Molecular neurology of prion disease

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Prions are infectious pathogens principally composed of abnormal forms of a protein encoded in the host genome. They cause lethal neurodegenerative conditions including CJD, GSS, and kuru in humans and scrapie and bovine spongiform encephalopathy in domestic animals. Remarkably, distinct strains of prions occur despite absence of an agent-specific genome: misfolded proteins themselves may encode strain diversity – with wide implications in biology. The arrival of variant CJD, and the experimental confirmation that it is caused by infection with BSE-like prions, has focussed research on early diagnosis and treatment. Recent advances lead to considerable optimism that effective human therapies may now be developed. While several drugs have been tried in small numbers of patients, there is no clear evidence of efficacy of any agent and controlled clinical trials are urgently needed. Importantly, there is increasing recognition that fundamental processes involved in prion propagation – seeded aggregation of misfolded host proteins – are of far wider significance, not least in understanding the commoner neurodegenerative diseases that pose such a major and increasing challenge for healthcare in an ageing population.
be viral was challenged, however, both by the difficulty to directly demonstrate a virus (or an immunological response) and because the transmissible agent was resistant to treatments which inactivate nucleic acids (such as ultraviolet radiation or treatment with nuclease). These remarkable findings led to suggestions in 1966 by Tikvar Alper and others that the transmissible agent may be devoid of nucleic acid and led John Griffith to suggest in 1967 that the transmissible agent may in fact be composed entirely of protein.1 In this remarkable letter to Nature, he proposed three hypothetical mechanisms for propagation of such an agent, one of which closely mirrors current thinking: indeed his model also presciently predicted the existence of distinct strains of agent. Needless to say, such a proposal met with great scepticism at the time, in what was the heyday of the "central dogma" of biology: that DNA encodes RNA that in turn encodes protein.

More than a decade later this remarkable proposal was lent biochemical credibility by intensive purification studies allied with laborious rodent bioassay. Progressive enrichment of brain homogenates for infectivity resulted in the isolation of a protease resistant glycoprotein, designated the prion protein (PrP) by Prusiner and co-workers in 1982. This protein was the major constituent of infective fractions and was found to accumulate in affected brains and sometimes to form amyloid deposits. The term prion (from proteinaceous infectious particles) was proposed to distinguish the infectious pathogen from viruses or viroids. Prions were defined as "small proteinaceous infectious particles that resist inactivation by procedures which modify nucleic acids".

The protease resistant PrP extracted from affected brains was of 27–30 kDa and became known as PrP27–30. Determination of some of the amino acid sequence of PrP27–30 enabled production of oligonucleotides that were used to screen cDNA libraries prepared from scrapie infected hamsters. These studies led to the recovery of cognate cDNA clones by Weissmann and colleagues in 1985. While it had been previously assumed that PrP would be virally encoded, remarkably, PrP27–30 was encoded by a single copy host chromosomal gene rather than by a putative viral nucleic acid in fractions enriched for scrapie infectivity. It then became clear that PrP27–30 was derived from a larger molecule of 33–35 kDa designated PrPSc (denoting the Scapie isoform of the protein).2 The normal product of the PrP gene however is protease sensitive and was designated PrPc (denoting the Cellular isoform of the protein). No differences in amino acid sequence between PrPc and PrPSc have been demonstrated. It is therefore possible that PrPSc is extracted from affected brains as highly aggregated, detergent insoluble, material that is not amenable to high resolution structural techniques. However, Fourier transform infrared spectroscopic methods show that PrPSc, in sharp contrast to PrPc, has a high β-sheet content.3 The underlying molecular events during infection which lead to the conversion of PrPc to the scrapie agent remain ill defined. The most coherent and general model thus far proposed is that PrPSc acts as a template which promotes the conversion of PrPc to PrPSc and that the difference between these isoforms lies in their conformation and state of aggregation.

The three dimensional fold or conformation of the cellular isoform was first established by NMR spectroscopy of recombinant mouse PrP.4 Subsequent studies on hamster, human, and other mammalian PrPs show that they have essentially the same conformation. Following cleavage of signal sequences, the mature PrPc consists of an N-terminal region of about 100 amino acids (which is unstructured in the isolated molecule in solution) and a C-terminal domain, also of around 100 amino acids. The C-terminal domain is composed largely of α-helical structure (with three α-helices and a short anti-parallel β-sheet) stabilised by a single disulphide bond linking helices 2 and 3. PrP is a glycoprotein with two asparagine linked glycosylation sites and is attached to the external cell surface via a glycosylphosphatidylinositol (GPI) anchor (fig 1).

The N-terminal region contains five repeats of an 8 amino acid sequence (the octapeptide repeat region). Mutations in this region, resulting in addition of integral numbers of additional repeats, lead to forms of inherited prion disease. While unstructured in the isolated molecule, this region is very highly conserved in evolution and contains two tight binding sites for Cu2+ ions.5 It is proposed that the unstructured N-terminal region may acquire structure following copper binding and a role for PrP in copper metabolism or transport is possible. Disturbance of this function by the conformational transitions between isoforms of PrP could be involved in prion related neurotoxicity.

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MOLECULAR BIOLOGY OF PRIONS

Nature of the infectious agent

A number of converging lines of experimental evidence now firmly support the idea that prions consist principally, or entirely, of abnormal isoforms of host encoded prion protein.
of misfolded PrP monomers. Once a stable “seed” structure is formed, PrP can then be recruited, leading to an explosive, auto-catalytic formation of PrPSc.

Little is known for certain about the molecular state of the protein that constitutes the self-propagating, infectious particle itself. There are examples of infectivity in the absence of detectable PrPSc,10–12 and different strains of prions (see below) are known to differ in their degree of protease resistance. A single infectious unit corresponds to around 10^5 PrP molecules.25 It is unclear whether this indicates that a large aggregate is necessary for infectivity, or at the other extreme, whether only a single one of these PrPSc molecules is actually infectious. This relationship of PrPSc molecules to infectivity could simply, however, relate to the rapid clearance of prions from the brain known to occur following their intracerebral inoculation.

The difficulty in performing structural studies on native PrPSc has led to attempts to produce soluble β-sheet rich forms of PrP, which may be amenable to NMR or crystallographic structure determination. It is now recognised that the adage regarding protein folding: “one sequence, one conformation”, is not strictly true. Depending on solvent conditions, probably any protein chain can adopt a variety of conformations in which there is a degree of periodic order (that is, extensive regions of secondary structure). However such alternative states do not have precisely and tightly packed side chains which are the hallmark of the native state of orthodox globular proteins.

Studies on a large fragment of the human prion protein (PrP91–231) have shown that at acidic pH PrP can fold to a soluble monomer comprised almost entirely of β-sheet in the absence of denaturants.24 Reduction of the native disulphide bond was a prerequisite for β-sheet formation and these observations of alternative folding pathways dependent upon solvent pH and redox potential could have important implications for the mechanism of conversion to PrPSc. Indeed, this monomeric β-sheet state was prone to aggregation into fibrils with partial resistance to proteinase K digestion, characteristic markers of PrPSc. Unusually for a protein with a predominantly helical fold, the majority of residues in PrP91–231 have a preference for β-conformation. In view of this property, it is possible that the PrP molecule is delicately balanced between radically different folds with a high energy barrier between them; one dictated by local structural propensity (the β-conformation) and one requiring the precise docking of side chains (the native α-conformation). Such a balance would be influenced by mutations causing inherited human prion diseases.

The precise sub-cellular localisation of PrPSc propagation remains controversial. However, there is considerable evidence implicating either late-endosome-like organelles or lysosomes.19–20 The environments of these organelles are evolved to facilitate protein unfolding at low pH prior to degradation by acid activated proteases. It is possible that the α-PrP to β-PrP conversion, caused by reduction and mild acidification, is relevant to the conditions that PrPSc would encounter within the cell, following its internalisation during re-cycling. Such a mechanism could underlie prion propagation, and account for the transmitted, sporadic and inherited aetiologies of prion disease (see table 1 and fig 2). Initiation of a pathogenic self propagating conversion reaction, with accumulation of aggregated β-PrP, may be induced by exposure to a “seed” of aggregated β-PrP following prion inoculation, or as a rare stochastic conformational change, or as an inevitable consequence of expression of a pathogenic PrP mutant that is predisposed to form β-PrP. It remains to be demonstrated whether such alternative conformational states of the protein are sufficient to cause prion disease in an experimental host or whether other cellular co-factors are also required.

**Figure 2 Possible mechanism for prion propagation.** Largely α-helical PrPSc proceeds via an unfolded state (i) to re-fold into a largely β-sheet form, β-PrP (ii). β-PrP is prone to aggregation in physiological salt concentrations. Prion replication may require a critical “seed” size. Further recruitment of unfolded PrP (iii) or β-PrP monomers (iv) then occurs as an essentially irreversible process driven thermodynamically by intermolecular interactions.

**Prion strains**

In common with other pathogens, distinct naturally occurring isolates or strains are observed. However, it has been unclear how such strain properties could be encoded within an agent devoid of nucleic acids. Dickinson and colleagues isolated multiple distinct strains of naturally occurring scrapie in mice. Such strains are distinguished by their biological properties: they produce distinct incubation periods and patterns of neuropathology in inbred lines of laboratory mice (for a review, see Bruce et al26). As they can be serially propagated in inbred mice with the same Prnp genotype, they cannot be encoded by differences in PrP primary structure. Furthermore, strains can be re-isolated in mice after passage in intermediate species with different PrP primary structures.27 Conventionally, distinct strains of conventional pathogen are explained by differences in their nucleic acid genome. However, in the absence of such a scrapie genome, alternative possibilities must be considered. The protein-only hypothesis,27 if correct, must be able to explain how a single polypeptide chain could encode multiple disease phenotypes. Clearly, understanding how a protein-only infectious agent could encode such phenotypic information is of wide biological interest.

Support for the idea that strain specificity may be encoded by PrP itself was provided by study of two distinct strains of transmissible mink encephalopathy prions which can be serially propagated in hamsters, designated hyper (HY) and drowsy (DY).28 These strains can be distinguished by differing physiochemical properties of the accumulated PrPSc in the brains of affected hamsters.29 Following limited proteolysis, strain specific migration patterns of PrPSc on polyacrylamide gels were seen which related to different N-terminal ends of HY and DY PrPSc following protease treatment and implying differing conformations of HY and DY PrPSc.30

Distinct human PrPSc types have been identified which are associated with different phenotypes of CJD.31–34 (fig 3). The different fragment sizes seen on western blots following treatment with proteinase K suggests that there are several different human PrPSc conformations. However, while such biochemical modifications of PrP are clearly candidates for
the molecular substrate of prion strain diversity, it is necessary to be able to demonstrate that these properties fulfill the biological properties of strains, in particular that they are transmissible to the PrP in a host of both the same and different species. This has been demonstrated in studies with CJD isolates, with both PrPSc fragment sizes and the ratios of the three PrP glycoforms (diglycosylated, monoglycosylated, and unglycosylated PrP) maintained on passage in transgenic mice expressing human PrP. Furthermore, transmission of human prions and bovine prions to wild type mice results in murine PrPSc with fragment sizes and glycoform ratios which correspond to the original inoculum. Variant CJD (vCJD) is associated with PrPSc glycoform ratios which are distinct from those seen in classical CJD. Similar ratios are seen in cattle BSE and BSE when transmitted to several other species. These data strongly support the “protein-only” hypothesis of infectivity and suggest that strain variation could be encoded by a combination of PrP conformation and glycosylation. Furthermore, polymorphism in PrP sequence can influence the generation of particular PrPSc conformers. As PrP glycosylation occurs before conversion to PrPSc, the different glycoform ratios may represent selection of particular PrPC glycoforms by PrPSc of different conformations. According to such a hypothesis, PrP conformation would be the primary determinant of strain type with glycosylation being involved as a secondary process. However, since it is known that different cell types may glycosylate proteins differently, PrPSc glycosylation patterns may provide a substrate for the neuropathological targeting that distinguishes different prion strains. Particular PrPSc glycoforms may map most favourably in neuronal populations with a similar PrP glycoform expressed on the cell surface. Such targeting could also explain the different incubation periods which also discriminate strains, targeting of more critical brain regions, or regions with higher levels of PrP expression, producing shorter incubation periods. Recent work has shown strain-specific protein conformation can be influenced by the binding of copper and zinc to PrPSc providing a novel mechanism for post-translational modification of PrP, and for the generation of multiple prion strains.

Molecular strain typing of prion isolates now allows molecular diagnosis of vCJD and a new classification of human prion diseases with implications for epidemiological studies investigating the aetiology of sporadic CJD (fig 3). Such methods allow strain typing to be performed in days rather than the 1–2 years required for classical biological strain typing. This technique may also be applicable to determining whether BSE has transmitted to other species, thereby posing a threat to human health, for instance to sheep. Such ability of a single polypeptide chain to encode information specifying distinct phenotypes of disease raises intriguing evolutionary questions. Do other proteins behave in this way? The novel pathogenic mechanisms involved in

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Phenotype</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>Sporadic</td>
<td>Unknown: random distribution worldwide; incidence of 1–2 per million per annum</td>
<td>Sporadic CJD: sub-acute myoclonic form and range of atypical forms; multiple distinct prion strains associated with distinct clinicopathological phenotypes which include sporadic fatal insomnia</td>
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<tr>
<td>Inherited</td>
<td>Autosomal dominantly inherited conditions with high penetrance; all forms have germline PRNP coding mutations</td>
<td>Extremely variable: readily mimics familial Alzheimer’s disease and other neurodegenerative conditions; over 30 mutations identified; includes GSS, familial CJD, and fatal familial insomnia</td>
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<tr>
<td>Acquired</td>
<td>Iatrogenic infection with human prions via medical or surgical procedures: human cadaveric derived pituitary hormones, tissue grafts, and contaminated neurosurgical instruments</td>
<td>Iatrogenic CJD: typical CJD when direct central nervous system (CNS) exposure; ataxic onset when peripheral infection</td>
</tr>
<tr>
<td>Environmental exposure (presumed dietary) to BSE prion strain; probable secondary transmission via blood transfusion</td>
<td>Kuru</td>
<td>Unique to small area of Papua New Guinea; major epidemic in 1950s with gradual decline since cessation of cannibalism</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Variant CJD</td>
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Figure 3 Molecular strain typing of human prions. Western blot of brain homogenate after treatment with proteinase K shows different apparent molecular mass and glycoform ratios in patients with forms of sporadic or iatrogenic (T1–3) or vCJD (T4).
prion propagation may be of far wider significance and relevant to other neurological and non-neurological illnesses. Indeed, other prion-like mechanisms have now been described and the field of yeast and fungal prions has emerged.40 41

The “species barrier”

Transmission of prion diseases between different mammalian species is restricted by a “species barrier”42. On primary passage of prions from species A to species B, typically not all inoculated animals of species B develop disease, and those that do have much longer and more variable incubation periods than is seen with transmission of prions within the same species, where typically all inoculated animals succumb with a relatively short, and remarkably consistent, incubation period. On second passage of infectivity to further animals of species B, transmission parameters resemble within species transmissions, with most, if not all, animals developing the disease with short and consistent incubation periods. Species barriers can therefore be quantitated by measuring the fall in mean incubation period on primary and second passage, or, perhaps more rigorously, by a comparative titration study. The latter involves inoculating serial dilutions of an inoculum in both the donor and host species and comparing the doses required to kill 50% of inoculated animals (LD50). The effect of a very substantial species barrier is that few, if any, animals succumb to disease at all on primary passage, and then at incubation periods approaching the natural lifespan of the species concerned.

Early studies of the molecular basis of the species barrier argued that it principally resided in differences in PrP primary structure between the species from which the inoculum was derived and the inoculated host. Transgenic mice expressing hamster PrP were, unlike wild type mice, highly susceptible to infection with Sc237 hamster prions.43 That most sporadic and acquired CJD occurred in individuals homozygous at PRNP polymorphic codon 129 supported the view that prion propagation proceeded most efficiently when the interacting PrPSc and PrPC were of identical primary structure.44 45 However, it has been long recognised that prion strain type affects ease of transmission to another species. Interestingly, with BSE prions the strain component to the barrier seems to predominate, with BSE not only transmitting efficiently to a range of species, but maintaining its transmission characteristics even when passaged through an intermediate species with a distinct PrP gene.46 For instance, transmission of CJD prions to conventional mice is difficult, with few if any inoculated mice succumbing after prolonged incubation periods, consistent with a substantial species barrier.47 48 In sharp contrast, transgenic mice expressing only human PrP are highly susceptible to CJD prions, with 100% attack rate and consistent short incubation periods which are unaltered by second passage, consistent with a complete lack of species barrier.49 However, vCJD prions (again comprising human PrP of identical primary structure) transmit much more readily to wild type mice than do classical CJD prions, while transmission to transgenic mice is relatively less efficient than with classical CJD.46 The term “species barrier” does not seem appropriate to describe such effects and “transmission barrier” may be preferable.47 Both PrP amino acid sequence and strain type affect the 3D structure of glycosylated PrP, which will presumably, in turn, affect the efficiency of the protein-protein interactions thought to determine prion propagation.

Mammalian PrP genes are highly conserved. Presumably only a restricted number of different PrPSc conformations (that are highly stable and can therefore be serially propagated) will be permissible thermodynamically and will constitute the range of prion strains seen. PrP glycosylation may be important in stabilising particular PrPSc conformations. While a significant number of different such PrPSc conformations may be possible amongst the range of mammalian PrPs, only a sub-set of these would be allowable for a given single mammalian PrP. Substantial overlap between the favoured conformations for PrPSc derived from species A and species B might therefore result in relatively easy transmission of prion diseases between these two species, while two species with no preferred PrPSc conformations in common would have a large barrier to transmission (and indeed transmission would necessitate a change of strain type). According to such a conformational selection model47 of a prion transmission barrier, BSE may represent a thermodynamically highly favoured PrPSc conformation that is permissive for PrP expressed in a wide range of different species, accounting for the remarkable promiscuity of this strain in mammals. Contributions of other components to the species barrier are possible and may involve interacting cofactors which mediate the efficiency of prion propagation, although no such factors have yet been identified.

Recent data has further challenged our understanding of transmission barriers.40 The assessment of species barriers has relied on the development of a clinical disease in inoculated animals. However, it is now clear that subclinical prion infections are sometimes established on prion inoculation of a second species.47 Such animals may harbour high levels of prion infectivity but do not develop clinical signs of disease during a normal lifespan. The barrier to primary passage may then be to the development of rapid neurodegeneration and the resulting clinical syndrome rather than a barrier to prion propagation itself. The existence of such subclinical carrier states of prion infection has important potential animal and public health implications and argues against direct neurotoxicity of prions.48

Pathogenesis

In some experimental rodent scrapie models, as in natural sheep scrapie, infectivity is first detectable in the spleen and other lymphoreticular tissues (for a review, see Fraser et al60). Spleen titres rise to a plateau early in the incubation period, long before neuroinvasion is detectable. Central nervous system (CNS) prion replication then rises to high levels and the clinical phase follows next. The route of entry of prions following oral exposure may follow invasion of Peyers patches and other gut lymphoid tissues; the relative protease resistance of prions presumably allows a significant proportion of infectivity to survive the digestive tract. It is unclear how prions transit the intestinal mucosa, although M cells may be involved.61 It has been suggested that myeloid dendritic cells mediate transport within the lymphoreticular system (LRS).62 While mature B cells are required for peripheral prion propagation, this appears to be because they are required for maturation of follicular dendritic cells (FDCs). PrPSc accumulates in FDCs which are a long lived cell type and it is thought that they are the site of prion propagation in the spleen.55 56 However, neuroinvasion is possible without FDCs, indicating that other peripheral cell types can replicate prions.54 57 Neuroinvasion involves the autonomic nervous system innervating lymphoid tissue with retrograde spread to the spinal cord or via the vagus to the brain stem.54 55 Prions have been detected in the blood at low levels in some rodent models and experimental BSE infected primates60 62 and transmission of BSE prions between sheep by transfusion has been reported.64 Several reports of infrquent transmission from human blood to rodents have been reported (for a review, see Brown64) and there is now evidence for transmission of vCJD prion infection by blood transfusion.57 64
While prominent lymphoreticular involvement is seen in some experimental models or natural prion diseases, it is undetectable in others (for a review, see Fraser et al). Both host and prion strain effects are relevant. For example, infection of sheep with BSE prions results in a wide tissue distribution of infectivity, while infection of cattle with this strain does not, infectivity being largely confined to the CNS. In humans infected with sporadic CJD prions, infectivity is largely confined to the CNS, while in vCJD there is prominent involvement of lymphoreticular tissues.

**APPLICATION TO CLINICAL NEUROLOGY**

**Molecular classification of prion disease**

While neurologists have until recent years had to rely largely on clinical features to differentiate neurodegenerative disorders, the major advances in molecular genetics and in understanding molecular pathogenesis increasingly enable diagnosis using criteria higher in the diagnostic hierarchy of pathology. While prion diseases have traditionally been classified into CJD, GSS, and kuru, we can now divide them aetiologically into inherited, sporadic, and acquired forms (table 1) with sub-classification according to molecular criteria.

The terms CJD, GSS, and kuru simply represent clinical and/or pathological syndromes within a wider spectrum of disease and are also becoming problematic. For example the term CJD, now widely associated in the public mind with BSE, may be misleading. vCJD and familial CJD may be clinically very different to classical CJD and to each other, and of course have radically different aetiologies, both from each other and from classical sporadic CJD.

Around 15% of recognised prion disease is an inherited Mendelian disorder associated with one of the more than 30 recognised coding mutations in PRNP (for a review, see Collinge). For a single gene inherited disorder of high penetrance such as inherited prion disease, the diagnostic supremacy of direct demonstration of causative mutation by DNA analysis is clear. Indeed, the availability of such definite diagnostic markers has long allowed diagnosis of inherited prion disease in patients not only atypical on clinical grounds, but in whom classical neuropathological features are absent.

Indeed, the inherited prion diseases were the first neurodegenerative conditions for which direct gene tests were available and definitive pre-symptomatic testing was performed.

Such definite molecular genetic diagnosis has allowed recognition of a wider phenotypic range than hitherto appreciated. Kindreds with inherited prion disease have been described with phenotypes of classical CJD, GSS, and also with other neurodegenerative syndromes including fatal familial insomnia. GSS is a pathological term referring to the presence of particular amyloid plaque morphologies now recognised to a variable extent in several different inherited prion diseases. Kindreds are documented in which some individuals have the classical syndromes of CJD and GSS, while others do not fit these rubrics at all.

Cases diagnosed by PrP gene analysis have been reported which are not only clinically atypical but which lack the classical histological features entirely. Neuropathology in such patients is no longer the gold standard: rather the recognised clinicopathological manifestation of a particular inherited condition simply widens. Significant clinical overlap exists with familial Alzheimer's disease, Pick's disease, frontal lobe degeneration of non-Alzheimer type, and amyotrophic lateral sclerosis with dementia. All the inherited forms can now be definitively diagnosed and sub-classified according to pathogenic mutation.

Sporadic CJD makes up around 85% of all recognised human prion disease. It occurs in all countries with an apparently random distribution and annual incidence of 1–2 per million; hypothesised causes include spontaneous production of PrPSc via rare stochastic events, somatic mutation of PRNP, or unidentified environmental prion exposure. An association with sheep scrapie is not supported by epidemiological studies, however the lack of such evidence does not exclude the possibility that a fraction of sporadic CJD is caused by environmental exposure to animal or human prions. There is marked genetic susceptibility in sporadic CJD in that most cases occur in homozygotes at codon 129 of PRNP, where either methionine (M) or valine (V) may be encoded. Heterozygotes (MV) appear significantly protected against developing sporadic CJD. Additionally, a PRNP susceptibility haplotype has been identified indicating additional, as yet uncharacterised, genetic susceptibility to sporadic CJD at or near to the PRNP locus.

The acquired prion diseases include iatrogenic CJD and kuru, and arise from accidental exposure to human prions through medical or surgical procedures or participation in cannibalistic feasts. The two most frequent causes of iatrogenic CJD occurring through medical procedures have arisen as a result of implantation of dura mater grafts and treatment with human growth hormone derived from the pituitary glands of human cadavers. Other iatrogenic cases have resulted from corneal transplantation and use of contaminated neurosurgical instruments. PRNP codon 129 genotype is also relevant to susceptibility and incubation period.

The occurrence of cases of apparently sporadic CJD in unusually young people in the UK in 1995 led to concerns that BSE transmission to humans may have occurred. Arrival of further cases in 1996 led to the recognition of a novel clinicopathological type of human prion disease, now known as vCJD, indicating the arrival of a new risk factor for CJD in the UK. A link with BSE seemed highly likely on epidemiological grounds and this was strongly supported by experimental data, firstly from molecular strain typing studies and later by transmission studies into both transgenic and conventional mice. PRNP mutations are absent in vCJD, and all cases studied to date have been 129 MM (also unpublished data). That vCJD is caused by the same prion strain as that causing BSE in cattle, raised the possibility that a major epidemic of vCJD will occur in the UK and other countries as a result of dietary or other exposure to BSE prions and also concerns of potential iatrogenic transmission of pre-clinical vCJD via medical and surgical procedures.

That only PRNP 129 MM individuals are susceptible to BSE infection is questionable, since the other acquired human prion diseases, iatrogenic CJD and kuru, occur in all codon 129 genotypes as the epidemic evolves, with MV having the longest mean incubation periods. Human BSE infection of other PRNP genotypes may simply have a longer latency and (may also have a different phenotype).

The acquired prion diseases, such as vCJD, although not contagious in humans, are infectious diseases. In infectious disease, while again clinical and histopathological features may be key, confirmation of diagnosis, not least in life threatening conditions, is by identification of the infectious pathogen itself or a specific immune response to it. Isolation and strain typing of the pathogen is at the apex of the diagnostic hierarchy. Strain typing in particular may allow the source of an outbreak to be identified and the best available prognostic and therapeutic advice to be provided.

While it is essential to balance the potential risks and discomfort involved in an invasive diagnostic test against the improved diagnostic accuracy, it will only be by progressing steadily to greater use of molecular analysis of neurological disease that we will be able to deliver the diagnostic and...
ultimately therapeutic advances to patients with neurodegenerative diseases that are so desperately needed.

The marked phenotypic heterogeneity observed in human prion diseases has yet to be explained. However, it is likely that a significant proportion of this relates to the propagation of distinct human prion strains. The identification of strain-specific PrPSc biochemical properties now allows an aetiology based classification by typing of the infectious agent itself. Four types of human PrPSc have now been reliably identified from patients with sporadic or iatrogenic prion disease using molecular strain typing and a preliminary molecular classification proposed (fig 3). Sporadic and iatrogenic CJD are associated with PrPSc types 1–3, while type 4 human PrPSc is uniquely associated with vCJD and is characterised by a fragment size and glycoform ratio that is distinct from PrPSc types 1–3 observed in classical CJD. The M/V polymorphism at codon 129 of PRNP is associated with different PrPSc types. PrPSc types 1 and 4 have so far only been detected in MM individuals and type 3 cases are predominantly associated with at least one V allele (MV or VV), while type 2 is seen in any PRNP codon 129 genotype.

Molecular strain typing has major implications for epidemiological surveillance of sporadic CJD, whose aetiology remains obscure. While spontaneous conversion of PrPSc to a rare stochastic event, or somatic mutation of the PrP gene, resulting in expression of a pathogenic PrP mutant, are plausible explanations for sporadic CJD, other causes for at least some cases, including environmental exposure to human or animal prions, has not been ruled out by existing epidemiological studies. Sub-classification of sporadic CJD based upon PrPSc type immediately allows a more precise molecular classification of human prion disease and re-analysis of epidemiological data using these molecular sub-types may reveal important risk factors obscured when sporadic CJD is analysed as a single entity. For example, it will be important to review the incidence of sporadic CJD associated with PrPSc type 2 and other molecular sub-types in both BSE affected and unaffected countries in the light of recent findings suggesting that BSE prion infection of “humanised” transgenic mice may result in propagation of either type 4 PrPSc or type 2 PrPSc. Individuals that propagate type 2 PrPSc as a result of BSE exposure may present with prion disease that would be indistinguishable on clinical, pathological, and molecular criteria from that found in classical CJD.

Epidemiology of vCJD and other possible forms of human BSE infection

Estimates of the mean incubation period of human-to-human prion transmission come from study of growth hormone related iatrogenic CJD and kuru: in both cases estimates are of around 12 years. In kuru, incubation periods can exceed 50 years (Whitfield et al, unpublished observations). The effect of a species barrier is to considerably increase both the mean and range of incubation periods seen, which may approach the usual lifespan of the species concerned. For example, the cattle-to-mouse barrier for the BSE strain results typically in a three- to fourfold increase in mean incubation period. Mean incubation periods of human BSE infection of 30 years or more should be considered. Furthermore, prion disease in mice follows a well defined course with a highly distinctive and repeatable incubation time for a given prion strain in a defined inbred mouse line. In addition to the PrP gene, several additional genetic loci with a major effect on incubation period have now been mapped.

It can be anticipated that the human homologues of such loci may play a key role in human susceptibility to prion disease, both following accidental human prion exposure and exposure to the BSE agent. By definition, the patients identified to date with vCJD are those with the shortest incubation periods for BSE. These in turn, given that no unusual history of dietary, occupational, or other exposure to BSE has been identified, would be expected to be predominantly those individuals with short incubation time alleles at these multiple genetic loci in addition to having the 129 MM PRNP genotype. The vCJD cases reported to date may therefore represent a distinct genetic sub-population with unusually short incubation periods to BSE prions. It is possible therefore that recent estimates of the size of the vCJD epidemic based on uniform genetic susceptibility may substantially underestimate the eventual size. In this context, it will be difficult to accurately predict a human epidemic until such loci are identified and their gene frequencies in the population can be determined.

Long term transmission studies have been carried out using genetically modified mice to both characterise the distinct prion strains causing human disease and to model human susceptibility to infection with BSE and other prions. These mice express human, but not mouse, PrP. While these transgenic mouse models have been able to faithfully propagate human prion strains and recapitulate the characteristic neuropathology of vCJD, there are important caveats in extrapolating from such animal models to human susceptibility. However, these studies have found a much higher infection rate in transgenic mice expressing human PrP M129 than mice expressing human PrP V129 when challenged with either BSE or vCJD prions, and demonstrated that BSE prion infection can produce disease phenotypes resembling sporadic CJD infection of these mice and also novel prion strain phenotypes. Most recently, these studies have argued that the vCJD phenotype may only be expressed in the presence of the M form of human PrP. While this would imply that only those humans expressing human PrP M129 may develop vCJD, this does not mean that VV individuals are completely resistant to BSE prion infection, but rather that if infected they would show a different phenotype.

Early diagnosis of human prion disease

Despite the currently dire prognosis of established prion neurodegeneration, it is important to establish a firm early diagnosis. This is firstly because the differential diagnosis of prion disease includes potentially treatable conditions, for example cerebral vasculitis, and cortical biopsy, with full infection control precautions, may be considered in some patients. Secondly, although a devastating diagnosis, early confirmation removes uncertainty, which is itself distressing, obviates the need for further investigation, and allows a care plan to be established including infection control measures and appropriate counselling of patient and their family. However, the advent of the first therapeutic trials in prion disease (see below) creates a new impetus to accelerate diagnosis. Drugs that retard, or even eliminate, prion infection will not reverse existing neuronal loss and there may be little benefit in arresting prion propagation at the advanced stage of neurodegeneration that diagnosis is often made currently (table 2). Advances in early diagnosis must therefore go hand in hand with therapeutic advances to deliver real benefit to patients in the years ahead. While such effective drugs are not yet available, much research is ongoing in both diagnostics and therapeutics. However, given both the rarity and speed of evolution of prion disease, such research can only effectively progress with a high level of early referral, at a stage where the diagnosis may be very unclear, to research centres. For this reason, a UK-wide early
referral system has been established in the UK with the support of the Department of Health and the Medical Research Council. While a confident clinical diagnosis of vCJD may be possible clinically when allied with a positive pulvinar sign on MRI, this sign is not always present, may not be an early feature, and false positives have been reported. Further, while the initial patients with vCJD had a relatively uniform clinical presentation, not only are more atypical presentations now becoming apparent, but larger variability can be anticipated with involvement of additional genotypes in BSE prion infection (see above). It will be important to remain open minded about such phenotypes. The early clinical features of vCJD: depression, anxiety, behavioural change, and sensory disturbances, are highly non-specific. Differentiation from much commoner psychiatric causes requires the arrival of overtly neurological features such as ataxia, chorea, and cognitive decline although pre-existing use of neuroleptics and other psychotropic drugs may initially delay their diagnostic recognition. Current clinically based diagnostic criteria for vCJD, undoubtedly important for disease surveillance, require the evolution of disease over at least 6 months and development of several signs indicative of extensive cerebral damage (http://www.doh.gov.uk/cjd/cjd_stat.htm). However, early diagnosis, before extensive irreversible brain damage has occurred, is crucial to exclude treatable alternative diagnoses: PrP immunocytochemistry or western blot for PrPSc types 1–3.

Iatrogenic CJD
Progressive cerebellar syndrome and behavioural disturbance or classical CJD-like syndrome with history of iatrogenic exposure to human prions (pituitary derived hormones, tissue grafting or neurosurgery)
May be young
EEG, CSF, and MRI generally less helpful than in sporadic cases
PRNP analysis: no pathogenic mutations, most are 129 homozygotes
Brain biopsy in highly selected cases (to exclude treatable alternative diagnoses): PrP immunocytochemistry or western blot for PrPSc types 1–3.

vCJD (Human BSE)
Early features: depression, anxiety, social withdrawal, peripheral sensory symptoms
Cerebellar ataxia, chorea, or athetosis often precedes dementia, advanced disease as sporadic CJD
Most in young adults; however, age at onset 12–74 years seen
EEG non-specific slow waves, CSF 14-3-3 may be elevated or normal
MRI: most show high T2 weighted signal in posterior thalamus bilaterally
PRNP analysis: no mutations, all 129 MM to date
Tonsil biopsy: characteristic PrP immunostaining and PrPSc on western blot (type 4)

Inherited prion disease
Varied clinical syndromes between and within kindreds: should consider in all pre-senile dementias and ataxias irrespective of family history
PRNP analysis: diagnostic, codon 129 genotype may predict age at onset in pre-symptomatic testing

*Clinical duration typically 6 months or less but high variability: type 1 PrPSc associated with short duration (~8 weeks); ~10% have duration >2 years.

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<thead>
<tr>
<th>Table 2</th>
<th>Diagnosis of prion disease</th>
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<tbody>
<tr>
<td>Sporadic (classical) CJD</td>
<td>Rapidly progressive dementia with two or more of myoclonus, cortical blindness, pyramidal signs, cerebellar signs, extrapyramidal signs, akinetic mutism</td>
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<tr>
<td>Most cases aged 45-75</td>
<td>Serum EEG shows pseudoperiodic complexes in most cases</td>
</tr>
<tr>
<td>CSF 14-3-3 protein usually positive</td>
<td>CT and MRI normal, or atrophy, or abnormal signal basal ganglia</td>
</tr>
<tr>
<td>PRNP analysis: no pathogenic mutations, most are 129 MM (VV and MV may be of longer duration, clinically atypical, and with EEG less often positive)</td>
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<tr>
<td>Brain biopsy in highly selected cases (to exclude treatable alternative diagnoses): PrP immunocytochemistry or western blot for PrPSc types 1–3</td>
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on the basis of clinical criteria, atypical forms, which present much greater diagnostic difficulty, are not uncommon. Relatively slowly progressive forms can be difficult to differentiate from a rapidly progressive form of Alzheimer’s disease with myoclonus for example, and CSF 14-3-3, a marker of neuronal damage, may be unhelpful or misleading in these situations. The large majority of patients with sporadic CJD will be of the PRNP codon 129 MM genotype. Patients with PRNP MV or VV genotypes are more likely to have an atypical course and negative EEG and this may also represent propagation of different prion strain types (see above). Other CSF markers have been proposed including NSE, S-100b, and Titin, but again none are specific for prion disease. There is a pressing need for a non-invasive diagnostic test, ideally of blood, which may allow screening of donated blood and tissue as well as identification of asymptomatically infected and affected individuals. Low levels of PrPSc have been detected in some peripheral tissues, including muscle, in a minority of patients with sporadic CJD at autopsy. Such patients were often of long clinical duration and the
diagnostic potential of muscle biopsy in the early clinical phase may be limited. Reports of infectivity of blood from patients with classical CJD are infrequent and have not been questioned, but there is now evidence that vCJD prions may be transmissible by blood transfusion. Such evidence for human prionemia suggests that detection of PrPSc in human blood may be possible but is below the detection limit of current methods. Much research is ongoing to develop more sensitive methods including promising developments with in vitro PrPSc amplification to increase diagnostic test sensitivity. Other genetic markers may allow more precise identification of risk groups and both transcriptome and proteome analysis in sporadic and vCJD may provide disease markers.

**Presymptomatic and ante-natal testing**

Since a direct gene test has become available, it has been possible to provide an unequivocal diagnosis in patients with inherited forms of the disease. This has also led to the possibility of performing pre-symptomatic testing of unaffected but at risk family members, as well as antenatal testing. Because of the effect of PRNP codon 129 genotype on the age of onset of disease associated with some mutations, it is possible to determine within a family whether a carrier of a mutation will have an early or late onset of disease. Most of the mutations appear to be fully penetrant, however experience with some is extremely limited. In families with the E200K mutation and in D178N (fatal familial insomnia), there are examples of elderly unaffected gene carriers who appear to have escaped the disease. Genetic counselling is essential prior to pre-symptomatic testing and follows a protocol similar to that established for Huntington’s disease. A positive PrP gene analysis has important consequences for other family members, and it is preferable to have discussed these issues with others in the immediate family before testing. Following the identification of a mutation, the wider family should be referred for genetic counselling. It is vital to counsel both those testing positive for mutations and those untested but at risk, that they should not be blood or organ donors and should inform surgeons, including dentists, of their risk status prior to significant procedures as precautions may be necessary to minimise risk of iatrogenic transmission.

**Prevention and public health management**

While prion diseases can be transmitted to experimental animals by inoculation, it is important to appreciate that they are not contagious in humans. Documented case-to-case spread has only occurred by cannibalism (kuru) or following accidental inoculation with prions. Such iatrogenic routes include the use of inadequately sterilised intracerebral electrodes, dura mater and corneal grafting, and from the use of human cadaveric pituitary derived growth hormone or gonadotrophin. As discussed above, there is now evidence that vCJD prion infection is transmissible by blood transfusion. UK policy for some time has been to leucodeplete all whole blood and to source plasma for plasma products from outside the UK.

A further possible route of transmission of vCJD is via contaminated surgical and medical instruments. Prions resist conventional sterilisation methods and neurosurgical instruments are known to be able to act as a vector for prion transmission; several cases of iatrogenic transmission of sporadic CJD prions via neurosurgical instruments are documented. Recent evidence suggests that classical CJD may also be transmitted by other surgical procedures. The wider tissue distribution of prions in vCJD together with the potential that significant numbers in the population may be silently infected has considerably increased these concerns. Prions adhere avidly to stainless steel and transmit the disease readily in experimental models. Tonsillar PrPSc is readily detectable in all cases of vCJD studied at autopsy and lymphoreticular involvement is a very early feature of natural prion infection in sheep and in experimental scrapie models, where replication in the LRS is detectable early in the incubation period and rises to a plateau, which considerably precedes, and is maintained in, the clinical phase. This suggests that tonsillar PrPSc has probably been present for a considerable period, perhaps years, before clinical presentation of vCJD in humans, thereby providing the basis of prevalence screening of the general population for infection. Two anonymous screens of tonsillar and appendix tissues, removed during routine surgery, have been performed. Tonsil appears a more sensitive reporter of vCJD prion infection than appendix, and it is of concern that three positives were reported from a screen of around 12,000 largely appendix samples. A pilot scale study of 2000 tonsils found no positives, despite use of high sensitivity methods. A national scale study is now being organised by the UK Department of Health to obtain more accurate estimates of prion infection in the UK population.

Certain occupational groups are at risk of exposure to human prions, for instance neurosurgeons and other operating theatre staff, pathologists and morticians, and histology technicians, as well as an increasing number of laboratory workers. Because of the prolonged incubation periods to prions following administration to sites other than the CNS, which is associated with clinically silent prion replication in the lymphoreticular tissue, treatments inhibiting prion replication in lymphoid organs may represent a viable strategy for rational secondary prophylaxis after accidental exposure. A preliminary suggested regimen is a short course of immunosuppression with oral corticosteroids in individuals with significant accidental exposure to human prions.

**Therapeutic strategies**

All recognised prion diseases are invariably fatal following a relentlessly progressive course. However, there have been significant recent advances in understanding prion propagation and neurotoxicity and clear proof of principle studies of several therapeutic or secondary prophylactic approaches in animal models suggesting effective therapeutics for human disease is realistic.

While recent attempts at therapeutics in experimental models have focussed on drugs that target PrPSc formation, the cause of cell death in prion neurodegeneration remains unclear. A key area of controversy is whether PrPSc is itself directly neurotoxic. There are prion diseases in which PrPSc levels in brain are very low and, conversely, subclinical prion infection occurs with high levels of PrPSc and no clinical signs. Also, PrPSc itself is not directly toxic to neurons that do not express PrPSc. However, the conversion of PrPSc to PrPSc is clearly central to prion pathogenesis as mice devoid of PrP (Prnp0/0) are resistant to prion disease and do not propagate infectivity. An alternative possibility is that neurodegeneration results, at least in part, from loss of normal PrP function as it is sequestered into PrPSc aggregates, or alternatively, that a toxic intermediate (designated PrPΔ) may be formed during prion propagation. Although PrP-null (Prnp0/0) mice are essentially normal, adaptive mechanisms during neurodevelopment might compensate for loss of PrP function, masking any phenotype, whilst loss of PrPSc function in the developed, adult CNS might be deleterious. However, this alternative has been effectively excluded by the demonstration that PrP depletion in the neurons of adult mice also results in healthy animals with no evidence of neuronal loss. Importantly, this model validates PrP as a therapeutic target in prion disease. Also

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the availability of this model, in which PrP expression in neurons is lost at around 12 weeks post-natally, allows the effect of neuronal PrP ablation during established CNS prion infection to be studied. Remarkably, such mice were completely protected from the development of clinical disease and early spongiform neuropathology was reversed. These animals continued to accumulate PrPSc in non-neuronal glial cells and indeed accumulated prions to reach infectious titres seen in end stage conventional prion disease, again arguing against direct neurotoxicity of non-neuronal PrPSc. It is conceivable that reversal of early spongiosis in human disease may even allow some symptomatic recovery. Clearly, the transgenic approach used in this model to interfere with neuronal PrP expression is not applicable therapeutically in humans, but provides a clear proof of principle for agents or other methods to target PrPSc directly. One approach is the isolation of small molecule PrP ligands which bind and stabilise PrPSc,74 rendering it less available for conversion, including antibodies that bind or sequester PrPSc, or methods to down regulate PrP transcription or translation.

While the precise molecular events involved in the conversion of PrP0 to PrPSc remain ill defined, it can be argued that any ligand which selectively stabilises PrPSc should block prion propagation. The use of hydrogen/deuterium exchange to measure the extent to which regions of PrP transiently unfold argues that the conversion of PrP0 to PrPSc must proceed through a highly unfolded state that retains little organised native structure and that compounds which can bind to any ordered region of PrP0 should therefore inhibit the conversion pathway.121 The maintenance of effective brain levels of such drugs, to reduce prion propagation rates to below those of natural clearance penetration.

The advent of clinical trials in human prion disease

A variety of drugs have been tried in individual or small numbers of patients over many years. With a notable exception,126 reports have been largely anecdotal. There is no clear evidence of efficacy of any agent, and there is no doubt that controlled clinical trials are urgently needed. Such trials will be highly challenging for several reasons. Prion diseases are rare, often rapidly progressive, and always fatal which may make randomisation to placebo unacceptable. Patterns of disease overall are, however, extremely variable with clinical durations varying from weeks to >2 years in sporadic CJD, and >20 years in some inherited prion diseases.24 As “first generation” treatments proposed for prion disease are likely to have only a modest effect on disease progression, even using survival duration as an outcome measure requires study of large numbers to reliably assess efficacy. There is a lack of systematic natural history studies of disease progression and an absence of biological markers of disease activity. The high profile of these diseases often leads to media reporting of putative prion therapeutics with optimistic headline coverage leading, understandably, to patient requests for immediate access and unrealistic expectations of efficacy, particularly in patients with advanced neurodegeneration. When such promise of dramatic effect is not realised, loss of interest in participation in controlled trials may then follow, which necessarily take considerable time to be planned, peer reviewed, and ethically approved, to assess if the drug actually has a worthwhile therapeutic effect. In the UK, at the request of the Government’s Chief Medical Officer, a clinical trial protocol (http://www.controlled-trials.com/
Effective means to decontaminate prions on surgical and medical instruments will become available over the next few years and should reduce the risk of iatrogenic prion transmission. Secondary transmission of vCJD will continue until a suitable screening test for the infected state arrives.

Proof of principle studies in animal models suggest that humanised anti-PrP monoclonal antibodies could be used for passive immunisation in early pathogenesis to block neuroinvasion. This treatment could be considered for known iatrogenically infected individuals. Active human immunisation may be feasible technically but is unlikely to be pursued commercially.

While the precise molecular events in prion propagation are not clear and the atomic level structure of prions is not yet determined, it is clear that PrPSc is the essential substrate. It is also clear that interference with PrPSc expression in adult brain is without serious effect and blocks onset of neurological disease in animal models. It should be possible to identify small molecules which penetrate the CNS to bind to PrPSc and to prevent its recruitment into prions, or to use one of a number of emerging technologies to reduce PrPSc expression in brain. If such methods are able to reduce prion propagation rates to below those of natural clearance mechanisms, it ought to be possible to cure prion infection. New methods for early diagnosis, and their timely use, will be crucial, as such methods will not reverse neuronal cell loss which is appreciable or severe by the time clinical diagnosis is typically reached.

Perspectives for the decade ahead

- Effective treatment of neurodegenerative disease is one of the major challenges facing biomedical research. The focus on prion diseases following the BSE epidemic, added to the intrinsic interest in their unique pathology, has led to these diseases being amongst the best understood causes of neurodegeneration.

- It is to be hoped prion diseases remain rare. If no epidemic of BSE related human infection emerges, such advances in understanding neurodegenerative processes should have far wider value in commoner conditions, such as Alzheimer's and Parkinson's disease, also associated with aggregation of misfolded protein. Conversely, if extensive human infection with BSE prions emerges in exposed populations, and silent secondary transmission is efficient, the failure to have developed a treatment for what is, albeit inadvertently, a man-made disease, during the window of opportunity provided by the remarkable latency of these infections would not enhance public perception of biomedical research.

- Considerable uncertainty remains over the number of people pre- or sub-clinically infected with BSE prions and what proportion of these will develop clinical disease in the years ahead: the natural evolution of this infection in the UK population is likely to span decades. Identification of the key genes controlling incubation period will allow better estimates.

- Clinicians should remain alert to alternate phenotypes of infection with BSE prions, which could also manifest as sporadic CJD or novel phenotypes as well as vCJD.

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ELECTRONIC-DATABASE INFORMATION


Competing interests: none declared

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Molecular neurology of prion disease


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