Contributions to Microbiology
Editors: A. Schmidt, H. Herwald
Vol. 11

Prions

A Challenge for Science, Medicine and the Public Health System

2nd, revised and extended edition

Editors
H.F. Rabenau
J. Cinatl
H.W. Doerr

KARGER

Prions (2nd, revised and extended edition)

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Vol. 11

Series Editors

Axel Schmidt *Wuppertal*
Heiko Herwald *Lund*

Prions

A Challenge for Science, Medicine and the Public Health System
2nd, revised and extended edition

Volume Editors

Holger F. Rabenau *Frankfurt/Main*
Jindrich Cinatl *Frankfurt/Main*
Hans Wilhelm Doerr *Frankfurt/Main*

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'Contributions to Microbiology and Immunology'

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Dedication in Remembrance of *Andreas Scheid* (1941–2001)

Andreas Scheid, PhD, MD, University Professor, virologist and physician, was an outstanding scientist in influenza research at Rockefeller University, USA, and later on the Director and Head of the Institute of Medical Virology at the Heinrich Heine University of Düsseldorf, Germany. He also had a lot of interests, especially in cultural aspects, such as being an dedicated musician.

Andreas, you left us far too early due to a severe, very long-lasting and painful disease. I am very indebted to you as my personal teacher in virology. You gave me a lot of advice in virology, especially in our exchanges from physician to physician and as a personal friend.

Axel Schmidt
Series Editor

Note of the Series Editor

New emerging pathogens and/or infectious agents are a main scope of the Karger book series *Contributions to Microbiology*.

Prion-associated diseases such as BSE and other spongiform encephalopathies are a major and worldwide focus of interest at the moment, ranging from sciences, over politics and public media, to the 'worried' consumer of meat and meat-associated products, as probably the majority of us are.

I would like to express my special thanks to the highly competent and experienced volume editors Holger F. Rabenau, Jindrich Cinatl and Hans Wilhelm Doerr, all at the Institute of Medical Virology, Johann Wolfgang von Goethe University, Frankfurt am Main, Germany, who already edited the first and extremely successful 7th volume of *Contributions to Microbiology*, published in 2001. They convinced and engaged outstanding and internationally recognised scientists and experts in the corresponding fields to contribute excellent chapters for the present volume. Due to the tremendous success and acceptance of the initial exciting volume of *Contributions to Microbiology*, the preparation of a second, completely revised, updated and extended edition became mandatory. Again to the editors: my personal thanks to you for once more taking on this new challenge. Moreover, I also personally thank Karger Publishers for their kind and very supportive and professional collaboration with the project.

This edition of the volume provides a very comprehensive and updated source of information on all aspects of prions and prion-associated diseases that has been derived from very different viewpoints concerning spongiform encephalopathies. Many novel and 'up-to-date' aspects are presented which have not been included in other textbooks so far and in such a comprehensive way, which in turn will inevitably lead to a higher and more sophisticated awareness of the present situation concerning the 'prion problem'.

Axel Schmidt, Series Editor
Witten/Herdecke, November 2003

Preface to the Second Edition

This book was initiated and edited by general microbiologists or virologists, who had numerous questions concerning prion origin and replication, virulence and pathogenicity, diagnosis and therapy, transmission and susceptibility, as well as inactivation and prevention. Although the rapid progress of research had outdated the 'state of the art' at the time of publication, those questions were of such common interest that the book was soon sold out. Now, three years later, a second approach to these issues is presented. The protein-only hypothesis on prions is now commonly accepted, although there are some reservations. Therefore, the contribution dealing with arguments against this hypothesis was not rewritten. While the BSE epidemic has declined, the number of new, i.e. variant Creutzfeldt-Jacob disease cases has risen. Although it will probably not reach the features of an epidemic in humans, the challenge for science, medicine and public health is always present. After the first American case of BSE emerged in a cow that had not been imported, the question about an endogenous or exogenous origin of disease has again been raised. Our knowledge of prion biology has considerably improved covering both human, animal and even microbe affections. Beside some insights in basic research, the second edition emphasizes practical aspects of fighting human and animal prion diseases. Additionally, chapters on public regulations of this issue and on veterinary measures were included. The volume editors wish to thank Karger Publishers and the series editor of *Contributions to Microbiology* for encouraging this second edition.

Frankfurt am Main, November 2003 — The Volume Editors

Preface to the First Edition

After the great successes of modern hygiene and medical microbiology, many people thought the threat of infectious diseases had disappeared. However, emerging and re-emerging infections with more or less pathogenic potential disproved this opinion. The onset of the AIDS epidemic has brought the old problem of *slow virus diseases* to general attention. For many years, it had been postulated that some of those diseases, particularly of the brain, were caused by unconventional viruses. Infections with these agents do not induce classical inflammations – acute or chronic encephalitis due to a specific immune reaction – but a slow and irreversible degeneration of the central nervous system presenting as encephalopathy. Those diseases had been considered rare events in animals and humans, only interesting medical and veterinary doctors and scientists – scrapie in sheep and Creutzfeldt-Jakob disease (CJD) in man were known only to specialists. The onset of the bovine spongiform encephalopathy (BSE) epidemic in Great Britain completely changed the situation. The fatal risk of consuming contaminated beef became a topic. And indeed, the fears were materialized when the first cases of new variant CJD (vCJD) were identified.

From the scientific point of view, it was a real sensation, when infectious agents without any nucleic-acid-based genome were hypothesized, which were later called proteinaceous infectious organisms (prions). The dogma that self-replicating biologic agents depend on genomic information conserved in the sequence of nucleic acids had to be given up. However, it should not be forgotten that prions are real ‘viruses’ (in the original sense of toxic material),

as defined by the very early virologists. A lot of questions arose: How do those agents replicate? Do they always harm the host? Which factors of virulence can be identified? What are the mechanisms of pathogenicity? How are the agents transmitted? What are the options of early laboratory diagnosis and development of therapy and prevention?

In this edition of *Contributions to Microbiology, leading scientists in different fields of biomedical research show once again how important it is to deal with the prion 'problem' in a multidisciplinary manner*. The long-lasting discussion about the virus or prion theory is getting a clearer background. Furthermore, the structures of prions and their molecular biological analysis, and the questions of strain variations and species barrier are discussed. Another chapter tells us what we can learn about prions from yeast experiments. The possibilities of inactivation and disinfection of prions are of great importance for public health. This includes the test methods and their problems as well as the recommendations for clinical use and official regulations.

Another part of this book concentrates on the causes and clinical diagnostic aspects of TSE. While previously vCJD could be diagnosed only postmortem, new tests now allow identification of the disease with some confidence while victims are still alive (at least 1 year before its clinical onset). The tests include tonsil and appendix biopsies and magnetic resonance imaging. Furthermore, the epidemiology of human and animal prion diseases, disease management and the risks to public health or biologists (e.g. in the pharmaceutical industry) are also discussed.

In summary, this book informs about the state of the art of prion infection and disease.

Frankfurt/Main, May 2000

Holger F. Rabenau
Jindrich Cinatl
Hans Wilhelm Doerr

Rabenau HF, Cinatl J, Doerr HW (eds): Prions. A Challenge for Science, Medicine and the Public Health System. Contrib Microb. Basel, Karger, 2004, vol 11, pp 1–13

Transmissible Spongiform Encephalopathies: The Prion Theory – Background and Basic Information

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Non-Virus Properties of the Scrapie Agent

As early as 1966, Alper et al. [1] suggested, based on the anomalous resistance of the scrapie agent against both ionizing and UV irradiation, that the target size is too small for a viral genome. Irradiation at different wave lengths of UV light showed that scrapie infectivity was equally resistant at 250 and 280 nm [2]. Because proteins are more sensitive to UV irradiation at 280 nm than at 250 nm [3], Alper et al. did not follow up their original idea, that the scrapie agent could be a protein. A nucleic acid genome with unconventional features, an abnormal polysaccharide with membranes and a purely membranous nature of the scrapie agent [4] were discussed in the literature. In 1967, Griffith [5] had published a model of a self-replicating protein, which, however, did not trigger further research.

Other features became known that pointed to subviral agents. For example, no viruses or virus-like particles could be identified in electron micrographs of highly infectious material; the simplest explanation was that the agent was too small to be detected. The target size from ionizing radiation was originally determined to be about 150 kD [1] and later revised to 55 kD [6]. As a further untypical feature of a virus, it was realized that the scrapie agent did not initiate an immune response.

It was Stanley Prusiner from the University of California in San Francisco who extended the early studies systematically and summarized his own and other results as follows:

(1) Chemical and physical procedures which modify or destroy nucleic acids (meaning also those of viruses, bacteria, fungi, etc.) do not deactivate the scrapie agent.

(2) Chemical and physical procedures which modify or destroy proteins, do, however, deactivate the scrapie agent.

Nobody except Prusiner was brave enough at that time to formulate the logical conclusion from the results outlined above: the scrapie agent is not a virus but a proteinaceous infectious agent, which he called a 'prion' in his corresponding publication in *Science* [7]. This however, triggered a worldwide controversy, and some of the arguments are summarized by Diringer [8]. An infectious agent without a nucleic acid as information carrier was – and for some researchers still is – too much of a violation of a central dogma of molecular biology. It was argued that most experiments have lent only indirect support to the new agent, and one might envisage an unidentified virus with unconventional properties that prevent its identification. The earlier data had argued that the scrapie agent was as small as a viroid, but the speculation that a viroid might be the cause of scrapie [9] was given up when the properties of viroids and those of the scrapie agent were compared systematically and found antinomic [10].

The Concept of Two Isoforms of the Prion Protein

If the agent consisted predominantly or solely of protein, the next task would be to isolate and characterize the protein. This took several years and resulted in a single protein of 33–35 kD, called the prion protein (PrP). It is glycosylated, has a C-terminal glycolipid anchor, is highly hydrophobic, and appears as a membrane-associated protein [11]. After purifying the protein, Prusiner together with L. Hood and Ch. Weissmann and their teams, identified and cloned the gene of the protein. Surprisingly or not, the gene of PrP is a single-copy gene of the host [12].

How could a host-encoded protein be at the same time harmless to the cell and act as a lethal infectious agent when invading from the outside? It was an important step in the development of the prion model, when it became evident, that the PrP from uninfected animals was sensitive against degradation by proteinase K (PK) whereas the highly purified scrapie agent was highly resistant against proteolysis [13]. This result clearly showed that the PrP was present in different conformations, states of aggregation, possibly complexes with other molecules, whatever one could imagine to explain the difference in PK resistance. Although the PrP from noninfected and infected animals was chemically identical [14] – i.e. same amino acid sequence and chemical modifications – one had to assume functional differences, so-called isoforms. Whereas the cellular isoform PrP^{C} is produced in the healthy organism, the abnormal or scrapie form PrP^{Sc}, or PrP^{CJD} and PrP^{BSE} is produced in the infected organism

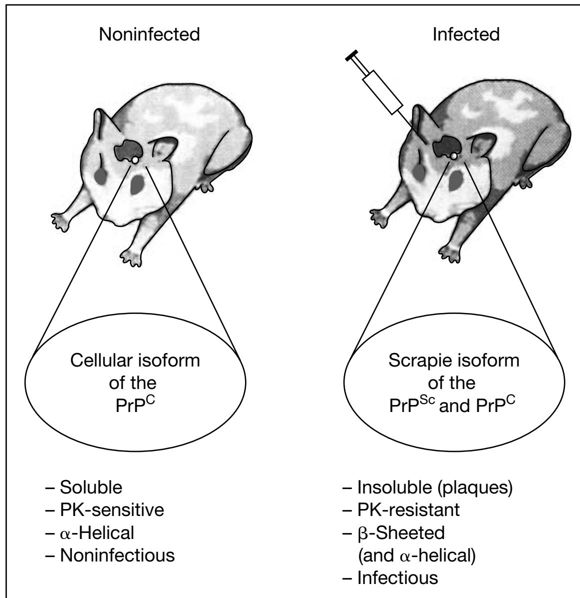

Fig. 1. Schematic presentation of PrP isoforms.

in cases of Creutzfeldt-Jakob disease (CJD) and bovine spongiform encephalopathy (BSE), respectively. PK resistance served indeed as marker of infectivity, and the term 'PrPres' was introduced to emphasize the particular feature of PK resistance. Some authors use PrPres synonymously with PrP^{Sc}, others point out that of PK resistance is a biochemical and infectivity a biological property. The situation is depicted in figure 1 of a healthy and an infected hamster.

The insolubility in aqueous solution is closely related to the PK resistance. PrP^{Sc} forms highly insoluble depositions in the infected brain which might have different forms, from compact plaques to quite diffuse depositions. As a consequence of the infection, PrP^{C} is transformed into PrP^{Sc} which is then present in a much higher concentration. It is noteworthy that the golden hamster became the experimental animal of choice, since the incubation time after intracerebral injection is fairly short (around 3 months) and which is even more important it is strictly related to the titer of the infectious material. During PK treatment of PrP^{Sc}, about 70 N-terminal amino acids are truncated, leading to the PK-resistant core of 27–30 kD, called PrP 27–30. Since infectivity is not reduced by the PK treatment, it is obvious that PrP 27–30 still possesses full infectivity and that the 70 N-terminal amino acids are not involved in the infectious process. Treatment with both PK and mild detergents like sarkosyl transforms

the infectious material into an even more compact fibrillar form with amyloid properties, which are called either prion rods or scrapie-associated fibrils (SAFs). Figure 1 also shows differences in the secondary structure of PrP^{Sc} and PrP^{C}, which will be discussed in more detail in another chapter of this book [Cappai et al., pp. 14–32]. For more details of the differences between PrP^{C} and PrP^{Sc}, the reader might refer to reviews by Prusiner [15–17].

Basic Experiments Pro and Contra Prions

Three experimental approaches should be mentioned which tackle the question of an informational molecule like a nucleic acid in prions in a very direct manner. In addition, there exists a wealth of experimental results from molecular biology, cell biology, genetics which are analyzed in another chapter of this book [Cappai et al., pp. 14–32].

The $PrP^{o/o}$ mouse is a transgenic mouse in which both alleles of the *PrP* gene have been knocked out [18, 19]. If such a mouse is inoculated with prions, no disease symptoms can be observed, and the disease cannot be passaged further from this animal. Such a result is in complete agreement with the prion model, since it shows that PrP is responsible for the pathogenic effect as well as for the infectivity. However, the results do not prove the prion model, since they only show that PrP is necessary but not that it is sufficient.

The phenomenon of scrapie strains is not so easily reconciled with the prion model. The first experiments were carried out by the Scottish scrapie researchers Bruce and Dickinson [20] and Kimberlin et al. [21]. The experiments can best be carried out with hamsters. Two different 'isolates' of infectious scrapie material – not considering the origin of different isolates – were used to inoculate genetically identical hamsters. In the first passage, a species barrier had to be overcome, and therefore the incubation time might be different after inoculation with different isolates. In the second passage, however, prions with an identical PrP sequence were used for inoculation, and still clearly different incubation times and different lesion patterns in the brain were obtained. Prions seem to contain more information than the mere sequence of PrP. This phenomenon is called 'prion strains', and several researchers take it as an indication of the presence of an informational molecule, most probably a hitherto undetected nucleic acid.

The Search for a Nucleic Acid

The existence of prion strains prompted several research groups to search systemtically for scrapie-specific nucleic acids in infectious material (for a

review, see Riesner [22]). One approach was to search for differences in the nucleic acid profiles of infected versus uninfected cells or tissue. Those studies were carried out by end-labeling all nucleic acids and comparing the patterns in two-dimensional gel electrophoresis. Another way was differential hybridization screening of cDNA libraries. It was also tried to analyze nucleic acids which co-purify with scrapie infectivity. Finally, nucleic acids still present in highly purified infectious material were cloned and screened for exogenous sequences. None of the approaches led to the detection of nucleic acid other than the host's, the cloning vector or bacterial or fungal impurity. One should, however, have in mind that the fact of not finding a nucleic acid does not prove its nonexistence.

Because of all the negative results, approaches were attempted either to find or to exclude scrapie-specific nucleic acids [22–26]. The concept was to start with highly purified prions and perform two types of measurement: (1) size and numbers of nucleic acid molecules present in the infectious material, and (2) number of infectious units. The ratio of the numbers of nucleic acid molecules and infectious units (P/I) might lead to two different conclusions. If the ratio were larger then unity, no definite conclusion would be possible. If, however, the ratio were smaller than unity, more infectious units would be present than nucleic acid molecules and therefore the nucleic acids would be excluded from being essential for infectivity. The numbers of infectious units were determined in a biotest using the incubation time assay; for the nucleic acid analysis a novel method, return refocussing gel electrophoresis, had to be developed. As outlined in the original literature, this technique was adapted to the very peculiar situation, i.e. that nothing was known about the nucleic acid to be analyzed, and DNA or RNA, double- or single-stranded and even heterogeneous nucleic acid had to be taken into consideration. As a summary of several experiments, the P/I ratio, depending on the length of the nucleic found in the infectious material is shown in figure 2. Only nucleic acids smaller than 50 nucleotides are present in amounts with $P/I > 1$, i.e. could still be essential for scrapie infectivity; larger nucleic acids, i.e. also those of viruses or viroids, are excluded. Thus, the nucleic acid analysis represents a completely independent proof that the agent of scrapie cannot be a virus.

Structural Transitions of the Prion Protein as Basis for the Replication Mechanism

The structures of PrP^C and PrP^{Sc} are presently the basis for any models of prion replication. Only schematic pictures are used here; molecular structures and models are described in one of the next chapters. In the noninfected organism, PrP^C is expressed in concentrations varying from tissue to tissue, and

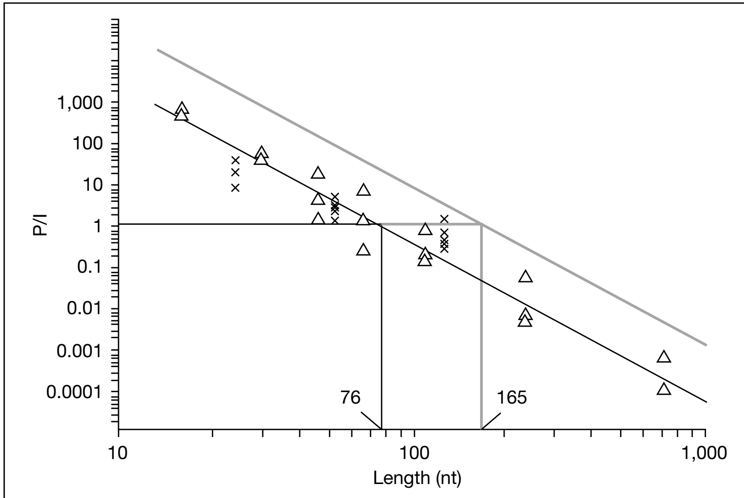

Fig. 2. Ratio P/I from five independent prion samples. Only for small nucleic acids (<76 nt) is the ratio P/I above unity. The calculation is based on the assumption that the hypothetical scrapie-specific nucleic acid is a well-defined molecular species among the heterogeneous nucleic acids. Data were taken from Meyer et al. [23] (x), and Kellings et al. [25] (Δ). The straight line is an interpolation in order to determine the average of the length of the nucleic acids at P/I of unity. The upper line represents a conservative estimation of the experimental errors.

is located on the outer membrane of the cell and anchored by its glycolipid anchor. It is degraded with a relatively high turnover rate. In the course of an infection, one might assume that the invading PrP^{Sc} comes into close contact with PrP^{C}, and due to this contact a conformational transition of PrP^{C} to PrP^{Sc} is induced. Such a mechanism as proposed by Prusiner's group is, depicted schematically in figure 3. The intermediate state of PrP^{C} and PrP^{Sc} being in contact is called a heterodimer; this is the reason why PrP^{Sc} alone has been assumed to be present as a dimer. Induced conformational changes of proteins are known from chaperone actions, and in this sense PrP might act as its own chaperone. The transition of PrP^{C} to PrP^{Sc} might go on as long as new PrP^{C} molecules are synthesized in the cell. The newly generated PrP^{Sc} will form new aggregates, or be incorporated into already existing aggregates; it will be stabilized by aggregation, thereby acquiring a much longer turnover time. The size and internal structures of PrP^{Sc}-aggregates vary from species to species and disease to disease as revealed by comparison of the different CJD disease variants (see Hill et al. [27] and Knight et al. [28], this volume].

It is presently not possible to perform the conversion of PrP^{C} into PrP^{Sc} in the test tube. Such an in vitro conversion would be the ultimate proof of the

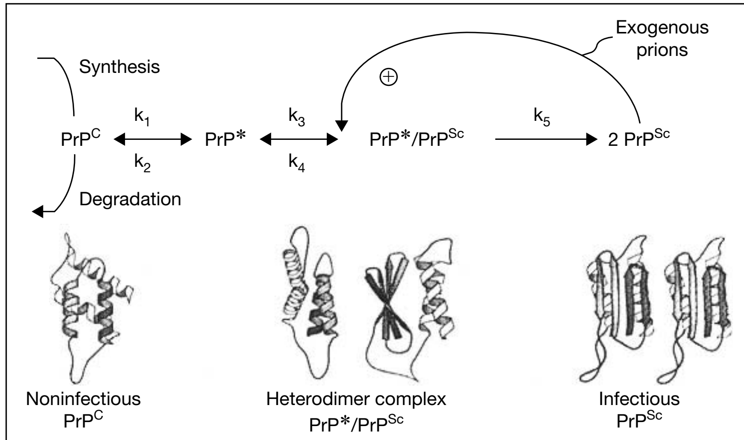

Fig. 3. The original Prusiner model for prion replication: PrP^C is synthesized and degraded as part of the normal metabolism. PrP* is an intermediate in the formation of PrP^{Sc}, which can revert to PrP^C or be degraded prior to its binding in PrP^{Sc}. In infectious prion diseases, exogeneous prions enter the cell and stimulate conversion of PrP* into PrP^{Sc}. One should note that the molecular structures depicted are from model calculations, not from NMR analyses. The figure is taken from Huang et al. [46].

prion model which is still lacking. In the following, a brief outline will be given on the features of conformational transitions underlying the PrP^C to PrP^{Sc} conversion and on different models of replication.

Besides studies on fragments of PrP and on recombinant PrP, experiments have been carried out on three natural forms of PrP, i.e. PrP^C, PrP^{Sc} and the N-terminal-truncated but still infectious form of PrP^{Sc}, called PrP 27–30. Biochemical and particularly biophysical experiments suffered from the fact that PrP^{Sc} and PrP 27–30 are insoluble in aqueous buffers and even mild detergents. Consequently, strong effort has been made to solubilize infectious prions, or biophysical methods were adapted to insoluble samples like in thin films or applying spectroscopy with microbeams.

As a summary of solubilization experiments, it became evident that prions lost their infectivity whenever they were solubilized. PrP^{Sc} or PrP 27–30 was either denatured, as for example in guanidine hydrochloride, or urea [29, 30], or its structure was shifted to a more PrP^C-like structure as in low concentrations of sarkosyl (2%) or sodium dodecylsulfate (SDS, 0.2%) [31].

Nevertheless, the different biophysical approaches led to a more complete picture of the different structures of PrP, as summarized in table 1. The molecular structure of PrP^C was derived from an NMR analysis [32, 33].

Table 1. Properties of PrP^{C} and PrP^{Sc}/PrP 27–30

PrP^{C}	PrP^{Sc}/PrP 27–30
Soluble	Insoluble, present in different forms of aggregates
PK-sensitive	PK-resistant
3 α-helices of 14–25 amino acids, small antiparallel β-sheet of 2 × 3 amino acids[1]	β-sheet formation (up to 30%) in addition/or partially in addition to the α-helices[2]
Noninfectious	Major or only component of infectivity

[1]The molecular structure of PrP^{C} in solution is known from an NMR-analysis [32, 33] with the structured C-terminal part (amino acid 110–231) and a more or less structureless N-terminal part.

[2]For PrP^{Sc}/PrP 27–30 secondary structure contents were estimated from circular dichroism (CD) and infra-red spectroscopic (IR) measurements [34, 35]; only rough structural models but no molecular structure are available.

Several approaches to in vitro conversion of PrP^{C} into PrP^{Sc} have been reported in the literature. Caughey et al. [34] incubated radiolabeled PrP^{C} with a large excess of PrP^{Sc} under partially denaturing conditions [36]. They could show that PrP^{C} acquired PK resistance after renaturation, and could induce even specific N-terminal truncation sites. However, because of the large excess of PrP^{Sc}, it could not be tested whether infectivity was newly generated; thus possibly PrPres but not PrP^{Sc} was induced. Later it was shown by utilizing strain-specific features (see chapter by Collinge et al. [27]) that infectivity was not generated [37]. Kaneko et al. [38] induced a transition from a soluble PrP^{C} into an insoluble, PK resistant structure by a large excess of the PrP peptide 90–145. PrP 27–30 which was solubilized by SDS could be retransformed into an insoluble state by adding 25% acetonitrile [31] or by mere removal of the SDS [39]. The secondary structure, too, was changed back to the characteristic β-sheeted structure of prions and partial PK resistance was induced. However, acquired infectivity as a consequence of the conformational change, was not found [39].

As a summary of the studies reported above, one has to conclude that the properties of β-sheeted structure, insolubility, and PK resistance are found in prions but are not strictly correlated with infectivity. PK resistance could be induced by several methods without, however, acquiring infectivity. PK resistance was correlated with aggregation and aggregation with β-structure, but these features are not sufficient for infectivity [30, 31, 40]. At present, it cannot be decided whether the right conditions for induced infectivity have not been

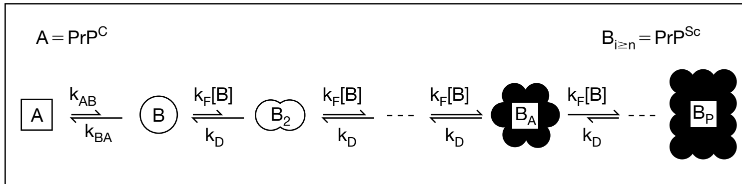

Fig. 4. The Lansbury model for prion replication PrP^C and the PrP^{Sc}-like structure are in fast equilibrium, with PrP^C being the favorable state. Several intermediates have to be formed before a stable nucleus of n PrP^{Sc}-molecules is generated. The stable nucleus is the first state with the typical prion properties. The figure is modified from Eigen [47].

found, a second, not yet identified component is still missing, or a principal feature of the $PrP^C \rightarrow PrP^{Sc}$ transition is not yet understood. It should be noted that a rigid chemical analysis of highly purified prion rods showed small amounts of specific lipids [41] and significant amounts, i.e. above 10% of a polyglucose scaffold [42]. It is, however, not clear whether these components are essential for infectivity.

Alternative models of prion replication have been reported in the literature by Prusiner et al. [43] and Come et al. [44]. Both are models of PrP conformational transitions and both are hypothetical in the sense that firstly they are derived from the ‘protein-only hypothesis’ and secondly assume conformational transitions occurring in PrP-free in solution.

As depicted in figure 3, Prusiner et al. [43] proposed the ‘heterodimer model’. A complex of PrP^C and PrP^{Sc} is formed, in which PrP^C is transformed into PrP^{Sc} similar to the enzymatic mechanism of an induced fit. In the mean time, the model was specified in that a protein X has to be involved in the complex [45]. Regarding an equilibrium, $PrP^C \leftrightarrow PrP^{Sc}$, PrP^{Sc} would be the favored state, otherwise there would be no driving force for the catalytic turnover which is reversible at equilibrium.

Another mechanism was proposed by Lansbury and his colleagues [44], in which fibril formation – as known for actin or β-amyloid and called ‘linear crystals’ – and generation of infectivity are closely connected. The model is depicted in figure 4. Monomeric PrP^C is in fast equilibrium with a PrP^{Sc}-like conformation, with PrP^C being the favorable state. A number of PrP^{Sc}-like molecules can form aggregates with decreasing concentrations, down to a nucleus of n PrP^{Sc}-like molecules. If the nucleus has formed, growth of the aggregates is faster than dissociation, and increasingly larger aggregates will be formed. In that mechanism – and in contrast with the heterodimer-model the first stable aggregate corresponding to a functional PrP^{Sc} will be the nucleus of n PrP^{Sc}-like molecules.

Eigen [47] has treated both models according to the exact law of chemical thermodynamics and kinetics. He concluded that the 'heterodimer model', which is in essence a linear autocatalysis, cannot be adapted to realistic thermodynamic and kinetic constants. Within the time span of a mammal's life an infection would either never happen or would always happen, in clear contrast to reality. If, however, several PrP^{Sc} molecules had to cooperate to transform one PrP^{C} molecule, a mechanism which would be similar to an allosteric enzyme action, threshold effects would come into play and such a mechanism would not contradict well-established thermodynamics and kinetics. With this extension, the Prusiner and the Lansbury models show some similarity in the sense that several PrP^{Sc} molecules are involved in the conversion process.

Infectious, Sporadic and Familial Etiology of Prion Diseases Based on the Prion Model

If the $PrP^{C} \rightarrow PrP^{Sc}$ transition is the primary event of the disease, essential conclusions can be drawn or else, remarkable experimental findings might be explained on the basis of this assumption. Firstly, a conformational transition which is catalyzed by the contact with other proteins or factors would also occur spontaneously according to the laws of thermodynamics, although the probability and the rate might be very low. If only a single or a few PrP^{C} molecules undergo the transition, they could start the autocatalytic cycle, and from the cell where it happened an infectious process could spread through the whole body or organ. This could be an explanation for the sporadic manifestations of CJD described in later chapters of this book [Knight et al., pp. 72–97; Zerr et al., pp. 98–116]. Secondly, if a protein has two or more structural alternatives, both of which are stable, or one of which is metastable, one should expect germline mutations which favor PrP^{Sc} over PrP^{C}. Favoring need not be limited to PrP^{Sc}, but could also be a higher incidence for the spontaneous $PrP^{C} \rightarrow PrP^{Sc}$ transition. Indeed, many prion diseases with hereditary manifestations are known, and all are connected with mutations in the PrP gene (see reviews by Prusiner [11, 48] and chapters on CJD and other human prion diseases in this volume [Knight et al., pp. 72–97; Zerr et al. pp. 98–116]). Finally, the sporadic as well as the familial forms of the disease were transmitted to experimental animals – or in the case of medical accidents to humans. These findings support the general concept of prion diseases. In summary, prion diseases are exceptional also in the sense that they can have an infectious as well as a sporadic or familial etiology. Thus, it is a great merit of the prion concept as well as convincing support for it that diseases of quite different etiologies can be reduced to a unique phenomenon.

Outlook

Since the transition of PrP^C to infectious PrP^{Sc} cannot yet be performed in the test tube, biophysical results on the structure and structural transitions of PrP will be rated as high, as they might lead to the desired in vitro $PrP^C \rightarrow PrP^{Sc}$ transition which then would represent the ultimate proof of the prion model. It will not matter whether a physical, chemical, enzymatic or any other treatment of PrP^C is applied as long as infectivity is newly generated from a noninfectious PrP^C sample. At present it is not clear whether the conformation of PrP^{Sc} is already present in the monomeric PrP, in an oligomeric state or how big the smallest size of the infectious entity might be. Infectious prions are only available as large aggregates; is this an artifact from the preparation or an intrinsic feature of the infectious state? The potential hidden difficulty is evident from the finding that about 10^5 PrP molecules are needed for one infectious unit. Are they all identical in conformation, and is the incidence of infection so low? Or do much fewer PrP molecules exist which are the really infectious PrP^{Sc} molecules which are protected by 10^5 PK-resistant PrP molecules? How could the large aggregates be dissociated to generate more infectious particles? – an essential question, particularly with respect to the Lansbury model? Do the lipids and the polysaccharides found in prions play an essential role for the infectious process, either by stabilizing the PrP^{Sc} conformation or by inducing contacts with the cell surface? As mentioned above, most experiments and models refer to conditions in solution; it might be that the transition works only in close contact with the membrane, i.e. closer simulation of cellular conditions would be required for a successful $PrP^C \rightarrow PrP^{Sc}$ transition.

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Structural Biology of Prions

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The transmissible spongiform encephalopathies (TSE) constitute a group of fatal neurodegenerative diseases occurring in humans (Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), variant CJD, kuru and fatal familial insomnia (FFI)) and animals (e.g. bovine spongiform encephalopathy (BSE) and scrapie). A considerable body of data supports the model that the TSE infectious agent, known as the prion, is a self-replicating protein devoid of nucleic acid [1, 2]. According to the protein-only hypothesis, prion propagation involves changing the conformation of the normal cellular prion protein (PrP^{C}) into an infectious isoform (PrP^{TSE} or also called PrP^{Sc}). The two conformers have distinct biochemical and biophysical properties but share the same primary sequence. While PrP^{C} is relatively detergent-soluble and sensitive to proteinase K digestion, PrP^{TSE} aggregates into rods or filaments and is detergent-insoluble and resistant to proteinase K digestion [3]. These biochemical differences are believed to reflect the different secondary and tertiary structures of PrP^{C} and PrP^{TSE} [4].

The majority of human TSE cases (>85%) are sporadic with an unknown etiology, and approximately 12–14% are caused by mutations in the PrP gene (PRNP). These mutations may promote prion formation by destabilizing PrP^{C} and facilitating the transition to PrP^{TSE}. The presence of different prion strains which manifest themselves as distinct clinico-pathological phenotypes has been difficult to accommodate within the protein-only hypothesis, but may relate to different PrP^{TSE} conformers. This and other in vitro experiments suggest PrP^{C} possesses considerable structural plasticity. Therefore, a detailed description of the structural properties and the cellular and molecular mechanisms of interconversion of PrP^{C} to PrP^{TSE} is central to understanding prion disease biology. Considerable advances have been made towards achieving this goal with the

determination of the three-dimensional structure of full-length PrP^C from a diverse range of species. However, major gaps still remain, with the most salient being the structure of PrP^{TSE} which is difficult to analyse because of inherent insolubility. However, recent progress utilizing electron crystallography is starting to yield some insights into the structure of the infectious form [5].

The Primary Structure of the Prion Protein

There are three commonly described PrP species, the normal non-infectious cellular isoform PrP^C, the full-length infectious form PrP^{TSE} and PrP27–30 which is obtained after partial proteolysis of PrP^{TSE}. The PrP27–30 protein is an N-terminally truncated molecule that commences most commonly at around residue 90 and represents the infectious protease-resistant core of PrP^{TSE}. The purification of PrP27–30 from infected hamster brain enabled the amino-terminus and internal peptide fragments to be sequenced [6]. Degenerate oligonucleotides based on these sequences were used to screen an infected hamster brain cDNA library and a PRNP cDNA clone was isolated [6]. Southern blot analysis of genomic DNA indicated that the PrP sequence was present as a single copy gene. A comparison of genomic DNA from normal and scrapie-infected hamster brain gave an identical restriction pattern, indicating that infectivity did not involve chromosomal rearrangements. The human PRNP gene is localized to the short arm of chromosome 20 [7]. The PRNP open reading frame is encompassed on a single exon of approximately 2 kilobases (kb) that is spliced to two small exons located 10 kb upstream [8, 9]. Expression occurs in most adult tissues, except liver, with the highest levels being in the brain. The promoter sequence of hamster PRNP resembles that of a house-keeping gene being GC-rich and lacking an apparent TATA box [8]. Multiple transcriptional start sites were identified 140–160 basepairs upstream of the ATG start codon. There are no differences in PRNP mRNA expression levels between infected and uninfected brains, or during infection [6, 10].

The nascent PrP molecule is approximately 250 amino acids in length. It has an amino-terminal signal peptide and a hydrophobic carboxy-terminal domain for membrane attachment via a glycosylphosphatidylinositol (GPI) anchor (fig. 1). There are four copies of an octapeptide repeat PHGGGWGQ (codons 60–91, numbering based on human sequence), with close analogy to a proximate nonapeptide PQGGGGWGQ (codons 51–59) in the N-terminal region. There is a single disulphide bond between cysteines 179 and 214 and two N-glycosylation sites at asparagines 182 and 198. A hydrophobic sequence (codons 113–135) in the middle of the protein may exist as a transmembranous region in some PrP isoforms [11]. Transgenic mice expressing PRNP with

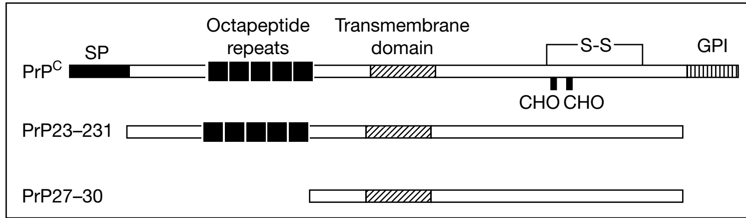

Fig. 1. Schematic representation of the main primary structural features of PrP and PrP27–30. SP represents the signal peptide, S-S the disulphide bond, CHO the N-linked glycosylation sites, and GPI the glycosylphosphatidylinositol anchor.

human mutations which favor the transmembrane form resulted in clinicopathological features that are found in some inherited human prion diseases. To understand the evolutionary conservation of PRNP, the gene was sequenced from a diverse range of species including chickens, rodents, sheep, primates and humans [12]. There is high amino acid sequence identity within mammals and birds, but low homology between birds and mammals. However, overall there was strong conservation of key structural elements between mammals and birds. The N-terminal region tends to differ by insertions and deletions in the octapeptide repeat domain. In contrast, the C-terminal region (91–231) varies by point mutations. Interestingly, there is high conservation of sequences (residues 23–90) which are not associated with either the structural elements or the core infectious portion of the molecule.

The precise function of PrP^C is unknown; however, considerable progress is being made towards understanding its physiological activities. PrP^C has been associated with maintaining cell survival [13, 14], promoting neurite outgrowth [15], modulating synaptic transmission [16] and copper homeostasis [17]. Potential PrP-binding proteins include the cell adhesion proteins N-CAMs [18], the laminin receptor [19, 20], stress-inducible protein 1 [21] and the intracellular signalling proteins synapsin Ib and Grb2 [22]. Its interaction with synapsin Ib and Grb2 may reflect it adopting a transmembrane topology [11]. If these binding proteins are bona fide PrP ligands, then PrP could function as a cell surface receptor involved in signal transduction to promote cell survival [21].

Ablation of PRNP by homologous recombination resulted in a line of PrP knockout mice ($PrP^{o/o}$) that were viable and overall phenotypically normal [23]. The $PrP^{o/o}$ mice are completely resistant to infection by PrP^{TSE}. Another line of $PrP^{o/o}$ mice was generated that developed late-onset ataxia [24]. The reason for the ataxia phenotype may be explained by the presence of a PRNP homologue located 16 kb downstream of the PrP gene [25]. The gene is designated Prnd and the protein product called doppel (downstream PrP-like).

It encodes a 179 residue protein with 25% amino acid identity to PrP. Prnd expression is upregulated in $PrP^{o/o}$ mice with late-onset ataxia. This is probably due to the deletion of the PRNP exon 3 splice acceptor site in these mice and this may alter transcriptional regulatory elements of Prnd. The tertiary structure of doppel is similar to PrP with high conservation of the main secondary structure elements [26]. However, the doppel protein has distinct biochemical properties compared to PrP with a lower global stability [27]. Doppel has a different physiologically role to PRNP as male Prnd knockout mice are infertile due to malformed spermatids which retards oocyte fertilization [28].

The Neurotoxic Model Peptide, PrP106–126

A peptide fragment encompassing residues 106–126 of human PrP is highly fibrillogenic and toxic to neurons in vitro [29, 30]. Since PrP106–126 toxicity mimics PrP^{TSE} by requiring the expression of PrP^{C} to cause cell death [31], this peptide has been utilized as a model to study the toxic properties of PrP^{TSE}. Treatment of cells with PrP106–126 induces the accumulation of truncated PrP^{TSE} [32] and membrane-associated PrP^{TSE} [33]. This PrP106–126 sequence contains the amyloidogenic palindrome AGAAAAGA from position 113 to 120 [29]. Based on the NMR structure (fig. 2), this sequence is part of, or adjacent to, the first of the two very short β-sheets in PrP^{C} [34–38]. This region of PrP^{C} is highly hydrophobic and has been shown to exhibit considerable flexibility and structural plasticity [35]. The structure of the PrP106–126 peptide is modulated by pH and has more β-sheet at pH 5 than at pH 7 [39–41]. Additionally, in the presence of lipids (at pH 7.4) it acquires a predominantly β-sheet conformation [40]. The hydrophobic AGAAAGA palindromic sequence is a key modulator of PrP106–126 toxicity and amyloidogenicity [41]. Hydrophilic substitutions in this hydrophobic core abolished neurotoxicity and this effect correlated with decreases in β-sheet content, aggregability and fibrillogenesis. The hydrophobic C-terminal valines, and the palindrome region from Ala-113 to Ala-120 of PrP106–126, are integral to the folding and/or stabilization of a β-sheeted aggregate. Another key modulator of PrP106–126 amyloidogenicity and neurotoxicity is copper and to a lesser extent zinc [32, 42]. The aggregation of PrP106–126 is completely inhibited in a metal-depleted buffer [42]. Histidine-111 and methionine-112 are key ligands for copper binding. Mutating these residues abolishes PrP106–126 neurotoxic activity. The behaviour of PrP106–126 is similar to the Alzheimer's disease amyloid beta (Aβ) peptide which binds copper and zinc, resulting in accelerated aggregation [43] and Aβ aggregation is also inhibited by metal chelators [44]. This supports

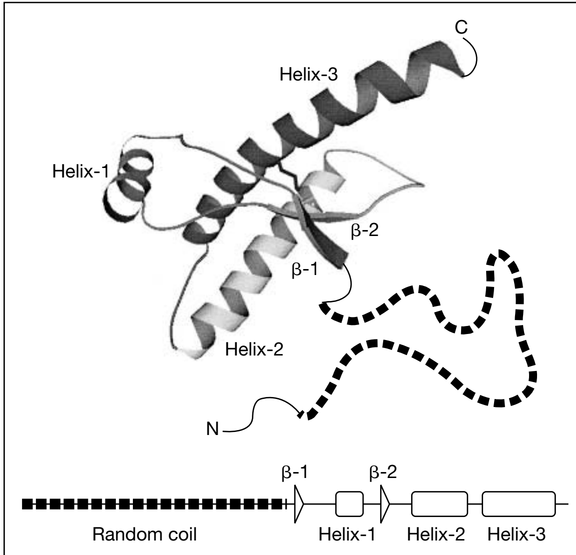

Fig. 2. Three-dimensional NMR structure of human PrP23–230 [38]. The three α-helices (Helix-1, Helix-2 and Helix-3) and the two β-sheets (β-1 and β-2) are labelled. The disulphide bond joining Helix-2 and Helix-3 is represented by the black angular line. The N-terminus from 23 to 124 is unstructured and contains the octapeptide repeats (residues 51–91) which are represented by the broken line. A schematic of the secondary structure elements is shown. The random coil is residues 23–124. β1 is 128–131 and β2 is 161–164. Helix-1, Helix-2 and Helix-3 are residues 144–154, 173–194 and 200–228 respectively.

the view of a common structure-function mechanism of amyloid generation in spongiform encephalopathies and Alzheimer's disease [45–47].

Metal-Binding Domains of the Prion Protein

Expansions in the PRNP octapeptide repeat number are linked to familial cases of prion disease. A two-repeat deletion causes CJD [48], whilst deletions of single octapeptide repeats are not associated with disease [49]. This iterative sequence binds copper [50–53] but the exact nature of this binding remains controversial as does the stoichiometry of copper binding to PrP^C, with values ranging from 1.8 to 5.6 [17, 52, 54, 55]. The interaction between octapeptide repeat peptides and copper was pH-dependent [56, 57] with no binding occurring below pH 6. The apo form of the octarepeat peptide encoding 2, 3 or 4 octarepeats is unstructured [53, 56], but the addition of copper can either result

in the formation of turns or structured loops [56] or promote α-helix formation [53]. Such differences may reflect buffer-dependent effects as similar studies using a Tris buffer, as opposed to phosphate buffers, did not alter octarepeat structure [51]. It was proposed that Tris-based buffers may be competing for copper at pH 7.4 [56].

PrP also possess high-affinity copper-binding sites in the C-terminal structured portion of the molecule [54, 58, 59]. The site is composed of histidine residues 96 and 111 [54, 55, 58] and shares part of the PrP106–126 metal-binding region [42]. A low-resolution structure of this PrP^C high-affinity copper site has been proposed based on X-ray solution scattering studies [58]. Methionine-109 and glutamine-98 could provide a sulfur and an oxygen ligand respectively. The site has an apparent Kd of 4×10^{-14} [54] indicating a high specificity for copper, further supported by its lower affinity by 6 orders of magnitude for other metals such as nickel and zinc. The crystal structure of a cognate octapeptide repeat (HGGGW) complexed with copper has been solved [60]. The copper is coordinated by a histidine imidazole, two deprotonated amides from the first two glycines with the tryptophan indole providing a hydrogen bond to the axially coordinated water molecule. The third glycine is not involved in direct binding to copper. The crystal structure was validated by EPR which confirmed that the histidine and glycine residues are direct ligands for copper [59]. The structure also identified potential intermolecular contact points between the octapeptide repeats which may allow the observed cooperative binding of the PrP repeats [56].

The physiological role of copper binding by PrP remains to be determined. In vivo studies in PrP knockout mice showed a decrease in synaptic copper levels [17]. However, these results have been disputed by another group who failed to detect a difference between PrP knockout and wild-type mice [61]. Treating cells with copper promotes PrP endocytosis [62]. This is dependent upon the octapeptide repeats as deleting or mutating the repeat sequence abolishes this response [63]. Supporting the possible disease-related importance of metals in prion biology is the finding that treating brain-derived PrP^{TSE} with chelators promoted the switching between glycoform types [64]. This suggests metals may play a role in prion biogenesis and strain types by modulating the conformation of PrP^{TSE} and/or PrP^C. This strengthens the proposal that metals can modulate the property of proteins that are central to a number of neurodegenerative diseases [65].

Secondary Structure Analysis of the Prion Protein

The purification of PrP^C and PrP^{TSE} under non-denaturing conditions allowed their secondary structures to be studied [4]. Fourier transformed

infrared spectroscopy (FTIR) and circular dichroism (CD) showed that PrP^C had a high α-helical content (~42%) and was almost devoid of β-sheet (3%). In contrast, PrP^{TSE} exhibited a high amount of β-sheet (43%) and decreased levels of α-helix (32%). The incorporation of PrP^C or PrP27–30 into liposomes caused major alterations to their protein structure. Liposome-encapsulated PrP^C had 34% β-sheet, 20% α-helix and the remaining 46% was both random coil and β-turns [66]. In contrast, liposome-incorporated PrP27–30 had 43% β-sheet, 57% β-turns and random coil, and no α-helix. The insertion of PrP27–30 into membranes leads to disruption of membrane integrity, presumably due to its partial insertion into the bilayer [67]. These PrP-membrane interactions are important given the apparent requirement for membrane fusion in promoting PrP^C to PrP^{TSE} synthesis [68].

Although the transition of PrP^C to PrP^{TSE} appears to involve major increases in β-sheet structure and reductions in α-helix [4], the same data indicates that this conformational change involves regions other than the α-helices as a considerable level of α-helix remains in PrP^{TSE}. Therefore, PrP^C to PrP^{TSE} biogenesis must also involve the conversion of the random coils to β-sheet. Alternatively the α-helices in PrP^C may be converted to β-sheet concomitant with other less ordered regions of PrP^C converting to α-helix. Analysis of PrP^{TSE} and PrP27–30 folding intermediates generated in guanidinium hydrochloride (Gdn-HCl) showed that PrP^{TSE} and PrP27–30 dissociate into monomers via a cooperative two-step transition from aggregate to monomer [66, 69]. In contrast to PrP^{TSE}, PrP27–30 appears to unfold via a stable intermediate(s) with a greater thermodynamic stability. Moreover, alterations to the regions surrounding the highly structured disulphide bonded C-terminal domain can also cause thermodynamic destabilization and structural disorder [70]. Therefore, the amino-terminal region affects the global secondary structure and behaviour of the carboxy-terminal segment.

Tertiary Structure of the Normal Prion Protein (PrP^C)

The determination of the three-dimensional structure of PrP^C was made possible by improvements in the expression of recombinant PrP in sufficient quantities to permit nuclear magnetic resonance (NMR) studies [71, 72]. The first structure to be solved, due to its solubility and high-level expression in bacteria, corresponded to mouse PrP (MoPrP) residues 123–231 [34]. This C-terminal fragment contained three α-helices and two short β-strands (128–131 and 161–164). The α-helices (helix-2, 179–193 and helix-3 at 200–217) corresponded to those predicted by the molecular modelling approach [73]. However, helix-1 (144–154) and the two β-strands were not previously

predicted. The second and third helices are linked by a disulphide bond to form a V-shaped pseudo-cyclic structure upon which the β-sheet and the first helix are anchored. The surface contains some hydrophobic patches near the β-sheet and the loop preceding helix-1 and an uneven distribution of positively and negatively charged residues. The invariant residues among the mammalian species were located in the hydrophobic core and presumably stabilize the overall fold of the protein. The familial point mutations are located within, or lie adjacent to the β-sheet and the second and third α-helical regions. This has obvious implications for their postulated effect on promoting PrP^C to PrP^{TSE} conversion by destabilizing these structural elements.

After this initial NMR report, the structure of PrP^C from other species and of longer lengths were solved, including full-length mature $MoPrP^C$ (PrP23–231) [37], Syrian hamster (SHaPrP) SHaPrP29–231 [35], SHaPrP90–231 [36, 74], bovine PrP [75] and human PrP (HuPrP) HuPrP23–230, HuPrP121–230 and HuPrP90–230 [38]. There was good overall consensus amongst the different structures with three α-helical and two short β-sheet regions. The N-terminal region (codons 21–120), which contains the octapeptide repeats, was shown to behave as a highly flexible tail with no apparent secondary structure. In SHaPrP, residues 113–128 formed a hydrophobic cluster with many side chain interactions [74] and this was adjacent to an irregular β-sheet region. Minor differences existed between the full-length and C-terminal fragments of SHaPrP and MoPrP, suggesting that there may be transient interactions between the structured core and the unstructured N-terminal domain [35]. Further refinement of the SHaPrP90–231 structure [74] extended helix-3 by 10 residues (200–227) and demonstrated many medium- and long-range NOEs (nuclear Overhauser effect) within the 113–128 hydrophobic cluster, indicating a definite structural inclination within this region. The presence of the N-terminal flexible tail stabilizes helix-3 and increases the length of this helix. The main difference between HuPrP, MoPrP and SHaPrP was in the backbone conformations of helix-3 and the loop joining the second β-strand and helix-2 [38]. Human PrP had features in common with both SHaPrP and MoPrP. The HuPrP and SHaPrP structures shared a longer helix-3, while the disordered loop in HuPrP from 167–171 is similar to that observed for MoPrP. The degree of disorder of the helix-2 and helix-3 residues is regulated by the length of the N-terminal tail. These species-based structural variations are located on the surface of PrP^C and may be responsible for modulating transmission across animal species-species barrier.

A comparison of the solved structures of human, bovine, mouse and hamster PrP showed that the C-terminal domains are similar, with the bovine and human PrP structures having the greatest resemblance [75]. Analysis of the region composed of helix-1, which is believed to be an important contributor to the species barrier, is so similar in human and bovine PrP as to yield no

barrier [75]. However, the differences in this region with mouse and hamster would clearly contribute to a species barrier. The PrP sequence from 23 different mammalian species was modelled onto the MoPrP121–231 NMR structure [76]. The 103 residue segment from 124 to 226 was highly conserved with substitutions at only 18 residues. These substitutions are localized to three distinct structural regions termed A, B and C. The A region is the loop between the second β-strand and helix-2 and includes a small portion of the C-terminal tail. Seven out of nine substitutions within or near the A region involved changes in either side chain polarity or changes leading to significant electrostatic modification. Since this region localizes to the PrP surface it may be involved in protein-protein interactions. Therefore, the sequence differences may alter intermolecular recognition by changing surface hydrogen-bonding patterns. The B region involves five hydrophobic residues located where helix-1 interfaces with the rest of the protein. This region has limited surface exposure and is not a typical binding site, but it is proposed that slight alterations in conformation may expose these hydrophobic residues and allow them to promote PrP self-association. The C region is located at the two ends of helix-1 and also represents a potential protein-protein-binding site. The variant amino acids in the C region could modify the specificity of the intermolecular interactions. The regions which lacked substitutions include the two β-sheets, the loop joining helices 2 and 3, the C-terminal two turns of helix-2 and the central two turns of helix-1. Conservation of these regions is presumably necessary to maintain the overall local three-dimensional structure and to facilitate intermolecular interactions to either itself or its ligand(s).

The crystal structure of a dimeric form of human PrP^{C} has been solved to 2 Å resolution [77]. The dimer revealed considerable structural rearrangement had occurred with swapping of the C-terminal helix-3 and rearrangement of the disulfide bond. The dimer interface is composed of residues from helix-2 forming an interchain two-stranded antiparallel β-sheet. Importantly, familial prion disease mutations are located in the regions directly involved in helix swapping. Therefore, PrP^{C} to PrP^{TSE} conversion could involve oligomerization via domain swapping [77].

Influence of Familial Mutations on the Structure of the Prion Protein

The pathophysiological effects of familial TSE mutations have been studied in both transgenic mice and cell culture models. Transgenic mice expressing MoPrP with the GSS proline to leucine mutation at residue 102 (residue 101 in mice) undergo spontaneous vacuolar neurodegeneration [78]. Furthermore,

brain extracts from affected mice could transmit disease to recipient mice expressing the MoPrP-P101L mutation suggesting the generation of de novo prion disease [79]. However, the successful modelling of other PRNP point mutations in transgenic mice has not been reported. The expression of MoPrP with an expanded octapeptide repeat resulted in neurodegeneration and PrP with altered biochemical properties but no transmission data was reported [80].

In cell culture, the expression of MoPrP harbouring the FFI-associated D178N mutation in Chinese hamster ovary (CHO) cells resulted in proteinase K-resistant and detergent-insoluble PrP [81]. In contrast, expression of HuPrP-D178N in the human neuronal M-17 cell line resulted in proteinase K-sensitive PrP [82]. This suggests differences in cell types and/or PrP species sequence can result in different biochemical properties. As already mentioned, based on the NMR structure of full-length or large fragments of mouse and hamster PrP [34–37], a number of the mutations are located close to the α-helical and β-sheet regions, suggesting they may be critically positioned to facilitate structural changes or alter the ligand-binding properties of PrP. Biophysical studies of recombinant proteins containing PRNP disease-associated mutations have shown effects on PrP structure and aggregation [83, 84]. Bacterially expressed MoPrP23–231-P101L was shown to alter the relative stability of the mutant protein as compared to the wild-type MoPrP23–231 protein [84]. The P101L mutation lowered the amount of α-helix and upon thermal denaturation the MoPrP23–231-P101L protein was more resistant to unravelling to random coil. Furthermore, wild-type MoPrP23–231 unfolded in a single-step process from helical to random coil as shown by the presence of an isodichroic point. In contrast, the MoPrP23–231-P101L protein did not have an isodichroic point indicating that a metastable intermediate is likely to exist along the PrP-P101L unfolding pathway.

The structural stability of recombinant HuPrP27–30 (residues 90–231) encoding either the P102L mutation, the D178N mutation, or the CJD-associated E200K mutation, was measured under different Gdn-HCl concentrations and pHs [83]. At neutral pH the E200K mutation caused a small reduction in the thermodynamic stability of the protein, but similar changes were not observed in the P102L and D178N proteins. At acidic pH the PrP molecules unfolded via a stable intermediate and there was no observable difference between mutant and wild-type PrP. This is different to the behaviour of SHaPrP90–231, which showed irreversibility of thermal denaturation [85]. It was suggested that the pathogenic mutations may not effect the thermodynamic stability of PrP^{C} and cannot be used to rationalize the destabilization of PrP^{C} and its conversion into PrP^{TSE} [83]. This highlights the variation that can occur when comparing different species and fragments of PrP, prompting caution when attempting generalized conclusions.

Tertiary Structure of the Infectious Prion Protein (PrP^{TSE})

The insolubility of PrP^{TSE} has impeded the determination of the three-dimensional structure of PrP^{TSE}. Secondary structure measurements of PrP^{TSE} have identified a high amount of β-structure compared with PrP^{C} which is predominately α-helix [4]. PrP27–30 contains even higher levels of β-structure correlating with its amyloidogenic propensity and subsequent increase in intermolecular hydrogen bonding. X-ray diffraction studies on hamster PrP27–30 shows β-sheet conformation, consistent with those observed by CD and FTIR [86]. The intersheet spacing of PrP27–30 is 8–9 Å which is small in comparison to other amyloids such the Aβ peptide of Alzheimer's disease. This suggests the PrP27–30 prion rods are packed via small side chains. Residues 113–127 of PrP are devoid of bulky aliphatic and aromatic residues and this region could form the majority of the amyloidogenic core. It was established that PrP27–30, following solubilization with different solvents and exposure to reverse micelles, could form two- and three-dimensional microcrystals in the presence of uranyl salts and in acidic pH [87]. Electron diffraction data indicated at least four different types of crystal lattices were present. A potential step forward in elucidating the structure of PrP^{TSE} has been the employment of electron crystallography to analyse two-dimensional crystals of PrP27–30 and the recombinant truncated miniprion PrPSc106 [5]. This yielded low-resolution projection maps to 7 Å. The N-linked sugar side chains were identified and by inference the location of the helices to which the sugars are attached. The helices would be located on the surface of PrP^{TSE} oligomers. Subtracting the density patterns of PrP27–30 and PrPSc106 allowed the region corresponding to the internal deletion (Δ141–176) in PrPSc106 to be localized towards the inside of the PrP27–30 crystals.

Deciphering the Structure of Infectious Prion Protein with Antibodies

PrP-specific antibodies are becoming increasingly useful tools in structural studies. The partial denaturation of PrP^{TSE} in Gdn-HCl increases its sensitivity to protease digestion and results in cleavage at codon 115 (as opposed to codon 90 without Gdn-HCl) and some proteolysis of the C-terminal residues 217–232 [88]. The partial denaturation of PrP^{TSE} was reversible since it could be refolded to a species which was only digested to residue 90 following dilution of the denaturant. The PrP27–30 protein displayed similar unfolding-refolding properties to PrP^{TSE} indicating that the N-terminal 89 residues are not necessary for the refolding reaction. The PrP sequence from

115 to 217–232 was the most resistant to unfolding, and the unfolding of this region beyond a threshold amount of denaturant prevented refolding. Recombinant antibody fragments (Fabs) raised against liposome-incorporated PrP27–30, reacted with epitopes encompassed by residues 90–120 [89]. These epitopes are largely inaccessible in PrP27–30 but were exposed following denaturation with 3 *M* Gdn-thiocyanate. In contrast, C-terminal epitopes are accessible in both PrP^{C} and PrP^{TSE}. This indicates that the N-terminal residues 90–120 are intimately involved in PrP^{C} to PrP^{TSE} conversion. This is supported by data showing that deleting any part of the 90–120 sequence perturbs PrP^{TSE} formation [90]. The reactivity of partially denatured PrP^{TSE} to the 3F4 monoclonal antibody formed the basis of a conformation-dependent immunoassay that could distinguish eight different prion strains propagated in Syrian hamsters [91]. This supports the model that strain types can correlate with different conformations of PrP molecules. If validated, this would confirm PrP as a molecule capable of assuming a number of different stable conformations.

Prion Protein Folding Pathways and Their Conformational Properties

The mobility of PrP^{C} as measured by hydrogen/deuterium exchange showed that in the unfolded state only a 10-residue region surrounding the disulphide bond retained its native structure [92]. There was no evidence of a highly organized folding intermediate. This suggests the conversion of PrP^{C} to PrP^{TSE} proceeds via a highly unfolded state which retains only a 10-residue nucleus rather than via a predominately organized folding intermediate which retains the α-helical regions. PrP conformation is influenced by pH as an acidic environment induces a dramatic increase in the exposure of hydrophobic regions on the surface of the protein [93]. At pH 5–7, HuPrP90–231 unfolds by a two-state pathway, but at lower pH a three-state unfolding is observed with a stable transition intermediate [93]. This intermediate is rich in β-sheet structure and maintains a high degree of solubility, which may be the precursor for the initiation of PrP^{TSE} aggregates. Notwithstanding that PrP may encounter lower pHs in a cellular environment [93], it was also proposed that the aqueous buffers used in biophysical studies are not physiologically relevant [94]. This was explored by measuring the interaction between PrP and lipid membranes and it was found that the membrane-bound form had a less structurally stable C-terminal domain and the flexible N-terminal became ordered [94]. This would be consistent with the N-terminal residues, 90–120, being necessary for PrP^{TSE} formation [89].

The folding kinetics of MoPrP121–231, as measured by tryptophan fluorescence, indicated that this fragment unfolded and refolded in the submillisecond scale at 22°C [95] which is extremely rapid for a protein containing a disulphide bond. In contrast, the kinetics of unfolding and refolding measured at 4°C showed that MoPrP121–231 folds with a half-life of 170 μs, with no obvious intermediates detected. It was concluded that the conversion of PrP^C to PrP^{TSE} does not involve a kinetic folding intermediate, but rather a thermodynamic folding intermediate. A possible folding intermediate was identified by reducing the disulphide bond of recombinant HuPrP91–231 in a low ionic strength buffer at acid pH. These conditions generated a highly soluble monomeric species which was rich in β-structure and partially proteinase K-resistant [96]. This species, termed β-PrP, contained two unstructured regions (91–126 and 229–230) which were also unstructured in PrP^C [34] and suggests the rearrangement of PrP^C to β-PrP occurs in the structured regions of PrP^C. Increasing the pH to 8.0 caused the slow (over a number of days) conversion of β-PrP back to PrP^C. Raising the ionic strength to physiological concentrations resulted in β-PrP to form fibrils with scrapie amyloid-like morphology and dimensions. The buffer conditions for β-PrP formation are similar to those of endosomal organelles, which suggests this may be a site of PrP^{TSE} propagation.

The dissociation and unfolding of PrP^{TSE} may occur via a sequential four-step pathway of aggregates involving dissociation to folded monomers, partially unfolded intermediates and finally an unfolded monomer [66]. The PrP27–30 intermediate species is a compact, metastable hydrophobic molecule with a significant proportion of non-β-sheet secondary structure and little tertiary structure interaction [69]. It was proposed that this structure has the characteristics of an aggregated molten globule folding intermediate [69]. The role and possible mechanism by which this intermediate is generated in vivo is not known, but its induction by low pH in the presence of salts implicates particular organelles, such as endosomes, or general cellular acidosis as being possible factors.

Conclusion

If the protein-only hypothesis of prion transmission proves correct then the above-mentioned studies will be invaluable for not only understanding the molecular processes which underlie the TSE, but also for understanding protein folding in general. Considerable detail is available on the structure PrP^C and its biophysical and protein folding properties. However, solving the solution structure of PrP^C provides one part of the equation, but our lack of knowledge of a complete three-dimensional structure of PrP^{TSE} remains a clear deficiency.

The available structural data on PrP^{TSE} and its distinct biochemical and biophysical properties to PrP^{C} has led to two main models, the template-assistance model [2, 97] and the nucleation-dependent pathway [98, 99], to explain PrP^{C} to PrP^{TSE} conversion. The template-assistance model proposes PrP^{TSE} exists as a monomer or small distinct oligomers, while the nucleation model suggests PrP^{TSE} can exist as a large polymerized unit [99]. In both models, PrP^{TSE} would act as a catalyst driving the conversion of PrP^{C} into PrP^{TSE}. However, a variation on the nucleated polymerization model proposes a non-catalytic conversion, whereby PrP^{C} can readily self-convert into PrP^{TSE} or an intermediate and is stabilized by incorporating into a PrP^{TSE} seed [99]. These models are consistent with the structural plasticity of PrP^{C} and its propensity to stabilize itself through self-aggregation. However, a unified model on PrP^{C} to PrP^{TSE} biogenesis requires elucidation of the physical size of the infectious particle, identification of the folding intermediates, deciphering the structure of PrP^{TSE} and the relative contribution of pH, lipids, metals, redox environment, primary sequence and protein cofactors. Ultimately, a defined PrP^{TSE} structure will help to explain the molecular basis of phenotypic strain variation and the nature of the 'toxic species'. This may permit the design of therapeutic agents that favorably perturb the transformation reaction and hence disease.

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Prion Strains and Species Barriers

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Prion diseases or transmissible spongiform encephalopathies are a group of neurodegenerative disorders affecting both humans and animals. These diseases include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Sheinker disease (GSS), fatal familial insomnia (FFI) and kuru. Prion diseases are transmissible by inoculation, have long incubation periods and share common histological features. One of the central features of prion disease is the conversion of the normal cellular form of the host-encoded prion protein (PrP^{C}) to an abnormal isoform designated PrP^{Sc}. This conversion occurs post-translationally and is thought to involve a conformational change rather than a covalent modification. PrP^{Sc} may be distinguished from PrP^{C} by its insolubility in detergent and partial resistance to protease degradation. Prion diseases in humans may be inherited through germ-line mutations in the human prion gene (*PRNP*), acquired through inoculation (including dietary exposure) or caused by rare events which convert PrP^{C} molecules to PrP^{Sc}. Much data exists to suggest that the sole or principal component of the transmissible agent, the prion, is an abnormal isoform of PrP and forms the basis of the protein-only hypothesis of prion propagation. This hypothesis suggests that PrP^{Sc} replicates itself by recruiting PrP^{C} molecules and inducing a conformational change, resulting in the accumulation of further PrP^{Sc} which may in turn convert more of the cellular isoform [1]. Ablation of the prion gene in transgenic mice (termed *Prnp*$^{0/0}$ mice) supports this hypothesis, with the animals being both resistant to experimental scrapie and failing to propagate prion infectivity, firmly supporting the role of PrP in these diseases [2–4].

Prion Strains

While there are several lines of evidence to support the protein-only hypothesis of prion propagation, the existence of distinct 'strains' or isolates which can be stably passaged in inbred mice of the same genotype has been a challenge to accommodate within this model. Prion strains can be distinguished by their different incubation periods and patterns of neuropathology ('lesion profiles') when passaged in inbred strains of mice. Three hypotheses have been proposed to explain the existence of prion strains. The first is the virus or virino hypothesis which assumes a mutation in an agent-specific DNA or RNA genome. However, no direct evidence for such an agent-specific genome has been produced. The second hypothesis tries to bridge the gap between the virus and protein-only hypotheses. Weissmann's unified hypothesis [5] states that while the protein is sufficient for infectivity, some small nucleic acid confers strain properties on the protein. The third is the protein-only hypothesis where PrP itself encodes the information required to determine the strain phenotype.

Evidence that strain specificity is encoded by PrP itself, and not a nucleic acid, was provided by studies on two distinct strains of transmissible mink encephalopathy (TME). Termed hyper (HY) and drowsy (DY), these strains can be serially passaged in hamsters. The strains can be distinguished by physicochemical properties of the PrP^{Sc} deposited in the brains of the infected hamsters. After treatment with proteinase K, strain-specific migration patterns can be seen on Western blots with DY PrP^{Sc} being more protease sensitive than HY PrP^{Sc} and producing different banding patterns [6]. The banding patterns are caused by different N-terminal cleavage sites for proteinase K which suggest the strains represent different PrP^{Sc} conformations, which has also been supported by infra-red spectroscopic studies [7]. Maintenance of these TME strains has also been shown in an in vitro conversion model when hamster PrP^{C} is mixed with HY or DY PrP^{Sc}, which also supports the view that prion strains involve different PrP conformers [8, 9].

Molecular Basis of Prion Strains

Several human PrP^{Sc} types have recently been identified which are associated with different clinicopathological phenotypes of CJD [10–12]. These types are distinguished by different fragment sizes seen after limited proteinase K digestion, suggesting different conformations of PrP^{Sc}. These types can be further classified by the ratio of the three PrP bands seen after protease

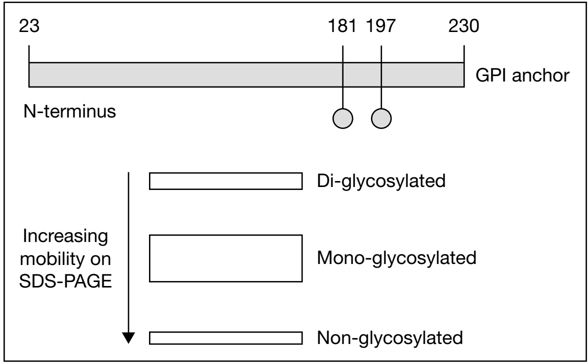

Fig. 1. Scheme illustrating the glycosylation of PrP^C. The addition of glycans at residues 181 or 197 of human PrP result in the production of di-, mono- or unglycosylated PrP fragments observed on Western blots.

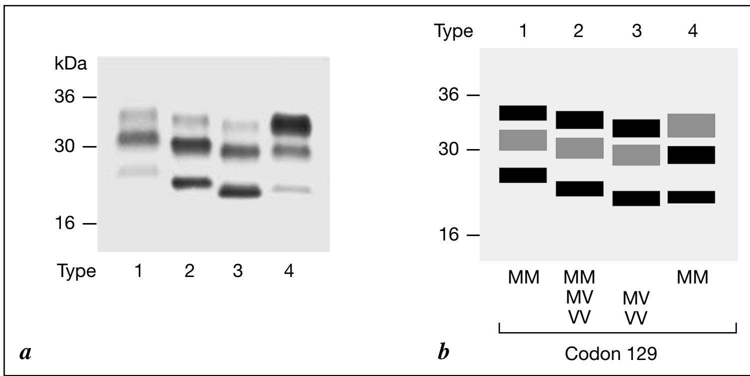

Fig. 2. ***a*** Western blot developed with monoclonal antibody 3F4 showing four types of PrP^{Sc} observed in cases of human prion disease. ***b*** Scheme illustrating human PrP^{Sc} types and their relationship with genotype at codon 129 of the prion protein gene.

digestion, representing di- mono- and unglycosylated fragments of PrP^{Sc} (fig. 1). PrP^{Sc} conformation and glycosylation are therefore plausible candidate as forming the molecular basis of prion strain diversity. Classical CJD is associated with PrP^{Sc} type 1–3, while type 4 human PrP^{Sc} is uniquely associated with vCJD and characterized by glycoform ratios which are distinct from those observed in classical CJD [10] (fig. 2). The glycoform ratios in vCJD are similar to those seen in BSE and cases of natural or experimental BSE

transmission to a variety of species with a relative abundance of the di-glycosylated PrP^{Sc} fragment.

As PrP glycosylation occurs before conversion to PrP^{Sc}, the different glycoform ratios may represent selection of particular PrP^{C} glycoforms by PrP^{Sc} of different conformations with inoculated prions preferentially recruiting and converting particular glycoforms of PrP^{C}. This effect can be illustrated in the transmission of classical CJD and BSE to wild-type mice where two different glycoform patterns and fragment sizes can be generated from the same pool of PrP^{C}. 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, PrP^{Sc} glycosylation patterns may provide a substrate for the neuropathological targeting that distinguishes different prion strains. Particular PrP^{Sc} glycoforms may replicate 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. High-resolution two-dimensional electrophoresis has been used to investigate the heterogeneity of PrP^{C} glycoforms and has revealed over 50 unique spots on immunoblot, each one representing distinct PrP^{C} species distinguished by differences in molecular mass and charge generated through glycan diversity [13].

Further supportive evidence for the involvement of PrP glycosylation in prion strain propagation has come from the study of transgenic mice expressing PrP with mutations interfering with N-linked glycosylation [14]. These mutations led to aberrant distribution of PrP^{C} which also affected the ability of these mice to be infected with prions. Mutation of the first glycosylation consensus sequence resulted in a restricted pattern of PrP^{C} expression which did not support PrP^{Sc} replication. Interestingly, mutation of the second glycosylation site resulted in a wider expression pattern of PrP^{C} which supported prion replication, however the incubation period for infection with hamster prions in these mice was over 500 days. Differences in the relative spatial expression of the glycoform mutants however compromise the use of this model in directly evaluating the precise role of glycosylation as a determinant of strain variation. Indeed, it has been established that there may be considerable heterogeneity among the carbohydrates attached to PrP^{C} (selected from over 400 distinct structures) [15] and as a consequence it will be an extremely complex task to determine whether particular carbohydrates are preferentially involved in the replication of distinct prion strains. Evidence that this may be the case is exemplified by the apparent superimposition of strain- and tissue-specific effects on PrP glycosylation seen in vCJD tonsil, spleen and lymph node PrP^{Sc}, which differs in the proportion of

the PrP glycoforms from that seen in vCJD brain [16, 17]. Transmission of PrP^{Sc} from these peripheral tissues will be useful in trying to assess the contribution of glycosylation in conferring strain-specific properties. Indeed, the cellular compartment in which prion propagation occurs may also have an effect on the glycosylation profile of PrP^{Sc}. This has been hinted to in experiments which have demonstrated that PrP^{Sc} glycosylation can be influenced by the cell and significantly altered by the glycosylation state of the pool of PrP^{C} which is converted [18]. Also, in a cell-free conversion assay it has been shown that the glycosylation of PrP^{C} affects its ability to bind to PrP^{Sc}, and that differences in primary sequence of the PrP^{C} and PrP^{Sc} affect the efficiency of conversion [19].

Since the strain-specific physicochemical properties of PrP^{Sc} can be maintained upon transmission to susceptible animals, variance of conformation and glycosylation represent plausible candidates for the molecular basis of prion strain diversity. However, as there are also strain-, species- and tissue-specific effects on PrP glycosylation, the superimposition of all of these factors will be involved in determining the molecular basis of a particular prion strain. Further evidence for PrP^{Sc} conformation enciphering prion strain comes from the study of eight different prion strains propagated in hamsters. Using a conformation-dependent immunoassay that quantitates the amount of antibody bound to denatured and native PrP molecules and plots this against the total PrP^{Sc} concentration, it was demonstrated that each of the strains tested had different concentrations of particular PrP^{Sc} conformers [20]. These data strongly support the ‘protein-only’ hypothesis of infectivity and suggest that strain variation is encoded by a combination of PrP conformation and glycosylation.

PrP primary structure is known to influence the conformation of PrP into a number of different folds which result in these distinct PrP^{Sc} conformers. That PrP primary structure influences which conformations may be thermodynamically preferred could therefore determine prion species barriers. Inoculated prions preferentially convert PrP^{C} into one of these preferred conformers, leading to prion propagation and disease. If the PrP primary structure of the host does not allow folding into the conformation of an inoculated prion, then there will be a barrier to transmission. However, should the PrP primary structure of the host allow folding into the conformation of an inoculated prion, whether from the same or a different species, then there will be transmission in the absence of a barrier. Therefore, in this model, a species barrier may be determined by the degree of overlap between the subset of PrP^{Sc} conformers allowed by the PrP of the host with those represented in the donor species [21].

While the function of PrP still remains unknown, its role in the development of prion disease is firmly established through transgenic mouse experiments. Conditional knockout of PrP in mice using a cre-loxP system at 9 weeks of age does not affect neuronal survival, but has an effect on the modulation of

neuronal excitability, suggesting that the pathogenic mechanism in prion diseases is not caused by a loss of PrP function [22]. A series of transgenic mice containing N-terminal deletions of PrP have been generated and assessed for the ability to propagate prion disease and probe structure/function studies of this protein. Transgenic mice expressing PrP with deletions up to residue 93 (on a *Prnp*$^{0/0}$ background) are phenotypically normal, develop scrapie when inoculated with mouse prions, and generate protease-resistant truncated PrP, which may be propagated [23]. Interestingly, these animals lack the octapeptide repeat region, and as insertions of additional repeat units are pathogenic in humans [24], raise some interesting questions as to the functional significance of this region of the protein which has been shown by solution NMR spectroscopy to have no discernible tertiary structure [25, 26]. Another transgenic mouse which contained deletion of the first α-helix, β-strand and part of helix 2 (PrPΔ23–88Δ141–176) were also susceptible to infection of prions and were able to propagate the infectivity generated [27]. Further experiments using transgenic mice overexpressing N-terminal deletions extending beyond mouse residues 106 to 121 and 134 develop an ataxic syndrome between 3 and 8 weeks of age. That this phenotype could be rescued by introducing a single wild-type PrP allele suggests that this phenomenon is not simply due to the expression of the truncated PrP protein, but rather its interference in an as yet unknown signalling pathway [23].

Species Barriers

Prion diseases are characterized by their transmissibility to experimental animals. This has been shown by the transmission of kuru to chimpanzees [28], and CJD [29] or GSS [30] to primates.

The transmission of prion diseases between different mammalian species, as measured by the appearance of clinical signs in the host, is limited by a ‘species barrier’ [31]. These barriers result in: (i) longer, more irregular incubation periods, which may approach or even exceed the lifespan of the animal; (ii) atypical clinical or histological signs in the recipient, and (iii) a decreased fraction of animals succumbing to disease. Transmission of prion diseases is dose-dependent, with an increase in the mean incubation period and decrease in the proportion of affected animals, as the inoculum is diluted. The inversely proportional relationship between incubation period and infectious titre can be useful in determining the infectious titre of a particular inoculum and also in the ‘cloning’ of prion strains using low dilutions of inocula, similar to principles used in classical virology.

Following the first transmission across a species barrier, upon further passage to the same species, the incubation period usually shortens and the clinical and histological signs become more consistent. The drop in incubation period from the first passage, between species, to the second and subsequent passage within the same species is often used to quantify the extent of these barriers. However, some species barriers, such as that between hamsters and mice, may be so great as to prevent the development of clinical disease upon first passage across species, even using the most efficient, intracerebral, route of inoculation with high-titre preparations.

One of the most intensively studied barriers is that limiting transmission of disease between hamsters and mice using the 263K strain of hamster scrapie [32, 33] (also referred to as Sc237 [34]). This strain of hamster scrapie was derived from the 'drowsy goat' source of scrapie which was passaged firstly through rats and then several times in hamsters. During the initial passage in hamsters, two pools were generated and inoculated into mice, resulting in the generation of two strains, 302K and 431K, which are pathogenic for both mice and hamsters. Following further passage in hamsters and cloning of the strain using limited dilutions of the inocula, the hamster-passaged scrapie from both pools lost the pathogenicity for mice and remained virulent in hamsters, producing disease in around 60 days post-inoculation. The reduction in incubation period in hamsters inoculated with the rat-passaged scrapie from 300 days to the 60 days observed after five further passages in hamsters suggests that the host influences the selection and adaptation of these strains. The 263K strain has long been regarded as non-pathogenic to mice, with no clinical signs of disease observed in inoculated mice at up to 735 days post-inoculation [33].

Advances in transgenic animal technology have proved invaluable in exploring this phenomenon, and abrogation of the species barrier was demonstrated in transgenic mice overexpressing PrP sequences from the donor species. It was shown that transgenic mice overexpressing Syrian hamster PrP transgenes showed no species barrier when infected with hamster prions; transgenic animals succumb to illness at around 75 days post-inoculation, while wild-type littermates remained symptom-free at over 500 days post-inoculation [34]. Additionally, the transgenic mice were found to harbour high levels of hamster and not mouse PrP^{Sc} which was found on passage to be pathogenic for hamsters and not mice [35]. Further experiments established that transgenic mice generated on a *Prnp*$^{0/0}$ background reduced the species barrier effect further, with a reduction of incubation period concomitant with removal of endogenous mouse PrP [36]. This led to the notion that primary PrP sequence is one of the main factors involved in the species barrier. Experiments using transgenic mice overexpressing human PrP genes have also been used to abrogate the species barrier between mice and humans [10, 37–39]. Transgenic mice

expressing human PrP have consistent incubation periods of between 180 and 220 days when inoculated with human prions from classical CJD cases. In these mice it has been possible to transmit a variety of human prion diseases including inherited, acquired, sporadic, variant CJD, and FFI [10, 38, 40, 41]. These mice have also been useful in modelling the risk posed to humans by prion diseases of other animals, in particular BSE from cattle. It was found that at prolonged incubation periods (>500 days) a proportion of the BSE-inoculated 'humanized' mice (which encode valine at codon 129, a common polymorphism in humans) showed clinical symptoms and neuropathology consistent with prion disease [38]. That these mice succumbed to disease only at long incubation periods with less than a 100% attack rate suggests that a substantial species barrier is present for this human PrP genotype. However, as cases of vCJD have only been observed in humans who are methionine homozygous at codon 129, it will be important to assess the susceptibility of transgenic mice expressing this genotype to infection with BSE.

Another study on the transmissibility of human prion diseases to transgenic mice suggested that expression of human PrP genes on a *Prnp*$^{0/0}$ background was necessary for transmission of human prions and that the presence of endogenous mouse PrP interfered with ablation of the species barrier [37]. Similar results were seen in experiments with transgenic mice expressing bovine PrP genes where the mice bred on a *Prnp*$^{0/0}$ background had much shorter incubation periods than those containing endogenous mouse PrP genes [42]. As the primary sequence alone was not the sole determinant of the species barrier, an as yet unknown factor termed 'protein X' was hypothesized to have an effect on the species barrier phenomenon. However, protein X could be PrP itself.

In exploring the role of the primary PrP sequence on the species barrier, several transgenic mice were generated containing chimeric mouse/hamster PrP genes. One such construct, where the central third of the mouse open reading frame is replaced with the corresponding section of hamster sequence (called MH2M), when expressed in transgenic mice generates prions with an artificial host range [43]. Such transgenic mice are susceptible to infection with both mouse and hamster prions and the PrP^{Sc} formed in these animals is chimeric in origin. These chimeric prions have been shown to be transmissible to both mice and hamsters and have proved useful in examining some of the many strains of mouse scrapie in the hamster by passage through this intermediate host, which shows no appreciable species barrier to either source of prions [44]. Similar experiments have been performed in transgenic mice expressing mouse-human [39] and mouse-bovine [42] chimeric PrP transgenes (MHu2M and MBo2M respectively). Interestingly, the MBo2M transgenic mice were not sensitive to inoculation with bovine prions and it was presumed that differences in amino

acid residues 180–205 between mouse and bovine PrP genes may be responsible, however this issue remains unresolved.

Importantly, there are other examples where the primary PrP sequence alone cannot explain such species barriers, perhaps best illustrated by the natural transmission of BSE to a wide variety of hosts, all with different PrP primary amino acid sequences. It has been clear for many years that prion strain type (see below) has a crucial effect on species barriers. A striking example of the impact of strain on species barriers has been provided by analysis of BSE prions. While classical CJD prions, propagated in humans expressing wild-type human PrP, transmit highly efficiently to mice expressing only human PrP with transmission characteristics consistent with complete absence of a species barrier [38], vCJD prions, also propagated in humans expressing wild-type PrP of identical primary structure, have transmission properties completely distinct from other human prions (as assessed either in transgenic or wild-type mice) but indistinguishable from those of cattle BSE [40, 45] and consistent with the presence of a transmission barrier. Transmission of two hamster prion strains (Sc237 and DY) to transgenic MH2M mice results in two different transmission profiles with a species barrier effect observed in the Sc237-inoculated mice whereas efficient transmission was observed in the DY-inoculated animals [46]. This data suggests, given the conformation of PrP^{Sc} in both of these hamster strains are different, that species barriers can be effected by PrP^{Sc} conformation, and not solely by PrP^{Sc} primary sequence, which in this case are identical for both strains.

Subclinical Prion Infection

Subclinical prion infection has been observed experimentally since early experiments attempting to transmit sheep and goat scrapie to rodents. These studies included the transmission of scrapie from Suffolk sheep via the intragastric route into mice [47]. No clinical signs of scrapie were observed after 13 months in the mice, however some histological lesions were present. In reviewing these experiments, Zlotnick [47] refers to passaging brain and spleen pools from these ‘subclinical cases’ into further mice, which developed symptoms some 5–6.5 months following intracerebral inoculation, and it is from these experiments that the ME7 strain of mouse scrapie was derived.

Recent studies using the transmission barrier between mice and hamsters with 263K hamster prions have provided further evidence that subclinical forms of prion disease exist. In one study, upon injecting wild-type mice with 10^7 ID_{50} (where ID_{50} represents the half-maximal infectious dose) units of 263K

hamster prions, no clinical signs of scrapie were observed, up to 782 days post-inoculation [48]. Brain and spleen tissue extracts taken from clinically normal 263K-inoculated mice (taken between 204 and 782 days post-inoculation) were intracerebrally injected into further mice and hamsters. All hamsters inoculated with the mouse-passaged 263K material from both brain and spleen, developed clinical scrapie, whereas mice inoculated with the same material were not affected. These authors estimated the titre of infectivity (calculated from the long incubation period seen in the hamsters) for the clinically normal mouse-passaged 263K prions to be about 10^2 ID_{50} per gram of tissue, which is much lower than that seen in clinically sick animals in the terminal stages of disease. Although this study provided evidence for the existence of subclinical prion infection in a species previously thought to be resistant, it was not possible to exclude persistence of the original inoculum rather than de novo prion replication as an explanation for these transmissions.

Other studies, which have included more detailed molecular and neuropathological assessment in clinically normal animals, have provided convincing evidence for replication of infectivity in mice inoculated with hamster prions [49, 50]. In one set of experiments, CD-1 wild-type mice were inoculated with approximately 8.5×10^6 LD_{50} of hamster Sc237 prions (purported to be the same strain as 263K [34]) or with saline alone [49]. No signs of clinical scrapie were observed in any of the animals during their lifespan and there was no significant difference in the ages at which animals in either inoculation group died. In all animals, brain tissue was examined for the presence of protease-resistant PrP^{Sc} by Western blotting with two different anti-PrP antibodies; one specific for hamster PrP, the other for both hamster and mouse PrP. Application of these antibodies revealed readily detectable levels of mouse PrP^{Sc} in all animals surviving $\geq$659 days post-inoculation with Sc237 prions. None of the animals displayed detectable levels of hamster PrP^{Sc} using the hamster PrP-specific antibody [49]. Second passage transmissions of infectivity to both Tga20 mice and hamsters resulted in consistent incubation periods with 100% of the animals succumbing to disease (fig. 3). These data argue against a low titre or persistence of the original inoculum as an explanation for the lengthy incubation periods and instead support the replication model. Indeed, the prion titres in the brains of these mice, when assayed in hamsters, were calculated to be approximately 10^8 LD_{50} units/g of tissue, which is considerably more than what was originally inoculated ($\sim 8.5 \times 10^6$ LD_{50}). These data point to the generation of a novel prion strain(s) infectious to both mice and hamsters whose properties contrast sharply with the limited host range of the original prion strain.

In a separate study, the kinetics of prion replication in hamster scrapie strain 263K-infected mice has been studied by transmitting 23 clinically normal

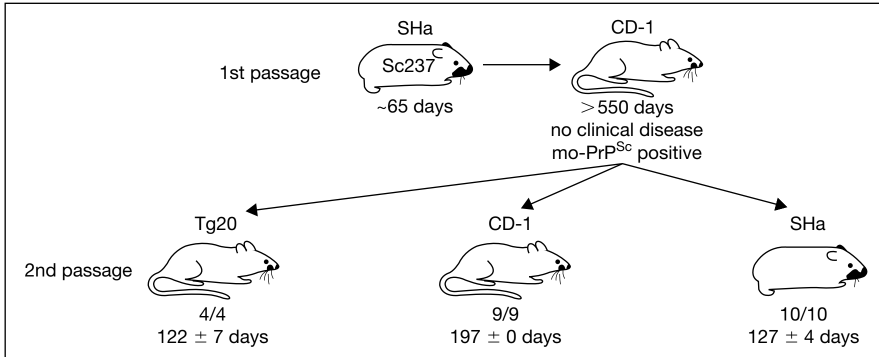

Fig. 3. Demonstration of subclinical prion disease in mice. Mice infected with hamster prions do not develop clinical disease. However, upon subpassage from clinically normal mice inoculated with hamster prions, clinical disease is observed in both mice and hamsters. Tg20 mice overexpress mouse PrP^C and have shorter incubation periods than wild-type counterparts. The numbers below the animals represent the numbers of mice affected/inoculated; the incubation periods (±SEM) are shown in days.

animals at various time-points into both mice and hamsters [50]. Western blotting was used with hamster- and mouse-specific antibodies at various time-points post-inoculation to characterize the species of PrP^{Sc} present in the brains of the inoculated mice. Interestingly, hamster PrP^{Sc} could be detected in the mice brains at 2 h post-inoculation, and then became undetectable during all of the time-points tested up to 782 days. That the brain tissue at the 2-hour time-point was positive for PrP^{Sc} and then became negative throughout the remainder of the time course is consistent with it being derived from the inoculum, which then cleared from the brain. Using an antibody specific to mouse PrP, mouse PrP^{Sc} was observed in 263K-inoculated mice from day 310 onwards. When brain extracts from mice were repassaged in hamsters it was demonstrated that as the age of subclinically infected mice increased, so did the infectious titre when material was repassaged in hamsters. The 263K-inoculated mice sacrificed at 2 h, and 5 days post-inoculation were shown to have significantly higher levels of hamster infectivity than mice tested at 140–390 days. This is consistent with persistence of the original 263K inoculum in the mouse brain at these early time-points following intracerebral inoculation. At extended periods from 463 to 782 days post-inoculation, however, brain material from 263K-inoculated mice were able to produce disease in hamsters with 100% attack rate and decreased incubation periods. However, as was seen in the previous study [49], the incubation period of mouse-passaged 263K prions in hamsters is

lengthened, even in the longest subclinically infected mice which would have the highest titre, from 70 days for hamster 263K to around 120 days for mouse-passaged 263K prions.

Subclinical prion diseases are not only restricted to instances involving crossing species barriers. In studies investigating the role of the lymphoreticular system in the pathogenesis of prion disease it was demonstrated that B-cell-deficient mice appeared resistant to peripheral prion infection [51]. These mice are susceptible to prion infection when inoculated intracerebrally, exhibiting incubation periods similar to those seen in wild-type control animals. While peripherally challenged immunodeficient animals showed no clinical signs of scrapie, marked accumulation of PrP^{Sc} in their brains was observed [52]. Inoculation of wild-type mice intracerebrally with brain extracts from these animals revealed that the subclincally infected animals harboured significant infectious titres which in some cases exceeded those seen in terminally sick wild-type control animals. While the above experiments have relied on the inoculation of high-titre preparations, subclinical prion infection has been observed in Tga20 mice injected with low doses of RML or ME-7 prions [53]. Clinical signs in some of these subclinically affected animals were found to oscillate between a healthy appearance and mild scrapie symptoms. However, animals that developed an ataxic syndrome always progressed to terminal stages of disease. Intriguingly, subclinically affected animals which were sacrificed at over 200 days post-inoculation contained similar levels of infectivity as terminally sick animals [53].

Implications of Prion Disease vs. Prion Infection

The demonstration of animals which can harbour high levels of prion infectivity and detectable levels of PrP^{Sc} without exhibiting any clinical signs of prion disease challenges our understanding of the pathogenic mechanisms involved in these diseases. That an animal may tolerate $>10^8$ ID_{50} units/g brain tissue in the absence of gross histological abnormalities also questions the nature of the neurotoxic molecule responsible for the widespread neuronal death and spongiosis which are hallmarks of these diseases. Several cases have been documented where there lacks a correlation between clinical disease and neuropathological features of prion disease [54], clinical cases in which levels of PrP^{Sc} are absent or barely detected [41, 55–57], and studies in mice which have low levels of PrP^C which after inoculation develop high levels of PrP^{Sc} and succumb to disease earlier than their wild-type counterparts [36]. However, the requirement of PrP for the development and to mediate the neurodegeneration

of prion disease still remains absolute. It has also been demonstrated that brain grafts producing high levels of PrP^{Sc} do not damage adjacent tissue in PrP knockout ($PrP^{0/0}$) mice [58].

The key to unravelling these issues may reside in the precise definition of the neurotoxic prion molecule at a molecular level. From the studies discussed here it is possible to hypothesize that the neurotoxic prion molecule may not be PrP^{Sc} itself, but a toxic intermediate that is produced during the conversion of PrP^{C} to PrP^{Sc}. In such a model, PrP^{Sc} represents a relatively inert end-product, and that the steady-state level of the neurotoxic intermediate determines the rate of neurodegeneration. The levels of the toxic intermediate may be regulated by factors such as the ability of the cell to clear this neurotoxic species as it is formed, such that once levels are reached at which the cell can no longer degrade this molecule, levels of the toxic species accumulate resulting in neurodegeneration. Such a cascade may stress nearby cells which also lose their ability to process the toxic species, possibly explaining the rapid degeneration which takes place once the disease takes hold. A possible candidate for such a toxic species could be a soluble conformer of PrP. Studies using recombinant prion protein have shown that at acidic pH PrP can fold into a soluble monomer composed predominantly of β-sheet structure that is protease-resistant and prone to aggregation into fibrils, suggesting such conformer may exist under physiological conditions [59]. Similarly, in Alzheimer's disease, which is also associated with the accumulation of insoluble protein aggregates, the soluble pool of the Aβ peptide (as opposed to the insoluble pool associated with plaque deposits in the brain) has been shown to be the principal determinant of the severity of neurodegeneration [60]. However, even if PrP^{Sc} is hypothesized as being an inert end-product of the conversion from the cellular isoform to the neurotoxic form, it still remains a strong diagnostic marker of prion infectivity.

The existence of subclinical prion infections has important public health implications with the possibility that iatrogenic transmission could occur from apparently healthy humans with high prion titres and from other species (such as sheep, pigs and poultry) which were exposed to BSE prions via contaminated feed and not developed clinical disease. As BSE prions are pathogenic in a wide variety of species and that the strain characteristics of BSE prions are retained upon transmission to new species suggests that BSE passaged in animals other than cattle will retain pathogenicity for humans. Such a possibility underlies the need to investigate the prevalence of subclinical prion infections in cattle and other species that have been exposed to BSE prions. This could include screening apparently healthy animals entering the food chain, which could be achieved using commercially available systems for detecting PrP^{Sc}.

Re-Evaluating Prion Strains and Species Barriers

The demonstration of prion infectivity in apparently resistant animals and those subclinically infected after same species passage questions our current understanding of prion species barriers and mechanisms of pathogenicity. Studies on the assessment of prion species barriers have relied on the development of clinical symptoms in inoculated animals. From the studies discussed above it now appears prudent to include molecular and neuropathological assessment of clinically healthy inoculated animals to exclude the possibility of subclinical prion infection. Defining any pathways involved in the long, clinically silent period of these diseases will be of great importance and could lead to early therapeutic and diagnostic strategies for these diseases. Identifying the neurotoxic prion molecule still remains one of the key factors in not only proving the protein-only hypothesis, but in enhancing our understanding of these diseases at a molecular level.

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Prions of *Saccharomyces* and *Podospora*

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Prion, a term coined to describe the infectious material found in scrapie-associated filaments, is now more broadly used to describe infectious proteins. Prions act as hereditary material in that they have the ability to convert a normal, cellular protein into an abnormal or prion form. This process proceeds autocatalytically and allows a cell to be in two different phenotypic states, either having only normal cellular protein or mostly prion form. Although the exact mechanism of prion propagation is not known, there is a growing body of evidence which suggests that for all prion proteins, known so far, a conformational change and aggregation into an amyloid-like form is required to alter the normal form into its prion form. However, in principle there are other mechanisms for prion formation possible, e.g. the autocatalytic covalent alteration of a protein [1]. On the other hand, not all proteins forming amyloid-like filaments are infectious proteins, e.g. Alzheimer's disease does not seem to be infectious.

In this review, we will mainly focus on the well-characterized yeast prion [URE3], but also shortly discuss [*PSI*] and other yeast and fungal prions (for an overview and direct comparison of prions, see table 1).

Three Genetic Criteria for Prions

Three genetic criteria that can distinguish yeast prions from nucleic acid replicons were described by Wickner [2] in 1994 (fig. 1):

(1) Reversible curability. If a prion can be cured, then it should be able to arise again in the cured strain. Curing a nucleic acid replicon however, such as

Table 1. Comparison of the well-characterized yeast and fungal prion systems

Prion system	[URE3]	[*PSI*]	[PIN]	[Het-s]
Organism	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	*Podospora anserina*
Phenotype	Nitrogen derepression	Translation read-through	Inducibility of [*PSI*]	Heterokaryon incompatibility
Protein	Ure2p	Sup35p	Rnq1p	HET-s
Function of normal (soluble) protein	Nitrogen repression	Translation termination	Unknown	Unknown Soluble form might have none
Modes of curing	Growth on GdmHCl Expression of Ure2p fragments Meiosis	Growth on GdmHCl Osmotic shock	Growth on GdmHCl	Meiosis
Influence of chaperones	Deletion of Hsp104 cures Overexpression of Ydj1p (Hsp40) cures Overexpression of Ssa1p (Hsp70) cures	Deletion of Hsp104 cures Overexpression of Hsp104 cures Hsp70s modulate	Deletion of Hsp104 cures	Unknown
Prion-inducing region	N-terminal domain Asn-rich	N-terminal domain Asn/Gln-rich	N-terminal domain Asn/Gln-rich	C-terminal domain (not rich in Asn or Gln)
Filament-forming region	N-terminal domain	N-terminal domain	N-terminal domain	C-terminal domain
Proteinase K resistance of prion	N-terminal domain resistant, leaves thin filaments	N-terminal domain transiently resistant	Unknown	Digestion leaves thin filaments

the mitochondrial DNA or a virus or plasmid, results in cells, which can only re-acquire that replicon by its introduction from outside.

(2) Overproduction of the normal protein increases the chances of a prion arising spontaneously.

(3) The gene for the protein is necessary for the propagation of the prion and the phenotypes for the mutant gene and prion are the same. The reason for this is that in both cases the normal form of the protein, and therefore its function, is absent or deficient. The second part of the third criteria is true as long as the phenotype of the prion form is a loss-of-function phenotype, which is apparently not the case for the mammalian PrP and [Het-s], as discussed later.

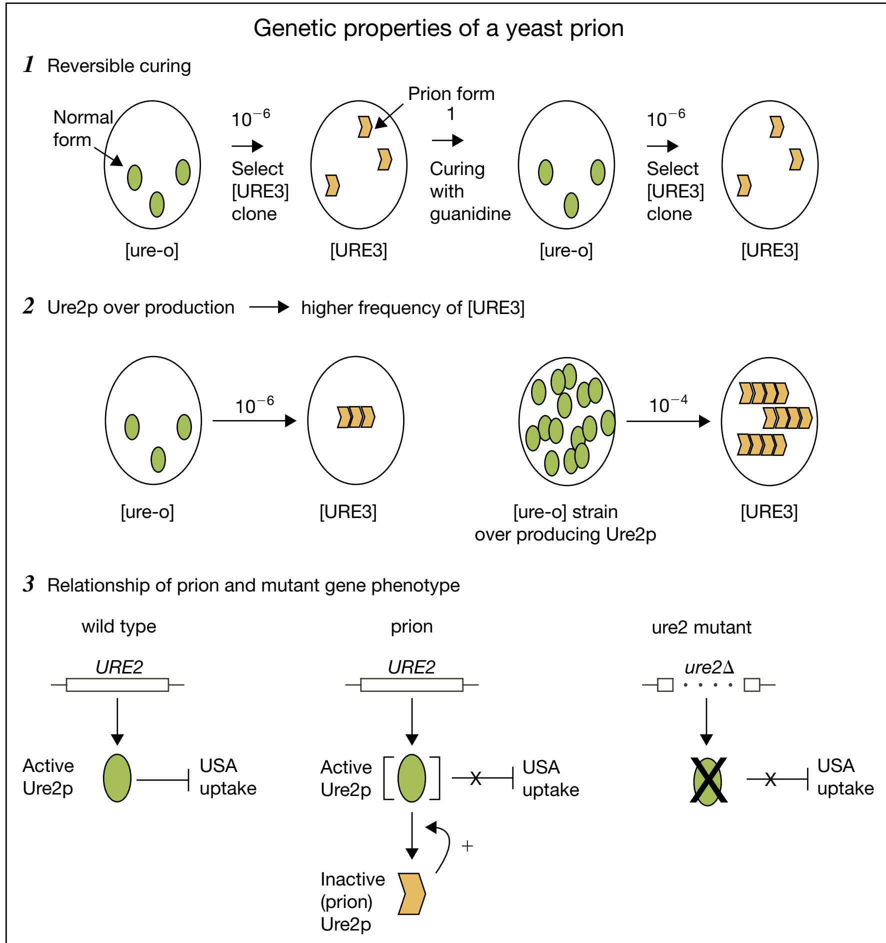


Fig. 1. Three genetic criteria for prions. A prion in yeast should, like yeast viruses and plasmids, behave as a non-chromosomal genetic element. Three unusual genetic properties were proposed to distinguish a prion from a nucleic acid replicon [2]. The figure outlines experiments showing that [URE3] satisfies all three genetic criteria as a prion of Ure2p.

[URE3] as a Prion of the Ure2 Protein

While studying uracil biosynthesis in *Saccharomyces cerevisiae*, Lacroute [3] isolated mutants by selecting for the ability to take up ureidosuccinate (USA, product of the second step in the uracil pathway) on media containing ammonia, a readily metabolized nitrogen source. Most isolates were chromosomal, defining

Table 2. A brief guide to yeast and fungal prion nomenclature

[URE3] nomenclature	
URE2	Wild-type gene
ure2	Mutant allele that is impaired in nitrogen catabolite repression
Ure2p	Full-length protein
[URE3]	Prion genotype, impaired in nitrogen catabolite repression
[ure-o]	Non-prion genotype, wild-type nitrogen catabolite repression
[*PSI*] nomenclature	
SUP35	Wild-type gene encoding a subunit of the translation termination factor
sup35	Mutant allele that impairs translation termination
Sup35p	Full-length protein
[*PSI*] or [PSI^{+}]	Prion genotype, causes non-sense suppression
[psi^{-}]	Non-prion genotype, wild-type translation termination
[Het-s] nomenclature	
het-s	Name of the locus and one of the possible alleles at this locus
het-S	Other possible allele at the *het-s* locus
HET-s	Protein product of *het-s* allele in the prion form
HET-s*	Protein product of *het-s* allele in the non-prion form
HET-S	Protein product of *het-S* allele
[Het-s]	Prion genotype of the *het-s* allele, producing incompatibility with [Het-S]
[Het-s*]	Non-prion genotype of the *het-s* allele, compatible with [Het-S]
[Het-S]	Genotype of *het-S* allele, incompatible with [Het-s]

the *ure1* and *ure2* genes [4, 5], while some were dominant, showed irregular segregation in meiosis and were transmissible by cytoplasmic mixing (cytoduction) implying that this was caused by a non-chromosomal gene that he named [URE3] (for prion nomenclature, see table 2) [3, 6]. Although the mutant *ure2* and [URE3] phenotypes were found to be the same, the molecular basis for this phenomenon remained unexplained.

The normal function of the *URE2* gene product, Ure2p, is to repress nitrogen catabolic genes that are regulated by transcriptional activator Gln3p [7, 8]. Under conditions in which Ure2p is not functional, Gln3p activates the transcription of many genes involved in nitrogen catabolism. One of these genes, *DAL5*, an allantoate permease, allows both allantoate and its structural homolog USA to be transported into the cell [9, 10]. Since USA is a biosynthetic precursor for uracil, USA must be directly supplied when this pathway is blocked by a *ura2* mutation in aspartate transcarbamylase. Thus, *URE2* mutation and [URE3] are assayed in the laboratory by the ability of cells containing the *ura2* mutation to grow on media containing USA in the presence of a good nitrogen source.

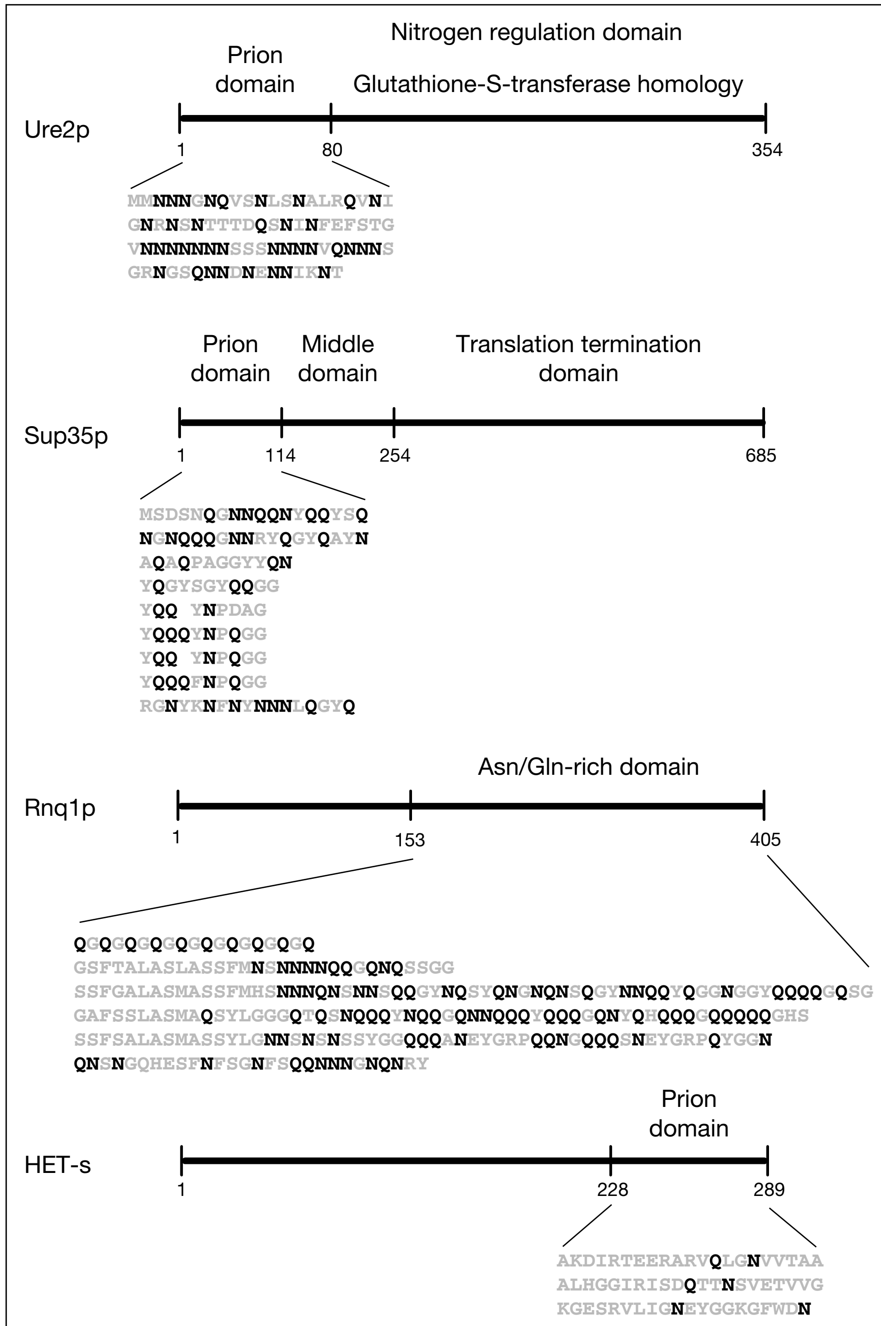
[URE3] colonies that spontaneously arise on minimal media containing USA, can be cured by growth of cells on low concentration of guanidine HCl [11]. [URE3] clones can be re-isolated from the cured strain by growth on selective medium [2]. When the normal form of Ure2p is overproduced, the frequency of [URE3] is increased by 20- to 200-fold [2], and this effect cannot be achieved by overproduction of the *URE2* mRNA or the presence of the gene in high copy number alone [12].

In the case of [URE3], *ure2* mutants and [URE3] strains are both able to take up USA on minimal media containing a good nitrogen source, and *URE2* is necessary for the propagation of [URE3] [2, 6]. Further studies ruled out the possibility that [URE3] was a stable transcriptional state based on nitrogen regulation [12]. These results show that [URE3] is fulfilling all genetic criteria for a prion. This finding has set the starting point for further investigations whether the similarities between yeast prions and PrP can be extended to the molecular level, e.g. whether yeast prion also form amyloid-like aggregates.

Structure of Ure2p

Genetic analysis has shown that Ure2p is comprised of two domains, a prion-inducing domain and a nitrogen regulation domain. The N-terminal prion-inducing domain (prion domain) has a sequence composed of about 40% asparagine residues (fig. 2). The start of the C-terminal domain is around residues 90–100: Ure2p$^{86–354}$, but not Ure2p$^{106–354}$, can complement a *URE2* deletion [13]. Furthermore, as will be discussed below, Ure2p$^{90–354}$ was found to be a stable dimer [14], the glutathione S-transferase homology starts at position 100 [13], and the first amino acid visible in the crystal structure of the C-terminal domain of Ure2p is residue 96 [15]. Overexpression of the first 65 residues increases the de novo formation of [URE3] by over 1,000-fold [16] and this rate is even higher when the first 80 residues are overexpressed [17]. Deletion of the first 65 residues of Ure2p leaves a C-terminal domain, the nitrogen regulation domain, which is able to complement *ure2*Δ [16, 18], but is not able to induce [URE3]. While the C-terminal domain appears less efficient in carrying out its role in nitrogen regulation in the absence of the prion domain, the prion domain induces [URE3] more efficiently when the C-terminal domain is absent [16]. This suggests physical interaction between the two domains.

Fig. 2. Prion domains of Ure2p, Sup35p, Rnq1p and HET-s. Asparagine and glutamine residues are shown in black in the sequences of the prion domains. Notice that the prion domain of HET-s is not rich in Asn and/or Gln in contrast to the yeast prions.



Nitrogen regulation domain
Prion domain
Glutathione-S-transferase homology
Ure2p
1
80
354
MMNNNGNQVSNLSNALRQVNI
GNRNSNTTTDQSNINFEFSTG
VNNNNNNNSSSNNNNVQNNNS
GRNGSQNNDNENNIKNT
Prion domain
Middle domain
Translation termination domain
Sup35p
1
114
254
685
MSDSNQGNNQQNYQQYSQ
NGNQQQGNNRYQGYQAYN
AQAQPAGGYYQN
YQGYSGYQQGG
YQQ YNPDAG
YQQQYNPQGG
YQQ YNPQGG
YQQQFNPQGG
RGNYKNFNYNNNLQGYQ
Asn/Gln-rich domain
Rnq1p
1
153
405
QGQGQGQGQGQGQGQGQ
GSFTALASLASSFMNSNNNNQQGQNQSSGG
SSFGALASMASSFMHSNNNQNSNNSQQGYNQSYQNGNQNSQGYNNQQYQGGNGGYQQQQGQSG
GAFSSLASMAQSYLGGGQTQSNQQQYNQQGQNNQQQYQQQGQNYQHQQQGQQQQQGHS
SSFSALASMASSYLGNNSNSNSSYGGQQQANEYGRPQQNGQQQSNEYGRPQYGGN
QNSNGQHESFNFSGNFSQQNNNGNQNRY
Prion domain
HET-s
1
228
289
AKDIRTEERARVQLGNVVTAA
ALHGGIRISDQTTNSVETVVG
KGESRVLIGNEYGGKGFWDN

However, folding studies comparing Ure2p and Ure2p C-terminal domain [14, 19–21] could not reveal a difference in their stability. This excludes a significant stabilization of the C-terminal domain by interaction with the prion domain. On the other hand, it does not exclude the possibility that the free energy of binding between the domains is rather marginal compared to the free energy of folding of the C-terminal domain, but might still be enough to support a native fold for the prion domain. Thual et al. did not observe any differences in the pattern of protease digestions of C-terminal domain and full-length Ure2p, which indicates that the prion domain is digested very fast without observable intermediates and therefore must be very flexible [20].

The C-terminal domain of Ure2p was crystallized and the structure determined [15, 22], but it was not possible to grow crystals from the full-length protein, due to proteolytic cleavage of the prion domain [22]. Based on sequence similarities, homology between the C-terminal domain of Ure2p and glutathione S-transferases (GSTs) was proposed [18], and the structure of the C-terminal domain of Ure2p is indeed nearly identical to that of GSTs. Similar to all GSTs, in the crystals Ure2p is a dimer. The contact between subunits is very similar to other GSTs with only slight changes of the relative orientation of the subunits [22] and ultracentrifugation studies have shown that Ure2p is also dimeric in solution [14, 23, 24], although one group detected monomeric and tetrameric states depending on the concentration [24].

Ure2p binds to glutathione with high affinity (dissociation constant in the micromolar range) and a crystal structure in complex with glutathione revealed that the glutathione molecule is bound as in other GSTs [25]. However, no GST activity could be found for Ure2p with standard substrates [18]. Although one conserved residue, which is involved in the GST activity, is missing in Ure2p it cannot be excluded that Ure2p possesses GST activity for some unknown substrates [15, 22].

Aggregation of Ure2p in vivo and in vitro

The first biochemical indication for the mechanism of [URE3] was the finding that Ure2p was partially protease-resistant in extracts of [URE3] strains. Ure2p in extracts of [ure-o] strains was completely digested very rapidly whereas in [URE3] extracts transient species of 32 and 30 kDa were formed. Complete digestion results in a relatively stable species around 7–10 kDa (fig. 3) [16]. The antibody used in these experiments was specific for the prion domain.

That aggregation might play an important role in the mechanism of [URE3] was further indicated by experiments using fusion proteins of Ure2p

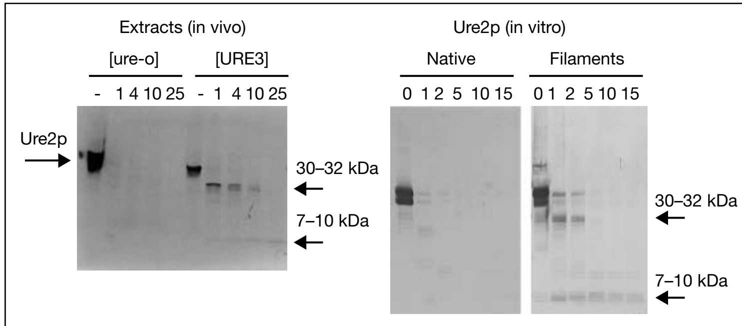


Fig. 3. Protease K resistance of Ure2p aggregates in vivo and in vitro. Extracts of [ure-o] and [URE3] strains were treated for the indicated times (in minutes) with proteinase K and antibody specific for prion domain of Ure2p was used to detect the protein and its N-terminal fragments on Western blots (left) [16]. Native, soluble Ure2p and in vitro formed filaments were treated with proteinase K for the indicated times (in minutes) and detected on Western blot with the antibody mentioned before (right) [23].

and green fluorescent protein (GFP). When fusion proteins of Ure2p$^{1-65}$ or full-length Ure2p with GFP were expressed in [URE3] cells the GFP localized in little spots in the cell, whereas expression of these fusion proteins in [ure-o] strains resulted in evenly distributed GFP throughout the cytoplasm (fig. 4) [26]. Recently, filaments of Ure2p were directly visualized in [URE3] yeast cells by electron microscopy using immunolabeling (fig. 5) [27]. These filaments can form larger networks, probably corresponding to the before mentioned little spots of GFP fluorescence (fig. 4, 5).

A similar aggregation process was shown to occur in vitro upon incubation under native or near native conditions with full-length Ure2p, N-terminal fragments of Ure2p, and fusion proteins with the prion domain (fig. 6) [23, 28, 29]. It has been shown that Ure2p$^{1-65}$ is able to form filaments on its own and to coaggregate full-length Ure2p, but not the C-terminal domain of Ure2p [23]. If unseeded, full-length Ure2p is relatively stable and does form filaments only after long incubation times, although amorphous aggregation can also be observed [23, 24]. Ure2p$^{1-65}$ is also able to form filaments when fused to the C-terminus of GST [28] or the N-terminus of various other enzymes [29]. Ure2p$^{1-65}$ filaments are rich in β-structure and an increase of β-structure from soluble Ure2p to Ure2p filaments was observed by Raman spectroscopy and from soluble GST-Ure2p$^{1-69}$ to filaments by FT-IR [23, 28]. Furthermore, all these filaments show green birefringence upon Congo Red binding [23, 28, 29]. These results led to the conclusion that parts of these proteins (the prion domains) form amyloid-like filaments, although this can only be conclusively

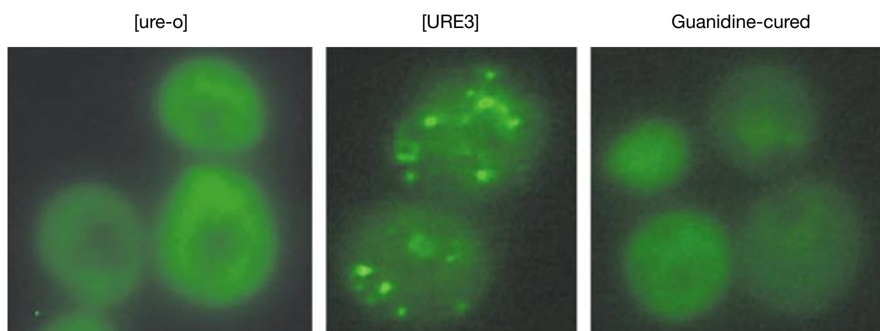


Fig. 4. Ure2p-GFP fusion proteins are aggregated in [URE3] cells. Aggregation of Ure2p was specifically detected in [URE3] strains when a Ure2p-GFP fusion was overexpressed from a single-copy plasmid with the *URE2* promoter and examined by fluorescence [26]. Wild type [ure-o] and guanidine-cured strains show the Ure2p-GFP fusion evenly dispersed in the cytoplasm.

proven by X-ray diffraction. Recently, Bousset et al. [30] using FT-IR were unable to confirm an increase in β-structure in the conversion of soluble full-length Ure2p to filaments. Although secondary structure calculations from FT-IR data for single proteins can result in substantial discrepancies compared to known crystal structures, this result could indicate that the filaments formed under the conditions used by this group are not amyloid-like and might differ from the filaments found by other groups and from the filaments responsible for the prion conversion in vivo.

Surprisingly, enzymes fused to the prion domain were found to be still active in the filaments [29] and filaments formed from full-length Ure2p could still bind glutathione [30]. This leads to the conclusion that the C-terminal functional domain of Ure2p or the enzyme domains in the fusion proteins still have their native conformation in the filaments. As we know that Ure2p filaments do not prevent Gln3p from traveling into the cell nucleus, this implies that this inability is either due to steric interference on the binding site or due to slow diffusion of Gln3p in the cytoplasm, so that more Gln3p can get into the nucleus before it is captured by Ure2p aggregated in only some spots in the cytoplasm (compare also fig. 5).

Proteinase K digestion of in vitro formed filaments of full-length Ure2p, Ure2p$^{1–89}$, and the fusions with prion domain results in protease-resistant, thin filaments that resemble filaments formed by Ure2p$^{1–65}$ (fig. 6c). They are composed of Ure2p$^{1–65}$ to Ure2p$^{1–70}$ and correlate with the appearance of a fuzzy band at about 7–10 kDa in SDS-PAGE (fig. 3) [23, and Baxa et al., unpubl. results]. This behavior upon protease treatment resembles the in vivo findings and

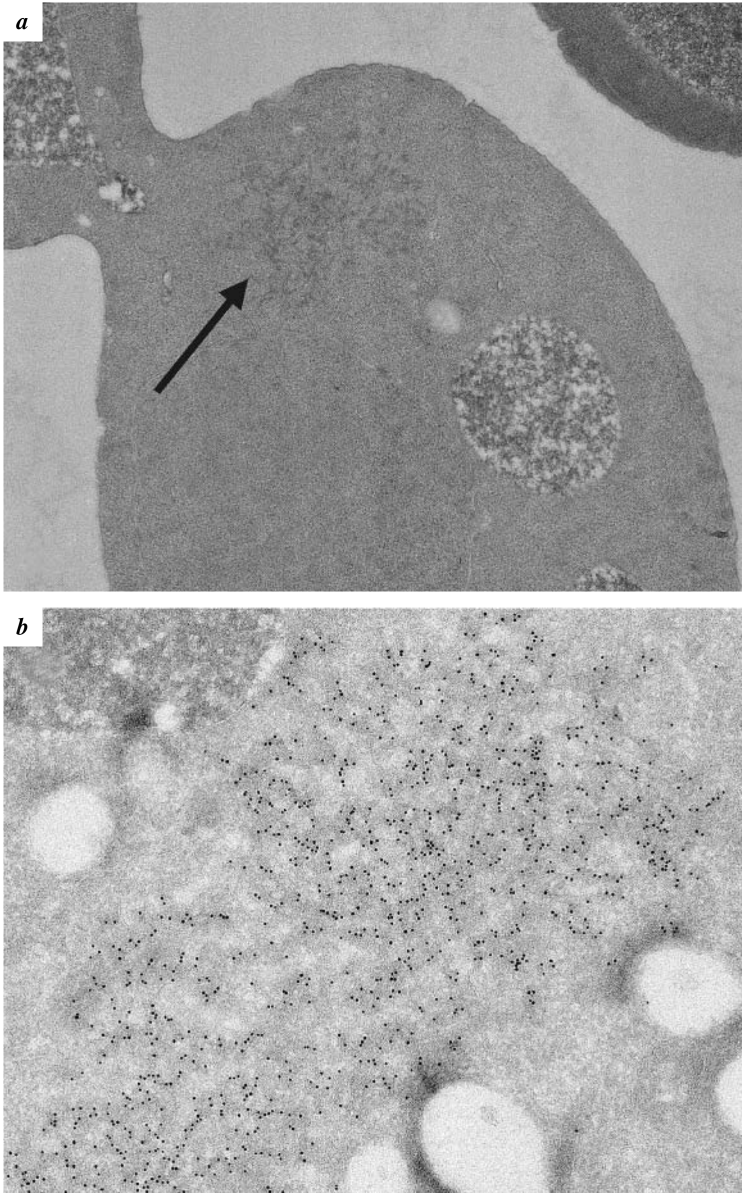


Fig. 5. Networks of Ure2p aggregates in vivo in [URE3] strains. ***a*** Transmission electron microscopy of thin sections of [URE3] strains show filamentous networks not seen in [ure-o] cells [27]. ***b*** Immunogold labeling shows that these filaments contain Ure2p [27].

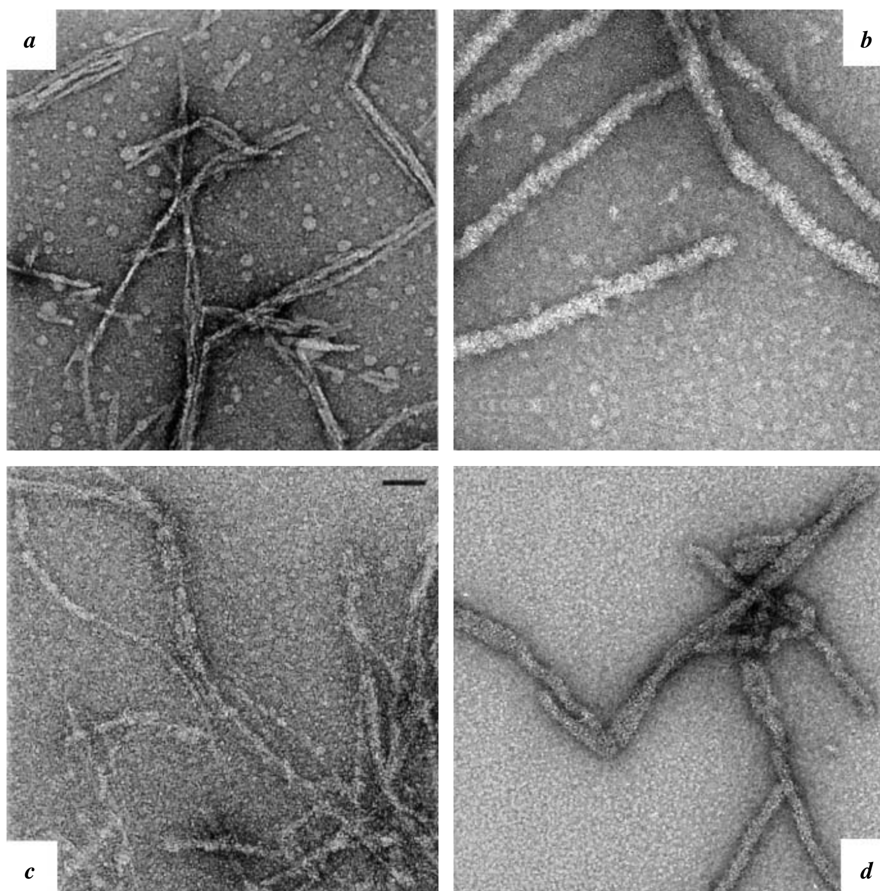


Fig. 6. Electron microscopy of negatively stained filaments of Ure2p, Ure2p$^{1-65}$, and the fusion protein Ure2p$^{1-80}$-GFP. ***a*** Filaments of Ure2p$^{1-65}$, consisting of parallel bundles of about 4.5 nm protofilaments [23]. ***b*** Filaments of full-length Ure2p with a diameter of about 30 nm [23, 29]. ***c*** Ure2p filaments observed after digestion with proteinase K resemble the Ure2p$^{1-65}$ filaments [23]. Bar = 50 nm. ***d*** Filaments of Ure2p$^{1-80}$-GFP with a diameter of about 20 nm [29]. Some thicker filaments consist of two protofilaments wrapped around a common axis.

supports the idea that the in vitro filaments have the same structure as the filaments that are responsible for the [URE3] phenotype in vivo. It has been shown that filaments that are still aggregated and are found at the top of SDS-PAGE gels can be stained with antibodies specific for the C-terminal domain, but not with antibodies specific for the prion domain [27].

Based on these in vitro data, a simple model for the Ure2p filament structure was proposed [23, 27]. In this model the prion domain forms a core fiber,

probably an amyloid-like core filament, and the C-terminal domain is arranged around this fiber, without changing its internal structure very much. The region between residues 65 and 95 makes the connection between the N-terminal core filament and the native-like C-terminal domains. This connection should be relatively flexible to avoid steric conflicts between the C-terminal domains. However, it should be taken into consideration that the region between residues 65 and 80 has weak [URE3]-inducing ability when residues 151–158 of the C-terminal domain are deleted, which makes the full-length Ure2p more efficient in [URE3] induction [17].

The C-terminal domain of Ure2p is relatively conserved in other yeasts and filamentous fungi, and most of the other *URE2* genes can replace *URE2* in *S. cerevisiae* [13]. However, only two of the other Ure2 proteins from very closely related *Saccharomyces* species could propagate [URE3] [13]. This might be explained by the substantial divergence in the N-terminal domains of the Ure2 proteins from different yeast species, although interestingly most of them are rich in asparagine and/or glutamine [13].

[*PSI*] as a Prion of the Sup35 Protein

Like [URE3], [*PSI*] was first described as a non-mendelian genetic element enhancing the efficiency of a weak non-sense suppressor, SUQ5 [31, 32]. [*PSI*] satisfies the genetic criteria as a prion form of Sup35p [2], a subunit of the translation release factor that recognizes termination codons and releases the complete peptide from the last tRNA [33, 34]. [*PSI*] can be lost by growing in the presence of low concentrations of guanidine hydrochloride or medium with high osmotic strength [35, 36]. From these cured strains, [PSI^+] clones can be isolated, which have arisen de novo [37, 38]. Overexpression of Sup35p increases the frequency of [*PSI*] by over 100-fold [38] and this is not caused by an excess of *SUP35* DNA or mRNA [39]. The phenotype of *sup35* mutants is similar to the phenotype of [*PSI*], and Sup35p is necessary for the propagation of [*PSI*] [40, 41].

Deletion experiments have shown that Sup35p can be divided into three major regions, the N-terminal, middle, and C-terminal domain (fig. 2). The C-terminal domain is essential for cell viability due to its function in translation termination, whereas the N-terminal and middle domains are not [41]. The N-terminal domain (1–114), however, is essential for propagation of [*PSI*] [41] and the same region is also sufficient, when overproduced, to induce the de novo appearance of [*PSI*] [39]. This region is very rich in glutamine and asparagine (28 and 18%, respectively) and contains several imperfect repeats of PQGGYQQYN [41, 42]. The glutamine residues were found to be particularly critical for prion induction and propagation [43]. An important role for the

repeats was demonstrated by showing that deletion of repeats decreases, while adding extra repeats increases the frequency of prion formation [41, 44].

Sup35p was found to be aggregated in extracts from [*PSI*$^+$] strains, but not in extracts from [*psi*$^-$] strains [45]. However, the aggregation of Sup35p was self-propagating in vitro, since adding a small amount of aggregated Sup35p from a [*PSI*$^+$] strain to an extract of a [*psi*$^-$] strain resulted in aggregation of the Sup35p from the latter [46]. Fusions of the N-terminal and the middle domain of Sup35p with GFP, when overexpressed in yeast cells, were shown to form visible aggregates in [*PSI*$^+$] cells, but were evenly dispersed throughout the cytoplasm in [*psi*$^-$] cells [47].

Recombinant N-terminal domain of Sup35p and full-length protein purified from *Escherichia coli* has been shown to form filaments in vitro [48, 49]. Neither the middle nor the C-terminal domain form filaments on their own [49], thus the N-terminal region is necessary and sufficient for fiber formation in vitro. In X-ray diffraction filaments formed from NM (N-terminal and middle domain) show a diffraction pattern that is typical for amyloid-like cross-β structures [50]. These filaments also bind Congo Red and show a yellow-green birefringence. Analysis of the kinetic behavior of filament formation revealed a surprisingly small dependence on concentration [43, 49, 50], and led to the proposal of an oligomeric intermediate in the assembly process [50]. Single molecule imaging experiments by fluorescence microscopy or AFM showed that filaments have a polarity and depending on conditions can have substantially different growth rates at the different ends [51–53].

Interaction with Other Proteins in the Cell and Influence of Chaperones

Although it is very plausible that the in vitro formed filaments constitute the molecular mechanism of the prion propagation in vivo, it should be noted that the prion protein finds a very different environment in the living cell, where interaction with natural binding partners and interactions with molecular chaperones might influence the prion propagation.

It was shown that Mks1p, a protein regulating Ure2p in the nitrogen regulation cascade, is necessary for induction, but not for the propagation of the [URE3] prion [54]. Even upregulation of the Ras-cAMP pathway (which downregulates the Mks1 activity) can reduce [URE3] induction [54]. When Sup45p (naturally occurring complexed to Sup35p in the translation termination complex) is overexpressed with Sup35p, the latter loses most of its ability to induce the appearance of [*PSI*] [55], which might be due to a stabilization of the normal form of Sup35p by being in a complex with Sup45p.

Although fusions of Ure2p and Sup35p with GFP have been used to examine the state of aggregation of these proteins in prion-containing cells [26, 47], the Ure2p-GFP fusion proteins have a tendency, particularly when overproduced, to cure the [URE3] prion [26]. In the same way, fragments of Ure2p (but not the full-length protein), when overproduced, cannot only induce [URE3] strains, but also cure [URE3] [26]. This is similar to the curing of scrapie-infected tissue culture cells by peptides derived from PrP [56]. It shows that induction and propagation are two separate processes and it is likely that these fusion proteins and peptides cure by incorporation into growing filaments poisoning their growth or changing their character to be less specific for Ure2p itself.

It has been shown that Hsp104 is necessary for the propagation of all characterized yeast prions [URE3], [*PSI*], and others, which are discussed later [42, 57–60]. Growth of yeast cells on low concentrations (3–5 m*M*) of guanidine hydrochloride was known for some time to cure prions [11, 36], but the mechanism remained unclear until it was shown recently that Hsp104 is inhibited by these small concentrations of guanidine [61, 62]. Furthermore, a single point mutation in Hsp104 can make [*PSI*] cells incurable by guanidine [63]. Hsp104 is thought to be involved in breaking up very large prion aggregates into smaller pieces, which can more efficiently act as seeds to recruit soluble protein and are more easily transmitted to daughter cells [45, 64, 65]. On the other hand, when Hsp104 is overproduced, the Hsp104 activity seems to be too strong for [*PSI*] propagation [57] and might prevent the formation of any seeds. However, in contrast to [*PSI*], [URE3] is not cured by overexpression of Hsp104 [58].

The Hsp70/Hsp40 system also has effects on prion propagation. Overexpression of Ydj1p, an Hsp40 homolog in yeast, results in complete loss of [URE3] [58] but has no effect on [*PSI*] [D.C. Masison, pers. commun.]. Overexpression of Ssa1p, an Hsp70 homolog, can cure [URE3] but not [*PSI*] [66], although a certain missense mutation in *SSA1*, resulting in loss of [*PSI*], has been described [67]. Deletion or overexpression of Hsp70 homologs (*SSA* and *SSB* gene families) in yeast does have variable effects on [*PSI*] [68], and can modulate the effect of Hsp104 overexpression [69, 70].

Generally the influence of molecular chaperones on prion induction and propagation is supporting the idea that the molecular mechanism is a conformational change in a protein, since chaperones are known to be involved in protein folding.

Prion Variants

For BSE and other TSEs, different strains have been found which express themselves in different disease latencies and brain pathologies [71, 72]. In the

protein-only hypothesis this phenomenon poses a challenge, because the information for prion propagation is encoded in the protein conformation rather than in a nucleic acid. So the strains or variants have to be explained by different conformations, one for each strain that lead to different phenotypes. Indeed different biophysical characteristics of the amyloid-like PrP aggregates have been detected for different strains [73–75].

Different variants have also been observed for yeast prions. After constructing a yeast strain in which Ure2p activity is expressed as a colored phenotype, Schlumpberger et al. [76] were able to describe two different, stable variants of [URE3], called type A and type B. Type B, which was characterized by a relatively high residual activity of Ure2p, could be induced by overexpression of either full-length Ure2p or Ure2p$^{1–69}$, whereas type A had less residual activity of Ure2p and could only be induced by overexpression of full-length Ure2p [76].

For [*PSI*], different strains were first described by Derkatch et al. [39], and here also the most obvious phenotype was a difference in residual activity of Sup35p. Weak [*PSI*] variants suppress non-sense mutations less efficiently (they have more Sup35p residual activity) and they are typically less stable than strong [*PSI*] variants in that they lose [*PSI*] more often [39]. The non-mendelian element [ETA] was shown to be actually a very weak [*PSI*] variant, which retains about 50-fold more Sup35p soluble than an isogenic strong [*PSI*] variant [77]. Once established, variants do not generally switch from weak to strong or vice versa, although an example of this has been reported recently [78]. These variants are not due to differences in the genome: when variants from the same genetic background are cured of [*PSI*], a broad spectrum of variants arises each time, when new [*PSI*] elements are induced [39]. It was possible to show that partially purified prion form of Sup35p from a strong [*PSI*] variant converted Sup35p much more efficiently in vitro than material from several weak [*PSI*] variants [79].

Generally it is assumed that prion variants result from transmissible conformational differences of the aggregated form of the prion proteins. There are some indications that these differences exist, but it could not be proven yet, that the existing differences are the molecular basis of variants. In vitro formed filaments of both Sup35p and Ure2p are polymorphic (wavy with varying repeat length or straight), but these features are normally constant throughout the length of the fiber [29, 49]. A chimera of the prion domains of Sup35p from two different yeast species (*S. cerevisiae* and *Candida albicans*) can form different conformations in vitro or in vivo depending on whether it is seeded with Sup35p from *Saccharomyces* or from *Candida* (by direct seeding in vitro or by overexpression in vivo) [80]. The two different conformations can be distinguished by their different effectiveness in seeding formation of filaments of *Saccharomyces*

or *Candida* Sup35p [80]. In a novel single-fiber growth assay using AFM it was possible to detect dramatic differences in polarity and growth rate between fibers. However, these differences are constant for one filament [53].

Other Prions in *Saccharomyces cerevisiae*

The non-mendelian element [PIN] ([*PSI*] inducibility) was identified as being necessary for the inducibility of [*PSI*] in yeast cells [42]. It was shown that this element has itself characteristics of a prion protein, but the gene encoding this protein was not known. In an attempt to identify this gene, high copy clones that could substitute for [PIN] were isolated [81]. Eleven genes were identified all of which had regions high in asparagine and/or glutamine, including Ure2p. However, the gene encoding for the protein that is necessary for the original [PIN] element was identified as Rnq1p, a gene that was found in a screen of proteins with regions high in Asn and/or Gln [60]. Rnq1p (fig. 2) was shown to have prion like behavior, but a phenotype could not be connected even with a knockout mutant until it was identified as the protein leading to [PIN].

Another asparagine-glutamine-rich region from the yeast gene called New1p gave the C-terminal functional domain of Sup35p a prion-like character, and so represents a potential prion domain [82]. However, it has been shown that the N-terminal part of the Sup35p prion domain can be replaced by a simple run of glutamine residues [43], and polyglutamine alone also can promote protein aggregation in yeast [83], so it is not clear if all proteins with regions rich in Asn and/or Gln are indeed behaving as prion proteins in vivo. The prion domains of Ure2p and Sup35p exhibit a 100-fold and over 1,000-fold lower frequency in prion conversion in their natural environment (N-terminal to Ure2p and Sup35p) [16, 84]. This suggests that some protein segments may be capable of acting as prion domains in the absence of the stabilizing influence of their natural environment, but incapable of doing so otherwise.

The data above strongly suggest that there exist more prion proteins than [URE3], [*PSI*], and [PIN] and that there is a positive interaction between different prions, in that the presence of one prion can increase the conversion rate for others. However, negative interactions between different prions are also possible. [URE3] can repress the propagation and de novo appearance of [*PSI*] and vice versa, in the presence of [PIN] [66].

[Het-s], a Prion with a Normal Cellular Function

When a mycelium of the filamentous fungus *Podospora* meets another mycelium of the same strain, the hyphae of the two colonies can fuse to share

nutrients. However, this process is stringently controlled because there are risks in fusing with another strain, which may be infected with a virus that could spread through the fusion process [85, 86]. In *Podospora anserina*, there are about 9 so-called *het* loci (heterokaryon incompatibility) that differentiate self from non-self. The first cells that meet carry out a trial fusion and if there is a difference at any one locus, the fused cells degenerate and a barrier to further fusion is established [86].

One of these incompatibility loci in *P. anserina* is *het*-s, with two possible alleles *het*-s and *het*-S encoding the two proteins HET-s and HET-S, both 289 amino acids long and differing only at 13 residues between the two [87]. It was found that *het-s* strains could have either of two phenotypes, depending on the presence or absence of a non-chromosomal genetic element called [Het-s] [88]. In the presence of [Het-s], the *het-s* cells could fuse with other *het-s* cells, but not with *het-S* cells. However, when [Het-s] was absent, *het-s* cells were neutral, fusing equally well with *het-s* and *het-S* cells. This non-chromosomal element could be lost in meiosis, but would arise again frequently in cells lacking it [89].

Coustou et al. [90] have found evidence that [Het-s] is a prion form of the HET-s protein and that it fulfills the genetic criteria for a prion protein, finding for the first time a prion protein with a cellular function. HET-s is more protease-resistant in [Het-s] cells [90] and when overexpressed HET-s was found in an aggregated form in [Het-s] cells, but not in cells lacking [Het-s] [91]. In [Het-s] cells overexpressing a HET-s-GFP fusion protein, the GFP fluorescence was detected in specific bright dots in the cytoplasm, whereas it was evenly distributed in cells lacking [Het-s] and in *het-S* strains. When *het-s* strains lacking [Het-s] are fused to [Het-s] cells, the GFP fluorescence changes from an even distribution to the bright dots [91]. For HET-s the C-terminal region (amino acids 228–289) was identified as necessary for prion formation (fig. 2) [92]. In contrast to all yeast prions known so far, the prion-forming domain of HET-s is not rich in asparagine and/or glutamine residues (fig. 2).

Recombinant HET-s protein purified under denaturing conditions was able to form filaments in vitro after refolding under different native-like conditions in about 4 days [93]. Seeding with preformed filaments could greatly decrease this time. Biophysical characterization of the filaments by CD and FT-IR spectroscopy indicated an increase of β-structure upon filament formation compared to the monomeric, soluble protein [93]. Furthermore, the filaments bound Congo Red and showed yellow-green birefringence indicating that these filaments are amyloid-like [93]. Digestion of in vitro filaments with proteinase K leads to a 7-kDa band on SDS-PAGE that is relatively resistant to further digestion resembling very much the behavior of in vitro Ure2p filaments. In contrast, soluble HET-s protein is digested totally. Dos Reis et al. [93] speculate that this 7-kDa band might correspond to the core region of the amyloid-like filaments,

the part of the protein that is directly involved in formation of the amyloid-like β-structure. Analyzed by electron microscopy, this protease-resistant material appears to be thin filaments (2–3 nm as opposed to 15–20 nm for the normal HET-s fibers) [94].

Are in vitro Produced Filaments Infectious?

The answer to this question is a very important missing link for the protein-only hypothesis of the TSEs. So far it was not possible to show that in vitro produced amyloid-like filaments of PrP are infectious and can induce disease. It might be easier to answer this question for the other prions from yeast and *Podospora* and a first attempt by Sparrer et al. [95] led to the conclusion that by introducing in vitro formed Sup35p filaments into cells by liposome transformation they could increase the rate of conversion of yeast cells from [psi^{-}] to [PSI^{+}] by about 100-fold [95]. However, the conversion rate was still very low (about 1–3%). The possibility that the increased amount of Sup35p in the cells is responsible for the effect was dismissed based on calculations of protein amounts rather than based on control experiments, e.g. with heat-denatured or soluble Sup35p. So, although the increase in conversion rate might be due to infectivity of in vitro formed filaments, this experiment still left some doubts. Maddelein et al. [94] were recently able to show that in vitro formed filaments from HET-s are able to convert colonies to the [Het-s] phenotype with very high efficiency (almost 100%). Soluble HET-s and unspecific aggregates (heat-denatured and TCA-precipitated) had only slightly higher efficiency than no protein or BSA controls [94]. This proves that the in vitro formed filaments resemble the prion form in vivo and that this aggregation is the molecular mechanism for the infectivity. Keeping the similarity of all fungal prion systems in mind, this conclusion can probably be extended to the yeast prions [URE3] and [*PSI*].

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Human Prion Diseases: Cause, Clinical and Diagnostic Aspects

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There are four predominant human prion disease phenotypes: Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI) and kuru (table 1). The commonest is CJD, varying in causation from genetic through acquired to unknown, with different epidemiological and clinical characteristics (table 2). However, all prionoses are progressive, invariably fatal neurodegenerative diseases, with broadly similar neuropathological features, most specifically in terms of PrP^{Sc} deposition, as well as their transmissibility to a range of laboratory animal hosts.

Our understanding of these diseases, especially that which has arisen from molecular biology, suggests a need for nosological reconsideration. CJD has become a generic diagnostic label for syndromes with different aetiologies (idiopathic, acquired and inherited) and with rather different clinico-pathological phenotypes (as is the case, for example, with sporadic and variant CJD). GSS, originally delineated as an autosomal dominantly inherited cerebellar ataxia with a characteristic pathology, is now something of an umbrella term, covering a group of illnesses with differing clinico-pathological features and varied underlying genetic abnormalities. In contrast, typical FFI remains a distinctive clinico-pathological entity linked to a specific genetic mutation. However, the same mutation may give rise to a clinico-pathological picture of CJD or FFI, depending on the background genotype at codon 129 of the prion protein gene (*PRNP*). Hence, GSS and CJD are perhaps redundant historical terms for forms of genetic human prion disease, with the term ‘CJD’ possibly regarded as somewhat anachronistic. Certainly, the different subtypes have been confused; a misleading uniformity arising from the single eponymous label. Kuru is perhaps the least ambiguous prion disease, because of its extraordinary cause and

Table 1. Types of human prion disease

Disease	Distribution	Cause	Notes
CJD	World-wide	Unknown	~1:1,000,000/year
GSS	Familial	Genetic	Rare
FFI	Familial	Genetic	Extremely rare
Kuru	Papua New Guinea	Ritual cannibalism	Vanishing

Table 2. Types of CJD

CJD type	Distribution	Cause
Sporadic	World-wide	Unknown
Iatrogenic	Reported in several countries	Accidental medical transmission
Variant	UK, France, Republic of Ireland, Italy, USA, Canada[1]	Probable BSE-contaminated food
Genetic	World-wide with particular foci	Genetic

[1] Country of attribution depends on country of residence at onset of disease and does not necessarily indicate country of infection.

epidemiology. While accepting these grounds for a terminological re-consideration, in the subsequent descriptions the traditional terms (such as 'FFI', 'GSS', etc.) have been employed. However, it is important to keep in mind that the clinical features of prion diseases in general depend on the agent strain, the specific cause or mode of exposure and the genotype of the affected individual.

Creutzfeldt-Jakob Disease

CJD is the most significant of the human prion diseases, partly because it is the commonest, but also because it includes variant CJD (vCJD) which is of such current medical, economic and political importance. The first description of CJD was published in 1921 by Dr A. Jakob. Most modern commentators regard the inclusion of Dr H. Creutzfeldt's 1920 paper as erroneous, but this has not eroded the use of the oddly compelling double eponym [1]. Perhaps inevitably, the original description was followed by a number of papers describing clinical and pathological variations, often given their own eponymous

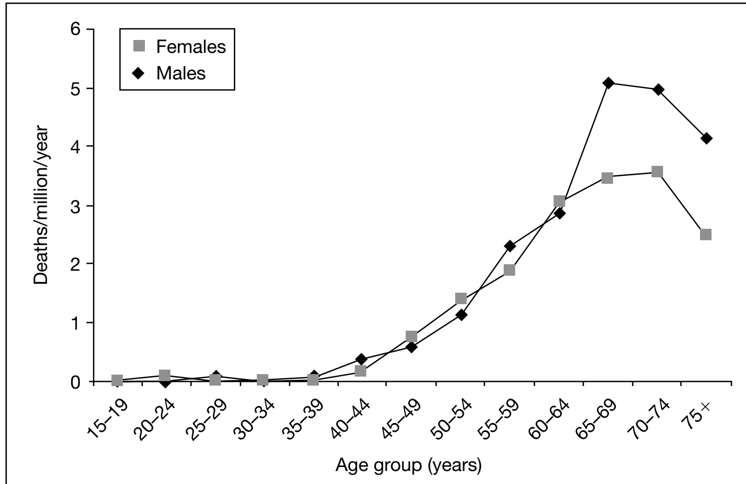

Fig. 1. Age- and sex-specific mortality rates from spCJD in the UK 1996–2002 (mortality rates calculated using 1991 Census).

names (such as Heidenhain's syndrome) [2]. In 1968, laboratory transmission to the chimpanzee was reported by Gadjusek and colleagues [3], prompting seminal unification of the disease subtypes and also raising the question as to whether this rare, apparently neurodegenerative illness, might be some peculiar sort of natural infection. The clinical and epidemiological studies that have followed this unexpected report have led to recognition of four main forms of CJD – sporadic, iatrogenic, variant and genetic (table 2).

Sporadic CJD (spCJD)

Epidemiology and Cause

This is the commonest form of CJD, but with a world-wide incidence of only approximately 1 per million of the population per year. Recent published incidence rates have tended to be higher than those reported in the 1970–1980s, but this probably reflects better case ascertainment and reporting consequent to current interest [4, 5]. Overall, male and females are affected approximately equally, although some studies have reported a slight female excess [6]. spCJD is predominantly a disease of late middle-age with a mean age at death in the late 60s (fig. 1). Cases with onset below the age of 50 are relatively rare. However, the incidence does not rise consistently with age and the often reported decline in incidence after the age of 70 years has been the subject of

some debate. Under-ascertainment in the elderly is one possible explanation, perhaps supported by the finding of recent particular rises in elderly incidence rates in the UK and other countries [4, 5, 6]. A number of studies in different countries have examined the incidence and characteristics of spCJD and have conducted case-control studies of potential risk factors [6–8]. The cause of spCJD is unknown. Research has failed to find any link with scrapie, either by country, diet or occupation. There is no convincing or consistent evidence of significant clustering of cases over time and direct case-to-case contact is not a likely mode of transmission. The possibility of unsuspected iatrogenic transmission via contamination of surgical instruments or blood transfusion is not supported by most surveillance data or by most case-control studies. However, two publications (one from Australia and one from four European countries) have reported an excess of surgical operations in cases compared to controls [9, 10]. Alternative explanations include a spontaneous conformational change of the normal prion protein (PrP^{C}) to PrP^{Sc} (the relatively protease-resistant, disease-associated isoform) or a somatic mutation of the *PRNP* gene. A spontaneous protein change or a somatic mutation should probably be reflected in a steadily increasing incidence with age and thus the observations on the age-specific incidence of spCJD have implications for the proposed theories of causation. It is, of course, possible that spCJD is not a homogenous entity with some cases arising spontaneously and some perhaps from hitherto unrecognized surgical contamination events.

In the normal human population, there is a common polymorphism at codon 129 of the *PRNP* gene open reading frame (situated on chromosome 20), whereby coding may be for methionine or valine, with individuals being MM or VV homozygotes or MV heterozygotes. In the normal UK population the distributions are approximately MM 40%, VV 10% and MV 50%. However, approximately 80% of UK spCJD cases harbour the MM genotype, with only about 10% being the MV genotype. This indicates that being an MM homozygote is, in some way, a risk factor for spCJD and that MV heterozygotes are relatively protected. This genetic factor has been found in other studied populations [6]. A recent publication reported a relatively high incidence of spCJD in Crete compared to the rest of Greece and found this was associated with a higher incidence of codon 129 methionine homozygosity in the Crete population [11].

Clinical Features

Various prodromal symptoms have been described (including headache, tiredness, sleep or appetite disturbance, and depression), but these are entirely non-specific, perhaps being given retrospective significance because of later developments. A case-control study in England and Wales between 1980

and 1984, using hospital patient controls, did not suggest that any of these symptoms were specifically related to CJD [12]. The presenting neurological symptoms often reflect a relatively focal neurological involvement but, in most cases, they are soon subsumed in a rapidly progressive global encephalopathy. Cognitive impairment and cerebellar ataxia are the commonest early features. Two particularly well-recognized, but relatively rare, presentations are the Heidenhain variant, with cortical blindness, and the Brownell-Oppenheimer variant, with a pure cerebellar syndrome [2, 13]. Whatever the mode of onset, the subsequent clinical course in most cases, is one of rapid progression, often surprising the clinician and dismaying the relatives. The rapidly evolving clinical picture always includes dementia, with ataxia and myoclonus being present in the majority of cases. Other features include primitive reflexes, pyramidal signs, cortical blindness and paratonic rigidity. The terminal state is often one of akinetic-mutism. Major pyramidal weakness, unequivocal lower motor neuron features and epileptic seizures are relatively uncommon. The median duration of spCJD is 4 months and around 65% of cases have an illness duration of less than 6 months. In 14% of cases, there is a relatively long duration of 12 months or more. However, durations of greater than 2 years are rare (5%) and this fact is reflected in the time limit embodied in the clinical diagnostic criteria for spCJD (discussed below).

The polymorphism at codon 129 of *PRNP* not only affects susceptibility but also influences the clinico-pathological expression of disease; the clinical and neuropathological features of spCJD varying somewhat according to whether the individual has the codon 129 MM, VV or MV genotype [14].

Diagnosis

In a typical case, the invariably progressive, rapid clinical evolution and the short illness duration, readily distinguish spCJD from most other dementing illnesses. However, the differential diagnosis can be problematic, especially if the onset of disease is relatively abrupt, the initial symptoms (such as ataxia) remain focal for a longer period, or there is an unusually long illness duration. In one UK study, about 6% of cases were initially misdiagnosed as stroke [15]. Alzheimer's disease with a less typical, relatively rapid myoclonic progression may occasionally cause confusion. As with many illnesses, a careful history is vital. The diagnosis of spCJD is presently categorized as definite, probable and possible (table 3). Definite spCJD requires neuropathological confirmation, usually obtained at autopsy but, occasionally, cerebral biopsy might be considered. Probable and possible diagnoses are clinical, and are based on the exclusion of alternative diagnoses, with the presence of requisite characteristic features and supportive findings in the EEG or CSF. A probable classification carries an approximately 95% certainty of spCJD and this reliability of the

Table 3. Diagnostic criteria for spCJD

I	Neuropathological (including immunocytochemical) confirmation
II	Rapidly progressive dementia
III	A Visual/cerebellar features
	B Pyramidal/extrapyramidal features
	C Myoclonus
	E Akinetic mutism
IV	Typical periodic EEG
V	Positive CSF 14-3-3
Definite:	I
Probable:	II + 2/4 of III + IV or possible + V
Possible:	II + 2/4 and duration <2 years

clinical criteria is one reason why biopsy confirmation is seldom necessary. However, it should be stressed that there is no infallible pathognomonic clinical diagnostic test, autopsy being necessary for diagnostic certainty. The clinical diagnostic criteria need careful application and the pre-mortem assessment of suspect cases requires neurological expertise. The EEG shows characteristic periodic discharges at some stage of the illness in about two-thirds of cases, but the abnormality may not be present until late in the illness and repeat recordings (usually at weekly intervals) may be necessary. The absence of this EEG pattern cannot exclude the diagnosis and the typical discharges are seen in conditions other than CJD, occasionally even in Alzheimer's disease. A persistently normal EEG, despite clinical progression, is incompatible with a diagnosis of spCJD [16]. The CSF 14-3-3 test is a valuable supportive investigation. 14-3-3 is a normal brain protein that may be present in the CSF in excessive amounts following neuronal injury. It is not intrinsically associated with CJD, and, in particular, is not specifically related to PrP. 14-3-3 may be elevated in a number of conditions including viral encephalitis, subarachnoid haemorrhage and recent cerebral infarction. It is therefore perhaps surprising to find that, in the appropriate clinical context, it has a high degree of sensitivity (94%) and specificity (94%) for spCJD [17]. This specificity is largely because the other conditions which characteristically lead to a positive14-3-3 CSF test are relatively easily distinguished from CJD on other grounds. However, it is important to note that the specificity of the test is context-dependent and the figure given here relates to the particular circumstances of CSF samples tested in a setting of an established CJD surveillance system. CSF 14-3-3 cannot be reliably used as a routine 'screening' test. Aside from an elevation in the total protein level, no other CSF abnormality is found and, in particular, a significant pleocytosis should prompt consideration of an alternative diagnosis. Cerebral imaging is

important in the investigation of suspect cases of spCJD largely in order to exclude alternative diagnoses and cerebral CT is characteristically normal or shows non-specific atrophy. Cerebral MRI is additionally important because, in some cases, a characteristic signal change is seen in the putamen and caudate. Occasionally, high signal is seen in the cerebral cortex and this may be focal, reflecting the particular clinical features of the case being investigated [18]. Significant atrophy is unusual if imaging is undertaken within 3 months of illness onset. Indeed, one early diagnostic clue may be the notable finding of normal brain imaging in the context of a rapidly progressive and devastating cerebral illness. MRI abnormalities in spCJD may be best seen on FLAIR sequences and a recent publication contains a recommended MRI protocol for the investigation of CJD [19, 20]. Routine blood tests show no relevant abnormality. Although modest disturbances in liver function tests may be seen in a number of cases, these probably reflect disturbances of general health consequent on the dementing illness or the effects of any symptomatic drug treatment.

Cerebral biopsy should be considered only if there is a reasonable possibility of an alternative, potentially treatable diagnosis. There are published guidelines concerning precautions to be taken during procedures involving potential CJD material [21].

Iatrogenic (iCJD)

iCJD is the accidental transmission of CJD during medical or surgical treatment.

Epidemiology and Cause

iCJD has resulted from neurosurgery (including the use of EEG depth electrodes), corneal grafting, human dura mater implants or exposure, and the use of human cadaveric growth hormone (hGH) and human pituitary gonadotrophin [22, 23, 24].

There are only 3 reported corneal grafting cases and 4 reported human gonadotrophin cases [24–26]. There are 7 reported neurosurgical/EEG depth electrode cases [23]. Numerically, the most important relate to cadaveric-derived hGH treatment and the surgical use of human dura mater grafts.

Some epidemiological features of iCJD tend to reflect the cause. For example, hGH cases tend to be relatively young. These cases resulted from the use of cadaveric-derived hGH manufactured from pituitary glands harvested at autopsy, with some glands presumably being obtained from CJD cases, with processing insufficient to eradicate infectivity. The hormone was derived from

pooled harvested glands with many thousands of glands in a single production lot. The treatments were administered over a period of time, with repeated injections increasing the risk of exposure to an infected batch. By 2000, 139 hGH cases had been described in a number of countries including Australia, Brazil, the Netherlands, New Zealand and particularly France, the UK and the USA [23]. The frequency of iCJD in the treated populations varies with country, being 0.3–0.8% in the USA, 1.9% in the UK, 4.4–5.9% in France and 2.7–10.9% in New Zealand [23]. The incubation period (calculated from the mid-point of therapy to disease onset) also varies with country, the mean being 26 years in New Zealand, 20 in the USA, 16 in France and 10 in the UK [23].

Human cadaveric dura mater has been used in surgical and interventional procedures, particularly in neurosurgical operations to repair dural defects. By 2000, 114 dura mater related CJD cases had been reported, from a number of countries, with 67 of these being identified in Japan alone [23, 27]. Nearly all have been associated with a particular brand, namely Lyodura and the biggest risk seems to be associated with grafts used between 1981 and 1987. Human dura mater grafts are no longer used in many countries, but they are still used in the USA, with donor screening and special processing precautions. The incubation period for dura mater cases seems to vary with the site of grafting and varies from 1.5 to 18 (mean 6) years [23].

Clinical Features

The clinical features depend somewhat on the route of infection. Human GH cases, resulting from the intramuscular injection of infected material, generally present with a progressive cerebellar syndrome and other features including dementia tend to occur later [22]. Human dura mater cases, resulting from infected material being placed relatively near to the central nervous system, tend to present with a rapidly progressive dementia along with other neurological features and may be clinically indistinguishable from sporadic cases, aside, of course, from the relevant past medical history.

Diagnosis

The principal indication of iatrogenic disease must come from the history, in particular a history of treatment with cadaveric-derived hGH or an operation involving the use of a human dura mater graft.

Variant (vCJD)

Much of the current interest in human prion diseases stems from the identification of this relatively new disease in 1996 [28]. Originally termed

'new variant CJD', it is now designated simply as 'variant CJD' although the organization set up by relatives of affected individuals in the UK has preferred the term 'human BSE'.

Epidemiology and Cause

Perhaps the most striking epidemiological feature of vCJD is its geographical distribution. In November 2003, 153 cases had been confirmed world-wide with only 10 outside the UK; 1 case being in the Republic of Ireland, 1 in Italy, 1 in the USA, 1 in Canada and 6 in France. It is important to note that cases are attributed to country according to the normal country of residence at the onset of symptoms. The USA case probably contracted the disease whilst in the UK. The relatively young age of the affected individuals is also notable. In stark contrast to the age distribution of spCJD, the median age of onset in vCJD is 28 (range 14–74) years. In the UK, vCJD is commoner in the north of the country than the south. This difference is unexplained [5, 29]. There is one statistically significant cluster of cases in Leicestershire, a region in the centre of England. This may be due to past local butchering practices but there is no definitive explanation currently [5]. Males and females are affected equally. There is now convincing evidence that vCJD is due to infection of humans with the BSE agent, most probably via contaminated food. Experimental studies have demonstrated that the same agent strain causes both vCJD and BSE. The available epidemiological evidence strongly indicates that the agent passed from cattle to man and it is obviously relevant that the UK had the greatest incidence of BSE. To date, it has not been possible to provide absolute proof that the zoonotic link was via food, but this is a very reasonable hypothesis, and the most likely route is via mechanically recovered meat (MRM) included in certain prepared products. During the relevant period, some MRM is likely to have contained spinal cord material from infected cattle. Other means of transmission might be relevant, such as occupational contact, surgical sutures derived from bovine gut, and drugs or vaccines prepared using foetal calf serum, but no specific evidence currently exists to implicate these routes [5, 30].

As with spCJD, the genotype of the individual appears to be important. All the cases tested to date (in September 2002: 105) have been codon 129 MM homozygotes, indicating that this particular genotype confers particular susceptibility to the disease. It also suggests that continued surveillance needs to be conducted with care: if BSE affects humans with MV or VV genotypes, the resulting clinical and pathological picture may be different.

It is still not known how many cases will eventually occur, as predictions must be based on rather uncertain assumptions about the population exposed to infection, the susceptibility of humans to the infection, and the resulting range of incubation periods.

The numbers of cases of vCJD are analyzed on a quarterly basis with an exponential increase up to 2001. However the data now suggest that the epidemic may have reached, or be close to reaching, a peak [5, 31]. The decline in BSE along with the relevant precautions and regulations in place should prevent further instances of cattle-to-man transmission; however, there is concern that secondary human to human spread may occur. There is evidence of lymphoreticular involvement in vCJD (spleen, appendix, tonsil and lymph node contain PrP^{Sc}) which is not seen in other forms of CJD and this raises the possibility of infection via contamination of surgical instruments and even via blood transfusion or the use of blood products [32].

There is experimental evidence of not only PrP^{Sc} deposition but also actual infectivity in reticulo-endothelial tissue in variant cases [33]. The potential existence of pre-clinical involvement of such tissues suggested that a retrospective survey of tonsils and appendices removed during routine surgical practice might give some indication of the future numbers of vCJD cases. To date, this survey has found 1 positive specimen out of 8,318 samples tested. On the basis of these results, the researchers estimated a detectable prevalence of prion protein accumulation of 120 per million among people aged 10–50, in the UK, between 1995 and 1999 [34].

The report of successful transmission of BSE from sheep to sheep via blood transfusion has increased concerns about possible blood infectivity in humans [35].

Clinical Features

Whereas spCJD presents with rapidly progressive symptoms that are usually clearly neurological in nature, vCJD tends to present with difficulties that are not unambiguously neurological and with a relatively slower progression. The commonest presentation is that of a psychiatric/behavioural disturbance and/or unpleasant sensory symptoms including pain, usually without neurological abnormality on examination [36–38].

In an analysis of the first hundred cases of vCJD, psychiatric symptoms preceded neurological symptoms in 63% of cases and were found in combination with them in 22% cases. In only 15% did neurological symptoms precede psychiatric symptoms [39]. Sensory symptoms affect nearly half the patients [40]. Many have seen a psychiatrist at some stage of the illness, some even being initially referred to psychiatry services by their general practitioner. In the majority of cases, features of depression are present and many were given antidepressant treatment [36, 37, 39]. Other features included anxiety, agitation, delusions and hallucinations.

The sensory features are usually persistent, unpleasant or frankly painful, and may be worse at night. There is usually no definite abnormality on clinical

sensory examination, although relevant signs are found occasionally. In some individuals, limb pain alone resulted in investigations such as an x-ray of relevant bones or joints. Nerve conduction studies were undertaken in some, but were normal. Where somatosensory-evoked potentials were performed, minor abnormalities were seen suggesting a relatively central disturbance of sensory pathways. In some cases there was lateralization which might suggest a cerebral origin. It is possible that the sensory symptoms reflect a thalamic disturbance [40]. At some point, other neurological features develop, with definite neurological abnormality developing at a mean of around 6 months from first symptoms, ataxia often being the most prominent problem. Chorea and dystonia may be seen, along with pyramidal features. The range of psychiatric and neurological features, along with their timing in the illness course, has been reviewed in the first hundred cases [39]. Eventually, the clinical picture is one of a dementia with multiple neurological features including myoclonus, although pre-terminal akinetic mutism is not as common as it is in spCJD. The illness duration is greater than that for spCJD, with a median of 14 (range 6–39) months.

Diagnosis

The differential diagnosis of a progressive neuropsychiatric disorder in relative youth is arguably wider than that of a rapidly progressive dementia in middle to late life. In addition, there is now considerable accumulated experience with spCJD and the clinical diagnostic criteria have been refined and validated over a number of years. Experience with vCJD is obviously relatively limited both in terms of numbers and time. The two investigations, namely the EEG and CSF 14-3-3, which have proved so helpful in the diagnosis of spCJD, are less so in vCJD. The typical periodic EEG pattern has never been seen in vCJD and the 14-3-3 test is less sensitive (around 41% of cases have a positive test) than in sporadic disease [41]. Fortunately, the cerebral MRI, which has had a rather limited role in spCJD, appears to be very useful in vCJD, with bilateral pulvinar high signal being seen in the majority of cases [19, 42]. Clinical diagnostic criteria have been developed, including the MRI features, and are being prospectively evaluated in continuing UK surveillance (table 4) [43].

Cerebral biopsy may be considered in selected cases. The main indication for this being significant concern about an alternative, potentially treatable, neurological illness.

Tonsillar biopsy has been proposed as a diagnostic test, but experience is very limited and this does not provide positive information about any possible alternative brain pathology [44]. With increasing experience of vCJD and the proposed clinical criteria, tissue diagnosis may not be necessary in life for the vast majority of cases.

Table 4. Diagnostic criteria for vCJD

I	A	Progressive neuropsychiatric disorder
	B	Duration of illness >6 months
	C	Routine investigations do not suggest an alternative diagnosis
	D	No history of potential iatrogenic exposure
	E	No evidence of a familial form of TSE
II	A	Early psychiatric symptoms[a]
	B	Persistent painful sensory symptoms[b]
	C	Ataxia
	D	Myoclonus or chorea or dystonia
	E	Dementia
III	A	EEG does not show the typical appearance of sporadic CJD[c] (or no EEG performed)
	B	Bilateral pulvinar high signal on MRI scan
IV	A	Positive tonsil biopsy[d]
Definite:		I A *and* neuropathological confirmation of vCJD[e]
Probable:		I *and* 4/5 of II *and* III A *and* III B
		or
		I *and* IV A[d]
Possible:		I and 4/5 of II and III A

[a]Depression, anxiety, apathy, withdrawal, delusions.
[b]This includes both frank pain and/or dysaesthesia.
[c]Generalized triphasic periodic complexes at approximately one per second.
[d]Tonsil biopsy is not recommended routinely, nor in cases with EEG appearances typical of spCJD, but may be useful in suspect cases in which the clinical features are compatible with vCJD and MRI does not show bilateral pulvinar high signal.
[e]Spongiform change and extensive PrP deposition with florid plaques, throughout the cerebrum and cerebellum.

Genetic (gCJD)

Familial instances of CJD have been recognized since 1930 and GSS was first described around the same time. Familial CJD, and GSS and FFI, are almost always related to an underlying *PRNP* mutation and should perhaps be grouped together but are considered separately below. The transmissibility of gCJD to non-human primates was first reported in 1973.

Epidemiology and Cause

In gCJD, there is an underlying mutation of the *PRNP* gene open reading frame leading to the production of a PrP protein molecule theorized to have a

Table 5. Haplotypes linked with genetic prion disease

E200K,129M	D178N,129V^{+}	D178N,129M$^{\#,+}$	A117V,129V*
V180I,129M	M232R,129M	P102L,129M*	P105L,129V*
T183A,129M	P102L,129M,219K	F198S,129V*	Q217R,129V*
H208R,129M	V210I,129M	Y145stop,129M*	Q212P*,†
P102L,129V			
ins24 bp,129M	ins48 bp,129M	ins96 bp,129M	ins96 bp,129V
ins120 bp,129M	ins144 bp,129M	ins168 bp,129M	ins216 bp,129M
ins192 bp,129V*			

$^{+}$Codon 129 type determines phenotype resulting from mutation.
*Linked to GSS phenotype.
#Linked to FFI phenotype.
†Codon 129 not specified.

heightened predisposition to adopting the configuration of PrP^{Sc}. Hence, the mutation is believed to be directly causative of a familial disease with an autosomal dominant pattern of inheritance. However, the mutation is conceivably a susceptibility factor rendering the affected individual particularly liable to the effects of some, as of yet, unidentified environmental agent. A number of different (point and insert) mutations have been identified (table 5).

gCJD tends to occur in clusters reflecting its familial nature. There are two very notable geographical concentrations of genetic disease, involving the E200K mutation. The first is in Israel, affecting Jews of Libyan origin, with an incidence of approximately 50 per million per year in this community. The second concerns two foci in Slovakia, with incidences ranging from around 4 to 17 per million per year.

Clinical Features

The clinical presentation varies with the underlying mutation and other factors (such as codon 129 genotype, which may affect the clinico-pathological phenotype resulting from any given mutation). For the Libyan Jewish E200K, *cis*-129M focus, the picture is not dissimilar to that of spCJD with a mean age at onset of 62 years, mean disease duration of under a year, and a broadly similar clinical profile. However, a sensorimotor polyneuropathy is a typical additional clinical feature. The codon 129 characteristic of the normal allele does not appear to affect the disease phenotype. This is not so in D178N-129V disease. Homozygous VV individuals have a mean age at onset is around 39 years with a mean illness duration of around 14 months, whereas the corresponding figures for heterozygous MV persons are 49 years and 27 months,

respectively. The disease phenotype associated with insert mutations is particularly variable and may, in some instances, involve young age at onset (for example, in the third decade) and very long duration illnesses (even exceeding 10 years). At least one determining factor is the number of repeats in the particular abnormal allele.

Diagnosis

The firm diagnosis of gCJD requires pathological confirmation of CJD in an individual proven to harbour an underlying *PRNP* mutation. DNA can be extracted from a blood sample and the open reading frame of the gene sequenced. A family history of the disease with an autosomal pattern of inheritance is the usual background, but apparently sporadic cases of CJD occasionally prove to have an underlying mutation, and *PRNP* gene sequencing is the only certain means of definitively excluding genetic disease.

Gerstmann-Sträussler-Scheinker Syndrome (GSS)

GSS is almost invariably genetically determined, associated with an underlying mutation in the *PRNP* gene open reading frame. Although this syndrome was intermittently reported under a variety of titles following its original description in a large Austrian family spanning a number of generations [45, 46], its nosological clarification as a prion disease was only achieved relatively recently [47]. Originally defined in terms of an autosomal dominantly inherited illness manifested predominantly as a progressive cerebellar ataxia, the term GSS also became associated with the common neuropathological denominator of numerous, widespread, multicentric amyloid plaques which demonstrate selective immunostaining by anti-PrP antibodies. However, with the passage of time, GSS neuropathology has been associated with a more diverse clinical spectrum, including dementia, spastic paraparesis and even extrapyramidal features which may respond to dopaminergic therapy. The neuropathological phenotype has also been extended to include the presence of neurofibrillary tangles. Divisions based on these varied features may have no intrinsic biological validity given the considerable clinico-pathological diversity described within families.

Epidemiology and Cause

The variety of genetic mutations associated with illnesses classified as GSS since 1989 further complicates the issue, and underscores the likely non-specificity of this diagnosis. This genetic heterogeneity may partly explain the described considerable phenotypic heterogeneity (table 6). Recent reports

Table 6. *PRNP* mutations associated with GSS (missense)

Codon	Amino acid change	Codon 129	Codon	Amino acid change	Codon 129
102	Proline to leucine	M[1]	198	Phenylalanine to serine	V
212	Glutamine to proline	?	145	Tyrosine to stop	M
217	Glutamine to arginine	V	105	Proline to leucine	V
117	Alanine to valine	V	232	Methionine to threonine	?
202	Aspartate to asparagines	V			
131	Glycine to valine	M			

[1]This mutation has also been reported in *cis* with valine at codon 129.
? = Not clarified in report.
Inserts:
8 octapeptide repeat (192 bp) insert starting at codon 84.
8 octapeptide repeat (192 bp) insert starting at codon 76.
8 octapeptide repeat (192 bp) insert specific site not stated but located within the codon 51–91 region.

continue to highlight intra-pedigree phenotypic variation with different members within one family having illnesses varying from typical CJD to classic GSS [48–56]. The normal polymorphism at codon 129 may also influence phenotype [57, 58]. The likely causative role of these mutations is strongly supported by the spontaneous development of spongiform degeneration in the brains of transgenic mice harbouring such changes [59].

In most instances a trans-generational neurologic syndrome is apparent within an affected kindred, usually following autosomal dominant transmission patterns, although uncommonly, GSS occurs sporadically [60, 61]. The first described and most common mutation occurs at codon 102 (P102L) which has recently been confirmed in members from the original family described by Gerstmann. This mutation is usually associated with the more typical 'ataxic' GSS clinico-pathological profile but exceptions have been reported, attributed to the modifying effects of non-pathogenic polymorphisms. The normal *PRNP* gene codes for a nonapeptide sequence which with only very minor nucleotide modifications is repeated as four octapeptides between codons 51 and 91. Inserts of varying multiples of these octapeptide repeats are commonly found in other familial prion diseases, especially CJD; the GSS phenotype appears linked to various 8 octapeptide repeat expansions of this gene region. Table 6 summarizes the currently recognized mutations associated with GSS.

Transmission studies, utilizing both non-human primates and rodents, have generally shown low success rates, with the most common causal mutation (P102L) transmissible in approximately 40% of cases; the other less frequent mutations have either not been assessed or did not transmit [62, 63].

Clinical Features

In the more typical GSS cases, symptoms begin in the fifth or sixth decade, but the onset may be as young as 25 years. The illness duration ranges from 3 months to 13 years with the mean around 5–6 years. Although early complaints are often vague or non-specific, there is inexorable, usually slow, progression, so that eventually patients come to manifest differing combinations of cerebellar, pyramidal, behavioural and cognitive difficulties.

Features of pan-cerebellar dysfunction typically include gait unsteadiness, limb ataxia with dysmetria, dysdiadochokinesis and intention tremor, titubation, nystagmus, and dysarthria, often accompanied by dysphagia. Although signs of corticospinal tract degeneration such as limb weakness, spasticity, hyperreflexia and positive Babinski's responses are present in many patients, in some families (especially with the P105L mutation) spastic paraparesis may dominate the clinical syndrome in the absence of cerebellar dysfunction. The unusual combination of absent lower limb tendon reflexes with extensor plantar responses is relatively frequent in later stages of the illness. Extrapyramidal motor disturbances are common and take the form of adventitious movements (such as myoclonus and athetosis) and rigidity.

Memory impairment is usually the first indication of cognitive decline and with the passage of time a more pervasive dementia invariably manifests. Along with the intellectual deterioration, changes in demeanour are common, ranging through aggression, irritability and emotional lability to apathy and withdrawal. Less common features are deafness, cranial nerve palsies and seizures. Death in a bedridden, akinetic-mute, totally dependent state is a frequent culmination.

An American (Indiana) kindred, associated with the F198S mutation, was remarkable for both the development of dopaminergic-responsive parkinsonian features (with minimal or absent tremor) and the presence of neocortical neurofibrillary tangles (NFTs) [49]. A Swedish family with later-onset cognitive decline and cerebellar dysfunction (associated with the Q217R mutation) also manifested neocortical NFTs in addition to the widespread PrP-positive plaques [49]. As already mentioned, the P105L mutation is recognized to cause a familial spastic paraparesis-dementia variant, without clinical evidence of cerebellar dysfunction or myoclonus [53]. An uncommon 'amber' stop mutation, causing termination of translation at codon 145 (Y145stop), was found in a patient with isolated progressive dementia spanning 20 years, who at autopsy had the combination of Alzheimer changes and numerous neocortical and cerebellar PrP-positive amyloid plaques [54]. A patient with P102L mutation, demonstrating moderate ataxia and sensory impairment, was found by haplotype analysis to carry a disease modifying polymorphism E219K on another allele. It is thought that this polymorphism delayed the onset of disease [64]; residue 219 of PrP is juxtaposed to the postulated binding site of 'protein X'

involved in conversion to PrP^{Sc}. Finally, the A117V mutation is associated with another 'telencephalic' variant of GSS wherein progressive dementia is the predominant clinical feature, often coexisting with lesser pyramidal and extrapyramidal findings but with minor cerebellar dysfunction [65].

Diagnosis

Neuroimaging (both CT and MRI scanning) may be normal [66] or show non-specific atrophy affecting the cerebral hemispheres and/or cerebellum. Most often the EEG only shows a non-specific excess of slower frequencies (which may appear in bursts) but can be normal even in relatively advanced disease [66]. In addition, the generalized, 1–2 Hz triphasic or periodic slow-wave complexes most often sought to aid the diagnosis of spCJD can be seen, usually in patients with more aggressive disease and shorter total illness durations, often less than 12 months [55].

Fatal Familial Insomnia

FFI is a genetically determined TSE with a relatively characteristic clinicopathological phenotype. Although FFI was first applied as a descriptive diagnosis in 1986 to describe an illness afflicting 5 members of a large Italian family [67], it was not until 1992 that the disorder was shown to be a genetically determined prion disease [68], and within a few years confirmed to be transmissible [69].

Epidemiology and Cause

Since its clarification as a prion disorder, a number of additional pedigrees manifesting FFI have been described [70–73]. However, it remains a very rare disorder. Consistent with its characterization as a D178N mutation in the *PRNP*, FFI shows inheritance patterns consistent with autosomal dominance [68, 70]. However, incomplete penetrance is possibly quite common as 11 members of the original large Italian FFI kindred were found to harbour the mutation (3 older than 60 years) and yet were asymptomatic at the time of the report. Within FFI pedigrees, onset is usually in the fifth decade, but ranges from 20 to 63 years, with illness durations averaging around 13–15 (range 6–42) months [68, 70–74].

In a retrospective genetico-pathological analysis of families previously given the generic diagnostic label 'selective thalamic degeneration', three of four demonstrated the same D178N *PRNP* mutation, prompting their more correct classification as FFI [75]. As already mentioned, the same D178N mutation had previously been described as a cause of familial CJD in a number

of unrelated pedigrees, stimulating discussion as to explanations for this apparent clinico-genetic dichotomy [76]. The modifying influence of the normal polymorphism at codon 129 on the mutant allele has been proposed as the explanation, with FFI occurring in individuals with the D178N-129M haplotype and familial CJD occurring in individuals with the D178N-129V genotype [77]. However, detailed studies of kindreds containing the D178N and other mutations have shown sufficient clinico-pathological diversity and overlap to suggest that FFI and CJD most likely represent somewhat artificial but frequently identifiable clinical phenotypes, and cast doubt on the claim that ultimate illness is governed entirely by associated codon 129 allelic polymorphisms [70, 71, 73, 78–80]. Cases of familial prion disease have been reported carrying the D178N-129M polymorphism without symptoms of insomnia [81]. Recently, clinico-pathological phenocopies of FFI unassociated with an underlying *PRNP* mutation (known as sporadic fatal insomnia) have been described [82–84].

Clinical

The core clinical features of FFI consist of profound disruption of the normal sleep-wake cycle (with complete disorganization of the electroencephalographic patterns of sleep), sympathetic overactivity, diverse endocrine abnormalities (particularly attenuation of the normal circadian oscillations) and markedly impaired attention.

The sleep disturbance may initially be relatively minor but usually progresses over weeks to months until normal sleep may not be possible, supplanted by stupor usually accompanied by frequent, vivid dreams which may be acted upon while still somnolent [67]. Prompt arousal with light stimuli remains characteristic but not invariable [73], and as cognition fails, patients may not be able to recall their intrusive dreams. A variety of probable (auditory, visual and tactile) hallucinations may occur in addition to the parasomnias, and further contribute to the bizarre nocturnal behaviours and oneiric automatisms which can be observed [70, 72].

Dysautonomia constitutes the other major distinguishing clinical feature of FFI, and may be noticeably episodic. Although often reflecting sympathetic overactivity, its broader manifestations include impotence, sphincteric dysfunction, salivation, rhinorrhea, lacrimation, hyperthermia, hyperhidrosis, tachycardia, and hypertension [67, 80]. Autonomic dysfunction tends to occur relatively early in the clinical course and may be the presenting symptom.

As the illness evolves, a range of motor abnormalities usually evolve in variable combinations. Cerebellar and pyramidal dysfunctions culminate in prominent limb, gait, and bulbar difficulties, accompanied by hyperreflexia, upgoing plantar responses, intention tremor and dysmetria. Spontaneous and

reflex myoclonuses are commonly present. Disorders of ocular movement and generalized hypertonia may also be seen. Respiration is frequently altered and may display tachypnoea, or an irregular noisy pattern with intermittent apnoea and hypoventilation [67].

Cognitive impairment usually develops later in the evolution of the illness, but may remain relatively mild and restricted to mnestic difficulties on formal neuropsychological testing [67, 80]. Invariably, patients eventually become confused and disoriented, progressing to stupor and coma with death from intercurrent pneumonia a common outcome. Seizures are not commonly seen during the course of typical FFI.

Diagnosis

Routine biochemical and haematological parameters are typically normal as are CSF findings, although oligoclonal banding of uncertain significance and relevance has been reported in a single patient [72].

Systematic monitoring of FFI patients typically discloses a range of hormonal irregularities, comprising alterations in basal blood levels, changes in the circadian pattern of secretion, or both [67, 72]. Serum cortisol is continuously increased, with or without preservation of circadian fluctuations. Circulating levels of both melatonin and thyrotropin (TSH) are reduced, with greatly attenuated or absent variations over a 24-hour period. Despite this, thyroid hormone levels are reported as normal and TSH levels increase in response to challenges with thyrotropin-releasing hormone. In addition, normal circadian oscillations are lost for growth hormone, prolactin and follicle-stimulating hormone (FSH), with impaired responses of FSH and luteinizing hormone (LH) to LH-releasing hormone. Basal gonadal hormones may be increased (progesterone) or decreased (testosterone and oestradiol) but can be partly stimulated artificially with human chorionic gonadotropin.

Polysomnographic recordings confirm markedly reduced total sleep time and gross electroencephalographic disorganization of sleep, including virtual absence of typical rapid-eye-movement (REM) periods and deeper non-REM phases characterized by K-complexes, spindles and slow waves [67, 72]. Instead, non-wakefulness may be replaced by something approximating poorly developed REM phases that coincide with periods of dreaming. Even pharmacologic agents such as benzodiazepines and barbiturates may be unable to induce sleep-like EEG activity [67] but promising results were reported in a single patient given γ-hydroxybutyrate [72].

The routine EEG is often normal in the early stages, but usually shows the progressive development of widespread, non-specific slower (θ and δ) frequencies as the disorder advances [67, 70, 72, 73, 80]. However, generalized less responsive α activity has been reported during the course of a patient’s illness

[67]. Periodic complexes or triphasic waves are usually not seen in typical FFI associated with the D178N-129M haplotype [67, 70, 72, 73, 80].

Kuru

Kuru constitutes a horizontally transmitted prion disease of declining incidence, geographically confined to Papua New Guinea. It was first identified by Western medicine in the mid-1950s, as the more remote parts of New Guinea came under external control through the provision of medical and other services [85, 86]. 'Kuru' in the Fore language means 'to shiver' (or 'to be afraid'), and along with cerebellar ataxia, constitute the salient clinical hallmarks of the disorder.

Epidemiology and Cause

After it was identified, kuru was recognized to be endemic amongst the Fore linguistic and cultural group resident in the Eastern Highlands of New Guinea [85, 87]. It had been extant since at least 1941 but was also seen in the neighbouring Keiagana, Kanite, Kimi, Usurufa and Auiyana tribes with whom the Fore often inter-married. Hence, kuru occurred in a circumscribed, remote, mountainous region populated by approximately 17,000 indigenes dispersed over more than 160 villages. Since the outlawing of ritualistic endo-cannibalism in the late 1950s, kuru has shown a steady decline and is now almost eradicated [88, 89]. However, at the time of its original description, kuru was estimated to have a general prevalence of approximately 1% within the geographically circumscribed area centred around the Okapa Patrol Post, but an annual incidence of up to 10% was seen in some tribes and accounted for over 50% of all deaths in certain communities.

Over three-quarters of the originally described kuru victims were adult women and children of either sex but symptoms never commenced prior to 4 years of age; adult males only rarely developed kuru. Over the first decade of detailed investigation, the prevalence of kuru declined significantly, with a progressive increase in the youngest age of onset in children, such that whereas the illness was initially observed in children as young as 4 years in 1957, by 1967, symptom onset was never seen under 14 years of age. Eventually, the confluence of scientific and epidemiological data culminated in the theory that kuru had been transmitted and sustained at endemic proportions, by cannibalistic rituals observed as part of the funeral rites mourning deceased relatives [88, 90]. Women and children at these ceremonies ate the less desirable (including the highly infectious central nervous system) tissues while adult males partook of the (relatively non-infectious) organs, such as skeletal muscle. In

addition to ingestion of infectious tissues, conjunctival, nasal and skin contamination were other likely modes of transmission. Many observations purport incubation periods of greater than two, and up to four decades [87, 91]. Although the ultimate cause of kuru remains uncertain, a cannibalistic serial passage of a sporadic case of indigenous CJD remains the most plausible hypothesis.

Despite its experimental transmissibility, there is no epidemiological evidence to support vertical transmission, with pregnant symptomatic women (at various stages of kuru) typically delivering healthy infants who, to this date, have never gone on to manifest the illness.

Clinical Features

The onset is typically insidious without antecedent illness, and the overall clinical picture is extremely uniform, predominantly manifesting as an inexorably progressive pan-cerebellar dysfunction [85–87]. Fever and seizures are not features and the general physical examination is normal until late in the disease when effects of undernutrition may become evident. Total illness duration is usually 6–9 months, but ranges from 4 to 24 months, with homozygosity (particularly for methionine) at the polymorphic codon 129 of *PRNP* apparently linked to younger age at onset and shorter duration of illness [92].

Subtle ataxia affecting gait and disequilibrium usually herald the first or ambulant phase and may be initially appreciated by family or friends rather than the patient. Once the patient cannot ambulate unassisted due to their progressive cerebellar dysfunction, they are considered to have entered the second or sedentary phase of the illness. Nystagmus is rarely evident but a convergent strabismus frequently occurs, usually late in the clinical course. Within a matter of weeks or a few months, all voluntary motor activity is so impaired that the patient cannot even sit unsupported and is then described as having entered the third or terminal phase during which they are bedridden, totally dependent for feeding and all personal care, and typically incontinent of urine and faeces. Decubitus ulcers are a common complication, and by this stage the patient's bulbar functions have usually deteriorated to the point where they are anarthric and aglutic, with inanition an invariable feature. Death quickly ensues as a consequence of static bronchopneumonia, infected pressure sores or starvation.

Irregular and coarse 'tremors' soon accompany the limb and gait ataxia and may give rise to body actions resembling shivering. The tremors are usually only observed during voluntary motor activity and also often take the form of an intention tremor affecting purposeful appendicular movements and/or titubation of the head and trunk. Additional involuntary movements having the appearance of chorea or athetosis may be seen although some authors ascribe this adventitious motor activity to misunderstood features of cerebellar ataxia

[93]. Significant features of pyramidal or extrapyramidal dysfunction (akinesia and rigidity) are usually not prominent. Muscle weakness and wasting are not seen until the secondary effects of malnutrition supervene, and somatic sensory functions appear maintained throughout the course of the illness.

Cognition tends to be relatively spared, at least until late in the course of the illness when its accurate assessment is very difficult due to the incapacitating impairments of motor function, including speech. The prevailing mood tends to be one of euphoria through the early phases of the illness and emotional lability and a pseudo-bulbar affect with inappropriate excesses of laughter or crying may be seen. Ultimately, the patient's demeanour becomes one of apathetic withdrawal.

Diagnosis

As for the other prion diseases, routine biochemical and haematological investigations are normal, as is the cerebrospinal fluid [85], with the clinical diagnosis appropriately restricted to indigines of the Eastern Highlands of New Guinea manifesting a typical illness. Neuropathological examination is required for unequivocal confirmation.

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Epidemiology and Risk Factors of Transmissible Spongiform Encephalopathies in Man

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Several forms of human spongiform encephalopathies have been described in the literature since the scientific world became aware of the first cases of Creutzfeldt-Jakob disease (CJD) in the early 1920s. Soon after the first cases had been described by German neuropathologists Hans Gerhard Creutzfeldt and Alfons Jakob, a report of a peculiar disease, which was named the Gerstmann-Sträussler-Scheinker syndrome (GSS), followed. In the following years a few case reports occurred repeatedly in the literature and the disease was considered as a rare neurodegenerative disorder which occasionally followed an autosomal dominant inheritance pattern (table 1). In the 1950s, an obscure epidemic occurred in Papua New Guinea and this disease, called kuru, became a starting point for epidemiological investigations of this disease. As soon as the transmissibility of kuru was shown and the scientists became aware of the similarities between kuru, CJD and scrapie, a well-known disease in sheep, the interest in epidemiology of CJD led to several case-control studies on CJD, which aimed to find risk factors for this disorder. The potential transmissibility of CJD between humans by iatrogenic procedures was first recognized in 1974. In recent years, genetic forms have been increasingly described. A general surge of interest in the incidence of CJD in the last 10 years is primarily associated with the occurrence of bovine spongiform encephalopathy (BSE) in the UK and later elsewhere in the world. Due to the fear that this disease might be transmitted to man through the consumption of beef products, epidemiological studies were initiated throughout Europe. Prior to these investigations, almost all mortality statistics were available worldwide (table 2).

Table 1. Human spongiform encephalopathies

Year of first description	Disease	Pathogenetic mechanism
1920	Creutzfeldt-Jakob disease (CJD)	Sporadic
1928	Gerstmann-Sträussler-Scheinker syndrome (GSS)	Genetic
1957	Kuru	Acquired
1974	Iatrogenic CJD	Acquired
1986	Fatal familial insomnia (FFI)	Genetic
1989	Familial CJD	Genetic
1996	Variant Creutzfeldt-Jakob disease (vCJD)	Acquired
1999	Sporadic fatal insomnia (SFI)	Sporadic

Table 2. Annual incidence rates of CJD (table derived from Brown [2], modified by inclusion of published data)

Country	Survey years	Incidence (cases per million)
Argentina	1980–1996	34 cases
Australia	1979–1992	0.75
Chile	1955–1972	0.10
	1973–1977	0.31
	1978–1983	0.69
Germany	1979–1990	0.31
	1993–1997	0.85*
France	1968–1977	0.34
	1978–1982	0.58
	1992–1995	0.96*
UK	1964–1973	0.09
	1970–1979	0.31
	1980–1984	0.47
	1985–1989	0.46
	1990–1994	0.70*
	1995–1996	0.74*
India	1971–1990	30 cases (0.002)
Iceland	1960–1990	0.27
Israel	1963–1987	0.91
	1989–1997	0.90

Table 2 (continued)

Country	Survey years	Incidence (cases per million)
Italy	1958–1971	0.05
	1972–1986	0.09
	1993–1995	0.56*
Japan	1975–1977	0.45
New Zealand	1980–1989	0.88
The Netherlands	1993–1995	0.81*
Austria	1994–1995	1.27*
Sweden	1985–1996	1.20
Switzerland	1988–1997	1.14
Slovakia	1993–1995	0.62
Czechoslovakia	1972–1986	0.66
Hungary	1960–1986	0.39
USA	1973–1977	0.26
	1979–1990	0.90
Belarus	1981–1989	21 cases

Cases of CJD are reported from Egypt, Belgium, Brazil, China, Finland, Greece, Indonesia, Iran, Yugoslavia, Canada, Colombia, Northern Ireland, New Guinea, Norway, Mexico, Oman, Peru, Poland, Portugal, Romania, Senegal, Spain, South Africa, Taiwan, Thailand, Tunisia, Uruguay, Venezuela, and West Bengal.

*Prospective studies.

According to these statistics, the total annual number of individuals who died of CJD was between 0.1 and 1 per million population (= mortality). A classification into sporadic, genetic and iatrogenic cases was not possible. So far, a systematic registration of the incidence over longer periods is only being performed by the UK, where retrospective studies were already started in the 1970s. After the occurrence of BSE in 1986, the investigations were continued using modified methods of data acquisition. Former investigations, which primarily included mortality statistics based on death certificates, revealed an incidence between 0.31 and 0.42 [1, 2]. In subsequent prospective studies, it could be shown that the increase in registered cases – the incidence was meanwhile reported to be 0.74 – was due to the different methodological approach. The improvement of diagnostics and, probably, awareness of CJD within the group

of patients aged >70 years are factors that influence the incidence [3, 4]. This finding was repeatedly observed in other countries as soon as active surveillance studies were initiated.

Molecular biological procedures have meanwhile enabled the detection of different mutations in the prion protein gene (*PRNP*) and thus to distinguish genetic from sporadic cases. In addition, genetic examinations allow the identification of patients who, formerly, would not have been diagnosed as CJD cases owing to the atypical clinical symptomatology, as has been shown for various mutations [5]. The physiological polymorphism at codon 129 (either as methionine or valine) of the *PRNP* was identified to be a major susceptibility factor for sporadic and iatrogenic cases and probably variant CJD. The homozygosity at codon 129 is considered to influence the incubation time in acquired cases and the disease duration and phenotypic diversity in sporadic CJD [6, 7].

Sporadic CJD (spCJD)

Descriptive Epidemiology

Incidence

Sporadic CJD cases occur in all age groups, but no case has ever been reported in a child. The youngest patient seen in the German CJD surveillance study was 19 years, the oldest 88 at disease onset. Analysis of the age-related cases in 1993–2002 shows a peak in the age group 70–74. A decline in the incidence of spCJD can be seen in patients older than 80 years (fig. 1).

The lower incidence of CJD in older patients was already reported in earlier studies and can be explained in several different ways. On the one hand, neurodegenerative dementias are frequent in this age group, and it was often not possible in the past to distinguish CJD from Alzheimer's disease or vascular dementia due to the lack of reliable diagnostic tests. On the other hand, it is also conceivable that a genetic cause is responsible for the decreased life expectancy. An atypical clinical picture in older patients that does not fulfill the classification criteria and therefore is not recognized as CJD might also be another explanation.

The disease occurs more frequently in women than in men (1.4:1). This finding remains even when demographic factors are taken into account. By calculating the age- and sex-specific ratios, the difference is still striking.

The incidence of spCJD in most European countries is stable over the years and varies between 1 and 1.5 cases per million per year. In Germany, this corresponds to around 100 new cases of sporadic cases in a country with 80 million inhabitants. In addition, 10 cases of genetic cases are observed each

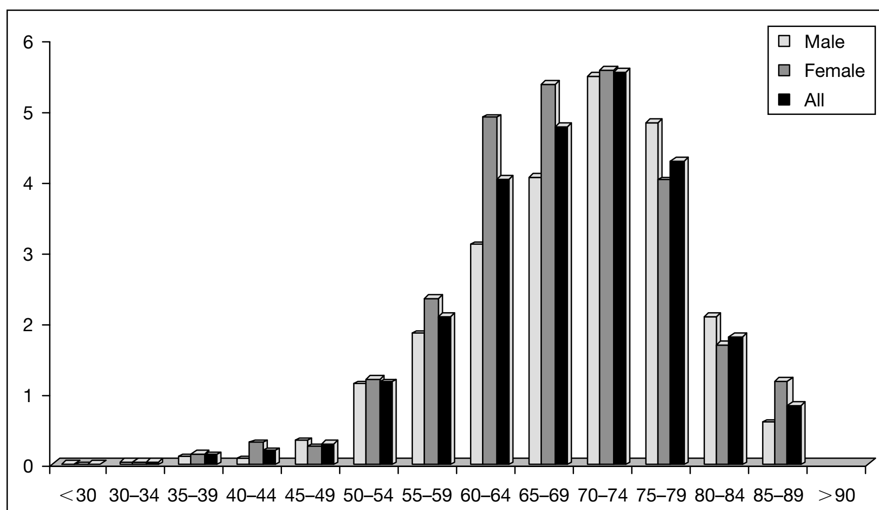

Fig. 1. Age-specific incidence of CJD in Germany, 1993–2002.

year. As the disease duration is generally short (median 6 months, range 1–86), the incidence and mortality rates are close. The extension of clinical diagnostic criteria by inclusion of the detection of 14-3-3 proteins in the cerebrospinal fluid as a sensitive pre-mortem test for spCJD led to more precise clinical diagnosis and had an important impact on surveillance studies. This modification, which was accepted 1998 by the WHO and the European surveillance programs, increased the accuracy of the incidence figures [8].

The median survival time until death in spCJD is 6 months, but may also vary to a great extent between 3 weeks and 7 years. Cases with longer duration were occasionally reported [9]. Patients who are homozygous for methionine or valine on codon 129 of the *PRNP* tend to a more rapid disease course than heterozygous patients (fig. 2). The age at onset determines the survival time too – young patients tend to have a longer survival time (fig. 3).

Cluster

Several studies in the past attempted to analyze possible clusters of spCJD (higher frequency of cases occurred than expected). Such studies are usually hampered by small numbers of observed cases. Nevertheless, apparent clusters were recently reported from France and Australia [10, 11]. A higher frequency of CJD in the metropolitan area of Paris was already described in the 1980s [12]. An occurrence of spCJD in a husband and wife remains a curiosity and a detailed analysis revealed that such a coincidence is statistically within the usual

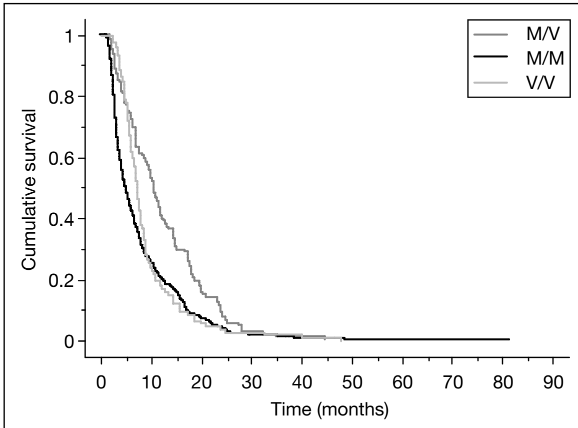

Fig. 2. Kaplan-Meier cumulative survival time stratified by codon 129 genotype (M = methionine; V = valine).

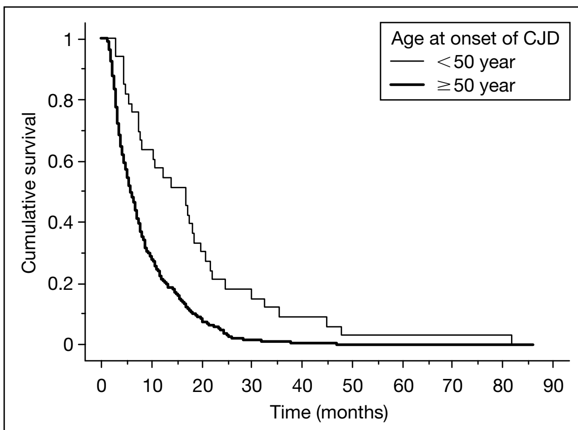

Fig. 3. Survival time of patients aged younger and older than 50 years.

expectancy [13]. In Germany, spCJD cases correlate with population density and no particular cluster has been observed up to now (fig. 4).

CJD in Europe

Sporadic CJD has been reported all over the world and the incidence figures are comparable among countries that use a similar methodology of case

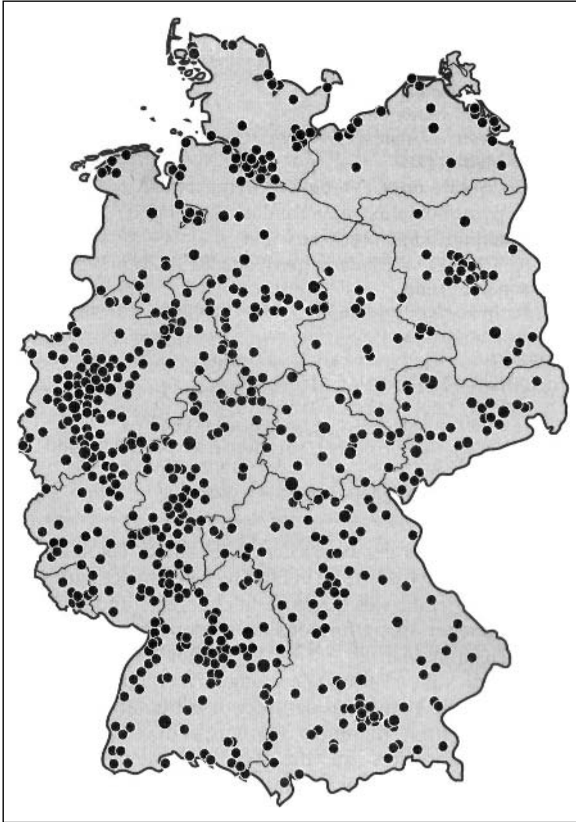

Fig. 4. Distribution of spCJD cases in Germany by place of residence.

ascertainment. Meanwhile, such programs have been initiated by all European countries. Between 1993 and 2002, data from 3,391 patients were analyzed throughout Europe. To increase the comparability, mortality rates are reported (table 3). A striking finding is a report of high incidence/mortality of spCJD in Switzerland. There, an incidence of 2.50 for the year 2001 and even 2.63 for 2002 was seen [14]. An analysis of possible influence of case ascertainment, change in the methodology etc. was discussed and was not confirmed. One possible explanation might be that active CJD surveillance in a country with a small population might be able to detect even atypical CJD cases. If such high incidence figures are confirmed in the following years, this might imply that the true incidence of spCJD might be twice as high than considered before.

Table 3. Annual mortality rates per million of spCJD in Europe and Australia

	Australia	Austria	France	Germany	Italy	Netherlands	Slovakia	Spain	Switzerland	UK
1993	0.96	0.77	0.60	0.44	0.48	0.79	0.40	0.54	1.44	0.63
1994	0.61	1.15	0.80	0.85	0.58	1.18	0.40	0.41	1.42	0.91
1995	1.04	1.15	1.02	1.00	0.49	0.52	0.40	0.48	1.27	0.60
1996	1.37	1.15	1.18	0.93	0.89	0.90	0.40	0.69	1.41	0.68
1997	1.07	0.77	1.38	1.30	0.83	1.21	0.60	0.79	1.41	1.03
1998	1.33	1.03	1.38	1.40	1.11	1.15	0.40	1.52	1.27	1.08
1999	1.32	0.77	1.53	1.18	1.35	1.14	0.20	1.27	1.13	1.06
2000	1.51	1.03	1.46	1.29	1.06	0.69	0.40	1.09	1.40	0.84
2001	0.98	1.36	1.84	1.54	1.45	0.88	0.40	1.52	2.50	0.96
2002	0.72	0.87	1.71	1.13	1.40	0.93	0.60	1.19	2.63	1.23

Role of the Codon 129 Genotype

The methionine/valine polymorphism at amino acid position 129 of the prion protein gene influences the neuropathological characteristics of the sporadic and hereditary variants of human transmissible spongiform encephalopathies [6, 15–20]. Homozygosity at codon 129 is considered as a susceptibility factor for CJD (fig. 5). While the genotype distribution among the general population is 42% homozygosity for methionine (MM), 13% homozygosity for valine (VV) and 45% heterozygosity (MV), methionine homozygosity predominates in patients with spCJD. All patients with vCJD examined so far have been homozygous for methionine at codon 129 [21]. Valine homozygous CJD patients tend to be younger than patients who are either MM or MV [16]. Epidemiological observations of iatrogenic cases revealed that homozygous patients have shorter incubation periods than heterozygous patients (first MM and VV cases occurred in 1989, MV cases in 1994; fig. 6) [22]. In homozygous patients with kuru, the disease occurred earlier than in heterozygous patients. Moreover, shorter disease duration was observed [23].

In spCJD, the phenotypic variability is determined by codon 129 genotype (table 4) [6, 7, 24]. Homozygous patients have the most rapid disease course, whereas the disease duration is twice as long in heterozygous cases. On the other hand, patients with at least one valine allele are younger. Another not well-explained observation is the predominance of women in patients who are homozygous for methionine (table 4).

Risk Factors

Due to the neuropathological similarity between kuru and scrapie [25], a possible link between scrapie and CJD [26] was examined in the past. From an epidemiological point of view, the numerous investigations performed

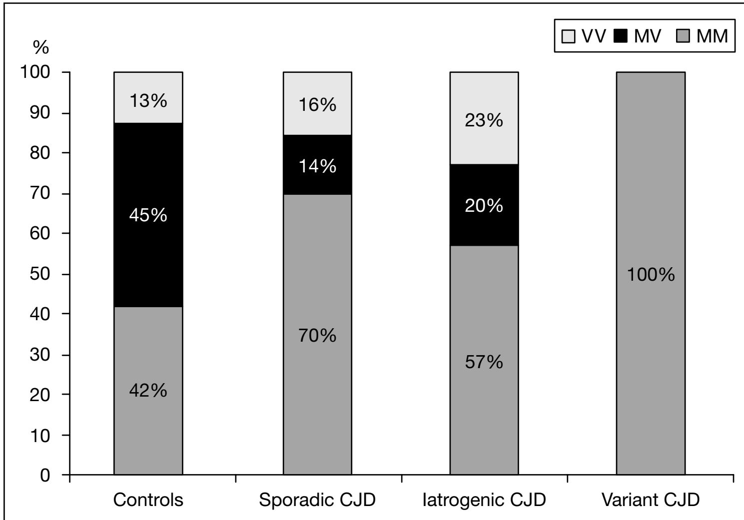

Fig. 5. Codon 129 genotype in CJD and controls (controls: population in Germany; sporadic CJD [15]; iatrogenic CJD [75]; variant CJD [21]).

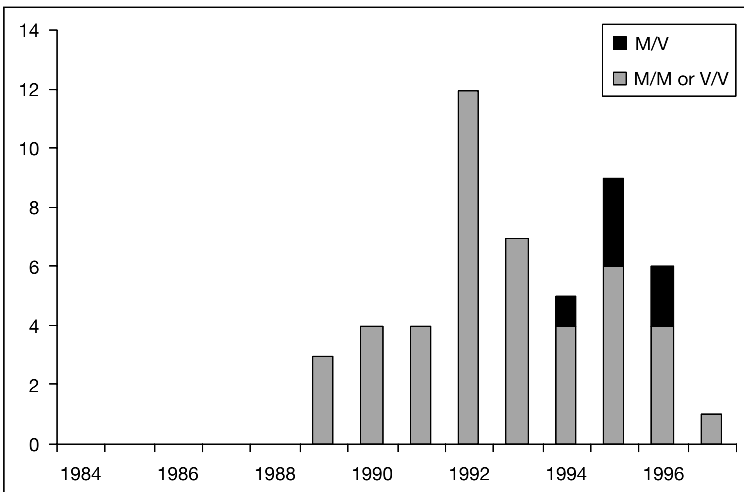

Fig. 6. Iatrogenic CJD after growth hormone treatment: year of disease onset and genotype at codon 129. Contamination period from January 1984 to June 1985 [modified according to 22].

Table 4. Characteristics of patients with spCJD and genotype at codon 129

Genotype at codon 129	n	Median age at disease onset (range)	Sex f:m	Median disease duration months (range)
M/M	515	67 (31–88)	1.6:1	5 (1–81)
M/V	127	64 (35–84)	1.2:1	11 (2–45)
V/V	120	63 (19–88)	1.3:1	7 (2–48)

revealed no connection: in scrapie-free countries the occurrence of CJD, as compared to those countries in which scrapie is endemic, is equally frequent [26]. Especially in France and Italy a different epidemiological distribution of scrapie and CJD was evidenced [27]. However, these conclusions are subject to the reservation that the worldwide trade with (contaminated?) sheep products is not taken into consideration.

Potential risk factors for CJD were examined in several case-control studies (table 5). The methodology of the individual studies is different, in particular with regard to the selection of the control group, which might bias the results [28]. Although significant factors have been found in each study (table 5), the attempt to identify one common environmentally related or endogenous factor has failed. However, two recent studies met the problem of possible bias and reassessed the data using population controls [29, 30]. In both studies, surgical procedures in the past were significantly associated with the development of spCJD. In the study from Australia, the risk progressively increased with the number of surgical treatments (odds ratio (OR) 2.13). This was confirmed by a re-analysis of a considerably larger pool data set of cases from the European CJD surveillance study [30]. A history of surgery was significantly associated with the risk of spCJD (OR 1.8, 95% CI 1.2–2.6), which was not dependent on the number of surgical procedures, and was stronger in females (OR 2.5, 95% CI 1.5–4.0).

In uncontrolled studies some risk factors were proposed to be related to CJD. Owing to the potential transmission of the disease to medical staff, the following case reports deserve special attention: one internist who, 30 years ago, had worked in a department of pathology for a period of 12 months developed CJD, although no direct contact with CJD was documented [31]. One pathologist [32], one neurosurgeon [33], and two assistants who worked in a department of histology [34, 35] developed CJD. Furthermore, one orthopedist is reported to have performed experiments with sheep dura mater 30 years prior to disease onset [36]. In spite of these reports, no increased CJD disease risk was revealed by controlled studies in medical professions [37].

Table 5. Case-control studies: CJD-associated findings

CJD-associated findings	Ref.
Surgery	29, 30, 70, 71
Physical injuries	71
Tonometry within 2 years before onset	70
Suture age 15 through 3 years before onset	70
Head-face-neck injury	70
Other trauma	70
Family history of dementia (excluding familial cases)	37, 72
Herpes zoster in adult life	72
Consumption of raw meat and brain	37, 73
Exposure to fish, squirrel, rabbit	74
Exposure to leather products, fertilizer consisting of hoofs and horns	37
Residence or employment on a farm or market garden	29

The consumption of brain tissue from various free-ranging animals was occasionally discussed to be associated with the occurrence of spCJD [38, 39]. The observation of an increased occurrence of spCJD in dairy farmers led to the assumption that these cases could be connected with BSE cases in their herds [40]. A comparison of the occupationally related incidence of CJD in dairy farmers showed an incidence beyond expectation of about 4 cases per million population per year in other European countries and did not show any difference as compared to the UK [4]. The occupationally related incidence is higher than the general incidence of CJD, but it is in conformity with the expected number of diseased individuals in the age group 60–70 years. Moreover, clinical symptoms, neuropathological findings and experimental transmission to mice did not differ from sporadic cases [41].

Iatrogenic Cases

A man-to-man transmission of the infectious agent has only been evidenced through direct iatrogenic exposure to infectious tissue (dura mater, cornea, and growth hormones derived from human cadaveric pituitaries, in rare cases by insufficiently sterilized surgical instruments or EEG electrodes; table 6). The first iatrogenic transmission ever reported was recognized only because of

Table 6. Iatrogenic cases of CJD (table modified according to Brown [47] and Cambridge Healthtech Institute's Conference on Transmissible Spongiform Encephalopathies, Washington, D.C., October 27–28, 1999)

Mode of infection	Patients n	Agent entry into brain	Mean incubation period (range)	Country of occurrence
Corneal transplant	3	Optic nerve	16, 18, 320 months	Germany, Japan, USA
Stereotactic EEG	2	Intracerebral	18 months (16, 20)	Switzerland
Neurosurgery (without dura)	4	Intracerebral	19 months (12–28)	France, UK
Dura mater graft	106	Cerebral surface	6 years (1.5–16)	Worldwide, most in Japan
Growth hormone	125	Hematogenous	12 years (5–30)	Most in France, UK, and USA
Gonadotrophin	5	Hematogenous	13 years (12–16)	Australia

the short incubation period in a patient with corneal graft 18 months before first symptoms. The donor – as was later found – had died of CJD. Another case was reported by a Japanese working group, but no data are on hand about the cornea donor [42]. The third known case of an iatrogenic transmission occurred in Germany [43]. Two patients developed CJD 16 and 20 months following examination with stereotactic EEG electrodes, which had been used in a patient with CJD and were insufficiently sterilized [44]. The infectivity of these electrodes has been demonstrated by transmission in chimpanzees [45]. Owing to the use of insufficiently sterilized neurosurgical instruments, the infectious agent was transmitted in 4 further cases [46, 47].

The transmission of CJD via dura mater grafts by lyophilized dura from a single manufacturer is well documented. Prior to 1987, no additional inactivation procedures with NaOH had been performed [48, 49]. More than 100 cases were reported worldwide [P. Brown, pers. commun.], most of them from Japan [75]. In the period studied, 1 out of 3,000 recipients of dura mater grafts developed CJD in that country. Only a few cases were seen after the use of non-commercial products [50]. Disease transmission also occurred after non-neurosurgical procedures following orthopedic surgery [49], cholesteatoma [51], after embolization of a nasopharyngeal angiofibroma [52], and of an intercostal artery [53].

Up to 1985, patients with primary hypopituitarism were treated with human growth hormones derived from human cadaveric glands. At that time, the first cases of CJD occurred in young patients who had received intramuscular injections of pituitary gland-derived hormones [54, 55]. The mean incubation time in these patients is 12 years (age range 5–30 years) [47]. More than 120 cases have meanwhile become known worldwide, 62 of these in France [56],

32 in the UK, and 28 in the USA [P. Brown, pers. commun.]. According to estimations, about half of the preparations produced in France between January 1984 and March 1985 might have been contaminated [56].

The incubation time of the disease depends on the portal of entry of the infectious agent which is shorter after intracerebral infection (several months, dura mater), but may range from years to decades after peripheral inoculation (12 years, growth hormones). Genetic factors (genotype at codon 129 of the *PRNP*) influence the incubation time. While so far primarily growth hormone-infected individuals with a methionine or valine homozygosity (shorter incubation time) have developed CJD, the number of persons with a methionine/valine heterozygosity is steadily increasing (longer incubation time) [22, 57] (fig. 6).

The occurrence of iatrogenic CJD cases, which have been observed since 1985 and which are caused by contaminated dura mater grafts and growth hormone preparations, has also contributed to an increase in the total incidence. In the course of the BIOMED study in the years 1993–1995 [58], 12% of all cases in France and 6% of the cases in the UK were of iatrogenic origin during this period, mostly after treatment with growth hormones derived from human cadaveric pituitary glands [58].

Variant CJD (vCJD)

The numbers of spCJD cases in the UK have remained stable over the periods of observation or have slightly increased for methodological reasons (fig. 7; also see www.cjd.ed.ac.uk). In the course of a cooperation between epidemiological surveillance studies in Europe, altogether 153 patients (143 in the UK, 6 in France and each 1 in Canada, Italy, in Ireland, and in the USA) with vCJD have been identified so far (http://www.doh.gov.uk/cjd/cjd_stat.htm; November 3, 2003) [59–61]. These cases differ clinically and histopathologically from the classic sporadic form of CJD in the young age at disease onset (median 28 years, range 14–74), early behavioral abnormalities, depression and anxiety [62], frequent dysesthesia, late dementia and longer disease duration (14 months). Neuropathological lesions of the brain are characterized by pronounced plaque-like prion protein depositions in the entire brain, showing a dense eosinophil center surrounded by spongiform changes (so-called florid plaques), which are not seen in spCJD. The epidemiological link of the novel CJD phenotype in the UK to BSE was soon substantiated by experimental transmission data [41, 63].

Meanwhile, diagnostic criteria allow the clinical diagnosis of probable vCJD in these patients [21, 64] (see table 4, p. 83, in the article of Knight et al.).

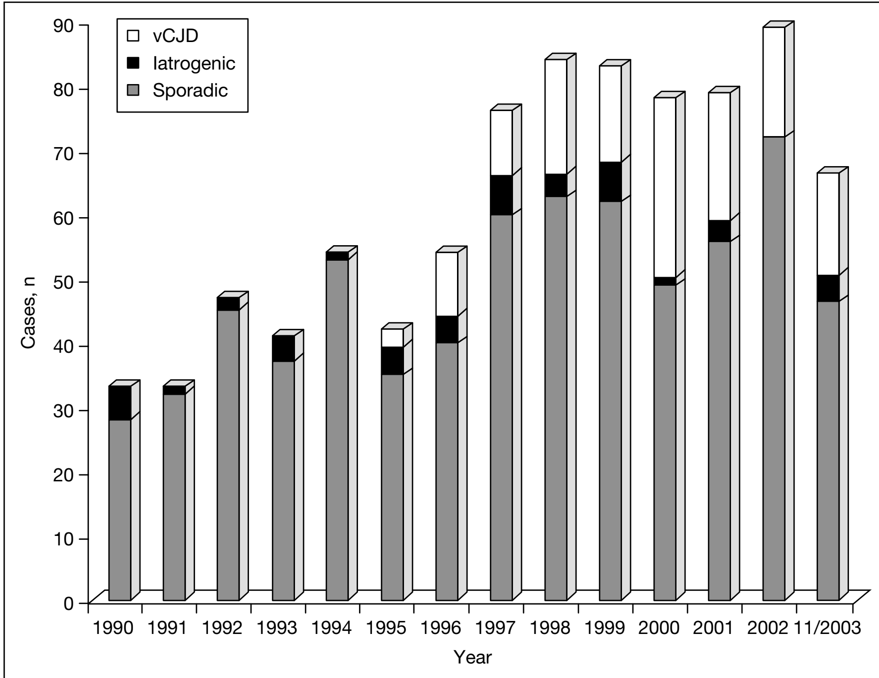

Fig. 7. CJD in the UK (source: http://www.cjd.ed.ac.uk/figures.htm).

The introduction of these criteria into surveillance studies was followed by inclusion of clinically probable cases into the statistics. The artificial rise in vCJD figures in the year 2000 is explained by this change in the methodology (fig. 8).

The possible incubation time in these patients can only be assessed. Presumably it is at least 5–10 years since the exposure to the BSE agent was highest in the 1980s and the first disease cases were observed in 1994. Some data pointed toward an increase in the numbers of new cases in recent years [65]. However, considering the currently available data, an exact prognosis regarding the further development of the vCJD cases is speculative. Depending on the postulated incubation time and transmission probability [66], the numbers range from several hundred to 80,000 diseased individuals. The risk factors remain unknown. Regarding life history, eating habits, or occupational exposure, no peculiarities have been reported of the vCJD cases observed in the UK [67]. A striking difference in incidence of vCJD has been observed with higher figures in the north compared with a dense populated south of the UK [68]. The nature of this is not known and remains a question in view of BSE

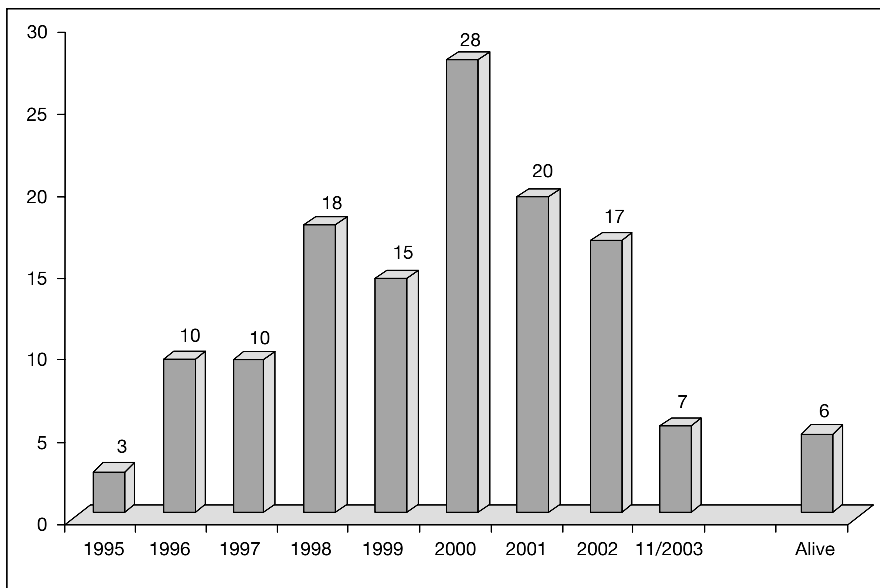

Fig. 8. Variant CJD in the UK (definite and probable cases).

cases, which occurred mainly in the south of the country. However, one cluster of vCJD was reported in Leicester and was discussed to be linked to the local traditional butchery practices [69].

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Aspects of Risk Assessment and Risk Management of Nosocomial Transmission of Classical and Variant Creutzfeldt-Jakob Disease with Special Attention to German Regulations*

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Creutzfeldt-Jakob disease (CJD) is a rare neurodegenerative disorder affecting humans which has been recognized for decades [13, 29, 34]. However, a few years ago, the emergence of a new form of CJD was observed – first in the UK and subsequently in several other countries. This new disease, originally reported in 1996 and now called 'variant' Creutzfeldt-Jakob disease (vCJD) [50–52], is caused by the same etiological agent as 'mad cow disease', i.e. bovine spongiform encephalopathy (BSE) [12, 26, 35, 42]. The causative agent of BSE and vCJD belongs to a family of pathogens which underlie a variety of related neurodegenerative diseases, collectively known as transmissible spongiform encephalopathies (TSEs).

As revealed by several different methods of 'strain typing', isolates of the infectious agent of BSE and vCJD consistently exhibit identical biological and biochemical properties whereas they can be clearly differentiated from their pathogenic counterparts associated with classical forms of human TSEs such as 'sporadic', 'familial' and 'iatrogenic' CJD [12, 26, 35, 42]. According to our

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current state of knowledge, the vCJD cases diagnosed so far can most likely be attributed to transmissions, probably via contaminated food, of BSE agent from cattle to man [11, 12, 15, 16, 42].

The comprehensive countermeasures which have been implemented in response to the BSE epidemic during the past few years should effectively prevent further spread of this disease to humans, thereby minimizing or even eliminating the risk of new primary vCJD infections [5]. However, further challenges in the area of public health arise from the hypothetical risk of human-to-human transmission (secondary infection) of vCJD as well as from iatrogenic or accidental transmission of classical forms of CJD. When addressing these problems, it is necessary to clearly distinguish between the topic of classical CJD and vCJD.

In the past, various – although relatively few – cases of iatrogenic CJD have been reported [9]. For these cases, which clearly demonstrate the existence of a 'real' risk, the sources and routes of infection have been identified. This made it possible to devise and implement protective measures in order to reliably prevent similar iatrogenic transmissions in the future.

In contrast, the risk of secondary vCJD transmission is less precisely assessable. Currently, this risk poses an only 'theoretical', but potentially very dangerous hazard. On the one hand, it is still unclear whether and to what extent secondary transmissions of vCJD do or may occur and whether they can be mediated not only by contaminated tissues but also by blood or blood products. On the other hand, both BSE in cattle and vCJD in humans are caused by a newly emerged strain of TSE agent with previously unknown phenotypic features. This agent is responsible for the BSE epidemic in the UK, where it led to more than 181,000 clinical cases in the cattle population, although most of the animals were probably infected through the comparatively inefficient oral route [2–5, 7, 29, 49]. The considerable infectious potential of the agent is further emphasized by the fact that it was able to leap – apparently again via the oral route – the species barrier between cattle and man and to establish vCJD in humans. These and other findings highlight the hazardous properties of the BSE/vCJD agent and have given rise to the implementation of precautionary measures particularly aiming at the prevention of secondary vCJD infections.

When focusing on hospital settings, the avoidance of patient-to-patient or patient-to-personnel transmission of CJD and its variant should be of utmost importance. This article presents a general overview about the risk of infection and strategies to prevent nosocomial transmission of CJD and vCJD through surgical instruments and other medical devices. A distinction should be made between these aspects and other possible problems which are associated with the potential transmission of CJD and vCJD via transplants, tissues or cells, blood or blood derivatives and other medicinal products of human (or bovine)

origin. The latter aspects will only be dealt with partially, as they are discussed in greater detail elsewhere [3, 6].

Transmissible Spongiform Encephalopathies

Characteristics of the Etiological Agent

Irrespective of whether TSEs are caused by prions [40, 41] or by another, as yet unidentified, infectious principle, TSE agents differ fundamentally from all other conventional pathogens, such as parasites, fungi, bacteria or viruses in their exceptional biological, biochemical and biophysical properties. Their high resistance to conventional methods of chemical, enzymatic, thermal and physical (UV or ionizing radiation) inactivation is particularly striking [30, 38, 45]. Almost all disinfectants, especially those acting on nucleic acids, fail to inactivate TSE agents (also see chapter by Taylor, p 136–145 in this volume). Because of their great heat stability, the same holds true for temperatures normally used for preparing food. For some TSE strains, in the case of a heavy initial infectious load, or following desiccation of contaminated organic material, a complete inactivation cannot be guaranteed even after steam autoclaving at 134°C for 18 min. Thus, the hazardous properties of TSE agents, as well as their high tenacity, warrant specific prophylactic measures and a specific choice of cleaning procedures, disinfectants and sterilization methods to minimize the risk of iatrogenic or accidental transmission of classical CJD or vCJD in nosocomial environments.

Risk Assessment

Reported Transmissions of Human TSEs

So far, transmission of TSE agents between humans, with the exception of kuru, has only been observed in the context of iatrogenic cases of classical CJD [9]. These cases can be attributed to transplants of infectious tissue (dura mater, cornea), treatment with contaminated medications such as growth hormones or gonadotropin extracted from pituitary glands of deceased human donors, or medical interventions using contaminated instruments. Until July 2000, the following cases of iatrogenic CJD were reported [9]: 139 following growth hormone treatment (74 of them in France), 4 following treatment with gonadotropin, 114 after transplantation of dura mater tissue (67 of them in Japan), 3 following corneal transplantations, 5 following neurosurgery with insufficiently sterilized surgical instruments and 2 after invasive implanting of insufficiently sterilized EEG electrodes. In Germany, 5 cases of iatrogenic CJD have been reported to date: 4 after dura mater and 1 following cornea transplantation.

No unequivocal cases of accidental occupational transmission in a nosocomial environment, including neuropathological laboratories, have been reported in the literature to date [31]. However, case reports of CJD in a neurosurgeon and in 2 laboratory workers imply that the disease (at least in these 3 cases of medical professionals) may be attributed to occupational exposure to the infectious agent.

There is no epidemiological evidence suggesting that the incidence of CJD is higher in medical professionals than in the general population [31]. The same holds true even for specific hospital environments and laboratories (including neurosurgical and neuropathological facilities), where exposure to TSE agents may be above average.

Iatrogenic and Accidental Transmission in Nosocomial Settings

To date, there is no evidence for transmission of human TSEs through normal social or nursing contacts [31, 43]. An essential prerequisite for transmission is the uptake of infectious material. With this in mind, nosocomial transmission of CJD or vCJD is principally possible: (1) when contact with tissues or body fluids containing TSE agent leads to a contamination (e.g. of instruments, parts of the body, etc.); (2) when this contamination is not completely removed or inactivated by cleaning, disinfection and/or sterilization, and when (3) residual infectious material is finally taken up by or brought into the body of a new host via (a) accidental occupational exposure of hospital personnel, or (b) during medical intervention on a patient (e.g. surgery).

Following transmission of the agent, the amount of infectivity transferred as well as the route of exposure will determine (i) whether an infection occurs at all, and (ii) the length of the incubation period.

Thus, the risk of an iatrogenic or accidental transmission in a nosocomial environment has to be ascertained for a wide spectrum of possible medical scenarios [1, 6, 18, 31, 43, 48]. This can be done by assessing patients, tissues and body fluids, as well as various routes of exposure, for their infectious potential and by segregating them into different risk categories. Based on the risk assessment, preventive measures specifically tailored to various treatment scenarios can be devised and implemented. This enables the risk management to minimize the hazard of nosocomial transmission of classical CJD and vCJD.

Stratification of Patients

Apart from kuru, which is not relevant in this context, a potentially elevated risk of transmission is inherent in medical treatments or interventions performed in patients with an evident risk for CJD (including GSS and FFI) or vCJD. In this group, several levels of risk should be differentiated: (a) patients with an established diagnosis of TSE, (b) patients with clinical symptoms of

a probable or possible TSE, and (c) asymptomatic patients who are at high or elevated risk of developing a TSE. In Germany, for instance, the latter group consists of patients who received hormonal extracts from human pituitaries or who underwent dura mater transplantation, as well as patients with a known risk for familial TSE [43].

In order to be able to identify patients with possible or clinically probable CJD or vCJD, physicians, and especially neurologists, should be familiar with the clinical presentation of human TSEs and with the criteria for their differential diagnosis. These are described in detail elsewhere [1, 37, 39, 53]. In the case of suspected TSE (e.g. unexplained dementia or unclear psychiatric disorder), in particular prior to performing elective invasive procedures, the expected benefit of a medical treatment should be weighed against the potential risk of transmission. This can be achieved through additional discussion with the referring physician or by a neurological consultation.

In comparison to the group of risk patients, the group of asymptomatic patients without any recognizable risk is much larger. The patients belonging to this collective may develop sporadic CJD with an incidence already previously quoted (i.e. 1–1.5 cases per million inhabitants and year), whereas the frequency of undetected vCJD infections in this group is at present almost impossible to reliably assess [6, 23, 28].

Stratification of Tissues

Additional questions arise in parallel with the risk assessment for different groups of patients. Which parts of the body of TSE patients or carriers serve as agent reservoirs? What are the concentrations of agent in these sites? The infectivity status of tissues helps to identify possible sources of contamination and to assess the potential risk of transmission associated with specific medical interventions and treatments.

According to a review published by the WHO [48], TSE agent has been detected in classical CJD, GSS and FFI by bioassay most frequently and at the highest titers in the brain, spinal cord and eye. Less frequently and in medium range or low titers, infectivity could also be detected in cerebrospinal fluid (CSF), lungs, liver, kidney, spleen, placenta and lymph nodes. In a variety of other tissues (such as heart, skeletal muscles, intestinal, peripheral nerves) and body fluids or in excrement, TSE agent could not be detected. Controversial results exist concerning the presence of CJD agent in blood. Human-to-human transmission of classical CJD (or GSS and FFI) through blood or blood products has not been observed to date. Inoculation of blood or blood components from CJD patients into animals revealed no or only an extremely low infectious potential. At present, the general consensus is that the blood of patients with classical CJD may be considered as non-infectious material, at least in the context of infection control [48].

vCJD has been transferred from humans to experimental animals through intracerebral inoculation of brain homogenate [12, 35]. The agent of vCJD has also been detected by bioassay in tonsils and in the spleen [11]. In contrast to classical CJD, in which pathological prion protein as a biochemical marker for infectivity is hardly ever detectable outside the central nervous system (CNS), it can be found in vCJD in a variety of peripheral tissues in addition to the brain, spinal cord and eye (retina and posterior segment). Here, PrP^{Sc} has been detected consistently in tonsils [25, 46] as well as in other components of the lymphatic system (spleen, lymph nodes, thymus and appendix-associated lymphoid tissue) [25, 46], the optic nerve [46] and in some parts of the peripheral nervous system [32]. Adrenal glands and rectum were also positively tested for PrP^{Sc} [46]. Most interestingly, pathological prion protein was also found in two preserved specimens of the appendices from 2 vCJD patients who underwent appendectomy 8 and 24 months prior to the outbreak of the disease [27, 28].

The involvement of the lymphoreticular tissue in vCJD patients, together with experimental results pointing to a crucial – and meanwhile more comprehensively elucidated [24, 36] – role of B cells in the neuroinvasion of TSE agents [33], and compelling evidence for the presence of infectivity in buffy coat or plasma of GSS-infected mice [8, 10], have raised fears that blood of vCJD patients could be infectious. However, so far, bioassay experiments have not been able to demonstrate infectious agent in the plasma or buffy coat of vCJD patients [11], nor was it possible to detect PrP^{Sc} in concentrated buffy coat by sensitive Western blotting [46].

In contrast to classical CJD, vCJD is characterized by a consistent and extensive involvement of peripheral tissues, predominantly in the lymphatic system, and, to a lesser degree, in the peripheral nervous system. It is therefore possible that medical treatments or invasive procedures performed in the context of vCJD are associated with a higher risk for iatrogenic or accidental transmission than in the case of classical CJD.

Efficiency of Transmission via Various Routes of Exposure

The probability of contracting a TSE infection as well as its incubation period depend on both the transferred quantity of TSE agent and the route of transmission [31, 43, 48]. Consequently, for a thorough risk assessment, it is crucial to review the potential sources of infectivity in close connection with the various routes of exposure along which the agent can potentially invade the body.

Exposure of intact skin or mucous membranes (except for the eye) to TSE agents can be considered as a 'negligible' risk, but should nevertheless be avoided. Transcutaneous exposure through non-intact skin or mucous membranes, contamination of the eye and the import of agents by means of needles,

scalpels or other surgical instruments represent a higher – though not exactly quantifiable – risk. The most efficient route of infection is intracerebral inoculation, closely followed by intravenous and intramuscular application. Provided that in the case of intra-species transmission 1 infectious dose of agent is necessary in order to intracerebrally infect a recipient with the probability of 50% (1 $ID_{50i.c.}$), then approximately a tenfold amount of infectivity (i.e. 10 $ID_{50i.c.}$) is needed for achieving the same effect intravenously; for intraperitoneal, subcutaneous and peroral infection 100, 10^4 and 10^5 $ID_{50i.c.}$, respectively, would be required [19, 20]. Thus, the oral route of infection is about 100,000 times less efficient than a direct application of infectious agent into the brain.

Retrospective Analysis of Iatrogenic CJD Cases

When reviewing the iatrogenic cases of CJD observed so far [9], there is a striking consistency concerning the type of treatments and interventions performed on patients who later became ill: they all underwent medical procedures which are potentially associated with a hazard for transmission as defined by the risk assessment criteria outlined above.

The following medical history is common to all iatrogenic CJD cases reported to date: either transplants (dura mater, cornea), medication with preparations derived from CNS-associated tissues of deceased human donors (such as growth hormone), or the application of neurosurgical instruments or invasive EEG electrodes previously used on the brains of CJD carriers. While it is impossible to specify the CJD risk status of the tissue donors, it can be assumed that those patients from whom the disease was transmitted via EEG needles or neurosurgical instruments had a recognizable risk for CJD, i.e. conspicous clinical symptoms.

In any case, the scenario of contamination through contact with tissues of potentially high or highest infectivity was common to all observed iatrogenic transmissions. Since covert contaminations of transplants, medications or instruments were not removed or inactivated by appropriate procedures, infectivity was inadvertently carried over to other patients. This occurred via highly efficient routes of transmission, i.e. through direct inoculation of the brain (neurosurgical instruments or invasive EEG electrodes), transplantation of contaminated tissues adjacent to the CNS (dura mater, cornea) or intramuscular injection of preparations such as growth hormone.

As prophylactic measures specifically directed against the identified sources and routes of transmission underlying iatrogenic CJD, it was recommended that pituitary hormones of human origin should be replaced by other therapeutics or alternative treatments if available (since 1987, growth hormones and gonadotrophins are being manufactured in vitro) [47] and that individuals with an elevated risk for CJD should be excluded as tissue or organ donors [14, 43]. This is expected to efficiently prevent new iatrogenic CJD infections.

Risk Management – Current Strategies to Minimize the Risk of Transmission

The knowledge about iatrogenic CJD and the emergence of vCJD have led to the formulation of various national and international recommendations and guidelines aiming at the prevention of nosocomial transmission of human TSEs [1, 6, 17, 18, 31, 43, 48]. Obviously, these safety measures need to be flexible, allowing constant updating and adaptation to new insights, scientific findings or changing circumstances and we advise to consult additionally to other resources the Internet for most recent information.

Principles for the Prevention of Transmission

All prophylactic measures in nosocomial environments pursue a central purpose: to prevent exposure of patients and personnel to possible sources of risk (such as contaminated tissues, medications, surgical instruments or medical devices). In this endeavor, the efficient decontamination (i.e. removal and/or inactivation) of TSE agents by cleaning, chemical disinfection or, if applicable, sterilization of potentially contaminated materials which may act as a source of infection or vehicle for transmission is of utmost importance.

In 1996 and 1998, recommendations for the treatment of and intervention in patients with a recognizable risk for CJD were summarized in Germany in reports about 'Desinfektion und Sterilisation von chirurgischen Instrumenten bei Verdacht auf Creutzfeldt-Jakob-Erkrankungen' (Disinfection and Sterilization of Surgical Instruments in the Case of Suspected Creutzfeldt-Jakob Disease) [18] and 'Krankenversorgung und Instrumentensterilisation bei CJK-Patienten und CJK-Verdachtsfällen' (Nursing and Sterilization of Instruments used on CJD patients and in Suspected CJD) [43].

These national recommendations, as one example, specify a comprehensive list of safety procedures for the prevention of nosocomial transmissions of classical CJD. They include guidelines for the avoidance of occupational exposure in the context of interventions in patients and during the handling of samples, measures for post-exposure prophylaxis, and differentiated instructions for the prevention of iatrogenic infections. Additionally, they detail preventive measures in special areas such as ophthalmology, neurology, transplantology or pathology, as well as methods for the disposal of waste in hospitals and the handling of corpses until burial. Special attention is given to the cleaning, disinfection and sterilization of contaminated materials. Comparable guidelines have been issued in several other countries and by international health organizations.

Hospital Care

Based on all data currently available, hospital care of CJD and vCJD patients is not associated with a risk of transmission, provided that standard

hygiene precautions are observed [31, 43]. This is also true for the handling of excreta of these patients.

Transmission via Surgical Instruments or Medical Products

In the context of invasive medical procedures the prophylactic challenge is far more complex, requiring a stepwise approach to maximally reduce the risk of nosocomial CJD or vCJD transmission. First, a distinction must be made as to whether (a) the planned intervention is to be performed on a patient with a recognizable risk of CJD or vCJD, or (b) such a risk cannot be identified.

Patients with a Recognizable Risk of Classical CJD (Including GSS and FFI)

Concerning invasive procedures on patients with an evident risk of CJD, readers are referred to the aforementioned recommendations summarized in 'Desinfektion und Sterilisation von chirurgischen Instrumenten bei Verdacht auf Creutzfeldt-Jakob-Erkrankungen' (Disinfection and Sterilization of Surgical Instruments in the Case of Suspected Creutzfeldt-Jakob Disease) [18] and 'Krankenversorgung und Instrumentensterilisation bei CJK-Patienten und CJK-Verdachtsfällen' (Nursing and Sterilization of Instruments used on CJD patients and in Suspected CJD) [43]. References on recommendations and guidelines in other countries and those issued by international organizations (such as the WHO) are summarized elsewhere [31].

Conventional methods of disinfection and sterilization do not sufficiently inactivate TSE agents [30, 38, 45], which, in addition, exhibit a high affinity for steel [22]. Therefore, in accordance with current guidelines, invasive procedures on patients with a recognizable risk of CJD should, if possible, be avoided. However, if an operation or invasive procedure on those patients is indispensable, the use of disposable[1] materials and instruments is recommended. In case that this is impossible, all instruments and medical devices used during interventions on the brain and eyes of patients with a recognizable risk of CJD, and which may have been in contact with the potentially infectious tissues, must be submitted to special decontamination procedures or, if this is not feasible, discarded. In principle, the same holds true for the handling of instruments and medical devices following operations on risk patients performed in tissues other than the CNS or eye. However, because the potential tissue infectivity is lower in this case, the decontamination may be carried out

[1]Medical products which should be used only once are: scalpel blades; (biopsy) needles or cannulas; CSF aspiration cannulas; equipment for spinal anesthesia and nerve blocks; knives and lancets (except diamond-made) for ophthalmic use; thermolabile endotracheal tubes; bone drills and screws that may have contact with the spinal cord or CSF; implantables.

under somewhat less stringent conditions. Treatment with 1–2 *M* sodium hydroxide solution (for 24 h), 2.5–5% sodium hypochlorite solution (for 24 h) as well as 3, 4, or 6 *M* guanidiniumthiocyanate solution (for 24 h, 1 h or 15 min, respectively) followed by steam sterilization at 134°C (for 1 h) [31, 43, 48] are considered appropriate for the decontamination.

Patients with a Recognizable Risk of vCJD

The broader distribution of infectious agent in the body of vCJD patients than in patients with classical CJD called for a reconsideration of the practices of cleaning and disinfection of instruments and medical devices. An overview of British safety guidelines and recommendations on sterilization issues in vCJD is given in a report on behalf of the vCJD Consensus Group by Spencer and Ridgway [44]. In Germany, a task force initiated by the Robert Koch Institute together with the Scientific Advisory Board of the Bundesärztekammer (Federal Chamber of Physicians) has recently developed recommendations for minimizing the risk of vCJD transmission [1].

In these recommendations the aforementioned and still valid guidelines concerning instruments and medical devices used in patients with an evident risk of classical CJD have been tightened for patients with a recognizable risk of vCJD, in that they require the disposal of all instruments and devices, irrespective of the tissue in which they were applied. In case that this may cause technical or economical problems, such as in the use of flexible endoscopes, pools of instruments should be established – and have already been done so in Germany (Institute of Neuropathology, University Hospital Göttingen, Creutzfeldt-Jakob lending devices program of the University of Göttingen in collaboration with Fujinon: Endo-Praxis 2001;2:38) – which are available for particular interventions on vCJD patients.

Patients without a Recognizable Risk of CJD or vCJD

For patients without an evident risk of classical CJD, the likelihood of harboring or developing the disease, and therefore the hazard of transmitting the causative agent, is extremely low (world-wide incidence of sporadic CJD: ~1–1.5 cases per 1,000,000 inhabitants and year). Concerning the prevention of iatrogenic or accidental transmission of classical CJD, it was therefore deemed unnecessary to submit instruments or medical devices formerly used in such patients after careful conventional decontamination to additional cleaning or inactivation procedures specifically aiming at the elimination of TSE agents.

However, in the light of the uncertain extent of alimentary exposure of the population to the BSE agent, the risk assessment and prevention of possible vCJD transmissions from patients with unrecognized carrier status must be considered from additional points of view. For various reasons (e.g. insufficient

epidemiological data) the hazardous potential of vCJD cannot yet be as clearly assessed as that of classical CJD. In particular, it is currently impossible, and will most probably remain so in the near future, to draw any accurate conclusions on the number of asymptomatic carriers or unrecognized cases of vCJD. On the other hand, PrP^{Sc} – and thus vCJD infectivity – is already present in lymphatic tissues such as the appendix and possibly the tonsils during preclinical phases [27, 28].

In 2001, the United Kingdom Department of Health published a risk assessment for the transmission of vCJD by surgical instruments [17]. This assessment is founded on the experiment-based premise that optimal decontamination procedures may lead to a reduction of infectivity titers by 5 orders of magnitude (i.e. by 5 'logs'). Accordingly, it must be assumed that certain kinds of medical interventions are associated with an elevated risk for iatrogenic and accidental transmission of vCJD. In particular, these include interventions performed on tissues which bear an inherent risk of contamination of surgical instruments or medical devices with an initial load of 10^5 ID_{50} or more infectivity, i.e. operations on the CNS, the posterior segment of the eye and the organized lymphatic tissue. According to the hazard analysis, the peak of potential iatrogenic transmissions associated with interventions on lymphatic tissue, first and foremost on tonsils, is to be expected in the UK for 2005. However, owing to the pathogenesis of vCJD, the maximum risk for transmission resulting from brain or ophthalmologic operations should occur about 10 years later, i.e. around 2015. The total number of cases that probably will have to be attributed to iatrogenic transmission is estimated at approximately 5–10% of the vCJD cases acquired by the alimentary route.

Bearing in mind the variety and the frequency of surgical interventions on organs and tissues potentially containing infectious agent, the German vCJD task force aimed to recommend a generally applicable decontamination procedure that takes into account the theoretical risk of vCJD transmission from unrecognized carriers on the one hand, without considerably compromising the conventional processes for cleaning, disinfection and sterilization of surgical instruments or medical devices, on the other. In addition, the suggested procedure should take into account the potential cross-contamination of instruments during the cleaning process and also be suitable for thermolabile medical devices. Thus, the maintenance of surgical instruments and other medical equipment should comply with the recommendations of the Kommission für Krankenhaushygiene und Infektionsprävention (Committee for Hospital Hygiene and Infection Prevention) and the Bundesinstitut für Arzneimittel und Medizinprodukte (Federal Institute for Drugs and Medical Devices) of November 2001 [21], and combine two or more methods suitable for an at least partial decontamination and/or inactivation of TSE agents.

In particular, such methods include cleaning under alkaline conditions ($pH > 10$)[2] and subsequent steam sterilization at 134°C. For details, please refer to the comprehensive report of the vCJD task force [1] and the technical appendices of this article.

Transmission through Blood and Blood Products

Currently, blood and blood products are considered as safe in the context of accidental or iatrogenic transmission of classical CJD [48]. However, the concern that vCJD might be transferred by this route has led to various precautionary recommendations and measures. In Germany, these include the removal or depletion of leukocytes from blood donations and the exclusion of blood or plasma donors who have spent more than a total of 6 months in the UK between 1980 and 1996. Details can be found in a recent report on the strategy of blood supply after the emergence of vCJD ('Bericht der Arbeitsgruppe Gesamtstrategie Blutversorgung angesichts vCJK' [6]), prepared by a commission consisting of representatives of the Paul Ehrlich Institute, of the Robert Koch Institute and of additional external experts and published by the Bundesministerium für Gesundheit (Federal Ministry of Health). This report also outlines new approaches aiming at further minimizing the risk of vCJD transmission, such as more stringent criteria for the 'optimal use' of blood and blood products.

Summary and Outlook

Despite the occurrence of classical CJD, which has consistently been observed during the past decades, only relatively few cases of iatrogenic transmission and no unequivocal case of occupational CJD infection have been reported to date. Over time, the sources of infection and the transmission routes of iatrogenic CJD have been identified. On this basis, it was possible to establish various measures of infection control in the context of classical CJD which are expected to efficiently prevent accidental or further iatrogenic transmissions of this disease.

At present, the risk of human-to-human transmission of vCJD is much less predictable. The development and implementation of preventive measures against the currently 'only' theoretical but potentially very dangerous risks of

[2]NaOH or KOH mixed with tensides are expected to be effective within 10 min when used at temperatures of ~50–60°C. However, the destabilizing effect on PrP^{Sc} should be confirmed by appropriate tests. The corrosive potential of NaOH or KOH to instruments and medical devices may be decreased by specific additives. In ophthalmology, potential risks associated with alkaline cleaning need to be excluded. In any case, changes in cleaning procedures must not affect the results of operative and other medical interventions [1].

vCJD transmission has been an urgent as well as demanding task for health policy-makers. When addressing this task, decision-makers have to weigh any expected benefits of preventive or precautionary measures against possible or predictable negative effects in other domains of the public health system. Such weighing is often difficult given the still existing gaps in the knowledge on vCJD. This emphasizes the importance of further research into the complex subject of TSEs, although vivid scientific activities have already provided a much better understanding of these diseases during the past few years.

The theoretical risk of vCJD transmission through blood or blood products and through surgical instruments or medical devices has led the health authorities to a series of prophylactic steps, which should provide, according to the best of current knowledge, effective protection against an inadvertent and unpredictable spread of vCJD. However, it should not be overlooked that there is no practical diagnostic test available to date which would allow the preclinical diagnosis of TSEs. In the absence of such a test, the tools for risk assessment and risk management in the context of classical CJD and vCJD disease remain inadequate. On the other hand, a preclinical test for human TSEs will inevitably constitute new challenges – last but not least in the area of medical ethics. Thus, as apparently always, the problem of TSEs remains complex.

Appendix 1

Destabilization and Inactivation of TSE Agents: Theoretical Approaches and Assay Systems (also see chapter by Taylor p 136–145 in this volume.)

TSE agents are unique pathogens which are essentially composed of an abnormal, misfolded form of the prion protein (PrP^{Sc}). Given that the misfolded state of PrP^{Sc} is required to maintain the infectivity, virtually all strategies to inactivate TSE agents aim at changes in the conformation, folding or aggregation of the pathological prion protein PrP^{Sc}.

The success of an attempted inactivation can be measured by comparing the infectivity of a treated sample with that of an untreated control by infecting mice or hamsters with serial dilutions of the material. However, besides ethical considerations, animal experiments are time-consuming and costly. Thus, it would be highly desirable to be able to examine a larger number of potentially active chemicals, drugs or other compounds for their capacity to inactivate TSE agents in an in vitro assay. This would allow specific selection of the most promising candidates for a subsequent evaluation of their inactivating potential in animals in a pre-screening procedure.

Due to the misfolded state of PrP^{Sc}, a 'core' region ranging from amino acids 90–230 of the human PrP^{Sc} is highly resistant to digestion with proteinase K. The truncated proteinase-resistant core (PrP27–30) is still associated with infectivity. All known and practically established methods for the inactivation of TSE agents, if they don't directly degrade the protein, render PrP^{Sc} and/or PrP27–30 sensitive to proteinase K, i.e. they denature the protein in such a way that it becomes accessible to complete enzymatic degradation by proteinase K. In addition, as observed in most studies on scrapie, BSE or CJD, TSEs are consistently associated with the appearance of

proteinase K-resistant PrP^{Sc} in vivo. For these reasons, it can be concluded that any reduction of the amount of proteinase K-resistant PrP^{Sc} achieved by adequate procedures correlates with a decrease of infectivity. Therefore, testing for proteinase K-resistant PrP^{Sc} appears as a suitable in vitro screening test for the potency of inactivating substances. However, as a final step in the evaluation of substances which inactivate TSE agents, any reduction of infectivity suggested by the loss of proteinase K-resistant PrP^{Sc} needs to be confirmed in animal experiments.

Probing the unfolding or denaturation of PrP^{Sc} by inactivating substances by a proteinase K assay as outlined above is easily accomplished using the Western blot technique. In fact, many research groups routinely check tissue samples for the presence of PrP^{Sc} by Western blotting and employ proteinase K to discriminate between PrP^{Sc} and its normal, proteinase K-sensitive, cellular isoform PrP^{c}. Furthermore, the proteinase K assay does not require highly purified PrP^{Sc} preparations but can be performed with crude brain homogenates obtained from scrapie- or BSE-infected mice, hamsters, sheep, or cattle. Cell cultures infected with TSE agents may serve as an additional or alternative source for PrP^{Sc}.

In principle, the unfolding or denaturation of PrP^{Sc} by inactivating substances could also be monitored by addressing other unique biochemical and biophysical properties of PrP^{Sc}. For example, the binding of conformation-dependent antibodies or Congo red to PrP^{Sc} should allow differentiation between different folding states of the prion protein. Also, spectroscopic methods may be applied to analyze the unfolding and denaturation of PrP^{Sc} in inactivation procedures. However, for any of these methods the relation between their respective readout and the reduction of TSE infectivity in vivo is less well established than for the proteinase K assay. In addition, these alternative approaches would usually require highly purified PrP^{Sc} which is tedious and potentially hazardous to isolate.

In summary, the proposed proteinase K-assay for the testing of the inactivation of TSE agents is a robust, cheap, well-established and rapid technique to ascertain the unfolding or denaturation of PrP^{Sc}. This and the predictive value of diminished or absent proteinase K resistance for the reduction of TSE infectivity predisposes it as a method of choice for the screening for substances capable of inactivating TSE agents.

On the basis of theoretical considerations and published data, the following chemical or physical principles may exert a destabilizing or inactivating effect on TSE agents: (a) pH (alkaline additives like NaOH, KOH, Na_2CO_3); (b) tensides, especially newer zwitterionic substances developed for the enhanced analysis of proteomes, or other cleaning components like phosphates; (c) chaotropic agents like guanidinium salts or urea; (d) reducing agents like thiols for the cleavage of disulfide bonds; (e) oxidizing agents like ozone, hydrogen peroxide, peracetic acid and sodium hypochlorite; (f) metal ions, especially cupric ions, to be either added or eliminated by chelating agents like EDTA; (g) heat-resistant, inactivating enzymes; (h) steam sterilization.

Although some of these principles have already been shown to be insufficient for the complete inactivation of TSE agents, beneficial effects of combining two or more of these principles remain to be investigated.

Appendix 2

General Principles Underlying Current Guidelines for the Reprocessing of Surgical Instruments and Medical Devices in Various European Countries

Based on the risk assessment described above, several European countries have developed risk management guidelines to minimize the hazard of nosocomial transmission of

CJD/vCJD through surgical instruments and medical devices. Due to the specific situation (e.g. exposure to potentially BSE-contaminated meat and meat products) and pre-existing safety standard regulations in various countries, these guidelines differ to some degree. However, in general they share the following safety principles:

(1) Follow the guidelines on the control of infection by TSE agents in nosocomial settings jointly published by the Advisory Committee on Dangerous Pathogens (ACDP) and the Spongiform Encephalopathy Advisory Committee (SEAC): 'TSE agents: Safe working and the prevention of Infection (1998)'. TSE agents are unusually resistant to conventional chemical and physical decontamination. Therefore, among other health authorities, the British Department of Health has issued special guidance on the importance of cleaning to minimize the risk of cross-contamination with TSE agents via non-disposable surgical instruments and medical devices ('HSC 1999/178 Variant Creutzfeld-Jacob Disease (vCJD) – Minimising risk of transmission', to be read in conjunction with 'HSC 1999/179' and 'HSC 1999/123').

(2) Consult specialists for infection control if you consider it possible that a patient on whom an operation or other invasive medical intervention shall be performed has, or is 'at risk' of having, CJD or vCJD.

(3) Use disposable surgical instruments and medical devices when practicable. However, this must not affect the results of the medical intervention or treatment. Discard surgical instruments and medical devices which are impossible to clean.

(4) Quarantine all non-disposable equipment applied on patients who have, or are 'at risk' of having, CJD or vCJD until a clear diagnosis has been established. In the case of classical CJD those instruments and devices must be submitted to special decontamination procedures, and if this is not feasible, discarded; in the case of vCJD, all quarantined equipment has to be discarded.

(5) Avoid any fixation of blood, mucous or tissues at the surface of non-disposable surgical instruments or medical devices by drying, exposure to fixating disinfectants (e.g. aldehydes, alcohol) or heat before thorough cleaning. Fixation can be avoided by immediate incubation in a cleaning solution (containing detergents and possibly additional non-fixating disinfectants) or by rapid transfer to reprocessing.

(6) Reprocess non-disposable instruments and devices in a (central) facility suitable for the cleaning, disinfection and sterilization of surgical instruments and medical devices which are (potentially) contaminated with TSE agents.

It has to be emphasized that the following guidelines refer to the cleaning, disinfection and sterilization of medical equipment used on patients without a recognizable risk for CJD or vCJD and aim to minimize the 'theoretical' risk of transmission from asymptomatic carriers. As outlined above, in the case of invasive interventions on patients with an evident risk for these diseases (including patients with an established diagnosis of human TSE) additional safety measures have to be observed:

(1) Clean all instruments thoroughly, preferably using a validated or at least standardized mechanical method before sterilization. Effectiveness may be enhanced by ultrasonification. According to the recommendations of the German vCJD Task Force [1], any maintenance of surgical instruments and medical devices (even in the absence of a recognizable risk) should comply with the 'Anforderungen an die Hygiene bei der Aufbereitung von Medizinprodukten' (Requirements to Hygiene in the Maintenance of Medical Devices) [21] and combine two or more methods suitable for an at least partial decontamination/inactivation of TSE agents. This could be achieved for example by mechanical cleaning/disinfection (validated or at least standardized) using a washer/disinfector and an alkaline medium (with a $\mathrm{pH} > 10$) at an elevated but protein non-fixating temperature (usually about 55°C, possibly up to 93°C in case that

strong alkaline substances are used), followed by thorough neutralization and rinsing and thermal disinfection (see below). The stringency of the recommendation to use alkaline cleaners varies for different European countries, since other approaches (e.g. increase of cleaning efficacy by enzymes) may result in an optimized cleaning process as well.

(2) Submit surgical instruments and medical devices to thermal disinfection. Critical (according to the Spaulding classification) thermostable medical devices shall generally be sterilized by steam sterilization at 134°C (a) for a period of at least 5 minutes, or (b) 18 min in case that alkaline cleaning is not feasible. The guidelines on the control of infection by TSE agents in nosocomial settings published by the ACDP and SEAC include a sterilization cycle of 18 min hold time at 134–137°C in a properly functioning porous load steam sterilizer. This step is generally suggested for the reprocessing of thermostable devices in France (Circulaire DGS/5C/E 2n° 2001-138; 14.3.2001) and Switzerland. Consequently, many benchtop steam sterilizers currently being placed on the market now feature a so-called ‘prion cycle’ (i.e., a cycle of 18 min hold time at 134–137°C). Ambitious claims have been made concerning the effectiveness of this cycle for the inactivation of TSE agents. However, it has to be noted that a sterilization cycle of 18 min hold time at 134–137°C alone cannot guarantee complete inactivation of the infectivity – last but not least, because thermostable variants of TSE agents are known to exist.

(3) Specific considerations. In cases in which proper steam sterilization is not possible, cleaning and disinfection provide the only risk-reducing steps: Semi-critical (according to Spaulding classification) devices, which have been used without contact to the CNS, ocular fundus or organized lymphatic tissue may be thoroughly cleaned by alkaline, enzymatic or other appropriate solutions with a high cleaning efficacy and subsequently disinfected. As exemplified in the decontamination of flexible gastrointestinal endoscopes, even a ‘fixating’ disinfectant such as glutaraldehyde may be used for this purpose because of its reliable activity against a variety of more widely distributed pathogens (like HBV, HCV or mycobacteria). In some countries, the use of peracetic acid as a disinfectant is favored. Critical (according to the Spaulding classification) thermolabile medical devices, which have been used without contact to the CNS, ocular fundus or organized lymphatic tissue may be thoroughly cleaned by alkaline cleaners and subsequently disinfected by a non-fixating procedure. The efficacy of plasma sterilization to at least partially inactivate TSE agents needs to be tested.

Thermostable medical devices to be used in contact with the CNS, ocular fundus or organized lymphatic tissue (e.g. in ENT surgery) which cannot be reprocessed by mechanical cleaning under alkaline conditions in a manner which provides reliable decontamination and thorough rinsing may be reprocessed by other suitable standardized and well-established (e.g. enzymatic) cleaning procedures, provided that the final step is a steam sterilization at 134°C for a period of at least 18 min. This is particularly relevant for devices to be applied in eye surgery, since here residues of alkali pose a considerable hazard. In case that for those or other medical devices the outlined proceeding is not applicable, a special procedure for reprocessing should be developed and validated.

Critical (according to the Spaulding classification) thermolabile medical devices, which generally come into contact with the CNS, ocular fundus or organized lymphatic tissue and which are difficult to clean, represent a considerable problem in the context of infection control. Manufacturers of such devices should take care of this problem, for example by improving the design of those devices with respect to better decontamination. In any case, the use of critical thermolabile medical devices requires a careful risk evaluation and well-balanced decision whether recycling is possible.

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Resistance of Transmissible Spongiform Encephalopathy Agents to Decontamination

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The transmissible spongiform encephalopathies (TSEs) include scrapie in sheep, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob disease (CJD) of humans. In TSEs, a normal host-protein (PrP^C) is converted to a disease-specific form (PrP^{Sc}) as a consequence of infection. PrP^{Sc} resists proteolytic digestion and forms pathological deposits, particularly within the central nervous system (CNS) where this is usually accompanied by spongiform encephalopathy. One theory is that TSE agents are simply PrP^{Sc} (perhaps accompanied by other host-specific proteins) which acts as a template for the conversion of PrP^C to PrP^{Sc} [1, 2]. Although there is no general disagreement with the idea that PrP^{Sc} is at least a component of such agents, there is an opinion [3–6] that the protein-only ('prion') hypothesis cannot explain (a) the variety of phenotypic characteristics of different strains of scrapie agent in mice of the same *PrP* genotype, or (b) the phenotypic stability of the BSE agent in mice, regardless of whether transmission is directly from cattle to mice, or via intermediate species such as kudu, nyala, domestic cats, pigs, sheep, goats, and even humans [7, 8]. Although the incidence of BSE in the UK is declining, concern has been heightened by its putative link with a variant form of CJD (vCJD) first reported in 1996 [9]. The phenotypic characteristics of this agent in mice are the same as the BSE agent which is unlike any other TSE agent that has yet been characterized [8]. Although TSE agents have not been fully characterized, they are known to have a high degree of resistance to inactivation, which has resulted in accidental transmission. CJD was transmitted accidentally by using inadequately decontaminated neurosurgical equipment [10, 11]. Scrapie was transmitted accidentally to sheep and goats through the survival of this agent in formol-treated vaccines

Table 1. Degree of inactivation of TSE agents achieved by various procedures[1]

No detectable infectivity	Significant titre reduction	Little titre reduction
Sodium hypochlorite (16,500 ppm available chlorine)	1 *M* or 2 *M* sodium hydroxide	aldehydes organic solvents hydrogen peroxide
Autoclaving at 121°C after 1 *M* sodium hydroxide treatment	sodium dichloroisocyanurate (16,500 ppm available chlorine)	phenolic disinfectants chlorine dioxide iodine and iodates
Autoclaving at 121°C in 1 *M* sodium hydroxide	chaotropes (e.g. guanidine thiocyanate)	peracetic acid protcolytic enzymes microwave irradiation
Boiling in 1 *M* sodium hydroxide	95% formic acid hot 1 *M* hydrochloric acid	UV irradiation gamma irradiation
	autoclaving for 18 min at 134–138°C	autoclaving after aldehyde, alcohol or dry heat treatments
	autoclaving for 1 h at 132°C	
	autoclaving at 121°C in 5% sodium dodecyl sulphate	
	dry heat at >200°C	

[1]Based upon data presented in this publication, and in Taylor [22].

[12, 13]. BSE was transmitted accidentally through the survival of infectivity after the cooking procedures used to manufacture meat and bone meal for inclusion in animal feed [14]; the failure of most of these procedures to completely inactivate BSE and scrapie agents has been demonstrated [15, 16]. It was considered by the late 1980s that a few reliable decontamination procedures for TSE agents had been established. In the UK, the recommended methods were porous-load (PL) autoclaving at 134–138°C for 18 min [17], or exposure to sodium hypochlorite solution containing 20,000 ppm available chlorine for 1 h [18]. In the USA, gravity-displacement (GD) autoclaving at 132°C for 1 h, or exposure to 1 *M* sodium hydroxide for 1 h, was preferred [19]. These recommended procedures were adopted worldwide, and have been incorporated into formal recommendations on how to deal with TSE infectivity [e.g. 19, 20]. However, further decontamination studies on BSE and scrapie agents have cast doubt on the reliability of three of these recommended methods. These, and other data will be discussed (table 1).

Chemical Methods of Inactivation

There was little diminution in BSE infectivity after a 2-year exposure to formol saline [21]. This accords with the knowledge that other TSE agents resist inactivation by formalin and other aldehydes [22]. BSE infectivity was inactivated by exposure for 30 min to solutions of sodium hypochlorite containing 16,500 ppm available chlorine [23]. In contrast, a sodium dichloroisocyanurate solution with an equivalent concentration of available chlorine was not effective [23]. Studies with BSE-infected bovine brain and scrapie-infected rodent brain showed that treatment with 1 or 2 *M* sodium hydroxide for up to 2 h did not completely inactivate these agents, and permitted the survival of up to 4 logs of infectivity [23]. This contradicts earlier data showing that a 1-hour treatment with 1 *M* sodium hydroxide was effective [24] but the sensitivity of these assays was substantially reduced by the need to dilute the samples before injection to render them non-toxic. In a more recent study, it was not found necessary to dilute the samples if the pH was carefully neutralized before injection, and the assays were therefore more sensitive [23]. Other reports record the detection of residual scrapie infectivity after treatment with 1 *M* sodium hydroxide for either 1 h [25, 26] or 24 h [27], and the survival of CJD infectivity after exposure to 1 *M* [28] or 2 *M* sodium hydroxide [29].

Heat Treatment

The 22A strain of scrapie agent was not inactivated by microwave irradiation [30]. Dry heat at temperatures up to 180°C for 1 h did not inactivate the ME7 strain of scrapie agent, and there was some survival of infectivity after exposure at 160°C for 24 h; a 1-hour treatment at 200°C was effective [31]. However, a significant degree of survival of the 263K strain of hamster-passaged scrapie agent and the 301V strain of mouse-passaged BSE agent after a 1-h exposure at 200°C has been demonstrated [32]. Other data have shown some survival of 263K infectivity after exposure to 360°C for 1 h [33]. However, lyophilized brain homogenate was heated under anoxic conditions in this study; prior drying is known to enhance the thermostability of conventional micro-organisms and TSE agents [34, 35]. In more recent studies carried out by Brown et al [36] it was reported that traces of scrapie infectivity could be detected after 263K-infected brain-tissue had been exposed to 600°C for fifteen minutes in a muffle-furnace. Because such a process would be expected to reliably destroy all forms of organic material, it was hypothesised that an inorganic 'fossilised' skeleton of PrP^{Sc} might retain sufficient structural integrity to trigger the conversion of normal PrP into the disease-specific form. Although this

is a matter of speculation, the survival of infectivity after exposure at 600°C has been confirmed in a second round of experiments (Brown, personal communication). Undiluted macerates (350 mg) and saline homogenates of BSE-infected bovine brain were exposed to GD autoclaving at 132°C. Survival of infectivity in both types of sample after a 30-min exposure was not surprising, given that CJD and scrapie agents had been shown previously to survive after a 30-min, but not 1-hour exposure [24]. After a 1-hour exposure, the BSE-infected macerate, but not the homogenate, was still infectious [37]. Others have reported some survival of scrapie infectivity after infected brain homogenates were exposed to GD autoclaving at 132°C for 1 h [26, 38].

Previously described differences in the thermostability of mouse-passaged strains of scrapie agent [39] were confirmed in the studies of Kimberlin et al. [18]. Although strain 139A was completely inactivated by exposure to GD autoclaving at 126°C for 2 h, strain 22A was not; a 4-hour exposure of 22A is required to inactivate 22A under these conditions [40]. However, the studies of Kimberlin et al. [18] also showed that PL autoclaving at 136°C for 4 min was completely effective with both of these strains of scrapie agent, and resulted in the UK recommendation to use PL autoclaving at 134–138°C for 18 min for inactivating CJD-contaminated materials [17]. Nevertheless, it was still recommended that instruments used in surgery involving the brain, spinal cord or eyes of known or suspected cases of CJD should be discarded rather than recycled after autoclaving (also see chapter Beekes et al., p 117–135 in this volume). This advice was later extended to include other categories of patients recognized to have a higher risk of developing CJD. These are blood relatives of families with a known predisposition to TSE, and individuals who had been recipients of (a) hormones derived from the pituitary glands of human cadavers (b) dura mater graft material derived from human cadavers, or (c) human corneal grafts. The continuing advice not to recycle surgical instruments after their use in neurosurgical or ophthalmological procedures was probably based upon the history of doubt about the effectiveness of autoclaving with TSE agents, and further studies did cast doubt on the reliability of the PL autoclaving standard. BSE agent and two strains of rodent-passaged scrapie agent survived exposure to such PL cycles even when the exposure period was increased to 1 h [23]. However, the average mass of the infected brain-macerates used in this study was 340 mg [23], compared with 50 mg in the earlier study [18]. The larger samples were used because similarly sized samples of intact (unmacerated) brain tissue had been previously inactivated by the 134–138°C PL procedure [37, 41, 42]. It was also considered that the larger samples might more realistically represent the maximum mass of TSE-infected tissue that might have to be disposed of by autoclaving during human and veterinary healthcare, but no official advice has ever been issued in this respect. As will be discussed, the degree of smearing and

drying onto the glass containers that occurred with the larger (340 mg) samples is the main explanation for the survival of infectivity in these samples. Therefore, partial drying of infected tissue onto glass or metal surfaces should be a prerequisite when trying to define effective standards for inactivating TSE agents by heat.

Because of the uncertainties relating to PL autoclaving introduced by the studies of Taylor et al. [23], further experiments were carried out to assess the effectiveness of PL autoclaving cycles at 134, 136 and 138°C for times ranging between 9 and 60 min using samples of infected brain macerates weighing either 50 or 375 mg. The agents used were (a) 22A, a mouse-passaged strain of scrapie agent that is known to be more thermostable than other strains of mouse-passaged scrapie agent [18, 39], (b) 263K, a hamster-passaged strain of scrapie agent that had more recently been shown to survive PL autoclaving [23], and (c) 301V, a mouse-passaged strain BSE agent that had not been tested previously. The data from these experiments [43] show that 301V can survive exposure to 138°C for 1 h. However, 50-mg macerates of 22A-infected brain-tissue in which the infectivity levels were $\geq 10^{7.2}$ ID_{50} were inactivated by all of the 136°C processes, which accords with earlier data [18]; the same is true for the 50-mg macerates exposed for four different time periods at 134°C. Paradoxically, 1 case was observed in mice injected with material from a 50-mg sample autoclaved at 138°C for 9 min. This might have been written off as an experimental aberration, had it not been that positive cases were also observed in mice injected with material from 375-mg macerates autoclaved at 136 or 138°C (but not at 134°C). These data suggest that the thermostability of the 22A strain was actually enhanced as the temperature of autoclaving was increased, and the difference between the 134 and 138°C samples was statistically significant ($p < 0.01$). With 263K the starting titre was $10^{8.3}$ ID_{50}/g, and there was much the same degree of survival of the agent whether autoclaving was carried out at 134, 136, or 138°C which would support the above hypothesis. For 301V which had a starting titre of $10^{8.6}$ ID_{50}/g the data are even more convincing in this respect; 60% of the animals injected with material autoclaved at 134°C developed disease; the ratio for samples exposed at 138°C was 72%. This is statistically significant ($p < 0.05$). These data indicate that simply increasing PL autoclaving temperatures and holding times would not necessarily be effective in achieving a reliable decontamination standard for inactivating TSE agents.

Observations on the Thermostability of TSE Agents

When scrapie agent is completely inactivated by autoclaving, destruction of the agent proceeds in an exponential fashion [44]. If the amounts of

infectivity remaining after increasing exposure times, through to the time when complete inactivation is achieved, are plotted on a logarithmic scale, a straight line is obtained which shows that the death rate is constant. In contrast, when a heating procedure is only partially inactivating, a tailing type of inactivation curve results which shows an initial decline and then flattens and persists with time [44]. After autoclaving at 134–138°C for 18 min, it has been shown that the amount of BSE or scrapie infectivity that survives is relatively constant regardless of either the starting titre, or whether the agents are present in bovine, hamster or mouse brain [45]. An example of the tailing type of inactivation curve can be derived from data relating to the inactivation of the 263 K strain of scrapie agent by autoclaving [23]. If, as is often the practice with conventional micro-organisms, the initial steep decline in infectivity is used to predict the time that it will take to achieve complete inactivation, this results in a gross underestimate for 263K. This reinforces the viewpoint that, although such estimates may sometimes by useful, there is no substitute for establishing full inactivation curves [46]. Tailing inactivation curves are not uncommon for conventional microorganisms; these may result from the protective effect of aggregation during the inactivation process, or be due to population heterogeneity where differing straight-line inactivation curves for two or more subpopulations combine to produce a tailing curve. Where there is no population heterogeneity, the same sort of tailing curve is usually obtained when the surviving organisms are recultured and retested [47]. One explanation for the presence of heat-or chemical-resistant subpopulations of scrapie agent might be the protective effect of aggregation which could occur in homogenates of infected tissue but not in undiluted tissue. This argument has been invoked to explain why 2% peracetic acid inactivated the scrapie infectivity in intact scrapie-infected mouse brain but not in 10% homogenates of brain tissue [48].

Although 7 logs of infectivity were lost, 2 logs of 263K in 340-mg samples of macerated hamster brain survived autoclaving at 134°C for 1 h. Similarly sized samples of infected brain tissue are completely decontaminated within 18 min by autoclaving if the brain tissue is undisrupted, as opposed to macerated. The lesser efficiency of inactivating macerates may result from the fact that some smearing and drying before autoclaving occur with this type of sample [34, 35]. It seems that PrP^{Sc} in the dried portions of the brain macerates is rapidly heat-fixed but retains its biological activity, despite the ensuing exposure to high-pressure steam. Protection by fixation has been shown to occur during the inactivation of poliovirus by formalin [51], and prior fixation in ethanol [37] or formalin [42] considerably enhances the thermostability of the scrapie agent. It has also been observed that the amount of scrapie infectivity inactivated after 4 h under vacuum at 72°C (resulting in low-temperature boiling) is greater than that achieved using the same equipment over the same

timescale at atmospheric pressure when an end temperature of 120°C is achieved due to the presence of fat [16]. The relatively heat-resistant subpopulation of scrapie agent retains its thermostability when reheated, suggesting that this is an acquired but stable characteristic of the heat-resistant subpopulation that differentiates it from the main population. After one PL autoclaving cycle at 134°C for 18 min, the titre of 263K was reduced by 3.3 logs, but only by a further 1.7 logs after autoclaving for a second time [49]. Evidence for the intrinsic and fundamental difference of this subpopulation comes from the fact that, at its limiting dilution, it produces an average incubation period which is well beyond that for unheated material at its limiting dilution [50]. Collectively, the data show that procedures which produce rapid and/or extremely effective fixation of PrP^{Sc} result in enhanced resistance of TSE agents to heat inactivation.

Combining Heat with Exposure to Sodium Hydroxide

Although autoclaving, or exposure to sodium hydroxide, do not per se completely inactivate TSE agents, inactivation can be achieved by combining these procedures. Taguchi et al. [52] and Ernst and Race [26] described the successful inactivation of CJD and scrapie infectivity respectively by exposure to 1 *M* sodium hydroxide followed by GD autoclaving at 121°C for 30 or 60 min. Inactivation of 263K has also been reported after GD autoclaving at 121°C for 90 min in the presence of 1 *M* sodium hydroxide [27]. More recently, it has been observed that if 22A is autoclaved at 121°C for 30 min in the presence of 2 *M* sodium hydroxide (without a prior holding period in sodium hydroxide), inactivation can be achieved [53]. There are practical problems relating to this procedure, such as the potential exposure of operators to splashing with sodium hydroxide, and the potential deleterious effect on the autoclave. There are data that show inactivation of the high-titre 301V strain of mouse-passaged BSE agent by boiling in 1 *M* sodium hydroxide for 1 min [54].

Current Concerns

At present, there is no way of determining the potential scale of the vCJD epidemic. In contrast to the situation with sporadic CJD, PrP^{Sc} has been detectable in lymphoreticular tissue such as tonsil, spleen and lymph nodes in vCJD [55]. In sheep with scrapie, it is known that such tissues become infected long before the onset of clinical signs. The same is true for scrapie in mice, and is likely to be the case with vCJD, as evidenced by the detection of PrP^{Sc} in the appendix of a pre-clinical case of vCJD [56]. Depending upon the

number of individuals in the UK currently incubating vCJD, instruments used in general surgery (not just neurosurgery) could increasingly pose a risk with regard to iatrogenic transmission. If there is a significantly increasing risk, it may be that processing surgical instruments through hot sodium hydroxide could be an effective option, given that this is a highly inactivating process. However, this would require the identification of types of stainless steel that would not be adversely affected. A non-damaging process that is under investigation is autoclaving at 121°C in 5% sodium dodecyl sulphate. Although a 15-min exposure was not completely effective, there was a significant reduction in the infectivity titre [57], suggesting that a 30-min exposure might prove to be completely effective. There is clearly a need to establish reliable methods for decontaminating CJD-infected surgical instruments without damaging them.

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Bovine Spongiform Encephalopathy and Its Relationship to the Variant Form of Creutzfeldt-Jakob Disease

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Bovine spongiform encephalopathy (BSE or 'mad cow disease') is a relatively new disease. It was first confirmed in Great Britain in November 1986 following microscopic examination of the brains from 2 cows in southern England [1]. The lesions were consistent with a diagnosis of a spongiform encephalopathy, though at this time it was not known if the disease was transmissible. Nevertheless, the features of these 2 cases, and several more presented during the course of 1987, showed a remarkable resemblance to the lesions found in scrapie of sheep.

Scrapie of sheep, a transmissible spongiform encephalopathy (TSE) that less frequently also affects goats and moufflon, was well known in Great Britain and had existed there and in France, Germany and in countries bordering the Danube Valley since the early 18th century [2].

In 1985, only six TSEs were known, three in animals (scrapie, transmissible mink encephalopathy of farmed mink and chronic wasting disease of some species of Cervidae) and three in man (Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease and kuru). With the possible exception of scrapie, all are rare diseases. Some are geographically localized: transmissible mink encephalopathy to North America and Northern Europe [3], chronic wasting disease to North America [4] and kuru to the Fore-speaking people in the Eastern Highlands of Papua New Guinea [5].

In the summer of 1986, also in Great Britain, a scrapie-like disease was identified in an adult captive nyala (*Tragelaphus angasi*) [6]. Subsequently, a total (to September 15, 2003) of 136 cases of scrapie-like spongiform encephalopathy have been reported, mostly in the UK, in a total of eight species of captive wild Bovidae, six species of captive wild Felidae and in domestic cats. Domestic cats contribute 93 cases to the total [7] and cheetah

10, including 3 cases of Feline spongiform encephalopathy (FSE) in cheetahs exported from the UK to the Republic of Ireland, France and Australia and 2 further cases in France. The latest incident has been a single confirmed case of FSE in an Asiatic golden cat (*Felis temmicki*) in Australia. This animal was born in a German zoo in 1992 and imported into Melbourne zoo in 1998 from The Netherlands under lifetime quarantine restrictions [8]. Most, or all, of these incidents can be attributed to probable exposure to the BSE agent from cattle. To appreciate how this may have occurred, see 'Epidemiology and Transmission' sections below.

Returning to BSE, it soon became clear that, like scrapie and other TSEs, the new disease was a prion disease. There were two reasons for this. First was the discovery, at an early stage, that disease-specific fibrils morphologically resembling scrapie-associated fibrils (SAF) were present in the brains of affected cows [1]. SAF are aggregates of a disease-specific neuronal membrane protein (called prion protein or PrP) that can be isolated from detergent extracts of unfixed brain material from the brains of sheep confirmed to have scrapie and also from the brains of other species affected with TSE.

Secondly, it was shown that the major protein of BSE fibrils is the bovine homologue of PrP as judged by its size, protease resistance, immunoreactivity, lectin-binding and partial N-terminal protein sequence [9]. The diagnosis and investigation in the research context of all TSEs have been enhanced by application of refined methods for PrP detection (e.g. by immunoblotting and immunocytochemistry) that have been developed in recent years. Most notable has been the improvement in methods of extraction and concentration of any PrP present and the generation of carefully designed and improved antibodies or techniques to detect PrP at low concentrations. These enable distinction to be made between the normal cellular form of PrP (PrP^{C}) and the disease-specific form PrP^{Sc} [10, 11]. Also of importance are the 'Rapid' tests for PrP [12] that have particular value in surveillance and in slaughter cattle for increasing consumer confidence in beef.

In March 1996, 10 cases of a new variant form of CJD (vCJD) were announced in the UK and subsequently the case reports were published [13]. The independent UK Spongiform Encephalopathy Advisory Committee (SEAC) stated that in the absence of any other plausible explanation, the most likely cause was exposure to BSE before the introduction of the specified bovine offals ban in 1989. The specified offals included those tissues from cattle that were thought most likely to contain the BSE agent even before clinical signs were evident. Neither at the time, nor subsequently, has a definite link been made between BSE in cattle and vCJD by virtue of occupation or the eating habits of affected individuals [14]. Thus the precise origin of infection for humans who develop vCJD is unknown.

Measures have been applied to protect animal and human health from BSE. The former (discussed briefly in the 'Epidemiology' section below) has resulted in a decline in the UK epidemic. New exposures of humans to the BSE agent via food have ceased in the UK. Uncertainties about key epidemiological parameters, such as the extent of effective exposure, patient susceptibility and the length of the incubation period, make predictions of the size and duration of the vCJD epidemic difficult [15]. There are currently (to November 3, 2003) a total of 140 definite or probable cases of vCJD in the UK of which 4 are still alive [16]. There are also 6 cases in France and 1 each in Canada, Italy, the Republic of Ireland and the USA. None of the cases in France or Italy had a history of residence in the UK.

In summary, BSE, several other new or newly recognized spongiform encephalopathies of animals and vCJD of man share the pathological features of TSE and molecular biological features of prion diseases. Diagnostic methods for BSE and vCJD are well developed, but at present confirmation of the diagnosis can only be made post-mortem. The BSE epidemic in the UK is declining towards obscurity as enforced measures take their effect. Prediction of the outcome of the vCJD epidemic is more difficult due to absence of key epidemiological information such as the length of the incubation period. The following sections will deal with the cause, clinical signs and epidemiology of BSE. Finally, there follow sections on the transmissibility of prion diseases in general, BSE and vCJD in particular and the relationships between them.

Cause of BSE

Properties of Agents That Cause TSE

There is dispute about the nature of the agents that cause TSE; they are variously described as prions, virinos or unconventional viruses [17]. All authorities agree that the agent whatever it may be, is unconventional and that the prion protein (PrP) plays a central role in the biology of TSE [42]. The uncoventionality of the agent is related to the fact that hosts mount no recognised immune response to infection and agents are extraordinarily resistant to physical and chemical inactivation using methods that are usually lethal to conventional bacteria, viruses and fungi. The former property means there is no currently available practical premortem in vitro test to detect infected cattle with BSE or humans with vCJD. In regard to the latter property, the infectivity is not indestructible. Methods that reduce infectivity to undetectable or negligible levels have been developed and are practically effective [18, 19]. These include incineration and autoclaving in strong alkali (sodium or potassium hydroxide), (e.g. for high-risk waste), conventional autoclaving at specific temperatures for defined times (e.g.

for surgical instruments), the use of sodium hypochlorite or sodium hydroxide solutions (e.g. for infected premises and surfaces or for instruments that cannot withstand autoclaving) and rendering (e.g. for low-risk animal waste). Conditions are specified for each kind of treatment and these must be strictly adhered to (see the chapters of Taylor, p 135–146 in this volume and Beekes et al. p 117–135).

The BSE Agent

Disease results from an interaction of the environment (in this case the agent and its genome if it has one) and the genome of the patient. Simply put, and in regard to the environmental component, the cause of BSE is exposure to an unconventional scrapie-like agent or prion, known as the 'BSE agent'. The strain of agent appears very stable and has been consistently isolated from cattle with the natural disease in the UK, in Switzerland [20], and also from a nyala, a greater kudu and domestic cats, all with TSE. It appears that there is a single major strain of agent in cattle and the biological strain type is different from any of the 20 or so strains of scrapie agent derived from natural cases of scrapie in sheep or from laboratory animals following passage [21]. In this regard, it is unique and has a distinct biological 'fingerprint' that enables field or experimental isolates to be identified with certainty. This fingerprint is determined by inoculating specific in-bred strains of laboratory mice and F_1 hybrids and determining the incubation period and lesion profile. The lesion profile is determined by microscopic examination of nine grey matter and three white matter areas of brain for evidence of vacuolation and assessing the severity of this vacuolation. However, this is a lengthy and costly procedure that is not practical for everyday use, so quicker and cheaper molecular methods have been and are being developed. The molecular methods are based upon the structure of PrP^{Sc}, notably the fragment size and glycosylation ratios following immunoblotting of this protein.

In regard to the host genetic component in BSE, this has been investigated in a number of countries. For example, although polymorphisms in the bovine *PrP* gene have been reported [22–25], none has been associated with BSE occurrence. At present, therefore, it can be concluded that variation in the sequence of the *PrP* gene in cattle is not a major risk factor for BSE in cattle, rather, the disease is associated only with exposure to the infectious agent probably via the oral route. How this occurs will be discussed in detail in the section on epidemiology.

Clinical Signs of BSE

A clinical case definition of BSE in the UK has been given by Wilesmith and Wells [26]. The clinical signs have remained constant during the UK epidemic [27] and are closely similar in the different breeds and countries where

BSE has occurred [27, 28]. These observations are consistent with the isolation of the same biological strain type of agent from different herds and countries, at least in regard to the UK and Switzerland [20].

The presenting signs may be subtle changes in behaviour such as seeking solitude, a changed order of entry into the milking parlour and hind limb gait ataxia. Occasionally, the presenting sign is recumbency that increases the difficulty of making a clinical diagnosis because recumbency (the so-called 'downer cow') is a common feature of dairy practice and associated with a wide range of causes [29, 30]. Furthermore, in the UK, because over 70% of dairy cows calve between July and December, there can be false associations with a seasonal occurrence of disease. The initial clinical signs may develop when cows are dry, separated from the lactating herd and out at grass during the summer months, so are less closely and less frequently observed. They are stressed less and initial signs may not be sufficiently clear to provoke suspicion of BSE. But signs become increasingly obvious with time and especially when they calve and rejoin the lactating herd when observation is increased. This results in an increase in reporting at this time.

The stress of transportation can also reveal clear clinical signs not observed before the event. There is also evidence from studies in the research environment that when removing or reducing the stress of lactation and standardizing the husbandry and feeding in a stress-free way, clinical signs may subside. However, given time, the signs usually do progress to become obvious, provided the suspect is kept for long enough.

The clinical signs of BSE are similar to those of scrapie and fall into the same categories of altered sensation, mental status, posture and locomotion. As in scrapie, the signs are insidious in onset, are progressive and lead to a fatal outcome. The predominating signs are neurological and include apprehensive behaviour, hyperaesthesia and gait ataxia. The duration of signs ranges from 7 days to 14 months, but is typically 1–2 months. This helps to distinguish most other acute, infectious diseases, including rabies. As BSE is a notifiable disease in all countries where it occurs, the full duration of the clinical phase is foreshortened by the need to compulsorily slaughter the animal once the disease is suspected or a clinical diagnosis has been made.

Sensation

The most frequently recorded signs are apprehension, changes in temperament and behaviour, abnormal ear position, nervousness of entrances, nervous ear and eye movements, teeth grinding and frenzy.

Mental Status

The most frequently recorded signs are hyperaesthesia to touch, sound and light, excessive licking of the muzzle and flanks and head shyness. Simple tests

can be applied to determine if individual cows have increased sensitivity to sound (e.g. banging a metal tray) or to light (putting the animal in a darkened room and then switching on the light). Kicking in the milking parlour is also a common sign. This can be very violent if the lower hind limbs are touched, whether in the parlour or not. It is unwise to examine a suspect case of BSE alone and without adequate protection or restraint.

Posture and Locomotion

In regard to posture, low head carriage, laidback ears, arched back and a wide-based stance at rest are common at-rest features. The most frequently recorded signs during movement are hypermetria, gait ataxia and falling (especially on slippery concrete and when turning). Permanent recumbency usually precedes death.

Signs positively correlated with BSE include hypersensitivity to touch or sound, teeth grinding, apprehension, kicking and ataxia. Negatively correlated signs are circling and blindness [31].

General signs include loss of bodily condition and weight, and reduced milk yield. The loss of bodily condition and weight might be attributed, at least in part, to reduced rumination that is very evident in cattle with BSE [31]. Bradycardia has been reported in cattle with BSE [33, 34]. Automated, remote methods have been adapted for measuring the heart rate in cattle suspected to have BSE, and their diagnostic usefulness has been confirmed [35]. Some cattle show disturbances in cardiac rhythm. By administration of atropine, it has been shown that the bradycardia is mediated by increased vagal influence, suggesting that the cardio-inhibitory reflexes in the caudal brainstem are functionally altered in BSE [35]. Healthy cattle deprived of food also exhibit bradycardia.

Less frequently encountered signs include drinking by lapping (like at cat) rather than by sucking. Some cattle may get apparent relief from assumed pruritus following rubbing of the middle of the back but the scratch reflex seen in sheep is not a prominent feature.

Epidemiology of BSE

During 1987, initial epidemiological studies [36] of 200 BSE-affected herds and cases eliminated several possible causes of BSE. These included: direct and indirect contact with sheep (20% of affected farms had had no sheep on them in living memory), imported cattle and semen, vaccines, hormones, herbicides, pesticides (including organophosphorous compounds) and other toxic substances. The only common feature was the use, in concentrate rations

prepared for cattle, of the end products resulting from the processing of the unwanted material of slaughter and was mostly derived from abattoirs and butchers. The process is called rendering. Although new chemical methods of rendering are beginning to appear, the traditional method involves cooking (with or without added fat) to remove water, followed by pressing or centrifugation to separate the tallow (fat) from the greaves (protein). Meat and bone meal (MBM) is produced from the greaves fraction by grinding. It soon became evident that, of the end products, MBM was the most likely vehicle of infection. This was because the BSE agent was more likely to partition with the protein than the fat fraction and because the use of MBM was more parochial than that of tallow that tended to be processed in a small number of specialist plants. If tallow had been the source there would have been a more even distribution of BSE occurrence [37]. A case-control study supported this view [38]. Meantime, experimental transmission of BSE to mice from brain material from affected cattle by the autumn of 1988 had established that BSE was unquestionably a TSE [39].

Thus it was concluded that BSE is an infectious prion disease transmitted mainly, if not entirely, via concentrate feed. The route of exposure from feed is presumed to be oral but conjunctival or nasal routes cannot be ruled out entirely given the proximity of the whole head to the feed source during feeding and especially when automatic feeders are used, e.g. in milking parlours. The vehicle of infection is MBM produced by rendering of unwanted waste tissues resulting mainly from slaughter.

Rendering of Animal Waste

Some rendering processes use hyperbaric conditions, others operate at atmospheric pressure or under vacuum. Some are continuous processes, others batch processes. The question arose as to why BSE was a new disease apparently caused by a TSE agent in the MBM suddenly evident in the period 1985–1986 when MBM had been used as a feed ingredient for cattle for several decades previously. Indeed in 1950 and onwards in Great Britain, MBM was used as a feed supplement in ‘national grain balancer feed’ and ‘national high protein concentrate feed for cattle’ [40]. Although it is difficult to authenticate by documentation, it is believed that MBM was used in cattle feed at least from the 1930s. So why did BSE suddenly appear for the first time in the mid-1980s?

A hypothesis that explained this was advanced by Wilesmith et al. [37], who conducted a detailed survey of rendering plants in Great Britain in 1988. This survey revealed that two major changes had been occurring in the rendering industry during the 1970s and earlier 1980s. The first was a gradual change from batch to continuous processing that had been operating since 1972 and was still continuing. However, the study did not reveal significant differences

in the mean maximum temperatures used in the two processes. Thus this change could not itself account for the sudden change in exposure of cattle in 1981–1982, to a dose of BSE agent sufficient to cause disease by the oral route. A sudden change at this time was required to explain the timing of the origin of the epidemic for two reasons. First, the mean incubation period of BSE was calculated to be 60 months [41]. This meant that the first effective exposures (i.e. those that would have resulted in clinical disease had the recipients lived long enough), resulting in the first cattle with BSE in 1985–1986, would have been in 1981–1982.

Second, at the outset, BSE was geographically widely distributed throughout Great Britain. Because MBM was mostly used on farms close to its point of manufacture and as there were approaching 50 plants operating in 1988, there must have been a change that affected either the majority of plants or, strategically located plants that produced a higher than average output of MBM. The survey revealed that a reduction in the use of hydrocarbon solvents for the recovery of extra tallow from MBM resulted in about a 40% reduction in the MBM produced by this method during the critical period. The hypothesis was supported by analysis of the data on the geographical distribution of BSE. This showed that the incidence in Scotland was relatively low compared with that in England and Wales. This was correlated partly with the fact that the only two rendering plants in Scotland used hydrocarbon solvents for extraction of tallow. Cases of BSE in Scotland were attributed mainly to the movement of cattle from south of the border where they had been exposed.

Two other reasons for the variable geographic distribution of BSE as the epidemic approached its peak were as follows. Some rendering plants produced only tallow: the other product, fat-rich greaves, was sent to larger rendering plants where it was added to locally collected raw material for a second rendering so two heat treatments were used and a dilution factor occurred. This presumably had the effect of reducing the amount, or availability, of infected material in the original raw material. There was a negative correlation between the geographical location of such plants producing MBM from this source and the occurrence of BSE in the same region.

The second reason was the variation in the market share of different feed manufacturers that varied the inclusion and amount of MBM in feed prepared for cattle. This was shown following epidemiological analyses of BSE occurrence in the Channel Islands. There was a much higher incidence of BSE in Guernsey than in Jersey. On both islands, dairy cows were fed high levels of concentrate feed because there were no milk quotas there (as there were in the rest of the EU) and the milk from these breeds was particularly rich in fat, thus requiring a high dietary input. The variation in BSE incidence between the two islands was positively correlated with the frequency with which MBM was used

in the feed prepared by different manufacturers in Great Britain that supplied the feed for the two islands.

It is important to note that the changes in the rendering processes that were adopted by the industry were commercially motivated and not directed by, or consequent upon, government action. The changes were partly stimulated by safety considerations. A large explosion of hydrocarbon solvents similar to those used in rendering had effectively destroyed a factory and part of a village with loss of life, thus sending a clear message about the risks involved. Also, a fall in the price of tallow and rising costs of energy made the process uneconomic for most producers of tallow. Some of the hydrocarbon solvents used were carcinogenic and toxic, so this was another reason to phase them out, even though they were removed by live steam stripping (for re-use) from MBM before sale.

No plausible alternative hypotheses have been put forward but a more recently revealed change in calf feeding practice undoubtedly would have contributed. As a result of a review of the origin of BSE [42], it was revealed that during the critical period 1970–1988, feed manufacturers introduced MBM into rations for calves from the first or second week of age, curiously a practice first reported following research in animal nutrition in Australia. Interestingly however, almost twenty years earlier, Miller and Robertson [43] recommended the feeding of MBM to calves over 8–10 weeks old at an inclusion rate of about 10% of the ration.

However, experimental studies to investigate, under laboratory conditions, whether the BSE and scrapie agent can be destroyed by hydrocarbon solvents used in rendering, either alone or in combination with dry or wet heat processes, have revealed little ($<$1 log) loss of infectivity [44]. It was proposed that the heat was more likely than the solvents to have caused the small amount of inactivation that was measured. Thus it was concluded that the hydrocarbon solvent treatment of MBM had little capacity to reduce infectivity. For sound experimental reasons, a mouse-passaged BSE agent (301V) was used in the study rather than the BSE agent itself. The experimental method was very sensitive, but it could just be that the BSE agent from cattle would have behaved differently. In any case, the small reduction in titre might still have been just enough to prevent an effective dose of BSE agent being delivered to cattle, in feed, via MBM produced by this method.

Rendering experiments have been completed and have shown that two processes used in the EU were unable to completely inactivate the BSE agent [45]. These processes were banned from use for processing ruminant protein in 1994. Similar studies using the scrapie agent revealed that only one of the tested processes was effective in inactivating the scrapie agent [46]. This hyperbaric process (133°C, 3 bar for 20 min or processes providing the same degree of

assurance) is now the only one permitted for rendering mammalian waste destined for use as animal feed in the EU [47]. Later in this document, the term 'defective rendering system' is used. In this document, it is used to indicate a process that did not effectively inactivate scrapie and/or BSE agents and implies no deficit in producing final products devoid of conventional agent infectivity, and no deficit of nutritional quality or of inadequate operation of the plant. Subsequent EC legislation prohibits the use of mammalian protein (with a few exceptions) in the feed for food animal species.

Origin of the Epidemic

For BSE to suddenly develop and escalate as it did, at least three factors were required. These included an infective agent that could replicate in cattle tissue, a large population of susceptible cattle and a means of recycling the agent. The recycling component was achieved by a combination of rendering ruminant waste from infected cattle, incorporation of infected MBM into feed and then feeding it back to cattle. Those cattle that were kept long enough to accommodate the full incubation period expressed clinical disease. The source of the agent that initiated BSE in cattle is explained below. Evaluation of the susceptibility of cattle was achieved by analysing the *PrP* gene from healthy control and BSE-affected cattle, and by conducting pedigree analyses.

PrP Genetics and Influence of Breed on the Occurrence of BSE

The bovine *PrP* gene has five or six copies of a short, G-C-rich element within the protein-coding exon [22]. The frequency of occurrence of these octapeptide repeat polymorphisms in healthy cattle and cattle with BSE in Scotland revealed no differences [25]. The structure of the *PrP* gene of Belgian cattle [24] and US cattle [23] seems no different from that of British cattle. These studies collectively show that the 6:6 repeat is the most frequent, the 6:5 is moderately frequent and the 5:5 repeat is rare. The *PrP* gene of cattle seems not to exert a detectable influence on the occurrence or incubation period of BSE. Hau and Curnow [48] studied the incidence of BSE in related animals and developed a statistical model. They concluded that there was still no evidence, molecular or statistical, for genetic variation in susceptibility. The incidence of BSE within dairy breeds is constant. However, the numbers of cases within each dairy breed varies directly with the breed size [49]. BSE predominates in the Holstein-Friesian breed because it is numerically by far the most common dairy breed.

An analysis for beef breeds cannot be done because there are no available denominators for the numerical size of the breeds. Dairy cattle are predominantly affected by BSE because of the system of feeding. Calves from dairy breeds are removed from the dam within 24 h or so of birth, fed artificial milk and are subsequently fed hay and a protein-rich concentrate ration often

containing MBM. Beef calves on the other hand are suckled and are less frequently fed concentrate rations containing MBM.

Transmission studies of BSE using two different dairy breeds [50] showed 100% susceptibility and closely grouped incubation periods. Furthermore, there has been a consistency in the neuropathology of BSE in all cattle throughout the epidemic, supporting the view that only one major agent strain is responsible. Collectively, these studies show there is no variation in susceptibility of cattle as a result of breed or *PrP* genetics.

Descriptive Epidemiology

Data on suspect cases of BSE have been collected throughout the epidemic by using a questionnaire, amended from time to time in the light of experience. This has enabled a detailed analysis of the epidemic to be made and an epidemiological model to be developed. The model permits future predictions to be made following various interventions (e.g. culling strategies) and the investigation of problems like the occurrence of cases born after the introduction of the ruminant feed ban (see below).

Once BSE was made a notifiable disease in the UK in 1988, data were collected on the suspects reported, those reported and then movement-restricted, those restricted then compulsorily killed, and of these, the number confirmed by microscopic examination of the brain. The last mentioned provide data for confirmed cases of BSE that can be plotted on the epidemic curve by month and year of clinical onset. This is the true scientific representation of the epidemic. Because there is inevitably a delay from the date of onset of clinical signs of any cow actually with BSE and the date when the case is officially confirmed, the epidemic curve is always representing the true picture as it was around 6 months previous to the current date. It is important to note that some reported and restricted animals make a complete clinical recovery and are not cases of BSE. Also, some cases that are compulsorily killed are not confirmed as BSE. Around 40% of the latter have other neuropathological lesions. Around 60% of the BSE-negative cases are presumed to have a metabolic origin as no morphological lesions are seen. The percentage of suspect cases that are histologically negative has on average been around 15%, but the percentage is now increasing as the epidemic declines.

There are seasonal changes in the negative rate. The rate tends to increase in the spring, as at this time of the year there are more cases of metabolic disease after cattle are turned out to grass following winter housing. Signs attributable to metabolic disorders can mimic the signs of BSE, but can often be discriminated by biochemical analysis of blood or milk samples. Although data from these studies are put into the public domain they are often misunderstood, partly for reasons given above and partly because reports prepared for different

purposes refer to cases in the UK, Great Britain or sometimes the British Isles, including the Channel Islands.

The epidemic reached a peak in early 1993 (when the incidence was about 1% of breeding animals) and since then there has been a sustained decline as a result of the introduction of the ruminant feed ban in 1988. The epidemic was expected to decline close to extinction by 2001 [51]. But since the incubation period may be as long as the lifetime of cattle, there could be a few cases occurring for some considerable time into the future. Such cases should be due to historical exposure via feed. In fact, the number of BSE-confirmed cases identified by passive surveillance in Great Britain in 2001, 2002 and 2003 to date (August 26, 2003) is 781, 445 and 107 respectively [DEFRA, pers. commun.]. BSE is predominantly a disease of dairy cattle wherever it has occurred. This is because it was associated with the feeding of MBM to calves born into dairy herds and destined for breeding. By October 1997, 60.2% of dairy and 15.9% of beef herds had experienced at least one BSE case [52]. Most cases in beef suckler herds originated as cross-bred calves in dairy herds where they were exposed to infected feed. These female calves were unsuitable as dairy animals and therefore were sold off to beef suckler herds for breeding. Thus there were more purchased cases of BSE in beef suckler than in dairy herds. The epidemic curve for herds has a similar pattern to the epidemic curve for cases. The incidence of BSE within herds has not altered very much and has always been below 3.6% [52]. However, this is misleading because for the most part in any one year, the significant exposure is to calves in a single birth cohort rather than to the herd as a whole. Wilesmith [53] calculated that on average, if one case of BSE occurred in a birth cohort, this represented an attack rate of 7% in the cohort. It is clear however that some cattle are first exposed to an effective dose as adults. Whereas most cases of BSE occur in cattle aged 4–6 years, there is a range, the youngest case being 20 months old and the oldest 19 years.

To explain the low within-herd incidence of BSE that could not be explained by genetic variation in susceptibility, a low average exposure hypothesis has been proposed for BSE [54, 55]. This suggested that the average exposure in affected herds could have been as low as 10 oral LD_{50} of cattle-adapted BSE agent per tonne of concentrate feed. Another feature proposed was that the infectivity was not evenly distributed through concentrate feed but occurred in packets. Thus the incidence of infection within a birth cohort would depend upon the frequency or concentration of packets within the batch. This hypothesis fits well with the other epidemiological observations such as the higher risk of BSE occurrence in larger herds compared with smaller herds. This is because a farmer would have a greater chance of buying an infected packet if he purchased a large amount of feed. On average, larger batches of feed were purchased by farmers with large herds than by those with small herds.

Hypotheses for the Origin of BSE

Three main hypotheses for the origin of the epidemic have been put forward [37]. The first, that it was initiated by a mutant strain of the scrapie agent from sheep, is not supported by the epidemiological findings. Results from these studies reveal an extended common-source epidemic. Had a mutant strain of scrapie been responsible, it would have to have been geographically widespread which is not plausible. A single mutation, if it had been a source, would have given rise to a point source, propagative epidemic that clearly did not happen. However, some people consider that the first introduction of BSE may have occurred much earlier than is supposed and that the visible epidemic was the result of a second passage of the agent.

The alternative two hypotheses, namely that it was due to infection with a scrapie-like agent from sheep or a cattle-adapted scrapie-like agent, are unresolved.

Scrapie occurs in sheep and goats at an unknown incidence in a wide range of countries especially in the Northern Hemisphere. In some countries like Argentina, Australia and New Zealand, scrapie is believed to be absent. Cattle and sheep, embryos and semen have been exported to other parts of the world from the UK and European countries over a long period of time. These and some other countries had strict control of imports of live sheep and goats before the BSE era because of the scrapie risk. But, restrictions on the importation of cattle and cattle products because of a TSE risk was not considered until the discovery of BSE. No case of BSE has occurred in Argentina, any other country of South America, Australia or New Zealand, despite these potential risks. All these countries have very strict import controls that virtually eliminate the risk of importing these diseases. It is also interesting to note that cattle challenged parenterally with brain material from sheep in the USA with natural scrapie developed a neurological disease that was unlike BSE as reported from the UK [54]. Furthermore, there was very little morphological pathology in the brain though PrP was found [56]. This disease – 'experimental scrapie in cows' – has not been produced by challenge by the oral route, neither has it been reported as a natural disease of cattle anywhere in the world.

Factors Influencing the Occurrence of BSE in the UK

Sheep Source Hypothesis

Since the epidemic started and has been focused on the UK there must have been special features peculiar to that country that enabled the epidemic to be initiated. One factor was the change in rendering ruminant waste as mentioned above, but some other countries used similar methods to those used in the UK. Assuming an origin from sheep that were a reservoir of scrapie infection in Great Britain, it was clear that there was a unique set of parameters operating

there. There was a large population of sheep that outnumbered cattle by about 4:1 and this was a much higher ratio than existed in any other country in Europe or North and South America. This population provided a relatively larger proportion of sheep waste for rendering than any other country. Because most plants rendering mammalian waste accepted material from all species, there would have been a high likelihood of sheep origin MBM getting into any feed for cattle in which MBM was incorporated. The next factor was the deficit in rendering that was revealed later, and finally the common use of MBM in the diets of dairy calves destined for breeding. Assuming an ovine source, the next ready-made step was the recycling of infected cattle tissues through the defective rendering system. This exacerbated the epidemic that then took off in an exponential fashion though it was only revealed later after the incubation period of the already exposed cattle was completely expressed. However, the early epidemiological work establishing ruminant-derived MBM as the vehicle for spreading BSE resulted in the imposition of a statutory ruminant protein feed ban in the UK in 1988. This was at a relatively early stage of the epidemic, and was immediately effective in reducing exposure via feed, though not completely so.

Cattle Source Hypothesis

The alternative potential source of infection from cattle is more difficult to explain though some of the factors influencing the occurrence of BSE, like deficits in rendering processes, are common to this hypothesis too. If cattle were the source there would have to have been a geographically widespread, self-perpetuating infection in cattle that did not, or did not frequently, give rise to clinical disease.

The Case for a 'Sporadic' BSE Origin

A 'sporadic' form of BSE akin to sporadic CJD operating at the same incidence (1 case per million per annum) could not have produced sufficient infection in MBM to initiate an epidemic on the scale that was observed [52]. There have been no pathologically confirmed reports of a scrapie-like disease in cattle prior to BSE (1985) anywhere in the world, even after examination of archived adult cattle brain material in several countries, including in the UK, Switzerland [R. Fatzer, pers. commun.], New Zealand [57], Tasmania [58] and Uruguay [59]. In other words, BSE was a new disease.

The Case for a Subclinical Carrier Origin

If one assumed a carrier state of subclinical bovine TSE, this could be a possible cause, but there is no positive evidence for it. One also has to explain why a subclinical carrier state suddenly changed into a clinical disease. There is no evidence at all for a change in the *PrP* gene structure in cattle so the only other potential change could be in the agent itself. There is abundant evidence [60] that scrapie-like agents mutate and strain selection occurs on crossing a

species barrier such that a different strain from that in the challenge dose is isolated after successive passages in the new host. Thus, it could be that a previously avirulent strain of BSE agent widely distributed within the cattle population and with a self-perpetuating mode of transmission mutated and was rapidly recirculated via the defective rendering system in the early 1980s. As tissues from cattle infected with the new agent entered the rendering system, the epidemic was increasingly effectively fuelled as explained above.

Conclusion

It is not possible to precisely identify the origin of BSE whether from sheep or cattle. On balance there is more support for a scrapie origin from sheep if only because there was a reservoir of sheep scrapie in the UK long before BSE was observed. The non-establishment of BSE following challenge of US cattle with brain material from US sheep with scrapie could be explained by the fact that the strains of scrapie in the challenge inoculum were not pathogenic for cattle by the oral route. There could well be different strains of scrapie present in British sheep that theoretically could be pathogenic for cattle by the oral route. Experiments to investigate this hypothesis are in progress but results have not yet been published. It is also clear that rendering processes that did not sufficiently inactivate the agent and the inclusion of MBM in the diets of dairy calves destined to be breeding cattle were critical factors in the initiation of the epidemic. Recycling of cattle infection was also very important in causing an escalation of the epidemic. It should be possible to eliminate BSE (the disease) from cattle if the following measures are in place and enforced. Control of the: (1) composition of ruminant feed, (2) specification of parameters for rendering that effectively inactivate TSE agents, and (3) removal and destruction of tissues likely to carry the BSE agent. Whether these measures will be sufficient to eliminate BSE infection remains to be seen.

Horizontal Maternal and Paternal Transmission

Horizontal Transmission

A case-control study was conducted in 1993 following the recognition of a number of cases of BSE that were born after the introduction of the ruminant feed ban in 1988 [61]. This study revealed that neither horizontal nor maternal transmission could account for the majority of BSE cases born after the ban was introduced. The low within-herd incidence of BSE (<3.6% throughout the epidemic and declining as the national incidence of BSE declines to 1.7% in 1997 [50]) does not support horizontal transmission being a cause. Furthermore, around 30% of herds that have had a case of BSE, have had only 1 case. Also, home-bred herds that have had very high incidences within a birth cohort rarely have such high incidences subsequently. All these features enable us to conclude that horizontal transmission is not a factor in BSE.

Maternal Transmission

In regard to maternal transmission, this cannot alone sustain the epidemic in the UK because the necessary contact rate cannot exceed 1:1. That is to say not all affected cows will produce a calf that itself reaches adulthood and also produces a calf. At best, maternal transmission, if it occurred, could only slightly lengthen the epidemic and not prevent eradication of the disease from the UK. A cohort study initiated in 1989 aimed to investigate the occurrence and incidence of BSE in two groups of animals – one group the offspring of BSE-affected cows (the cases) and the other offspring of healthy cattle 6 years old or more (the controls). Each member of a pair came from the same birth cohort and the same farm. These animals were then collected together in pairs and accommodated on three neutral farms where they were all managed and fed identically. Unfortunately, the experiment was confounded by the fact that most, if not all the cattle had potentially been exposed to BSE-infected feed due to the accidental cross-contamination of the diet with MBM. Detailed analyses of the results [62–65] enabled the SEAC to conclude that there is some evidence for direct maternal transmission at a low level. However, variation in genetic susceptibility to BSE following feed-borne exposure might occur. The risk of transmission from dam to calf is likely to be less than 10%. This appears to be confined to animals born after the onset of BSE in the dam and up to 2 years beforehand, with an increasing risk the closer the calf was born to the time of onset of clinical signs in the dam [66].

The results of a comprehensive investigation of the role of bovine embryos in the transmission of BSE have recently been published [67]. Semen from 13 bulls, 8 with clinical BSE, was used to inseminate 167 cattle with clinical BSE in the UK. Embryos were collected using protocols established by the International Embryo Transfer Society and were transferred according to these protocols into 347 BSE-free cattle imported from New Zealand for the purpose. No BSE resulted in any recipient cow or in the offspring after 7 years or in mice inoculated intracerebrally with embryos as determined by clinical signs, microscopic examination of the brain, immunohistochemistry and examination for scrapie-associated fibrils. Although this does not absolutely refute the occurrence of maternal transmission of BSE, it does support the view that if it occurs at all, it is not frequent and unlikely to be via the embryo.

Paternal Transmission

There is no evidence for the transmission of BSE via the sire either by natural service or via semen. BSE has occurred in around 500 bulls. Semen and reproductive organs from some bulls with clinical BSE have been bioassayed in susceptible mice and found to contain no detectable infectivity [7]. A comprehensive review of the risks of vertical transmission of BSE is included in an Opinion of the Scientific Steering Committee [68].

Cattle with BSE Born after the 1988 Feed Ban

In Great Britain August 28, 2003 there have been 178,971 confirmed cases of BSE detected by passive surveillance (officially confirmed cases following reporting of cattle suspected clinically to have BSE). A further 1,208 cases have been detected by targeted active surveillance (officially confirmed BSE after 'Rapid' testing of brain from fallen stock, emergency slaughter animals and others). As at July 31, 2003 44,485 confirmed cases were born after the feed ban of July 18, 1988. Born-after-the-ban (BAB) cases have occurred in all countries with BSE in native-born cattle. In the UK, neither horizontal nor maternal transmission could be responsible for the majority of these cases (see above). The majority of BAB cases of BSE occurring in the approximate 6-month period between mid-July 1988 and the end of 1988 were attributed to the use of ruminant feed manufactured before the ban, but still in the supply chain or on farm. After this date, almost all of this feed would have been used and so another source must have been responsible. At this time, MBM was still legally being used in feed for monogastric species in the UK. Thus, ruminant feed prepared in mills that also manufactured feeds for monogastric species was potentially at risk from cross-contamination with MBM destined for non-ruminant feed.

A detailed epidemiological study in Great Britain revealed that there was a changing geographical distribution of home-bred cases of BSE in the country with time. Whereas the percentage of cases in the Southwest (the region with the largest percentage of cases) was declining in cattle born between 1985/1986 and 1990/1991, the percentage in the Northern and Eastern regions was increasing. These latter regions have relatively much higher pig and poultry populations than other areas, and MBM was used in the diets for these species but not of course for cattle. Following further investigation and the positive detection by an ELISA of ruminant-derived protein in rations prepared for cattle, it became clear that accidental cross-contamination or ruminant rations with MBM was responsible for the continuing, but reduced exposure of cattle to MBM following the 1998 feed ban.

This accidental cross-contamination was believed to occur because common equipment was used in mills preparing feed for ruminants and non-ruminants. The specific sites could be the auger pit and auger at the point of delivery, during the manufacture of the feed, during transportation (i.e. by the use of vehicles contaminated with small amounts of MBM) or on farm. Another way this could occur was the 'cascade' phenomenon with pelleted feed. It is most important for poultry and pigs that the quality of the pellet is sound. Sometimes, the pellet was not well made and so new feed was manufactured to replace it. The malformed pellets could have been used as ingredients for pig feed, adjusting the recipe as appropriate. Even if the poultry pellets contained MBM this would not be a problem as it was normal practice to include MBM in some feeds for pigs.

The same type of 'cascade' could have followed if pellets prepared for feeding to pigs failed the quality checks, but this time the malformed pellets would have more likely been passed into the ruminant feed and may have contaminated it with MBM. In an emergency, farmers might temporarily also have fed non-ruminant rations to cattle when the store of cattle feed had run out before a new delivery, or in inclement weather when access to the ruminant feed in store might have been impossible. The level of cross-contamination was probably small but the amount involved could have been decisive in permitting a lethal dose of BSE infectivity to be delivered. How small was determined by a quite separate experiment called the 'attack rate' study that was in progress. The objective of this was to determine the attack rate and incubation period of cattle exposed orally to four different doses of untreated brain material from cows with confirmed BSE. The amounts used were 100 g on three occasions, 100 g on one occasion, 10 g on one occasion and 1 g on one occasion. The dose of infectivity received by the cattle in each group would be in a similar proportion to the challenge amount of brain, the titre of which is being determined but likely to be in the region of 3.5 mouse i.c. (intracerebral) ID_{50}/g. The attack rate study indicated that some cattle in the 1-gram dose group have succumbed to BSE (and of course cattle in the other groups did too but with a higher attack rate and generally a shorter mean incubation period). Although the experiment used brain material from clinical cases, field cases of BSE could only be exposed to brain from subclinically affected cattle in the form of MBM, as clinically affected cattle were all incinerated after August 8, 1988. The message was very clear. A very small amount (e.g. 1 g) of fresh brain could contain sufficient infective agent to kill a calf by the oral route 4 years later [G.A.H. Wells, pers. commun.]. This put an entirely different complexion on the cross-contamination issue since relatively trivial amounts of MBM in cattle diets could not be dismissed as harmless. They could carry, if infected, lethal doses of BSE agent. It is now clear that these types of accidental cross-contamination were likely to be the single most important cause of BAB cases of BSE.

Of course, feed for pigs and poultry containing MBM should have carried no infectivity if the ban on specified bovine offals (later specified risk material) had been completely effective. As it turned out, it was not, and thus any deficit there may have been in the rendering process would have allowed infectivity through into the MBM. Once all the weak links in the chain were identified and corrected through modified legislation and improved compliance and enforcement, the feed source of BSE agent disappeared. From March 29, 1996 in the UK, it is prohibited, and becomes a criminal offence, to offer for sale or supply, or to feed to any farm animal species including horses and fish, MBM derived from mammalian species. The effective date of implementation of this order in the UK, following the completion of a feed recall scheme, was August 1, 1996.

No food animal species born after this date in the UK should be exposed to BSE or scrapie agent via MBM. The law is effectively enforced continuously by a number of checks, and the use of an ELISA and other tests to detect mammalian protein in all food animal feed. Despite the new law and strict enforcement of the ban, 55 cases of BSE, the latest a solitary case born in 1999, have been confirmed in cattle in Great Britain born after August 1, 1996 – so-called BARB or born-after-the real-ban cattle with an even smaller number in Northern Ireland. In none has a definite cause been found. Maternal and horizontal transmission appear unlikely and infected feed seems still to be the culprit, perhaps resulting from imported feed contaminated with the BSE agent. This may have been transported to the UK in ships or vehicles from continental sources at least until January 1, 2001 since when an EU wide ban on the feeding of processed animal protein to any food animal species has been imposed. Epidemiological studies are in progress to attempt to explain the occurrence and develop a hypothesis for the origin of this fortunately small subpopulation.

Variant Form of CJD and Its Consequences

On March 20, 1996, an announcement was made by the Secretary of State for Health in the UK, of 10 cases of a new variant form of CJD attributed to exposure to BSE. As a result, a ban on exports from the UK of live cattle and cattle products (other than milk) was applied by the European Commission (EC) by the demand of the Member States on March 27, 1996. The UK SEAC advised amongst other things that the feeding of mammalian MBM to all species of farm animals reared for food, including horses and fish, should cease. This ban was in place on March 29, 1996.

Negotiations with EU Member States and the EC eventually resulted in the agreement that exports of deboned meat and meat products from cattle born after August 1, 1996 could recommence from August 1999. This was called the Date Based Export Scheme. The date of August 1, 1996 was chosen because it was the date by which it was considered that cattle would be no longer exposed to BSE infectivity via feed in the UK. France subsequently challenged the decision, but the challenge was rejected by the EC on October 29, 1999 following a thorough examination of the French case and the latest data from the UK by the Scientific Steering Committee of the EC.

Geographical Distribution of BSE

Small numbers of cases of BSE have been confirmed in cattle exported in the pre-clinical phase from the UK to other countries without BSE in their native stock. These countries include (number of confirmed cases), Canada (1), Denmark (1), the Falkland Islands (1), Germany (5 + 1 case imported from Switzerland), Italy (2) and Sultanate of Oman (2). Such cases are of little

importance as long as they are identified clinically, disease is confirmed by microscopic examination of the brain and the carcase is totally destroyed so that no part can enter any food or feed chain.

To September 8, 2003, (table 1), countries with BSE in native-born cattle identified by active and passive surveillance and reported to the Office International des Epizooties, have included all Member States of the EU except Sweden; Canada, the Czech Republic, Israel, Japan, Liechstenstein, Poland, Slovakia, Slovenia and Switzerland. It is important to note that the factors of importance are the incidence of disease rather than total number of cases and whether epidemics are rising, level of falling (table 1). Cattle in all these countries appear to have been infected by feeding imported or home-generated BSE-infected MBM to young cattle, accidentally or by design. A very small number of cases of BSE (<40) have been identified in animals in countries with BSE in native-born cattle including the UK. These have all been imported from countries with BSE in their native stock but mostly from the UK.

Because the origin of BSE in all countries with the disease in their native-born stock is believed to have been MBM, a Commission Decision of June 27, 1994 prohibited the feeding of mammalian-derived protein to ruminant animals throughout the EU (although some countries had already introduced national legislation to this effect for cattle or all ruminants from 1990). Since January 2001 it is prohibited to feed processed animal protein (including MBM) to any species of food animal. Some of the earlier cases of BSE have been attributed to MBM originating in the UK. However, it is possible that MBM prepared in some countries could have arisen from native-born infected cattle tissues, especially for the more recently exposed cases. This is because the export of specific bovine offals (SBO) and their derived products, including MBM and feed containing MBM was prohibited in 1990.

In 1999 three 'Rapid' tests have been evaluated and approved by the EC for use in clinically suspect cattle [12] though they are used also for active surveillance of BSE and scrapie and to improve consumer confidence in beef. Additional tests have also been recently approved. It is now a requirement in the EU that the central nervous system (CNS) of all fallen stock and emergency slaughter animals under 24 months and of all cattle for normal slaughter over 30 months are tested using an approved 'Rapid' test for PrP^{Sc}. Carcases and offals from slaughter cattle that are positive by the 'Rapid' test are withdrawn and destroyed so they can enter no food or feed chain. The same applies to the carcase and offals from the immediately preceding carcase on the slaughter line and the two following. The use of 'Rapid' testing and active surveillance has revealed relatively large numbers of cattle with BSE in some countries including some with no clinical signs of the disease. Disease is however confirmed by use of one or the other of confirmatory tests.

Table 1. Annual incidence rate of BSE (number of native-born cases per million cattle over 24 months old) in countries that have reported cases for the years 2000–2002 inclusive and total number of cases to date. (Data courtesy OIE, updated to 12 June 2003)

	2000	2001	2002	Total number of native-born cases since 1987
Austria	0	0.96	0	1[a]
Belgium	5.53	28.22	25.75	114
Canada	0	0	0	1
Czech Republic	0	2.85	2.50	5
Denmark	1.14	6.77	3.35	12
Finland	0	2.39	0	1[a]
France	14.73	19.70	20.96	838
Germany	1.07	19.97	17.02	267
Great Britain	298.33	258.24	247.80	180.166*
Greece	0	3.3	0	1
Ireland	38.17	61.8	88.39	1,276
Israel	0	0	6.25	1
Italy	0	14.1	10.60	86[a]
Japan	0	1.44	0.97	7
Liechtenstein	–	–	–	2[a]
Luxembourg	0	0	14.54	2
The Netherlands	1.07	10.25	13.19	63
Northern Ireland	97.78	95.36	126.45	2076*
Other British Isles	0	0	–	1290*
Poland	0	0	1.28	9
Portugal	186.95	137.88	107.80	801
Slovakia	0	18.34	18.73	12
Slovenia	0	4.34	4.44	3
Spain	0.59	24.23	37.95	317
Switzerland	40.6	49.1	27.93	446

–Data not listed.
*Data courtesy DEFRA UK as at 31 July 2003 and including pre 1988 cases.
[a]Data for 2003 excluded.
Note: Data are constantly changing and should be checked with the State Veterinary Service of the country concerned before use is made of them.

The introduction of compulsory rapid testing of targeted risk populations and of all slaughter cattle over 30 months of age throughout the EU since 1 January 2001 resulted in an increase in the reporting of positive cases of BSE. Switzerland and some countries of the EU had instituted such testing earlier and some countries tested slaughter cattle over 24 months of age (rather than >30 months as required by law). These collective actions resulted in rising epidemics as previously unsuspected cases were revealed.

Concurrent with the introduction of formal active surveillance was a progressive tightening of the TSE controls on SRM, feed and animal by-product processing and use. In turn, as these controls bite, new exposures of cattle to BSE in feed have progressively declined in the EU and in many countries there is now a decline in incidence. Table 1 shows the number of native-born cases of BSE per million cattle over 24 months of age for the last three years (data courtesy OIE website updated to 12 June 2003). The incidence for the UK in 2002 is 228.24. However, for this country there is the complication of the over 30 months rule which prohibits, with a few exceptions, cattle over 30 months of age entering the food or feed chains. These cattle are not subject to complete rapid testing. It has recently been recommended to government following a review of the current risks from BSE, that cattle in the UK born after 1 August, 1996 should be permitted into the human food chain again, provided they pass an approved 'Rapid' test for PrP^{Sc} as in the rest of the EU. In Great Britain the incidence of BSE has been declining continuously since 1992 in which year the incidence was 7596.19 (Source OIE website) and in 2002 is 247.8.

Table 1 reveals that the incidence of BSE is declining also in Portugal and Switzerland. Some countries have such a low number of cases that the incidence figure is not revealing (Austria, Finland, Israel and Greece for example) that to date have reported only one case each. Although some countries show an increasing incidence there is evidence that it is levelling off prior to decline. Reference to the most recent monthly data provided for Member States in the EU confirms that no country within the EU now has serious risk from BSE provided all the measures are well enforced. In 2002 over 10 millions of rapid tests were carried out on cattle in the EU, a 20% rise in the numbers tested in 2001. Despite the increased testing the ratio of positive cases found in tested animals dropped by 22%. The determining feature for future cases is the effective enforcement of the January 2001 feed ban that prohibits processed animal by-products being fed to any food animal in the EU. Because of the long incubation period of BSE (about 5 years on average) judgement cannot be passed on how effective this has been until at least 2006.

Transmissibility of Prion Diseases

In regard to transmission of prion diseases, there are five issues to consider. The first is the natural host range and the second the experimental host range. The third issue is the tissue distribution of the agent in the terminal phase of the natural clinical disease. The fourth issue is the distribution and quantitation of infectivity within tissues from the time of exposure until death in the clinical phase. This is usually regarded as part of the study of pathogenesis that

seeks to determine also how the infection gets from the point of entry (often the gut in natural disease) to the brain. Finally, there is the issue of biological strain typing and this will be discussed in the last section of this chapter.

Natural Host Range for BSE

BSE has, or is assumed to have, transmitted from cattle to eight other species of Bovidae, all wild species held captive in zoos and wildlife parks in the UK, to domestic cats [69, 70] and to five captive wild species of Felidae. The cases in Bovidae are presumed to have contracted the disease from the same feed source as cattle. The 1988 ban on feeding ruminant protein to ruminants gave protection to these species and reported cases have declined since, though a small number of BAB cases have occurred in these species too. The cases in captive wild Felidae are presumed to have occurred following consumption of TSE-infected, uncooked carcase material from cattle that contained central nervous tissue [71], probably in the form of bovine heads (large cats) and vertebral columns (small cats). This practice was prohibited as a result of the SBO ban in 1990. The total number of cases in these species to date has been 37 [DEFRA, pers. commun.]. In regard to domestic cats, there has been a total of 90 in the UK with 1 case each in Norway [72], Liechtenstein and Switzerland to May 2003. The origin of these cases is more likely to have been from infected MBM or tissues in proprietary pet food but other sources cannot be ruled out. The epidemic curve for cats reached a peak in 1994 (16 cases) but has declined to zero since 2002.

The concurrent appearance of a case of conventional sporadic CJD in a man in Italy and a case of suspected TSE in his pet cat has been reported [73]. However, the feline case has not been confirmed to be FSE [G.A.H. Wells, pers. commun.]. The cause of all the cases in the UK is likely to be infection with the BSE agent that has been formally confirmed in three domestic British cats, the nyala (*Tragelaphus angasi*) and a greater kudu (*Tragelaphus strepsiceros*) following biological strain typing in mice [21, 74]. A scrapie-like spongiform encephalopathy in red-necked ostriches (*Struthio camelus*) in a German zoo has been reported [75, 76]. However, the real nature of this disease is in doubt, particularly as brain material inoculated into experimental rodents susceptible to scrapie did not develop spongiform encephalopathy [H. Diringer, pers. commun.].

TSE in Primates Resulting from Exposure to the BSE Agent

In France, captive primates (a single rhesus monkey (*Macaca mulatta*) [77], macaque monkeys and lemurs [78]) have been reported to have developed a TSE naturally. It is suggested that this has occurred as a result of dietary exposure to TSE infection in feed imported from the UK before controls were in place [78]. Some doubts have been expressed about this because of the atypical and independently unconfirmed clinical and pathological features of the

'natural' disease. Nevertheless, experimental transmission of BSE to some of these primates has been achieved with similar clinical signs and distribution of PrP^{Sc} in the brain. It is important to note that no natural TSE has been reported in any captive primate in the UK, despite at least some of them being fed on feed containing high inclusion rates of MBM derived from UK abattoir waste [79].

These authors were successful in experimentally transmitting BSE and scrapie to each of two marmosets by parenteral inoculation [80]. BSE has also been transmitted experimentally by parenteral routes to cynomolgus macaques [81] and to squirrel and capuchin monkeys [C.J. Gibbs, Jr., pers. commun.]. In regard to the transmission of BSE to man, see below.

Experimental Host Range of the BSE Agent

As far as is known, BSE has been successfully transmitted to all the species that have been challenged except chickens and hamsters. Successful transmissions by parenteral routes of challenge include: cattle [50], sheep and goats [82], pigs [83], mink [84] and mice [39] (for primates, see above). All of these species except pigs are also susceptible by the oral route using high-titre brain material from cattle confirmed to have BSE post-mortem.

Tissue Distribution of BSE Infectivity in Cattle with Natural and Experimental BSE

Over 50 different tissue types from cattle with confirmed BSE, sampled at necropsy of cows in the terminal phase of clinical BSE, have been bioassayed in susceptible mice [7, 85–89, D.M. Taylor, pers. commun.]. Infectivity has been confirmed in central nervous tissues namely the brain, spinal cord (cervical and terminal segments) and the retina. It was not detected in any other tissue including nerves, muscles, milk [86] and male and female reproductive tissues including embryos [88] and semen [7, 85].

Infectivity titres measured in mice have only been reported for brain tissue from cows with confirmed BSE. Despite the indistinguishable clinical signs and extent of the neuropathology in the donor cows, a range of titres has been reported from a maximum $10^{5.2}$ i.c. ID_{50} mouse infectious units per gram [90] down to about $10^{3.0}$ mouse i.c. ID_{50}/g, or perhaps even lower. Field cases of disease are now being killed earlier in the clinical phase of disease because of earlier recognition and because of animal welfare considerations. This could account for the lower titres observed in more recent cases.

Sub-passage of BSE in mice (i.e. from cow to mice to mice) has resulted in the selection of a strain called 301V. This can be grown to high titre (10^{10} mouse i.c. ID_{50}/g) in brain and 10^7 in spleen [91].

Due to the species barrier effect there is an obstacle to transmission when crossing a species barrier. This is recognized as a reduced sensitivity of the new

species to detect low titres of infectivity. To determine the quantitative difference in sensitivity between mice and cattle for measuring infectivity in cattle, a comparative bioassay was set up. This comparative bioassay uses mice and cattle to measure infectivity titres in the pooled brainstem of cows with natural, clinical BSE. The titration in mice reveals a final titre of $10^{3.3}$ mouse i.c. ID_{50}/g of pooled brainstems. The titre in the same brainstem pool when measured in cattle by the intracerebral route from which it is, concluded that the cow has about 500 times greater ability (sensitivity) to detect infectivity in cow brain than mice [92]. This is important because most bioassays of tissues for infectivity have used mice that may not detect low levels of infectivity, if present. To overcome this difficulty, certain selected tissues from cattle with experimental BSE have been inoculated into cattle by the intracerebral route (see next section).

Pathogenesis of TSE

Pathogenesis of Experimental Scrapie in Rodents

Much of what we know about the principles of pathogenesis comes from the study of experimental scrapie in mice, e.g. by Kimberlin [93]. Collectively, these studies have indicated that following infection by a peripheral route, there is a lag, zero or eclipse phase following challenge when no infectivity can be detected in any tissue. Replication follows in lymphoreticular system (LRS) tissues like the spleen, lymph nodes, and after oral infection, the organized LRS tissue of the gut. A competent immune system may be required to effect neuroinvasion [94].

Neuroinvasion is a critical phase that in mice is controlled by the *Sinc* (*PrP*) gene. The methods of investigation have involved titration of infectivity in different tissues in experimental scrapie in mice by Kimberlin and Walker [95] in the 1970s and 1980s. Subsequently, the hypothesis advanced, namely that neuroinvasion of the CNS was accomplished via the peripheral nervous system, was supported by Diringer and co-workers [96–98], who detected and measured the amount of PrP^{Sc} in the CNS in experimental hamsters during the incubation period. The results from the investigations of both groups indicate the plausibility and importance of a neural spread of infectivity. A summary is given here. Following peripheral challenge, infection is first established in the LRS, probably in fixed follicular dendritic cells [99], including in the spleen, lymph nodes and, if the infection is oral or intragastric, Peyer's patches of the gut. If challenge is by the oral route migrating intestinal dendritic cells transport PrP^{Sc} from the gut to lymphoid tissues [100]. Lymphocytes may assist fixed follicular dendritic cells to achieve functional maturity and enable replication of the agent. The role of the immune system in prion diseases has been reported [101, 102]. Infection of the CNS is established by several neural routes. In mice, one is via the autonomic splanchnic nerve that enters the spinal cord in the mid-thoracic region from whence infection transmits caudally to the distal spinal

cord and rostrally to the brain. Another route is via the vagus nerve, which in hamsters may be the only route following oral challenge. Two minor routes in hamsters are via the cervical and thoracolumbar cord. When organs are assayed for infectivity during incubation and in the clinical phase, it is clear that infectivity is largely restricted to the LRS and to the CNS, with tissues like muscle being free of detectable infectivity at all times. In the terminal phase of clinical disease the infectivity titre in the CNS exceeds that of the LRS at any time. Very early after exposure, no infectivity in any tissue may be detected. After a lag period of weeks or months (the zero phase), infectivity is detectable in the LRS, and typically detection of infectivity in the CNS is achieved when approximately half the incubation period has elapsed.

Pathogenesis of Natural Scrapie in Sheep

In regard to the pathogenesis of natural scrapie in sheep, seminal studies have been conducted in the USA by William Hadlow and co-workers in the 1970s and early 1980s. They studied goats with natural clinical scrapie [103] and Suffolk [104] and other breeds of sheep [105] with natural clinical scrapie, or incubating the disease. These studies assisted greatly in determining some of the protection measures used in the UK soon after BSE was discovered, notably the selection of tissues (SBO) that should be removed from healthy cattle for destruction. It seems from these studies in Suffolk sheep that there is no major conflict with the pathogenesis as described for rodent models. Deductions about the pathogenesis of scrapie in Suffolk sheep are consistent with those outlined above for experimental scrapie in mice and hamsters. However, in sheep, maternal and horizontal transmissions are important features of the epidemiology of the disease [106]. The placenta harbours infectivity [107–109] and PrP [108]. Since maternal transmission of experimental scrapie in the experimental animal species (mice and hamsters) does not occur, the important issues are how the placenta becomes affected in sheep and what tissue element therein harbours this infectivity. There are no published reports of infectivity titres in the placenta of sheep, so we do not know how infectious it is in comparison with brain.

This outline of the possible pathogenesis of natural scrapie in sheep is important for the control of scrapie and even more so for the control of BSE should it ever occur in small ruminants. This is because experimental transmission of BSE to sheep has been achieved [82]. In subsequent studies, infectivity was found in both brain and spleen [111]. Since in cattle with natural and experimental BSE no infectivity can be detected in the spleen (see below), it suggests that the pathogenesis of BSE in sheep were it to occur in nature, would be more likely to mimic that of scrapie in sheep than BSE in cattle. This is supported by experimental studies following challenge of sheep with the BSE agent from cattle by the oral route and determination of the distribution of PrP^{Sc} [112]. As a

result, the CNS and certain offals of sheep that contain significant LRS tissue could theoretically contain infectivity in animals incubating the disease, whereas other tissues like muscle, mammary gland and milk do not. Major risk tissues like brain and spleen can be removed in the abattoir and be destroyed so they can enter no food or feed chain. This approach has been taken in some European countries as a precautionary measure as part of a risk reduction policy for the occurrence of BSE in sheep.

Pathogenesis of Experimental BSE in Cattle following Oral Challenge

Because the within-herd incidence of BSE is very low and because BSE is not a familial disease as is natural scrapie, the pathogenesis of natural BSE could not be determined from the study of natural cases. However, it was important to identify the organs and tissues in cattle incubating BSE that carried infectivity. This was done using an experimental model of BSE in which 30, 4-month-old calves were orally challenged with 100 g of untreated brain tissue from cattle clinically affected with the natural disease and confirmed to have spongiform encephalopathy post-mortem. There were also 10 undosed control cattle. The cattle were sequentially killed at roughly 4-month intervals (3 challenged cattle and 1 control) commencing 2 months after dosing and over 40 tissues collected from each for study, the majority of which were bioassayed in susceptible mice. Tissues from each of the 3 challenged cattle at each 'kill' stage were pooled before inoculation. Clinical signs were not evident in the animals remaining in the challenged group until cattle had reached 35–37 months post-challenge. The shorter period of incubation than in the natural epidemic (mean 60 months) can be attributed to the use of uncooked brain material (not MBM) and the large amount (100 g) and presumed large dose administered. The results of the above studies have been reported [113–116]. No infectivity was detected in any tissue in cattle killed 2 months after challenge. From 6 to 18 months and from 36 to 40 months, infectivity was confirmed in the distal ileum, an area rich in LRS tissue comprised of Peyer's patches. This finding supports the decision to include intestine from duodenum to rectum inclusive in the specified bovine offals bans in various countries, despite not detecting such infectivity in the natural disease. No infectivity was otherwise found in any non-neural tissue other than in a single instance in bone marrow during the clinical phase of disease. Possible explanations for this finding, which include possible cross-contamination, have been given [116].

The detection of infectivity in the brain and spinal cord by mouse bioassay, the first occurrence of diagnostic histopathological changes in the brain and the detection of PrP^{Sc} in the brain, are essentially coincident at 32 months post-challenge and antedate the onset of clinical signs by at least 3 months.

Of considerable practical importance was the detection of infectivity in dorsal root ganglia from 32 months post-challenge when the cattle were clinically

normal. This finding has resulted in a proposal by the EC to prohibit the use of the vertebral column from cattle (sheep and goats) for the production of mechanically recovered meat throughout the EU from January 1, 2000. Several countries, including the UK, have already adopted this precaution or gone beyond it.

Because mice are less effcient at detecting BSE than are cattle (see above), some of the tissues collected from the experimental cattle have been inoculated intracerebrally into cattle in order that low levels of infectivity that might be present in tissues 'negative' in mice might be detected. This study is ongoing and results are not yet complete but has revealed that the tonsil of cattle shows low levels of infectivity during the incubation period of BSE. This has prompted a small modification of the SRM ban and specification for the preparation of the tongue for human consumption. In addition a pool of third eyelids (nictitating membrane) from confirmed clinical cases of BSE has revealed infectivity following intracerebral inoculation in cattle. This tissue has not previously been bioassayed. This latter tissue is classified as SRM in the UK and Portugal as the whole head is SRM.

Relationship of Variant CJD to BSE

On March 20, 1996, as a result of the surveillance activities of the UK CJD Surveillance Unit, the SEAC reported the occurrence of 10 cases of a new variant form of CJD that had affected young people. By November 2003, that number of definite or probable cases had risen to 143 cases in the UK (of which 6 remain alive). There are 6 cases in France and 1 case each in Canada, Italy, the Republic of Ireland and the USA. None of the cases in France or Italy had a history of residence in the UK. There is current evidence that the annual incidence of vCJD is declining in the UK and there is some cautious optimism that this may continue. Caution is necessary for at least one reason, namely that so far all genetically analysed cases have been associated with methionine: methionine homozygosity at codon 129 of the *PRNP* gene. Another is the uncertain length of the incubation period.

Not only is this variant form of CJD (vCJD) new, it is also unusual because of the early age at onset (mean 28 years). Other unusual features are a prolonged clinical phase (just over a year), clinical presentation, progression and neuropathology (see also chapter Zerr and Pose, p 98–116 in this volume).

A dozen or so animal species developed TSE for the first time between 1986 and October 1999. From 3 of these an agent has been isolated with the same biological properties as the BSE agent. Epidemiological investigation showed that it was possible, and indeed likely, that either MBM or uncooked CNS tissues derived from cattle was the source of infection for these species.

It was thus very clear that consideration had to be given to the possibility that BSE had arisen in yet another species, *Homo sapiens*, following exposure to the BSE agent. This idea was strengthened because vCJD had followed on soon after the outbreaks of BSE in cattle in the six countries where cases had occurred, the UK, France, the Republic of Ireland, USA, Canada and Italy.

Origin of Infection in Humans

The important question to answer was how did this infection arise? Could it have been due to high exposure to the BSE agent, which might be more likely in people working with high-risk tissues such as abattoir workers, knackermen and veterinary pathologists? Or could it have been because high-risk tissues from cattle infected with the BSE agent were knowingly, or unknowingly, part of the human diet? MBM was not known to be part of the human diet. The alternative source, CNS tissue from cattle infected with the BSE agent, was a possibility, particularly historically.

By 2001, analyses of epidemiological data did not provide evidence to suggest that there is an increased risk of vCJD through past surgery, previous blood transfusion, occupation or a range of dietary factors [14]. Thus it is still epidemiologically unproved that cattle are the source of the infection the patients acquired, or if they are, that it was an exposure resulting from consumption of cattle tissues.

In March 1996, the risk from SBO was considered to be very low because of the effects of the SBO ban and its subsequent and recent modifications. Thus new exposures from this source would also be expected to be very low. However, at the time the SEAC announcement was made on March 20, 1996, the Committee stated that: 'Although there is no direct evidence of a link (*between BSE and vCJD*), on current data and in the absence of any credible alternative, the most likely explanation at present is that these (*ten*) cases are linked to exposure to BSE before the introduction of the SBO ban in 1989.' As a result of the new findings, the statement and the ensuing debate, the EC, at the behest of Member States of the EU, imposed a ban on exports from the UK of cattle and cattle products with the exception of milk and semen. The Commissioner made it clear that no action was to be taken at Commission level in regard to consumption of authorized beef products within the UK. That was the responsibility of the UK Government. The ban was for reasons of public confidence in the safety of beef. Subsequently, a set of conditions was agreed which the UK had to satisfy before beef could be exported again. The ban was lifted by Commission Decision in August 1999, challenged subsequently by France and the challenge rejected on scientific grounds by the EC Scientific Steering Committee on October 29, 1999.

Returning to the period following the discovery of vCJD, epidemiological, genetic, molecular and transmission experiments were set up or extended.

In regard to the molecular genetic investigations, results showed that there was no evidence for the existence of a *PrP* gene mutation in any confirmed case of vCJD. But each patient so far studied has exhibited methionine homozygosity at polymorphic codon 129 of this gene. This is a common polymorphism that occurs at a frequency of around 39% in the normal populations so far studied [117]. There was no evidence at all for the familial occurrence of vCJD. Iatrogenic transmission of CJD was also ruled out [14].

Experimental Transmission

Transmission studies were critical. The objectives were to demonstrate whether or not vCJD was an experimentally transmissible disease, to identify an experimental host as a model for further studies and to define the agent biologically. Most important of all was to compare the strain type of agent isolated from human brain with that of the BSE agent derived from cattle brain, or with the strain type of any other agents that were relevant.

Transmission to Conventional Mice and Biological Strain Typing

Even before the experiments started, it was clear that the agents could not be precisely the same. This is because the amino acid sequence of the PrP from cattle was known to be different from that derived from humans. The significance of this fact depends on the viewpoint of the reader in regard to the nature of TSE agents, i.e. whether they are prions, virinos or unconventional viruses.

Notwithstanding the different hypotheses of agent structure, it was already known from several previous transmissions to mice (from brains of cattle with BSE, cats with FSE, a nyala and greater kudu with TSE, and pigs, sheep and goats with experimental BSE) that they all showed the same incubation period and lesion profile. That is to say the strain type isolated from each of these species was biologically indistinguishable. But it was different from any of the known 20 or so strains of scrapie agent [21, 74]. This was despite the fact that the amino acid sequence in PrP from each of these species was different. The authors also suggested that some additional informational molecule, such as a small as yet unidentified nucleic acid, might be required to define the strain. Since that time much work has been done to show how the PrP might confer strain-specific properties by virtue of its conformation [118–121]. However, the differing views about the agent structure have not yet been resolved. Using the same kind of in-bred mice as referred to above, it was shown that isolates from brains from 2 patients each with sporadic CJD, the first 2 confirmed before the BSE era, 2 during the BSE era and 2 from farmers who had BSE in their herds, were all quite unlike the BSE agent [122]. However, when isolates from the brains of 3 patients with vCJD were inoculated and transmitted to the same kind of mice [122], three conclusions were drawn. First vCJD was an experimentally

transmissible disease. Second the mice were uniformly susceptible. They were thus potentially useful as a model species to titrate the infectivity and investigate its distribution in body tissues. This was a matter of some importance considering the magnitude of surgical interventions in the general population and the potential risks that might ensue from them or other interventions, such as transfusion of blood derived from clinically healthy patients incubating the disease. Finally, it was conclusively shown that the strain of agent was effectively identical in 3 patients with vCJD and the strain was indistinguishable from the BSE strain from cattle.

Transmission to Transgenic Mice

A number of valuable studies using transgenic mice, commenced before vCJD was discovered, have helped to elucidate the strength of the species barrier between cattle and man with respect to the BSE agent. Mice that express both mouse and human PrP when challenged with CJD agent (excluding the vCJD strain that was not used) can generate human PrP^{Sc}. However, when BSE agent is inoculated, only mouse PrP^{Sc} is detected. This is not surprising since BSE from cattle from widely separated geographical locations readily and consistently transmits to conventional mice and is characterized by the deposition of PrP^{Sc} in brain tissue. The transgenic studies suggest that human PrP^{C} is less susceptible to being changed to human PrP^{Sc} by bovine prions than by human prions [123].

In a second study using transgenic mice that only expressed human PrP, the incubation period following challenge with CJD was shorter than when transgenic mice expressing mouse and human PrP were used, suggesting some degree of interference by the former, if PrP from both species was expressed. Furthermore, infectious mouse brain material sub-passaged into the same kind of mice showed no change in incubation period, thus indicating that the species barrier had been abolished. When the challenge was with BSE agent to transgenic mice that only expressed human PrP, the incubation period was very long indeed. This suggests that human PrP^{C} is considerably more difficult to convert to PrP^{Sc} using bovine prions than human prions [123]. The fact that the mice used in the study expressed valine and not methionine at codon 129 means that predictions about the strength of the species barrier between cattle and mice cannot be firmly made. This is because cattle all express only methionine at this codon as do all human patients with vCJD so far tested. CJD prions such as those used in the above experiments have been transmitted to wild-type mice in the past but often with some difficulty and sometimes not at all. However, vCJD prions transmit much more readily to the wild-type (FVB) mice used in the reported study, whereas transmission to transgenic mice is less efficient [124]. The outcome of a further series of transgenic mice studies, especially involving those with the codon 129 methionine homozygosity, is awaited with interest.

Molecular Strain Typing

Biological strain typing, generally regarded as the gold standard method for confirming identity between agents, is expensive, time-consuming (at least 18 months to 2 years to get a result and sometimes longer) and of little practical value other than for research. Molecular strain typing [125], however, does not suffer from any of these criticisms and supports the view that the BSE agent from cattle could be responsible for vCJD [124, 126]. Assuming the prion hypothesis of agent structure is correct, strain variation is proposed to be dependent upon the conformation (shape and folding pattern) of PrP and the glycosylation ratios. Thus it is proposed that the PrP protein itself may encode the phenotype of the disease [126]. Molecular analysis of prion strain variation (molecular, as distinct from biological strain typing) is based on distinguishing between the biochemical properties of PrP derived from the brains of patients with different CJD phenotypes. Following extraction, treatment with proteinase K, Western blotting and determination of the glycosylation ratios in PrP^{Sc} derived from human brain, at least four types of PrP (types 1–4) can be identified [126]. There is a characteristic abundance of the diglycosylated form of PrP derived from the brain of vCJD cases. This profile has been seen also in transgenic mice to which the disease has been transmitted. Types 1–3 occurred in brains from sporadic, familial and iatrogenic CJD patients. Type 4 PrP was found only in the brains from patients with vCJD. It was found in all brains examined and in the tonsil too when it was sought. No PrP was found in the tonsils from patients with other forms of CJD. Thus there are possibilities to distinguish vCJD from other forms of CJD both in life (by tonsil biopsy) and after death [127].

Because the molecular strain type of agent identified in all cases of vCJD so far examined is consistent (type 4) and this type has not been seen in other forms of CJD, it is now regarded as a valuable aid to confirm the aetiological diagnosis. This is currently impractical by using mice for biological strain typing. Some authors nevertheless have aired a view of caution about the use of the method in its current form for determining the molecular strain types of agent isolated from other species [128–131]. This is because previously existing biologically typed strains can have molecular profiles similar to vCJD and BSE [128, 131]. Furthermore, PrP^{Sc} also varies independently in the amount and pattern of glycosylation according to brain region [130]. In addition, the molecular signature of PrP^{Sc} from 42 French isolates of natural scrapie from 21 flocks in different regions of France could not be distinguished from that in French cattle with BSE or from a cheetah imported from the UK with FSE [132]. There is no evidence for the occurrence of BSE in sheep in any country so it seems unwise at present to assume that molecular typing of isolates from different species is robust enough to certainly distinguish biological strains. Technical differences between laboratories could account for some of the anomalous results so far

reported [133, 134]. More technical development is required together with a harmonization of methods between laboratories.

Comparative Neuropathology

As mentioned in a previous section, BSE has been experimentally transmitted to macaque monkeys [81]. The clinical, and particularly the neuropathological features, resemble those seen in vCJD [81, 135]. However, these features have not been reported in three other species of primate challenged with BSE [80, C.J. Gibbs Jr., pers. commun.]. Furthermore, there are similarities between the TSE observed in mule deer with chronic wasting disease and vCJD [4], particularly in the occurrence of PrP-positive plaques.

Tissue Distribution of Prion Protein-Scrapie Isoform

In regard to the tissue distribution of PrP^{Sc} in vCJD, this has been found in the tonsil [127], appendix [136] spleen and lymph nodes [135]. Results from tissue infectivity studies have yet to be reported for tissues other than brain. Since PrP is not detected in the tonsils of patients with other forms of CJD, and infectivity or the presence of PrP^{Sc} is infrequently reported from LRS tissues of patients with other forms of CJD, it is possible that the pathogenesis of vCJD is different from the other forms too. For a review of vCJD, see Collinge [136].

Effect of the Occurrence of Variant Form of CJD

The potential links of vCJD to the BSE agent from cattle via consumption of infected offals like brain and spinal cord before measures were put in place in 1989 have been sufficiently convincing to have caused severe repercussions on the beef industries of countries with BSE in native-born cattle. The occurrence of BSE in cattle in the UK alone has been estimated to have cost more than GBP 4 billion to 1999 [137].

The proposed linkage between BSE and vCJD has resulted in the imposition of SBO bans, or amendment to offals bans in existence. In some countries, including the UK, the SBO ban was extended to a specified bovine materials ban and thereafter to a specified risk materials ban that included some sheep tissues. An EC Decision on the exclusion of specified risk materials was adopted in June 2000 and amended in January 2001. It is intended that this is graded in severity depending upon the geographical BSE risk (GBR) assessment ascribed to the source country. A GBR for EC Member States and countries wishing to trade with the EU has been published by the Commission following analysis of dossiers submitted by trading countries. A second round assessment is in progress to take account of the dynamic nature of BSE risk and the extension to the number of countries that have reported the disease.

The UK ban included the CNS of sheep and goats of certain ages and the spleen of sheep and goats of all ages, as a hypothetical risk from BSE in sheep

was perceived. This was because some sheep had been exposed to MBM in their diet before the feed ban (and perhaps by accident afterwards) and BSE had been experimentally transmitted to both sheep and goats by the oral route [83]. No natural case of BSE has been found in sheep anywhere in the world, even after limited investigations using biological strain typing of isolates or molecular typing or PrP from sheep [138, 139].

The potential for sheep to harbour the BSE agent and for it to be transmitted maternally and horizontally has been one of the most difficult to come to terms with. Adopting the precautionary principle and excluding all at-risk sheep and even tissues from healthy sheep would have very serious consequences for the sheep industry of Europe and especially the UK and its environment. Sheep play a major role in the rural scene and the consequences from a socio-economic point of view are incalculable. On the other hand, doing nothing could put consumers at risk from exposure to BSE agent if it was in fact present and if it is pathogenic for man. At present, some countries in the EU, including the UK, have opted for a sensible risk reduction policy. This may have to be modified if ever BSE was found in sheep. Humans have been widely exposed to sheep PrP over centuries without any evidence of risk for human health. We cannot be totally confident that that could not happen in the future as a result of exposure to the new (currently potential) hazard of BSE in sheep. However, as we acquire new knowledge of prion diseases and how they work, it seems that it may become possible to control, and eventually eliminate, clinical scrapie from sheep. How that might be done and whether infection will be eliminated too is another story beyond the compass of this chapter.

Concluding Remarks

BSE is well on the way to elimination in the UK, albeit with some hiccups on the way. The UK experience of BSE shows how, with perseverance and using multidisciplinary techniques, a serious epidemic of a new unconventional disease can be first brought under control and then eliminated. There is clear evidence that the situation in the rest of the EU is improving rapidly and that consumers are unlikely now to be exposed to the BSE agent in food. The occurrence of BSE in North America and Asia is a worrying phenomenon that must be tackled with vigour. We now have the experience and the tools to do it. There are other countries that may be at risk. The introduction of 'Rapid' testing in the EU has revealed relatively large numbers of BSE-infected cattle that otherwise would not have been detected. Active surveillance targeted to high BSE-risk populations on the one hand and to slaughter cattle over 30 months of age on the other, combine to reveal the 'Real' incidence of disease and inspire consumer confidence in bovine products respectively. Eradication of BSE now seems a realistic possibility but to

achieve this desirable state of affairs all countries must follow the recommendations of the WHO and OIE, conduct risk analyses and rigorously enforce the regulations they put in place to manage any risks identified. The disastrous effects on public health (fortunately now perhaps rather less than previously envisaged), animal health and to international trade in bovine products are salutory lessons that must be learned. One lesson is that animal feed must be prepared from the same wholesome materials that are used for human food. Another is that infected materials must be removed and totally destroyed. If this approach had been appreciated earlier BSE would never have happened in the first place.

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Possibilities to Manage the BSE Epidemic: Cohort Culling versus Herd Culling – Experiences in Switzerland

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Bovine spongiform encephalopathy (BSE), a disease of cattle first reported in 1986 in the UK [1], has been found in the cattle populations of most European countries. To date, Japan, Israel and Canada are the only non-European countries where BSE has been reported in indigenous cattle [2].

Given all available evidence, the BSE agent is not transmitted horizontally between cattle; rather it is primarily transmitted through feed. Meat-and-bone meal (MBM) is the main source of infectivity and cattle [3] are usually infected during calf-hood. Although it is likely that the other calves in a herd of a similar age to a BSE case also consumed feed from a contaminated batch of MBM, the incidence of BSE within most herds is very low. The most probable explanation is that exposure to the BSE agent was, on average, very low. Recycling of the BSE agent is likely to have resulted in an increased number of 'packets' of infectivity rather than an increase in the average titre of infectivity within a contaminated batch of MBM. As a result, the average within-herd incidence would be unlikely to increase [4] (see also the chapter of Bradley).

Several strategies, including those that focus on feed as a source of infection and various culling programmes, have been adopted with the aim of eradicating BSE. Culling strategies vary from culling the affected animal only, to culling all animals within the birth cohort of the BSE case, to culling the entire herd.

Objective of a Culling Strategy

The primary objective of a culling strategy is to eliminate animals epidemiologically linked to an index case so that the number of BSE-infected animals

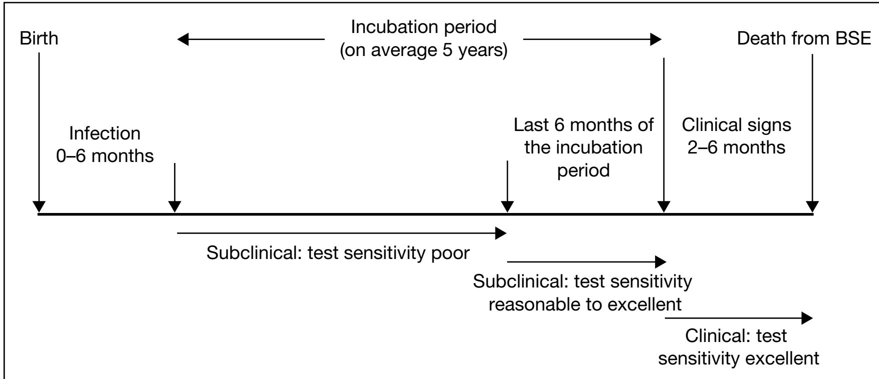

Fig. 1. An outline of the course of BSE infection in an animal and the relative sensitivity of currently available laboratory tests for detecting a case of BSE.

entering the human food or animal feed chain is minimised. The most efficient strategy would be one that maximises the number of BSE cases eliminated from the food and feed chain while minimising the number of uninfected animals that are culled. To achieve this, a culling scheme should target those animals that have the highest chance of being infected.

The Likelihood of Additional Cases of BSE in an Infected Herd

Following the detection of BSE in a herd, how many additional cases linked to the index case are there likely to be? This is not an easy question to answer. Currently available tests, which are all undertaken post-mortem, are only able to detect clinical cases and animals within approximately the last 6 months of their incubation period [5] (fig. 1). With the exception of the UK [6], in those herds where more than 1 case of BSE has been detected, the additional case(s) have inevitably been born within ±1 year of the index case [7, 8]. Just how many of these 'birth cohort' animals were originally infected is likely to be related to the size and stage of the BSE epidemic during their exposure to contaminated MBM. The number of infected animals that are likely to still be in the herd when an index case occurs depends on the probability of them surviving as herd members. They may be slaughtered, culled or die from non-BSE-related causes prior to the occurrence of the index case. As a result, the number of cattle originally infected with BSE is likely to be much greater than the number of confirmed cases. In fact, a simulation model, based on Swiss data, showed that almost 90% of BSE cases from an original birth cohort are likely

to have left the herd before an index case is detected. Of the 10% of BSE cases that are likely to still be present, approximately one third would be detectable if they were immediately culled after the detection of the index case and tested. The remainder would not be detected by currently available tests, as they would be more than 6 months from the end of their incubation period. If they were allowed to remain alive until the end of their productive lives, approximately 75% would be detectable.

Alternative Culling Programmes

Each country has adopted a different approach, which has changed over time. The main culling strategies that have been applied are as follows:

Culling: (a) the index case only; (b) all cattle on the case farm where the index case was diagnosed; (c) all cattle on the farm where the index case was born and raised; (d) all cattle on the index case farm and on the farm, where the index case was born and raised; (e) all susceptible animals on the index case farm (including sheep, goats and cats); (f) 'feed cohort' (cattle that could have been exposed to the same feed as the index case), and (g) 'birth cohort' (all cattle born 1 year before or 1 year after the index case and raised on the same farm).

Experiences Gained in Switzerland

In the first 6 years following the diagnosis of BSE in Switzerland in November 1990, only BSE cases were culled. During this period, 12 farms had more than 1 case of BSE (11 farms with 2 cases and 1 farm with 3 cases). All of these animals were born within 1 year and were raised on the same farm as their respective index case. As a result, they could be assigned to a defined risk group, that is a birth cohort, which is made up of all animals born in the period from 1 year before to 1 year after the birth of the index case.

In December 1996, the Swiss parliament introduced a herd-culling programme in response to concerns surrounding the cases of the variant Creutzfeldt-Jakob disease (vCJD) reported in Great Britain, an increasing number of BSE cases born after a feed ban (BAB) introduced in 1990, and, most importantly, the measures imposed in export markets. Once a BAB case was reported, the whole herd was culled. If the index case originally came from another farm, all the cattle on the farm where it was born and raised were also culled. Herd culling was adopted retrospectively, so that all Swiss herds that had ever had a case of BSE were included.

Herd culling was restricted by the Swiss parliament to run until June 30, 1999. During the 30 months it was in effect about 3,000 animals that were

culled and disposed of. Most were examined clinically and brain tissue samples from a total of 1,761 cows were tested. Four of these cows, which were all clinically normal, were positive. Since they were all born within the period from 1 year before to 1 year after the birth of their respective index case, they could also be assigned to the same risk group (birth cohort) as the additional cases that were seen before herd culling began. As a result, from July 1999, it was decided to cull birth cohort animals rather than the whole herd. A cohort is defined as all animals that were either born or reared as calves on the farm within the year prior to or following the birth of the index case. It also includes those animals from the original cohort that no longer reside on the farm where the index case was born and reared.

Since cohort culling began, more than 600 animals from 115 cohorts have been culled and tested for BSE with only 1 additional case being detected. Additional cases of BSE, that were not part of the birth cohort of an index case, have never been observed in any herds in Switzerland.

Experiences gained in Switzerland support birth cohort culling on the farm where the index case was born and raised as a targeted risk management strategy that focuses on those animals within a herd that have the greatest chance of having BSE. On average, about one-fifth of a herd is likely to be culled. The rationale of such a program is easier to explain and much more acceptable to farmers. Since its introduction, markedly more suspect cases have been reported in Switzerland, although part of this increase may be attributable to a concurrent enhancement in disease surveillance activities including the introduction of targeted surveillance in 1999.

Figure 2 shows that most of the additional cases of BSE that have been detected in Switzerland were born in the two time periods (1988–1991 and 1994/1995) when exposure to the BSE agent was highest. It appears that the chances of finding additional cases amongst birth cohort animals born after approximately 1996, by which time exposure was at its lowest, is likely to be negligible. As a result, there may be little advantage in culling cohort animals born in the later stages of the epidemic when exposure to the BSE agent would have been minimal.

Choosing a Culling Strategy

A number of factors need to be considered when choosing an appropriate culling strategy including a science-based risk assessment that takes account of the full range of measures applied to BSE, the epidemiology of BSE, the stage of the BSE epidemic, international agreements and standards, societal expectations and political imperatives.

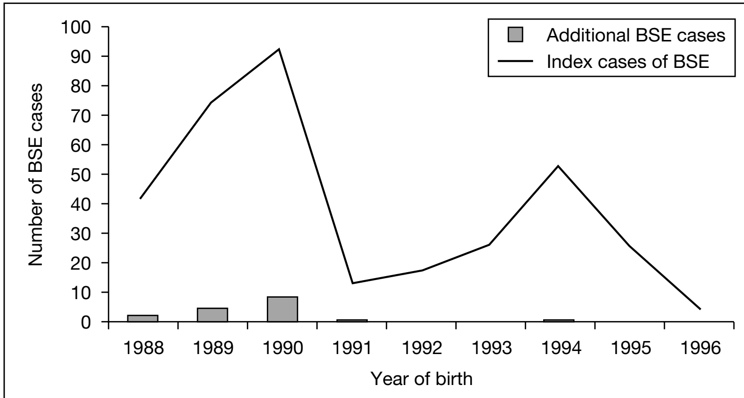

Fig. 2. The number of cases of BSE and the additional cases linked to an index case detected in Switzerland by year of birth.

It is important to recognise that a culling strategy is part of a continuum of measures that complement each other to effectively manage the risks associated with BSE for both the human and animal population. The impact of other measures, such as a mammalian animal feed ban, an SRM ban, the incineration of BSE cases and a surveillance program, together with a consideration of the level of exposure to the BSE agent amongst birth cohorts as the epidemic progresses, all need to be considered when deciding on a realistic culling strategy. While it is legitimate 'to do everything possible to protect consumers', each of the measures finally chosen needs to be reasonable, otherwise it is likely that the effectiveness of the overall BSE programme will be compromised.

Ideally, all the animals exposed to the same feed as an index case, should be culled [7]. Unfortunately this is rarely, if ever, possible as there are considerable, if not insurmountable difficulties in identifying potentially contaminated batches of feed, determining the distribution of infectivity within contaminated batches and establishing which herd(s) may have been exposed.

While herd culling may be a politically expedient means of increasing consumer confidence and facilitating exports, it is unlikely to be an efficient risk management measure. There are significant problems in implementing such a strategy as the available evidence indicates there is likely to be a considerable wastage of uninfected animals, farmers see it as a radical approach and they may be less willing to notify suspect cases. Although there may be sufficient compensation for culled animals, farmers are less likely to report suspects if they do not believe it is reasonable to cull apparently healthy, productive animals. In addition, they are likely to lose valuable genetics and/or their 'life's work'.

Evidence from a number of countries [7, 8] indicates that, in those herds where more than 1 case of BSE has been detected, the additional case(s) were born within 1 year of the index case. As a result, culling a birth cohort is a more rationale risk management strategy as it focuses on those animals within a herd that have the greatest chance of having BSE. Even so, depending on the initial level of exposure and the original size of the cohort, it is likely that relatively few additional cases of BSE will still be present in the cohort when an index case is seen. Despite this, it is likely to be much more acceptable to farmers compared with herd culling.

It is important to note that even if an infected birth cohort animal was culled immediately and entered the food chain it is likely that it would be in the preclinical phase of BSE. For example, a simulation model based on Swiss data predicts that when an index case is seen, approximately 70% of infected cohort animals are likely to be more than 6 months from the end of their incubation period. Together with an SRM ban and a feed ban, these animals are unlikely to pose any significant risks for human or animal health.

Since BSE is not transmitted by milk [9], semen and embryos [10–12], it is questionable whether birth cohort animals need to be culled immediately following the detection of an index case. If they were allowed to remain in the herd and live out their productive lives before being culled, approximately three-quarters of them would either be within 6 months from the end of their incubation period or have entered the clinical phase of BSE. There is an excellent chance that they would be detected by the currently available tests allowing the BSE status of the remaining animals to be assessed with greater accuracy. As a result, by letting cohort animals remain in the herd until the end of their productive lives, both the impact of BSE on herd management is minimised and the BSE status of the original cohort can be assessed with greater accuracy. This can all be achieved without any additional risk of cohort animals entering the human food or animal feed chain.

Conclusion

A culling strategy is part of a continuum of measures that can be employed to effectively manage the risks associated with BSE. The most efficient strategy is one that takes adequate account of the epidemiology of BSE and complements other measures, such as an SRM ban, a feed ban and a surveillance program, to maximise the number of BSE cases eliminated from both the human food chain and the animal feed chain while minimising the impact of BSE on herd management. It should target those animals that have the highest chance of being infected. Following the detection of an index case in a herd, the culling strategy

that best fulfils these objectives is one that focuses on the birth cohort of the index case. Cohort animals should be allowed to live out their productive lives before being culled or slaughtered. In the later stages of an epidemic, when exposure to the BSE agent is likely to be minimal, the chances that there will be additional cases amongst birth cohort animals born during this time is likely to be negligible. As a result, it may be reasonable to modify the culling strategy so that cohort animals born after a particular date are no longer culled.

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Regulatory Aspects of BSE and CJD with Special Emphasis on Germany

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Almost no other disease has caused as many discussions and had such a significant impact on society as well as on experts as bovine spongiform encephalopathy (BSE), also known as 'mad cow disease'. Hereby, a term with a high public impact has been generated and established which is known by almost everyone worldwide. BSE has changed the nutritional attitudes of an almost inestimable part of the worldwide human population. Of course, this has also had a significant impact on the global and/or European customer attitudes on the food market with changes in sales that are in the range of billions of Euros and/or US dollars. Probably only the HIV/AIDS pandemic was comparable from the viewpoint of its social impact. Comparably to HIV/AIDS, BSE also gained high political implications rapidly apart from the scientific rationale. These political attempts – more or less significant and/or effective – also led to very contradictious debates which were often severely prohibitive for consensus finding and/or import/export interactions between different countries.

It would exceed the possibilities of this chapter to re-analyze, re-capitulate and review all the activities in association with BSE. Besides, it would not even make too much sense, as many activities were fairly distant to the scientific rationale and were merely activities for promoting public and/or questionable scientific relations. In such cases, real problems became targets for differences in international and national communications and disputes: A real political impact was also underlined by activities of the different available media in official communications such as television, newspapers, etc. Objectivity and scientific rationale sometimes became of minor importance and recently self-declared 'opinion leaders' used the opportunity for personal profiling by (knowingly) disinforming and/or unjustified frightening of the community.

Due to official obligations, we have to differentiate between laws, orders, rules, guidelines, official announcements, statutes, consensus papers, commandments and official lists; and many community representatives do not even know about the corresponding juridical obligations in accordance to these obligations. Such activities were often differently handled, even among the EU member states. Consecutively, early in 2000, EU standards became harmonized between the different EU member states. In many cases, some member states eased their statutes and had to revise these activities under the pressure of the EU/EC again. There are recommendations originating from the World Health Organisation (WHO) for the worldwide management of BSE and guidelines of several institutions responsible for processing of material of bovine origin. Additionally, worldwide, many countries established regulations for the prevention of endangering with BSE and/or Creutzfeldt-Jakob Disease (CJD) into their issue of federal responsibility.

Apart from this mainly geographical assessment, activities for the management of BSE and CJD should also be seen under the viewpoint of its estimation, such as:

- Activities supporting the epidemiological supervision
- Export prohibitions and/or limitations for the international trade associated with living/slaughtered cattle and/or associated embryos, and sperm
- Export prohibitions and/or limitations for the international trade with any material with putative ingredients of bovine origin including food for human and/or animal purposes
- Requirements for the keeping of cattle including the prohibition of feeding animal feed which could be a potential resource for acquisition of BSE
- Recommendations for the elimination of (putatively) BSE-infected animals and for the decontamination of agricultural facilities
- Recommendations for slaughtering and processing of meat-associated food
- Guidance for the processing of material derived from ruminants, especially for pharmaceutical and/or cosmetic products. Furthermore, restrictions for the usage of cell cultures of bovine origin, for vaccine production, and diagnostic reagents/diagnostics
- Requirements concerning the production of feed for animal purposes containing material, associated with ruminants, and other products such as animal fats, or gelatin
- Safety regulations for staff working with agricultural equipment and/or in accordance to meat processing

Not all of these processes were restricted to cows and materials of bovine origin. They are also nearly valid and transposable for other ruminants such as sheep and goats. The management of BSE also had a significant impact on the handling and ranking of other spongiform encephalopathies, especially CJD in

man, where extensive programs for disease management and prevention were also established. Those activities are specified below:

- Introduction of a lawful obligation for case reports
- Exclusion of a defined group of individuals as blood donors
- Introduction of higher sophisticated and 'up-to-date' procedures for processing sterilisation regimens for medical equipment and/or clothing
- Temporary introduction of disposable instruments for invasive medical applications (e.g. tonsillectomy, appendectomy, neurosurgery, etc.)
- Safety regulations for medical staff

In order to supply detailed information on this topic, the items have to be reviewed on the basis of each corresponding country. This would exceed the possibilities of this chapter, and therefore the chapter is mostly focused on European conditions with the primary aim being Germany. In so far as it would be beyond the scope of the chapter to review and evaluate the activities of the responsible national and/or international commissions and committees responsible for disease management and prevention of BSE in order to estimate if significant failures occurred during this process. Such commissions were for example established on the EU level but also in some single EU member states, as in the UK by the British Government in 1997.

Reference should also be given here concerning the apparently important and very detailed parliamentary questionnaires, e.g. from the German Party 'Bündnis 90 – Die Grünen', directed to the German Government. The German chancellor also assigned the 'Bundesrechnungshof' in December 2000 to analyze the problems and open questions associated with the BSE situation with special focus on Germany.

A complementary field is the guidance of consumer associations, the establishment of quality declarations as well as voluntary obligations and restrictions from various institutions and/or providers of food and animal feed. Furthermore, consumer councils gave their hints for more or less appropriate handling of the problem to the customers.

EU subvention programs in accordance to regulatory strategies were very effective. Especially the strategically optimally organized acquisition of older cattle in order to initiate an organized slaughtering process and appropriate elimination for a temporary consolidation of the critical market price situation concerning products derived from beef in order to stabilize the market price for beef. At this point it should be taken into special consideration that aspects of disease prevention were only of minor importance in this context. In order to demonstrate the hectic public and/or governmental situation with this process, as for example in Germany, some actions are exemplarily listed in appendix 1. Of course, this process can also be transferred to many, if not almost all other countries involved.

Special Regulations for the Prevention and Management of BSE

Epidemiological Supervision

The epidemiological supervision does not only cover the official registration of BSE cases in cattle. It also covers the registration of spongiform encephalopathies in all other animals, included related animals. Special emphasis has to be laid on ruminants such as sheep and goats. In the UK, BSE has been under an official registration regulation since 1988, followed by the other EU member states in 1990. In Germany, a national reference center ('Nationales Referenzzentrum für Spongiforme Enzephalopathien') was established in 1992, allocated at the 'Bundesforschungsanstalt für Viruserkrankungen der Tiere' (BFAV Federal Research Center for Viral Infections in Animals) in Tübingen.

In June 2000, the EU member states had to undergo the obligation for the performance of diagnostics concerning BSE in cattle older than 2 years which were slaughtered because of a poor or questionable medical condition. The EU also decided on a stronger supervision of sheep and goat herds, where a BSE test also became mandatory for animals older than 18 months. This EU-wide obligation was established and became mandatory on January 1, 2002. A very important part of the official epidemiological prevention program is the total documentation of the life history of each animal. This was ensured by introducing a 'cattle passport', which ensures that the whole life of an animal from birth until being slaughtered can be exactly recapitulated and accompanies each individual animal during its lifetime.

Trading Limitations for Living Cattle

The limitations for the trade with living cattle were organized in a multistep cascade. One of the first activities was an export prohibition for cattle originating from the UK which were born before July 18, 1988. This was also transferred to calves from cattle where BSE infection could not be excluded. Later, in 1990, the EU also introduced export prohibitions for cattle originating from the UK which were older than 6 months. Some EU member states, including Germany, even introduced a total import prohibition for cattle coming from the UK. Further restrictions were felt necessary concerning the trade with sperm and embryos of bovine origin.

Restrictions for the Trade with Food and Other Materials of Bovine Origin Including Animal Feed

Trade with animal feed was restricted very early after the transmission route of BSE has become evident. Germany banned these imports already in 1989 in case of feed imports from the UK. The first import ban for British beef

was already executed in 1988 by the US Government. In Europe, the first ban followed 1 year later, although it was temporarily cancelled again a few weeks later. A worldwide export prohibition for materials of bovine origin from the UK was released by the EU/EC authorities in 1996. Also in this case, the EU/EC took over worldwide responsibilities.

Requirements for the Keeping of Cattle Including the Ban of Feed Containing Material Derived from Ruminants

Already very early after the first occurrence of cases of BSE, animal feed was taken into consideration for the establishment of the disease due to epidemiological observation. Despite the prohibition of the application of feed derived from ruminants such as cattle, sheep and goats, within cattle keeping was handled very differently in the different EU member states, and especially worldwide. As the first European state, Sweden established a detailed feeding prohibition already in 1987, followed by the UK in 1988. Despite a general EU directive, also including Germany, it was pronounced relatively late and became an obligation in 1994.

The feeding of companion animals for example only came under the pressure of the official authorities in a very late stage of the BSE scenario. In the UK the first attempts were made in 1996. In Germany such obligations were introduced in November 2000, at the time when the first cases of BSE were becoming evident. At that time it was also recognized that animal species almost incapable of being infected by spongiform encephalopathies due to the BSE epidemiology may also act as vectors/vehicles for transferring the contagious agent. Apart from feed derived from meat, materials such as bonemeal, blood and fat of ruminant origin were placed under a registration regulation. This also covered feed for the breeding of calves instead of milk of bovine origin. In Germany these fat-containing materials were only heated up to $<100°C$ until the end of 2000.

Appropriate Eradication and Elimination of BSE-Infected and/or Endangered Animal Collectives Including the Decontamination of Agricultural Facilities

Regulations for slaughtering BSE-suspicious animals were introduced in the UK already in 1989. The market value for animals slaughtered by this regulation was supported by the British Government for the half of the corresponding market price. Since 1990, British farmers have received the full corresponding market price in this disease management program. Due to a consequent and systematic prophylactic disease prevention program introduced in the UK in 1996, all cattle older than 30 months were ordered to be slaughtered.

Consecutively, other European countries also declared regulations for sacrificing cattle imported from the UK and/or other 'high-risk countries' such as Northern Ireland and Switzerland, although these recommendations were juristically suspicious and often not possible to realize.

In contrast to these procedures, regulations were established for farms where cases of BSE were observed. In these cases the whole cattle cohort/population had to be sacrificed in case that breeding of cattle was the main purpose of animal keeping in these populations. For Germany a juristical rationale for this recommendation was urgently decided in 2001 with extremely high priority. Guidelines for the decontamination of agricultural facilities accomplished this process. In Germany for example, guidelines of the 'Bundesministerium für Ernährung, Landwirtschaft und Forsten' (German Ministry for Nutrition, Agriculture and Forest Protection) were defined and executed. Regulations concerning the elimination of BSE-suspicious cadavers should also be mentioned in this context.

Requirements Concerning the Production of Materials of Bovine Origin Including Slaughtering and Human Food Production

The first steps for consumer protection concerning BSE-contaminated food were initiated in the UK in 1988 – these included banning the sale of inner organs and milk of BSE-infected animals and/or BSE-suspicious animals. Already at this time-point, the potential risk of the different tissues and meat products was very controversially discussed in the scientific community. This basic attitude hindered and also accomplished the BSE prevention strategies like a 'red thread' over a long period of time covering many years. It is reflected in the classification of declared 'high-risk materials' and/or 'specific risk materials'. This confusion interacted with a lot of significant decisions felt in the consecutive period. Eyes, brain, spinal cord and the lungs were declared as materials with the highest risk impact. Spleen, gut, tonsils, lymph nodes and the uterus were declared as materials of secondary risk. For both categories, on October 1, 2000 the EG/EU/EC declared a general prohibition of their usage. In some cases, different EU countries were much more restrictive, such as the sale of T-bone steaks was prohibited in France in November 2000 due to federal regulations.

In this context it is striking that a statement of the EU commission defined specific 'high-risk materials' of bovine origin not to be used as food for human purposes or animal feed already in 1996, although this recommendation was ignored and not realized within the first years after declaration. In case of animals older than 12 months, these 'high-risk materials' are generally to be removed and to be eliminated by burning and/or another appropriate method for disease prevention. Most of the countries consider burning the material as the first choice of an appropriate elimination strategy. In some countries, especially in the UK and Portugal, these regulations were even strengthened as the age limit of cattle was

lowered to 6 months. Additionally, the whole head of the animal was declared as a ‘high-risk material’ with all its consequences, e.g. usage of tongue. The strategic concept of the EU declared the member states – due to the BSE epidemiology – into five different risk categories. The obligation for disease management and prevention programs in these countries is orientated according to this classification.

The EU commission reviewed rapid diagnostic procedures in 2000 and introduced them as mandatory in 2001. In some cases the introduction of such diagnostic testing procedures was already established at an earlier time-point, for example, in Germany such test methods were already established in 1999 on a non-mandatory basis, where North Rhine-Westphalia was the first state in the country to follow this concept. The obligation for these tests depended on the age of the slaughtered animals. In Germany for instance, in the first step, only cattle older than 30 months prior to being slaughtered were tested, at a later time-point this age margin was lowered to 24 months.

Since 2000, an additional EU-wide obligation for the labeling of beef became mandatory. This gives the customers the chance to recognize where the animal was slaughtered, portioned and further processed. Until now, this obligation still has a potential for optimization such as the often missing declaration of ingredients in meat products and/or sausages. Furthermore, a mandatory declaration of the countries from which these components originate is in preparation and as well as the current activities in the EU at the moment.

Requirements for Processing of Rough Materials of Bovine Origin in Pharmacology, and Cosmetics and Toiletries

Numerous regulations for the production of pharmaceuticals and pharmaceutical additives of bovine origin have been defined. Many active substances and/or ingredients such as insulin, glucagon, aprotinin, heparin, cholesterin, albumin, corticosteroids, urosodeoxycholic acid, collagen, catgut, and many other materials are involved. Also involved are additives such as gelatin, lactose, talc and different oils of bovine/ruminant origin, casein, and/or nutrients for in vitro purposes such as microbiological media as for example peptone-containing preparations and/or brain heart infusion media.

In Germany, a cascade for the evaluation of the danger going out from materials derived from bovine, ovine, or caprine origin was initiated in 1994. For a sufficient drug safety, special emphasis was laid on the country of origin of the corresponding drug material/additives. Furthermore, the production process in which the drug was manufactured and/or further processed, was individually reviewed. Additionally, an estimation of the daily dose of this material by appropriate use of the drug was evaluated, the period of time of taking the drug as well as the route of drug application. Countries responsible for the origin of such materials were reviewed by the officially established

‘Office International des Epizooties’ (OIC) and were classified into six different risk categories.

The evaluation of processing techniques was primarily based on data available from literature concerning the stability and/or possibility to inactivate the contagious agent and/or declarations from validation studies originating from the manufacturers. Aspects derived from the literature were also the rationale for ranking the rough materials into five different risk categories. The route of application of a drug especially takes into consideration the efficacy of contagiousness tested by different experimental assessments such as application of a contagious substance by the intracerebral, intravenous, intraperitoneal and/or oral route. A standard was defined which included a quantification index. Only if the drug was in the range of the proposed index was the drug then allowed to be sold on the market. As described above, this system was also transferred to cosmetic products several months later in Germany. The registration regulation was not only applied to drugs having direct contact to risk materials. The usage of cell cultures of bovine origin, e.g. for use in the production of vaccines and/or diagnostics, was also reviewed in a similar process, and restrictions were defined and introduced. Some countries even spoke out a total prohibition for the use of materials of bovine origin originating from ‘high-risk BSE countries’ for the production of pharmaceuticals. As an example, Japan proceeded this way already in 1996 and later even extended this prohibition to all pharmaceutical ingredients derived from living animals. In December 2000, more than 28 countries were affected by these exclusion prohibitions.

Requirements for the Production of Animal Feed Containing Material of Animal Origin

In the UK, until 1980, all feed derived from animals had to be heated up to 115°C for 1 h. Since 1981, this regulation was eased and the minimal requirement for processing the feed concerning the thermic inactivation was declared to be 82°C for 1 h. The EU’s recommendations for the processing and preparation of feed of animal origin were then defined as a minimal requirement of 133°C at an atmosphere of 3 bar over a period of at least 20 min. This decision became mandatory for all EU member states in 1996 with an extension possibility until April 1, 1997. Despite all these definitions, there was still a tremendous chance for interpretation in these guidelines and consecutively a big difference among the different facilities preparing animal feed.

Even in Germany, not all of the waste derived from slaughtering processes was always treated in accordance to the EU recommendations for preparing animal feed over a period of several years. Such activities mainly focused on materials which demonstrated an important risk for hygiene as evaluated and interpreted by the official veterinary agencies. Further guidelines were

established for the preparation and processing of fats of animal origin or gelatin. For the guidelines it was not of importance if the materials were used for human food or animal feed, or if they were processed for other purposes and products.

Safety Regulations in Agriculture and Meat Procession

The overall official national and international activities for disease management and prevention of BSE range from considerations concerning cleaning and disinfection procedures in slaughtering facilities to very well defined guidelines concerning the safety assurance of employees engaged in the slaughtering process up to requirements in case of having to take specimens, transport of the material and the processing of the material for BSE diagnostics. Special guidelines for laboratories performing rapid BSE diagnostics were also established apart from the normal regulations concerning the handling of infectious materials.

Special Regulations for the Prevention and Management of CJD

Law-Enforced Obligations to Report Human CJD Cases to the Official Authorities

A 'National Surveillance Unit' was established in the UK in 1990 and an obligation to report CJD cases was introduced. Other European countries followed this strategy with a delay, including Germany in 1994.

Exclusion of a Defined Group of Individuals as Blood Donors

In 1999, the American Food and Drug Administration (FDA) advised to exclude people as potential blood donors in case they had stayed in the UK for more than 6 months between 1980 and 1996.

Introduction of Higher Valanced Procedures for Processing Medical Equipment and/or Laundry in the Hospital Setting

Due to the BSE pandemic, autoclaving regimens were reviewed and revised. Steam autoclaving at 134°C over a period of 20 min was also introduced in many clinical facilities as it is much more clinically appropriate and effective than the older autoclaving regimen at 121°C. In Germany – due to a recommendation of the Robert Koch Institute – instruments for neurosurgical purposes used in a patient suffering or infected by CJD have to be autoclaved for at least 1 h. Additionally, in such cases, cleaning and desinfection regimens of the instruments involving, e.g. NaOH, NaOCl or guanidium isothiocyanate are required. Newer recommendations of the Robert Koch Institute generally

recommend alkaline pretreatment of instruments for neurosurgical purposes, in order to ensure – apart from the cleaning efficacy – the destabilizing effect of these substances on the contagious agent (see also Beekes et al., p 117–135 in this volume).

Preference of Disposable Instruments

In case appropriate procedures – as described above – cannot be performed (e.g. due to missing stability of the material), further regulations were established and became mandatory. A prohibition for the use of disposable contact lenses in ophthalmology was pronounced in this context. In case of the use of trial contact lenses, they should not be re-used by other persons or be given to other patients. Further, a central processing/decontamination of endoscopes which have been used for CJD patients has been initiated and was done in specially skilled reference centers by using guanidium isothiocyanate. In some European countries a general introduction of disposable instruments, e.g. for tonsillectomies or appendectomies, was required. In the UK this requirement was even applied only temporarily.

Safety Regulations for Medical Staff

Apart from the official and highly sophisticated recommendations of the WHO and/or National Ministries of Health, scientific associations and boards were also involved in the process of establishing guidelines and giving advice and/or reviewing and commenting official requirements. Very detailed comments were given on the protection of employees working in pathology, with special emphasis on the performance of autopsies in patients that died of CJD. Further comments and guidance were elaborated for the pretreatment of teeth derived from cattle/ruminants which are unavoidable for learning and demonstration purposes, e.g. in dental medicine.

It cannot be the aim of this chapter to judge how the total 'BSE story' was handled, as the accomplishing political dimensions are tremendous too. Furthermore, many responsibilities are very individual in each separate country. Nevertheless, at the moment, the efficacy of all the activities cannot be extensively interpreted. An analysis of the whole BSE pandemic, including scientific, ethical, public, political, economical and media aspects, is due to be performed on an international basis where all groups interested in this subject will be able give their comments and opinions to a scientific committee for a previous overall evaluation. The only issue which really became evident is that regulatory aspects with official national and international administrative activities – to the knowledge of the authors – never developed into such a tremendous dimension in the

management and prevention of infectious diseases as in the case of the BSE pandemic.

Appendix 1

Source of information concerning regulatory attempts exemplarily for Germany as the largest EU member state (listed chronologically concerning the date of publication).

Richtlinie 90/667EWG des Rates **27. Januar 1990**; (Tierkörperbeseitigung). Amtsblatt der EG L363/51.

Bundesgesundheitsamt: Bekanntmachung über die Zulassung und Registrierung von Arzneimitteln vom 12. August 1991. Abwehr von Arzneimittelrisiken – Stufe II. Beim Bundesgesundheitsamt zugelassene und fiktiv zugelassene Arzneimittel, die unter Verwendung von Ausgangsmaterial der Spezies Rind, Schaf und Ziege hergestellt werden und zur parenteralen Applikation oder zur Anwendung auf offenen Wunden oder am Auge vorgesehen sind. Bundesanzeiger Nr. 153, S. 5541 vom **17. August 1991**.

Bundesgesundheitsamt: Bekanntmachung über die Zulassung und Registrierung von Arzneimitteln vom 17. August 1990. Abwehr von Arzneimittelrisiken – Stufe I. Human- und Tierarzneimittel, die unter Verwendung von Ausgangsmaterial der Spezies Rind, Schaf und Ziege hergestellt werden (alle Parenteralia sowie Externa, soweit sie für die Anwendung am Auge oder auf offenen Wunden bestimmt sind). Bundesanzeiger Nr. 157, S. 4323 vom **23. August 1990**.

Bundesministerium für Gesundheit: Empfehlungen zur Minderung des Infektionsrisikos durch Zoonosenerreger und sonstige Erreger von Tierinfektionen bei der Herstellung von Arzneimitteln (Zoonosen-Empfehlung) vom 15. August 1991. Bundesanzeiger Nr. 164, S. 6120 vom **3. September 1991**.

Council of the European Communities: Council Directive 93/42/EEC of 14 June 1993 concerning medical devices. Official Journal of the European Communities No. L 169/1 of **12 July 1993**.

Bundesgesundheitsamt/Robert Koch Institut für Infektionskrankheiten: Bekanntmachung der Sicherheitsanforderungen an Arzneimittel aus Körperbestandteilen von Rind, Schaf oder Ziege zur Vermeidung des Risikos einer Übertragung von BSE bzw. Scrapie vom 16. Februar 1994. Bundesanzeiger Nr. 40, S. 1851 vom **26. Februar 1994**.

Bundesgesundheitsamt, Pressedienst: Hohe BGA-Sicherheitsanforderungen an Arzneimittel, Massnahmen zur Verminderung des BSE-Risikos: Referat Presse und Öffentlichkeitsarbeit 10/1994 vom **28. Februar 1994**.

Bundesministerium für Gesundheit: Empfehlungen für Sicherheitsanforderungen an kosmetische Mittel aus Körperbestandteilen von Rind, Schaf oder Ziege zur Vermeidung des Risikos einer Übertragung von BSE bzw. Scrapie vom 09. Mai 1994. Bundesanzeiger Nr. 96, S. 5500 vom **25. Mai 1994**.

Bundesgesundheitsamt: Bekanntmachung über die Registrierung, Zulassung und Nachzulassung von Arzneimitteln (Sicherheitsanforderungen an Arzneimittel aus Körperbestandteilen von Rind, Schaf oder Ziege zur Vermeidung des Risikos einer Übertragung von BSE bzw. Scrapie) Aufruf Nr. 1 zum Einreichen von wissenschaftlichem Erkenntnismaterial vom 6. Juni 1994. Bundesanzeiger Nr. 116, S. 6567 vom **24. Juni 1994**.

Bundesministerium für Gesundheit: Verordung über fleischhygienische Schutzmassnahmen gegen die Bovine Spongiforme Enzephalopathie (BSE-Verordnung) vom 3. Februar 1995. Bundesanzeiger Nr. 25, S. 1061 vom **4. Februar 1995**.

Bundesinstitut für Arzneimittel und Medizinprodukte: Bekanntmachung über die Zulassung und Registrierung von Arzneimitteln – Abwehr von Arzneimittelrisiken, Stufe II (Zulassungs- oder registrierungspflichtige Fertigarzneimittel, die in die Zuständigkeit des Bundesinstituts für Arzneimittel und Medizinprodukte fallen und Bestandteile aus Gehirn, Rückenmark, Auge, Milz, Tonsillen, Lymphknoten, Illeum, proximalem Colon, Hypophyse, Zirbeldrüse, Dura mater, Cerebrospinalflüssigkeit, Nebennieren, Plazenta oder Peritoneum vom Rind enthalten) vom 25. September 1995. Bundesanzeiger Nr. 210 vom **9. November 1995**.

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The Challenge for the Public Health System

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Creutzfeldt-Jakob disease (CJD) and the transmissible spongiform encephalopathies (TSEs) of animals are characterized by very long asymptomatic incubation periods. There is currently no applicable preclinical diagnostic test, and once symptoms develop there is as yet no treatment. If a suspected transmission route is blocked, no effect on numbers of cases can be expected for many years; for example while the ban on feeding ruminant-derived protein to cattle was introduced in July 1988 to stop the transmission of bovine spongiform encephalopathy (BSE), the peak incidence of disease was towards the end of 1992 and the beginning of 1993. These are the challenges of the TSEs to public health and medical practice.

Properties of Transmissible Spongiform Encephalopathies

While there is controversy over almost every aspect of the details of TSEs, there is a consensus on a number of general features. Firstly, TSEs involve a slowly progressing non-inflammatory neurological degeneration, and once symptoms develop they are invariably fatal. The disease has features of both genetic and infectious disease; TSEs are clearly transmissible, as was shown in the 1940s when a vaccine against the sheep disease Louping Ill, prepared from the spinal cords of infected sheep, transmitted scrapie at high frequency [1]. On the other hand, there is equally clearly a strong genetic element to the diseases; CJD can occur in humans in a familial form with 100% penetrance, and flocks of sheep of certain genotypes are particularly susceptible to the development of scrapie [2]. Despite this, the diseases are immunologically silent; no antibody or

Table 1. TSEs

Disease	Natural host
Scrapie	sheep and goats
Transmissible mink encephalopathy	mink
Chronic wasting disease	mule, deer and elk
Bovine spongiform encephalopathy	cattle
Feline spongiform encephalopathy	cats
Exotic ungulate encephalopathy	Kudu, nyala, oryx, gemsbok, eland
Kuru	humans – Fore tribe
Creutzfeldt-Jakob disease	humans
Gerstmann-Sträussler-Schinker syndrome	humans
Fatal familial insomnia	humans

cellular immune response has been observed in animals or humans incubating the disease and the clinical presentation is of an encephalopathy with no inflammatory response rather than an encephalitis. There is as yet no reliable surrogate marker for infection, and the only satisfactory assay system still involves measurement of infectivity in vivo. Infectivity is extremely difficult to destroy completely, being refractory to conventional autoclaving techniques and most disinfectants; autoclaving in the presence of 1 *M* sodium hydroxide or soaking in hypochlorite with adequate levels of free chlorine are currently thought to be the only certain methods of disinfection [3, this volume]. Disease is associated to a greater or lesser extent with the deposition of the normal cellular protein PrP^{C} in an abnormal isoform, designated PrP^{Sc}. The ratio between infectivity and PrP^{Sc} deposition depends on the strain of the agent concerned, of which there may be more than twenty in the case of sheep scrapie. Each strain has its own distinct and inheritable properties, including pathogenesis and susceptibility to inactivation procedures. Finally, infectivity is found in the CNS, especially the brain, but is not confined to it, also appearing in lymphoid tissues, depending on the strain and model considered.

Types of TSEs of Concern

The commonly considered TSEs are listed in table 1. Kuru was transmitted as a result of certain funerary practices of the Fore tribe of Papua New Guinea, and BSE was transmitted at the height of the UK epidemic by feeding the rendered carcasses of infected cattle to cattle. Neither of these mechanisms

is a natural means of maintaining infection in populations as conventionally understood. The only TSEs shown in table 1 that are known to be naturally self-sustaining are scrapie of sheep and chronic wasting disease of mule deer and elk. The remainder present as diseases restricted to the affected animal or human unless experimentally transmitted by artificial routes.

It is impossible to be sure that an infectious disease will not change its host range or other properties; for example, BSE is believed by most workers to have originated from sheep scrapie although it is clearly distinct from known strains in its properties. However, there is no evidence that classical scrapie of sheep and goats is capable of transmission to humans as CJD. The incidence of CJD does not follow exposure to sheep-derived materials or the presence of scrapie in a country, being surprisingly uniform throughout the world at about 1 case per million head of population per year [4]. Not all TSEs are therefore of major proven importance to human health. The cause of sporadic CJD is not known, although it is believed to be noninfectious, while familial CJD and Gerstmann-Sträussler-Schinker syndrome are both genetic in origin rather than transmissible. Both forms are relatively rare, so that the problems they pose are those of a distressing clinical illness rather than a major public health problem. However, one or more of these diseases was responsible for the occurrence of iatrogenic CJD transmitted by surgical instruments, corneal transplantation, dura mater or hormones derived from human cadaveric pituitaries (see table 6 in Zerr and Poser [5], in this volume).

The most effective strategy to prevent iatrogenic transmission has involved alternative or highly selective sourcing. For example, in the United Kingdom dura mater is no longer used, having been replaced in neurosurgical procedures by autologous fascia, and growth hormone is now produced by recombinant DNA technology rather than from cadaveric pituitaries. However, dura mater continues to be used in the USA and there is no substitute for human cornea. Safety is sought by careful examination of the donors to ensure freedom from neurological disease, and in the case of dura mater, the use of unpooled preparations of raw materials then subjected to particular processes.

The first case of the variant form of CJD termed vCJD was identified in 1995, and all cases of vCJD examined so far have the same strain characteristics as BSE and strains derived from BSE either experimentally by laboratory passage or by feeding material from infected cattle to domestic cats or zoo animals [6, 7]. The conclusion that the agent of BSE and of vCJD are the same seems inescapable, and the most likely explanation for the occurrence of vCJD is that it resulted from exposure of the victims to BSE by some route, probably oral. Thus BSE is believed to be transmissible to humans in a way in which scrapie is not, and moreover human exposure to beef and beef products in

various forms is far greater than the corresponding exposure to ovine material; consequently, BSE is a major concern for human health.

By the same token, vCJD is a major concern as it is a new disease, and would almost certainly be more readily transmitted to humans than BSE by the same route.

Finally, in the UK and elsewhere during the BSE epidemic, ruminant derived protein was used as a feed supplement for sheep. BSE can be transmitted to sheep experimentally by the oral route where the tissue distribution has been shown to be the same as in classical scrapie. Consequently, the theoretical possibility exists that BSE has been introduced into the general sheep population, where it might be expected to be self-sustaining, as is scrapie, while retaining the strain characteristics of BSE, including the ability to transmit to humans. Scrapie is now considered by some as a potential cause for concern, although transmission of BSE to sheep in an agricultural rather than experimental setting has not been demonstrated.

Minimizing the Risks of Transmission of Spongiform Encephalopathies by Medicinal Products

In 1991, the Committee for Proprietary Medicinal Products (CPMP) of the European Union issued a Note for Guidance on Minimizing the Risks of Transmission of BSE by Biological Medicinal Products for Human Use. After the first public recognition of vCJD in 1996, the document was revised somewhat, although the principles on which the first document was based were considered to be sound. The strategy chiefly involved sourcing from animals not at risk from BSE because they came from BSE-free countries, and secondly from tissues expected to have little or no risk of infectivity. Thus brain was regarded as a potentially highly dangerous tissue while serum was not, based on early studies of scrapie in sheep [8]. In addition, while it was recognized that the agents are difficult to destroy, it was considered that the manufacturing process could contribute to safety by removal or inactivation of infectivity. Studies to demonstrate the effect of the process are however difficult to perform convincingly. The revised document also indicates that other TSEs, specifically those of ruminants are of concern.

Objections to the approach taken could include the degree of certainty with which any of the strategies can be documented. The definition of a country as BSE free has been given by the Organisation Internationale des Epizooties (OIE) and the Scientific Steering Committee (SSC) of DG24 of the European Commission, but there are major debates surrounding the adequacy of surveillance and the control of feeding practices required. There are also problems associated with the movement of cattle and their traceability; for example the

producers of Brain-Heart Infusion broth used in bacterial culture were unable to identify the country of origin of the source animals with certainty. Similarly, the classification of tissues used can pose problems where there is a possibility that a low risk tissue such as bone can be contaminated by a high risk tissue such as brain or spinal cord. The difficulties of relying on the manufacturing process unless it involves total chemical degradation are obvious from the hardy nature of the agents. Consequently, products have tended to be considered on a case by case basis.

The issues raised by BSE in particular are very broad, covering a variety of foods and medicines, and the responsibilities for regulation are equally widespread. This has led to problems where solutions have been proposed in one area which have caused enormous difficulties in others. An example was the Commission Decision 97/534/EC to ban the use of all specified risk materials (SRMs) from use for any purpose. SRMs included the skulls and spinal cords of cattle, sheep and goats as well as certain lymphoid tissues of sheep and goats. This approach made good sense from the agricultural point of view, and would have bypassed most of the difficulties associated with uncertainties of geographical sourcing, but it caused great difficulty for the pharmaceutical industry which uses large amounts of tallow derivatives such as magnesium stearate. Tallow derivaties are in fact produced by harsh chemical treatments capable of inactivating TSEs and are therefore considered safe despite the possible inclusion of SRMs in their starting materials. The regulatory approach has evolved subsequently, and continues to do so.

Implications of the Variant Form of Creutzfeldt-Jakob Disease: Blood and Blood Products

Blood and its cellular components are used in a number of life-saving medical interventions, and blood-derived proteins, such as albumin, immunoglobulin and clotting factors such as factor VIII and factor IX are used to treat patients who would otherwise have a very poor life expectancy. Studies on the pathogenesis of scrapie in sheep [8] and in a variety of animal models lead to the conclusion that there is a stage of the disease outside the nervous system, specifically involving the immune system although the cell type remains controversial [9, 10]. In a number of animal models and in scrapie itself, infectivity has been identified in blood [11]. Experiments to detect CJD infectivity in human blood have been equivocal and epidemiological studies of recipients of blood and blood products have failed to identify any demonstrable risk [11]. It remains possible that infectivity is found in the blood of classical CJD victims, but at such a low level, or in so few donors that the effect is undetectable by

epidemiological or experimental methods. Those at risk of CJD because of a family history or previous medical treatment are therefore excluded from donation in both Europe and the USA. However, there was until recently some disagreement about the action to be taken should it be found after donation that an individual was in fact at risk or developed classical CJD. In the USA, the decision taken was that all products to which such a donor contributed should be recalled as a precautionary measure. While very few donors were involved every year, this caused major disruptions of supply, because a single donation contributes to many products. Up to 20% of some products were the subject of recall. In Europe, the reasoning was that there was no evidence that blood or blood products have ever transmitted infection, so that any risk must be extremely small, that in view of the assumed prolonged incubation period of the disease if there were infectivity in the blood, considerably more is likely to come from asymptomatic than symptomatic individuals, so that withdrawal would not in any case contribute to safety significantly, and that the problems of supplying products would cause clinically unacceptable failures to treat patients. Withdrawal after the event was therefore not undertaken in Europe, and the current USA policy has now been modified to essentially the same approach. However the situation with vCJD could be entirely different. It is known that the abnormal isoform of PrP can be detected more easily outside the CNS in lymphoid tissue in vCJD than in classical CJD so that levels in the blood could be higher. Furthermore estimates of the size of the epidemic to be expected in the UK range up to over 100,000 [12] so that exposure could be far higher than with the classical form. Consequently, it was decided to stop the production of blood products from UK plasma, although the use of UK-sourced blood and cellular components would continue as they could not be supplied from other countries.

A second decision made by the United Kingdom government has been to introduce leucodepletion to remove white blood cells from the components used, as a means of reducing infectivity. While the factual basis underlying this decision could be questioned [13], there are likely to be other benefits of leucodepletion which also justify it. The steps taken to counter the possible risk of transmission of vCJD by blood or blood products are very costly. There remains some debate as to whether they are either necessary or effective, a problem which is associated with many aspects of the public health impact of TSEs.

Implications of the Variant Form of Creutzfeldt-Jakob Disease: Other Medical Interventions

The peripheral distribution of the abnormal form of PrP, which is believed by many to be the infectious entity itself raises questions concerning surgical

interventions. It is already well known that classical CJD can be transmitted by neurological instruments such as electrodes, and that they cannot be adequately sterilized by standard methods. Only disposable instruments of this type are therefore used. However, if vCJD infectivity is in fact widely disseminated throughout the infected but asymptomatic individual, it implies that instruments used in more general surgery could also be able to transmit infection. Many such instruments are not readily disposable. Investigations to address the problem include studies of sterilizing cycles; for example autoclaving in the presence of 1 *M* sodium hydroxide is likely to be effective, although its effect on the instruments may be severe (see also Beekes et al., p 117–135 in this volume).

The Future

Many of the problems surrounding the TSEs come from the absence of a satisfactory preclinical diagnostic which can be used in the short term to establish the size of the problem and the effect of steps taken to solve it. There is a great deal of work in progress to develop diagnostic methods applicable to readily obtained specimens. Most are based on attempts to detect the abnormal form of PrP, ultimately in blood [14]. The approach assumes that PrP^{Sc} and infectivity will be found in the blood of preclinical cases, and will require an assay of very high sensitivity, probably more sensitive than an infectivity assay. A final question concerns the ethics of testing individuals for a disease for which there is no treatment and which is believed to be invariably fatal. Similar ethical problems arose in testing for HIV, and they can be addressed.

A second issue is the possible development of treatments or prophylaxis. Once symptoms associated with neuronal destruction have developed it may be difficult to reverse them, and until symptoms have developed there is currently no evidence of infection. Nonetheless, there are a number of approaches which have been applied in animal models, including the use of dextran sulphate, and the related pentosan sulphate has been proposed as a human treatment [15]. The obstacles are that it is not known what dose by what route, given how soon after infection and for how long will be required to protect a human subject. The potential treatments are also likely to have effects: pentosan sulphate is an anticoagulant which may give problems if taken over a long period. The development of such treatments would clearly be facilitated by the simultaneous development of diagnostics which could monitor their effects on the incubation of CJD development.

In summary, the challenges to public health posed by TSEs are complex, and arise from the nature of the agents concerned and the difficulties of diagnosis, disinfection and treatment they present. Procedures are in place which

can be expected to minimize possible transmissions to humans, but the scale of the problem will only be clear when the current efforts to develop a better understanding of the diseases and their diagnosis bear fruit.

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