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Prion biology relevant to bovine spongiform encephalopathy

J. Novakofski*, M. S. Brewer†, N. Mateus-Pinilla‡, J. Killefer*, and R. H. McCusker*

Departments of *Animal Sciences and †Food Science and Human Nutrition, University of Illinois at Urbana-Champaign 61801-4737; and ‡Illinois Natural History Survey, Center for Wildlife and Plant Ecology, Champaign, IL 61820

ABSTRACT: Bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD) of deer and elk are a threat to agriculture and natural resources, as well as a human health concern. Both diseases are transmissible spongiform encephalopathies (TSE), or prion diseases, caused by autocatalytic conversion of endogenously encoded prion protein (PrP) to an abnormal, neurotoxic conformation designated PrPsc. Most mammalian species are susceptible to TSE, which, despite a range of species-linked names, is caused by a single highly conserved protein, with no apparent normal function. In the simplest sense, TSE transmission can occur because PrPsc is resistant to both endogenous and environmental proteinases, although many details remain unclear. Questions about the transmission of TSE are central to practical issues such as livestock testing, access to international livestock markets, and wildlife management strategies, as well as intangible issues such as consumer confidence in the safety of the meat supply. The majority of BSE cases seem to have been transmitted by feed containing meat and bone meal from infected animals. In the United Kingdom, there was a dramatic decrease in BSE cases after neural tissue and, later, all ruminant tissues were banned from ruminant feed. However, probably because of heightened awareness and widespread testing, there is growing evidence that new variants of BSE are arising “spontaneously,” suggesting ongoing surveillance will continue to find infected animals. Interspecies transmission is inefficient and depends on exposure, sequence homology, TSE donor strain, genetic polymorphism of the host, and architecture of the visceral nerves if exposure is by an oral route. Considering the low probability of interspecies transmission, the low efficiency of oral transmission, and the low prion levels in nonnervous tissues, consumption of conventional animal products represents minimal risk. However, detection of rare events is challenging, and TSE literature is characterized by subsequently unsupported claims of species barriers or absolute tissue safety. This review presents an overview of TSE and summarizes recent research on pathogenesis and transmission.

Key Words: Bovine Spongiform Encephalopathy, Chronic Wasting Disease, Prion

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Introduction

Bovine spongiform encephalopathy (BSE) is one of several transmissible spongiform encephalopathies (TSE), or prion diseases, which are progressive neurological disorders thought to be caused by conversion of endogenous, host-encoded prion protein (PrP) to an abnormal conformation designated PrPsc (Prusiner, 1982; Legname et al., 2004). Prion diseases may be genetic, sporadic, or transmitted, although in all cases, progression of the disease is associated with accumulation of abnormal PrPsc conformer in the brain and subsequent neurodegeneration (Prusiner, 2004). Both the disease process and disease transmission are related to stability of the PrPsc conformer, which is markedly resistant to biological or chemical degradation. The abbreviation PrPsc refers to scrapie, the prototypical prion disease, whereas an alternative, PrPRes, refers to protease resistance. The mechanisms of prion disease transmission are of great interest because spontaneous occurrences of prion disease are rare and most cases in domestic and wild animals result from transmission. Moreover, based on DNA analyses, the AA sequence of PrP is well conserved in mammalian species; therefore, most, if not all, mammalian species are susceptible to prion diseases (Table 1) and the disease may be transmitted between species. Most prion diseases may be transmitted to laboratory animals, and substantial evidence indicates that a variant form of human Creutzfeldt-Jakob Disease (vCJD; sporadic CJD is the proto-
Table 1. Examples of transmissible spongiform encephalopathies (TSE)

<table>
<thead>
<tr>
<th>TSE disease</th>
<th>Primary occurrence</th>
<th>Known secondary occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine spongiform encephalopathy (BSE)</td>
<td>Cattle</td>
<td>Bison, eland, gemsbok, kudu, nyala, oryx, (all Bovidae); cats, goats, lab rodents, mink (repassage), pigs, sheep, humans, other primates&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Scrapie</td>
<td>Sheep, goats</td>
<td>Other primates, cattle, lab rodents, Rocky Mountain elk</td>
</tr>
<tr>
<td>Creutzfeldt-Jakob (CJD)</td>
<td>Humans</td>
<td>Other primates, lab rodents</td>
</tr>
<tr>
<td>Fatal familial insomnia (FFI)</td>
<td>Humans</td>
<td>Lab rodents</td>
</tr>
<tr>
<td>Kuru</td>
<td>Humans</td>
<td>Other primates, lab rodents</td>
</tr>
<tr>
<td>Gerstmann-Straussler-Scheinker syndrome (GSS)</td>
<td>Humans</td>
<td>Other primates, lab rodents</td>
</tr>
<tr>
<td>Variant CJD (vCJD)</td>
<td>Humans</td>
<td>Lab rodents</td>
</tr>
<tr>
<td>Chronic wasting disease (CWD)</td>
<td>Mule deer, Rocky Mountain elk, white tail deer (wild and domestic)</td>
<td>Cattle, ferrets, lab rodents (repassage), mink, squirrel monkeys, goats</td>
</tr>
<tr>
<td>Feline spongiform encephalopathy (FSE)</td>
<td>House cats, cheetah, ocelot, puma, tiger</td>
<td>Lab rodents</td>
</tr>
<tr>
<td>Transmissible mink encephalopathy (TME)</td>
<td>Domestic mink</td>
<td>Cattle, ferrets, raccoons, lab rodents</td>
</tr>
</tbody>
</table>

<sup>a</sup>Lab rodents include gerbils, guinea pigs, hamsters, mice, and rats.
<sup>b</sup>Repassage means secondary transmission after adaptation in an intermediate species.
<sup>c</sup>Other primates may include chimpanzees, macaque monkeys, marmosets, prosimians, squirrel monkeys.

Basis of Prion Disease

Transmission of TSE seem to result from three unusual characteristics of PrP<sup>Sc</sup>, although many specifics are unclear. First, PrP<sup>Sc</sup> is autocatalytic in that PrP<sup>Sc</sup> promotes conversion of additional PrP molecules into the PrP<sup>Sc</sup> conformation (Raymond et al., 2000; Prusiner, 2004). Second, PrP<sup>Sc</sup> is resistant to proteases (intracellular, intestinal, and environmental) that would normally destroy the protein (Telling et al. 1996; Riesner, 2003). This results in accumulation of degraded PrP<sup>Sc</sup> in vivo and persistence ex vivo. Third, accumulated PrP<sup>Sc</sup> results in neuronal damage and spongiform changes in the brain (Unterberger et al., 2005).

The prnp gene is present in most, if not all, wild-type mammals and is highly conserved across species (van Rheede et al., 2003). Endogenous PrP is encoded by a single exon of the prnp gene, which codes for a 256- to 264- AA precursor of approximately 28 kDa that is processed by cleavage of a 22- to 24- AA signal peptide yielding a mature protein of 231 to 253 AA (Riesner, 2003; Van Rheede et al., 2003). Species differences in PrP size are primarily a function of differences in the number of octapeptide repeat regions.

Normal PrP is a glycoprotein primarily found on nerve and immune cell membranes, although the protein is found at lower concentrations on plasma membranes. The PrP protein has O- and N-linked glycosylation sites in its precursor form (Riesner, 2003) and is found as a mixture of nonglycosylated, monoglycosylated and diglycosylated forms. Cell surface attachment occurs via a glycosyl phosphatidyl inositol anchor (Riesner, 2003). PrP is a metal-binding protein having at least one site that binds Cu<sup>2+</sup>, with a K<sub>d</sub> of 10<sup>-14</sup> M (Jackson et al., 2001). Other transition metal ions (Zn, Mn, and Ni) bind with lower affinity. Metal binding alters PrP biochemical characteristics, including protease resistance (Brown et al., 1997; Lehmann, 2002). The PrP mutations that have additional copies of the metal-binding octapeptide repeat have properties similar to PrP<sup>Sc</sup> (Lehmann and Harris, 1996). Copper binding stimulates PrP endocytosis (Pauly and Harris, 1998). In addition to extracellular function, intracellular PrP may modify properties of intracellular proteins, including antiapoptotic Bcl-2 (Kurschner and Morgan, 1995), several heat shock proteins (Edenhofer et al., 1996; Zanata et al., 2002), neuronal synapsins, and the growth factor signal adapter Grb2 (Spielhaupter and Schatzl, 2001). Knockout mice without PrP often seem to be normal, suggesting that normal PrP function may be redundant with an unidentified protein. Neurological abnormalities in some strains of PrP knockout mice result from an incomplete construct, which upregulates the adjacent dpl gene (Aguzzi, 2003). The dpl gene prod-
Figure 1. Cellular processing of PrP. 1) Cellular PrP is processed like many other membrane-associated or extracellular proteins (1). The PrP can be internalized before degradation by proteosome or lysosomal proteases. In contrast, PrP<sup>sc</sup> processing results in limited proteolysis (2). Limited degradation produces PrP<sup>sc</sup> fragments, which accumulate and presumably play a role in cell death. These fragments lead to propagation of the PrP<sup>sc</sup> infection in adjacent cells. Conversion of PrP to PrP<sup>sc</sup> occurs sequentially. A) Normal PrP can refold into PrP<sup>sc</sup> in the extracellular space. B) Fragments of PrP<sup>sc</sup> may remain within the cell or may be externalized by transport vesicles or by cellular rupture upon death. C) Intracellular PrP<sup>sc</sup> could interact with PrP during intracellular processing resulting in conversion of PrP to PrP<sup>sc</sup> in the infected cell. D) Intracellular PrP may spontaneously change conformation to PrP<sup>sc</sup>. Endogenous, host-encoded prion protein = PrP; abnormal PrP conformation = PrP<sup>sc</sup>.

Pathogenesis

Disease-associated PrP<sup>sc</sup> has undergone a conformational change from predominantly helical to predominantly β-pleated sheet (Nguyen et al., 1995; Legname et al., 2004). This modification is posttranslational, so the sequence of AA in PrP and PrP<sup>sc</sup> within an individual is identical. Spontaneous conformational change of PrP to PrP<sup>sc</sup> is thermodynamically unfavorable (Baskakov et al., 2004). Variation arising from mutations in the PrP AA sequence may make the conversion more favorable. The altered conformation renders PrP<sup>sc</sup> resistant to proteinase K activity, such that it is incompletely cleaved, resulting in several products with decreased solubility and increased tendency to aggregate (Telling et al., 1996; Riesner, 2003). Similarly, the infective PrP<sup>sc</sup> conformer is resistant to heat and chemical sterilization processes (see Decontamination section below). Protease resistance is the basis for most current methods of PrP<sup>sc</sup> detection.

Conversion of PrP to PrP<sup>sc</sup> may occur in several steps (Figure 1). The PrP<sup>sc</sup> in the extracellular spaces can interact directly with PrP, causing refolding of the latter to produce additional PrP<sup>sc</sup>. Strong evidence for this was provided by studies showing conversion of PrP by PrP<sup>sc</sup> even in the absence of cells (Raymond et al., 2000). In addition, intracellular PrP<sup>sc</sup> could interact with PrP during intracellular processing, resulting in conversion within the infected cell. Either of these processes propagates PrP<sup>sc</sup>. Experimental models indicate that elevated intracellular PrP results in incomplete degradation of PrP and conversion to PrP<sup>sc</sup>-like proteins (Chiesa and Harris, 2001). Improper or elevated retrograde transport of PrP within the cells or altered proteasomal degradation can result in accumulation of intracellular PrP, independent of the presence of PrP<sup>sc</sup> (Ma et al., 2003). However, ubiquitination of protease-resistant PrP<sup>sc</sup> is probably a late event in transmitted TSE pathogenesis rather than an important early step (Kang et
al., 2004). Extrapolating from these findings, it is possible to speculate that sporadic TSE may arise by a combination of these intracellular events.

The neurotoxic mechanism of PrP_{sc} remains unclear, with different lines of evidence supporting some combination of PrP_{sc} toxicity and alteration in normal PrP function. The presence of both PrP_{sc} and synthesis of PrP are required for pathogenesis. Inoculation of a dose of PrP_{sc} that is infectious in normal mice does not cause disease in PrP knockout mice (Prusiner et al., 1993), indicating that endogenous PrP conversion to PrP_{sc} is necessary to cause pathology. Loss of normal PrP function also is not the cause of pathology because PrP knockout mice do not develop disease. Crosslinking of PrP with antibodies in the absence of any PrP_{sc} results in rapid onset of neuronal disease (Solforosi et al., 2004), suggesting that disruption of normal PrP function or accumulation alone may result in a neurotoxic process.

Clinical signs of TSE are primarily neurological: behavior changes, impaired coordination, muscle spasms, and repetitive movements. Body wasting in spite of normal food intake is common, although obesity and abnormal glucose metabolism are observed in some strain/host combinations (Ye and Carp, 1995; Wadsworth et al., 2003). Neurological deterioration is progressive after clinical symptoms appear. Specific combinations of symptoms, referred to as TSE phenotype, reflect different pathogenic TSE strains. The TSE typically have long incubation periods of months (rodents, cats), years (cattle, sheep, deer), or decades (man), so clinical signs usually are evident in older animals. Because the sequence of PrP protein is highly conserved and most of the PrP_{sc} is eventually derived from converted host-encoded PrP, infection does not evolve a substantial immune response or inflammatory reaction in host animals (Aguzzi, 2003; Prusiner, 2004); however, chronic inflammatory conditions may modify natural PrP_{sc} transmission (Heikenwalder et al., 2005). All TSE cause spongiform changes in the brain associated with some degree of amyloid plaque development. Amyloid plaques consist of PrP_{sc} aggregates, which may be detected histologically as diffuse, condensed, or florid plaques in infected brain tissue. Specific combinations of clinical symptoms reflect the specific regions in the brain where PrP_{sc} accumulates, the characteristic pattern of damage and the nature of plaque deposition (Brown et al., 1994; DeArmond et al., 1997).

Animals may develop PrP_{sc}-based disease 1) by ingestion of PrP_{sc}; 2) by peripheral exposure to PrP_{sc}, most commonly by an iatrogenic route (surgery, cadaveric growth hormone injection, corneal transplantation); 3) by hereditary transmission as an autosomal, dominant trait; or 4) sporadically by unknown origin (Wadsworth et al., 2003; Prusiner, 2004).

Oral infection by ingestion involves transfer of PrP_{sc} from the digestive tract to the spleen or lymphoreticular system, and then to the peripheral nervous system and eventually to the brain. Infectivity is established in peripheral lymphoid organs before infective PrP_{sc} is found in the central nervous system (CNS), indicating that peripheral conversion of PrP to PrP_{sc} is necessary step for infection. There seem to be two potential routes of neuro-invasion by PrP_{sc} via lymphoid tissue: one path involving the spleen, and the other path involving visceral lymph nodes (Kimberlin and Walker, 1989). Following ingestion, PrP_{sc} may be degraded by digestive enzymes, leaving a pathogenic fragment similar to that of a proteinase K-resistant PrP_{sc} fragment. The PrP_{sc} fragment may be cotransported across the intestinal epithelial with ferritin (Mishra et al., 2004). The PrP_{sc} or PrP_{sc} fragments are transported from the intestine to secondary lymphoid organs by intestinal dendritic cells, which are specialized to acquire antigen from peripheral tissues (Huang and MacPherson, 2004). Dendritic cells from the intestine present PrP_{sc} to T and B lymphocytes within lymphoid tissues such as Peyer’s patches of the intestine or follicular dendritic cells (FDC) of the spleen, thymus, and tonsils (Mabbott and Bruce, 2003; Prinz et al., 2003). In the lymphoid tissues, PrP_{sc} molecules accumulate following conversion from the PrP conformation. Accumulation of PrP_{sc} and progression to the CNS nervous system requires B-cell cytokines, although expression of PrP on the B-cells is not necessary for transmission (Klein et al., 1997; 1998). The spleen is a rich source of B-cells, and B-cell cytokines enable splenic FDC cells to mature (Prinz et al., 2003; Aguzzi and Miele, 2004). Splenic FDC may serve as the source of PrP for peripheral PrP_{sc} formation as they express a high level of PrP (Aguzzi, 2003). Subsequent transfer of PrP_{sc} from FDC to sympathetic nerves depends on the distance between the two structures (Prinz et al., 2003). Chronic lymphocytic inflammation, which upregulates cytokines, enables PrP_{sc} accumulation in otherwise PrP_{sc} free tissues (Heikenwalder et al., 2005).

After oral inoculation, PrP_{sc} can be detected in a portion of the follicles of Peyer’s patches in the distal ileum of cattle for much of the disease progression (Terry et al., 2003). Detection of PrP_{sc} in lymphoid tissue of experimentally infected animals is possible before clinical signs are observed. In the CNS, detectable PrP_{sc} is first seen in the spinal cord, then the brain stem, and then in higher brain areas. Levels of PrP_{sc} in the brain increase exponentially following infection before the development of clinical symptoms. By the onset of clinical symptoms, PrP_{sc} titers in spleen and lymph tissues have either plateaued or decreased, although in the later stages of disease, PrP_{sc} levels are increased in Peyer’s patches. (Kimberlin and Walker, 1988; Anderson et al., 1996). Oral infectivity demonstrates that PrP_{sc} is resistant to stomach acids, mammalian digestive proteases, and in the case of ruminants, bacterial proteases.

Neural invasion by PrP_{sc} does not require exposure to lymphoid tissue. Peripheral exposure, for example through damaged skin (Taylor et al., 1996), the tongue (Bartz et al., 2003), or vascular system (Kimberlin and Walker, 1989), results in direct PrP_{sc} exposure of the
nervous system with subsequent retrograde transport to the CNS and rapid infection. Infection seems to be particularly rapid and effective by peripheral exposure of heavily innervated tissue such as the tongue (Bartz et al., 2003). There is also retrograde transmission by nerves as PrP\textsuperscript{sc} is found in sensory nerves and neuromuscular junction in the tongue of intracerebrally (i.c.) inoculated animals (Bartz et al., 2002; Mulcahy et al., 2004). Iatrogenic infection by medical procedures results from central or peripheral neural inoculation. It is worth noting that there have been more than twice as many iatrogenic CJD cases as vCJD cases. Iatrogenic prion disease transmission has been documented from exposure to neuro-surgical instruments, from injection of cadaver-derived growth hormone or gonadotrophin, and from corneal or dura tissue transplants (Brown et al., 2000; Weissmann et al., 2002).

Another route of peripheral infection is maternal transmission to the fetus or newborn. The traditional view is that scrapie is maternally transmitted to the fetus; however, it is clear that separating fetal (vertical) transmission from postnatal lateral transmission is difficult (Ridley and Baker, 1995). Transmission of PrP\textsuperscript{sc} in utero across the maternal-fetal barrier requires a placental architecture permitting relocation of infective PrP\textsuperscript{sc} and genetic susceptibility of the fetus.

**Genetics of Prion Disease**

Specific TSE phenotypes result from the combined effects of pathogenic mutations, genetic polymorphisms of \textit{prnp}, and the specific strain of infectious PrP\textsuperscript{sc}. Sporadic (spontaneous) and hereditary forms of prion disease share a common pathogenesis except that the initiating events are unknown in the first case and pre-determined by a pathogenic mutation in the second. “Sporadic” refers to development of prion disease with no familial history or apparent exogenous infection. The spontaneous conversion of PrP to PrP\textsuperscript{sc} is thermodynamically unfavorable, as indicated by the low incidence of sporadic CJD (Baskakov et al., 2004). Variation in the PrP AA sequence may make the conversion more or less favorable, thereby altering susceptibility to prion disease. There are 86 reported mutations or polymorphisms of the \textit{prnp} sequence, with at least 25 that increase risk of spontaneous PrP\textsuperscript{sc} formation, predispose the individual to exogenous infection, or both (Heaton et al., 2003; Wadsworth et al., 2003). Susceptibility may be reflected by minimal infectious dose, incubation time following exposure, or relative statistical risk in a population.

A common protein polymorphism of human PrP, methionine or valine at residue 129 (129M/V), seems to be predictive of CJD susceptibility (Figure 2). Heterozygosity at 129 protects individuals from CJD, whereas 129V homozygotes are strongly overrepresented in individuals with iatrogenic or sporadic CJD (Collinge et al., 1991; Palmer et al., 1991). All vCJD patients have the 129M homozygous genotype (Collinge et al., 1996). Besides susceptibility, PrP polymorphisms may result in different symptoms. In sporadic CJD, homozygous 129M individuals developed no amyloid plaque, whereas homozygous 129V individuals developed extensive plaques (Pickering-Brown et al., 1995).

Approximately 10 to 15% of human prion diseases are familial, meaning the individual has inherited a \textit{prnp} mutation that confers a high susceptibility. All families identified with inherited forms of prion diseases have either point mutations or insertions in the \textit{prnp} gene (Collinge and Palmer, 1994; Prusiner, 2004). Mutation D178N is found in fatal familial insomnia and familial CJD (Gambetti et al., 2003). The clinical and pathological differences between familial CJD and fatal familial insomnia, which have the same D178N mutation, are the result of the 129M/V polymorphism (Goldfarb et al., 1992). Mutations responsible for Gerstmann-Straussler-Scheinker syndrome include P102L, A117V, F198S D202N, Q212P, and Q217R (Piccardo et al., 2001).

**PrP\textsuperscript{sc} Strains: Conformation and Glycosylation**

Besides the AA sequence variation that contributes to phenotypic variation of TSE, a single PrP sequence can change into multiple PrP\textsuperscript{sc} conformers, each producing a distinct pathologic phenotype (Telling et al., 1996; Safar et al., 2000). Strain differences result from variation of the α helix to β sheet conversion within PrP\textsuperscript{sc}, as well as variation in degree of PrP glycosylation. In general, transmitted TSE strains retain their phenotypic fingerprint, so the existence of different strains of PrP\textsuperscript{sc} is an important key to understanding the risk for interspecies transmission.

At least 20 strains of scrapie have been differentiated by their incubation period, clinical symptoms, and brain lesion pattern (Bruce et al., 1994, 2003). Different strains of TSE can be serially passaged in inbred mice with no change in the TSE strain characteristics, demonstrating that strain differences may be independent of PrP amino acid sequence in the host (Bruce, 2003). Characteristics of both the hyper and drowsy hamster-adapted scrapie PrP\textsuperscript{sc} strains also can be reproduced in a cell-free conversion system, indicating the importance of PrP\textsuperscript{sc} conformation (Bessen et al., 1995). Different strains of PrP\textsuperscript{sc} generated from the same PrP precursor are cleaved at distinct sites by proteinase K, demonstrating differences in tertiary structure of PrP\textsuperscript{sc}. Variation in glycosylation patterns of PrP\textsuperscript{sc} may play a role in TSE phenotype (DeArmond et al., 1997; Hill et al., 1998), and because of the different cleavage sites, the size distribution of glycosylated proteinase K digestion fragments provides another signature of strain type (Wadsworth et al., 2003). The BSE PrP\textsuperscript{sc} contains high levels of diglycosylated and low levels of monoglycosylated protein. Some scrapie strains contain primarily monoglycosylated protein, whereas the glycosylation profile in other strains is more similar, but not identical, to BSE (Baron et al., 2004). Human prion disease also
Figure 2. Sequence alignment of mature prion proteins. AA sequences were obtained from the NCBI (2004) protein database and the Swiss-Prot/TrEMBL (2004) Protein Knowledge database and consensus sequences within a species and comparisons between species were generated in ClustalW. Sequence is indicated using single letter AA coding and a period (.) indicates an AA is not present in that species. Amino acids that are identical to those in ovine PrP are not shown. Shaded bars indicate notable domains of PrP. Proteinase K digestion of PrPsc removes much of the octapeptide repeat and a portion of the metal binding domain to leave the resistant PrP 27-30 fragment. The α1 helix is important as a probable binding site for PrPsc during catalytic conversion. The region at 165-177 is also important for PrPsc conversion. It may play a role in interspecies transmission and this region binds the putative X protein chaperone (Kiyotoshi et al., 1997; Gossert, et al., 2005). The H/R/Q polymorphism in this region at AA 171 in sheep is a major determinant of scrapie resistance. Endogenous, host-encoded prion protein = PrP; abnormal PrP conformation = PrPsc.

Diagnosis and Potential Treatment

There currently is no definitive antemortem test for TSE. Initial diagnosis of TSE is achieved through clinical examination. However, observation of neurological symptoms, such as abnormal motor movement, behavioral, and cognitive changes are indicative but not definitive because they may reflect other neurodegenerative processes (Cockcroft, 2004). In humans, ancillary techniques that can be employed to support a clinical diagnosis include electroencephalography, brain imaging, cerebrospinal fluid screening, tonsil biopsy (Kübler et al., 2003; Soto, 2004). Electroencephalography is used to identify changes in brain wave patterns that are common to, but not unique to TSE. Imaging brain structure is primarily useful to exclude other encephalopathies. Elevated 14-3-3 protein in cerebrospinal fluid is indicative of TSE but also may be increased by other causes of neurodegeneration.

Postmortem examination of brain samples is required to confirm the presence of TSE (Table 2). Histological techniques can identify the characteristic spongiform lesions, astrocytic gliosis, and (usually) plaques found in TSE. Although the antibodies used are not conformer specific, immunohistochemical staining can identify pathological accumulation of PrP, which is assumed to be PrPsc because it has accumulated. Both approaches are labor intensive. Biochemical tests using brain samples are based on differential sensitivity of PrP vs. PrPsc to proteinase K digestion. After proteinase K digestion, the remaining immunoreactive PrP is considered to be PrPsc and may be assayed by conventional techniques such as Western blotting or ELISA (Kübler et al., 2003; Soto, 2004). Commercial tests that have been approved for diagnosis of BSE or CWD may be sold only to federal or state diagnostic laboratories and are not available for research purposes.

can be phenotyped by glycosylation patterns. Sporadic and iatrogenic forms of CJD exhibit a high proportion of monoglycosylated PrPsc, whereas vCJD, exhibits a higher proportion of diglycosylated PrPsc similar to BSE (Collinge et al., 1996; Wadsworth et al., 2003). Different PrP glycoforms, as well as different amounts of PrP, are expressed in different areas of the brain and may contribute to TSE variation in neuropathology (Beringue et al., 2003). In addition, host PrP genotype has a major influence on the degree of PrPsc glycosylation contributing to variation in neuropathology on passage (Somerville et al., 2005).

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### Table 2. Prion detection methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Assay</th>
<th>Species</th>
<th>Country approval</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immunohistochemistry</strong></td>
<td>Microscopic examination of brain sections stained with antibodies for PrP protein. Antibodies are not conformation-specific but PrP does not accumulate except under pathological conditions.</td>
<td>All</td>
<td></td>
<td>Gold standard for TSE detection. Completion time: 2 to 3 d. Low sensitivity to postmortem autolysis. Detects PrP&lt;sub&gt;sc&lt;/sub&gt; deposits in brain before histopathological identification of prion-induced vacuoles.</td>
</tr>
<tr>
<td><strong>Bioassay</strong></td>
<td>Suspect brain sample injected into the brain or periphery of test animal (usually mouse). Infectivity diagnosed by clinical signs of infection and incubation period. Test results confirmed by other methods.</td>
<td>All</td>
<td></td>
<td>Completion time: several months. Lowest detection limits of all tests. Sensitivity depends mouse strain used.</td>
</tr>
<tr>
<td><strong>Histopathology</strong></td>
<td>Microscopic examination of brain sections for changes indicative of spongiform encephalopathy, vacuoles, or presence of prion-associated fibrils.</td>
<td>All</td>
<td></td>
<td>Completion time: 2 to 3 d. Proper fixation required to prevent postmortem autolysis. Visualization techniques vary from chromogenic precipitate to radio isotopes.</td>
</tr>
<tr>
<td><strong>Western blotting</strong></td>
<td>Sample is homogenized, treated with proteinase K, which degrades normal PrP, electrophoretically separated with SDS-PAGE, blotted onto a membrane, and probed with an antibody to detect the protease-resistant fragments (PrP&lt;sup&gt;sc&lt;/sup&gt;).</td>
<td>All</td>
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Animal bioassay remains the most sensitive method for detection of PrP\textsuperscript{sc} (Soto, 2004). Although time consuming and labor intensive, it is necessary to detect very low levels of infectivity. Use of animals overexpressing PrP has shortened bioassay time, whereas use of animals transgenic for another species of prion is central to interspecies transmission research. Cell culture bioassays with various cell lines, including common fibroblasts, may be used to detect PrP\textsuperscript{sc} that is not measurable with biochemical techniques (Weissman et al., 2002; Vorberg et al., 2004).

A variety of substances have been investigated to prevent transmission or arrest progress of TSE. Inhibitory compounds could interfere with PrP conformational change, PrP\textsuperscript{sc} aggregate formation, or PrP\textsuperscript{sc} protease resistance. In addition, primary therapeutic considerations are toxicity and ability to cross the blood brain barrier. Early interest focused on compounds similar to Congo red, which is used as a histological stain for PrP\textsuperscript{sc}. Compounds showing activity in vitro include Congo red (Caughey and Race, 1992; Rudyk et al., 2000), porphyrins (Caughey et al., 1998), lysosomotropics or protease inhibitors (Doh-ura et al., 2000), pyridine-based compounds (Perrier et al., 2000), branched polyamines (Supattapone et al., 2001), and phenothiazines or \textit{bis}-acridines (May et al., 2003). Several compounds have shown promise in vivo including tetraclione (Forloni et al., 2002) and quinolines (Murakami, 2002). Quinacrine (an acridine) and chlorpromazine depress PrP\textsuperscript{sc} aggregation, but they are ineffective in treating animal models (Barret et al., 2003).

Transmissible Spongiform Encephalopathies

\textit{Scrapie}

Scrapie was first reported in 1732, and it is the prototypical spongiform encephalopathy found in ovines and caprines. Scrapie refers to the pruritus-induced rubbing and scratching (scraping) that results in wool loss. It is an acquired infection with an incubation period of 2 to 5 yr and an average age of onset of clinical symptoms of 2.5 yr (Schreuder, 1994). Typically, no more than 5\% of the animals in a herd show clinical symptoms of infection.

Scrapie epidemics are self-sustaining because of horizontal transmission (animal-to-animal) and apparent environmental persistence. Contaminated pastures have caused numerous outbreaks of scrapie (Chatelain et al., 1983; Redman et al., 2002). In Iceland, eradication attempts failed when scrapie-free sheep were re-stocked onto pastures that had been kept free of sheep for 3 yr (Sigurdarson, 1991) indicating the stability of PrP\textsuperscript{sc} even in the soil. Infectious material buried in the soil loses only approximately 50\% of its infectivity in 3 yr (Brown and Gajdusek, 1991). There is limited evidence for specific mechanisms of environmental transmission, although it clearly occurs. Likely possibilities include saliva or feces (Miller et al., 2004), urine (Shaked et al., 2001), or decay residue of infectious tissues such as placenta (Table 3). Because PrP\textsuperscript{sc} is stable in the soil, animals could become infected by consumption of soil or inhalation of contaminated dust.

Scrapie PrP\textsuperscript{sc} is recoverable from lymphatic tissue, tonsils, spleen, and thymus, as well as nervous tissue. Accumulation of PrP\textsuperscript{sc} in placental tissue depends on susceptible fetal genotype and stage of gestation (Tuo et al., 2001, 2002; Andreoletti et al., 2002); however, the presence or absence of PrP\textsuperscript{sc} in placentomes of fetuses does not correlate with time to postnatal infection (Andreoletti et al., 2002). This suggests that infection does not occur in utero but is postnatal. Infection may occur from direct postnatal contact with placental tissue or from pasture contaminated with placental tissue. Scrapie has been experimentally transmitted (by i.c. inoculation) to a number of animals, including hamsters, mice, mink, ferrets, goats, monkeys, and cattle. Oral infection has been successfully demonstrated in mice (Chandler, 1963), sheep, goats (Pattison et al., 1972), and squirrel monkeys (Gibbs et al., 1980).

Variation in the coding region of the \textit{prnp} gene, previously known as the scrapie gene \textit{Sip}, influences susceptibility to scrapie. The major AA polymorphisms are A136V, H154R, and H171Q/R (Tranulis, 2002). Ani-

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**Table 2 (Continued). Prion detection methods\textsuperscript{a}**

<table>
<thead>
<tr>
<th>Method</th>
<th>Assay</th>
<th>Species</th>
<th>Country approval\textsuperscript{b}</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDI test (Improbio Technology, South San Francisco, CA)</td>
<td>Conformation-dependent immunoassay.</td>
<td>Cattle</td>
<td>European Union, United States, Japan</td>
<td>Dilution accuracy: 1:30. Detection limit: 0.5 to 5.0 pmol. Completion time: 8 h. Does not involve proteinase K.</td>
</tr>
<tr>
<td>Herd-check (IDEXX Laboratories, Westbrook, ME)</td>
<td>Conformation-dependent immunoassay.</td>
<td>Cattle, deer</td>
<td>United States</td>
<td>Completion time: 4 to 5 h Does not involve proteinase K.</td>
</tr>
</tbody>
</table>

\textsuperscript{a}See Table 1 for abbreviations. Endogenous, host-encoded prion protein = PrP; abnormal PrP conformation = PrP\textsuperscript{sc}.

\textsuperscript{b}Tests approved by the European Commission for BSE detection are based on immunodetection of the pathological PrP\textsuperscript{sc} conformer.

\textsuperscript{c}Dilution accuracy is a reflection of test accuracy with known positive samples.
Table 3. Potential infectivity of food-animal tissues$^{a,b}$

<table>
<thead>
<tr>
<th>High infectivity</th>
<th>Brain, eyes, spinal cord, skull, vertebral column, tonsil, trigeminal ganglia, dorsal root ganglia, distal ileum$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium infectivity</td>
<td>Lymph nodes, proximal colon, spleen, dura mater, pineal gland, placenta, cerebrospinal fluid, pituitary, adrenal</td>
</tr>
<tr>
<td>Low infectivity</td>
<td>Distal colon, nasal mucosa, peripheral nerves, bone marrow, liver, lung, pancreas, thymus, blood, feces, uterus$^{a,b}$</td>
</tr>
<tr>
<td>Minimal infectivity</td>
<td>Heart, kidney, mammary gland, milk, ovary, saliva, salivary gland, seminal vesicle, serum, skeletal muscle, testis, thyroid, bile, bone, connective tissue, hair, skin, urine</td>
</tr>
</tbody>
</table>

$^a$DEFRA (2004b). Based on scrapie-infected sheep and goats.
$^b$Tonsil and distal ileum classified by USDA (2004a).

mals with $prnp$ genotypes, A$^{138}$R$^{154}$Q$^{171}$/ARQ, ARQ/VRQ, and VRQ/VRQ seem to be the most susceptible to scrapie. Heterozygous AHQ or ARR animals are partially susceptible, whereas homozygous ARR sheep are clinically resistant (Hunter, 2003; Baylis and Goldmann, 2004). Sheep with a R171Q polymorphism are resistant, but not immune, to scrapie (Goldmann et al., 1991).

Scrapie is a reportable disease in the United States, and the Animal and Plant Health Inspection Service has had a voluntary scrapie-free flock certification program since October 1992. The national prevalence of scrapie is 0.2% in the United States, with approximately 84% of cases occurring in the Suffolk or Hampshire breeds (USDA, 2004d).

Bovine Spongiform Encephalopathy

First observed as a progressive neural degenerative disease in the United Kingdom in 1984, BSE was specifically diagnosed in 1987 (DEFRA, 2000). By June 1990, there were some 14,000 confirmed cases out of an estimated population of 10 million cattle in the United Kingdom. Since 1986, nearly 200,000 cases of BSE have been identified in the United Kingdom (DEFRA, 2004a). The geographic distribution of BSE has increased to 24 countries, including the United States (Office International des Epizooties, 2004).

Symptoms of BSE include abnormal gait and stumbling, hyperresponsiveness to stimuli, tremors, loss of BW (>75% of cases), aggression, licking or rubbing, and decreased milk yield (roughly 50% of cases; Cockcroft, 2004). Unlike scrapie, however, pruritus (itching or scratching) is not a common symptom (Wilesmith et al., 1988). The median age for clinical signs of natural BSE infection is 42 mo, with an incubation period of 2 to 8 yr. Animals as young as 20 mo have tested positive for BSE (DEFRA, 2004a). The anatomical locations and severity of brain lesions are similar in most cases of BSE. Vascular changes are prominent in the nucleus of the solitary tract (hindbrain, glossopharyngeal nerves), nucleus of the spinal tract of V (hindbrain, trigeminal nerves), vestibular nuclear complex (medulla; the obex is the portion of medulla or brainstem just distal to the cerebellum), central gray matter, rostral colliculus (mesencephalon; orientation reflex), and hypothalamus (Simmons et al., 1996).

Bovine spongiform encephalopathy is considered a “common source” epidemic, meaning that animals contract the disease from a common element, most likely eating feed containing rendered meat and bone meal (MBM) contaminated with bovine brain and spinal cord from infected cattle. Development of infection is related to dose and tissue PrP$^\text{sc}$ content. Semen, chemicals, autosomal inheritance, biologics, and pharmaceuticals have been ruled out as the common source (Wilesmith et al., 1988, 1991, 1992).

There is little evidence for vertical transmission of BSE from dam to fetus (Foster et al., 1999; Wrathall et al., 2002); however, calves born in close contact with BSE-infected cows may be infected within days after calving (Hoinville et al., 1995; Donnelly et al., 1997). Calves and mice fed placenta from confirmed BSE cases remained healthy (Middleton and Barlow, 1993; Hoinville et al., 1995), suggesting that maternal transfer is postnatal in cattle, although the route remains unclear. No PrP$^\text{sc}$ has been demonstrated in bovine colostrum, although PrP$^\text{sc}$ infectivity has been demonstrated in human colostrum (Tamai et al., 1992).

In the United Kingdom epidemic, BSE occurred predominantly in Friesian and Friesian crossbred cattle, although this is likely to be a function of management practices rather than genetics. There are two sequences of the bovine $prnp$ gene, one with five repeats of an octapeptide metal binding sequence and one with six (Goldmann et al., 1991), but this genotype difference does not influence the occurrence of BSE (Hunter et al., 1994). An analysis of 370 cattle in Scotland indicated similar frequencies of the two genotypes between healthy cattle and animals with BSE; however, the number of octapeptide repeats may influence rate of progression or age at onset (Castilla et al., 2004; Croes et al., 2004). Besides the octapeptide repeat, there is a 23-bp insertion/deletion polymorphism in the noncoding $prnp$ promoter region that is associated with BSE susceptibility (Sander et al., 2004). Other members of
the Bovidae family, including captive nyala, eland, kudu, gemsbok, and oryx, also are susceptible to BSE or very similar diseases (Kirkwood and Cunningham, 1994).

Specified bovine offals, which included brain, spinal cord, spleen, thymus, tonsils, and intestines, were banned from use in ruminant feed in the United Kingdom in 1989. The BSE epidemic peaked in 1992 at almost 1,000 cases per week. Subsequently, the number of positive animals decreased by 1993, which is consistent with a 2- to 8-y incubation in cattle. A large part of the decrease is attributed to the ban on feeding ruminant-derived MBM to other ruminants (Anderson et al., 1996); however, BSE cases continued to occur at a lower rate, which was attributed to cross-contamination of ruminant feed. This led to complete exclusion of MBM from all farm animal feeds in 1996. The United Kingdom stopped exporting MBM in 1996. Currently, there are approximately 5 to 10 BSE-positive cases/wk being confirmed in the United Kingdom (DEFRA, 2004a). Of these cases, more than 95% were born after the initiation of the ban on feeding ruminant-derived MBM. The small number of BSE cases continuing to occur in animals born more than 15 yr after the feed ban suggests additional routes of infection, such as horizontal transmission or environmental reservoirs.

There are several hypotheses concerning the origins of BSE. One theory is that BSE existed at very low, and therefore undetected, levels in the cattle population before 1988, as a rare sporadic disease, and that its increase in incidence in the United Kingdom resulted from coincidental changes in the industry or in the environment. Given the delay between exposure and development of clinical symptoms, British cattle were likely exposed to a TSE that resulted in disease in 1981 or 1982. This exposure corresponded to cessation of solvent extraction of fat from MBM in most rendering plants (Wilesmith et al., 1991, 1992; Figure 3). Downer cattle or cattle with “staggers” may have been infected with BSE before its recognition as a transmissible disease. A second theory suggests that BSE initially appeared as a new TSE in the 1980s, which was spread by the contemporary industry practices of low-temperature rendering and inclusion of bovine-derived MBM in bovine feed. A third theory suggests that scrapie reached infective levels through recycling in MBM ultimately adapting to the strain currently recognized as BSE. A fourth theory suggests that a new strain of scrapie that was more readily transmitted to cattle arose in sheep (DEFRA, 2004b). In fact, sheep-passaged BSE has strain characteristics similar to the original BSE, and sheep infected with BSE are not clinically distinct from sheep infected with scrapie (Hill et al., 1998).

**Atypical Strains of BSE.** Unlike scrapie, most cases of BSE seem to be a single strain based on three characteristics: 1) a typical BSE case has a high concentration of lesions in the brain stem with unremarkable olfactory bulb involvement; 2) a typical BSE case has an overrepresentation of high-molecular-mass/diglycosylated PrPsc and a lower proportion of monoglycosylated glycoforms; and 3) a typical BSE case develops very little amyloid plaque in damaged tissues.

Recently, three cases of BSE in France (Biacabe et al., 2004) and two cases in Italy (Casalone et al., 2004) have been described that were atypical based on standard strain typing using Western blotting of proteinase K-digested brain stem samples. The atypical cases from Italy also had a distribution of brain lesions different than expected from BSE. The French atypical BSE cases occurred in cattle that were older than 24 mo and that did not have clinical symptoms when tested. In these cattle, PrPsc was characterized by an unglycosylated proteinase fragment with a greater molecular weight than typical BSE, a characteristic similar to the PrPsc from cattle experimentally infected with scrapie. In contrast, the two Italian atypical cases were characterized by lower-molecular-weight unglycosylated PrPsc, similar to sporadic CJD. Moreover, mouse passages of BSE PrPsc suggest that two distinct BSE strains may exist in British cattle as well (Lloyd et al., 2004). These observations strongly suggest that BSE of cattle exists in multiple sporadic strains, as do TSE in other species. Therefore, increased surveillance is likely to detect additional BSE strains and additional cases in spite of rigorous preventative measure.

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**Figure 3.** Meat and bone meal production from rendered animal by-products.
Chronic Wasting Disease

The first recognition of CWD occurred in 1967 in captive mule deer and hybrid mule deer × white-tailed deer at a Colorado research facility (Williams and Young, 1980). By 1985, it had been identified as a TSE in free-ranging cervids, including mule deer, white tail deer, and elk in Colorado and Wyoming. Currently, CWD is found in 12 states and two Canadian provinces in both farmed-raised and wild cervid populations.

When clinical signs of CWD infection appear, cervids present behavioral changes (loss of fear to humans), progressive weight loss, excessive salivation, polydipsia, polyuria, ataxia, weakness, inability to stand, dehydration, dull hair coat, drooping head and ears, and emaciation (Williams and Young, 1980; Spraker et al., 1997). The age at onset of clinical symptoms in elk ranges from 2 to 8 yr, and the duration of symptoms ranges from 5 to 12 mo before death (Miller et al., 1998). The youngest naturally infected mule deer reported was approximately 17 mo old, whereas the youngest elk was approximately 24 mo old (Miller et al., 2000; Ball, 2002). Based on dentition, white tail deer may be infected as young as 5 to 7 mo of age (Illinois Natural History Survey, unpublished data). Amino acid polymorphisms of cervid prnp occur at Q95H, G96S, A116G, M132L, and S138N (O’Rourke et al., 1999, 2004; Johnson et al., 2003). No genotype seems to be resistant, although Q95G, S96A,116G,138 deer are less likely to have CWD, whereas elk homozygous for M132 are overrepresented among CWD-positive animals.

In CWD, microscopic lesions and pathological changes are limited to the CNS, characterized by neuronal degeneration and spongiform encephalopathy with no inflammatory response, intracytoplasmic vacuoles in neurons, and astrocytic hypertrophy and hyperplasia with occurrence of amyloid plaques (Williams and Young, 1993). Abnormal PrPSc has been demonstrated by immunohistochemistry in the brain, palatine tonsils, lymph nodes, along the small and large intestine, Peyer’s patches and spleen of affected deer (Siegurdsen et al., 1999). Accumulation in the alimentary tract and lymphoid tissue precedes accumulation in brainstem (Williams and Miller, 2002).

The origin of CWD is undetermined. Like BSE, it has been suggested that CWD originated with the transmission of scrapie from domestic sheep to cervids. Scrapie inoculation results in a TSE of elk indistinguishable from CWD (Hamir et al., 2004b); however, countries such as the United Kingdom, with more prevalent rates of scrapie, have not reported CWD. Another theory suggests that it is the result of a spontaneously occurring TSE of cervids (Williams and Young, 1980; Miller and Williams, 2004).

Like scrapie, CWD epidemics are self-sustaining in both captive and free-ranging cervid populations. It is unlikely that the primary route of CWD transmission occurs by consumption of infected tissue, although artificial feeding stations for cervids could exacerbate the problem because of environmental contamination. Epidemics in captive deer and elk provide strong evidence of horizontal transmission similar to scrapie epidemics (Miller and Williams, 2003; 2004). It is likely that transmission occurs by both direct and indirect contact. Contaminated pastures have resulted in CWD in captive herds similar to outbreaks of scrapie. Early involvement of lymphoid tissue of the alimentary tract is consistent with reported horizontal transmission from saliva or feces in contaminated pens (Miller et al., 2004).

Placental transmission remains a possible route for vertical transmission of CWD in deer (Miller and Williams, 2003); however, the specific mechanisms for transmission, including source of PrPSc, reservoirs, route of infection, and infective dose, remain unknown.

Sources of Transmissible PrPSc

The degree to which PrPSc in different tissues can transmit infection is critical to understanding risk to the livestock industry and human population because PrPSc is not destroyed by cooking or proteinases and may be infectious if consumed (Taylor, 1999). Many nonneuronal tissues, including abomasum, adrenal gland, heart, intestine, kidney, lung, lymph nodes, mammary gland, parotid gland, skeletal muscle, tonsils, spleen, and uterus, express PrP; however, PrPSc accumulation is not a direct function of PrP expression, nor is it tissue-specific (Table 3). In animals with clinical TSE symptoms, levels of PrPSc are greatest in the CNS but rarely are found in peripheral tissues other than lymph nodes, spleen, and tonsil (Horiiuchi et al., 1995; Wadsworth et al., 2001).

Infectious dose usually is expressed in terms of LD50, the amount of agent required to infect or to kill 50% of the population exposed, or in terms of infectious units, the amount required to kill one animal from a group. One picogram of PrPSc contains approximately 100 infectious units, or 10 LD50 (Brown et al., 2001). An oral LD50 of brain tissue is in the milligram range (Bolton et al., 1991). The infectivity of PrPSc-containing tissue varies depending on the route of exposure and the PrPSc content, which increases with disease progression, peaking when clinical symptoms appear (Kimberlin and Walker, 1988; Anderson et al., 1996). Intraperitoneal injection is approximately 104 times more efficient, and i.c. inoculation is approximately 109 times more efficient than oral ingestion (Prusiner, 2004). Intravenous injection requires a five- to sevenfold greater dose compared with an i.c. inoculation (Brown et al., 2001).

Meat and Bone Meal

Although there are different hypotheses concerning the origins of BSE, clearly the BSE infective agent spread through the cattle population via ruminant feeding of rendered ruminant MBM. Meat and bone meal is the rendered product from mammal tissues, including
bone, exclusive of any added blood, hair, hoof, hide, manure, stomach, and ruminal contents. It must contain a minimum of 4.0% P, and the Ca level must not exceed 2.2 times the P level. (AAFCO, 2004; CFIA, 2004). Rendering methods are not capable of fully inactivating PrP<sub>Sc</sub> (Taylor et al., 1997), and it is plausible that changes in the rendering industry concurrent with a rare event of scrapie adaptation to a bovine host resulted in the BSE epidemic. Specifically, a shift from batch rendering to continuous processing, elimination of solvent extraction with steam stripping, or a reduction in rendering temperature may have resulted in infectious MBM (Taylor et al., 1995b; Anderson et al., 1996). Before 1970, high-temperature batch rendering (of sheep carcasses) was the predominant practice in the United Kingdom. In the mid-1970s, rendering temperature was lowered, and then, during the late 1970s and early 1980s, solvent extraction, typically applied to “greaves” after the primary cooking process, was abandoned on a large scale by the United Kingdom’s rendering industry (Wilesmith et al., 1992; Taylor et al., 1997; Figure 3). This may have resulted in lower inactivation of the infectious PrP<sub>Sc</sub>, allowing it to concentrate through recycling of infected animals in MBM (Phillips et al., 2000; Horn et al., 2001).

The U.S. Food Safety and Inspection Service (FSIS) has amended meat inspection regulations to designate the brain, skull, eyes, trigeminal ganglia, spinal cord, vertebral column (excluding the vertebral and lumbar vertebrae, and the wings of the sacrum), and dorsal root ganglia of cattle 30 mo of age and older, and the tonsils and distal ileum of the small intestine of all cattle, as specified risk materials, which are inedible and prohibited for human food (USDA, 2004a). Specific methods for disposition are not prescribed by FSIS, although processors must outline methods in their HACCP plans. In addition, protein derived from mammalian tissues, with some exceptions, such as blood, milk, or any product composed entirely of porcine or equine protein, must be excluded from ruminant feed (Code of Federal Regulations, 2000).

**Stunning**

It has been shown that muscle foods may become contaminated with CNS tissue or other high-risk tissue during the slaughter or processing of cattle. Contamination can result from stunning with pneumatic or cartridge-fired penetrating bolt devices, advanced meat-recovery systems (mechanically deboned meat), inadvertent inclusion of nervous tissue during fabrication, or from contaminated equipment during fabrication (Kelley et al., 2000; Daly et al., 2002; USDA, 2004c). Air injection stunners fragment CNS tissues, which can enter the circulatory system as emboli and lodge in the capillaries of peripheral tissues, such as lung and heart, which are heavily vascularized (Schmidt et al., 1999; Horlacher et al., 2002). Experiments suggest captive-bolt stunning could distribute CNS material into lymph nodes, spleen, liver, kidney, and muscle (Daly et al., 2002). Stunning also has been shown to increase CNS content of jugular blood samples (Love et al., 2000). Central nervous system tissue has been detected at low levels (μg/g) on subprimal cuts, in ground meat, and in tissue derived from advanced meat-recovery systems in U.S. commercial plants (Schmidt et al., 2001). Nervous tissue also has been detected in whole muscle cuts from cattle harvested in European Union commercial facilities (Prendergast et al., 2004). Emboli containing CNS tissue were detected in just under 1% of slaughtered cattle (Lucker et al., 2002). Therefore, FSIS prohibited the use of penetrating captive-bolt stunning devices that inject air into the cranial cavity (USDA, 2004b).

The sawdust from carcass splitting clearly has the potential to distribute CNS tissue onto both equipment and carcasses (Schmidt et al., 2001; Lucker et al., 2002). Distribution of CNS tissue onto operators and equipment also may occur via other routes (Daly et al., 2002). Removal of cervical vertebrae and spinal canal from chuck bones before advanced meat-recovery-system processing decreases contamination with CNS tissue (Schmidt et al., 2001). Commercial kits that detect CNS tissue at levels of 1% or more in meat products (Hughson et al., 2003) will not be useful for detection because research suggests levels present in meat from commercial processing plants are in the range of 0.001% (Schmidt et al., 2001).

**Muscle Tissue**

It is generally thought that transmission of prion disease by muscle foods is unlikely (Table 3). Attempts at experimental transmission by feeding muscle from BSE-infected animals have failed (European Commission 2002a,b); however, ground meat products carry some risk from inadvertent nervous tissue contamination during slaughter or processing. The use of mechanical deboning or advanced meat recovery systems to recover tissue from bones containing nervous tissue represents the greatest risk of transmission.

Like most tissues, muscle contains PrP at roughly 2 to 10% of the concentration in brain (Moudjou et al., 2001). The PrP is likely produced by the muscle cells rather than by other cellular components because cultured myoblasts and myotubes express PrP (Brown et al., 1998). The presence of PrP<sup>Sc</sup> in muscle is unusual. Muscle containing PrP<sup>Sc</sup> has been reported from i.c.-infected transgenic mice that overexpress prnp, from infected outbred wild-type mice (Bosque et al., 2002), in orally infected Syrian hamsters (Thomzig et al., 2003; 2004), and in a subset of CJD patients (Glatzel et al., 2003; Kovacs et al., 2004). Accumulation of PrP<sup>Sc</sup> also has been reported in the skeletal muscle of tongue from hamsters following either intralingual or i.c. inoculation with transmissible mink encephalopathy (Mulpay et al., 2004). Several muscles (psoas major, supraspinatus, semimembranosus) from genetically susceptible
scrapie-infected sheep have tested positive for PrP\textsuperscript{Sc} (Andreoeletti et al., 2004). The PrP\textsuperscript{Sc} was located in muscle spindles, part of the neural reflex mechanism in muscle. Immunodetectable PrP\textsuperscript{Sc} has not been found in muscle from cattle, sheep, elk, or raccoons experimentally infected with scrapie, CWD, or mink encephalopathy by other groups (Hamir et al., 2004a). Furthermore, in small trials, PrP\textsuperscript{Sc} has not been demonstrated in muscle from BSE-infected cattle (European Commission, 2002b). Other investigators have failed to observe PrP\textsuperscript{Sc} in muscle from either CJD or vCJD patients (Head et al., 2004).

The issue of possible transmission from muscle foods is a critical one and needs additional investigation to define actual risk. Pathogenesis of PrP\textsuperscript{Sc} must be established specifically for a host species, PrP\textsuperscript{Sc} strain, and infection route because extrapolation of results from animal models and across interspecies combinations is not necessarily predictive of transmission rates (Thomzig et al., 2003). To date, no infectivity has been detected in skeletal muscle of food animals using bioassays, the current gold standard for infectivity detection, although this does not rule out detection of rare events in the future.

**Tallow and Gelatin**

Edible tallow is rendered only from the tissue of healthy bovine animals (Codex Alimentarius, 1998); however, because tallow might be rendered from infected animals without clinical symptoms, there is reason for concern. The risk seems low because epidemiological studies have failed to connect tallow consumption with BSE occurrence (Wilesmith et al., 1988). Moreover, rendering of BSE-spiked material produced tallow that was not infectious, although the MBM produced from the same material was highly infectious (Taylor et al., 1995b). Continued vigilance is warranted in light of a report that inactivation of PrP\textsuperscript{Sc} by autoclaving is less effective in the presence of lipid (Appel et al., 2001).

Two-thirds of the gelatin produced worldwide comes from cattle by-products (Schrieber and Seybold, 1993). The European Commission evaluated whether changes in temperature, pH, and fat removal during the manufacturing process produce “safe” gelatin. Both acid and alkaline processing methods to produce gelatin reduce the BSE infectivity by 10\textsuperscript{2} to 10\textsuperscript{3}-fold (Grobben et al., 2003); however, the European Commission (2002a) concluded that the process could not be modified and still produce a functional product. Because questions remain, the FDA (1997) issued *Guidance for Industry, The Sourcing and Processing of Gelatin to Reduce the Potential Risk Posed by Bovine Spongiform Encephalopathy (BSE) in FDA-Regulated Products for Human Use* to ensure the safety of gelatin. The FDA stated that there is no scientific basis for banning the use of gelatin in consumer products intended for oral consumption or cosmetic use, even if the gelatin is derived from countries reporting cases of BSE. However, products regulated by the FDA that are used for human injection, for use in or on the eye, or implantation should not be manufactured from gelatin produced using cattle from BSE-positive countries.

**Milk**

Because infective PrP\textsuperscript{Sc} is found in the lymphoreticular system and lymphocytes are found in milk, it is possible that milk may be infective. High concentrations of PrP have been found in the secretory vesicles of epithelial cells in the digestive tract, suggesting this also may occur in mammary gland epithelial cells (Fournier et al., 1998). Normal PrP is found in the mammary gland (Horiuchi et al., 1995). Taylor et al. (1995a) demonstrated that mice receiving milk from BSE-infected cows (orally, i.c., or intraperitoneally) showed no signs of neurological disease, spongiform encephalopathy, or other specific pathology after 2 yr. Colostrum, which contains different proteins from milk, also might be considered an increased risk. Human colostrum of a CJD patient was able to transmit PrP\textsuperscript{Sc} to mice (Tamai et al., 1992), although the presence of PrP\textsuperscript{Sc} in bovine colostrum has not been reported. Milk, colostrum, and mammary tissue are not currently considered to be infective (DEFRA, 2004b; Table 3). Nonetheless, this conclusion is based on relatively low sensitivity assays, so investigations began in United Kingdom in 2000 to test milk and milk components for the presence of PrP\textsuperscript{Sc} using more sensitive methods (SEAC, 2002).

**Blood**

Experiments to demonstrate the infectivity in blood from animals with prion disease have produced mixed results. Not all infected animals exhibit detectable levels of PrP\textsuperscript{Sc} in the blood nor does blood from infected animals consistently transmit disease (Ironside and Head, 2003). Transmissible PrP\textsuperscript{Sc} has been demonstrated in blood and blood components from infected mice, hamsters, guinea pigs, chimpanzees, and monkeys (Brown et al., 1998; 2001). Experiments examining BSE infectivity from blood indicate a strong likelihood that blood or blood components from some, but not all, donor animals are infectious. Hunter et al. (2002) found that sheep-adapted BSE was transmitted to host sheep by transfusion of whole blood in 17% of exposed animals, whereas scrapie transmission by the same route was approximately 19%. Llewelyn et al. (2004) found that one of 48 people became infected after receiving a transfusion from asymptomatic individuals who subsequently died with vCJD. The recipient developed vCJD 6.5 yr after transfusion and 3 yr after the donor developed symptoms. Infectivity of blood components increases with disease progression (Cervenakova et al., 2003). Transmission in animal models shows that the greatest infectivity is associated with blood cells and, in lesser amounts, with plasma fractions (Brown et al.,
1998; Cervenakova et al., 2003). Both PrP and PrP\textsuperscript{sec} bind plasminogen (Fischer et al., 2000; Maissen et al., 2001). The potential transmission of prion disease by blood is clear, although the actual risk to humans under routine medical circumstances is low (Table 3). The FDA (2002) has issued a Guidance for Industry. Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease (CJD) and Variant Creutzfeldt-Jakob Disease (vCJD) by Blood and Blood Products, which was intended to decrease the risk of prion disease transmission by blood transfusion.

**Urine**

A protease-resistant form of PrP has been found in urine of animals and humans infected with PrP\textsuperscript{sec} (Shaked et al., 2001). The protein found in urine, which seems to differ from brain-derived PrP\textsuperscript{sec}, was entitled \textit{UPrP}\textsuperscript{sec} by the authors. The \textit{UPrP}\textsuperscript{sec} was found in the urine of hamsters inoculated with scrapie, cattle with BSE, and humans with CJD but was not found in the urine of uninfected hamsters, cattle, or humans. The \textit{UPrP}\textsuperscript{sec} was found in the urine as early as 17 d after i.c. inoculation of hamsters with scrapie PrP\textsuperscript{sec} and long before clinical or pathological changes occurred. Clinical signs appeared at d 105; however, in contrast to the original brain-derived PrP\textsuperscript{sec}, the \textit{UPrP}\textsuperscript{sec} did not cause overt clinical infection, although inoculated hamsters did have \textit{UPrP}\textsuperscript{sec} in their urine, implying that \textit{UPrP}\textsuperscript{sec} was transmitted and replicated within the new host.

**Interspecies Transmission**

There is overwhelming evidence that TSE can be transmitted between species. This occurs primarily because there is a very high homology across all mammalian PrP protein sequences (Figure 4). Glycosylation sites, disulfide bonds, and trans-membrane regions are perfectly conserved in 26 mammalian species, spanning all placental orders, whereas sequences responsible for secondary structure, processing, and membrane attachment are well conserved (Krakauer et al., 1998; Van Rheede et al., 2003). The conservation of PrP among mammals implies that the protein has a useful role, although its physiological function is unclear (Oesch et al., 1991).

Interspecies infection is generally less efficient than intraspecies transmission, which is reflected by a higher LD\textsubscript{50} or delay in onset of symptoms in the first passage from a donor species to a new host species. Transmission of BSE to mice requires 1,000 times more infectivity than transmission to cattle (Bradley, 1999). These differences are referred to as a “species barrier.” However, subsequent passages within the new host species are usually more efficient, indicating PrP\textsuperscript{sec} adaptation. Species barriers are dependent on several factors, including PrP protein sequence, and the physiology of the digestive tract, immune, and nervous systems. The species barrier is most protective if the delay in onset of symptoms approaches the normal life expectancy of the host species. The likelihood of interspecies transmission may be very low or result in subclinical disease, but it is unlikely there are absolute barriers between mammalian species. There may be a substantial species barrier, but it is important to recognize that the number of documented species barriers is small, whereas the demonstrated examples of interspecies transmission are clearly increasing.

**Bovine to Livestock Transmission**

Experimental transmission of BSE to a variety of animals, including pigs, sheep, and goats, has been demonstrated (Table 1). Exposing pigs to BSE brain homogenate by intracranial, i.v., and i.p. routes resulted in TSE in approximately 1 to 3 yr, whereas short-term feeding of infected MBM did not result in disease (Wells et al., 2003). Not all exposed pigs developed clinical disease, although preclinical histopathology suggested this was a function of a long incubation period rather than complete resistance. Sheep succumb to experimental BSE challenge in approximately 500 d (Foster et al., 1993). This is similar to the time required for intraspecies scrapie transmission between sheep where no PrP sequence difference exists. Ovine PrP differs from bovine PrP at several AA besides known polymorphic sites, which suggests that susceptibility is not entirely controlled by overall sequence similarity between donor and recipient PrP. Sheep with the R171Q polymorphism are resistant but not immune to BSE (Jeffrey et al., 2001; Houston et al., 2003). Moreover, human PrP contains R171, indicating that this particular residue is not an important locus for resistance.

**Cervid to Livestock Transmission**

Circumstantial evidence as well as experimental exposure data (Hamir et al., 2001) indicate the likelihood of transmitting CWD to cattle or humans is low. Strain typing indicates that PrP\textsuperscript{sec} from BSE-infected cattle and CWD-infected deer or elk are different (Stack et al., 2004), however, interspecies transmission has been experimentally demonstrated by CWD mule deer brain tissue inoculation into cattle (Hamir et al., 2001). Similarly, CWD can be transmitted to raccoons and ferrets. Moreover, ferret-passaged CWD demonstrated an increased host range and pathogenicity. Ferret-adapted CWD PrP\textsuperscript{sec} was subsequently transmissible to hamsters and became more pathogenic with a shorter incubation period as the number of passages increased in ferrets (Bartz et al., 1998). A comparison of in vitro PrP conversion to PrP\textsuperscript{sec} suggests the likelihood of cervid-to-bovine transmission may be similar to that of bovine-to-human transmission (Raymond et al., 2000). Conversion of PrP to PrP\textsuperscript{sec} by cervid PrP\textsuperscript{sec} is most efficient for cervid PrP, moderate for ovine, and least efficient for humans or bovine PrP (Raymond et al., 2000). In this case, the number of cattle tested with CWD may be...
too small to be conclusive. Interspecies transmission of BSE occurs between bovine and human with PrP sequences that are 88% identical, whereas bovine, porcine, and cervid PrP sequences are 94% identical (Figure 2). The greater sequence similarity of cervid to livestock species, which may share pasture space and ecologies, compared with the similarity between humans and bovines, suggests the potential for CWD transmission livestock species. The threat that CWD poses was formalized in a declaration by the U.S. Secretary of Agriculture that “the possibility [that] CWD from deer and elk could cause disease in humans or in domestic livestock ... is an emergency that threatens the livestock industry of this country” (Federal Register, 2001).

Livestock to Human Transmission

Transmission of BSE to humans as vCJD may never be proven unequivocally. It is clear from TSE strain analyses that BSE can be transmitted to humans, causing vCJD, which is distinct from sporadic CJD (Collinge et al., 1996; Bruce et al., 1997; Hill et al., 1997). Of the 144 cases of vCJD reported, all the individuals that have been genotyped were homozygous 129M, which also is known to increase the risk for CJD. Evidence from transgenic mice expressing human PrP (129V and/or 129M) suggests that BSE may also be transmitted to humans but manifest as sporadic CJD (Asante et al., 2002; Wadsworth et al., 2004). Transmission of BSE to...
humans must be very inefficient considering the number of vCJD cases relative to the number of exposures. It also is likely that scrapie transmission to man is extremely inefficient because scrapie is common in sheep, whereas CJD, the most similar human TSE, occurs in roughly one in one million individuals.

Decontamination of PrP<sup>sc</sup>

One of the most striking characteristics of PrP<sup>sc</sup> is its resistance to inactivation by common cleaning and sterilization techniques (Brown et al., 1982; Taylor, 1999). Contamination with PrP<sup>sc</sup> has two components; PrP<sup>sc</sup> in organic material, and PrP<sup>sc</sup> that is tightly bound to surfaces such as stainless steel and plastic. Cleaning with conventional detergents removes organic material, and any associated PrP<sup>sc</sup>, which would otherwise inactivate or dilute sterilizing agents. Therefore, cleaning before disinfection can result in greater decontamination, even though cleaning agents do not inactivate remaining PrP<sup>sc</sup>. Inactivation of PrP<sup>sc</sup> has not been accomplished by treatment with detergents, formaldehyde, hydrogen peroxide, ethanol, ether, or acetone (Rutala and Weber, 2001).

Decontamination of PrP<sup>sc</sup> requires a one log reduction greater than the LD<sub>50</sub>. For example, if the concentration of PrP<sup>sc</sup> in infectious brain is 10<sup>6</sup> i.c. LD<sub>50</sub> per gram, a 10<sup>7</sup>, or seven-log reduction of infectivity would be required for safe disinfection. Treatment with chlorine at 1,000 to 5,000 ppm (full strength commercial bleach) for 30 min produces less than a four-log reduction in infectivity (Brown et al., 1982). Longer exposure produces minimal improvement in decontamination, whereas higher levels of chlorine (10,000 ppm) result in greater than a four-log reduction (Kimberlin et al., 1983; Rutala and Weber, 2001). Standard steam sterilization (121°C for 15 min) results in less than a three-log reduction. To effect a greater than three-log reduction requires autoclaving at 121°C or higher for more than 30 min (Kimberlin et al., 1983; Taguchi et al., 1991). Treatment with sodium hydroxide (1 N) for 15 to 30 min is effective at decontamination (Rutala and Weber, 2001). High concentrations of phenol or guanidine thiocyanate also inactivate PrP<sup>sc</sup> (Ernst and Race, 1993; Manuelidis, 1997). If conventional cleaning before disinfection can produce a four-log reduction, subsequent sterilization must produce an additional three-log reduction to produce minimally adequate decontamination of high-risk material.

Metal Surfaces

Binding of PrP<sup>sc</sup> to surgical instruments is believed to be a major source of iatrogenic transmission of CJD (WHO, 1999). The PrP<sup>sc</sup> binds to surfaces (metal and plastic) without losing infectivity (Weissmann et al., 2002). Instruments that have undergone multiple cycles of cleaning and sterilization have demonstrably resulted in cases of iatrogenic CJD (Weissmann et al., 2002). Stainless-steel-bound PrP<sup>sc</sup> may be more infective than soluble PrP<sup>sc</sup>. Stainless-steel wire (0.5 × 5 mm) exposed to PrP<sup>sc</sup> retained 10<sup>7</sup> LD<sub>50</sub> infective units and required only 5 min of i.e. exposure to cause infection (Flechsig et al., 2001). Metal-bound PrP<sup>sc</sup> seems particularly resistant to decontamination. Using a bioassay, wire contaminated with PrP<sup>sc</sup> remained infectious after cleaning with sodium hydroxide and autoclaving, conditions that would have inactivated PrP<sup>sc</sup> in tissue (Yan et al., 2004).

Environmental Persistence

It is apparent that PrP<sup>sc</sup> is extremely resistant to degradation and that environmental reservoirs capable of transmission exist; however, because laboratory detection of infectious PrP<sup>sc</sup> at low levels is very difficult, there is little additional experimental information about environmental persistence of infectious PrP<sup>sc</sup>. The first published report of PrP<sup>sc</sup> persistence is that of Brown and Gajdusek (1991), showing that scrapie-infected fluid, mixed with soil, packed into perforated Petri dishes, and embedded within soil-containing pots, retained infectivity after being buried for 3 yr. Anecdotal evidence suggests that environmental decontamination is exceedingly difficult. Facilities that have been exposed to scrapie remain infectious for long periods, and there are no published reports describing successful decontamination of a facility infected with CWD. Miller et al. (2004) recently demonstrated CWD infection from pens contaminated by carcasses or fecal material. Published risk assessments for environmental transmission of scrapie and BSE, either within or between species, are based almost exclusively on assumptions because little experimental data are available (Ridley and Baker, 1999; Cummins et al., 2002).

Implications

Most mammalian species are susceptible to transmissible spongiform encephalopathies caused by aberrant prions. Transmission between animals and species occurs via exposure to infected tissue or residue. In all cases, infectivity is concentrated in nervous tissue, which has been banned from the food and feed supply. Interspecies transmission is rare, but like intraspecies transmission, it is more likely to occur between genetically susceptible individuals. Although bovine spongiform encephalopathy may have arisen from interspecies transmission of scrapie, increasing evidence suggests that it continues to arise spontaneously, and continued surveillance will eventually detect these cases as well. Similarly, strain phenotype is largely maintained with passage through different species, suggesting that many of the multiple strains of transmissible spongiform encephalopathies in domestic animals (>20 scrapie, >3 bovine spongiform encephalopathy) probably arose independently. Together, these observations indicate that transmissible spongiform
encephalopathies cannot be eradicated. Considering the low probability of interspecies transmission and the low prion levels in nonnervous tissues, consumption of conventional animal products represents minimum risk to humans.

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