP^{Sc} but exposed for PrP^{c} (Safar *et al.*, 2002). Native and denatured samples containing PrP are subjected to an enzyme-linked immunosorbent assay (ELISA) with recFab that recognize amino acid residues 132–156 of bovine PrP for capture, and then detected with a fluorescence-labeled recFab that recognizes residues 95–105. Ratios of time-resolved fluorescence signals from a native sample and a denatured sample allow the determination of whether the sample contains only normal PrP or a mixture of PrP^{c} and PrP^{Sc}. The CDI demonstrated sensitivity similar to that of animal bioassays in transgenic mice (Safar *et al.*, 2002). The fluorescence signal ratios between native and denatured PrPs from BSE-infected cattle and transgenic mice expressing bovine PrP infected with BSE and vCJD were indistinguishable, suggesting that these PrPs possessed similar conformational characteristics (Safar *et al.*, 2002). The CDI also indicated that PrP from CWD-infected deer and elks was different from that of BSE-infected cattle (Safar *et al.*, 2002).

Commercial diagnostics

Commercially available diagnostic tests for prion diseases rely heavily on the biochemical properties of the pathogenic form of PrP and immunochemical reagents raised against PrP. An automated version of the CDI, aCDI™, became available through InPro Biotechnology (South San Francisco, CA, USA) in 2003. aCDI can analyse over 1000 samples a day. In November 2003, IDEXX Laboratories (Westbrook, ME, USA) announced the availability of a new test kit for chronic wasting disease, IDEXX HerdChek® CWD. This test uses tissue homogenates without proteinase K digestion in an ELISA format.

Other commercial tests currently available include Prionics®-Check Western (western blot) and Prionics®-Check LIA (ELISA), distributed by Prionics AG in Switzerland, the Platelia™ test (ELISA; Bio-Rad Laboratories, Hercules, CA, USA) and the Enfer test (ELISA; Enfer). The Prionics®-Check Western was the

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first rapid BSE test officially validated by the Swiss Federal Veterinary Office and the BSE Reference Center of the University of Bern in the spring of 1998. Since then, many European countries have adopted it as an official test for the purpose of BSE surveillance, and it has been widely used for BSE detection all over the world. Bio-Rad offers the Platelia BSE test, which detects the protease resistant PrP by a sandwich ELISA with a color-converting, enzyme-coupled detection antibody. The Platelia BSE test was validated by the European Commission in July 1999. Since the test became available at the end of 2000, it has been used for BSE detection throughout the European continent and Japan.

In Japan, the Platelia BSE test has been chosen for the national BSE screening program, which initiated widespread systematic BSE testing in 2001. In the winter of 2003, two TSE-affected cows at the unusually young ages of 21 and 23 months were identified in Japan. BSE surveillance systems typically focus on cows aged 30 months or older, but the Japanese findings may suggest a need for testing cows under the age of 24 months in a potentially heavily exposed population. In March 2004, the Bio-Rad TeSeE® test became licensed by the USDA for use in the National Veterinary Service Laboratories (NVSL) in Ames, Iowa and the NVSL network laboratories as part of the USDA's expanded BSE surveillance program. The Enfer TSE test was originally developed by Enfer Scientific (Tipperary, Ireland) and has been approved for cattle testing by the European Union and Japan. The Enfer test is a rapid post-mortem test to detect PrP^{Sc} by an ELISA technique with enhanced chemiluminescent technology. In March 2001, the diagnostics division of Abbott (Abbott Park, IL, USA) and Enfer Scientific reached a collaborative agreement for marketing and distributing the Enfer TSE test worldwide to detect BSE and scrapie. In April 2004, Abbott received approval from the USDA to sell and distribute the Enfer test for the detection of BSE.

Currently, the BSE tests approved by the USDA are an immunohistochemical test that takes several days to perform and obtain results, and two rapid tests, the Bio-Rad TeSeE test and the Enfer TSE test. The first case of BSE was identified in the USA late in December of 2003, resulting in the recall of affected beef. There is a serious need in the USA for reliable, rapid and accurate BSE tests that make large-scale screening possible within the established workflow of the beef industry.

Orchid BioSciences Europe (Abingdon, UK) provides genotyping test services for scrapie in Europe with their tag-array single-nucleotide polymorphism technology. The company performs genotyping services for the UK government's National Scrapie Plan and the Northern Ireland Scrapie Plan. GeneSeek (Lincoln, NE, USA) has also been providing genotyping tests for scrapie in sheep. When the first BSE-positive cow was identified in the USA in December 2003, USDA chose GeneSeek to analyse the DNA from brain tissue of the affected cow, and GeneSeek confirmed the results with a MassARRAY system (Sequenome, San Diego, CA, USA), a mass spectrometry platform for high-throughput, single-nucleotide polymorphism-based DNA analysis and identity testing. Q-RNA (New York, NY, USA) is developing a new blood-based test with patented single-stranded RNA ligand technologies (Zeiler *et al.*, 2003) to detect BSE in live animals. Since current BSE tests are post-mortem, such an ante-mortem test would be a significant tool for a BSE surveillance and eradication program in live cattle. There is an urgent need to detect prion disease before clinical symptoms appear in order to reduce the risk of contamination of biological products, including blood supplies and bovine-derived consumables.

Surrogate markers

To facilitate ante-mortem diagnosis, considerable efforts have been made to investigate surrogate markers associated with prion disease. The clinical diagnosis of CJD can be supported by quantitative measurements of cerebrospinal fluid (CSF) or serum for neuron-specific enolase (Zerr *et al.*, 1995), erythroid differentiation-related factor (Miele *et al.*, 2001), 14-3-3 protein (Hsich *et al.*, 1996; Zerr *et al.*, 1998; Kenney *et al.*, 2000) S-100 protein (Otto *et al.*, 1998a, b, c) and a tau protein (Otto *et al.*, 1997).

Neuron-specific enolase concentrations were significantly higher in 58 patients with definite and probable CJD (median 94.0 ng/ml) than in 26 control patients (median 9.5 ng/ml). Measurements of neuron-specific enolase in CSF used for the diagnosis of CJD had a sensitivity of 80% and a specificity of 92% at a cut-off of 35 ng/ml (Zerr *et al.*, 1995). Similarly, an erythroid-specific marker, erythroid differentiation related factor, was measured by quantitative reverse transcriptase-PCR amplification from blood samples, and has been shown to be down-regulated in individuals infected with vCJD (Miele *et al.*, 2001).

Another assay system involves the quantification of 14-3-3 protein, which is usually absent in CSF in normal individuals but elevated in CJD patients. However, elevated levels of this protein are also found in the CSF of patients with viral encephalitis, and in the first month after stroke (Hsich *et al.*, 1996). A study tested 215 CSF samples including controls for all herpes viral encephalitides, and several other neurological diseases (Thor *et al.*, personal communication). All autopsy-confirmed cases of CJD were positive by immunoblot for the isoform of 14–3-3, as were two suspected CJD cases. Cases of herpes simplex virus encephalitis, but not other herpes family viruses, were also positive for 14-3-3. These results yielded a positive predictive value greater than 0.60 for this test. However, in a large cohort of patients with progressive dementia without CSF pleocytosis, the sensitivity of the assay was 96% and the specificity was 99% for the detection of sporadic CJD (G. Thor *et al.*,

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personal communication). Another study, involving 297 patients with a differential diagnosis that included CJD, demonstrated sensitivity of 90% and specificity of 88% for the 14–3-3 assay. In this investigation, the 14–3-3 immunoblot was negative for the one iatrogenic and all eight genetic cases, but positive for the sporadic cases of CJD (Otto *et al.*, 2002).

Another surrogate marker for prion diseases that is being investigated actively is the protein S-100 (Otto *et al.* 1998a, b, c; Beaudry *et al.*, 1999). S-100 is elevated in the plasma of CJD-infected individuals. A case–control study involving 224 patients with suspected CJD and 35 control patients without dementia suggested that serum measurement of S-100 was a valuable and relatively easy tool to detect CJD. Patients with CJD had a median serum S-100 concentration of 395 pg/ml, which was significantly higher than that found in patients with other diseases (median 109 pg/ml). At a cut-off point of 213 pg/ml, the test had a sensitivity of 77.8% and specificity of 81.1% (Otto *et al.*, 1998b).

Promising results for the diagnostic potential of tau protein measurement in CSF have also been reported (Otto *et al.*, 1997). Confirmed CJD patients had significantly higher concentrations of tau protein in CSF (median 13 153 pg/ml) than control patients with other dementing diseases (median 558 pg/ml) and those without dementing diseases (median 296 pg/ml). A more recent study with 297 patients with CJD indicated a diagnostic sensitivity of 94%, a diagnostic specificity of 90% and a positive predictive value of 92% for tau protein in CSF at a cut-off of 1300 pg/ml (Otto *et al.*, 2002). Several other tests based on increased plasma levels of interferon-γ (Murphy *et al.*, 2001), laminin receptor or laminin binding protein (Rieger *et al.*, 1997; Graner *et al.*, 2000) are being investigated.

Treatment of prion diseases

Currently, patients with prion diseases are provided with symptomatic treatments and affected animals are being destroyed. Despite several attempts at treatment or therapeutic modalities, all prion diseases still remain fatal. Major challenges in prion disease prophylaxis and therapy include the unsolved pathogenesis of the diseases, the lack of highly sensitive detection methods to identify affected individuals in early asymptotic stages, and difficulty in delivering drugs through the blood–brain barrier. In recent years, some promising outcomes have been demonstrated among dozens of compounds tested under experimental conditions (for review, see Brown, 2002; Koster *et al.*, 2003). These include Congo red (Caspi *et al.*, 1998; Demaimay *et al.*, 1998), anthracyclines (Tagliavini *et al.*, 1997), amphotericin B (Adjou *et al.*, 1999), tetracyclines (Forloni *et al.*, 2002), acridines (Korth *et al.*, 2001; May *et al.*, 2003) and pentosan polysulfate (Doh-ura *et al.*, 2004).

Therapeutics need to be effective when they are administered to clinically ill patients; however, protective effects were obtained only if the agents were given at the time of infection and in an early stage of the infection. Many tested compounds showed unacceptable toxicity for clinical use and poor penetration of the blood–brain barrier. An acridine-derived antimalarial drug, quinacrine, and an antipsychotic drug, chlorpromazine, were shown to inhibit PrP^{Sc} formation in chronically scrapie-infected mouse neuroblastoma cell culture (Korth *et al.*, 2001). Both drugs have been used as human medicines and are known to cross the blood–brain barrier. Quinacrine was ten times more effective than chlorpromazine. It was also demonstrated that tricyclic derivatives of acridine and phenothiazine were potent in reducing PrP^{Sc} formation in cell culture (Korth *et al.*, 2001).

Quinacrine was administered to three sCJD patients and one iatrogenic CJD patient at 300 mg/day for 3 months (Nakajima *et al.*, 2004). Although the treated patients demonstrated some improvement in their arousal level, clinical improvement was transient. Side-effects of quinacrine, including liver dysfunction and yellowish pigmentation, were modest and tolerable. A dimeric motif of acridine heterocycles, bis acridines, has shown approximately ten-fold increased bioactivity compared with the monomeric acridine-based compound quinacrine (May *et al.*, 2003). Bis acridines inhibited PrP^{Sc} formation in scrapie-infected mouse neuroblastoma cell culture at 25 nM concentration, and the compounds were not cytotoxic to the cells at the concentration of 500 nM.

Pentosan polysulfate, a compound generally used to treat bladder conditions, has also been interrogated as a candidate for therapy. Pentosan polysulfate alters physiological properties of PrP by binding to heparan binding sites of the proteins, and potentially inhibits further PrP^{Sc} production (Dealler and Rainov, 2003). The drug has to be administered directly into the brain because it does not penetrate the blood–brain barrier. Although the Committee on the Safety of Medicines in the UK did not recommend the use of pentosan polysulfate to treat vCJD because of insufficient clinical and safety data and the absence of relevant studies in animal models (Gould, 2003), a few patients with vCJD in UK, including a teenager in Northern Ireland (Dyer, 2003) have been treated with the drug. A recent study demonstrated an incubation time 2.4 times longer for scrapie-infected mice than for control mice when the mice were given pentosan polysulfate intracerebrally during the early stage of infection (Doh-ura *et al.*, 2004). No significant prolonged effect was found for quinacrine. Intracerebral pentosan polysulfate infusion also decreased neurodegenerative changes, PrP^{Sc} deposition and infectivity of the brain from affected mice (Doh-ura *et al.*, 2004).

Even though beneficial effects were found in cell culture for certain compounds, application of these

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compounds in animal models and ultimately to humans requires considerable safety and efficacy testing. A treatment for prion diseases seems to be remote and there are many obstacles; however, effective modalities are being investigated for other neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. These disorders and prion diseases share common pathobiological themes of aggregated protein deposition in the central nervous system; therefore, improved treatments against these diseases may also be applicable in prion disease therapy.

Prion inactivation

That prions are highly resistant to most physical and chemical methods used to inactivate conventional pathogens is now well established (Taylor, 1999, 2000; Taylor and Woodgate, 2003). It has long been recognized, for example, that boiling alone is ineffective in rendering the agent non-infectious and that higher temperatures are more efficient when combined with steam under pressure (i.e. autoclaving). As a means of decontamination, dry heat is used only at the extremely high temperatures achieved during incineration, usually in excess of 600°C (Brown *et al.*, 2000). Incineration at lower temperatures has been demonstrated to be ineffective in completely eliminating the infectious nature of the TSE agent. Although initial prion inactivation studies demonstrated that exposure to sodium hydroxide (alkaline pH) alone for 1 h was sufficient to completely destroy prion infectivity, its efficacy has been questioned (Taylor, 2000; Taylor and Fernie, 1996). However, sodium hydroxide exposure followed by gravity displacement autoclaving at 132°C for an hour seems to destroy prion infectivity adequately (Taylor, 1996). Formaldehyde fixation increases resistance to autoclave inactivation (Taylor and McConnell, 1988; Brown *et al.*, 1990).

As examples of thermal resistance to inactivation, studies on extremophiles that are able to replicate in unconventional ecosystems, including at high temperatures (hyperthermophiles), give clues about the survival of macromolecules under adverse conditions (Stetter, 1996, 1999). These organisms exist with optimal growth temperatures around 100° (Stetter, 1996, 1999). Enzymes and proteins from such hyperthermophiles have been shown to retain activity at these replication temperatures. The upper thermal stability limit for biological macromolecules from these organisms *in vitro* is 150–160°C (Brown *et al.*, 2000). Denaturation of proteins and nucleic acids occurs at temperatures well below their decomposition temperatures because of thermal disruption of hydrogen and hydrophobic non-covalent bonds (Brown *et al.*, 2000). Studies with indicator organisms such as *Bacillus subtilis* and *Serratia marcescens* have also shown that these bacteria resist brief exposure to temperatures between 270°C and 340°C, and viability of some dry spores persists after exposure to 370°C (Brown *et al.*, 2000). None of the organic molecules or microorganisms has been shown to retain biological or replicating activity after exposure to temperatures above 400°C, and certainly none would be expected to survive 600°C. Nevertheless, the amyloidogenic agents of TSE not only withstand chemical decomposition but also retain the power to nucleate their own replication after exposure to temperatures that should decompose or volatilize most organic molecules (Brown *et al.*, 2000).
These studies clearly demonstrate, through a series of experimental challenge methods using animal models to measure the level of inactivation, that the TSE agents are resistant to conventional decontamination methods and need special attention. As a result there is public concern over safety of several biological products, including blood, pharmaceuticals (such as hormones, vaccines andamins) and food products of animal origin. The fate of prions in animal carcasses on farms, and even in manures and in manure products, is also of concern. Whether these pathogenic agents are decontaminated under conventional manure management practices is still unknown. Composting, a relatively new treatment process for farm manures and even for animal carcasses on farms, might possibly have an effect on these agents. The pH during composting of manures and of low C:N ratio wastes, such as animal carcasses, easily reaches values of 8.5–9.0 over several weeks to months. The entire mass being composted typically reaches temperatures of 55–75°C. It is speculated that prion infectivity may not survive these processes. Other composting systems, such as lime stabilization, might be even more effective in reducing the infectivity of PrP^{Sc} because of the same elevated temperatures and even higher pH values (10.5–12.0).

Further studies on high-pressure and ozone inactivation methods are under way in several leading laboratories. These studies will further aid in the development of simpler, proven methods of PrP inactivation and carcass disposal.

Remarks

Despite considerable research efforts over the past several decades, the precise mechanisms of prion disease, as well as the normal functions of PrP, remain to be elucidated. There is a need to standardize the reagents and animals, including transgenic and PrP knockout mice, used in prion research. Standardization will increase the ability to reproduce the experimental results of one laboratory in other laboratories, providing more opportunities to validate key discoveries. The infectious particles associated with prion disease deserve further investigation as to whether they act independently in causing disease or whether additional components are

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involved in infectivity. The molecular and biochemical typing of PrP indicates the presence of multiple PrP strains in TSEs. The underlying mechanisms of PrP strain variation and the determinants of interspecies transmissibility need to be further identified. Cumulative evidence suggests that there are risks for humans of acquiring TSEs as a result of the consumption of food products contaminated with TSE-causing agent(s) or the receipt of a tainted therapeutic product, including blood transfusion. However, the best knowledge available to date indicates that these risks are small or non-existent. This assessment might have arisen from incomplete knowledge of PrP strains or their behavior in hosts and across species, the detection limits of current assays, and the limitations of bioassays in measuring PrP infectivity. Thus, there is an urgent need to develop high-precision ligands that are specific to PrP species, strains (conformations), allelic variants and infectious isoforms. The development of novel methods and reagents that detect PrPs may lead to better diagnostics as well as to better therapeutic and prophylactic strategies against prion diseases.

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