PRION DISEASE: ADVANCES IN DIAGNOSIS AND TREATMENT

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Steve Dealler is a medical microbiologist with Morecambe Bay Hospitals NHS Trust. His work on the diagnosis, epidemiology and potential treatment of transmissible spongiform encephalopathies has brought him international recognition. He has been at the forefront of work on the epidemiology of human food containing the vector for bovine spongiform encephalopathy (BSE), and the development of prophylaxis against variant Creutzfeldt-Jakob disease (vCJD). He is currently working on a potential treatment, pentosan polysulphate. Here he describes the current state of knowledge in the battle against this devastating disease and the political inertia that frustrated earlier attempts to prevent the epidemic.

INTRODUCTION

Before bovine spongiform encephalopathy (BSE) appeared in 1987 a few scientists chatted around the world about a rare set of diseases, and fought amongst themselves as to their source. Now, however, in 2005, things are very different and we are fighting to handle animal and human diseases that may (or may not) cause major epidemics that are to some degree our own fault (Table 1). By 1996, the United Kingdom (UK) government was fully aware that almost the entire population had been exposed to a fatal animal condition, for which it had knowingly avoided all research into treatment or diagnosis.

Transmissible spongiform encephalopathies (TSEs)⁽¹⁾ are a group of pathological animal conditions (Table 2) in which an infectious agent gives rise, after logarithmic growth in the brain, to cerebral damage but to little sign of inflammation. No antibodies are produced to the agent in the body, and TSEs were not realised to be infective until around the middle of the last century. Transmission experiments showed that they had extremely long incubation periods, often 20% of the normal life expectancy of the animal, that the infectious agent was similar in size to a virus, and that it

could be filtered out. The rapid expansion in research due to the epidemic rise of BSE in the UK widened the understanding of all TSEs and made us realise why any treatments or diagnosis techniques we find for them may also be true for other conditions that may be infective in similar ways (eg Alzheimer's disease (AD), Parkinson's disease (PD)).

BACKGROUND AND HISTORY

It is now widely accepted that the infectious agent for TSEs (and possibly for AD and PD) is largely proteinaceous; as the misfolding of a normal protein of the body. In prion disease, the prion protein labelled as PrP^C changes to an abnormal ('disease-associated') form called the PrP^d that is not destroyed adequately by body enzymes, and this abnormal form will cause the further alteration of PrP^C to PrP^d, and build up within tissues. There is still argument as to the mode of infectivity of the agent but it can be stated that PrP^C is required for infection to take place and without it further infectivity is not found. A similar protein is found in AD called the Alzheimer precursor protein (APP) and with PD called α -synuclein (α -S).

The protein infectious form is called the prion, which is resistant to irradiation, ultraviolet light, a wide range of enzymes (including powerful peptidases), antiseptic chemicals, heat, and antiviral agents. The PrP^C is found throughout the animal kingdom and it has a series of well-preserved segments, two chains of sugars attached, two heparin binding sites, and has retained a section normally associated with Cu⁺⁺ ions. PrP^d, however, is hydrophobic in nature and tends to form into crystalloids within the tissues in which it is present. Various forms and strains of prion disease are seen for different species; these varying by incubation period, brain and peripheral tissues distribution, chemical glycoform (ie nature of the PrP^d that is produced), and different ranges of further species (usually about 50%) that may be infected by the strain experimentally.

The prion is derived from the normal PrP^C form of the protein, which is found throughout the body except on red cells, but to a much greater degree on various leukocytes, and brain cells. As such it may infect the body not only through the brain but also through peripheral tissues, which themselves become infectious. However, because of cellular turnover, the quantity of prions present in these tissues remains low and it is only in the brain, where late in the disease, PrP^d builds up to such a degree that stimulation is

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seen of microglia, the release of cytokine peptides along with other inflammatory intermediates, and as a result progressive neurocytic apoptosis takes place leading to death of the animal. If we want, we can find a similar story in both PD and AD but the certainty of infectivity is not so clear. For instance, transgenic mice carrying abnormal forms of the human PrP, the APP or α -S that are associated with familial forms of the diseases develop the pathologies spontaneously.

Diagnostics for TSEs have become important following the realisation that several of them may infect humans (or should be assumed to do so), and that, because of modern agricultural and medicinal techniques large numbers of people may be put at risk. Also, prophylactic agents and progress in potential methods of treatment for Creutzfeldt-Jakob disease (CJD) patients suggest that diagnostics may be necessary to permit the therapeutics to be used early in a symptomatic phase.

Number of infected cattle diagnosed and so not eaten in UK	Over 188,000
Number of infected cattle eaten	Approximately 800,000
Number of meals using meat from infected cattle eaten per person in UK	50 (by 1996)
Number of countries with BSE cases	23 (plus some exported from UK)
Number of countries with vCJD cases	147 in UK, 2 in USA, 1 in Canada, 8 in France
Proportion of cattle in UK from infected herds	95% (by 1995)

Table 1 BSE infection

Bovine spongiform encephalopathy, variant CJD and blood transfusion: the worries that made rapid and effective diagnostics worthwhile

BSE was first diagnosed in 1987 and was quickly realised to be rapidly rising in the UK bovine community. Epidemiology showed that the condition was spread through the feeding of infective material made from the carcass of one infected bovine to another and this was stopped in the UK in 1988. Unfortunately, because cattle die (generally) at around the age of three to six years due to BSE, but are commonly slaughtered much earlier, most of the infected animals were eaten presymptomatically. Ultimately, it was possible to say that over a million infected cattle were present in the UK, at a peak around 1991, that the maximum number of animals dying of BSE was in 1993, that over 95% of cattle in the UK were from infected herds by 1995, and that the average consumption of meals made from infected cattle was 50 per head of population by 1996. The recurrent denial by the UK Ministry of Agriculture Fisheries and Food (MAFF) that there was any risk to humans because BSE was simply scrapie in cattle (and hence would have the same infective range among other species) during this period was followed by evidence that BSE infected cats (considered immune to scrapie), monkeys, but not hamsters (common research tool for scrapie infection). The UK government also decided not to fund research into the field adequately in that it said that if it did fund aspects including diagnostic or treatment research then the people in the UK would question MAFF's policy of BSE being of no risk to humans and stop eating beef. In 1990, under pressure from the scientific society, MAFF banned the availability in food of a progressive range of central nervous system (CNS) and lymphoid tissues that were expected to carry the highest titre of infectivity. Also, all cattle with symptoms of BSE automatically became the property of MAFF and, as such, no tissues were available for research to any group outside the UK Government control.

The incubation period of a TSE is considered to be much longer when passing from one species to another, and when the dose is low or given orally, as may have been the case in BSE in human food, it was thought to be longer again. Also, the incubation period rises in proportion to the normal life expectancy of the recipient animal. BSE was considered to have infected the cattle shortly after birth (within the first seven months) and the peak incidence was at five years of age. Bovines have a natural life expectancy of 20-30 years, and humans one of 70 years (ie between two and four times higher). Hence, if passing from other humans we might expect a human incubation period of 10-20 years, but because it was passing from cattle at a low dose and by mouth this was expected to be between 20 and 40 years. As such, a peak of clinical disease in humans transferred from cattle was calculated to occur in 2010-2030.

In March 1996, a variant form of CJD (vCJD) was reported in young humans in the UK and was realised to be BSE in humans. The disease could be passed back to animals to produce the same tissue distribution of prion infection as seen when BSE was inoculated into them with the same typing of glycosylation in the prion proteins that were extracted from humans, and from the animals with BSE; a factor not true for any known form of CJD.

The initial cases of vCJD were worrying in that it was not at all clear how the first case could appear so early without representing the beginning of a very large epidemic in the UK. It was this factor, and the realisation that BSE must have been exported by the UK to countries all around the world, that caused major research progress and political action taken. At the point of writing, only 146 cases of vCJD have been reported in the UK and the case numbers are no longer rising and all but one are of a single PrP genotype. The cause for this is unclear. Unfortunately, there have been now two cases of vCJD derived from blood transfusion, one of which was of a different, more common PrP genotype, and it is realised that a further epidemic of vCJD in that genotype must be expected as a result of oral transmission of BSE.

It is because of these factors that diagnostic techniques in cattle to remove asymptomatic BSE-infected bovines from the human diet were required urgently, human diagnostic techniques were needed in that vCJD did not have classical EEG changes or its initial clinical symptoms, and blood testing systems were needed to avoid the transmission of vCJD in blood and other medical practices.

Age distribution of CJD: total number of cases since 1988.

Natural TSE	Natural host	Incubation period	Age of infection	World distribution
Scrapie	Sheep and goats	2 years	<6 months	Widespread except Australia and New Zealand
Sporadic Creutzfeldt-Jakob disease (CJD)	Humans	?>30 years	Unknown. Familial forms seen in less than 50%	Worldwide
Gerstmann-Straussler-Scheinker syndrome (GSS)	Humans	30–50 years	Familial condition	Worldwide
Fatal Familial Insomnia (FFI)	Humans	40 years	Familial condition	Worldwide
Kuru	Humans	6–>40 years	Neonate-adult	Papua New Guinea
Variant-CJD (vCJD)	Humans	?5–?40 years	Unclear, possibly teenage or younger	UK, France, Canada, USA
Bovine spongiform encephalopathy (BSE)	Bovines (+other animals fed BSE in error)	3–10 years	<7 months	UK and countries importing cattle from UK
Chronic wasting disease (CWD)	Deer and elk	?2–10 years	Various points in life	USA, Canada
Transmissible mink encephalopathy (TME)	Mink	<2 yrs	<6 months	USA, Russia, Finland

Table 2 Natural forms of prion disease

Chronic wasting disease (CWD)⁽²⁾

CWD was originally reported at Fort Collins, Colorado, in captive mule deer in the late 1960s but case numbers have expanded in deer and elk in the Rocky Mountains and are spreading both north and eastwards. This slow epidemic of what now appears to be an epidemic disease becoming endemic raises several problems in that there is no proof that the disease is not infectious to humans, and there is no clear mechanism of natural spread. A widespread attempt has been made to follow its growth by the testing of all slaughtered and 'fallen' (ie found dead) deer and elk using rapid testing methods, and the eating of nervous tissue in these animals has been warned against by officials in specific states.

SOME LIGHT AT THE END OF THE TUNNEL

For many years, the possibility of any diagnostic method before death, or treatment after symptoms had appeared, seemed minimal. The determination by the UK government not to fund any research into these aspects was changed dramatically with the appearance of vCJD in 1996, but the scientists in the field expected few opportunities of treatment or diagnosis for these cases without many years of research: it just seems that they were wrong, and that both AD and PK may well find answers from the same research.

DIAGNOSTICS⁽⁴⁾

Diagnostics for prion disease using transmission of the condition to further animals

In the past, this was looked on as the only reliable method and the minimum quantity of tissue that was found to transmit the disease to another animal of the same species by inoculation directly into the brain was said to contain 1 infection unit (IU). The reason for this technique was that the transmission from one species to another was commonly found to require 10^4 times the quantity of tissue (the 'species barrier') and when inoculated into peripheral tissues again a much higher amount might be needed (Table 3). When inoculated into the brain a short incubation period is seen, and this still represents the most sensitive method for measuring prion infectivity.

Attempts were made to pass the 'species barrier' and permit mice to be used to measure human tissue infectivity with CJD using the transgenic development of the human PrP gene in the mouse embryo. Currently this process has proved inadequate but progress is being made⁽⁵⁾.

Tissue infectivity varies during the incubation period of the disease. Following peripheral inoculation of infection into the body, infectivity is found in peripheral tissues, starting at a concentration that is dependent on the dose that was inoculated but dropping over a short period to a generally unmeasurable level. Following this, lymphoid tissue and reticuloendothelial tissues become infectious and rise relatively early in the incubation period to reach a plateau at a low level compared with the final infectivity of brain tissue. After half the incubation period the infectivity is also found in the CNS, building up in a logarithmic manner until the death of the animal.

Tissue into which inoculum injected	Dose required for transmission
Brain	1 IU
Blood	5–>10 IU
Peritoneum	>400 IU
Given orally	> 10^4 IU (lower possibly in neonates)

Table 3 Dose of infection required to transmit the disease when inoculated into various sites

Electron microscopy (EM)

Several methods are claimed to show tubulofilamentous particles present in brain that are specific to TSEs and start to appear in the tissues at around half the incubation period of the disease. However, it now seems that they do not contain immunoreactive PrP⁽⁶⁾ and hence probably show an early brain secondary pathological process. The build up of PrP^d in the brain can be shown at a later point using immunogold EM staining of 65nm sections etched in sodium periodate for 60 minutes, immunogold labelled to detect PrP using specific PrP monoclonal antibodies, and grids counterstained using uranyl acetate and lead citrate. The forms then seen under EM are called scrapie-associated fibrils (SAF), which are seen as long thin crystalloid structures.

Histopathological methods⁽²⁾

Standard staining methods can be used for the demonstration of specific histopathological changes in the brain (eg haematoxylin and eosin stains). However, tissue can be decontaminated in 96% formic acid for one hour prior to processing into paraffin wax. A problem with this technique is that, as with BSE where approximately 15% of cases were falsely misdiagnosed, the spongiform changes appear late in the incubation period and, although this is unlikely to be a problem with humans dying of CJD, it cannot be reliable with slaughtered animals that are killed when symptoms start. Also, the development of congo red stained amyloid as PrP plaques is only seen in 10% of the cases, particularly in the cerebellum in CJD and is late in disease. Many samples of the brain must be taken to be sure that spongiform changes are not present, as some parts of the brain may have no changes. In CJD, spongiform change is relatively reliable in various layers of the cerebral cortex; fine vacuole-like holes appear in the neuropil as 20-200 microns in diameter vacuoles but becoming confluent at times to create larger ones substantially distorting the cytoarchitecture. Similar vacuoles in the cytoplasm of larger cortical neurons may be seen. Cortical involvement is usually accompanied by spongiform change in the cerebellar cortex and basal ganglia. Microvacuolar change may be seen in the cerebellum. In clinical cases that have died after long periods of symptomatic disease, neuronal loss may be severe and a *status spongiosus* appears where collapse of the cytoarchitecture appears in the cortex leaving a distorted edge of gliotic tissues. The neurons also die in the basal ganglia and there is a dramatic drop in granular and Purkinje cell populations. Gliosis involving astrocytes and microglia is present throughout areas associated with neuronal loss, and microglia also increase around PrP amyloid plaques.

Immunostaining for PrP in brain and tonsil is of particular value in that an excess of PrP amyloid plaques would create a certain diagnosis. Ultrastructural and immunocytochemical studies in both human and animal prion diseases have demonstrated that microglial cells are intimately involved in PrP plaque formation, and may perhaps play a role in the processing of PrP into an amyloid structure. The appearance of PrP by immunostaining in the human tonsils or those of deer are also important in that they are found in early clinical cases of vCJD and CWD⁽⁷⁾, whereas this is not true with other forms of TSE. Third eyelid tissue biopsy in CWD is an effective and relatively simple confirmatory test.

EEG changes

A gradual loss of normal EEG patterns is seen in sporadic CJD and in 60-80% of cases generalised bi- or triphasic periodic sharp wave complexes appear with a frequency of around 1-2 per second. With clinical signs similar to sporadic CJD this would be a useful confirmation of the diagnosis. Initially, EEG changes may be unilateral, as may periodic complexes. Unfortunately, this pattern is not universally found and many other dementing illnesses also may show some similar signs⁽³⁾. The point during the clinical progression of the disease at which this periodic pattern is found may vary, and indeed may not be until very late, and hence weekly EEG tests may be needed, often an unrealistic requirement.

This periodic pattern appears less frequently in genetic or human growth hormone related CJD. It is not seen in vCJD, which even in itself may help to separate a case of sporadic from variant CJD in an older patient.

MRI scanning

Late in the clinical period of vCJD it has been noted that a reliable, symmetrical high intensity signal is seen in the pulvinar by high intensity MRI scanning⁽⁸⁾. This is not seen in

MR Scan: symmetrical high intensity signal is seen in the pulvinar

Company source of test	Speed of test	Complexity of test	Specificity	Cost	Experience in veterinary use
Enfer (Abbott, Abbott Park, Illinois, USA)	Slow	Complex because of proteinase K step	++	Medium	Medium
BioRad (Hercules, California, USA)	Slow	Complex because of proteinase K step	++	Relatively high because of equipment	Very high
Idexx (Westbrook, Maine, USA)	Rapid	Simple because of Seprion ligand used	+++	Relatively low price because of complex equipment not needed	Medium (large amounts in CWD)
InPro (San Francisco, California, USA)	Slow	Complex	+++	Computerised equipment needed	Low
Prionics (Zurich, Switzerland)	Slow	Relatively simple because of specific anti-PrP ^d used	+++ because Western blot available also for confirmation	Relatively high because of antibody cost High	High

(Poor comparison-related assessments have been carried out. All have high false-negative rate due to low level of prions present in early stages of disease)

Table 4 Comparison of licensed rapid brain tissue tests for PrP^d that are used commercially for the testing of asymptomatic cattle for BSE

Change that is reported	Tissue source or method	Change seen	Availability of test
14-3-3 protein (23)	CSF	Increased in CJD but not all TSEs	Neuropathology Case Western Reserve University Cleveland, Ohio or CJD Surveillance Unit, Edinburgh
Glial fibrillary acidic protein (GFAP), protein S-100B, neuron specific enolase (NSE), myelin basic protein (MBP)	CSF	GFAP is more available than the others by ELISA assay, all of which are associated with brain damage and gliosis	Neurological research groups
Fatty acid binding protein (FABP)	Blood/CSF	May be increased in TSE conditions: further research required	Research groups
PrP or interferon gamma (IFN gamma), or the laminin receptor (LR) or the laminin receptor precursor (LRP)	Blood	Altered in clinical conditions of TSE as a range of changes	Research groups (Proteome Sciences, UK)
Erythroid differentiation related factor (EDRF)	Blood	Low in scrapie and possibly other TSEs	Not commercially available. Research at Roslin Institute, Edinburgh.
Infra-red spectra for plasma	Blood	Plasma from BSE asymptomatic cattle shown to have changes in the IR spectra of their plasma	Roche Inc (the test is unavailable currently)
Vaso-vagal reflex		Loss of heart rate changes with breathing during progression of TSE	Tsens Ltd, UK

Table 5 Non-specific tests for TSE also used to follow disease progression

other forms of human disease although MRI sequences, only as diffusion-weighted images (DWI) of the cortex, showed unequivocal pathology in CJD and clear atrophy was only seen late in disease with a long clinical period.

Rapid specific diagnostic techniques

a) Brain samples⁽⁹⁾

It was realised that cattle incubating BSE were still reaching human food in Europe and that the prions would not be destroyed by cooking processes. As such, several technical methods were developed that would indicate PrP^d in the brain of the slaughtered animal in such a manner that would permit the carcass, kept overnight, to be destroyed the next day when the result was ready, and not reach the human diet. Several companies are now selling assessed and licensed methods for testing brain tissue derived from the base of the brain and obtained as a sample through the foramen magnum (Table 4). The tests are expected to become positive probably some time between 50% and 75% of the clinical incubation period.

b) Blood samples

No rapid technique is currently commercially available in indicating PrP^d in blood. However, several methods are

currently well advanced in development. For further information on these, contact Microsens Biotechnology in the UK or BioMerieux from France. We now realise that the tests become positive relatively early in the incubation period of the disease, and that extremely small amounts of blood can be used for the tests. Modifications of these tests may actually be able to look for AD and PD.

Non-specific diagnostic techniques^(10,11)

Late in the incubation period of prion disease, the pathological processes in the brain and elsewhere cause local release of cytokines and alterations in the relative quantities of proteins in the cerebro spinal fluid (CSF) and blood (Table 5). The major problem for all of these tests is that the findings may also be found in other conditions that may give similar symptoms to CJD. However, they may be of value in following the progress of the patient's illness, and potentially the value of treatment. Also, because the tests in blood and the vasovagal response test are so easily carried out, they may be used to help in diagnosis in clinically ill patients.

Genetic PrP changes association with prion disease and glycoside changes found in some clinical strains

In humans, the prion protein with 253 amino acids is coded on the short arm of chromosome 20 from its specific gene (PRNP), and small changes in amino acids of the PrP^C are seen between mammals. Small, but specific, changes are seen in familial forms of the disease, Gerstmann-Straussler-Scheinker syndrome (GSS) and Fatal Familial Insomnia (FFI). In humans, there is a specific polymorphic mutation at codon 129 giving rise to either a valine or methionine amino acid at the peptide site and both a wide change in the incubation period and clinical symptoms of the CJD results.

Glycosylation changes⁽¹²⁾ in the ratio of PrP^d carrying 2, 1 or 0 glycosyl chains have been reported in different strains of CJD, vCJD, TME and scrapie. The importance of this glycoform research data is realised as a change in some sheep scrapie, which has now been suggested to be BSE transmission to sheep.

PRION DISEASE TREATMENT AND HOPES FOR THE FUTURE

Prions and the amyloids associated with AD and PD only seem to have their effect when relatively large amounts are present in the brain, and hence, by the time that we see symptoms starting to appear, any treatment would need, not only to stop their production, but also to stop the inflammatory reaction to the ones that had already formed.

As such it was not surprising that no treatments had been found to have any effect on the progress of the symptomatic disease. Anti-viral drugs, anti-cancer drugs, and anti-bacterials all showed no effect.

Of the few pharmaceuticals that appeared to make some difference were a small range of anti-inflammatory cyclooxygenase-1 inhibitors (eg indomethacin), and anti-superoxide agents (eg vitamin E). In CJD, an effect of the German equivalent of memantine is seen to slow the progression of the disease and now has shown a similar effect in AD.

One drug, quinacrine, was found to stop the production of prions *in vitro* and was rapidly used to treat CJD patients. At this point, over 500 have received treatment and no advantage has been seen from it.

A second drug, pentosan polysulphate, also seen to work *in vitro* but also in mice with scrapie when inoculated directly into the lateral ventricle of the brain, has now been used in around 12 CJD cases⁽¹³⁾. Encouraging results are being seen; it seems to stop the progress of the disease or at least slow it down, but further research is required.

A RAY OF HOPE?

In the Morecambe Bay area we are not seeing very many cases of vCJD and it will be a long time before any certainty in treatment in the use of pentosan polysulphate is with us. However, the mechanism by which it works is through attachment of the drug to the heparan binding site of the prion and stopping it from causing malformation of further proteins. As it happens, both APP and α -S also have similar heparan binding sites and would be affected in a similar manner.

Pentosan polysulphate inhibits cytokine activity within the brain and hence is expected to stop the cellular damage that is seen as the prion condition progresses and, as this is a similar process that takes place in both AD and PD, the hope is that this drug will work to some degree in these conditions also.

So, things are progressing with an obscure illness seen in small numbers, but it may shed light onto the widespread conditions that we all see regularly, and the tests and treatments that may come forward are not all that far away.

Prion n.

An abnormal form of a constituent protein (PrP) of brain cells. Prions are produced by mutations in the gene that codes for PrP and are very stable: they are not removed by the normal cellular processes of degradation and are resistant to radiation. They are believed to interact with normal PrP in such a way as to convert it to the abnormal form, which accumulates in the brain.

*Oxford Concise Colour Medical Dictionary. 3rd Ed.
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FURTHER READING

For further images and comparisons with other neurological pathology see <http://www.cjd.ed.ac.uk/path.htm>. However, it should be remembered that there is a poor relationship between the course of disease and pathology distribution.