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Preface

Prion disease is a lethal degenerative disorder of the central nervous system, infecting humans and animals. The disease has been of scientific interest because protein itself was thought to be an infectious agent. Now prion disease is becoming a social issue as well because of the bovine spongiform encephalopathy (BSE) epidemic and the outbreak of variant Creutzfeldt-Jakob disease (vCJD) in the United Kingdom.

BSE has begun to attack humans by an oral route, through food. But now we must also think about a second line attack of BSE, i.e., infection via transfusions. In addition to transfusions, we must be concerned about the safety of therapeutic drugs and medical practices against BSE.

As editor, I have compiled this book mainly from papers presented at the meeting of the International Symposium of Prion Diseases for Food and Drug Safety, held October 31–November 2, 2004, in Sendai, Japan. Sendai is a city that is historically associated with the prion hypothesis. The International Congress of Virology was held there in 1984. The 1984 meeting was the first time that there was intense discussion about whether the etiologic agents of transmissible spongiform encephalopathy (TSE) are prions or scrapie-associated fibrils (SAF). In this 2004 symposium, invited speakers comprised those at the cutting edge of current prion research who had not taken part in the 1984 arguments. These scientists thus represent the second generation of prion researchers. One of the purposes of this symposium was to spotlight a third generation of prion researchers whose work can continue over the next two decades.

After our very productive meeting, I was saddened to hear that Dr. Elizabeth Williams had suffered an accident. At the symposium, Dr. Williams had made a valuable contribution in her report on chronic wasting disease (CWD). I offer my prayers and best wishes for Dr. Williams and her husband.

In closing, I express my deep appreciation for the support of the Japan Intractable Diseases Research Foundation in the publication of this book.

Tetsuyuki Kitamoto, M.D.

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Nikolai G. Rainov	University of Liverpool, UK

Acknowledgment

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Contents

Pathology of Variant Creutzfeldt-Jakob Disease

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Summary. Variant Creutzfeldt-Jakob disease (CJD) is a novel form of human prion disease that appears to result from oral infection by the bovine spongiform encephalopathy (BSE) agent. Variant CJD is also unique in human prion diseases in that infectivity and accumulation of the disease-associated isoform of prion protein are readily detectable outside the central nervous system, perhaps reflecting the peripheral pathogenesis of this disorder following an oral infection with BSE. The neuropathological features of variant CJD are unique in terms of the histological features and the biochemical features of the abnormal isoform of prion protein in the brain and in lymphoid tissues. This peripheral accumulation of infectivity has also resulted in the apparent iatrogenic transmission of variant CJD on 2 occasions, following a transfusion with non-leucodepleted red blood cells from donors who subsequently died from variant CJD. All clinical cases of variant CJD have so far occurred in individuals who are methionine homozygotes at codon 129 in the prion protein gene. However, one of the iatrogenic infections occurred in an individual who is heterozygous at this locus, indicating the future clinical cases might also occur in this group. Continuing surveillance of all forms of CJD is required to address this possibility, not just in the UK but in other countries either with or at risk of cases of BSE in the cattle population.

Key words. neuropathology, variant CJD, prion protein, immunocytochemistry, biochemistry

Introduction

Transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders occurring in mammals, which include scrapie in

sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk and Creutzfeldt-Jakob disease (CJD) in humans [1,2]. Prion diseases are associated with conversion of the normal isoform of prion protein (PrP^{C}) in the brain to an abnormal disease-associated isoform (PrP^{Sc}). PrP^{Sc} has a different conformation from PrP^{C}, with a higher beta-sheet content. PrP^{C} is also relatively resistant to proteolytic degradation; this property is used to distinguish the two isoforms of the protein on Western blot analysis. The prion hypothesis states that the transmissible agent in these disorders (the prion) is composed entirely of PrP^{Sc} and is devoid of nucleic acid [2].

Since CJD was initially described in the 1920s, an ever- widening spectrum of human prion diseases has been reported (Table 1) [1]. This includes sporadic, familial and acquired diseases, the commonest of which is the sporadic form of CJD. The naturally occurring polymorphism at codon 129 of the prion protein gene (*PRNP*) influences susceptibility to sporadic CJD (Table 2). In comparison with normal population there is an excess of homozygotes at codon 129 in the *PRNP* in sporadic CJD (particularly methionine homozygotes), with a reduction in the percentage of heterozygotes [3,4]. The neuropathological phenotype of sporadic CJD is variable and appears to be influenced by the isotype of PrP^{Sc} in the brain as determined by Western blotting studies and the *PRNP* codon 129 genotype [4,5].

Table 1. Classification of human prion diseases

Idiopathic:	Sporadic Creutzfeldt-Jakob disease
	Sporadic fatal insomnia
Inherited:	Gerstmann-Sträussler-Scheinker syndrome and variants
	Familial Creutzfeldt-Jakob disease
	Fatal familial insomnia
Acquired:	Human source: Iatrogenic Creutzfeldt-Jakob disease
	Kuru
	Bovine source: Variant Creutzfeldt-Jakob disease

Table 2. Codon 129 PRNP polymorphisms in CJD and normal Caucasian population in the UK

Codon 129 Polymorphism	Methionine/methionine	methionine/valine	valine/valine
Normal	37%	51%	12%
Sporadic CJD	66%	17%	17%
Variant CJD	100%	-	-

Surveillance of CJD in the UK was reinstated in 1990 following the identification of a novel prion disease in cattle, known as BSE or "mad cow disease". BSE was first reported in 1987 [6], and early epidemiological studies indicated that it was spread by contaminated meat and bone-meal animal feed [7]. A ban on the use of this feed allowed the disease to come under control, but it has still not been eradicated in the UK and has occurred in many other countries [8]. Around 180,000 clinical cases of BSE have been identified in the UK [8], but the total number of infections (including animals slaughtered in the preclinical stage of the illness) is likely to have been much higher [9]. The recent widespread use of BSE testing of slaughterhouse cattle Europe has identified cattle infected with BSE, but had not exhibited any clinical symptoms prior to slaughter. Until the identification of BSE, there was no evidence that other prion diseases occurring in animals (particularly scrapie in sheep) were pathogenic to humans. However, the observation that BSE had transmitted by the oral route to other species (including domestic and wild cats and antelopes [8]) renewed concerns that it might represent a hazard to human health by the consumption of BSE-contaminated meat products.

All cases of suspected CJD in the UK are referred to the National CJD Surveillance Unit in Edinburgh by clinicians and pathologists. These cases are investigated and subject to detailed clinical and neuropathological assessment whenever possible. In 1996, the National CJD Surveillance Unit in the UK described a novel form of human prion disease in series of 10 patients; this disease has subsequently become known as variant CJD [10]. By the end of January 2005, 154 cases of variant CJD had been identified in the UK on the basis of clinical criteria and/or neuropathology. In contrast to sporadic CJD, the clinical and neuropathological features of variant CJD are relatively uniform. The clinical features of variant CJD are described in the chapter "Clinical Aspects of Variant CJD" by R. Knight in

this volume. The neuropathological features of variant CJD are summarised in Table 3 and discussed in detail below.

Neuropathology of Human Prion Diseases

Human prion diseases are characterised neuropathologically by spongiform change, neuronal loss, glial proliferation, and (in some cases) amyloid plaques [3]. A histological diagnosis of prion disease can be confirmed by employing techniques to identify PrP^{Sc} in the brain by Western blotting, paraffin-embedded tissue (PET) blotting, or immunocytochemistry. Western blot analysis on fresh or frozen brain tissues and immunocytochemistry on paraffin sections of the brain are the commonest techniques used to identify PrP^{Sc} [4,11,12]. Since all generally available antibodies to PrP recognise both the PrP^{C} and PrP^{Sc}, a limited protease digestion (usually with Proteinase K) is required to degrade PrP^{C}, leaving the partially digested PrP^{Sc} to react with the antibody [4,5].

Immunocytochemistry allows the identification of different patterns of PrP accumulation in the brain in prion diseases [11], which have enabled the identification of different pathological subtypes of sporadic CJD [4]. In most human prion diseases there is little evidence that PrP^{Sc} is present in tissues outside the brain, although this has been demonstrated recently in the spleen and skeletal muscle in a subset of patients with sporadic CJD [13]. In contrast, PrP accumulation and infectivity in lymphoid tissues is readily detectable in other prion diseases, particularly scrapie in sheep [14], and this feature is an important part of the pathology of variant CJD.

Neuropathology of variant CJD

The diagnostic neuropathological features of variant CJD are summarised in Table 3.

Macroscopic features

Macroscopic examination of the brain in variant CJD shows no specific abnormalities. Both cerebral and cerebellar cortical atrophy may occur in cases with a prolonged clinical history (usually 2 years or longer), but these features may be absent in cases with a short clinical duration of illness [15]. The central white matter, basal ganglia, thalamus, hippocampus, hypothalamus, brain stem, spinal cord and cranial nerves show no features

of note. Mild or moderate dilatation of the ventricular system may be found as a secondary phenomenon in cases with cerebral cortical atrophy.

Table 3. Diagnostic neuropathological features of variant CJD

1. Multiple florid plaques in H&E sections of the cerebral and cerebellar cortex; numerous small cluster plaques in PrP stained sections of these regions, along with amorphous pericellular and perivascular PrP accumulation

2. Severe spongiform change; with perineuronal and linear periaxonal PrP accumulation in the caudate nucleus and putamen

3. Marked neuronal loss and astrocytosis in the posterior thalamic nuclei (particularly the pulvinar) and midbrain

4. Perineuronal and synaptic PrP accumulation in the grey matter of the brainstem and spinal cord

5. PrP accumulation around follicular dendritic cells and macrophages within germinal centres in lymphoid tissues throughout the body

6. Predominance of di-glycosylated PrP^{Sc} on Western blot examination of central nervous system and lymphoid tissues

Microscopic features

In the cerebral cortex, spongiform change occurs in a microvacuolar pattern in a widespread, often in relation to amyloid plaques, although all layers of the cortex may be involved. The occipital cortex is most severely affected. The entorhinal cortex may show patchy microvacuolar spongiform change, but this is usually absent n the hippocampus. In contrast, the caudate nucleus and putamen are severely affected by spongiform change, which is often confluent and not apparently related to the distribution or number of amyloid plaques in these nuclei. Focal spongiform change is usually present in the globus pallidus, hypothalamus and most of the thalamic nuclei. The posterior thalamic nuclei (particularly the pulvinar) are usually spared, or at the most show only mild patchy spongiform change. Mild spongiform change is also detected in the periaqueductal grey matter in the midbrain and in the pontine nuclei. The cerebellar cortex is variably affected by spongiform change, which is occasionally confluent and often associated with amyloid plaques, neuronal loss gliosis and cortical atrophy, especially in cases with a lengthy clinical history.

In the cerebral cortex, neuronal loss is generally most severe in the primary visual cortex within the occipital lobe. There is a severe loss of neurones and astrocytosis throughout the cerebral cortex in cases with a lengthy clinical history, but the hippocampal neurones are usually well preserved. Neuronal loss in the basal ganglia is most evident in cases with severe and confluent spongiform change. However, in the thalamus, neuronal loss and astrocytosis are most severe in the posterior nuclei, particularly in the pulvinar [16]. These features are not conspicuous in the brainstem and spinal cord, but occur in the cerebellum, and usually most severe in the vermis in cases with a lengthy clinical history, resulting in cerebellar cortical atrophy.

The most distinctive neuropathological feature of variant CJD is the large fibrillary amyloid plaques in the cerebral and cerebellar cortex, known as florid plaques (Fig. 1a), which are defined as a fibrillary amyloid structure with a dense core surrounded by a pale region of radiating fibrils, and surrounded by spongiform change in an otherwise intact neuropil [10]. Florid plaques can also be identified using periodic acid/Schiff and Alcian blue stains and are strongly stained by the Gallyas silver technique [15]. Florid plaques occur in all layers of the cerebral cortex, but are most numerous at the bases of the gyri. They tend to be present in largest numbers in the occipital and cerebellar cortex (particularly in the molecular layer). Fibrillary amyloid plaques can also be identified in the granular layer of the cerebellum, but without surrounding spongiform change.

Ultrastructural studies of the amyloid plaques in variant CJD have demonstrated masses of radiating fibrils at the periphery of the plaques, with abnormal neurites similar to those seen in Alzheimer's disease [17]. Paired helical filaments and neurofibrillary tangles are not present in variant CJD, and immunocytochemistry for tau gives negative results. PrP accumulation at the ultrastructural level has been demonstrated by immunocytochemistry in plaque amyloid fibrils and some abnormal cell membranes surrounding the plaques [18].

Immunocytochemistry for PrP

The florid plaques in the cerebral cortex and cerebellum give an intense positive reaction on immunocytochemistry for PrP (Fig. 1b) [15]. Smaller "cluster plaques" (which cannot be identified in sections stained by haematoxylin and eosin) are also revealed by immunocytochemistry for PrP in these regions, along with a widespread amorphous pericellular deposition of PrP around glial cells and small neurones.

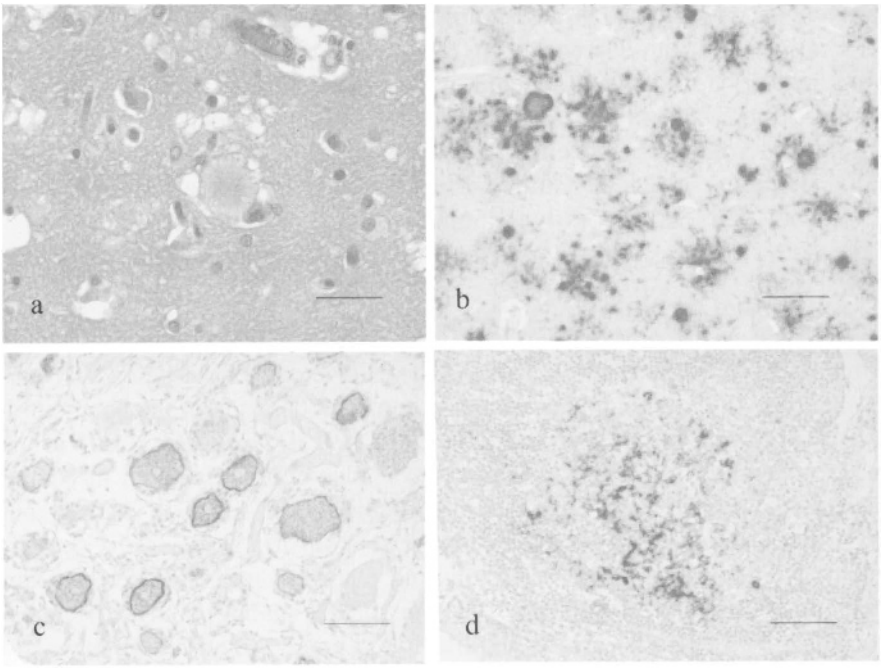

Fig. 1a-d. a. A florid plaque in the occipital cortex in a 20 year old male with a 20-month clinical history of variant CJD. The plaque has a dense eosinophilic core with a pale fibrillary periphery and is surrounded by a rim of spongiform change. Scale bar is 25μm. b. Immunocytochemistry for PrP in the occipital lobe in variant CJD (same case as Fig. 1a) shows intense labelling of the florid plaques, but also (KG9 anti-PrP antibody). Scale bar is 50 μm. c. Accumulation of PrP is revealed by immunocytochemistry in sensory ganglion cells within the dorsal root ganglia in variant CJD (6H4 anti-PrP antibody). Scale bar is 25 μm. d. Immunocytochemistry for PrP shows labelling of follicular dendritic cells within a germinal centre within the tonsil from an autopsy case of variant CJD. This finding is also present in tonsil biopsies on patients with variant CJD (12F10 anti-PrP antibody). Scale bar is 50 μm.

In the basal ganglia, there is a predominantly perineuronal pattern of PrP immunoreactivity which is often linear and apparently periaxonal. A synaptic pattern of PrP accumulation with occasional plaques is detected in the thalamus, but the linear pattern of PrP accumulation is usually absent. There is a dense synaptic accumulation in the dentate fascia, in the hippocampus and also in the subiculum and entorhinal cortex. PrP positivity in a synaptic pattern is present in the brainstem and spinal cord in the grey

matter, particularly in the substantia gelatinosa. The leptomeninges (including the arachnoid granulations) and dura mater give a negative reaction for PrP on immunocytochemistry.

The severe astrocytosis in the posterior thalamic nuclei is best demonstrated on immunocytochemistry for glial fibrillary acidic protein [16]. This technique also demonstrated astrocytosis in other areas of severe neuronal loss, and less frequently around the margins of florid plaques.

Quantitative pathology

Quantitative histological studies on the first cases of variant CJD confirmed that the measurable accumulation of PrP deposits in the cerebellum was far greater than in sporadic CJD cases [19]. The severe gliosis in the pulvinar within the posterior thalamus was also confirmed, with measured levels of astrocytosis far in excess of sporadic CJD cases [19]. Subsequent quantitative studies have shown that the relationship between the spongiform change and the presence of amyloid plaques varies in different brain regions in variant CJD [20,21]. Analysis of the spatial patterns of abnormal PrP deposition in variant CJD has found no significant differences between different regions of the cerebral cortex [22]. Textural analysis techniques to investigate the differences in patterns of abnormal PrP deposition in the brain in variant CJD are currently under development [23].

Non-CNS tissues

PrP accumulation is identified in the retina and optic nerve, spinal dorsal root ganglia (Fig. 1c) and trigeminal ganglia in variant CJD. However, peripheral sensory and motor nerves contain no detectable PrP [24,25]. The pineal gland and the posterior pituitary gland usually show synaptic positivity for PrP, but the anterior pituitary gland shows no PrP accumulation. PrP immunocytochemistry in other main organs (including the adrenal gland, thyroid gland, parathyroid gland, skeletal muscle, bladder, testes, female pelvic organs, heart, lung, liver, kidney, oesophagus, stomach, pancreas, gall bladder, salivary gland and skin) is negative [5,15,24].

In contrast, PrP accumulation is identified around follicular dendritic cells and macrophages within many germinal centres in the tonsils (Fig. 1d) and in gut-associated lymphoid tissues in the appendix and Peyer's patches in the ileum, spleen, lymph nodes from the cervical, mediastinal, para-aortic and mesenteric regions and the thymus [5,15,26]. Quantitative studies of PrP accumulation in lymphoid tissues in variant CJD indicate that lymph nodes and the tonsil are most likely to contain a high percent-

age of PrP-positive germinal centres than the spleen or gut-associated lymphoid tissues [24].

Biochemistry

Biochemical studies of PrP^{Sc} by Western blot analysis can be used to sub-classify cases of CJD by comparing the relative abundance of the non-glycosylated, mono-glycosylated and di-glycosylated forms of the protein and measuring the mobility of the non-glycosylated form [4,15]. In sporadic CJD, two major PrP^{Sc} isoforms have been described, termed type 1 and type 2 [4] (Fig. 2). The non-glycosylated fragment of type 1 has a molecular weight of 21kDa and the non-glycosylated fragment of type 2 has a molecular weight of 19kDa. In variant CJD, the mobility of the non-glycosylated portion of PrP^{res} is similar to that of type 2 [15,24]. In sporadic CJD type 2, the mono-glycosylated form predominates and is referred to as type 2A [4,15]. However, the di-glycosylated form of PrP^{Sc} predominates in variant CJD, so the isoform is termed type 2B (Fig. 2). A similar PrP^{Sc} glycosylation pattern to that type 2B PrP^{Sc} isoform in variant CJD has been identified in BSE and in other BSE-related conditions in other species [27].

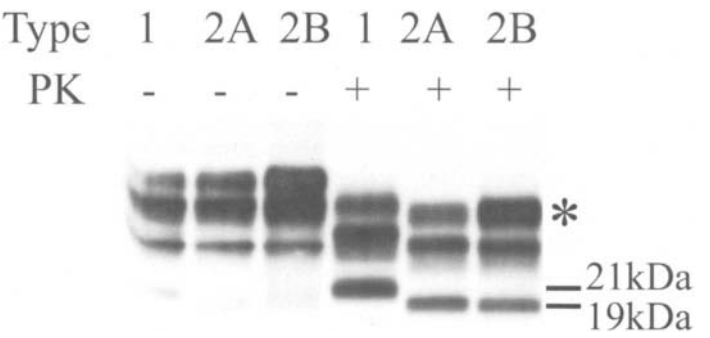

Fig. 2. Western blot analysis of prion protein in samples of cerebral cortex from two cases of sporadic CJD (type 1, type 2A) and a case of variant CJD (type 2B). The samples are shown before (-) and after (+) digestion with proteinase K(PK). The protease-resistant prion protein is classified as type 1 (21kDa non-

glycosylated lower band) or type 2 (19kDa non-glycosylated lower band). The type found in variant CJD is characterised by the predominance of the upper (*) diglycosylated band, and termed type 2B to distinguish it from that found in sporadic which is characterised by the predominance of the middle monoglycosylated band and termed type 2A.

Discussion

Variant CJD is the only human prion disease which appears to result from an acquired infection from a non-human species [27-29], namely from BSE in cattle. Experimental transmission studies have confirmed that the transmissible agent in variant CJD is very similar to the BSE agent, but different from the agent in sporadic CJD [27-29]. It is likely that most human exposure to BSE occurred by the consumption of contaminated beef products. Variant CJD is also distinct from other human prion diseases in terms of the widespread distribution of the infectious agent in the body, possible reflecting an oral exposure to the BSE agent responsible for this disorder. PrP^{Sc} has been identified by immunocytochemistry and Western blot examination in lymphoid tissues in variant CJD, and experimental transmission studies have confirmed that infectivity is present in these tissues, although at levels which are lower than in the brain [30]. This finding has caused concerns that variant CJD may be transmitted accidentally, by surgical instruments used on lymphoid tissues (such as in tonsillectomy procedures), or by blood transfusion or blood products [31].

The concerns over potential infectivity in blood in variant CJD were recently reinforced by the experimental transmission of BSE by blood transfusion in a sheep model, at a preclinical stage in the infection [14]. A case of variant CJD has been identified in the UK in an individual who received a single unit of non-leucodepleted red blood cells from a donor who had died from variant CJD 7.5 years previously, representing the first possible case of "iatrogenic" variant CJD [32]. The clinical, neuropathological and biochemical features of this case were entirely typical of variant CJD and the patient was a methionine homozygote at codon 129 in the *PRNP* gene. Recently, a second case of apparent iatrogenic transmission of variant CJD was reported in an elderly female who died with no history of neurological disease 5 years after receiving a single unit of non-leucodepleted red blood cells from a donor who subsequently died from variant CJD [33]. The recipient was a heterozygote at codon 129 in the *PRNP* gene, and showed no pathological or biochemical evidence of variant CJD in the central nervous system. However, PrP^{Sc} was identified by immunocytochemistry in the spleen and a cervical lymph node and Western blot analysis of the spleen

showed a biochemical profile similar to that in variant CJD [33]. Interestingly, PrP accumulation was not present in the tonsil or appendix, perhaps reflecting an intravenous (rather than oral) route of infection. This case also indicates that *PRNP* codon 129 heterozygotes are susceptible to infection with variant CJD, but may have a different incubation period before the onset of clinical disease.

The diagnostic pathological features of variant CJD are summarised in Table 3. It is noteworthy that the presence of florid plaques alone is not diagnostic of variant CJD, since florid PrP amyloid plaques have been described in cases of iatrogenic CJD following dura mater graft procedures [34], although the number and distribution of these lesions in the brain is more restricted than in variant CJD. The biochemical features of PrP^{Sc} in the brain in variant CJD on Western blot examination is relatively uniform [5], in contrast to sporadic CJD, where multiple PrP^{Sc} isoforms have been identified, and can co-exist even within a single case [35]. However, a similar brain PrP^{Sc} isotype to that found in variant CJD has been reported in a recent atypical case of sporadic CJD [36], reinforcing the need for the detailed study of human prion diseases by a combination of clinical, pathological, genetic and biochemical studies. As BSE continues to spread across the world, it can be anticipated that future cases of variant CJD will be identified in other countries.

Prediction of the future numbers of variant CJD cases in the UK and elsewhere remains difficult because of the uncertainties concerning the number of individuals incubating the disease and the likely incubation period, which may be influenced by genetic factors including the codon 120 polymorphism in the *PRNP* gene. Although there was earlier evidence of an increase in the incidence of the disease in the UK, this has not been sustained and the rate of increase in the number of cases is now declining [37]. A recent retrospective immunocytochemical study of PrP accumulation in a series of over 12,00 appendicetomy and tonsillectomy specimens in the UK found three positive cases, indicating that the prevalence of BSE infection in the UK population may be higher than the numbers of clinical cases of variant CJD would so far indicate [38]. This unexpected finding might possibly result from different incubation periods in different genetic subgroups at codon 129 in the *PRNP* gene and could indicate that further rises in the numbers of variant CJD cases may occur in the future. Continued surveillance for all forms of CJD is required to answer these questions, and neuropathology is a key part in the investigation and characterisation of such cases.

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Clinical Aspects of Variant CJD

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Summary. Variant CJD is one of the human prion diseases and has relatively uniform clinico-pathological characteristics, reflecting its presumed single cause and the uniform genotype of affected individuals. Typically, it presents with predominantly psychiatric features (often relatively non-specific ones suggesting depression) and any accompanying neurological features are often interpreted as part of a primarily psychiatric illness; variant CJD may be very difficult to diagnose in the early stages. Psychiatric features may remain as part of the overall picture even following the development of clear-cut neurological signs (after a median of around six months). The obviously neurological clinical picture is typically one of a rapidly progressive dementia with cerebellar ataxia, involuntary movements and painful sensory disturbances leading to death at a median of 14 months from onset. Definite diagnosis requires neuropathology but clinical diagnostic criteria have been developed allowing a very confident premortem diagnosis in the majority of cases. These criteria depend on the exclusion of other diseases, the presence of a typical clinical picture and the results of certain supportive investigations, most notably the cerebral MRI and tonsil biopsy.

Key words. CJD, variant CJD, clinical features, diagnosis

INTRODUCTION & PRION DISEASES

Prion diseases (or Transmissible Spongiform Encephalopathies) are a group of illnesses affecting animals and man unified by certain neuropathological features, transmissibility properties and the central role of the prion protein (PrP) in the disease process (Table 1) [1]. The main human form is Creutzfeldt - Jakob disease (CJD) and this is now sub classified

into four types according to causation and other characteristics: sporadic, genetic, iatrogenic and variant (Table 1).

Table 1. PRION DISEASES & CJD

1a: THE PRION DISEASES OF ANIMALS AND HUMANS

ANIMALS	HUMANS
Scrapie	Kuru
Chronic Wasting Disease	GSS & FFI
Transmissible Mink Encephalopathy	SFI
Bovine Spongiform Encephalopathy Cattle Felines Exotic Ungulates	Creutzfeldt-Jakob Disease Sporadic Genetic Iatrogenic Variant

1b: THE DIFFERENT TYPES OF CJD

TYPE	DISTRIBUTION	CAUSE
Sporadic	Worldwide	Unknown
Genetic	Many countries Certain foci (e.g. Israel)	Prnp mutations
Iatrogenic	Several countries	Medical & Surgical treatments Especially hGH & Dura Mater
Variant	Mainly UK	BSE infection in diet

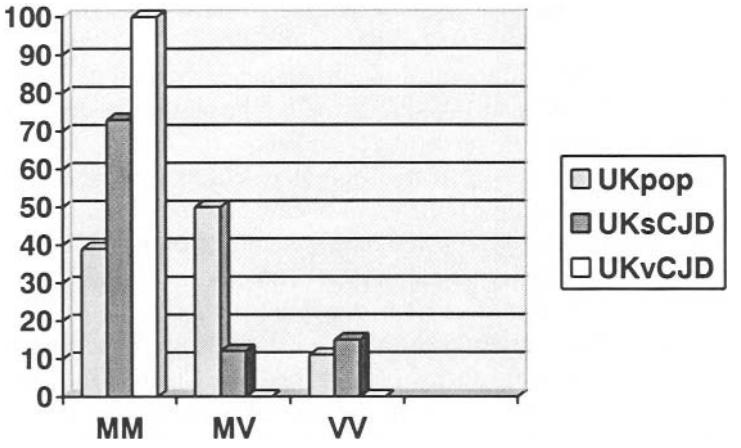
GSS: Gerstmann Sträussler Scheinker Syndrome
FFI: Fatal Familial Insomnia
SFI: Sporadic Fatal Insomnia
Variant CJD previously known as 'new variant CJD' and sometimes called 'Human BSE'

While PrP plays a vital role, the pathogenesis of prion diseases is unclear and the precise nature of the infective agent involved in transmissibility remains uncertain. Given the pivotal role of PrP (which is beyond the scope of this discussion), it is perhaps not surprising that the gene responsible for PrP (In humans, Prnp, on chromosome 20), has significant effects on the disease [1].

In the case of genetic prion disease, many underlying mutations have been reported, but the gene plays an important role in all forms of CJD [1,2]. At codon 129 of Prnp, there is a common polymorphism involving methionine (M) and valine (V) and thus the normal population is divided into three groups: homozygotes (MM or VV) and heterozygotes (MV) (Fig 1). This polymorphism has three potential effects: it affects susceptibility to CJD, it may affect incubation period in acquired forms and it may affect the clinico-pathological phenotype of the resulting disease in all forms of CJD. The effect on susceptibility is indicated by the overrepresentation of MM individuals and the under representation of MV individuals in sporadic CJD (Fig 1)[3]. Most strikingly, all cases of variant CJD with genetic analysis have proved to be MM (Fig 1). The Prnp 129 genotype distribution varies with country and there is an East-West gradient of MM genotype (Fig 1) [3,4].

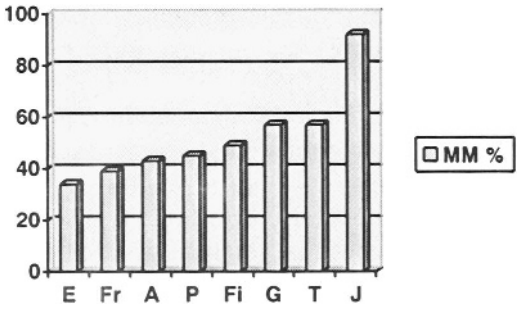
Figure 1. Prnp-129 GENOTYPE DISTRIBUTIONS

1a: UK FIGURES



Percentage distributions of Prnp-129 genotypes in the UK normal, sporadic CJD and variant CJD populations. Normal data based on 5 Caucasian studies. Sporadic CJD data from cases 1990-1999. Variant CJD data from 130 analysed cases.

1b: VARIATIONS WITH DIFFERENT COUNTRIES



E: Eire, Fr: France, A: Austria, P: Poland, Fi: Finland, G: Greece, T: Turkey, J: Japan. Percentage of normal population with Prnp-129 MM genotype.

CJD, like all prion diseases, essentially affects the central nervous system and the main clinical features reflect brain dysfunction. The underlying neuropathology is 'neurodegenerative' without features of inflammatory nature, there are no systemic features such as pyrexia and there is no antibody response. All prion diseases are progressive and ultimately fatal.

The precise clinico-pathological picture depends on a number of factors: the cause, the strain of agent, the PrP protein type and the codon 129 genotype (Table 2). There may be as of yet undiscovered disease determinants. Variation with cause may reflect different disease mechanisms (such as spontaneous versus acquired), different routes of acquisition (such as oral or intramuscular in variant CJD and hGH-related CJD respectively) and, of course, may reflect different agent strain. Agent strain is a complex issue beyond the scope of this discussion; however, there is good evidence for the existence of different strains of infective agent with different biological behaviour in transmissible forms of disease [5]. Clearly, the concept of 'agent strain' is difficult to apply in the case of illnesses that are currently thought not to be acquired (sporadic and genetic CJD) and the definite characterization of the molecular nature of 'strain' must await the definite characterization of the agent itself. PrP is classifiable into different protein types (I, IIA and IIB) on the basis of molecular size and glycosylation patterns [6]. Different PrP types are, to some extent, associated with different clinico-pathological pictures, but this is a developing complex area and the precise nature of this association is not clear. Whether or not PrP protein type can be taken as a simple surrogate for agent strain is very doubtful. However, in humans, the unique association of PrP IIB with variant CJD

(vCJD) is very helpful diagnostically. Codon 129 genotype is also associated with clinic-pathological phenotype.

The typical clinical features found in CJD are given in Table 2; the precise pattern seen in different forms and in different individuals depend on the factors delineated above.

Table 2.

TYPICAL CLINICAL FEATURES OF HUMAN PRION DISEASES AND KNOWN DETERMINANTS

DISEASE	TYPICAL FEATURES	KNOWN DETERMINANTS or ASSOCIATIONS
Human Prion Diseases	Dementia Cerebellar Ataxia Involuntary movements Pyramidal signs	Cause Route of infection Agent strain PrP protein type Prnp-129 genotype
Sporadic CJD*	Onset with rapidly progressive dementia Cerebellar Ataxia Cortical blindness Pyramidal signs Myoclonus	Prnp-129 genotype PrP protein type
Variant CJD	Onset with psychiatric features Cerebellar Ataxia Dystonia/chorea Pain/other sensory symptoms Occulomotor symptoms/signs Pyramidal signs Dementia Myoclonus	(All tested individuals have been Prnp-129 MM and the PrP protein type has been IIB)

*There is clinico-pathological heterogeneity in sporadic CJD and the known determinants of this are listed in the table.

SPORADIC CJD

Sporadic CJD (sCJD) is important in any consideration of vCJD. Aside from being the commonest form of CJD with a world-wide distribution, it is a very important differential diagnosis of vCJD. Surveillance for vCJD in any country must involve surveillance for all forms of CJD and this will be heavily based on sCJD.

sCJD is essentially a disease of the middle-aged and elderly, although young cases occur occasionally. The typical clinical picture consists of a rapidly progressive, short duration illness dominated by dementia along with other neurological features, especially cerebellar ataxia, cortical visual dysfunction and myoclonus (Table 2) [7]. The typical case has a progression that is, in fact, rather unlike that of a 'neurodegenerative' disease, and is a fulminating brain illness, sometimes progressing almost day-by-day and can lead to death over just a few weeks. Although the actual diagnosis may not be immediately made, the illness is usually obviously neurological and encephalopathic at onset and early referral to a clinical neurologist often results.

However, while around 80% of cases follow this typical, rather uniform course, there is significant clinico-pathological heterogeneity in sCJD. The clinical variation is expressed particularly, but not only, in the presenting features. Two well-recognised variations are those of Brownell-Oppenheimer and Heidenhain [7]. In the first, there is a progressive cerebellar ataxia for some weeks or more before the emergence of other problems. In the second, there is an initial progressive visual disturbance culminating in cortical blindness. In the early stages, these are difficult to recognise as sCJD and there are other, more common, possible diagnoses that need consideration and investigation. The basis of this heterogeneity of clinical onset is uncertain. If favoured current theories of sCJD are correct, suggesting it is due to spontaneous PrP conformational change or a somatic Prnp mutation, then the process has to start somewhere and different onsets may then simply reflect the anatomical site of the first pathology. The clinico-pathological variations certainly reflect, in part, codon-129 genotype and PrP protein type. A clinico-pathological-molecular classification of sCJD was suggested by Parchi et al, dividing the disease into groups based on MM, MV, VV, PrP type I and type II [8]. However, the precise significance and validity of this classification is unclear. There are some particularly difficult, but very rare, variations in sCJD, including courses characterised by a relatively slowly progressive dementia [8].

VARIANT CJD: BASIC EPIDEMIOLOGY

152 individuals have been diagnosed as having definite or probable vCJD in the UK (figure at December 2004). The mean, median and range of ages of onset of illness are 28, 26 and 12-74 years, respectively. The figures at death being 30, 28 and 14-74 years. Of the 152, 85 are male and 67 female (no statistically significant difference). Outside the UK, 14 individuals have been affected. France: 9, Eire: 2, USA: 1, Canada: 1, Italy: 1. The French, Italian and one of the Irish cases are thought to have contracted the illness in those countries; the others probably contracted vCJD whilst spending time in the UK.

It is notable that the age of onset has not changed significantly over the UK epidemic, despite a presumed limited time period of exposure to BSE infection in diet. This suggests a number of possibilities: an age-related exposure, an age-related incubation period, or, perhaps most likely, an age-related susceptibility.

The incidence of vCJD in the UK appears to have peaked around the middle of 2000 and is now declining [9]. However, it should be noted that all tested cases to date (130 in total) have been of the Prnp 129-MM genotype and there could be other peaks related to other genotypes. In addition, the two reports of possible blood transmission indicate the possibility of further secondary transmission cases [10,11].

The cause of vCJD is considered to be due to BSE infection in diet. While there is no absolute proof of the dietary theory, this is a strong theory, with good supportive evidence and with no reasonable alternative explanations for the occurrence of vCJD [5].

VARIANT CJD: CLINICAL FEATURES

The known general determinants of the clinico-pathological features of prion disease were discussed in the introduction and are listed in Table 2. The heterogeneity of sCJD has been related in part to variations in two of these factors: Prnp-129 genotype and PrP protein type. Variant CJD is thought to be due to one cause via one route (BSE in diet, with the exception of secondary blood transmission), one agent (the BSE agent), is associated with one PrP-protein type (IIB) and has affected one genotype (MM). It is, therefore, perhaps not surprising that vCJD has a relatively homogenous clinical profile and neuropathology.

The early clinical features in the first 100 cases were analysed in a study by Spencer et al [12]. While sCJD typically presents with clearly neuro-

logical features, vCJD typically presents with psychiatric or behavioural problems. This is reflected in the fact that the commonest first specialist referral in the first 100 cases was to a psychiatrist (38%). Psychiatric symptoms dominate the early phase of the illness and, although some neurological symptoms may be present, they are often relatively non-specific, often unaccompanied by abnormal neurological signs and can be taken as part of a psychiatric problem or even side-effects of psychotropic medication. Psychiatric features continue throughout the illness and 63% of the first 100 cases were referred to a psychiatrist at some point in the illness [12].

The early psychiatric features are ones relatively common in medical practice including social withdrawal, apathy, anxiety, and insomnia. More specific features such as delusions and hallucinations occur but are less common. Many individuals were initially diagnosed as depression and treated accordingly. Sensory symptoms, typically unpleasant or painful, may occur and are usually not associated with sensory signs [13]. After this initial period, clear neurological abnormalities develop, typically involving cerebellar ataxia and memory/cognitive impairment. Involuntary movements (such as chorea or dystonia) and eye movement abnormalities (sometimes with diplopia) are not uncommon. Signs of pyramidal dysfunction, such as spasticity, hyperreflexia and extensor plantar responses are common, but major specific weakness is not. Convincingly abnormal neurological signs appear at a mean of around 6 months, but can be late, occurring only at around 2 years in one case.

As the disease progresses, there is increasing gait disturbance, speech, language and swallowing dysfunction along with dementia and other neurological features leading to increasing need for care. The affected individual becomes immobile and demented, usually with myoclonus, and dies of intercurrent illness (such as bronchopneumonia) after a median illness duration of 14 months (range: 6-40).

There is an important question: Is there a characteristic psychiatric picture or a pattern of early neurological features that should make earlier clinical diagnosis possible?

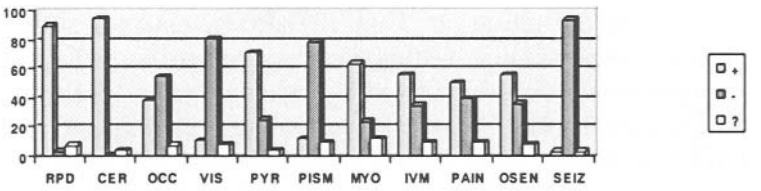
This question was specifically considered in the paper by Spencer et al, analysing the first 100 cases [12]. The authors divided the clinical features into 'psychiatric' and 'neurological' ones and then noted their frequency at different illness stages. A feature was classed as 'Common' if it affected 50% or more, 'Less Common' if it affected over 25% but less than 50% and 'Rare' if it affected less than 25%. A feature was classed as 'Early' if it first appeared in less than 4 months from onset, 'Later' if between 4 months but under 6 months and 'Late' if at 6 months or later. As far as the psychiatric picture is concerned, the 'Common Early' features were non-

specific (dysphoria, withdrawal, anxiety, irritability, insomnia, loss of interest). More 'organic' features (such as disorientation) or more unusual features (such as hallucinations or delusions) tended to occur 'Late' and/or be 'Rare'. As far as the neurological picture is concerned, there were no 'Common Early' features and only one 'Less Common Early' feature, namely pain, a rather non-specfic symptom. More specific neurological features generally occurred 'Late'. The differentiation of cognitive impairment and psychiatric dysfunction is, of course, not always easy. Looking at potentially 'organic' cognitive complaints in vCJD, 'poor memory' was a feature in 90%, 'impaired concentration' in 68% and 'disorientation' in 72%. However, while both impaired memory and impaired concentration first appeared at a mean of 4 and 5.5 months respectively, they are relatively non-specific complaints that may be features of primarily psychiatric problems (such as depression). Disorientation is arguably more indicative of an 'organic' problem but was not reported until a median of 7.5 months into the illness. Formal neuropsychological testing might be helpful. Kapur and colleagues reported an analysis of 24 cases that had had such testing during their illness [14]. Specific deficits were found (interestingly, somewhat different from those typically seen in sCJD), but the tests were generally undertaken because of a prior suspicion of cognitive dysfunction and at a relatively later stage of illness (means: 8 months after onset and 59% of illness duration).

It is difficult to conclude anything other than that the early diagnosis of vCJD is difficult and, at least in some cases, presently impossible.

As for the typical neurological picture, as the illness develops, Fig 2 shows the frequency of signs seen in 100 cases of vCJD at the time of referral to the UK National CJD Surveillance Unit (NCJDSU). This is not the frequency of signs at presentation nor the accumulative totals over the whole illness, but the general clinical picture at the time of referral in the UK surveillance system. In general, this has been around the time when the clinical neurologist has started to suspect vCJD. Dementia and cerebellar features predominate. Occulomotor problems had been present in 38%, but visual impairment (including cortical blindness) was present in only 11%. Chorea, dystonia and myoclonus were relatively common, but specifically Parkinsonian features uncommon. Sensory symptoms, including pain, affected just over half (UK NCJDSU data).

Figure 2. SELECTED CLINICAL FEATURES IN 100 CASES OF vCJD (FEATURES RECORDED AS HAVING BEEN PRRESENT AT TIME OF REFERRAL AS A SUSPECTED CASE TO UK NCJDSU)



(+ Present, - Absent, ? Uncertain or no data)

RPD: Rapidly Progressive Dementia, CER: Cerebellar ataxia, OCC: Occulomotor symptoms/signs, VIS: Other visual problems (including cortical blindness), PYR: Pyramidal features (usually hyperreflexia & extensor plantar responses), PISM: Parkinsonian extra-pyramidal features, MYO: Myoclonus, IVM: Other involuntary movements (chorea, dystonia, tremor), PAIN: Pain, OSEN: Other sensory disturbances (dysaesthesia, paraesthesisia, numbness, cold feelings), SEIZ: Seizures.

VARIANT CJD: DIAGNOSIS

The definite diagnosis of any prion disease requires neuropathological confirmation and, in humans, this usually means autopsy examination, although cerebral biopsy is occasionally performed. The clinical diagnosis of a prion disease is based on three steps: The consideration of a prion disease, the exclusion of other possible diagnoses and the results of various supportive investigations. This needs a familiarity with the features of prion diseases, a general knowledge of clinical neurology and an understanding of the roles of the supportive tests. In general, this implies the diagnosis is best undertaken by a clinical neurologist.

In the instance of vCJD, the features have been described above and the differential diagnosis is potentially wide, being discussed below. There are three tests that may provide support for the diagnosis: CSF analysis, cerebral MRI and tonsil biopsy.

The CSF should not contain an excess of cells but may show a non-specific protein elevation. A positive 14-3-3 test may be helpful but the sensitivity is less than in sCJD, being positive in only around half of vCJD cases [15]. The specificity is relatively high in UK surveillance practice

but it must be remembered that 14-3-3 is a normal neuronal protein that is released into the CSF following neuronal damage and its specificity for CJD is critically dependent on the context within which the test is undertaken. CSF tau testing has a higher sensitivity in vCJD cases (with 'positive' defined as >500pg/ml: sensitivity is around 80%) [16]. Again, it is necessary to stress that the specificity will depend on testing context (in UK surveillance: 92% for vCJD) [16]. It follows, therefore, that a positive CSF 14-3-3 (or tau) test may be helpful in supporting the diagnosis of vCJD, if appropriate alternative causes of a positive result are first excluded, however, a negative test is entirely compatible with a diagnosis of vCJD.

The EEG is particularly helpful in the diagnosis of sporadic CJD, showing (in most, but not all cases) a characteristic pattern of periodic complexes. This has not been reported in vCJD. Indeed, the EEG may be essentially normal or only mildly non-specifically abnormal even at a relatively late stage of illness. Eventually it shows deterioration in the normal background rhythms and excessive slow wave activity.

The cerebral MRI is extremely helpful. Its first role is, naturally, in the exclusion of other possible diagnoses, however it can be positively useful in that a characteristic high signal is seen the posterior thalamus in most cases. This so-called 'Pulvinar sign" is present in over 85% of cases. It is seen on T2, PD and, especially, FLAIR sequences. It is not absolutely unique to vCJD but is clearly of great supportive value in the appropriate clinical context. It is not present in all cases; there are no obvious differences between the individuals with and without this MR finding [17].

There is involvement of the reticulo-endothelial system (RES) in vCJD and a tonsil biopsy may be helpful in supporting the clinical diagnosis. A positive finding allows a high degree of confidence in the clinical diagnosis but, currently, is not taken to as support for a definite diagnosis in the absence of neuropathological confirmation. This is important as preclinical involvement of RES tissue is known to occur and subclinical involvement is at least possible [11,18].

Clinical diagnostic criteria have been developed, based on the exclusion of other diseases, the typical clinical picture and the results of tests (MRI and tonsil biopsy and they are given in Table 3 [19]. They allow diagnoses to be classified as 'definite', 'probable' or 'possible' vCJD (see Table 3). To date, all cases that have been classified as 'probable vCJD' on the basis of these criteria, and in whom neuropathological examination was undertaken, have proved to have vCJD.

Table 3.

THE ESTABLISHED DIAGNOSTIC CRITERIA FOR VARIANT CJD

I	A	PROGRESSIVE NEUROPSYCHIATRIC DISORDER
	B	DURATION OF ILLNESS > 6 MONTHS
	C	NO ALTERNATIVE DIAGNOSIS AFTER ROUTINE TESTS
	D	NO POTENTIAL IATROGENIC PRION EXPOSURE
	E	NO EVIDENCE OF GEBNETIC/FAMILIAL PRION DISEASE
II	A	EARLY PSYCHIATRIC FEATURES
	B	PERSISTENT PAINFUL SENSORY SYMPTOMS
	C	ATAXIA
	D	MYOCLONUS or CHOREA or DYSTONIA
	E	DEMENTIA
III	A	NOT EEG TYPICAL PERIODIC ACTIVITY OF sCJD (or NO EEG PERFORMED)
	B	BILATERAL PULVINAR SIGN ON CEREBRAL MRI
IV	A	POSITIVE TONSIL BIOPSY

Further details are given in Will et al [19]

DEFINITE: IA + Neuropathological confirmation of vCJD

PROBABLE: I + 4/5 of II +IIIA + IIIB
or
I + IVA

POSSIBLE: I + 4/4 of II + IIIA

VARIANT CJD: DIFFERENTIAL DIAGNOSIS

The differential diagnosis of a progressive neuro-psychiatric condition, ultimately involving cognitive impairment and involuntary movements, in a young person, is potentially wide and a full discussion is beyond the scope of this article. However, a list of conditions that have been at least initially suspected as being vCJD in the UK is given in Table 4. Clearly, some of these conditions (e.g. Wilson's disease) have relatively straightforward diagnostic tests but others, especially sCJD may present more difficulties. It may seem surprising that sCJD (characteristically a short duration illness, typically presenting with dementia in the middle-aged and elderly) represents the most important differential diagnosis of vCJD in the UK, however the two diseases clearly have features in common and there are a number of specific reasons why their differentiation is not always straightforward. Firstly, sCJD occasionally occurs in unusually young people. Secondly, there are unusual clinical presentations of sCJD, including, rarely, psychiatric or sensory symptoms. Thirdly, there are atypical clinical courses or unusually long duration illnesses in some individuals with sCJD. In this differential diagnosis of sporadic from variant forms, a number of factors may be helpful. The EEG typically shows a characteristic pattern of periodic discharges in sporadic CJD; however this is not seen in all cases and less often in the clinically atypical forms. Although all tested cases of vCJD to date have been of the Prnp-129 MM genotype, the finding of an alternative genotype could not be taken as an absolute indication that an illness is not variant CJD, especially given the recent report of an apparent transmission via blood to an MV individual (although this individual had not actually developed vCJD). The cerebral MRI is the most useful non-invasive test, the 'pulvinar sign' being an extremely helpful finding in relation to vCJD. Tonsil biopsy can be performed in particularly difficult cases and disease-related PrP has not been found in cases of CJD other than variant, using the standard laboratory methods. However, neither the absence of the pulvinar sign, nor a negative tonsil biopsy, can exclude the possibility of vCJD. In instances where real uncertainty remains, differentiation rests with histological examination of the brain and PrP protein typing. The ultimate test might be regarded as agent strain typing via animal transmission experiment, but this is obviously not a routine, nor a quick procedure.

Table 4.

DIFFERENTIAL DIAGNOSIS OF vCJD
Other conditions *initially* suspected as being vCJD in the UK

SPORADIC CJD	The most important differential diagnosis in the UK
IMPROVED or RECOVERED	A number of referred Individuals recovered or Improved with no definite diagnosis
ALZHEIMER'S DISEASE	
INFLAMMATORY CONDITIONS	Including: Limbic Encephalitis Cerebral vasculitis
WILSON'S DISEASE	
FRONTO-TEMPORAL DEMENTIA	
SUBACUTE SCLEROSING PANENCEPHALITIS	

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Dura mater related Creutzfeldt-Jakob disease in Japan: Relationship between sites of grafts and clinical features

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Summary. A nationwide survey documented 117 cases of Creutzfeldt-Jakob disease (CJD) transmitted from cadaveric dura mater grafts in Japan to September 2004. Of these, 110 patients were identified to have received the same type of lyophilized cadaveric dura mater graft during the period between 1978 and 1991. Incubation period from grafting to onset of symptoms varied from 16 months to 23 years, with most patients developing neurological symptoms after 2 to 15 years. We conducted q retrospective review of the full medical records of 107 of dura-related CJD (dCJD) patients. Patients were divided into two groups by site of neurosurgical or orthopedic procedure (supratentorial vs. infratentorial). Hemiparesis or hemianopsia developed as an initial manifestation in 31.9% of 47 patients with supratentorial grafts but did not develop among any of the infratentorial group ($p<0.0001$). Conversely, brainstem symptoms (nystagmus, diplopia, ipsilateral hearing loss, facial paresis or paresthesia) were noted in 25.0% of the infratentorial group, but were not seen in the supratentorium group ($p<0.0001$).

Key words. Creutzfeldt-Jakob disease, prion, dura mater, iatrogenic, transmissible spongiform encephalopathy

Introduction

Prion disease or transmissible spongiform encephalopathies (TSE) encompass a number of fatal neuroinfectious diseases in human and animals. Animal prion disease includes scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk, and transmissible encephalopathy in mink. In humans, prion disease includes classical spontaneous Creutzfeldt-Jakob disease (CJD), familial CJD, and infectious prion disease [1].

Sporadic CJD is the most common prion disease, accounting for approximately 85% of human prion disease. Mean age at onset is 63 years (range: 30 – 92 years). Clinical features are characterized by rapidly progressive dementia, myoclonus, and periodic synchronized discharges (PSDs) on EEG.

Infectious prion disease includes kuru in Papua New Guinea, transmitted by cannibalism, variant CJD in the United Kingdom, transmitted from BSE [2], and iatrogenic prion diseases.

Worldwide, CJD has been transmitted via cadaveric dura mater graft in 175 patients [3-5]; as a result of human pituitary growth hormone or gonadotropin in 174 [6,7]; through corneal grafting in 2 [8], and via intracerebral stereotactic EEG electrodes in 2 [9]. There have been an additional 6 cases in which the disease was thought to have been transmitted via neurosurgical procedures [9].

Dura-related CJD in Japan

Epidemiology and inactivation procedure

The first case of dura-related CJD was reported in 1987 in U.S. [10], and the number of cases has since been increasing steadily, reaching 175 in 18 countries to date. In Japan, since the first case was documented in 1991 [11], 117 cases of CJD resulting from dural graft have been identified during the period between 1978 and 1991. Most of these patients underwent grafting between 1982 and 1987 [5,12,13](Fig.1).

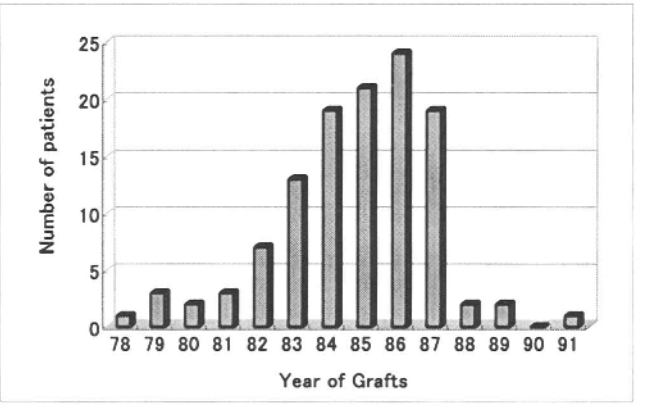

Figure 1. Number of Creutzfeldt-Jakob disease (CJD) patients vs. year of dura mater grafting

The incidence of sporadic CJD is 0.6 to 1 per million inhabitants in Japan. Based on data regarding approximately 12,000 to 20,000 grafts/year used in Japan, two thirds of imported dura mater was Lyodura, and one third was Tutoplast. Of the 117 patients who have developed dural graft-related CJD, 110 received the same type of Lyodura, a brand of dura mater graft processed by B. Braun Melsungen AG of Germany [12]. In addition, development of CJD in a patient grafted with Tutoplast has been reported in the U.S. [14]. However, in 7 patients in Japan, the brand of dura mater transplanted during neurosurgical procedures has not been identified. The period between 1984 and 1987 represented the peak transmission period of CJD via dural graft; with a very high incidence of 19 cases / 8,000 recipients to 21 cases / 13,000 recipients (i.e. 0.15 to 0.3% of neurosurgical patients receiving dura mater) [13].

A number of patients in Japan received dura mater grafts during neurosurgical and orthopedic procedures (meningioma or other brain tumors (n=36); aneurysm, subdural or intracerebral bleeding (n=30); acoustic neuroma (n=12); Jannetta's operation for decompression of facial nerve spasm or trigeminal neuralgia (n=22); Arnold-Chiari malformation (n=4); spinal tumor (n=2); and ossification of the posterior longitudinal ligament (n=1)).

Mean age of onset for dural graft-related CJD was younger (54.8±14.0 years) than for sporadic CJD (64±10 years).

Of the 107 dural graft-related CJD patients, 100 were identified to have received the same type of lyophilized cadaveric dura mater graft, Lyodura®, which was sterilized with gamma irradiation without NaOH treatment. From May 1978, the supplier began supplementing the stan-

dard sterilization procedure with immersion in1N NaOH for 1 hour. Following this change in procedure, no new CJD cases were seen among patients receiving dura mater grafts, with the exception of one patient who received a dura mater graft from another supplier [14]. However, even immersion in 1N NaOH does not completely eliminate the infectivity of cadaveric dura mater [16]. Hence, the recommendation that all existing cadaveric dura mater products be banned was finally endorsed in 1997 by both the World Health Organization and the Japanese Ministry of Health and Welfare.

While incubation period from grafting to the appearance of the first symptoms varied from 16 months to 23 years, it seems that most patients developed neurological symptoms after 2 to 15 years from the grafts (Fig.2). A long CJD incubation period was observed in a 37-year-old patient, who developed unsteady gait and dementia in 2001, 23 years after receiving a dura mater transplant. In another CJD patient in Switzerland who was suspected to have contracted the disease from a dural graft, an incubation period of more than 22 years before the onset of dementia has been reported. This patient exhibited abnormal prion protein in the brain and spleen on Western blot analysis of protease-resistant prion protein after phosphotungstate precipitation [17].

The cumulative number of confirmed cases was seen to increase concomitantly with increase in the length of the incubation period. However, the number of new CJD patients decreased markedly for incubation periods of 16 years or longer (Fig. 3).

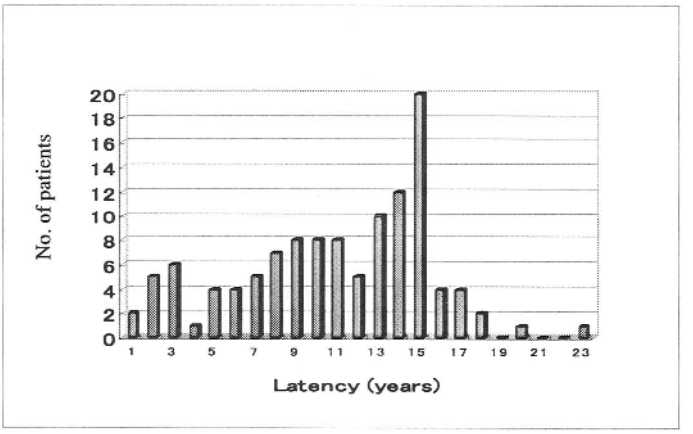

Figure 2. Incubation period from dura mater grafts to the appearance of first symptoms varied from 16 months to 23 years

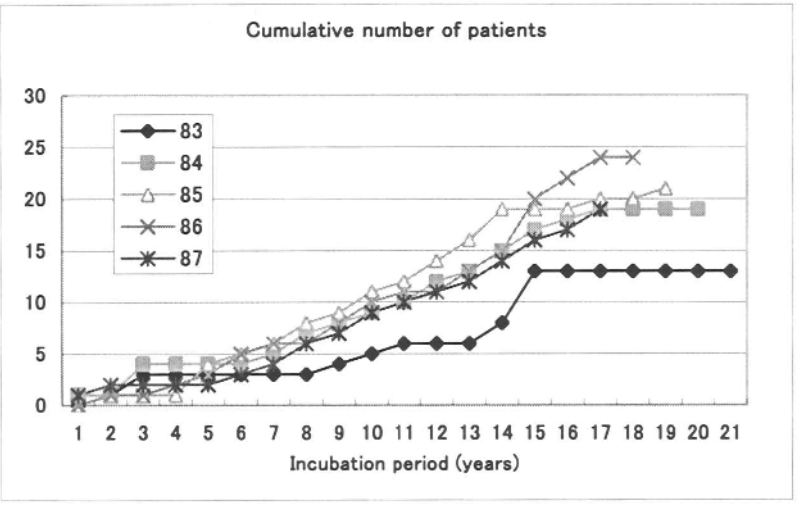

Figure 3. Cumulative number of patients vs. period from grafting to onset of symptoms

The cumulative number of patients increased concomitantly with an increase in the incubation period from 1 to 23 years. However, the number of new patients decreased markedly when the period was 16 years or over.

Atypical dura-related CJD with slowly progression

As survival period from symptom onset to death varies according to patient care, we analyzed clinical course according to the period from the onset of symptoms to development of akinetic mutism. Almost 85% of dural graft-related CJD cases exhibited a rapidly progressive clinical course with myoclonus and PSDs on EEG deteriorating into akinetic mutism within 2 to 6 months from the onset of symptoms. Immunohistochemical findings in such cases are similar to those of sporadic CJD, characterized by fine granular deposits of prion protein (termed synaptic type dural graft-related CJD). The remaining 15% of dural graft-related CJD cases exhibited showed a slow deterioration and atypical clinical features. These cases are characterized by the late occurrence or absence of myoclonus and PSDs on EEG and the development of akinetic mutism over a period longer than one year from onset (Fig.4). Neuropathological findings are characterized by the presence of many florid plaques in the brain [18,19]; hence this form is termed plaque type dural graft-related CJD.

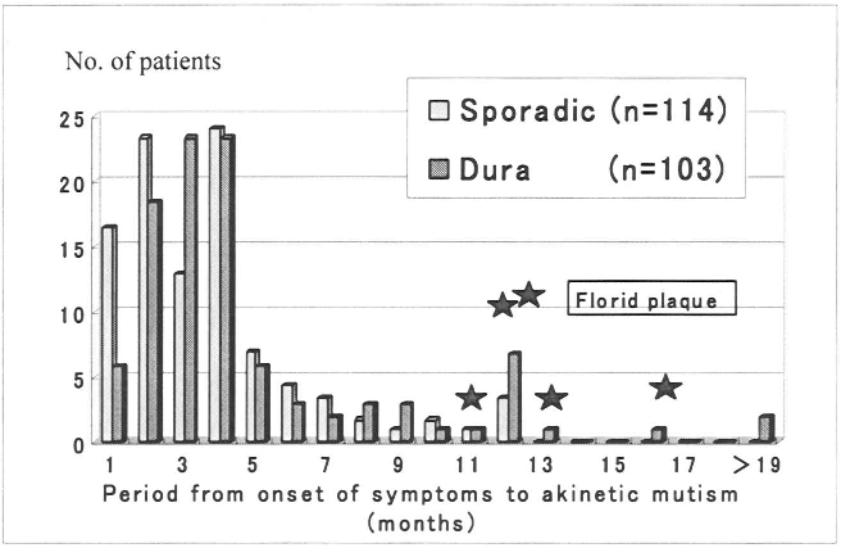

Figure 4. Period from onset of symptoms to akinetic mutism in sporadic and dura mater graft-related CJD patients

In sporadic CJD, MRI reveals marked progressive cerebral atrophy even at 8 months from the onset of symptoms. In contrast, in the slowly-progressive form of dural graft-related CJD, MRI showed very mild atrophy even after 13 months from the onset of symptoms.

Satoh et al. reported that while the major PrPSc species from plaque type and synaptic type dural graft-related CJD exhibited a 21 kDa structure after deglycosylation (type 1 PrPSc), a C-terminal PrP fragment of 11-12 kDa was associated with synaptic type-dural CJD but not with the plaque type. It was speculated that the difference in these two subtypes of CJD may result from the properties of prions in the contaminated dura mater grafts. However, to confirm this speculation, information on the PrP genotype of the donor will be required [19].

Initial manifestations related to the site of grafts

We recently encountered a 74-year-old female patient who underwent dura mater grafting in 1986 during the course of neurosurgical treatment for right parietal cortex meningioma. She had been well for 17 years until developing the tremor and weakness in the left upper extremity in April 2003. Neurological examination revealed left hemiparesis with myoclonic jerks and mild rigidity in both extremities. Magnetic resonance

diffusion-weighted images (DWI) revealed a clear increase in signal intensity surrounding the postoperative site in the right parietal cortex. In August 2003, serial DWI showed continuous spread of the high signal intensity area along the cerebral cortex, as well as in the caudate head and putamen. These findings suggested that the abnormalities associated with prion protein developed from the regional area of the dura mater implantation [5].

It has been reported that regions with increased signal intensity on magnetic resonance imaging are correlated with a dramatic accumulation of pathological prion protein, as shown by immunohistochemistry of autopsied brain [20]. The host prion protein implicated in the pathogenesis of prion disease is abundantly expressed in the central nervous system, where it may be conformationally modified during the course of the disease into a protease-resistant form [1]. Our observations suggest that the prion protein spread from the infected dura mater graft to the adjacent cerebral cortex and might have continued to spread to the caudate nucleus and putamen via nerve pathways. In the advanced stage, the prion protein may spread cell-to-cell via nerve pathways and throughout the perivascular space.

Our previous report suggested that initial manifestations of dura-associated CJD may be related to the grafted sites; ataxia was the initial manifestation in 48% of 29 patients with infratentorial dural grafts, but in only 7% of 14 patients with supratentorial grafts [21]. Moreover, a CJD patient in whom dura mater was implanted in the posterior fossa during Jannetta's operation developed vertigo and nystagmus as presenting symptoms, and neuro-otological examination suggested an initial lesion occurring adjacent to the grafted site [22]. In the presented case, the initial manifestation may have developed directly from the site of the dura mater graft, as evidenced by the area of high signal intensity on DWI. It has been reported that DWI appears to be much more sensitive than conventional MRI with respect to detecting early abnormalities in sporadic CJD [23], and this is consistent with the MRI and DWI findings in the presented case of dural graft-related CJD in which the initial manifestation correlated well with the abnormalities seen on DWI. Thus, careful observation of the initial symptoms and early DWI must be performed in order to make an early diagnosis of dural graft-related CJD.

We conducted a retrospective review of the full medical records of 107 cases of dural graft-related CJD patients. Particular attention was paid to the first neurological assessment and to the onset of symptoms as reported by the family.

The 107 patients with dural graft-related CJD were divided into a supratentorial group and an infratentorial group according to grafts site.

Forty-seven patients in the supratentorial group received grafts during neurosurgical procedures (for tumor or aneurysm/hematoma), while 60 patients in the infratentorial group received grafts during neurosurgical or orthopedic procedures (for tumor, aneurysm, Jannetta's operation for trigeminal neuralgia or facial spasm, Chiari malformation, or spinal cord tumor).

Our results suggested that initial manifestation of dural graft-related CJD could be related to graft site. Initial focal signs and symptoms reflecting hemiparesis or hemianopsia developed contralateral to the dural graft in 31.9% (15 of 47) of patients with supratentorial dural grafts including those in the frontal, parietal, occipital or temporal cortex. In contrast, such symptoms did not occur in the infratentorial group ($p<0.0001$). On the other hand, symptoms of cranial nerve impairment at the graft site, such as nystagmus, diplopia, hearing impairment or hemiparesis/paresthesia in the facial nerve distribution were significantly more common in the infratentorial group than the supratentorial group, affecting 25.0% (15 of 60) vs. 0% (0 of 47, $p<0.0001$). These findings suggested a significant correlation between initial manifestations and graft site.

We anticipate that our results will contribute to the early diagnosis of dural-graft-related CJD, particularly atypical slowly progressive cases.

Conclusion

A national survey indicated 117 CJD patients who had received dura mater grafts. These cases were divided into two groups on the basis of clinical course. The rapidly progressive group showed similar clinical features to sporadic CJD, while the slowly progressive group were clinically atypical in exhibiting no myoclonus, no PSD on EEG, and mild changes on MRI. In the slowly progressive group, akinetic mutism took over 1 year to develop from onset of illness. The most striking feature in this subtype was the presence of many florid plaques in the brain.

Initial manifestations were related to graft site. Hemiparesis or hemianopsia developed as an initial manifestation in 31.9% of the supratentorial group, but did not occur in the infratentorial group. On the other hand, brain stem symptoms, such as nystagmus, diplopia, ipsilateral hearing difficulty or facial paresis) were more common in the infratentorial group.

Acknowledgements

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Treatment Options in Patients with Prion Disease - the Role of Long Term Cerebroventricular Infusion of Pentosan Polysulphate

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Summary. Prion diseases (PrD), also known as transmissible spongiform encephalopathies, are believed to be caused by accumulation of an abnormal isoform of the prion protein (PrP^{sc}) in the central nervous system. Creutzfeld-Jacob disease (CJD) in its sporadic and variant form is the most frequent and clinically important PrD. At present there is no proven specific or effective treatment available for any form of CJD, although some oral agents, such as quinacrine or flupirtine, are being investigated in clinical trials.

Pentosan polysulphate (PPS), a large polyglycoside molecule with weak heparin-like activity, has been shown to prolong the incubation period of PrP^{sc} infection when administered to the cerebral ventricles in a rodent scrapie model. PPS also prevents the production of further PrP^{sc} in cell culture models. However, PPS penetrates poorly the blood-brain barrier and only a minor fraction of orally administered drug may reach the CNS. These properties of PPS prompted its cerebroventricular administration in patients with vCJD and other PrD, such as iatrogenic CJD and Gerstmann-Sträussler-Scheinker syndrome (GSS). Long-term continuous infusion of PPS at doses of up to 110 μg/kg/d did not cause serious drug-related side effects. Follow-up CT and MRI imaging demonstrated that brain atrophy may progress further during PPS administration, while the neurological status may remain stable. Proof of clinical efficacy has not been the aim of the current clinical studies of PPS, however one patient with vCJD survived for 23 months after initial symptoms and 39 months after diagnosis, while the median duration of illness with vCJD is 13 months (range 6-39).

Some lessons have been learned from the early studies of application of PPS in PrD patients. Surgery in a brain affected by PrD may result in a higher rate of surgical complications than might be expected in analogous cases with other conditions. Secondly, efficacy of PPS or any other treatment option in advanced PrD cases will be very difficult to assess, due to the lack of specific and objective criteria for measurement of response. Overall survival may remain therefore one of the few objective ways of assessing outcome in treated patients. Finally, if clinically significant benefits to patients are to be expected, PPS administration should start as early as possible in the course of the respective disease and before irreversible loss of neurological function has occurred. Further clinical, neuroradiological and laboratory investigations of cerebroventricular PPS administration in the setting of a prospective clinical study will be essential for the assessment of possible clinical benefits of PPS in PrD.

Key words. GSS syndrome, pentosan polysulphate, prion disease, sporadic CJD, transmissible spongiform encephalopathy, quinacrine, variant CJD

Introduction

Significant research interest has been attracted recently by human pathological conditions related to transmitted or intrinsically generated pathologic prion protein. Prion diseases (PrD) have thus a common and unique biological background, but a variable clinical manifestation of the presumably common pathogenetic mechanism. PrD, also known as transmissible spongiform encephalopathies (TSE), are fatal neurodegenerative disorders with different clinical forms including sporadic, inherited, and acquired diseases, the latter including transmissible and iatrogenic forms [1]. All forms of PrD have in common an abnormal metabolism of the prion protein, PrP^{c}, which results in the production of uncleavable, protease-resistant isoforms, PrP^{sc}, accumulating mostly in the CNS and causing neuronal dysfunction and eventually death. Prion diseases can affect both humans and animals and include such conditions as Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome, kuru, and fatal familial insomnia (FFI) in humans, bovine spongiform encephalopathy (BSE) or "mad cow disease" in cattle, scrapie in sheep, and chronic wasting disease in mule, deer, and exotic ungulates [1].

The normal physiologic isoform of the prion protein, PrP^{c}, is found in the body of all mammals. The *Prnp* gene encodes a polypeptide which undergoes glycosylation in the rough endoplasmic reticulum and then gly-

cosyl modification in the Golgi apparatus of the cell. Because of this glycosylation, it is expected that the glycosyl structure of the protein in different cells would be different [2]. The glycosylated PrP^c protein, associated at the C-terminus with a glycosyl phosphatidyl-inositol (GPI), is transported to the surface of the cell membrane. The half-life of PrP^c on the membrane of cells grown in culture is 3-6 hrs, after which it is internalised and degraded in the endolysosome compartment [3, 4]. A shorter peptide form of PrP^c may be recycled to the surface of the cell before lysosomal destruction. Conversion between PrP^c and PrP^{sc} occurs likely during the internalisation process. The specific physiologic function of PrP^c is largely unknown, although studies suggest it may play a role in copper binding and oxidative metabolism [5], interactions with the extracellular matrix, apoptosis, and signal transduction [6]. It is evolutionary conserved, which suggests an important physiologic role, but mice lacking PrP^c appear to grow and function normally [7].

The pathologic form of the prion protein, which is the causative agent "prion" of all PrD, is an abnormally folded isoform of the cellular prion protein PrP^c, known as PrP^{sc}. PrP^{sc} accumulates mostly in the brain of affected mammals [8, 9]. The underlying pathological process involves a post-translational conformational change PrP^c into PrP^{sc} [10, 11]. PrP^c is usually present in an α-helix conformation, in pathologic conditions only a small fraction of PrP^c is folded as α-helix, while the vast majority is present in an unfolded β conformation (β-helix PrP^{sc}). The precise molecular mechanism responsible for this unfolding process is not known, but two models are favoured (for review see [12]). One model suggests that PrP^{sc} acts like a crystal seed for the further addition of converted PrP^{sc} molecules and the subsequent formation of PrP^{sc} aggregates [13], whereas the other model postulates conversion intermediates involving a putative PrP^c-PrP^{sc} heterodimer complex [8, 14]. The claim that the PrP^{sc} is an infective agent involves the demonstration that the PrP^{sc} form can itself modify the structure of PrP^c to PrP^{sc} *in vitro*, however, this modification is inefficient [15]. Recent results suggest that single-stranded RNA molecules are necessary for PrP^{sc} amplification, and that RNA from invertebrates fails to support pathologic prion amplification *in vitro* [16].

PrP^{sc} is only found in infective animals, and mice without PrP^c production cannot become infected or infective [7]. PrP^{sc} appears to accumulate within lysosomes, growing insoluble crystalloid fragments. Initially, PrP^{sc} builds up intracellularly and then it is seen extracellularly as amyloid in histopathologic sections stained with Congo red. Microglia is activated by contact with amyloid plaques and insoluble extracellular PrP^{sc}, which results in local production and release of cytokines, reactive oxygen species, and

glutamate [17, 18]. These compounds give rise to specific local neuronal damage and apoptosis, seen as spongiform defects in the brain. Apoptosis and oxidative damage in neurons seem to follow their local exposure to cytokines [19, 20], and physiological neuronal activity is expected to be severely impaired well in advance of histopathologic changes. The progressive, slow build-up of PrP^{sc} may mean that only tissues where cells are not involved in a continuous turnover are likely to exhibit functional and morphological damage. Although cells of the immune system are also infected by PrP^{sc}, their cellular turnover is considered to prevent the body showing any immunodeficiency; whereas neurons infected with and accumulating PrP^{sc} are damaged but not replaced, and hence long term neurological deficits become clinically manifest.

While the prime target of PrP^{sc}-caused damage seems to be neuronal, massive neuronal loss is not always seen in PrD. On the other hand, activation of astrocytes occurs very early in the course of prion infection of the CNS in a consistent fashion. It can be reproduced easily in experimental models and leads to significant physiological effects such as impairment of the blood-brain barrier [21]. In addition, astrocytes are one of the few cell types capable of supporting prion replication [22]. Microglial cells are another cell type increasingly implicated in brain damage due to prion infection. Experiments indicate that activation of microglia may be essential in causing neuronal damage in PrD, and that this phenomenon is dependent on the expression of PrP^{c} [17]. Moreover, microglial activation and accumulation in affected brain areas precede neuronal cell death and parallel the temporal and spatial pattern of PrP^{sc} deposition [23]. Histologically, common late stage lesions in the CNS are neuronal loss, spongiosis and astrogliosis, accompanied by an accumulation of microglia and, occasionally, the presence of amyloid plaques and various small deposits of prion protein [24]. For a definitive diagnosis of human PrD, histopathologic assessment of the CNS is essential [25].

Sporadic CJD was originally described in 1921 and occurs mostly in individuals between 40-80 years of age, with an incidence of approximately one case per million per year. Patients suffering from CJD show a wide spectrum of clinical symptoms within a few distinctive forms of the disease [26]. While most of the CJD cases at present are sporadic, CJD may also occur as a familial form in no more than 10% of sporadic cases [27]. It follows an autosomal dominant pattern of transmission, with 70% of the patients having mutations in codons 178 or 200 of the *Prnp* gene.

Iatrogenic transmission of CJD has been proven in more than 200 cases in relation to corneal transplants, dura mater grafts, and hormones purified from human glands [28, 29].

FFI and GSS are also inherited by autosomal dominance. Both are very rare, with no more than 10 known families with FFI and 50 with GSS [30, 31]. GSS, unlike other PrD, may have a longer clinical course [32]. It is characterised by missense mutations of the *Prnp* gene, by specific neuropathological lesions and multicentric amyloid plaques.

The most recently recognized form of PrD in humans, new variant CJD (vCJD), was first described in 1996 as linked to BSE [33]. What distinguishes vCJD from sporadic cases is that the age of patients is much lower (vCJD age range 19-39 years, versus sCJD age range 55-70 years) and the duration of illness is longer (vCJD 7.5-22 months, versus sCJD 2.5-6.5 months). Variant CJD displays a distinct pathology characterised by abundant florid plaques surrounded by vacuolation [34]. Most cases of vCJD have been observed in the UK. In addition, all investigated cases of vCJD showed homozygosity of methionine at codon 129 [35, 36].

The clinical features of PrD are extremely heterogeneous and may include rapidly progressive dementia, psychiatric symptoms (mostly in vCJD, less in CJD), cerebellar syndrome (in kuru, GSS, CJD), movement disorders (myoclonus, dystonia, chorea, mostly in vCJD), encephalopathy (in CJD), pyramidal signs, cortical blindness, and sensory symptoms (hyperpathia, mostly in vCJD) [26, 32, 37, 38].

Treatment options in prion disease

PrD are still uniformly fatal, some within weeks to months from diagnosis, while vCJD patients may survive for more than a year, and GSS patients for up to 6 years. No specific treatments for PrD are known, although some prophylactic and neuroprotective agents have been proposed on the basis of cell culture experiments and animal studies [39-42]. Animal studies indicate that substantial neuropathological changes in PrD are already present before the onset of symptoms and are spatially related to PrP^{sc} deposits. Ideally, an effective intervention should start during the preclinical stage of disease, and be aimed at preventing PrP^{sc} neuroinvasion or propagation in the CNS. Unfortunately, no tests are available currently to detect asymptomatic PrD, except for carriers of pathogenic mutations of the *Prnp* gene.

Inhibition of PrP^{sc} formation may be achieved, at least in laboratory experiments, through one of the following strategies:

- Abrogation of PrP^{c} synthesis or prevention of its transport to the cell surface
- Stabilisation of the PrP^{c} structure to make its conformational change unfavourable

- Sequestration of PrP^{sc}
- Reversion of PrP^{sc} to a protease-sensitive form
- Interference with the interaction between PrP^{c}, PrP^{sc}, and other macromolecules involved in the conversion process (for review see [43]).

However, most compounds that have shown some effectiveness in cell culture or in animal models of PrD only work when administered at the time of infection or shortly thereafter. The heterogeneity and complexity of PrD suggest that a combination of several compounds with different modes of action may be necessary for their prevention and treatment. Preclinical diagnostic tests for PrD are urgently needed and deemed crucial in the success of an early treatment.

Antibiotics, dyes, and NMDA receptor ligands

The polyene macrolide antibiotic, amphotericin B [44, 45], and its less toxic derivative, MS-8209 [46], have been shown to delay scrapie agent propagation and PrP^{sc} accumulation in mice or hamsters. The amyloid-binding dye, Congo red, is able to inhibit PrP^{sc} accumulation and replication, most likely by overstabilising the abnormal conformational isoform [47, 48]. The anthracycline 4-iodo-4-deoxy- doxorubicin has been found to delay hamster scrapie progression via binding to amyloid fibrils [49]. Suramin, a highly sulphated urea based compound, and dapsone, a sulphone, were also tested against mouse scrapie and found to increase the incubation period when given continuously [50, 51]. Porphyrins and phthalocyanins as sulphated forms also were shown to inhibit the production of PrP^{sc} in neuroblastoma cell cultures [52, 53].

The neurotoxic effect displayed by PrP^{sc} and its fragments was found to be prevented *in vitro* by antagonists of NMDA receptor channels, such as memantine [17, 54]. Moreover, flupirtine, a triaminopyridine compound clinically used as non-opiod analgesic drug, which acts like an NMDA receptor antagonist, but does not bind to the receptor, was found to display a strong cytoprotective effect on neurons treated with PrP^{sc} or with a toxic fragment [54, 55]. A double-blind placebo-controlled study has been carried out in 28 CJD patients [56]. Patients treated with flupirtine showed significantly less cognitive changes (dementia) than placebo patients, which led the authors to conclude that flupirtine may have beneficial effects on the cognitive function of patients with CJD [56]. The study did not investigate other aspects of neurological deterioration in progressive CJD, the results appear therefore of limited usefulness.

Active and passive immunisation

Several studies have suggested that antibodies (Ab) might have beneficial anti-prion properties in infected cells [42, 57]. Auto-antibodies can be induced in PrP^c-expressing mice and have the potential to cure cells after prion infection [58, 59]. Furthermore, in transgenic mice expressing both PrP^c and a defined anti-PrP^c antibody, prion infectivity within the spleen is significantly reduced [60]. Peretz et al. (2001) investigated seven different recombinant antibodies raised to various parts of the normal PrP^c protein [61]. They exposed a mouse neuroblastoma cell line (ScN2a) infected with PrP^{sc} to varying concentrations of each Ab and measured the amount of PrP^{sc} protein. The most potent Ab prevented conversion of PrP^c to PrP^{sc} and also cleared pre-existing PrP^{sc} in a dose-dependent manner. Removal of Ab after 2 weeks of treatment left cultures free of prion infectivity for an additional 4 weeks [61].

Recent work by White et al. (2003) suggested that another approach, passive application of anti-PrP^c antibodies, could be effective [62]. Monoclonal antibodies (mAb) were generated in non-tolerant PrP^c-knockout mice and exhibited different specificities towards PrP^c and PrP^{sc}. Mice were infected with PrP^{sc} by the intraperitoneal (i.p.) or intracerebral routes (i.c.), and mAb were injected i.p. twice per week, starting a few weeks after infection. In mice infected with prion by the i.p. route, mAb treatment produced a dose and time dependent reduction in PrP^{sc} and prion infectivity, and a significant prolongation of survival. Interestingly, the largest effects were obtained with the mAb mainly reacting with PrP^c. However, the protective effect of mAb was only observed when prions were applied by the i.p. route, whereas prion infections caused by i.c. inoculation were not influenced by mAb treatment. This suggested that the antibody cannot cross the blood-brain barrier at a sufficient concentration to exert a protective function [62].

It remains however unclear whether the above findings in cell culture and mice are transferable to humans, given the fact that the exact mode and time point of prion infection are usually unknown. In addition, in most of the mouse studies, a very high dose of mAb was applied in a continuous fashion (for review see [42]), which may produce allergic reactions and also result in inactivating antibodies in human patients.

Quinacrine and chlorpromazine

The antimalarial drug quinacrine (mepacrine), a cyclic tetrapyrrole, and the antipsychotic chlorpromazine (Figure 1) were shown to prevent the conversion of PrP^{c} to PrP^{sc} in cell culture. Doh-ura et al. (2000) reported that lysosomotropic agents (e.g. quinacrine or chloroquine) inhibited protease-resistant prion protein accumulation in scrapie-infected murine neuroblastoma cells (ScNB). The inhibition occurred without apparent effects on normal PrP^{c} biosynthesis or turnover, and without direct interactions with prion protein molecules [63]. Similar effects of quinacrine were reported later by Korth et al. (2001). These authors cultured mouse neuroblastoma cells (ScN2a) infected with PrP^{sc} to show that 6 days of treatment with quinacrine or chlorpromazine was able to reduce the conversion of PrP^{c} to PrP^{sc} [64].

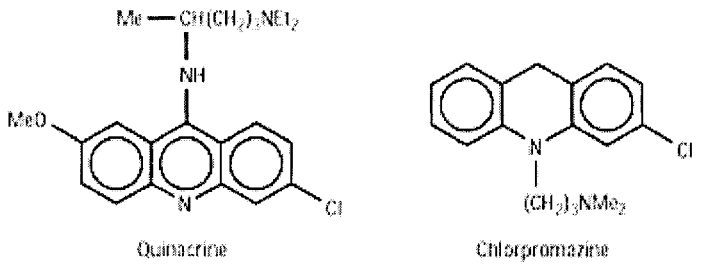


Figure 1. Chemical structure of quinacrine and chlorpromazine

Quinacrine has been used in humans for over 60 years to treat malaria, and can be administered orally at high doses on a daily basis. The currently suggested oral dose for CJD patients is however higher than the antimalarial dose and may produce significant side effects in a considerable proportion of the treated patients. Chlorpromazine, although less potent than quinacrine in cell culture, crosses the blood-brain barrier to a larger extent.

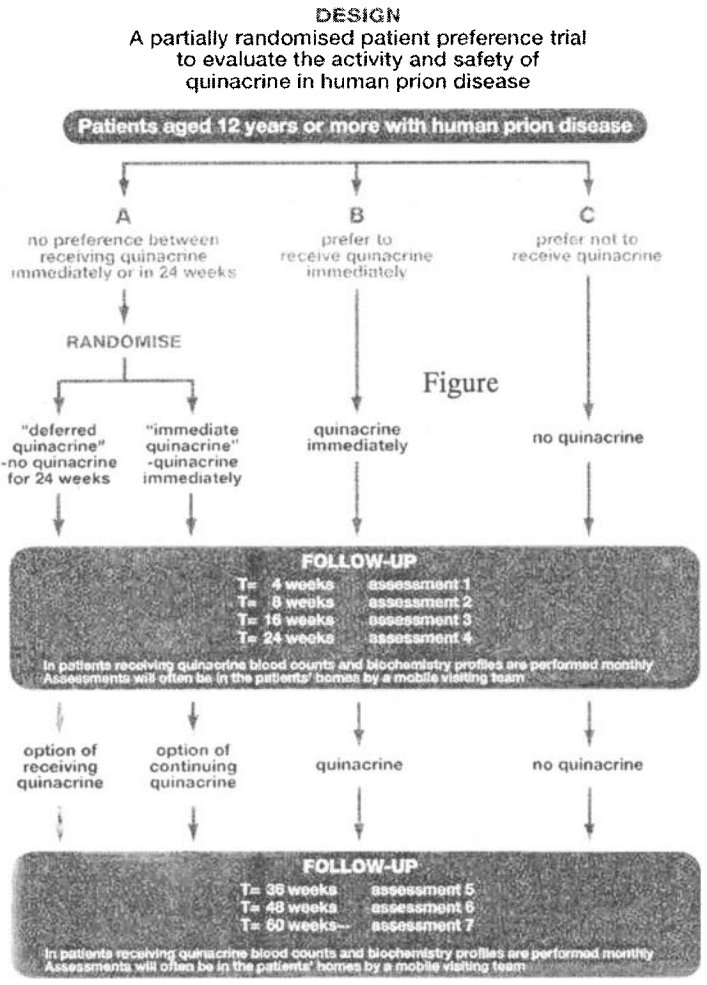
Turnbull et al. (2003) showed that quinacrine also may act as an effective antioxidant, readily scavenging hydroxyl radicals generated during incubation of toxic PrP^{sc} fragments with murine neurons [65]. Quinacrine also significantly reduced toxicity of the $PrP^{106\text{-}126}$ peptide fragment in these cells. On the other hand, Collins et al. (2002) evaluated oral quinacrine in mice infected with PrP^{sc}, but were not able to demonstrate any significant effect of the drug on overall survival of treated animals compared to controls [66]. Barret et al. (2003) also examined the efficacy of quinacrine and chlorpromazine in different *in vitro* models and in an experimental murine

model of BSE [67]. Despite the inhibition of PrP^{sc} accumulation in ScN2a cells, quinacrine was unable to produce a detectable effect in the animal model.

Japanese researchers are carrying out an ongoing clinical study of oral quinacrine in patients with sporadic and iatrogenic CJD. Results in the first 4 patients have been published recently [68]. Quinacrine (300 mg/d) has been administered for 3 months. Improved arousal level of patients with akinetic mutism, and restored eye contact or voluntary movements in response to stimuli were described. Clinical improvement was however transient, lasting 1-2 months. Quinacrine at the above dose caused liver dysfunction and skin pigmentation in all cases [68]. Further results in a larger patient population should be presented in near future.

A prospective clinical study of quinacrine in PrD, the PRION-1 study [69], is currently enrolling patients in the UK (Figure 2). Patients aged 12 years and older with all types of PrD are eligible. The study protocol features a partially randomised design, with patients who opt for quinacrine treatment split in two arms according to their preference for immediate vs. deferred (by 24 weeks) treatment. Treated patients will receive a loading dose of quinacrine (1 g on the first day), followed by 300 mg/d as a long term dose. The primary efficacy endpoints are mortality and the proportion of responders overall and at 24 weeks. Response is defined as independently rated lack of deterioration, global impression of change (based on the Clinician's Interview Based Impression of Change, CIBIC-plus), and patients score on the Brief Psychiatric Rating Scale (BPRS). Secondary efficacy endpoints are neurological and neuropsychological changes, including changes in markers of disease activity, MRI, and EEG [69].

Routine follow-up will be identical for all patients participating in the PRION-1 trial, with the exception of patients with inherited PrD, who have longer disease duration and will be followed up less frequently. Follow-up assessments are taking place monthly for the first 6 months, then every 3 months until end of study. Assessments will include medical history, physical examination, liver function and blood clotting, neurological examination recorded on video, and a series of neurological assessments. The study accrual target is 160 patients. Currently 18 patients have been enrolled [69].



Figure

Figure 2. Flow chart of the PRION-1 trial of quinacrine in patients with PrD [69].

Polysulphonated glycosides (glycans)

Several polysulphonated polysaccharides, including pentosan polysulphate (PPS) (Figure 3) and dextran sulphate (DS), have been shown to prolong the incubation period in PrP^{sc} infected rodents if given before infection [70-72], and to inhibit PrP^{sc} accumulation in neuroblastoma cells [73]. The effects of these polyanions may be due to an inhibition of the formation of PrP fibrils [74] or to reduction of the amount of PrP^{c} on the cell surface by stimulating

endocytosis of PrP^c [52]. Sulphonated polyglycosides are not known to penetrate the CNS, and hence the first attempts to demonstrate their effects were made in peripheral organs [75].

Caughey and Raymond (1993) tested various polysulphonated glycosides (PG) and found PPS, carrageenan, and dextran sulphate 500 (DS500) to be highly active in the inhibition of PrP^{sc} production [73]. PPS was most active, showing half of its maximal activity at 1 ng/ml. Shyng et al. (1995) reported PPS and related compounds to cause a decrease of PrP^c on the surface of cultured chicken and mouse neuroblastoma cells. PPS caused a redistribution of PrP^c from the surface to the interior of the cell (intracellular late endosomes). The differences in the binding strength of PrP^c to PPS and to other PG were found to parallel their *in vivo* and *in vitro* anti-PrP^{sc} formation potency [52]. Ehlers and Diringer (1984) inoculated mice i.p. or i.c. with scrapie agent and treated them systemically with DS500 [76]. None of the i.c. inoculation experiments were affected by the treatment. With i.p. inoculations, however, it was seen that DS500 did decrease (by approximately one order of magnitude) the infectivity found in the spleen at various times after single injection of the drug, and significantly prolonged incubation times. Treated mice also showed a significant increase in the mean incubation period compared with controls. It was noted that DS500 remained in the body of an inoculated mouse for up to 7 months. A maximum effect was seen when the drug was given at the same time with the infection, and no effect was seen when it was given 35 days after infection [76].

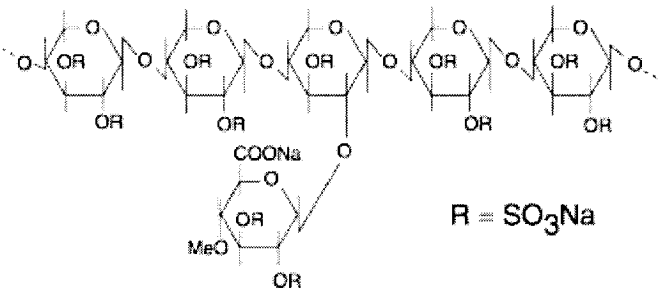


Figure 3. Chemical structure of pentosan polysulphate (PPS)

This work was followed by the study of Farquhar and Dickinson (1986), who carried out a series of murine experiments to inoculate i.p. scrapie [75]. This was associated, at various times before and after the scrapie inoculum, with various quantities of DS500 as a single i.p. injection. It was found that DS500 reliably increased the incubation period of the disease, and that this did not seem to depend on the strain of disease or the inbred strain of mouse

used. The effect seemed to be present when DS500 was given up to 4 weeks before and up to 3 weeks after the scrapie inoculum. The incubation period was extended by 5-19% at this dose, but if increased doses of DS500 were used, the incubation period could be prolonged by up to 62% [75]. Kimberlin and Walker (1986) gave various inocula of scrapie to mice either i.v. or i.p., and various drugs were tested before or after the scrapie infection [77]. DS500 proved effective in reducing the titre of the scrapie inoculum. Little effect was seen with heparin, dextran, or DEAE dextran. Diringer and Ehlers (1991) inoculated mice i.p. with scrapie and administered PPS i.p. on different days (84 to 50) before the PrP^{sc} infection. PPS increased the incubation period of mice by up to 75% [70]. Hamsters were also inoculated i.p. with various quantities of DS500 or PPS and with scrapie, separated by 2 to 24 hrs [78]. As the dose of DS500 increased, the incubation period also increased, but the maximum achieved with non-toxic doses of the drug was 21%. It was noted that a single i.p. administration of PPS increased the incubation period of i.c. inoculated scrapie by around 18% [78]. Farquhar et al. (1999) injected i.p. PPS immediately after scrapie infection of mice. Depending on mouse strain, a single PPS dose of 250 mg increased the scrapie incubation period by up to 66%. A single 1 mg i.p. dose of PPS protected mice completely from simultaneous scrapie infection. On the other hand, oral PPS was ineffective in delaying disease [79].

Doh-ura et al. (2004) recently infected transgenic mice (Tg7) expressing hamster prion protein with i.c. scrapie, and different agents were infused cerebroventricularly starting on day 10 or day 35 after infection [80]. The infusion was continued for 4 weeks. Infused drugs included lysosomotropic chemicals, such as E-64d cysteine protease inhibitor, chloroquine, quinacrine, amphotericin B, and PPS. Lysosomotropic agents demonstrated marginal effects in prolonging the incubation time when administered on day 35 after infection, and either no effect or less effect at the earlier stage (day 10 after infection). Amphotericin B and PPS demonstrated remarkable effects either early or late in the disease course. Amphotericin B resulted in around 30% prolongation of the incubation time when administered at the early stage, and 12% prolongation at the late stage. PPS showed more beneficial effects than amphotericin B, and mice which received PPS at the early stage survived 173% longer, and at the late stage 92% longer. Maximal effects of PPS at a later stage (day 42 of infection) were obtained at 230 μg/kg/day. Analysis of detailed relationship between the initiation time of the infusion of PPS and the outcome revealed that the effects of PPS were quite dependent on the timing of infusion initiation, with earlier initiation of treatment rendering a better prognosis [80]. Analysis by either immunohistochemistry or immunoblotting demonstrated that PPS potently inhibited PrP^{sc} deposition in the brain. It also showed that amount or dis-

tribution of deposited PrP^{sc} in the brain of mice treated with PPS was modified and did not return to the same level observed in the control animals, even when they were at a terminal stage. Immunohistochemical analysis demonstrated that mice treated with PPS from the early stage did not show any PrP^{sc} deposits in the brain on day 52. On the other hand, control animals demonstrated PrP^{sc} deposits in the parahippocampal white matter on day 35, and later also in the thalamus. No notable adverse effects were observed in experimental mice treated with up to 230 μg/kg/day intraventricular PPS for two months. In a separate set of experiments in normal dogs, higher doses, such as 345 μg/kg/day and 460 μg/kg/day, did show adverse effects such as partial or generalized epileptic seizures, which began within 24 hours after PPS infusion at the above high doses was initiated [80].

Both heparin and PPS are rapidly taken up into RES cells by a saturatable pathway. Low doses are cleared quickly into the RES, whereas higher doses saturate the RES and are excreted in urine [81]. PPS is metabolised by cellular non-specific desulphation in many organs and tissues, including vascular endothelium [82]. Renal excretion of desulphated PPS from plasma takes place over 6 days following a single dose, which also involves partial polyxylose chain breakdown. PPS can be administered orally, but only a low proportion (0.5-4%) of the drug is detected in the blood circulation [83, 84]. When PPS is given orally, anti-inflammatory effects are seen in the bladder after long-term administration [85]. It is considered that this is due to accumulation of the drug in cells of the RES with slow break-down and excretion. When used for anticoagulation and given s.c. or i.v., PPS may cause an early, benign, reversible thrombocytopenia and a rise in lipoprotein lipase activity [83]. Similarly to heparin, a rare, immune, severe form of thrombocytopenia has been also reported [86]. No significant neurological symptoms or signs have been reported in humans or animals treated orally or parenterally with PPS.

There has been no penetration in the CNS demonstrated with peripherally administered PPS, which is not surprising with the hydrophilic nature of the drug. On the other hand, direct intracerebral administration of PPS may afford high compartmental concentrations of the drug in the CNS, but no pharmacokinetics is available for this specific mode of administration. Direct administration of PPS to the CNS would be expected to allow PPS to concentrate inside cells, entering them via ubiquitous heparan-binding sites, and to exert biological effects on those cells infected by PrP^{sc}. In analogy to other therapeutic molecules, e.g. recombinant proteins delivered directly into the primate and human brain [87, 88], it is considered likely that cerebroventricular infusion of PPS may have the highest ratio of local versus systemic drug concentration.

Rationale for local administration of drugs to the CSF

The clinical and late preclinical phase of PrD with PrP^{sc} formation in the brain requires drugs that can cross into brain parenchyma and be present in the brain in a biologically active concentration [32, 42, 43]. However, in the early stages of PrD, with an intact blood-brain barrier (BBB), there is severe limitation of the penetration of drugs from blood into brain interstitium, and from there into glial and neuronal cells. Even at late stages of the disease, tight junctions of the brain capillaries may remain at least partially intact and therefore selectively limiting the entry of most molecules.

Compounds that are highly lipid soluble, such as alcohol, barbiturates, and some anticonvulsants, may easily pass through the endothelial cells forming the inner layer of the BBB. Lipid solubility is measured by the oil/water (octanol/water) partition coefficient, and molecules with a high coefficient usually permeate efficiently the BBB (for review see [89]). Such highly lipid soluble compounds with a high partition coefficient are phenytoin and methadone, and they cross the BBB in large quantities under normal conditions. Not all lipid soluble molecules, however, easily traverse the BBB. Compounds highly bound to plasma proteins have restricted access to the brain. For these substances, the degree of dissociation of the protein complex in transit through the capillary bed determines the degree of penetration across the BBB. Furthermore, there are special transport systems responsible for enhanced passage of certain compounds with low lipid solubility across the BBB, such as the physiologically important molecules D-glucose and phenylalanine [90]. The BBB can be subjected to pharmacological or osmotic modifications aimed at temporarily increasing its permeability to certain therapeutic molecules. These approaches are however invasive and have the potential for serious side effects [91, 92].

The CSF-brain barrier (CBB) seems to be more permeable because of its anatomical structure lacking tight junctions between the neuroependymal cells lining the cerebral ventricles. Substances administered to the CSF have been shown to penetrate into brain tissue by diffusion. The physical process of diffusion is gradient-driven, and penetration of the CBB will be enhanced by higher concentration of a molecule in one compartment [93, 94]. This fact points at an important advantage of the local application of drugs to the CSF - high local concentration in the CNS compartments, as opposed to negligible systemic concentration due to low reabsorption in the blood stream.

Continuous CSF circulation is physiologic process which lends itself to dissemination of substances throughout the CNS. CSF is continuously produced and completely replaced in the brain approximately every 8 hours.

In normal adults, the rate of CSF removal by reabsorption is equal to the rate of CSF production by filtration of blood through the intraventricular choroid plexus. CSF circulates from the sites of production, the lateral ventricles and third ventricle, into the cerebral aqueduct and into the fourth ventricle. From there CSF escapes the internal ventricular system of the brain by the foramina of Luschka and Magendie into the subarachnoid space around the brain and the spinal cord. Arachnoid granulations and dural sinuses are the route for CSF reabsorption to the blood circulation [95].

Animal models support findings in humans. In a model of cerebroventricular infusion in rats, radioactive sucrose was infused into one lateral ventricle. Within minutes after infusion, sucrose moved into the third ventricle, the aqueduct, fourth ventricle, and the subarachnoid space of the quadrigeminal, ambient and interpeduncular cisterns. About 15% of the injected sucrose entered these large cisterns. In contrast to most other CSF-brain interfaces, little sucrose moved from CSF into the medulla next to the lateral recesses and tissues adjacent to the large CSF cisterns. A thick, multilayered *glia limitans* visible on electron micrographs seemed to form a CSF-brain barrier at these interfaces [96].

Evidence exists also for the bulk flow of brain interstitial fluid via preferential pathways through the brain, which is closely related to CSF. This bulk flow of interstitial fluid has implications for drug delivery, drug distribution, and drug clearance [97].

Preliminary results with continuous long-term cerebroventricular administration of PPS in human PrD

The first objective of cerebroventricular PPS administration in PrD patients was to evaluate the short and long term safety and tolerability of escalating doses of PPS administered by continuous long term infusion. A secondary objective was to assess efficacy of PPS in delaying disease progression and improving existing neurological deficits. Patients with probable sporadic, iatrogenic, or variant CJD, or with hereditary syndromes such as GSS or FFI were eligible to receive PPS infusion. Informed consent was obtained where possible. If patients were not fit and able to consent, a legally appointed representative signed the consent forms. The primary endpoint of PPS administration studies was maximum tolerated dose of PPS as assessed by occurrence of serious toxicity resulting from PPS administration. Dose-limiting toxicity (DLT) was defined as any one of the following occurring in two or more patients:

- Any grade 4 toxicity attributed to PPS

- Grade 3 toxicity for neurological symptoms or for symptoms in other organ systems lasting longer than 5 days and attributed to PPS

Patients considered for PPS administration had to have a probable diagnosis of one of the above PrD in accordance to WHO criteria. Normal haematological, renal, and liver function was also a requirement. Because of the surgical procedure for implantation of the ventricular catheter and subcutaneous pump and infusion system, ongoing treatment with anticoagulants such as warfarin, heparin, clopidogrel, or aspirin was not allowed. Also the presence of any active infection or any viral syndrome within two weeks prior to treatment was an exclusion criterion.

Patients undergoing surgery had standard ventricular catheters placed in the anterior horn of the right lateral ventricle or in a few cases in both frontal horns, unless clinical reasons dictated another point of access to the ventricular system. In the first case with PPS administration, the catheter was connected initially to an external pump for trial administration of PPS, and later attached to a subcutaneously programmable pump (Synchromed EL, 18 ml reservoir, with side port, Medtronic Inc.) (Figure 4) permanently implanted in the abdominal wall. Later cases had simultaneous implantation of the catheter system and the infusion pump in the same surgical session. After a period of time after the surgical procedure (3-14 days) in which the pump was not active and scar tissue formation was expected to occur, PPS infusion commenced at a low dose level. The decision to proceed to the next higher dose level was based on the absence of clinical side effects and on normal findings on non-enhanced CT scans (e.g. exclusion of hydrocephalus or intracranial blood).

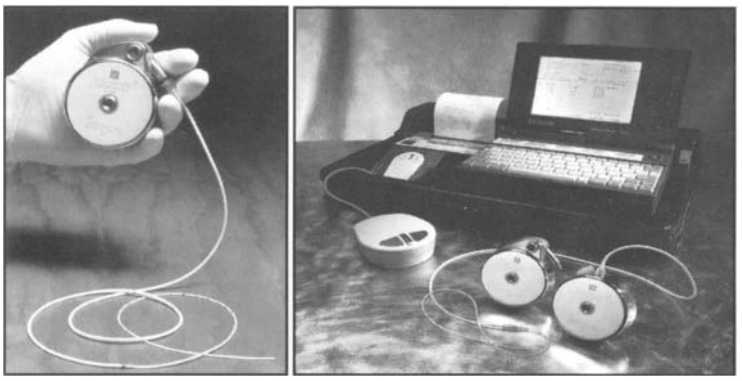


Figure 4. The implantable externally programmable pump (Synchromed EL with side port, Medtronic Inc., Minneapolis, MN) is shown with attached catheter (**A**). (**B**) shows a portable pump programming unit (telemetry unit) with printer.

There are no previously published data on a safe or potentially effective dose of cerebroventricularly infused PPS in human patients with PrD. Based on preclinical animal work, a dose escalation schedule was set up starting at 1 μg/kg/d and escalating on a daily basis until a target dose of 11 μg/kg/d was reached. This represented a 10-fold dose reduction, based on body surface area and weight differences, from the lowest effective PPS dose used in scrapie-infected mice in a preclinical study of intraventricular PPS [80]. The maximum daily dose of cerebroventricular PPS administered to the first 6 patients on a long term basis was 11 μg/kg/day. All further cases have received a maximum dose of 110 μg/kg/d in 10-20 μg/kg/d escalation steps, but long term follow up with this dose is still limited (Table 1).

The clinical source of PPS is *Pentosanpolysulfat SP54* in sterile 1 ml vials, supplied by the pharmaceutical company Bene Arzneimittel GmbH (Germany). Each vial contains 100 mg of Sodium-PPS (100 mg/ml) with 1% sodium-4-oxopentanate as a stabiliser. For filling of the pump reservoir, PPS SP54 100 mg/ml is diluted with 0.9% NaCl to a final concentration of 1-10 mg/ml. The pump is then programmed to deliver the total daily dose in a continuous simple infusion mode (constant volume and infusion rate over time).

There are no standardised or widely accepted criteria for assessment of treatment efficacy in PrD. Surrogate criteria for efficacy were thus adopted and included overall survival, speed of disease progression before PPS infusion compared with disease progression after start of PPS, neuroradiological imaging, and changes in the general and neurological condition of the patients.

The first patient to receive PPS infusion was a young man suffering from vCJD [98]. He presented initially with subjective signs of behavioural disturbance, followed a few months later by progressive ataxia, pyramidal signs and myoclonus, which led to the clinical diagnosis of possible vCJD. The clinical picture combined with abnormal MR findings in the FLAIR sequence (pulvinar sign) and positive tonsil biopsy allowed the diagnosis of probable vCJD 8 months after the occurrence of initial clinical symptoms. At the time of first administration of PPS, the patient had symptoms of advanced vCJD, such as ataxia, dementia, dysphagia, dysphasia, myoclonus, and was confined to bed and unable to care for himself. He was fed via percutaneous gastrostomy. The initial PPS dose of 1 μg/kg/d was escalated without drug-related complications to the target dose of 11 μg/kg/d. Continuous infusion of PPS for 23 months has not caused any drug-related side effects. Cerebroventricular PPS at the above dose did not have any measurable systemic anticoagulant activity in serum, as confirmed by unchanged INR (international normalised ratio) before and during PPS infu-

sion. Follow-up CT scans demonstrated no intracerebral haemorrhage, and there were no seizures. Subdural fluid collections first over the right hemisphere and subsequently over the left hemisphere necessitated surgical (burr hole) evacuation of fluid. Repeated surgical revisions of the fluid collections were necessary (Figure 5).

This first patient is currently alive and in a stable condition. Although there were no major improvements in the neurological and general condition, there were a few notable changes. The patient is now able to fix his eyes on persons, to obey simple one stage commands, and to make verbalization attempts in response to stimuli. The sleep/wake cycle and the reflex swallow are restored and the myoclonus is reduced. The patient has gained 5 kg of weight compared to pre-PPS baseline, while on the same nutritional regime. Regular follow-up CT scans have shown progressive brain atrophy during PPS administration, which could not be correlated to any worsening of the clinical condition (Figure 5).

Since January 2003, a total of 13 patients with PrD have undergone surgery and continuous cerebroventricular administration of PPS. Anonymised clinical and follow-up data are presented in Table 1. The most important clinical finding is the safety of PPS administration to the cerebral ventricles. The maximum tolerated dose of PPS has not been reached. There were no cases with side effects attributable to PPS, even in patients receiving 110 μg/kg/d of PPS.

Focal seizures have been observed in one patient on 11 μg/kg/d, and generalised tonic-clonic seizures in one patient with 110 μg/kg/d. It remains to be clarified if these seizures were a side effect of PPS or of surgery, since in both cases they occurred months after start of PPS and during infusion with a stable dose of PPS.

It is currently unclear if a higher dose of PPS has a stronger effect, and if dose escalation should be continued above 110 μg/kg/d. In most cases it seems that PPS administration results in a temporary halt of disease progression, but this conclusion is not based on hard evidence or objective measurements. PPS administration seems not able to reverse the clinical course of advanced disease and to achieve functional recovery of established neurological deficits.

Furthermore, surgery in the brain affected by PrD may result in a higher rate of surgical complications than usually encountered in comparable non-PrD cases. Brain atrophy may progress while PPS is administered, and there is no apparent correlation between degree of atrophy and clinical status of the patients. Therefore, in accordance with results from the preclinical animal studies [80], cerebroventricular infusion of PPS should be commenced as early as possible after disease diagnosis and, if possible, before the occurrence of fixed neurological deficits.

In conclusion, despite the encouraging preliminary results in PrD patients receiving long term cerebroventricular PPS, further clinical, neuroradiological and laboratory investigations in the context of a prospective clinical study will be essential for the evaluation of genuine clinical benefits of PPS administration.

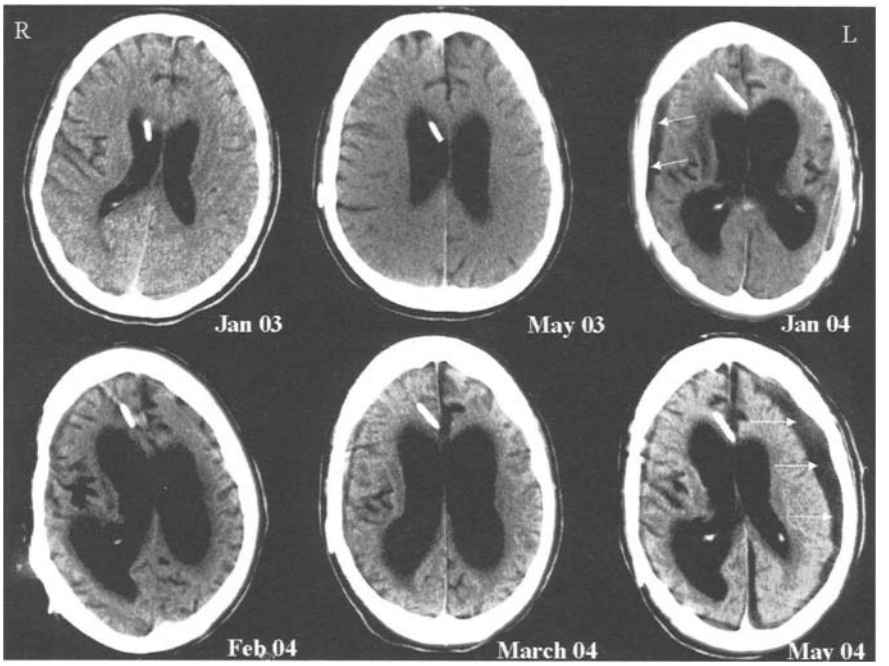


Figure 5. Serial CT scans of patient #1 demonstrating sequential occurrence of right parietal subdural fluid collection (upper right, arrows) and left parietal subdural collection (lower right, arrows). Note the progression of brain atrophy over time.

Table 1. Summary of clinical data of all current patients with PPS administration.

Patient number	Sex	Age at Dx[a] (years)	Diagnosis and clinical course after start of PPS	Survival (months after Tx[a])	Maximum PPS dose (μg/kg/d)
1	M	17	vCJD. Stable disease, swallowing and myoclonus improved, brain stem function improved. PPS started at very advanced stage of disease.	23	11
2	M	19	sCJD. Initially neurological improvement, later slow progression. Weight gain 10 kg. Reduction of myoclonus. Partial seizures occurring a few months after start of PPS, currently on phenytoin.	10	11
3	F	12	vCJD. Stable disease, wheelchair bound. Currently speech deficit, stable weight, swallowing remained intact.	13	11
4	M	15	vCJD. Stable after PPS, but disease progressed rapidly before PPS started.	9	11
5	F	34	GSS Stable disease, but surgical complications (brain haemorrhage) giving rise to neurological deficits.	10	11
6	F	32	GSS Stable disease. Initially only very mild neurological symptoms present.	3	11
7	M	37	Iatrogenic CJD (GH[b] administration) Cerebellar syndrome, initially stable condition. Rapid deterioration despite PPS.	6	110
8	F	27	Iatrogenic CJD (GH[b] administration) Stable disease, but rapid progression before start of PPS. Alive but in state of limited awareness.	9	110
9	F	39	vCJD. Presented with psychiatric syndrome. Continuous neurological deterioration while on PPS. Generalized seizures occurring 2 months after start of PPS. Died of disease progression.	4[c]	110
10	M	44	GSS Continued neurological deterioration. Increase in mental symptoms and disorientation while on PPS.	4	110
11	M	34	Iatrogenic CJD (GH[b] administration) Stable disease.	1	110
12	F	39	GSS Mild neurological deficits at start of PPS.	-[d]	110
13	F	66	sCJD	-[d]	110

[a] - Dx/Tx - Diagnosis/Therapy.

[b] - Growth hormone.

[c] - Patient deceased.

[d] - Follow-up period < 1 month.

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Human prion diseases: novel diagnostic principles

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Summary. Prion diseases include a wide range of diseases affecting both humans and animals. One commom feature of this diverse group of diseases is the deposition of PrP^{Sc}, the abnormally folded form of the host encoded prion protein. The proposed replicative cycle of prions is relatively simple. It encompasses misfolding of a single protein, the cellular prion protein PrP^{C}, into a disease-associated form termed PrP^{Sc}. This is followed by PrP^{Sc} aggregation, and possibly fragmentation of aggregates which may augment the number of replicative units. While there is no formal proof of the correctness of the above model, a wealth of evidence indicates that pathogen-encoded informational nucleic acids are dispensable for prion replication. The detection of PrP^{Sc} in various tissue compartments including the central nervous system, the lymphoreticular system and skeletal muscle is the principle of the majority of diagnostic test aimed to verify the clinical suspicion of a prion disease. This article discusses current concepts surrounding the basic biology of prions and the diagnosis of prion diseases.

Key words. Prions, CJD, PrP^{Sc}, strain, diagnosis

Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are inevitably fatal neurodegenerative conditions which affect humans and a wide variety of animals [1, 2]. Although prion diseases may present with certain morphological and pathophysiological similarities to other progressive encephalopathies, such as Alzheimer's and Parkinson's disease [3], they are unique in that they are transmissible by inoculation or ingestion of prion-contaminated material.

Primary signs of prion diseases in humans are impaired cognitive functions and ataxia. Upon histological analysis of tissue, spongiform degeneration of the brain accompanied by activated astrocytes and microglia is observed [4]. These changes are accompanied by the accumulation of a protease-resistant form of host derived prion protein (PrP^{Sc}, Sc=scrapie). The cellular form of the prion protein (PrP^{C}, C=cellular) is a protease-sensitive sialoglycoprotein which is anchored to the membrane via a glycosyl phosphatidyl inositol residue. Much evidence suggests that abnormally processed PrP may represent an intrinsic component of the infectious agent causing prion diseases [2, 5].

The most common human TSE is Creutzfeldt-Jakob disease (CJD), which has been classified as sporadic (sCJD), familial (fCJD), iatrogenic (iCJD), and variant (vCJD) [6]. Sporadic CJD is very rare, and appears to be evenly distributed worldwide: countries which carry out surveillance uniformly report an incidence of approximately 0.6-1.2 x 10^{-6} x $year^{-1}$ [7] although higher incidences have been reported [8]. The etiology of sCJD is unclear: no exogenous or endogenous causes have been identified yet. Familial forms of the disease are inherited as autosomal dominant traits, and co-segregate with mutations in the *PRNP* gene, which encodes the prion protein [9]. Instead, iatrogenic cases are attributed to neurosurgical intervention, transplantation of tissues, or administration of hormones derived from deceased individuals suffering from TSEs [10]. In 1996 a novel form of a human TSE emerged in the United Kingdom and was thus termed (new) variant CJD (vCJD). Biochemical and histopathological evidence suggests that vCJD represents transmission of bovine spongiform encephalopathy (BSE) prions to humans [11-13].

TSEs have been observed in a wide variety of animals and include scrapie of sheep, BSE of cattle, TSE of farmed mink and chronic wasting disease of deer and elk. TSE in cats, zoo bovids, and non-human primates is most likely a result of transmission of BSE to these species [14].

Biology of PrP^{C} and PrP^{Sc}

The gene encoding PrP (*PRNP*) is a single copy gene located on chromosome 20 in humans. *PRNP* has three exons: only exon three is coding for PrP. Human PrP is a protein of 253 aminoacids. The first 22 aminoacids encode a signal peptide that is cleaved off during translation. Residues 51 to 91 contain a nonapeptide followed by four identical octarepeats, which

may function as copper binding sites. *PRNP* is polymorphic at codon 129 encoding either valine or methionine. Homozygosity for methionine has been shown to constitute a risk factor for the development of prion diseases [15]. PrP^C is expressed at highest levels on neurons and other cells of the central nervous system (CNS). Besides the CNS, PrP^C is expressed in the lymphoreticular system and in skeletal or heart muscle [16].

PrP^C consists of a highly structured C-terminal part, containing three alpha-helices plus two short anti-parallel beta-strands, and an unstructured N-terminus of 120 amino acids length. Following translation, PrP^C is modified by N-linked glycosylation at residues 181 and 197 and the addition of a C-terminal glycosylphosphatidylinositol anchor at residue 230. The mature protein is attached to the cell surface in specialized detergent-resistant microdomains referred to as rafts [17] via its glycosylphosphatidylinositol anchor, and may cycle between the cell surface and early or late endosomes [17, 18].

The nature of the infective agent

In the 60' it became apparent that prions are fundamentally different from conventional agents, as they cannot be sterilized by damage to nucleic acids [19]. The idea that the agent that causes TSEs is entirely made up of proteins was first brought up by Griffith in 1967 [20]. Subsequently, it was shown that a relatively protease resistant form of the prion protein is a major component of the infectious fraction [21]. The protein-only hypothesis was formulated and in a simplified form states that the infective agent is devoid of nucleic acids and principally consists of PrP^{Sc}, an abnormally folded, protease-resistant, beta-sheet rich isoform of a normal cellular protein termed PrP^C [22]. According to this theory, infectivity propagates simply by recruitment and "autocatalytic" conformational conversion of the cellular prion protein into disease-associated PrP^{Sc} [23]. The exact mode of propagation of PrP^{Sc} remains a mystery to this day. At least two possible explanations exist. The first one is referred to as the "template-directed refolding hypothesis". According to this theory, monomeric PrP^{Sc} imparts its conformation onto monomeric PrP^C, the result being two molecules of PrP^{Sc} [24]. This would imply that one protein would be able to induce a change in the tertiary structure of another protein. Although theoretically conceivable, this theory is not fully supported by experimen-

tal evidence. The second theory, the "seeded nucleation hypothesis" states that PrP^{Sc} and PrP^{C} co-exist in equilibrium [25]. In a healthy organism the equilibrium would be heavily shifted towards PrP^{C} with only diminutive amounts of PrP^{Sc} present. In the case of prion-disease, highly ordered aggregates of PrP^{Sc} molecules would function as the infectious agent and would be able to recruit monomeric PrP^{Sc} molecules into the "infectious" PrP^{Sc} aggregate. According to this theory, PrP^{Sc} is only infectious as a highly ordered aggregate. Although this theory is as well far from proven, there is experimental evidence favoring this mechanism, particularly in the yeast model of prion replication [26, 27] .

Distribution of PrP^{Sc} within prion-diseased organisms

The accumulation of PrP^{Sc} in the central nervous system is feature that is common to all forms of prion diseases. Studies focusing on the temporal and spatial distribution of PrP^{Sc} in experimentally infected rodents have demonstrated that accumulation of PrP^{Sc} in the central nervous system is a relatively late event which occurs only shortly before animals develop clinical signs of prion disease [28, 29]. Peripheral organ systems seem to be involved at earlier timepoints and it could be shown that PrP^{Sc} accumulates in lymphoid tissues such as spleen, lymph nodes or in skeletal muscle well before the animals show clinical signs of prion disease [30]. Interestingly, the presence of PrP^{Sc} within lymphoid tissues seems to be depended on factors such as the route of prion exposure or the type of prion strain [31].

Molecular diagnosis of human prion diseases

Molecular diagnosis of human prion diseases relies on the combination of genetic, biochemical and neuropathological investigations in conjunction with the clinical data.

Genetic investigations

Sequencing of *PRNP* enables the exclusion of genetically caused CJD [32]. In addition, this investigation provides information on codon 129

polymorphism. There is compelling evidence from studies on genetically modified mice and from clinical studies on patients suffering from human prion diseases that homozygosity for methionine on codon 129 constitutes a risk factor for the development of prion disease [33]. Notably, methionine homozygotes are clearly overrepresented among sCJD patients. Furthermore all individuals affected by vCJD are codon 129 methionine homozygotes. Besides constituting a risk factor for the development of prion diseases, this polymorphism has a considerable effect on the clinical, biochemical and neuropathological presentation of prion-diseased individuals [33].

Biochemical investigations

The basis of biochemical characterization of PrP^{Sc} resides in the relative resistance of PrP^{Sc} towards proteolytic degradation. Whereas PrP^{C} is entirely digested by Proteinase K, identical treatment leads to removal of a variable number of N-terminal aminoacids in the case of PrP^{Sc}. This results in the appearance of three distinct bands, corresponding to the di-, mono-, and unglycosylated form of PrP^{Sc}, upon western blotting [34]. The molecular classification of PrP^{Sc} takes two parameters into account: The first one is the size and thus mobility of the unglycosylated band of PrP^{Sc} on polyacrylamide gel electrophoresis, whereas the second parameter includes information of the relative abundance of the signal intensity produced by the di-, mono-, and unglycosylated form of PrP^{Sc}. The resulting information is then used to establish the "type" of PrP^{Sc} that may be classified according to proposed schemes (Figure 1) [35, 36]. Depending on the exact conditions under which the protease digestion and the western blotting procedure is performed, between 3 and 6 different PrP^{Sc} types can be distinguished [35, 37]. Distinct PrP^{Sc} types are thought to represent the molecular correlate of distinct prion strains and the fact that the PrP^{Sc} type that can be found in patients suffering from vCJD is identical to the PrP^{Sc} type present in BSE diseased cattle, is one of the main arguments supporting the theory that BSE prions are responsible for the vCJD epidemic in humans [11].

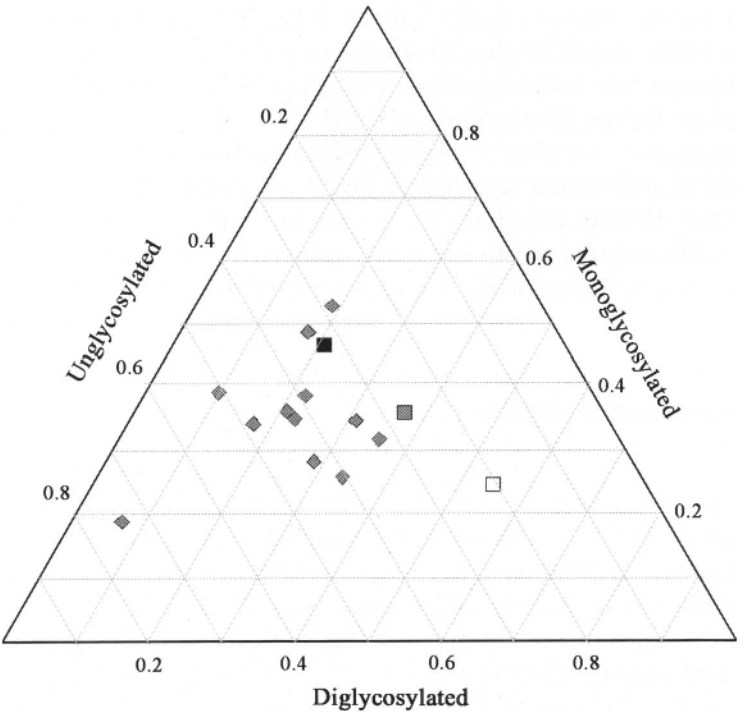

Figure 1. Glycoform profiles of Swiss sCJD patients.

The triangular plot correlates the intensities of the upper (diglycosylated), middle (monoglycosylated) and lower (unglycosylated) bands of PrP^{Sc}. Swiss CJD cases of the year 2002 are depicted in grey diamonds. As controls, sCJD PrP^{Sc} type 1 (MM1, black box), sCJD PrP^{Sc} type 2 (VV2, grey box) and vCJD, PrP^{Sc} type 4 [35] (type 2b [34]) (empty box) are depicted. Others have previously plotted the ratio of the diglycosylated/total signal intensity on the ordinate to the ration of the monoglycosylated/total signal intensity on the abscissa, although there is no biological reason to assume that ratio of the diglycosylated/total signal intensity to the ratio of the monoglycosylated/total signal intensity is more meaningful than the ratio of the monoglycosylated/total signal intensity to the ratio of the unglycosylated/total signal intensity. In contrast, the ternary method of presentation depicts unprocessed data, thereby avoiding any implicit hierarchy between the three bands. As the ternary plot combines rigorous objectivity with a synthetic and intuitive illustration, it could be generally used in representation of biochemical prion strain typing data. A Microsoft Excel spreadsheet for automated generation of ternary glycoform graphs can be freely downloaded from our Web site (http://www.unizh.ch/pathol/neuropathologie/d/glycotypetriplot.xls).

Although the amount of PrP^{Sc} present in the central nervous system exceeds the amount of PrP^{Sc} that can be found outside the CNS by orders of magnitude, in terms of tissue accessibility non-neuronal tissue compartments offer obvious advantages [38].

Recent studies have demonstrated the diagnostic utility of peripheral PrP^{Sc} detection, and depending on the type of human prion disease PrP^{Sc} can be demonstrated in lymphoid tissue and skeletal muscle of prion-diseased individuals (Figure 2).

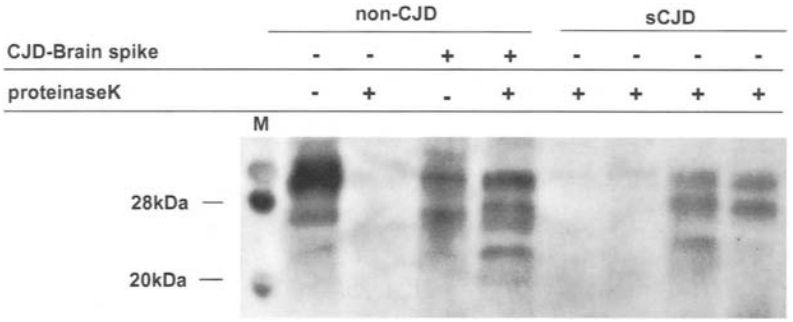

Figure 2. Western blot analysis of phosphotungstate-precipitated PrP^{Sc} from muscle samples of patients with sporadic Creutzfeldt–Jakob Disease.

Lane 1 shows molecular weight marker, Lanes 2 and 3 show muscle homogenate from a patient without a prion disease, spiked with brain homogenate from a control. Lanes 4 and 5 show muscle homogenate from a patient without a prion disease, spiked with brain from a patient with sporadic Creutzfeldt–Jakob disease. Lanes 6, 7, 8 and 9 muscle homogenate from patients with sporadic Creutzfeldt–Jakob disease. PrP^{Sc} is present in two samples (lanes 8 and 9). Lanes 3, 5 through 9 show results after proteinase K digestion.

Histological investigations

Routine neuropathological investigations include sampling of defined regions within the CNS and immunohistochemical demonstration of PrP. Special emphasis is put on the investigation of distinct deposition pattern of PrP in various regions of the CNS such as the cerebellum and the thalamus [4]. Histopathological features of prion diseases include spongi-

form change, neuronal loss, and gliosis (astro- and microglia). Spongiform change is characterised by diffuse or focally clustered, small, round or oval vacuoles in the neuropil of the deep cortical layers, cerebellar cortex or subcortical grey matter. The degree of spongiform change and the pattern of deposition of PrP is dependent on the type of prion disease and it could be shown that in sporadic CJD, these patterns are correlated to the codon 129 genotype of *PRNP*. Characteristic patterns of PrP^{Sc} deposition are synaptic, patchy/perivacuolar, or in the form of plaques. Certain genetic forms of human prion disease such as fatal familial insomnia show very little deposition of PrP^{Sc} when assayed by immunohistochemistry for PrP.

Conclusions

In subsequent years, we will see the implementation of several therapeutic approaches aimed to decelerate the clinical course of prion diseases. In order to select patients for these studies, and in order to monitor the efficiency of treatment, it will be essential to develop sensitive and specific assays able to confirm the diagnosis of a prion disease from tissue compartments which are easily accessible. Ideally, these assays should be able to distinguish various forms of human prion diseases. Given the fact that previous years have seen the discovery of a wide range of sensitive methods for the detection of pathological prion protein in non neuronal tissue compartments such as lymphoid tissue or muscle, one could hope that these aspirations will be met.

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History and state of the art of PrP-res "typing" in Creutzfeldt-Jakob disease

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Summary. Much progress has been made in understanding the molecular basis of phenotypic variability in Creutzfeldt-Jakob disease (CJD) in the last ten years. The most significant advance was the discovery that the genotype at polymorphic codon 129 of *PRNP* and the "type" of the protease-resistant prion protein fragment, PrP-res, have a major influence on the disease phenotype in all forms of CJD, irrespective of their etiology. The most widely accepted CJD classification includes six clinico-pathological phenotypes and two major types of PrP-res, types 1 and 2, which can be distinguished on the basis of a ~2 kDa difference in relative molecular mass of the protein fragment. However, alternative classifications of human PrP-res types distinguished three patterns of PrP-res molecular mass instead of two, thereby creating significant confusion in the field. Fortunately, progress has been recently made in clarifying these disparities. Most significant in this regard, has been the finding that pH variation among CJD brain homogenates in standard buffers influences the size of PrP-res. Thus, some of the PrP-res heterogeneity used to identify putative strain-specific PrP-res types simply represents a technical "artefact" related to the experimental conditions. On the other hand, recent data have also shown that PrP-res types 1 and 2 are heterogeneous biological species, which can be further distinguished into molecular subtypes that fit the current histopathological classification of sporadic CJD in 6 subtypes. Finally, novel truncated PrP-res fragments of smaller size than PrP-res types 1 and 2 have recently been identified in CJD. Although more studies are needed to fully characterize the presence, characteristics and biological significance of these peptides, preliminary results indicate that their search and characterization may be useful in the molecular diagnostics of CJD subtypes.

Key words. prion, classification, immunoblotting, protease, *PRNP*

Introduction

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders of humans and animal that are largely infectious. The pathogenesis of these disorders is linked to the prion protein (PrP), a host-encoded, copper and membrane bound glycoprotein of unknown function [1]. There is no agent replication or transmission of infectivity in the absence of PrP expression. Moreover, an abnormal, partially protease-resistant isoform (PrP^{Sc} or PrP-res) of the normal cellular prion protein (PrP^{C}) specifically accumulates in the nervous system during infection and represents the hallmark of the disease [1].

Creutzfeldt-Jakob disease (CJD), the most common human TSE, and scrapie, the prototype of animal TSEs, comprise a broad spectrum of clinico-pathological variants [2]. The existence of multiple strains of the infectious agent in conjunction with host genetic factors such as *PRNP* mutations or polymorphisms are thought to be responsible for TSE phenotypic heterogeneity [2,3]. Although uncertainties remain on the molecular basis of TSE strains and the relationship between strains and PrP, several lines of evidence indicate that PrP-res exists in a variety of molecular "types" showing distinct physicochemical properties, and representing genuine biochemical signatures of individual strain-host genotype interactions [2]. Given that PrP-res is the only molecule with a proven link to the prion strains and that agent bioassays in mice are very time consuming, unraveling the physicochemical properties of PrP-res associated with each TSE strain or phenotype (i.e. PrP-res "typing") has undoubtedly assumed crucial importance for strain typing and the molecular classification of TSEs, with wide implications for both disease diagnosis and epidemiologic surveillance. In this work we critically review the history and current knowledge of PrP-res "typing" in CJD.

PrP-res type 1 and type 2: A tale of two proteins

The first evidence of a physicochemical heterogeneity of PrP-res came from studies on experimental scrapie and transmissible mink encephalopathy (TME). In 1986, Kascsak et al [4] originally demonstrated that PrP-res from several strains of murine scrapie show significant differences in biochemical and physical properties such as glycosylation or molecular mass. Subsequently, Bessen and Marsh [5] identified and characterized two strains of transmissible mink encephalopathy, that differed in the electrophoretic mobility (i.e. relative molecular mass) of the PrP-res fragment.

The first demonstration of PrP-res by Western blot in a CJD affected subject dates back to 1986 [6,7], but the existence of different human PrP-res isoforms was only shown in the early nineties. In depth analysis of PrP-res in the post-mortem brains of individuals with Fatal Familial Insomnia (FFI) and familial CJD linked to the D178N *PRNP* mutation (CJD178), whose *PRNP* sequence only differs for the genotype at codon 129 in cis with the D178N mutation (M in FFI, V in CJD178), demonstrated that the size of the protease resistant core differs in the two TSE subtypes [8]. In addition, the glycoform ratio of PrP-res was distinct in the two diseases [8]. Modification of the N-terminal proteinase K cleavage site resulted in fragments of different size [8]. From these results it was concluded that development of either FFI or CJD178 requires the codon 178 mutation in concert with the codon 129 polymorphism and, therefore, that the primary PrP sequence specifies the phenotype of the disease and the physico-chemical properties of PrP-res. The interpretation at that time was that the codon 129 polymorphism would modify the conformation of the D178N mutated, pathogenic prion protein molecule which would result in the two distinct N-terminal cleavage sites in the protease resistant molecules associated with FFI and CJD178 [8]. One year later, however, two different types of PrP-res were also found in sporadic CJD subjects syngenic in the *PRNP* (Fig. 1) [9,10]. For the first time in a naturally occurring human TSE it was shown that distinct clinico-pathological variants and PrP-res molecules may form independently from the primary structure of PrP [9,10]. This observation therefore provided the first strong evidence for the existence of prion strains in humans. The two PrP-res molecules of sporadic CJD, named types 1 and 2, differed from each other both in relative mobility in SDS-polyacrylamide gels, which is indicative of size, and in their glycoform ratio [10]. Type 1 and type 2 PrP-res, in conjunction with the codon 129 polymorphism, were shown to largely determine the clinico-pathological variants of sporadic CJD [10,11]. Interestingly, type 1 and type 2 PrP-res had a molecular mass of either 21 kDa or 19 kDa, indistinguishable from those associated with FFI and CJD178 [7,12]. These observations were followed by the startling finding that the two PrP-res types are found in all forms of CJD independently from the apparent etiology of the disease, i.e. sporadic, inherited or acquired by infection [12,13]. This result strongly suggests a common mechanism of PrP-res formation in all forms of prion diseases and indicates that probably only a limited number of structures or conformers can be adopted by PrP.

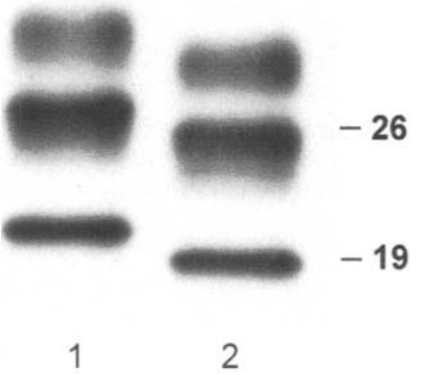

Fig. 1. PrP-res types 1 and 2 in sporadic CJD homozygotes for methionine at *PRNP* codon 129. Western blot of proteinase K-treated brain homogenates stained with the 3F4 monoclonal antibody, which recognize the PrP residues 109-111. The three bands are composed of (from the top to the bottom) diglycosylated PrP, monoglycosylated PrP, and unglycosylated PrP. The figure show two distinct pattern of human PrP-res, which differ for the electrophoretic mobility of the protein core. Type 1 (lane 1) has a relative molecular mass of 21 kDa, whereas type 2 (lane 2) has a mass of 19 kDa. The mobility of the core varies between the two PrP-res types because different numbers of amino acids are removed from the N-terminal of PrP by proteinase K.

As in FFI and CJD178, the PrP-res molecules associated with other subtypes of human prion disease showed distinct glycoform ratios, characterized by an overrepresentation of the diglycosylated form [8, 12-15]. They include CJD linked to the E200K-129M haplotype [12,16] and, as the most publicized example, new variant CJD [14,15]. This pattern of glycosylation dominated by the fully glycosylated form has been named pattern B to be distinguished from the most common pattern A, characterized by an overrepresentation of the monoglycosylated form [13]. Since glycosylation is a co-translational process that is known to differ among cell types, the different glycoform ratios may reflect the involvement or the targeting of distinct neuronal populations. Alternatively, different prion strains may preferentially convert certain PrP-res glycoforms. However, in inherited prion diseases, the glycoform ratio of PrP-res is also reflected by altered PrP^C processing induced by specific *PRNP* mutations, and therefore, it var-

ies independently from the agent strain [16,17]. The E200K and the D178M mutations represent 2 examples of such an influence on PrP^C metabolism [16,17].

Human PrP-res typing: a matter of controversy

After the original proposal of two major types of PrP-res in sporadic CJD, two alternative classifications of human PrP-res types, also based on the electrophoretic mobility of the unglycosylated form, were proposed [14,18]. Unfortunately, in both instances, the same numerical nomenclature was used to refer to putatively different biochemical "types" of PrP-res, creating further confusion in the scientific debate. To tackle this problem, this review identifies the classification referred to by adding the initial of the first author of the original publication, after the number indicating the type of PrP-res [i.e. (P) for Parchi, (C) for Collinge, (Z) for Zanusso].

In 1996, one year after the type 1 (P), type 2 (P) original proposal, Collinge et al. reported a classification of four human PrP-res types [14]. They observed types 1 (C) and 2 (C) in sporadic CJD and iatrogenic CJD subjects homozygous for methionine at codon 129, type 3(C) only in iatrogenic CJD linked to MV or VV at codon 129, and type 4 in variant CJD. Types 3 and 4 had the same gel mobility but showed a distinct ratio of the three glycoforms. The reported differences in electrophoretic mobility among the putative PrP-res isoform were less than 1 kDa between types 1 (C) and 2 (C) and from 2 to 3 kDa between types 2 (C) and 3. It must be emphasized that, at variance with Parchi et al. [9-11], the classification by Collinge et al [14] was originally presented as a pure molecular classification, whereas the complete study, including the clinico-pathological data, was only recently published (see below) [19].

When Parchi et al extended their analyses to iatrogenic CJD, kuru and variant CJD they confirmed the presence of only two major PrP-res types, corresponding to those previously described in sporadic CJD. The five samples from iatrogenic CJD subjects carrying MM at codon 129 had PrP-res type 1 (P), whereas the samples from one iatrogenic CJD subject carrying VV, 2 kuru cases, and 1 variant CJD case had PrP-res type 2 (P) [13]. It is noteworthy, however, that Parchi et al reported a certain degree of heterogeneity within both types 1 and 2, particularly among type 1 samples [10,13]. These, however, were subtle biochemical differences compared to the type 1 / type 2 difference in relative molecular mass, and failed to show a consistent reproducibility or a correlation with the disease pathological phenotype, or both. This observation prevented these authors

from a further distinction of PrP-res types, despite the fact that the characterization of at least 6 distinct pathological sporadic CJD subtypes [11] argued for the existence of more than two prion strains and two PrP-res types. Based on these results and considerations, it was concluded that: i) Collinge et al had erroneously matched their types 1 and 2 with those described by Parchi et al.; ii) PrP-res types 1 (C) and 2 (C) may represent technical "artifacts" unrelated to the agent strain or the disease phenotype; iii) PrP-res type 2 (P) has the same electrophoretic mobility as type 3 (C); iv) the finding of a distinct PrP-res type in sporadic and iatrogenic CJD carrying VV at codon 129 was puzzling and inconsistent with the finding of Parchi et al. [9-11].

In 1999, Wadsworth et al [20] found type 3 (C) in sporadic CJD carrying VV at codon 129, and therefore acknowledged that PrP-res type 3 (C) is not specific to the acquired form, as they had stated in their original report [14]. Furthermore, the result corroborated the interpretation that PrP-res type 2 (P) has indeed the same electrophoretic mobility as type 3 (C). In the same study [20], however, Wadsworth et al, provided evidence of a biochemical difference between types 1 (C) and 2 (C) and claimed the validity of their classification in 3 types of PrP-res, based on the analyses of electrophoretic mobility. Wadsworth et al. claimed that PrP-res type 1 (P) from MM subjects can be distinguished into 2 subtypes based on a 0.5 kDa difference in the relative molecular mass. Moreover, they observed that when pK digestion is performed in the presence of at least 20 mM EDTA, the two PrP-res subtypes show the same electrophoretic mobility, with an estimated shift, defined as type 2^-, of about 1.100 and 0.650 kDa for the first and second type respectively [20]. Finally, they were able to produce an interconversion of PrP-res types 1 (C) and 2^- (C), as well as the formation of an intermediate form with a electrophoretic mobility similar to type 2 (C), by simply changing the buffer concentration of Zn^{2+} and Cu^{2+} before the PK digestion. It was concluded that PrP-res types 1 (C) and 2 (C) represent distinct conformations acquired by PrP-res in the presence of metal ions such as copper and zinc and that the dependence of PrP-res conformation on the binding of copper and zinc represents a new mechanism of post-translational modification of PrP and for the generation of multiple prion strain [20].

In a subsequent study [21], the precise sites of PrP-res PK cleavage were determined, using N-terminal sequencing and mass spectrometry, in 36 cases of sporadic, iatrogenic, and genetic human TSEs including all known CJD subtypes as well as FI and kuru. Although each sample showed a ragged N-terminus, only two primary cleavage sites were detected: the N-terminal species starting at Gly-82 (G82) was shared by all samples classified as type 1 (P) by immunoblotting, whereas the species

beginning at Ser-97 (S97) was present in all those classified as type 2 (P) [21]. The other cleavage sites clustered around the primary site were not randomly distributed and were at least partially related to the codon 129 genotype. Within sporadic cases with PrP-res type 1 (P), those carrying the codon 129 MM had only one additional variant beginning at G78, whereas the subjects carrying VV had variants started at residues G86 and G90. In contrast, all the sporadic CJD with the PrP-res type 2 (P) [21] had both elongated and truncated variants flanking the invariable S97 cleavage site, regardless of the 129 genotype. However, in the MM2 (P) subjects the G92 was the only elongated variant, whereas the 129 MV2 (P) and VV2 (P) subjects showed additional N-terminally elongated species that extended to residues G90 and G86 in the VV2 (P) and up to residue G82, thereby overlapping with PrP-res type 1 (P) in the MV2 (P). Similar findings were obtained in the forms acquired by infection. Given that protein sequencing allows a more accurate mapping of PrP-res fragments than those based on the analyses of gel migration, the finding of only two primary cleavage sites, at G82 in type 1 (P) and at S97 in type 2 (P) confirmed that two major types of PrP-res characterize sporadic, genetic, and acquired forms of CJD. Furthermore, the study confirmed that the major PrP-res fragment associated with variant CJD is indistinguishable from those of the PrP-res type 2 (P) associated with sporadic CJD. Finally, by showing that either PrP-res type 1 (P) or 2 (P) is a slightly heterogenous form depending on the codon 129 genotype and possibly other unknown factors, the study paved the way for a further characterization and classification of human PrP-res subtypes.

In 2001, in an attempt to solve the debate and reach a worldwide consensus and standardization of human PrP-res typing, the World Health Organization set up a multicentric study in which thirteen specimens from brains of sporadic and variant CJD cases were distributed to twelve laboratories around the world for molecular characterization by defined protocol and classification according to the (P) and (C) scheme. The results of the study showed the following: all laboratories obtaining a result were able to reproduce the types 1 (P) and 2 (P) scheme, whereas none of the groups except Collinge's were successful in distinguishing types 1 (C) and 2 (C). It was concluded that whereas the results obtained by the (P) criteria were in agreement for all laboratories, the (C) scheme was of no practical use other than in the laboratory which developed it [Minor et al, manuscript in preparation].

In 2001, Zanusso et al [18] further distinguished PrP-res type 1 (P) into 2 subtypes showing a less than 1 kDa difference in mobility. However, at variance with Wadsworth et al [20], they distinguished the 2 PrP-res subtypes based on their sensitivity to pH variations, rather than on their pre-

sumed degree of binding to metal-ions. According to this study [18], about 60% of type 1 (P) samples show a pH-dependent shift in electrophoretic mobility, whereas the remaining 40% appear unaffected by pH variations. Although the details of the analyses of clinico-pathological features was not included in the study, the following differences between the two groups of patients were reported: the subjects who carried the type 1 (Z) had a distinct clinical course with early dementia or cortical visual disturbances, myoclonus, and periodic sharp wave electroencephalographic activity, whereas those carrying the type 2 (Z) had an ataxic-dementing CJD variant of longer duration. It was concluded the two putative PrP-res subtypes represent two distinct, strain-specific, protein conformations that show a different response to pH variations (i.e. one conformation is pH dependent, the other is not). More recently, however, the same authors abandoned their proposed nomenclature of three types of PrP-res in sporadic CJD and adopted that of Parchi et al. of only two types [22]. Similarly, they apparently did not confirm the existence of two distinct clinico-pathological phenotypes, possibly related to two prion strains, in MM subjects carrying PrP-res type 1 (P) [22].

In 2003, Hill et al [19] re-proposed their "3 types (C)" molecular classification of the PrP-res associated with sporadic CJD but, for the first time they also presented the results of the neuropathologic examination and the correlation with the molecular findings. Furthermore, compared to previous studies by the same group [14,20], the molecular characterization included 2 novel findings: i) the type 3 (C) pattern was also detected in a sporadic case carrying MM at codon 129; ii) a new PrP-res type, named type 6 (C), with a 2 kDa slower electrophoretic mobility than type 1 (C) was described in a single case [19]. From a careful analysis of the neuropathological data included in the study, the following considerations can be drawn. First of all, both MM1 (C) and MM2 (C) showed histological features consistent with the MM1 (P) pathological phenotype, the only reported difference between the MM2 (C) and MM1 (C) being a moderate compared to mild spongiform change in the basal ganglia and a predominant frontal and occipital compared to predominant occipital involvement of the cerebral cortex. Secondly, the pathological features of the VV3 (C) and MV3 (C) subtypes largely corresponded to those previously described by Parchi et al in the VV2 (P) and MV2 (P) groups. Finally, the pathological features associated with the single type 6 (C) case were also consistent with the MM1 (P) phenotype. It appears reasonable to conclude that the spectrum of pathological features of the CJD population examined by Hill et al was largely representative of that described by Parchi et al [10,11] in their population, and that there is no significant disagreement between the two groups in the pathological classification of CJD subtypes.

Thus, the major differences lie in both the experimental approach and the interpretation of the biochemical data. Parchi et al's original molecular classification [10,13] was reached after a systematic analysis of immunoblot as well as pathological data obtained from several brain regions, which led them to interpret as potential technical or chemical artefacts the relatively minor variability in PrP-res gel mobility that was observed within the same case or pathological phenotype (i.e MM1). In contrast, the original study by Collinge et al [14] was based only on the Western blot analysis of individual samples which led them to interpret as strain-specific the whole spectrum of observed PrP-res variability.

In 2004, Notari et al [23] added a critical, possibly final, piece to the puzzle by providing the demonstration that, indeed, there is a significant variability in electrophoretic mobility associated with each PrP-res sample that only depends on the experimental conditions used. Notari et al. demonstrated that the heterogeneity of human PrP-res within specific groups [MM1 (P) or MV1 (P) and, to a much lesser extent, in the other CJD subtypes] strictly depends on pH variations among brain homogenates (Fig. 2).

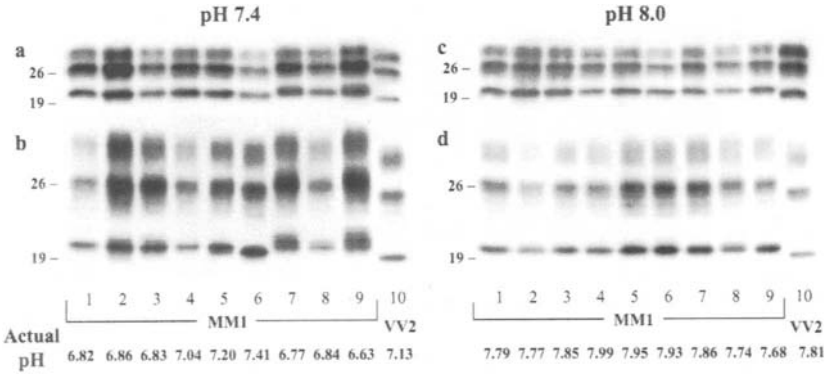

Fig. 2. Electrophoretic mobility of PrP-res type 1 (P) (MM subjects) generated by PK digestion in standard lysis buffer at pH 7.4 (a and b) and pH 8 (c and d). Immunoblot analysis of frontal cortex homogenates from 9 MM1(P) (lane 1-9) and 1 VV2 (P) (lane 10) subjects stained with the 3F4 monoclonal antibody. Homogenates were prepared in standard lysis buffer pH 7.4 (a and b) or 8.0 (c and d). Aliquots were digested with 100 μg/ml of PK for 1 h at 37°C. The actual pH values for each homogenate are shown at the bottom of each lane. a and c, samples were run in a Tris-glycine PAGE 12% mini-gel (5,5 cm long). b and d, samples were run in a Tris-glycine PAGE 12% gel (15 cm long). Approximate molecular masses are in kilodaltons. There is a certain degree of heterogeneity in the eletctrophoretic mobility of PrP-res type 1 (P) extracted from different MM subjects. The heterogeneity is much higher among samples prepared in LB pH 7.4

than in those prepared in LB pH 8.0. Samples prepared in LB pH 7.4 show heterogeneous pH values. There is a significant correlation between the pH value of the homogenate and the immunoblot profile of PrP-res in each sample. Reproduced with permission from Notari et al 2004 J Biol Chem 279:16797-16804.

They found that due to the lack of sufficient buffer capacities of standard Tris or PBS buffers, there is a significant heterogeneity in pH among CJD brain homogenates even when they are prepared from the same brain. The homogenate pH in turn influences the electrophoretic mobility of the PrP-res core generated by protease digestion, due to the combination of at least 3 factors: i) protease digestion of full length PrP-res is a step-by-step process yielding fragments with various degrees of resistance (Fig. 3); ii) PK activity is pH dependent (Fig. 3); iii) in sporadic CJD MM1 (P), MV1 (P) and, to a lesser extent, in VV1 (P) the size of PrP-res further changes depending on whether protease digestion is performed at a pH below or above 7.2.

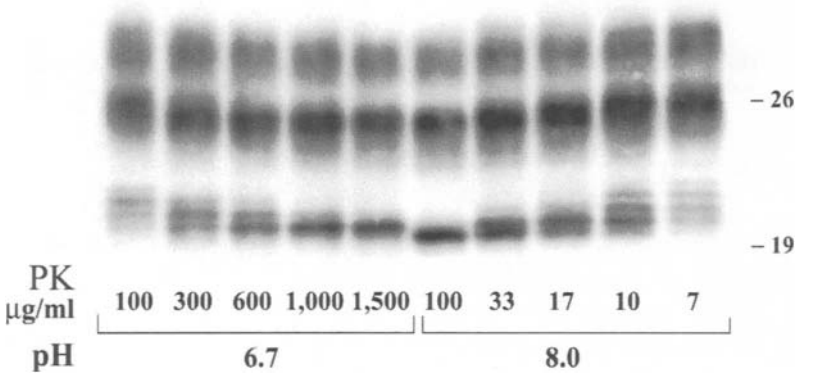

Fig. 3. Effect of PK concentration on the electrophoretic mobility of PrP-res type 1 (P) (MM subject). Immunoblot analysis of frontal cortex homogenates from a MM1 (P) subject, stained with the 3F4 monoclonal antibody. Homogenates were prepared in lysis buffer with strong buffer capacity (100 mM Tris) at pH 6.7 (lane 1-5) and 8.0 (lane 6-10). Aliquots were digested at 37° C for 1 h using different PK concentrations. Samples were run in a Tris-glycine PAGE 15% gel (15 cm long). Molecular masses are in kilodaltons. The same heterogeneity in PrP-res fragments that is generated by performing the PK digestion at different pH values can be obtained by changing the PK concentration at a given pH value. Reproduced with permission from Notari et al 2004 J Biol Chem 279:16797-16804.

Taken together, these and other (see below) results strongly suggest that there is no basis at present for claiming that more than one CJD subtype or CJD strain is associated with either the molecular combination 129 MM and PrP-res type 1 (P) or VV and PrP-res type 2 (P) as suggested by Collinge et al or Zanusso et al. In this respect, it is also noteworthy that experimental transmission of prions from pure MM1 (P) and VV2 (P) subjects to non-human primates and mice have given, within each group, uniform results in terms of incubation time or lesion profile [24,25].

Given the results on the effect of pH and that EDTA, according to the protocol of Wadsworth et al [20], is prepared in a basic solution at pH 8 and then added to the homogenate, Notari et al [23] also investigated whether a change in pH rather than the EDTA itself could be responsible for the observed shift in gel mobility of PrP-res type 1 (P) (see also above). Indeed, their results indicate that this is the case (Fig. 4). They measured the homogenate pH before and after the addition of EDTA and found that the addition of EDTA prepared stock solution at pH 8.0 to standard PBS or LB pH 7.4 significantly increased the pH value of the homogenate (about 0.7 pH units) (Fig. 4). Furthermore, they were not able to reproduce the effect of EDTA when the EDTA solution was adjusted to pH 7.0 before being added to the homogenate (Fig. 4). A potential influence of pH on the copper chelation activity of EDTA was considered unlikely, since the potential-pH diagram for the copper-EDTA system indicates that EDTA forms stable copper complexes over a wide range of pH values [23]. In conclusion, the data argue that EDTA at a 20mM concentration has no direct effect on the pattern PrP-res type 1 (P) cleavage by PK.

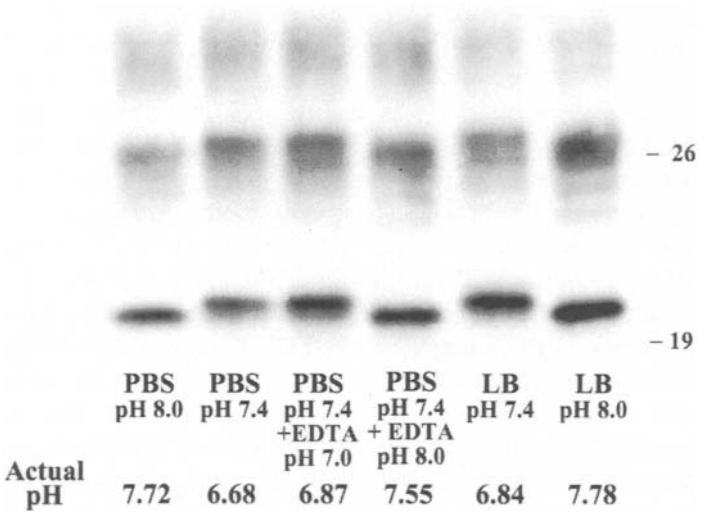

Fig. 4. Effect of EDTA on the electrophoretic mobility of PrP-res type 1 (P) (MM subject). Immunoblot analysis of frontal cortex homogenates from a MM1 (P) subject stained with the 3F4 monoclonal antibody. PK digestions were performed in PBS, pH 8.0 (lane 1), PBS, pH 7.4 (lane 2), PBS, pH 7,4, 20 mM EDTA (using a 250 mM EDTA stock solution tritiated at pH 7.0) (lane 3), PBS, 20 mM EDTA (using a 250 mM EDTA stock solution tritiated at pH 8.0) (lane 4), lysis buffer (LB) pH 7.4 (lane 5) and LB pH 8.0 (lane 6). All aliquots were digested with 150 μg/ml of PK for 1 h at 37°C. The actual pH values of the homogenates are shown at the bottom of each lane. Samples were run in a Tris-glycine PAGE 12% gel (15 cm long). Approximate molecular masses are in kilodaltons. EDTA at a 20mM concentration has no direct effect on the electrophoretic mobility of the PrP-res fragments generated by PK digestion. However, the addition of EDTA prepared stock solution at pH 8.0, to standard PBS or LB pH 7.4, induces an increase in pH that is sufficient to cause a shift in gel mobility of PrP-res core fragment. Reproduced with permission from Notari et al 2004 J Biol Chem 279:16797-16804.

It is difficult to provide a definite explanation for the finding of Zanusso et al, who also observed a pH dependent shift in electrophoretic mobility, but only in about 60% of their MM1 subjects. It is of significance, however, that they did not perform any direct measurement of actual pH in the homogenates. Thus, they may unwittingly have compared PrP-res core fragments generated at different pHs. It is also noteworthy that Wadsworth et al [26], in agreement with the results of Notari et al, re-

ported a shift in electrophoretic mobility in all their sporadic CJD MM subjects with either PrP-res type 1 (C) or type 2 (C) when they were treated with 20 mM EDTA at pH 8, before PK digestion. However, according to the results of Notari et al the change in PK cleavage depends on homogenate pH variations rather than on EDTA.

To conclude this summary of the disparities that have characterized PrP-res "typing" in humans, it is worth mentioning, that, although the classification proposed by Collinge et al [14] is still widely cited and acknowledged, there have been no studies by other groups replicating the finding of 3 distinct patterns of PrP-res electrophoretic mobility which correlate with the disease phenotype in sporadic CJD. The only exception is represented by Zanusso et al whose classification, however, as discussed before, has been already at least partially modified [22], wait to be reproduced by others and, above all, significantly diverge from the classification of Collinge et al. In contrast, both the distinction between types 1 (P) and 2 (P) and the classification of sporadic CJD in 6 pathological subtypes [11] have been reproduced by many European, North American and Japanese groups [26-38].

Progress towards the characterization of "real" subtypes of PrP-res types 1 (P) and 2 (P)

As stated above, several lines of evidence indicate that the pathological heterogeneity of human TSEs is related to the physicochemical properties of PrP-res [10,11,13]. However, whether each phenotype is indeed related to a specific PrP-res tertiary or quaternary structure is still unknown. In sporadic CJD 6 phenotypes have been characterized but only two major PrP-res types have been consistently reproduced, based on differences in the relative molecular mass of PrP-res [10,11].

By using gels with improved resolution and different experimental conditions, Notari et al [23] recently provided evidence that PrP-res types 1 (P) and 2 (P) may indeed be "biologically" heterogenous and include further subtypes. They showed that: i) PrP-res type 1 (P) in codon VV homozygotes shows a slightly faster electrophoretic mobility compared to type 1 (P) in 129 MM subjects (Fig. 5); ii) PrP-res type 2 (P) from MV subjects with kuru plaques is clearly distinguishable from the other type 2s (P) because it uniquely resolves as a doublet, even when a relatively high pK concentration is used (Fig. 5), and iii) PrP-res from VV2 (P) subjects as well as variant CJD display a significantly higher protease resistance than the PrP-res from MM2-cortical cases. Thus, analysis of protease resis-

tance may allow the distinction between VV2 (P) and MM2-cortical cases without *PRNP* genotyping and further helps, in combination with the study of PrP-res glycoform ratio [36], to distinguish variant CJD from other CJD MM2 (P) subtypes.

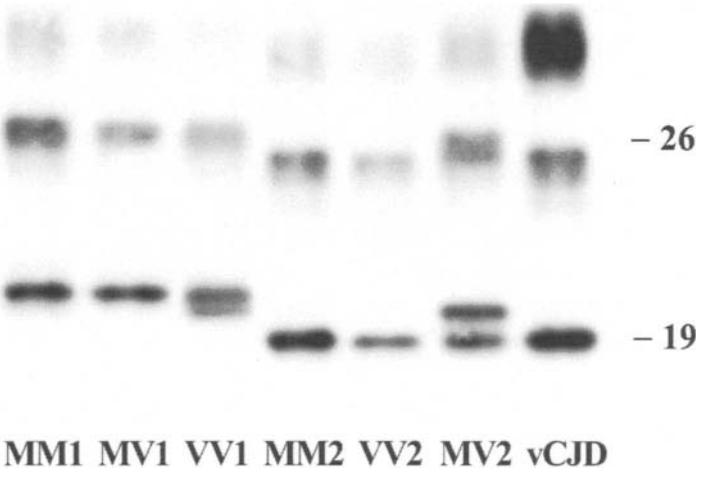

Fig. 5. Distinction of subtypes of PrP-res type 1 and type 2. Immunoblot analysis of frontal cortex homogenates from MM1, MV1, VV1, MM2-cortical, VV2, MV2 (classified as in reference 11) and variant CJD subjects is shown. Homogenates were prepared in lysis buffer with 100 mM Tris at pH 6.9 (a). Aliquots were digested with 2000 μg/ml of PK for 1 hour at 37°C. Samples were run in a Tris-glycine PAGE 15% gel (15 cm long). Approximate molecular masses are in kilodaltons. Based on the analysis of PrP-res electrophoretic mobility it is possible to distinguish 4 out of 5 sporadic CJD pathological subtypes. Reproduced with permission from Notari et al 2004 J Biol Chem 279:16797-16804.

Beyond PrP27-30: N- and C-terminal PrP-res fragments

For many years, full-length PrP-res and its truncated, protease-resistant core, PrP27-30 were thought to be the only TSE specific PrP components. However, additional PrP-res fragments were found between the early and mid nineties. These included unglycosylated PrP-res fragments of 7-8kDa, truncated at both N- and C-termini that characterize all Gerstmann-Sträussler-Scheinker (GSS) disease subtypes [39-41], and either a 16- or a

7 kDa C-terminal fragment found in scrapie-infected hamsters [42,43]. More recently, two novel C-terminal fragments of PrP-res, originally described in GSS subjects carrying the P102L mutation [25], and in familial CJD [16] linked to the E200K-129M haplotype, have been extensively characterized in sporadic CJD [44]. These PrP-res fragments have a relative molecular mass of about 12 and 13 kDa and include glycosylated and unglycosylated forms (Fig. 6). N-terminal sequence of the two peptides by automated Edman degradation indicated that they begin at residues 162/167 and 154/156 respectively [44].

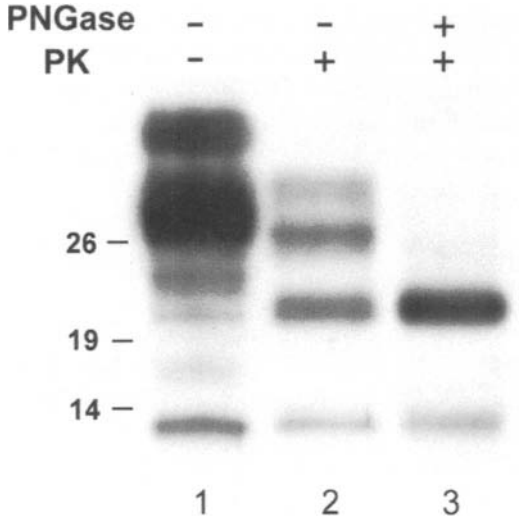

Fig. 6. Proteinase K-resistant C-terminal fragments 12/13. Immunoblot analysis of frontal cortex homogenates from a MM1 (P) subject stained with anti-PrP antibody 2301, a rabbit antiserum specific for C-terminal residues 220-230. Homogenates were prepared in lysis buffer 100 mM pH 6.9. Aliquots were treated with PK (lane 2 and 3) or PNGase (lane 3). Samples were run in a Tris-glycine PAGE 15% gel (7 cm long). Approximate molecular masses are in kilodaltons. In these experimental conditions PrP-CTF12/13 run as a single band in the immunoblot.

Additional characterization of the two fragments has shown that they have an intact C-terminus which likely contains the GPI anchor [44].

At variance with PrP27-30, the electrophoretic mobility PrP-CTF12/13 is comparable among the different sporadic CJD subtypes [44]. Similarly, the relative amount of PrP-CTF 12/13, although varying considerably from case to case, accounting for between 0- and 25% of the whole PrP-res sig-

nal, showed no apparent correlation with the disease subtype [44]. An interesting exception, however, seems to be the dura graft associated iatrogenic CJD with PrP plaques. Indeed, Satoh et al [45] recently found the 12/13 kDa PrP-CTF associated with iatrogenic CJD without plaques, whereas it was not found in the subtypes with plaques.

Very recently, Zanusso et al [22] reported the existence of two novel PrP-res fragments in sporadic CJD brain tissue. They found that whereas all sporadic CJD cases with PrP-res type 1 (P) as well as MM subjects with type 2 (P) PrP-res were characterized by the presence of unglycosylated PrP fragments of 16-17 kDa, the VV and MV subjects with type 2 (P) contained fully glycosylated PrP fragments, which, after deglycosylation, migrated at 17,5-18 kDa. A similar truncated fragment of about 17kDa has been recently detected in sporadic CJDMM2 and variant CJD by another group [46]. Although of interest, these recent data should be considered preliminary and await a more systematic characterization.

Acknowledgement

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Chronic Wasting Disease in Cervids in North America

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Abstract

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy of free-ranging and farmed cervids in North America that is distinct from scrapie of domestic sheep, bovine spongiform encephalopathy, and Creutzfeldt-Jacob disease of humans. The purpose of this paper is to review the current status of CWD in North America. The natural host range of CWD includes mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*). Experimentally, by intracerebral or oral exposure, the host range is wider, but there appears to be a significant barrier to infection of cattle and humans. The exact mechanism of CWD transmission is not known but recent studies indicate that direct transmission, indirect transmission via environmental contamination, and transmission associated with carcasses are possible. Maternal transmission does not appear to play a significant role in CWD. Although still under investigation, polymorphisms in the prion protein influence CWD pathogenesis in mule deer and elk. Studies of CWD pathogenesis following oral exposure demonstrate early widespread distribution of abnormal prion protein in the lymphoid tissue prior to invasion of the central nervous system. These data have lead to techniques for CWD surveillance in deer based on testing retropharyngeal lymph node. However, both brain and lymph node must be tested in elk for highest sensitivity due to differences in pathogenesis in this species compared to deer. The unique nature of a transmissible spongiform encephalopathy occurring in free-ranging cervids is a serious challenge to wildlife managers and animal health agencies in North America.

Bovine spongiform encephalopathy (BSE) in Japan

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Summary. Bovine spongiform encephalopathy (BSE) has become an important problem not only for the animal industry, but also for public health. BSE was first recognized in Japan in September 2001 as a result of fallen stock surveillance. Since October 2001, all cattle slaughtered at abattoirs have been tested for BSE. From April 2004, examination of all dead cattle aged over 24 months has been conducted at livestock hygiene service centers. Samples positive on enzyme linked immunosorbent assay (ELISA) are further subjected to western blot (WB) and immunohistochemistry (IHC). Fourteen BSE cases had been reported by November 2004; thirteen were classified as the typical form while the other was atypical. Variant forms of BSE with atypical histopathological and/or biochemical phenotype have also been reported in Italy and France. Further study is required to characterize the BSE prion, and to this end, brain homogenates from Japanese BSE cases were intracerebrally inoculated into wild-type mice. The first case diagnosed in Japan (BSE/Chiba) has been successfully transmitted to rodents. Mean incubation period (409.0 ± 28.2 days) was considerably longer than that previously reported. PrP^{Sc} distribution, possibly due to prion titer, recipient susceptibility and/or sample storage conditions. Transgenic mice that overexpress a bovine PrP gene were recently introduced to overcome the species barrier problem, hopefully enabling accelerated transmission of BSE prions. These transgenic mice are also being used in ongoing transmission studies of the atypical BSE case.

Key words. BSE, surveillance, transmission

Introduction

Bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in cervids, and Creutzfeldt-Jakob disease (CJD) in humans are neurodegenerative disorders known as prion diseases or transmissible spongiform encephalopathies (TSE) [1]. BSE, suspected to have been caused by contaminated meat and bone meal (MBM) [2, 3], was first reported in the United Kingdom (UK) in 1987 [4], and over 180,000 cases have thus far been reported. Furthermore, contaminated animal feed was exported from the UK to other countries, and new cases continue to appear in several European Union (EU) countries, and non EU countries. As BSE is considered to be a man-made disease.

In 1996, the occurrence of variant CJD (vCJD) was reported and a possible link with BSE was suggested [5, 6, 7]. Until the occurrence of vCJD, no relationship had been observed between animal and human prion diseases, and BSE was considered solely as a novel animal disease. Today, BSE has become an important problem not only for the animal industry, but also for public health.

In Japan, the first case of BSE was observed in 2001 [8]. Various control measures have since been taken to prevent human and animal exposure to BSE prions, including prohibition of the use of MBM in feed, removal of specified risk materials (SRMs), testing of all cattle at abattoirs, and fallen stock surveillance of all dead cattle aged over 24 months. This paper focuses on the diagnosis of BSE and the current status of BSE testing in Japan.

Clinical features of BSE

Prion diseases have a slowly progressive and invariably fatal course. They are characterized by a long incubation period in affected animals and absence of detectable immunological response in the host. BSE is characterized by 1) apprehension, behavioral changes, fear, increased startle response, or depression; 2) hyper-reactivity or hyper-reflexia: to touch, sound, and light; 3) ataxia of gait, including hypermetria and paresis, resulting in falling; 4) adventitial movements: muscle fasciculations, tremor, and myoclonus; 5) autonomic dysfunction: reduced rumination, bradycardia, and cardiac arrhythmia; and 6) loss of body weight and deterioration in general condition, and reduction in milk yield [2, 4, 9, 10, 11]. These features can also arise in other central nervous system disorders and clinical features are not evident until the terminal stage of the illness. There-

fore, considering the currently low incidence of BSE in Japan, the disease can be difficult to diagnose from clinical signs alone and laboratory tests are required, particularly to detect the pre-clinical stage.

Laboratory diagnosis of BSE

An abnormal isoform of prion protein (PrP^{Sc}) accumulates in animals affected with TSE. This is generated by a posttranslational modification of the cellular isoform of prion protein (PrP^{C}), and is generally thought to be the causative agent [1]. Recent evidence demonstrating that an in-vitro form of the prion protein (PrP) can be transformed and is infectious to mice gives support to this theory [12]. Detection of PrP^{Sc} and differentiation of this isoform from PrP^{C} are therefore crucial for the diagnosis of prion diseases. Conversion of PrP^{C} to PrP^{Sc} is the central event in prion propagation; however, the mechanism of this conformational change remains obscure.

PrP^{C} is a glycoprotein with a molecular mass of 33-37 kDa, contains two N-linked sugar chains, and is attached to the cell membrane by a glycosyl phosphatidyl inositol (GPI) anchor. Though the nature of PrP^{Sc} remains obscure, it exhibits conformational differences when compared to PrP^{C}; PrP^{Sc} has a larger number of β-sheets and diminished α-helical content compared to PrP^{C}, despite the fact that both consist of identical amino acid sequences [13]. Hence PrP^{Sc} is relatively resistant to protease digestion, easily aggregated into amyloid fibrils, and demonstrates insolubility. The protease resistance of PrP^{Sc} is widely accepted as the physicochemical basis by which PrP^{C} and PrP^{Sc} can be distinguished.

Diagnosis of BSE has been performed using histopathology [4], immunohistochemistry (IHC) [14, 15], and biochemical analyses (Western blot [16, 17, 18, 19] and enzyme linked immunosorbent assay (ELISA) based tests [20, 21]). Other than histological examination, all these tests aim to measure PrP^{Sc} as proteinase K (PK) resistant PrP [22, 23]. Recently however, a new technique has been introduced for BSE diagnosis, which is aimed at detection of PrP^{Sc} as conformationally different PrP [24].

The above-mentioned methods of BSE diagnosis are all post-mortem diagnostic procedures, and are applicable only in brain tissue (obex region) [25]. As PrP^{Sc} only accumulates in the central nervous system (CNS) during the middle to latter stages of disease progression, none of the available BSE tests can detect affected cattle in the early stage of the disease. To address this problem, tissues that could harbor BSE prions are defined as SRM and removed from BSE negative cattle to avoid human consumption.

BSE surveillance in Japan

In Japan, the first case of BSE was confirmed in September 2001 in a cow culled in an abattoir due to ataxia, after the introduction of fallen stock surveillance. From this point on, various control measures were taken to prevent human and animal exposure to the BSE prion. From October 2001, all cattle slaughtered in abattoirs have been tested, and to date cases of 11 BSE have been detected from over 3.7 million examined cattle. From April 2004, all dead cattle over 24-months old have been subjected to BSE testing. In terms of fallen stock surveillance, sampling tests were implemented and another BSE case has been detected as a result. Furthermore, complete fallen stock surveillance detected another BSE case at October 2004. Details of BSE cases in Japan are shown in Table 1. Screening is performed using ELISA, and 3 commercial kits (Bio-Rad, Enfer, and Fuji-Rebio) are now available in Japan. ELISA tests are conducted at meat sanitation inspection centers and livestock hygiene service centers controlled by each local government. If ELISA yields positive results, the sample is subjected to confirmatory testing (histopathology, IHC and western blot). Japanese BSE cases, including one of atypical BSE, can be classified into 3 groups according to test results [26] (Table 2). On the basis of the established guidelines, if PrP^{Sc} is detected either by WB or by IHC, cattle are diagnosed with BSE and excluded from human consumption.

BSE confirmation tests are performed at the National Institute of Infectious Disease (NIID), Hokkaido University, Obihiro University, and the National Institute of Animal Health (NIAH). These laboratories employ uniform methodology, including antibodies, reagents, and share information in order to standardize BSE diagnosis in Japan.

Table 1 BSE cases in Japan

No.	Date of confirmation	Farm location	Birth place	Birth date	Age at diagnosis (months)
1.	2001.9.10.	Chiba	Hokkaido	1996.3.26.	64
2.	2001.11.21.	Hokkaido	Hokkaido	1996.4.4.	67
3.	2001.12.2.	Gunma	Gunma	1996.3.26.	68
4.	2002.5.13.	Hokkaido	Hokkaido	1996.3.23.	73
5.	2002.8.23.	Kanagawa	Kanagawa	1995.12.5.	80
6.	2003.1.20.	Wakayama	Hokkaido	1996.2.10.	83
7.	2003.1.23.	Hokkaido	Hokkaido	1996.3.28.	81
8.	2003.10.6.	Fukushima	Tochigi	2001.10.13.	23
9.	2003.11.4.	Hiroshima	Hyogo	2002.1.13.	21
10.	2004.2.22.	Kanagawa	Kanagawa	1996.3.17.	95
11.	2004.3.9.	Hokkaido	Hokkaido	1996.4.8.	94
12.	2004.9.13.	Kumamoto	Kumamoto	1999.7.3.	62
13.	2004.9.23.	Nara	Hokkaido	1996.2.18.	103
14.	2004.10.14.	Hokkaido	Hokkaido	2000.10. 8	48

Table 2 Summarized test results for Japanese BSE cases

BSE cases	ELISA	WB	IHC	histopathology
1-6, 10-14	+	+	+	+
7	+	+	+	–[a]
8[b], 9[c]	+	+	–	–

[a]: no spongiform change was observed
[b]: atypical BSE case
[c]: case of BSE in a young animal
[b,c]: No PrP^{Sc} on IHC, and no spongiform change on histology

Fallen stock surveillance

Testing of slaughtered cattle was implemented immediately after the first BSE case was confirmed. On the other hand, setting up the complete fallen stock surveillance system has been a more lengthy process but has proven worthwhile, with 2 cases of BSE having been detected in 2004. The importance of fallen stock testing in understanding the current status of BSE in Japan has thus been confirmed.

In some cases, carcasses have been left for a long period under poor storage conditions. While it has been reported that PrP^{Sc} can be detected in autolyzed and/or deteriorated samples using WB [21, 27, 28], precise sampling of the obex region is problematic in liquefied brain tissue and it could therefore be difficult to perform confirmatory IHC under these conditions. To avoid or minimize such problems, tissue specimens must be collected as soon as possible when fallen cattle are found.

In Japan, rendering plants have accepted only BSE-negative cattle, with carcasses being kept refrigerated in so called "stock points", until test results are obtained. BSE was demonstrated in two of these refrigerated carcasses, enabling us to examine PrP^{Sc} distribution in these cattle; detailed reports of which are anticipated in the near future. It is clear that surveillance of fallen stock can provide detailed information about the current status of BSE in Japan and the distribution of PrP^{Sc} in BSE cattle.

Transmission of Japanese BSE prion to rodents

Previous pathological studies of BSE in UK have provided evidence for a uniform vacuolation pattern and PrP^{Sc} deposition in affected animals [29]. It has been thought that the BSE epidemic has been sustained by a single agent. However, variant forms of BSE, with atypical histopathological and/or biochemical phenotypes, have been reported in Italy [30], France [31], and Japan [26]. Furthermore, two distinct prions were isolated in a mouse after transmission from a typical BSE case [32]. In sheep scrapie, multiple prion strains have been described that are distinguished by their incubation periods and lesion profiles when passaged in inbred lines of mice [7, 34]. These results suggest that extensive studies on BSE prion transmission to rodents are required. The first case of BSE in Japan (BSE/Chiba) was successfully transmitted to rodents (Table 3), with a mean incubation period (409.0 ± 28.2days) considerably longer than that previously reported [7, 33, 34]. This might have been influenced by PrP^{Sc} distribution, prion titer, susceptibility of the mice, or storage conditions of samples. However, incubation periods have decreased during successive passaging. To overcome the species barrier in inter-species transmission, mice with overexpression of bovine PrP gene (TgBoPrP) have been bred and have been reported to be useful in BSE transmission experiments [35]. When inoculated intracerebrally with Japanese BSE brain samples, TgBoPrP mice exhibited clinical features at 261.2 ± 12.2 days post inoculation (Table 3). Transmission experiments of materials from other cases of Japanese BSE to TgBoPrP mice, including atypical BSE (case 8) and

BSE in young animal (case 9; 21 months old), are in progress, in order to amplify BSE prions. Further studies are required to clarify the strain variation of BSE prion.

Table 3 Incubation periods of BSE prion in mice

Passage history	mice	inoculum	diseased /inoculated	incubation period[a] (days)
1st	RIII	BSE/Chiba/1	5/5	409.0 ± 28.2
2nd	RIII	BSE/RIII	8/8	221.0 ± 4.7
2nd	CD-1	BSE/RIII	21/21	196.8 ± 8.4
3rd	CD-1	BSE/RIII/CD-1	22/22	173.6 ± 7.9
1st	TgBoPrP	BSE/Wakayama/6	5/5	261.2 ± 12.2
1st	CD-1	BSE/Wakayama/6	10/10	415.3 ± 52.3
1st	TgBoPrP	BSE/Fukushima/8[b]	0/5	>339
1st	TgBoPrP	BSE/Hiroshima/9[c]	0/6	>216

[a]: mean ± standard deviation
[b]: atypical BSE case
[c]: young BSE case

Conclusion

Ante-mortem diagnostic procedures for BSE are needed. However, PrP^{Sc} as an autologous specific structural protein does not evoke the host immune response and PrP^{Sc} accumulation is almost entirely limited to the CNS. Hence no practical ante-mortem methods of detecting BSE-infected cattle are currently available. Moreover, the pathogenetic mechanisms of prion disease are not fully understood. The time course of PrP^{Sc} accumulation in the brain has yet to be determined, and the question of whether this protein exists in other tissues has yet to be answered satisfactorily; resolution of these issues will help in evaluating the current BSE control program. Furthermore, current studies into transmission of cases of BSE that are atypical or that develop in young cattle are expected to amplify the BSE prion. These rodent transmission experiments may also help to clarify the existence of different BSE strains. Nonetheless, further research is required to solve the enigma of the BSE prion.

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The role of host PrP in control of incubation time

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Summary. PrP is central to TSE disease and has been hypothesised to be the infectious agent. Polymorphisms in the PrP gene are associated with different incubation times of disease following exposure to an infectious agent and mutations in the human PrP gene can apparently lead to spontaneous genetic disease. Strains of TSE agent are proposed to be generated and maintained through differences in glycosylation or conformation of PrP and the barrier to infection between species is thought to be due to the differences in the sequence of PrP between different species. To test these hypotheses, we have introduced specific modifications into the endogenous mouse *Prnp* gene by gene targeting. The mutated PrP gene is in the correct location under the control of the endogenous *Prnp* regulatory sequences and thus expressed in the same tissues and amounts as the wild type *Prnp* gene. By altering the murine PrP coding region to that of another species we have established that increasing overall identity between host and donor PrP can lead to either an increase or a decrease in incubation time of disease in a strain dependent manner. We have introduced a point mutation (101L) into the N-terminus of the host PrP and shown that it dramatically changes the susceptibility of the host to infection from different species. We have in addition demonstrated that polymorphisms in the N terminus (L108T) and C-terminus (F189V) of host PrP both alter the incubation time of disease. We have in addition introduced mutations into the *Prnp* gene which prevent glycosylation at each or both of the two N-linked glycosylation sites of PrP. Inoculation of these mice with infectivity has established that glycosylation of host PrP can influence incubation time of disease, vacuolar pathology and strain determination.

Key words. PrP, prion, transgenic models

The mechanism by which the PrP gene influences host susceptibility is not understood. The prion hypothesis proposed that identity between host PrP and the PrP sequence in the donor of infectivity leads to short incubation times and high susceptibility of the host to infection. Indeed this was demonstrated in early transgenic models where transgenic mice expressing multiple copies of the hamster PrP gene were shown to be more susceptible to a hamster strain of scrapie than wild type mice (Scott *et al.*, 1989). However subsequent transgenic experiments have demonstrated that the mechanism is very much more complex. While overexpression of a bovine PrP gene in transgenic mice leads to a model which develops disease rapidly when inoculated with BSE (Scott *et al.*, 1999), replacement of the murine PrP gene with a bovine PrP gene by gene targeting leads to longer incubation times in the transgenic mice than in wild type (P.Hart, unpublished results). This increase in incubation time is also observed on inoculation of gene targeted transgenic mice expressing human PrP with BSE and vCJD, despite the apparent sequence compatibility with vCJD. However isolates of sCJD have been shown to transmit efficiently to the same human PrP transgenic mice, suggesting that the observed long incubation periods with vCJD are not due to incompatibility between some host factors and the transgene, but rather to a specific effect of the individual TSE strain. Thus increasing the sequence homology between host and donor PrP can increase or decrease incubation time but not in a predictable way.

A single amino acid alteration in PrP may have a more pronounced effect on the susceptibility of a host to a particular agent than overall identity between host and donor PrP. Susceptibility to transmissible spongiform encephalopathies (TSEs) is strongly associated with PrP polymorphisms in humans, sheep and rodents. Polymorphisms at murine PrP codons 108 and 189 can have a dramatic effect on the incubation time of disease in mice, and provide an excellent model system for the dissection of the role of PrP gene variation in TSE biology. To define the role of codons 108 and 189 in the control of disease we have introduced each polymorphism separately into the murine *Prnp*^{a} gene. Inbred lines of mice expressing all allelic combinations were inoculated with TSE agents. The L108T and/or F189V polymorphisms in murine PrP were shown to be the major factor influencing incubation time of scrapie in mice (Moore *et al.*, 1998), and more recently it has been demonstrated that while the 189 polymorphism has the major influence over incubation time, homozygosity at codon 108 leads to shorter incubation times than heterozygosity (Barron *et al.*, 2005). The influence of homozygosity in the N terminus of PrP can also be demonstrated in CJD where the majority of cases occur in indi-

viduals homozygous for methionine or valine at codon 129 (Palmer *et al.*, 1991; Zeidler *et al.*, 1997a; Alperovitch *et al.*, 1999). In sheep the V136A, R154H and R171QH polymorphisms in sheep PrP have been used to establish a scale of susceptibility to scrapie, where VRQ/VRQ animals are most at risk of developing scrapie and ARR/ARR are thought to be resistant to disease.

It has been know for some time that point mutations in the human PrP gene are associated with familial TSE, where the mutation itself is apparently sufficient to cause a spontaneous TSE to develop (Hsiao *et al.*, 1989; Petersen *et al.*, 1992; Kong *et al.*, 2004). It has been hypothesised that the mutations lead to an instability in PrP and thus make it more likely to misfold into the disease associated form of PrP. This hypothesis was given considerable weight when it was demonstrated that over-expression of a murine PrP gene carrying a proline to leucine mutation at codon 101 (equivalent to the P102L mutation in human PrP associated with Gerstmann Straussler Syndrome) could produce a spontaneous neurodegenerative disease in mice, and that this disease could be passed on to other transgenic mice expressing a lower level of the same transgene (Hsiao *et al.*, 1990; Hsiao *et al.*, 1994; G. C. Telling *et al.*, 1996). However when the same 101L mutation is introduced into the endogenous murine PrP gene by gene targeting, a somewhat different outcome is obtained. Mice heterozygous (101PL) or homozygous (101LL) for the transgene do not develop a spontaneous disease, nor can they transmit disease to either wild type or 101LL transgenic mice (Manson *et al.*, 1999). While this result in itself may not be that surprising, since it could be anticipated that high expression levels of PrP are required to produce a spontaneous disease, what is remarkable about this mutation in the gene targeted mice is the way in which it alters susceptibility to disease. With the exception of vCJD, most human TSE isolates are difficult to transmit to wild type mice. However 101LL mice show 100% susceptibility and short incubation times when inoculated with infected brain homogenate from P102L GSS patients (Manson *et al.*, 1999). While this may demonstrate that donor and host identity at 101/102 increases the efficiency of transmission, a more surprising effect is seen when these mice are inoculated with SSBP/1 experimental sheep scrapie and 263K hamster scrapie (Barron *et al.*, 2001). These two agents, derived from hosts with proline at the equivalent position, have shorter incubation times in the 101LL mice than in the wild type mice (101PP). The 101L mutation has therefore altered the susceptibility of the host to TSE agents from three different species and the mechanism by which this is achieved is not dependent on overall PrP se-

quence compatibility between host and donor, or specifically at codon 101/102.

The prion or protein only hypothesis defines the TSE infectious agent as a protease resistant form of PrP which can self replicate (Prusiner, 1982). The existence of multiple strains of TSE agents with different incubation times, clinical features and neuropathology has proved a challenge to the prion hypothesis. In other infectious diseases, strains arise from mutations or polymorphisms in the infectious agent's nucleic acid genome (DNA or RNA) which encodes the heritable strain-specific information of each agent. However in order to accommodate strains in an infectious agent which may be composed entirely of protein, it has been proposed that each TSE strain represents a different stable conformation of abnormally folded PrP that can faithfully replicate and produce the diversity observed in disease (G. Telling *et al.*, 1996; Safar *et al.*, 1998). This concept has been given some support by *in vitro* conversion assay systems in which the characteristics of PrP-res can be conferred to PrP^C, demonstrating that pathological forms of PrP have some capacity to propagate themselves (Bessen *et al.*, 1995). However the failure of this cell-free conversion system to produce infectious material (Hill *et al.*, 1999) has hampered the ability to fully establish whether there is a link between TSE strain and PrP conformation.

While TSE strains have been shown to be associated with differences in conformation (Safar *et al.*, 1998), degree of protease resistance (Tremblay *et al.*, 2004) and glycoform ratios (Collinge *et al.*, 1996; Parchi *et al.*, 1996), the exact relationship between these characteristics and the TSE strain are still to be defined. The three bands of PrP-res correspond to the three different glycoforms of PrP, produced by differential N-linked glycosylation at amino acids 180 and 196 and detection by immunoblot analysis using a PrP antibody is increasingly being used to define a TSE strain. Understanding the relationship between PrP glycosylation and TSE disease has therefore become an important issue in the diagnosis of these diseases. We are addressing this question using transgenic mice (produced by gene targeting) which have altered patterns of PrP glycosylation. Initial results indicate that glycosylation pattern of host PrP can influence the incubation time of disease, the targeting of pathology and can modify the TSE strain as it is passaged through the host (N. Tuzi, unpublished results). The results from these experiments also raise questions concerning the use of PrP glycoform as a sole criteria for determining TSE strain. Further analysis of these models will allow the precise relationship between TSE strain and PrP glycosylation to be established

The idea that a protein alone may be the source of infectivity in the TSE diseases was first put forward in 1967 (Gibbs, 1967; Griffith, 1967) and later developed as the prion hypothesis by Stanley Prusiner (Prusiner, 1982). Over three decades this hypothesis has gained much support, but the exact nature of what this elusive infectious agent is remains to be defined. While infectivity is generally associated with protease resistant PrP in purified preparations from infected brain there are some reports which claim to separate PrP and infectivity (Manuelidis *et al.*, 1987; Somerville & Dunn, 1996). The resistance of PrP knock-out or null mice to TSE infectivity clearly demonstrates that PrP is required for the disease process but does not provide proof that PrP itself is the infectious agent (Bueler *et al.*, 1993; Manson *et al.*, 1994).

While the genetic linkage studies in human TSEs provide strong support for the prion hypothesis, the 102L mutation originally thought to be 100% penetrant does not now appear to be so, with a recent report of monozygotic twins carrying the 102L mutation where one twin developed GSS and the other remained unaffected (Hamasaki *et al.*, 1998). Moreover the expression of this mutation in murine PrP has shown a dramatic alteration in the susceptibility of 101LL mice to TSE infection, raising the possibility that the mutations are susceptibility factors rather than the primary cause of disease (Manson *et al.*, 1999; Barron *et al.*, 2001; Barron *et al.*, 2003). Indeed sheep scrapie was originally considered to be a genetic disease, as certain PrP genotypes were always associated with the development of scrapie. However the existence of such sheep in scrapie free countries such as New Zealand have shown scrapie to be an infectious rather than a genetic disease (Hunter *et al.*, 1997), and embryo transfer experiments have shown that sheep which would normally be expected to develop disease can survive if conditions of high sterility are maintained around time of birth and thereafter (J.Foster, personal communication).

Cell free conversion assays have demonstrated that PrP-res can induce transformation of PrP^{C} into a protease resistant form (Kocisko *et al.*, 1994). Moreover many aspects of the disease process such as the species barrier and faithful replication of the abnormal form of PrP could be demonstrated in this system (Kocisko *et al.*, 1995; Raymond *et al.*, 2000). However a final link between PrP-res and infectivity has not been achieved since it has not been possible to demonstrate the generation of infectivity in this system (Hill *et al.*, 1999). *In vitro* conversion assays based on cyclic amplification of the misfolded protein have also demonstrated in-

creased production of PrP-res but not to date an increase in infectivity (Saborio *et al.*, 2001; Soto *et al.*, 2002).

Recently it has been reported that an abnormally folded fragment of recombinant PrP (amino acids 89-230) produced disease when inoculated into mice expressing a transgene of the same truncated protein at 16 times the level of wild type PrP (Legname *et al.*, 2004). Subsequent passage of brain tissue from these mice produced disease in both wild type and transgenic mice. While this experiment continues to add evidence to the prion hypothesis, the absence of soluble PrP and other aggregates of PrP as controls in this study leaves the association of PrP and infectivity still unresolved. Moreover the abnormally folded protein used in these experiments did not demonstrate the normal protease resistance associated with disease specific PrP. In addition there are a number of descriptions of TSE disease occurring in the absence of a protease resistant form of PrP (Lasmezas *et al.*, 1997; Manson *et al.*, 1999). If therefore PrP is the TSE infectious agent, these models suggest it is a form other than PrP-res. Understanding the exact relationship between disease-associated forms of PrP and infectivity is one of the major problems in assessing the reliability of PrP-res based diagnostic assay systems. This issue has been further compounded by the identification of TSE disease in animals which do not appear to have a protease resistant form of PrP (Lasmezas *et al.*, 1997; Manson *et al.*, 1999) Indeed a model of disease has been shown to have high levels of infectivity in brain with undetectable levels of PrP-res by either immunoblot or ELISA based assays, suggesting that PrP-res is not always a reliable diagnostic marker of disease (R. Barron, unpublished results). These laboratory models are now being used to reinvestigate the nature of the infectious agent. These studies will determine whether infectivity is associated solely with PrP, or if other factors/molecules are involved. If PrP is shown to be the agent, these models may allow the identification of the specific form of PrP which is associated with infectivity.

We are thus still some way from defining the mechanisms by which host PrP sequence influences susceptibility to disease but it is hoped that *in vivo* studies using these well defined PrP gene targeted models and *in vitro* studies using cells lines derived from these models will allow the mechanisms by which host PrP controls susceptibility to disease to be unravelled. The species barrier and mechanisms of host resistance and susceptibility are not yet understood. Moreover we have yet to establish exactly what defines a TSE strain and what determines how that strain targets specific areas of the brain. Importantly the exact nature of the infectious agent still remains to be determined. Our ability to diagnose, control, eradicate or

treat these diseases is still dependant on providing answers to these fundamental questions.

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The role of the immune system in TSE agent neuroinvasion

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Summary. Many natural infections with transmissible spongiform encephalopathy (TSE) agents are likely to be acquired peripherally for example, following ingestion of contaminated feed. Following peripheral exposure TSE agents accumulate in lymphoid tissues before spreading to the brain. Many studies have attempted to identify the cells and their components that are required for the delivery of the TSE agent from the site of inoculation to the brain, a process termed *neuroinvasion*. In the lymphoid tissues of TSE-affected hosts these agents, as identified by disease-specific prion protein accumulations, usually accumulate on follicular dendritic cells (FDCs). Studies of mouse TSE models have shown that mature FDCs are critical for replication of infection in lymphoid tissues and subsequent neuroinvasion. Although examples of FDC-independent neuroinvasion have been described, treatments that interfere with the integrity or function of FDCs reduce TSE susceptibility by blocking the spread of disease to the brain. For example, temporary depletion of FDCs before oral inoculation with TSE agents blocks the accumulation of disease-specific PrP in Peyer's patches and mesenteric lymph nodes, and prevents neuroinvasion.

Studies in mice have shown that skin scarification is also an effective means of TSE agent transmission. Following inoculation via the skin the agent accumulates in the draining lymph node in association with FDCs. The accumulation of TSE agents in association with FDCs is also critical for the transmission of disease from the skin to the brain, as disease susceptibility is reduced in their absence. The mechanisms through which TSE agents are transported from the site of inoculation such as the gut or skin to lymphoid tissues are not known. Bone marrow-derived migratory dendritic cells have been proposed as a potential method of TSE agent transport from the gut lumen. Langerhans cells (LCs) reside in the epidermis and migrate to the draining lymph node after encountering antigen, suggesting these cells might play a role in TSE agent transportation from

the skin. To investigate the potential role of LCs in TSE agent transportation, mouse models have been utilized in which their migration was blocked. Experiments show that the early accumulation of TSE agents in the draining lymph node and their subsequent neuroinvasion was not impaired in mice with blocked LC migration. Thus although LCs have the potential to acquire TSE agents they are not involved in their transportation to draining lymphoid tissues.

The identification of cell populations critical for TSE pathogenesis provides cellular targets to which therapies can be directed. Described below are current understandings of the involvement of the immune system in the neuroinvasion of TSE agents.

Key words. Scrapie, prion, follicular dendritic cell, lymphotoxin, dendritic cell, Langerhans cell

TSE agents and the prion protein

Transmissible spongiform encephalophathies (TSEs), or prion diseases, are a group of sub-acute, infectious, fatal, neurodegenerative diseases, which affect humans and both wild and domestic animals. Diseases include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in mule deer and elk, and scrapie in sheep and goats. Although the precise nature of these infectious agents is uncertain, an abnormal, detergent insoluble, relatively proteinase-resistant isoform (PrP^{Sc}) of a host cellular prion protein (PrP^{c}), co-purifies with infectivity in diseased tissues [1]. To maintain infection, host cells must express the cellular isomer of the prion protein as mice deficient in PrP^{c} (*Prnp*$^{-/-}$ mice) do not develop disease after inoculation with TSE agents [2, 3]. The prion hypothesis argues that disease-specific PrP^{Sc}, or an intermediate between PrP^{c} and PrP^{Sc}, constitutes a major, or possibly the sole component of the infectious agent and mediates the conversion of PrP^{c} to PrP^{Sc} [4]. Indeed, recombinant PrP refolded into the disease-specific form *in vitro*, has the potential to transmit disease *in vivo* [5].

TSE agents accumulate in lymphoid tissues before the brain

Most transmissions of TSE agents, including natural sheep scrapie, BSE, CWD, and kuru and variant (v) CJD in humans, occur by peripheral expo-

sure. Although the main route of transmission of the BSE agent to cattle and other species is considered to be oral (ingestion), other routes of TSE agent transmission have been identified. Accidental iatrogenic transmissions of sporadic (s)CJD to patients have occurred through transplantation of tissues or pituitary-derived hormones contaminated with the sCJD agent [6]. Recent evidence also indicates that the vCJD agent in humans has been transmitted via blood transfusion [7, 8]. Studies in mice have shown that skin scarification is an effective means of scrapie agent transmission highlighting another potential route of accidental transmission [9]. Thus it is possible that some cases of natural sheep scrapie might be transmitted through skin lesions either in the mouth [10] or during close contact [11], or be passed from mother to offspring through sites of skin trauma at birth. Surgical instruments contaminated with the sCJD agent have also been shown to have the potential to transmit disease [12]. Together, these examples highlight important health and safety issues concerning risks to patients, health workers and scientists of acquiring TSE agents. For example, biopharmaceutical and cosmetic products derived from TSE agent-contaminated sheep and cattle tissues might also have the potential to transmit disease when applied to skin lesions [13, 14].

Most of our understanding of TSE disease pathogenesis has come from the study of experimental sheep or rodent scrapie models. Following experimental peripheral inoculation with the scrapie agent, infectivity and PrP^{Sc} usually accumulate in lymphoid tissues [15-19], long before either is detectable in the central nervous system (CNS). However, in some sheep with natural scrapie, PrP^{Sc} is also detected first in Peyer's patches and gut-associated lymphoid tissues [19, 20] and is later detected within other lymphoid tissues and the CNS [21] implying this disease is acquired orally. The detection of disease-specific PrP within the gut-associated lymphoid of tissue of a human vCJD patient 8 months before the onset of clinical signs is also consistent with the transmission of this disease by the oral route [22]. Despite the accumulation of huge titres of TSE agent infectivity with the lymphoid tissues during disease the host fails to mount a detectable specific immune response to the agent. This is perhaps not too surprising, since host tolerance to cellular PrP^{c} may prevent immunity PrP^{Sc}.

Lymphoid tissues play an important role in the transmission of some TSE agents. For example, genetic asplenia or splenectomy of mice shortly before or after a peripheral inoculation with the scrapie agent significantly extends survival time [23]. The early involvement of lymphoid tissues in disease pathogenesis prior to neuroinvasion might be prion strain-dependent, as the BSE agent in cattle [24] and the sCJD agent in humans [25] appear to be confined to nervous tissues. Using a highly sensitive as-

say system PrP^{Sc} has been detected in the spleens of some Swiss patients with sCJD at the end stage of disease, but the relevance of these observations to disease pathogenensis are unclear [26]. However, in patients with vCJD [22, 25, 27], most sheep with natural scrapie [28] or rodents with experimentally inoculated with the scrapie agent [17, 18, 29-31], infectivity accumulates in lymphoid tissues and abnormal forms of PrP are readily detected within the germinal centres (GCs) prior to the onset of clinical disease.

TSE agents accumulate on follicular dendritic cells

Over the years several lines of evidence have implicated FDCs as likely targets for TSE agent accumulation in lymphoid tissues. The initial indications came from studies using sub-lethal whole body γ-irradiation, which eliminates actively dividing lymphocytes and monocytes, but not stromal-derived cells such as FDCs. In these experiments, γ-irradiation before or after peripheral inoculation with the scrapie agent did not affect disease pathogenesis [32]. The precise ontogeny of FDCs is still debated [33-35], but at least in adult mice, they most likely derive from stromal precursor cells in lymphoid organs, rather than from haemopoietic stem-cells. Thus, as FDCs are mitotically inactive they would have survived the effects of the γ-irradiation treatment in the above study [32] and continued to sustain scrapie agent accumulation in the spleen. Of course, this hypothesis does not exclude a contribution from other radiation-resistant cell populations including terminally differentiated lymphocytes or macrophages.

Immunohistochemical analysis of murine lymphoid tissues shows that FDCs appear to express high levels of cellular PrP^{c} in uninfected mice [29], and accumulate strong levels of disease-specific PrP^{Sc} in mice inoculated with TSE agents [17, 29-31, 36]. In the lymphoid follicles of other TSE-affected species including sheep with natural scrapie [28], patients [25] with vCJD and mule deer [37] with CWD, disease-specific PrP accumulations also occur in association with FDCs. However, FDCs are not the only cells in lymphoid tissues that express PrP^{c} [38-41] or accumulate disease-specific PrP [31, 37, 42-44]. FDCs are specialized to trap and retain antigens on their surfaces in native form for long periods of time. Therefore, these cells might simply be acquiring TSE agents from other cellular sources and have little role in pathogenesis.

TSE pathogenesis in the absence of FDCs

Studies have shown that severe combined immunodeficient (SCID) mice which lack B lymphocytes and T lymphocytes do not develop disease following peripheral inoculation with TSE agents [45], but susceptibility can be restored following allogeneic bone marrow transplantation from a normal mouse [46]. Further studies using a variety of immunodeficient mice initially suggested B lymphocytes were the critical cells for efficient TSE pathogenesis as mice lacking them (μMT mice, RAG-$1^{-/-}$, RAG-$2^{-/-}$) were resistant to peripheral disease transmission, whereas mice lacking functional T lymphocytes alone ($CD4^{-/-}$, $CD8^{-/-}$, β2-$\mu^{-/-}$, *Perforin*$^{-/-}$, TCR$\alpha^{-/-}$) were not [47]. However, FDCs require on essential maturation signals from B lymphocytes to maintain their differentiated state [48, 49]. As a consequence, mice deficient in mature B lymphocytes are indirectly deficient in FDCs. Some of the signals between lymphocytes and FDCs are mediated via cytokines including tumour necrosis factor α (TNFα) which signals through the TNF receptor-1 (TNFR-1; Ref. [34]), and membrane lymphotoxin (LTα/β) which signals through the lymphotoxin β receptor (LTβR; Ref. [35]). Each of those receptors is expressed by FDCs or their precursor cells. In the absence of either of those cytokines, or their corresponding receptors, FDCs do not develop in lymphoid tissues [48, 49]. Studies utilizing mice with deficiencies in either cytokine signaling pathway have been particularly useful in discriminating between the cellular components of lymphoid tissues required for the accumulation of TSE agents.

We have shown that TNFα-deficient (TNF$\alpha^{-/-}$) mice are less susceptible than wild-type mice to peripheral inoculation with the ME7 scrapie agent strain, and due to an absence of FDCs, fail to accumulate the agent in the spleen [30]. Others have shown that mice deficient in components of the LTβR signaling pathway (LT$\alpha^{-/-}$, LTß$^{-/-}$ or LTβR$^{-/-}$ mice) are similarly less susceptible to peripheral challenge with the RML scrapie agent isolate [42, 50] or mouse-passaged CJD agent strain Fukuoka-2 [51]. These observations are congruent with a key role for FDCs, and also suggest that lymphocytes are unlikely to be directly involved in pathogenesis as both T and B lymphocytes are present and functional in these immunodeficient mice [52, 53].

The expression of PrP^c by FDCs [17, 54, 55], and their ability to capture complement-bound complexes [56-58] appear to be critical properties for the accumulation of scrapie prions on FDCs.

FDCs depletion reduces susceptibility to TSE agents

Specific blockade of lymphocyte-derived LTα/β or TNFα in immunocompetent hosts causes the rapid de-differentiation of FDCs [59, 60]. Reagents which block these signals provide tools to investigate the timing of FDC involvement in TSE disease pathogenesis and to explore their potential use in therapeutic intervention. A single treatment with LTßR-Ig [61] blocks LTβR-signaling and temporarily dedifferentiates FDCs for approximately 28 days [59]. FDCs trap and retain antigens on their surfaces through interactions between complement components and cellular complement receptors [62, 63]. Furthermore, complement components C1q and C3, and cellular complement receptors are considered to play an important role in the localization of the scrapie agent to FDCs [56-58]. Following treatment with LTßR-Ig expression of FDC-associated complement receptor 1 and bound complement components (C1q, C3 and C4) in Peyer's patches, lymph nodes and the spleen is absent [59, 64, 65]. Therefore, if any immature FDCs remain after LTßR-Ig treatment, they will be unable to trap and retain complement-opsonized antigens or TSE agents during the period of dedifferentiation.

The fate of the FDCs following LTβR-Ig treatment is not known, but several mechanisms could be responsible either singularly or in combination. First, FDCs could de-differentiate to an immature state that lacks their antigen trapping characteristics. Secondly, in the absence of LTβR stimulation, FDCs may undergo apoptosis. Finally, as recovering FDCs appear in a more diffuse pattern than the compact networks present in control mice [60], it is possible that LTβR-dependent chemokine gradients that are responsible for the localisation of FDCs within the germinal centre, are disturbed resulting dispersing the FDCs to atypical locations.

A single LTßR-Ig treatment shortly before an intraperitoneal inoculation of mice with the scrapie agent blocks the accumulation of infectivity and PrP^{Sc} in the spleen and reduces disease susceptibility [64, 66, 67]. Single treatments with LTβR-Ig up to 6 weeks after intraperitoneal inoculation with the scrapie agent result in progressively smaller but still significant delays in neuroinvasion [66, 67]. These effects are most likely due to the temporary loss of mature PrP^{c}-expressing FDCs. Treatment with huTNFR:Fc to specifically block TNFR-signalling also significantly extended survival time when compared to control treated mice, but had little influence on disease susceptibility [68]. The differing effects of LTßR- or TNFR-signaling blockade on disease susceptibility are most likely related to the differing duration of the FDC de-differentiation caused by these reagents: treatment with LTßR-Ig de-differentiates FDCs for approximately

28 days, whereas these cells reappear within 14 days of treatment with huTNFR:Fc [59, 64, 67, 68].

The suggestion that consumption of BSE agent-contaminated meat products is the most likely cause of vCJD in humans [69, 70] has focussed attention on the gastrointestinal tract as an important route of TSE agent transmission. Peyer's patches are the primary inductive sites in gut-associated lymphoid tissues that actively acquire antigens from the lumen of the intestine. Following intragastric or oral inoculation of rodents with the scrapie agent, infectivity and PrP^{Sc} accumulate first in Peyer's patches and gut-associated lymphoid tissues before their detection in the CNS [18, 71-73]. Within the gut associated lymphoid tissues of orally inoculated rodents [18] or some sheep with scrapie [20, 74], and mule deer inoculated with the CWD agent [37], disease-specific PrP accumulations occur in association with FDCs. Temporary FDC depletion prior to oral inoculation of mice with the scrapie agent prevented the development of clinical disease and blocked the accumulation of PrP^{Sc} in Peyer's patches and mesenteric lymph nodes [64]. These data demonstrate that FDCs are critical for the transmission of some TSE agents from the gut lumen to the CNS, and exclude the possibility of direct uptake of infectivity from the gut lumen into enteric nerves [75].

Previous studies have shown that a functional immune system is critical for scrapie agent neuroinvasion following inoculation by skin scarification as SCID mice do not accumulate PrP^{Sc} and infectivity in their spleens or develop clinical disease when inoculated by this route [9, 55]. However, whether FDCs or other components of the immune system are required for scrapie agent neuroinvasion following inoculation via the skin was not known. In an experimental system, migratory bone marrow-derived dendritic cells have been shown to have the potential to deliver the scrapie agent directly to the nervous system [76]. As skin is highly innervated neuroinvasion might occur via an FDC-independent pathway. For example, lymphocytes or Langerhans cells might acquire TSE agents within the skin and transport them directly to local peripheral nerves. However, FDCs are the critical cells for efficient TSE agent neuroinvasion from the skin as their temporary depletion prior to inoculation by skin scarification blocks the accumulation of PrP^{Sc} in the draining lymph node and reduces disease susceptibility [64].

Although the effects of LTβR-Ig treatment on scrapie agent neuroinvasion following peripheral inoculation are most likely due to the temporary depletion of mature FDCs, potential effects of treatment on other cell populations should not be overlooked. LIGHT is a transmembrane protein produced by activated T-lymphocytes that also binds to the LTßR [77]. However, the effects of LTßR-Ig treatment on disease pathogenesis are

unlikely to be due to impaired LTβR- or LIGHT-mediated T-lymphocyte responses as scrapie agent neuroinvasion is unaffected in T-lymphocyte-deficient mice [23, 47, 54, 78]. Signaling via LTßR has been shown to be important for the presence of migratory dendritic cells in the spleen [79]. Therefore, it is plausible that blockade of the LTßR-signaling pathway might have affected cell trafficking or the delivery of the scrapie agent to lymphoid tissues. However, as treatment with LTßR-Ig 14 days after scrapie agent inoculation via the skin or by intraperitoneal injection extended survival time [65, 66] the effects of treatment on scrapie pathogenesis are unlikely to be due to affects on cell trafficking as dendritic cells migrate to draining lymphoid tissues within the first few hours of antigen encounter [80]. Collectively these observations suggest it is highly unlikely that the major affect of LTßR-Ig-treatment on disease pathogenesis is independent of its effects on FDC maturation.

Treatments that inactivate FDCs may have therapeutic application in other pathological conditions. For example: FDC inactivation may reduce the severity of some autoimmune diseases [81]; FDC-associated HIV particles comprise a major viral reservoir in infected patients [82] and removal may improve the efficacy of antiviral therapies; elimination of FDCs may reduce the survival of some lymph node-resident lymphomas which require FDCs for their survival [60, 83]. Current evidence suggests the human TSE agent, vCJD, shares a similar requirement for FDCs in its pathogenesis, as PrP^{Sc} is associated with FDCs in lymphoid tissues from infected patients [25] and has been detected in these tissues before the onset of clinical disease [8, 22, 84]. Thus manipulation of FDCs may offer a potential approach for early intervention in some TSE diseases. But treatments that specifically interfere with the maturation status of FDCs will only be effective during the time interval between exposure to prion infectivity and its subsequent spread to the peripheral nervous system. While little is known about the precise timing of these events, comparisons of the effects of LTßR-Ig-treatment on scrapie agent pathogenesis following inoculation by different peripheral routes indicate this period varies considerably according to the route of exposure.

Neuroinvasion is likely to occur rapidly from the gastrointestinal tract as LTßR-Ig treatment 14 days after oral inoculation is ineffective [64]. The velocity of prion neuroinvasion from FDCs has been shown to be inversely proportional to the distance between the FDCs and local peripheral nerves [85]. In the Peyer's patch, FDC networks lie in close association with nerve fibres that run along the gut wall [18]. Therefore it is plausible that following accumulation and/or replication upon FDCs, TSE agent neuroinvasion occurs rapidly via the enteric nerves in the gut wall or Peyer's patch adjacent to FDCs [18, 73].

Following of inoculation of mice via the skin, scrapie agent neuroinvasion appears to occur directly from the draining lymph node between 14 to 42 days after inoculation [65]. However, LTßR-Ig treatment remains effective even when administered up to 42 days after intraperitoneal inoculation [66, 67]. Thus if manipulation of FDCs where ever to be used therapeutically against TSE diseases, the time interval available for intervention would depend critically on the route of exposure.

FDC-independent neuroninvasion

Several examples of natural or experimental TSE agent transmissions have been reported in which neuroinvasion appears to occur via FDC-independent pathways. How infection spreads to the CNS in these instances is not known. Infectivity might reach the CNS after direct uptake by nerve fibres at the exposure site, or following association with other cell types in lymphoid tissues. For example, whereas the susceptibility of FDC-deficient $TNF\alpha^{-/-}$ mice to peripheral inoculation with the ME7 scrapie agent strain is reduced [30], their susceptibility to inoculation with the RML scrapie agent isolate is not [42]. In the lymph nodes of RML scrapie-inoculated $TNF\alpha^{-/-}$ mice disease-specific PrP accumulations were detected in macrophages [42]. Therefore, in some circumstances macrophages might play a role in TSE agent neuroinvasion. Other studies indicate that the RML scrapie agent, unlike the ME7 scrapie agent, may target both PrP-expressing FDCs and haemopoietically-derived cells [54, 86, 87].

Some TSE agents in their natural hosts also appear to display similar variations in their tissue and cellular targeting outside the CNS. Infectivity and heavy PrP^{Sc} accumulations have been detected upon FDCs in the lymphoid tissues of patients with vCJD [22, 25, 27, 70], but not of patients with iatrogenic sCJD, even though the agent is introduced via the periphery [25]. In a study of Swiss sCJD patients, PrP^{Sc} was only detected in the lymphoid tissues of some patients during the clinical phase of the disease [26]. In natural cases of BSE in cattle, infectivity appears to be restricted entirely to nervous tissue. However, in some experimentally inoculated cattle infectivity has been detected in the distal ileum [88]. Within the Peyer's patches of the distal ileum, disease-specific PrP accumulations were confined to macrophages [44]. The cellular mechanism of TSE agent neuroinvasion in these examples is not known.

Transportation of TSE agents to lymphoid tissues

Following oral inoculation of rodents with the scrapie agent, disease-specific PrP accumulations can be detected in M cells within the follicle-associated epithelium overlaying the Peyer's patches [18]. M cells are key sites of antigen sampling in the gut mucosa and can provide portals of entry for enteric pathogens [89]. Experiments using an *in vitro* cell culture model, suggest that M cells have the potential to transcytose TSE agents across the gut epithelium [90]. But once TSE agents have crossed the gut epithelium it is not known how they are then delivered to the FDCs. FDCs could directly trap cell-free PrP^{Sc}, or other agent associated molecules, in a complement-bound complex [56-58], but it is also possible that mobile cells transport TSE agents to lymphoid follicles [91]. Several cells have the potential to transport TSE agents including macrophages and migratory bone marrow-derived dendritic cells (DCs). The evidence that macrophages degrade TSE agents makes them an unlikely and inefficient transport candidate [92-94].

Migratory dendritic cells

Migratory bone marrow-derived DCs are a distinct cell lineage from tissue-fixed, stromal-derived, FDCs which do not have a bone-marrow precursor [35, 95]. DCs continually circulate throughout the host's tissues and tissue fluids where they sample antigens and transport them to lymphoid tissues [80]. Unlike macrophages, DCs can retain some protein antigens in their native, non-degraded form [96]. Several studies have recently implicated the DC as a candidate TSE agent transport mechanism. For example, myeloid DCs appear to express high levels of PrP^{c} [40], and the prion protein fragment $PrP_{106\text{-}126}$ is a chemoattractant for monocyte-derived DCs *in vitro* [97]. Bone marrow-derived DCs can also acquire PrP^{Sc} *in-vitro* [91, 98]. Within the intestine, DCs are present in the lamina propria [99], and in the Peyer's patches they form a dense layer of cells in the subepithelial dome, just beneath the follicle-associated epithelium [100]. A sub-population of migratory DCs has been shown to have the potential to acquire PrP^{Sc} from the intestine and transport it to mesenteric lymph nodes via the lymph [91]. Therefore, following oral exposure, DCs might acquire TSE agents after transcytosis by M cells, or by direct uptake across the mucosal epithelium as demonstrated for the transport of bacteria [101].

Despite these observations direct demonstration of the involvement of DCs in the initial delivery of TSE agents to lymphoid tissues is lacking.

Studies have attempted to address whether $CD11c^+$ DCs aid the neuroinvasion of TSE agents by blocking their function by co-infection with lymphocytic choriomeningitis virus [50]. In these experiments specific DC suppression did not affect TSE pathogenesis [50], but as the mice were inoculated with high doses of the scrapie agent, the requirements for replication in lymphoid tissues may have been bypassed [42, 46].

Langerhans cells

Langerhans cells (LCs) are a subset of migratory DCs that reside in the epidermis and migrate to the draining lymph node following antigen encounter [80]. DCs, including LCs, are considered to provide potential mechanisms for the transmission of some pathogens into the skin such as dengue virus [102], HIV [103], maedi-visna lentivirus [104] and venezuelan equine encephalitis virus [105]. These characteristics suggested to us that LCs were plausible candidates for TSE agent transport from the skin to draining lymph nodes. However, antigens acquired by DCs usually quickly enter the lysosomal compartment where they are broken down into peptides for presentation to lymphocytes in association with MHC class II [80]. Thus these cells may handle TSE agents in a similar manner, as bone marrow-derived DCs [98] and LC-like cells [106] are able to process and degrade PrP^{Sc} following extended *in vitro* exposure.

LCs require a number of stimuli to induce their mobilization out of the skin [80]. The cytokines interleukin (IL)-1β, IL-18 and tumour necrosis factor (TNF)-α play key roles in regulating the migration of LCs from the epidermis to draining lymph node [107, 108]. LC migration from the epidermis is blocked following specific inhibition of these cytokines [107]. IL-1β and IL-18 are synthesized as inactive precursors which are cleaved to release the active cytokine molecule by the cysteine protease caspase-1 [109]. In mice deficient in caspase-1 LC migration from the skin is impaired [110]. Furthermore, treatment of skin with an irreversible inhibitor of caspase-1 [111, 112], potently blocks both spontaneous and induced LC migration from the skin [110].

To investigate the potential role of LCs in TSE agent transportation from skin to draining lymphoid tissues we studied pathogenesis in models where the migration of these cells from the epidermis was impaired. Blockade of LC migration from the epidermis through caspase-1 inhibition prior to inoculation did not effect the early accumulation of the scrapie agent in the draining lymph node or delay subsequent neuroinvasion [106].

To exclude the possibility that the magnitude of the pharmacological inhibition of LC migration [106, 110] was insufficient to observe a measur-

able effect on disease pathogenesis deserves consideration, an additional mouse model was used where LC migration from the skin was permanently blocked. In mice deficient in CD40 ligand ($CD40L^{-/-}$ mice), the number and morphology of LCs in the epidermis is normal but they fail to migrate from the skin and do not accumulate in the draining lymph node [113]. However, when inoculated with the scrapie agent via the skin, the early accumulation of scrapie in the draining lymphoid tissue was likewise not impaired in $CD40L^{-/-}$ mice whose LCs could not migrate out of the epidermis [106].

Our experiments suggest the initial transportation of TSE agents from the skin to draining lymphoid tissue occurs through an LC-independent mechanism [106]. The precise mechanism by which TSE agents are transported from the skin to lymphoid tissues remains to be identified. As macrophages are most likely involved in the degradation of infectivity [92-94], it is possible that TSE agents are transported in a cell-free manner. After entering the host antigens are rapidly opsonised by complement components. The complement system plays an important role in the localization of scrapie to lymphoid tissues [56-58, 114] suggesting TSE agents might be delivered to lymphoid tissues as cell-free complement-bound complexes.

How do TSEs agents enter the CNS after accumulating in lymphoid tissues?

Although infectivity has been detected in the blood-stream in some TSE models [115-117], there is no evidence to suggest a major route by which TSE agents access the CNS from peripheral tissues is via the blood-stream. Neuroinvasion is crucially dependent on PrP-expression in a non-haemopoietic tissue compartment between the lymphoid tissues and CNS, such as peripheral nerves [86, 118, 119] which express PrP^c in humans and animals [19, 75]. Following intragastric or oral inoculation of rodents, the scrapie agent first accumulates in Peyer's patches, gut-associated lymphoid tissues and ganglia of the enteric nervous system [18, 71]. The agent then most likely spreads to the CNS via the enteric nervous system, or splanchnic or vagus nerves [18, 71, 120-123], as neuroinvasion is impaired when sympathectic neurons are depleted [124]. A similar involvement of gut-associated synmpathetic neurones in the propagation of the vCJD agent in humans has also been proposed [125].

Although lymphoid organs are predominantly innervated with sympathetic nerve fibres, the follicles within which FDCs reside are poorly in-

nervated [126]. Thus how TSE agents bridge the gap between FDCs and sympathetic nerves is not known [127]. In an experimental system, TSE agent transportation by DCs to neurones has been proposed as a possible mechanism [76]. When splenic FDCs are artificially repositioned in close association with peripheral nerves (eg: in $CXCR5^{-/-}$ mice) neuroinvasion occurs faster [85]. Analysis of sheep Peyer's patches shows they contain a substantial network of nerve fibres that make close contacts with FDCs and tingible body macrophages [128]. Thus it is tempting to speculate that neuroinvasion from sheep Peyer's patches may also occur rapidly.

Conclusions

Efficient neuroinvasion of some TSE agents is critically dependent upon FDCs in lymphoid tissues. FDCs are long-lived cells that express high levels of PrP^c and are specialized to trap and retain native antigens on their surfaces. These characteristics make FDCs ideal targets for TSE agent accumulation in lymphoid tissues. Several studies have explored the potential of treatments that interfere with the integrity or antigen-trapping function of FDCs as an early opportunity for intervention against natural TSE diseases. However, these studies suggest that the time window in which FDC-targeted treatments may be effective is likely to vary widely according to the route of exposure. Although significant advances in the understanding of TSE pathogenesis in lymphoid tissues have been made, several questions remain to be answered. There is growing evidence from natural cases and experimental models to show that in some circumstances TSE agent neuroinvasion can occur through FDC-independent pathways, but the cellular mechanisms through with this occurs is unknown. Little is also known of how TSE agents are transported from the site of exposure to FDCs. Once TSEs agents have accumulated on FDCs the agent spreads to the CNS along sympathetic nerves, but how they bridge the gap between FDCs and peripheral nerves is likewise unknown. Understanding the earliest peripheral mechanisms involved in the transportation, accumulation or degradation of TSE agents is important for the development of therapeutic strategies to combat these fatal neurodegerative diseases.

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Prion protein interactions and TSE infections in cell culture models

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Summary. The process by which transmissible spongiform encephalopathy (TSE) agents, or prions, infect cells is unknown. There are also no effective treatments available for TSE diseases. Studies of cultured cells persistently infected with TSE agents have greatly contributed to understanding these and many other aspects of TSE disease. New cell lines have been developed to increase the repertoire of TSE strains that can be investigated in ex vivo models. Candidates for TSE therapeutics have been identified. Initial events involving the internalization and trafficking of TSE agents and the effect of membranes on the infection process have been examined. Recent progress in these areas is discussed below, which together illustrate the value of cell culture models in the study of prion diseases.

Key words. Exosomes, fluorescent PrP, microsome, prion, SN56

Cell culture models of TSE infection

Early in the study of transmissible spongiform encephalopathy (TSE) or prion diseases, as is typical in the study of infectious agents, investigators sought to develop cell culture models of infection. These defined ex vivo systems allow the study of a variety of features of infectious agents without the complexity and variability that is often observed in animal models (Table 1). Granted, such models do not always fully recapitulate all aspects of in vivo infections and cell culture models of TSE infection have been no exception in this regard. This has led to an under-appreciation of the value of these systems in some instances. However, it is clearly unreasonable to expect these models to reproduce all the nuances associated with disease processes as they occur in whole organisms. Nevertheless,

when considered in their entirety, cell models of infection have proven their merit as a tool in the study of TSE diseases.

Table 1. Applications of cell culture models of TSE infection

1.	Understand cellular mechanisms of PrP-res formation and degradation
2.	A more defined system to characterize the nature of the TSE agent
3.	Determine the basis for TSE strains
4.	Assays for TSE infectivity
5.	Mechanisms of neurodegeneration
6.	Diagnostics: find infection-specific markers other than PrP-res
7.	Screening for therapeutics
8.	Determine mechanisms of infection and intercellular spread

The earliest documented culture of prion-infected cells was reported by Clarke and Haig [1,2]. A scrapie-infected cell line was generated by culturing brain cells from a Chandler-infected mouse. The resulting cell line, called SMB for "scrapie mouse brain", was apparently of mesodermal origin and thought to be derived from microglia [3]. SMB cells were later developed into a cell line suitable for ex vivo infection studies after curing by treatment with pentosan polysulfate [4]. However, the first cell culture model of prion infection was reported by Clarke and Millson [5]. These investigators found that mouse-adapted scrapie (Chandler) infectivity could be propagated in mouse L fibroblasts, a perhaps surprising result given that the pathology of TSE disease is primarily restricted to neurons. We now know that TSE agents can infect other non-neuronal cell lines including NIH-3T3 fibroblasts [6], Schwann cells [7,8], and a rabbit kidney epithelial cell line overexpressing ovine PrP [9].

Nevertheless, neuronal cell lines still prevail as the cell types of primary interest and a number of varieties are now available. The N2a mouse neuroblastoma cell line is still the most commonly used, though for rea-

sons not yet understood these cells display substantial clonal differences in susceptibility to infection [10,11]. Overexpression of PrP in N2a cells has been reported to enhance the detection of infection [12], but this observation has been disputed by others [11] and it is not yet known whether this may rather be due to a clonal effect. A hypothalamic cell line, GT-1, has also been used extensively as these cells appear susceptible to infection with two biologically distinct strains of mouse-adapted scrapie (Chandler and 139A are very closely related, if not the same, strains [13]) and infection can be assessed by detection of PrP-res formation without cloning [14,12]. When differentiated into neuron-like cells by treatment with nerve growth factor (NGF), PC-12 rat pheochromocytoma cells are also susceptible to infection with the 139A and ME7 strains of mouse scrapie [15,16]. An exciting new model based on infections of primary cultures of neurons and astrocytes was very recently reported by Cronier and coworkers [17]. Recently, we have found that SN56 cells, a differentiable mouse septal neuronal cell line, are also readily infected with multiple strains of mouse scrapie (see below). It is interesting to note that relatively small scrapie infection-dependent increases in cell death have only been noted in the GT-1 and primary neuronal culture models, raising questions about mechanisms of neurotoxicity in TSE disease.

When considered together, several points become clear regarding properties and limitations of cell culture models of TSE infection. First, most models are based on infection of cells of mouse origin with mouse-adapted scrapie agents. This limits the study of the properties of TSE agents from other species, especially those associated with large animals or humans. The recent development of cell lines susceptible to sheep scrapie represents a great advance in this regard [9,8], especially by allowing evaluation of the contribution of PrP polymorphisms to susceptibility [18]. Another problem is that many cell lines are susceptible to infection with only one or two strains of agent, and in fact certain strains (e.g. ME7) seem capable of replicating in only select cell types and with reduced efficiency compared with the Chandler/139A strains [19]. However, this problem may rather be an opportunity to investigate the basis for the strain-dependent properties of TSE agents. Vorberg et al. have shown that acute PrP-res formation occurs in cells treated with strains that do not persistently infect the cells, thus showing that the barrier to long-term infection of the cells resides at the level of maintaining ongoing formation of new PrP-res [20]. In spite of these issues, the biological properties of TSE strains can be faithfully propagated in cell culture [4] thereby validating ex vivo models as a tool for the analysis of some aspects of scrapie strains.

Screening for therapeutics

At present there are no practical treatments for TSE diseases, which remain invariably fatal. A number of points of attack for therapeutic intervention are conceivably available. These include blocking initial infection, blocking entry into the brain, inhibition of PrP-res formation, and abrogation of neurotoxic events in the brain. Of these various targets, blocking PrP-res production has been the major assay employed to date likely due to the fact that it is the most readily testable. Typically, compounds of interest are evaluated for their ability to reduce PrP-res formation in scrapie-infected cells after treatment for one or more passages. These assays have now been adapted to high-throughput formats [21] to facilitate the screening of large libraries. A long and varied list of compounds that efficiently inhibit PrP-res formation in infected cells has been described and when subsequently tested in animal models of infection, many show prophylactic anti-TSE activity [reviewed in 22].

A recent screen of a library of 2000 FDA-approved drugs and natural products identified 17 inhibitors of PrP-res formation with activity at submicromolar concentrations, 15 of which were newly identified [21]. Importantly, these compounds were active against N2a cells infected with two different strains of scrapie agent, Chandler and 22L, suggesting that they might exhibit activity against a wide range of strains. A larger group of compounds was identified that showed activity only against Chandler-infected cells, highlighting the importance of testing candidate compounds against cells infected with as many different strains as possible [21]. Six of these compounds, including 3 anti-psychotics known to penetrate the blood-brain barrier, have been tested for anti-TSE effects in rodents [23]. Unfortunately, none were effective in either therapeutic or prophylactic regimens [23] but testing of other candidate compounds from this study are ongoing.

Since inhibitors identified in cell culture studies could act by either direct or indirect inhibition of PrP-res formation, it is of interest to be able to rapidly acquire mechanistic data regarding their activity. A high-throughput solid phase cell-free conversion assay has been developed to determine which compounds might act by directly interfering with interactions between PrP^C and PrP-res molecules [24]. The assay has been adapted to both radioactive and non-radioactive (biotin) formats. A screen of the above 17 inhibitors revealed that only three compounds, all polyphenols, were capable of directly inhibiting PrP^C/PrP-res interactions apparently by blocking binding of PrP^C to PrP-res [21].

Two other studies have used targeted approaches to inhibit PrP-res formation in infected cells. Since PrP^C is essential for susceptibility to in-

fection [25] and post-natal PrP^C knockout mice exhibit normal morphology and behaviour [26], inhibition of PrP^C expression has been considered a possible TSE therapeutic strategy. Although evidence supporting this idea had already been reported using transgenic approaches to control PrP^C expression [27,28], these were not directly amenable to a therapeutic application. However, small interfering RNA (siRNA)-mediated inhibition of PrP^C expression has been shown to effectively inhibit PrP-res formation [29]. Extending from their earlier work identifying laminin receptor precursor (LRP) as a receptor for PrP^C, Leucht and co-workers found that LRP-specific anti-sense treatments as well as an anti-LRP antibody could efficiently block PrP-res formation in two different cell culture models of infection [30]. These data would suggest a role for LRP in PrP-res formation, although it is difficult to determine whether this might have been due to an indirect effect via reduction of PrP^C levels that also occurred during these treatments. In any case, these studies point to anti-sense methodologies as a promising new avenue for the development of anti-TSE treatments.

PrP^C conversions in membranes

To aid in the study of the process of conversion of PrP^C to PrP-res, a cell-free version of this reaction was developed several years ago using purified PrP^C and PrP-res [31]. Since that time, a collection of modified versions of this reaction have been reported including two that are based on the use of brain homogenates [32,33]. However, all these forms of the reaction involved the use of either purified substrates and/or the presence of detergents/denaturants, conditions where the PrP^C molecules would be extracted from cellular membranes and hence potentially in a non-native state. Conceivably, membrane association of PrP^C could affect its interactions with PrP-res in a number of ways such as conformational alterations in PrP^C, as has been shown with recombinant PrP^C [34,35], or steric effects. Thus, to model the molecular processes that might occur during the initial infection of cells with TSE agents and to examine the effect of membrane association on the conversion of PrP^C to PrP-res, a cell-free conversion reaction was developed that more closely approximates physiological conditions [36]. These reactions contained isolated rafts, or detergent resistant membranes (DRMs), as a source of membrane-bound PrP^C and a crude microsome fraction from scrapie-infected mouse brain as a source of PrP-res. Interestingly, DRM-associated PrP^C was not converted to PrP-res until the PrP^C was released from DRMs by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) or the combined membrane frac-

tions were treated with the membrane-fusing agent polyethylene glycol (PEG).

To determine whether membrane association itself inhibits conversion of PrP^C by exogenous PrP-res as opposed to some other molecule localized to raft membranes, a more defined model system using purified PrP^C reconstituted into model raft liposomes was used [37]. Liposome studies have also shown that PrP^C can bind to model membranes in a manner independent of the GPI anchor [34,35,37]. GPI anchor-deficient PrP^C, in contrast to PrP^C bound via its GPI anchor, is readily converted by exogenous PrP-res molecules without PEG or PI-PLC treatment showing that PrP^C membrane association *per se* does not prevent conversion [37]. Together, these studies suggest that membrane association directed by the GPI anchor inhibits conversion of membrane-bound GPI-anchored PrP^C by exogenous PrP-res. It is possible that when tethered to the membrane via the GPI anchor, the PrP-res binding site on PrP^C is occluded and incapable of binding to PrP-res until either the PrP-res is inserted into a membrane contiguous with PrP^C or the PrP^C is removed from the membrane. These results raise the possibility that generation of new PrP-res during the spread of TSE infection between cells requires: (i) removal of PrP^C from target cells; (ii) an exchange of PrP-res containing membranes between cells; or (iii) insertion of incoming membrane-free PrP-res aggregates into the raft domains of recipient cells.

SN56 cells: a new model of TSE infection

In a search for new and improved cell lines capable of propagating TSE infectivity, a cholinergic mouse septal neuronal cell line called SN56 [38] has been tested (Baron and Caughey, unpublished data). These easily cultured cells are well characterized and possess a number of neuronal features including synaptic vesicle proteins [39], neuronal type calcium channels [40], and production of complex intercellular neuritic networks [38], which are induced upon differentiation of the cells. SN56 cells were highly susceptible to infection with at least three different strains of mouse scrapie (Baron and Caughey, unpublished data). Cell blot analysis showed a very high percentage of the cell population became infected under optimized infection conditions (Baron and Caughey, unpublished data). Differentiation of infected SN56 cells resulted in decreased production of PrP-res (Baron and Caughey, unpublished data). This decrease was associated with the serum deprivation component of the differentiation conditions. PrP-res formation was reduced in a serum concentration-dependent manner suggesting a serum factor(s) may modu-

late levels of PrP-res synthesis (Baron and Caughey, unpublished data). Thus, we have discovered a new neuronal cell culture model of TSE infection that may prove useful for the studies of TSE-dependent neurotoxicity and intercellular spread of TSE agents.

Effect of PrP-res membrane association on infection

Observations from previous studies using a membrane-based cell-free conversion system (see above) led us to propose two scenarios for initiation and propagation of PrP-res formation [36]. One mechanism involved the intercellular transfer of PrP-res via membrane microparticles (e.g. exosomes) released from infected cells which insert into the membranes of recipient cells, a process shown to mediate the intercellular transfer of other proteins [41,42] including PrP^C [43]. An alternate possibility involved the release of PrP-res aggregates free of membranes with subsequent insertion into host cell membranes, a process called "GPI painting" [44,45]. Both brain membrane fractions containing membrane-associated forms of PrP-res [46] and purified PrP-res preparations that lack membranes (e.g. [47]) contain high titers of infectivity. However, it is unclear which of these forms of PrP-res might be most efficient at initiating PrP-res formation and infection in host cells.

These two infection scenarios were evaluated in the SN56 cell model of infection (Baron et al., unpublished data). The effect of PrP-res association with membranes on infection efficiency was examined by comparing the levels of sustained PrP-res production in cells treated with either scrapie brain microsomes or purified, detergent-extracted PrP-res. When normalized for quantity of input PrP-res, scrapie brain microsomes induced dramatically enhanced persistent PrP-res formation compared to purified PrP-res (Baron et al., unpublished data). As has been shown with some cell lines [14,48], infected SN56 cells released low levels of PrP-res into the culture supernatant, which also initiated infection in recipient cells (Baron et al., unpublished data). Interestingly, microsomes labeled with a fluorescent marker were internalized by SN56 cells in small vesicles, which were apparently trafficked to neuritic processes (Baron et al., unpublished data). These observations suggest that efficient infection of cells may involve a transfer and/or internalization of membranes containing PrP-res.

Mechanism of enhanced infection by membrane-bound PrP-res

The mechanism behind the enhanced infectivity associated with membrane-bound PrP-res has not yet been determined. Considering our models of the infection process, it is possible that membrane-associated PrP-res is more efficiently "painted" into host cell membranes, perhaps via membrane fusion, as compared with membrane-free PrP-res, which would be restricted to a "GPI painting" mechanism that is known to be poorly efficient in vitro [45]. The infecting PrP-res would then be located in a membrane continuous with the host cell PrP^C thereby allowing for efficient conversion of membrane-bound PrP^C [36,37]. It is also possible that there is a more efficient binding/uptake of microsomes and the associated PrP-res than with purified PrP-res.

An enticing consideration is that it may be related to the different aggregation states of the PrP-res in the two preparations. When associated with membranes either naturally as produced in the brain [49] or after reconstitution of purified material into synthetic liposomes [50,51], PrP-res forms diffuse aggregates. However, detergent extraction of these membranes can result in the formation of larger rod-like polymers of PrP-res [49,51,50]. In the context of nucleated polymerization models of PrP conversion (for review see [52]), PrP-res preparations containing smaller aggregates would have a higher number of seeds per mole of PrP to initiate conversion than those comprised of larger aggregates and thus the former might be expected to have a higher specific infectivity (infectivity per unit PrP). Consistent with this concept, Gabizon et al. found that infectivity titers of purified PrP-res are 10 to 100-fold higher when the PrP-res is reconstituted into phosphatidylcholine liposomes [51,50]. Thus, the above SN56 infection data now provides a biochemical explanation for these observations.

Exosomes as mediators of intercellular transfer of PrP-res

The observation that very low amounts of PrP-res released from infected SN56 cells are required to initiate infection in recipient cells, suggested the secreted PrP-res may have a very high specific infectivity and mediate the intercellular transfer of PrP-res (Baron et al., unpublished data). This raised the question as to whether the secreted PrP-res might be membrane-associated. One form of released membrane particle is called an exosome (for reviews see [53,54]). Exosomes are small membrane vesicles released from cells by fusion of multivesicular bodies (MVBs) with the plasma membrane. MVBs are a late endosome-like compartment in which vesicles destined to become exosomes form by invagination of the

MVB membrane. Working independently from us, Fevrier et al. [48] reported that exosomes containing PrP^C and PrP-res are secreted by epithelial and glial cell lines. Culture supernatants as well as exosomes isolated from the culture media were infectious for cultured cells and animals [48]. Although it has not yet been formally shown, it is likely that the PrP-res released from infected SN56 cells is associated with exosomes, and this is currently under investigation. These observations are especially interesting in light of the proposed role for MVBs in PrP-res biosynthesis several years ago [55]. Thus, the high efficiency of infection induced by PrP-res released from infected cells reinforces the observation that membrane-associated PrP-res exhibits a high specific infectivity.

Visualizing PrP-res trafficking during acute infection

Although the intracellular trafficking of PrP^C has received much attention, little is known about the uptake, intracellular trafficking, and intercellular spread of PrP-res. Such studies require the unambiguous detection of the input PrP-res over the background of new PrP-res synthesized during the infection process. One solution to this problem is to conduct infections using purified PrP-res preparations that have been covalently tagged with a fluorescent dye. Fluorescent PrP-res preparations have recently been used in infections of differentiated SN56 cells under conditions that permit establishment of persistent infection (Magalhães et al., unpublished observations). SN56 cells processed large cell-surface PrP-res aggregates into smaller aggregates that were subsequently internalized into vesicles positive for markers of fluid phase endocytosis and acidic organelles (Magalhães et al., unpublished observations). These vesicles were then trafficked along neuritic projections and accumulated at the termini of the projections where contacts with neighboring cells occurred (Magalhães et al., unpublished observations). Similar observations have been made in various primary neuronal cultures (Magalhães et al., unpublished observations). These results represent the first visualization of the initial uptake and trafficking of PrP-res during the infection process.

Conclusions

As the collection of susceptible cell lines grows and becomes further characterized, ex vivo models of TSE infection will continue to improve. Until better assay targets are developed, screens for inhibitors of PrP-res formation in cell culture are likely to remain the mainstay in the search for

TSE therapeutics. Even if additional screening methodologies are created, it is quite likely that they will ultimately involve the use of persistently infected cells. Among the list of cells supporting infection, SN56 cells appear to be a promising new model for the study of many aspects of TSE disease.

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Semi-classical Quantization of Protein Dynamics: Novel NMR Relaxation Formalism and its Application to Prion

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Summary. Novel nuclear spin relaxation mechanism in proteins on a timesclae of micro to milliseconds has been proposed using the Berry-phase interference between periodic orbits within the semi-classical quantization formalism. In order to examine the proposed formalism, susceptibility has been represented by an analytical formulation as a function of the frequency of the periodic orbits, using a Riemann ζ function. Numerically obtained profile of the susceptibility has been quantitatively compared with that of experimentally obtained slow exchange rates in a stable globular protein, $p8^{MTCP1}$ and in a hamster prion protein. Behavior of amide nitrogen atoms in $p8^{MTCP1}$ was almost uniform between residues, and consistent with that of theoretically obtained ones. On the other hand, that of hamster prion was not uniform and quite different than that of theoretical one, suggesting that the slow dynamics of a hamster prion might not be well described by the periodical orbits around the unique saddle point. Local trajectories in the phase space around the native conformation of the hamster prion may be connected to the complicated geometry, corresponding to multiple conformational states, for instance, PrP^{C}, PrP* or PrP^{Sc}.

Key words. prion, semiclassical quantization, NMR, trace formula, Riemann zeta function

Introduction

Rigorous treatment of the quantization of protein's dynamics may be the critical procedure to understand its biological function. Normal modes at the local phase space around the energy minima representing the dynamics

on the time scale of picoseconds, are relatively well defined. However, we do not have much insight on the geometry of the phase space outside of these integrable harmonic modes. Recent single molecule observation techniques, such as the single channel recording [1], the fluorescence detected single molecular observation in the evanescent field [2], the CPMG relaxation dispersion methods [3,4] strongly indicate that a protein undergoes the very slow fluctuation on a timescale of micro- to milliseconds between a few discrete multiple conformational states. First, we will propose just phenomenological representation of such a phenomenon using a simple kinetic model.

$\Xi_{ji}(t)$ denotes the non-covalent bond interaction energy at site j. Here it is assumed that exchange reaction occurs between conformations i, c.f. A and B:

$$\Xi_{ji}(t) = \begin{cases} \Xi_{jA}(t); \textit{when the protein is in conformation A at time t} \\ \Xi_{jB}(t); \textit{when the protein is in conformation B at time t} \end{cases} \quad (1)$$

The expected value for the macroscopic observable, $\Theta_i(t)$ is given by

$$\Theta_i(t) = \sum_{j=1}^{n} \left\langle \Psi_j(t) \middle| O_{ji} \middle| \Psi_j(t) \right\rangle, \quad (2)$$

where

$$\left|\Psi_j(t)\right\rangle = e^{-i\Xi_{jB}(t-t_m)} e^{-i\Xi_{jA}(t_m-t_{m-1})} \cdots e^{-i\Xi_{jB}(t_2-t_1)} e^{-i\Xi_{jA}t_1} \left|\Psi_j(0)\right\rangle \quad (3)$$

and O_{ji} is the observable.

Protein dynamics on a timescale of micro- to milliseconds or thermodynamical stability is directly related to its biological function or its pathogenicity [5]. However, the nature of the slow motion is essentially different than that of the fast motion on a timescale of pico- to nanoseconds. Since the first folding reactions of proteins, such as the prion protein [6], occur within ~100 microseconds at room temperature, the phase trajectories during micro- to milliseconds essentially include the transition between the unfolded and the folded states. CPMG relaxation dispersion measurements [3,4] could elucidate such a dynamics of proteins on a timescale of micro- to milliseconds. In general, slow movements occurring really gradually during micro- to milliseconds can be detected through the changes of the electronic states around nuclei (chemical shifts). However, we cannot exclude the possibility that the fast movements on a timescale of pico- to nanoseconds rarely occurring almost randomly during micro- to milliseconds, because in the NMR relaxation measurements, population changes are essentially observed. On the other hand, accumulated data of single molecular measurements suggest that the former is more realistic. Overall it may be reasonable to assume that slow dynamics is potentially

complicated, recursive, transitive and possibly chaotic [7], and may be characteristic to proteins.

So far magnetic relaxation processes of nuclei in a protein have been interpreted and analyzed based on the linear response theory created by R. Kubo [8] or A.G. Redfield [9]. Although several convenient dynamical models were proposed, such as the model-free system [10], there have been actually no theoretical attempts to include the inherent complexities of the protein dynamics on a timescale of micro to milliseconds. Here we first propose the novel strategy based on the quantization of protein dynamics within a semi-classical approximation, which is then represented by a prime number theory in order to obtain the explicit analytical formulation.

Characteristics of protein dynamics

Protein-water system is generally considered as the typical chaotic system [7] defined by the characteristics of Lyapunov Exponents. In the energy conservation system of degree of freedom s, there are 2n-1 Lyapunov exponents which are positive: ($\lambda_1 = \lambda_2 = \sim = \lambda_q$) or negative ($\lambda_{q+1} = \lambda_{q+2} = \sim = \lambda_{2n-1}$) with $\sum_{i=1}^{2n-1} \lambda_i = 0$. Especially in the Hamilton system, $\lambda_{2n-i+1} = -\lambda_i$.

In such a complex system, we can define the Kolmogorov-Sinai entropy. Phase space is divided into m cells ($i_0, i_1, \ldots i_{m-1}$) with volume δv. If the probability that the trajectory is along the chain of cells is $P_{i0i1\ldots im-1}$, the entropy of the set of trajectories is

$$K_m = -\sum_{i_0 \cdots i_{m-1}} P_{i_0 i_1 \cdots i_{m-1}} \ln P_{i_0 i_1 \cdots i_{m-1}} \tag{4}$$

In the KAM(Kolmogorov-Arnold-Moser) torus, $K_m = 0$, while in chaotic set, entropy generation rate is defined as

$\Delta K_m/\Delta t = (K_m - K_{m-1})/\Delta t$. Then Kolmogorov-Sinai entropy is:

$$h_{KS} = \lim_{\Delta v \to 0} \lim_{\Delta t \to 0} \lim_{N \to \infty} N^{-1} \sum_{m=1}^{N} \Delta K_m / \Delta t = \lim_{\Delta v \to 0} \lim_{\Delta t \to 0} \lim_{N \to \infty} K_N /(N \Delta t) \tag{5}$$

It is known that for periodic motion, $h_{KS} = 0$, for Brownian motion, $h_{KS} = \infty$ and for chaos, $0 < h_{KS} = \infty$. For example, in a protein system (open Hamilton system), it is reported that the Lyapunov exponent > 0 and the KS entropy, $0 < h_{KS} = +\infty$ using molecular dynamics simulation [7].

Gutzwiller's Trace Formula

Let us consider a particle of mass m moving in a d dimensional space. x and y represent the coordinate and the momentum, respectively. Its Hamiltonian is

$$H(x) = -(\hbar^2/2m)\sum_{j=1}^{d} \partial^2/\partial x_j^2 + V(x_1, x_2, \cdots, x_d) \tag{6}$$

and the Schrödinger equation is

$$H(x)\Psi_n(x) = E_n\Psi_n(x). \tag{7}$$

Its solution can be obtained by the following Green function

$$G(x'', x'; E) = \sum_n \Psi_n(x'')\Psi_n(x')/(E - E_n), \tag{8}$$

where E_n can be obtained by summing up the poles of the trace

$$TrG(E) = (\equiv \int dq G(x, x; E)) = \sum_n \frac{1}{E - E_n}. \tag{9}$$

Since

$$(x + i\varepsilon)^{-1} = \wp x^{-1} - i\pi\delta(x), \tag{10}$$

density of state is

$$-\pi^{-1}\,\mathrm{Im}(TrG(E)) = \sum_n \delta(E - E_n)(\equiv \rho(E)) \tag{11}$$

On the other hand, Green function is the Laplace transformation of the propagator describing the temporal evolution,

$$K(x'', x'; t) = \langle x''|e^{-iHt/\hbar}|x'\rangle. \tag{12}$$

According to the Feynmann's path integrals, propagator can be expressed as

$$K(x'', x'; t) = \int_{x(0)=x'}^{x(t)=x''} D[x]\exp\{(i/\hbar)W[x]\}, \tag{13}$$

where L is the Lagrangian,

$$W[x] = \int_0^t L(x, \dot{x})dt. \tag{14}$$

K can be also represented as:

$$K(x'', x'; t) = \lim_{N\to\infty}[mN/(2\pi i\hbar t)]^{dN/2}$$
$$\int_{-\infty}^{\infty}\cdots\int_{-\infty}^{\infty}\prod_{i=1}^{N-1} dx_i \exp\{\frac{i}{\hbar}\frac{t}{N}\sum_{i=1}^{N}[\frac{m}{2}(\frac{x_i - x_{i-1}}{t/N})^2 - V(x_i)]\} \tag{15}$$

In the semi-classical limit ($\hbar \to 0$), we may count only the contribution around the saddle point,

$$\delta W[x] = 0 . \tag{16}$$

Fluctuation around the saddle point can be expressed as

$$K_{scl}(x'',x',t) = (2\pi i\hbar)^{-d/2} \sum_j \sqrt{T_j} \exp\{iW_j/\hbar - i\mu_j\pi/2\} . \tag{17}$$

j is the classical trajectory originates from x(0)=x' and ends at x(t)=x", and T_j is the periodicity,

$$T_j = \left| \det(-\partial^2 W_j / \partial x'' \partial x') \right| . \tag{18}$$

μ_j is the Morse Maslov index (number of singular point between x' and x").

Laplace transformation of the propagator K is equal to the Green function,

$$G_{scl}(x'',x';E) = (i\hbar)^{-1} \int_0^\infty dt K_{scl}(x'',x';t) e^{-iEt/\hbar} . \tag{19}$$

In a stationary phase approximation,

$$G_{scl}(x'',x',t) = 2\pi(2\pi i\hbar)^{-(d+1)/2} \sum_j \sqrt{|\hat{T}_j|} \exp\{iS_j(x'',x';E)/\hbar - i\mu_j\pi/2\} \tag{20}$$

$$\hat{T}_j = \hat{T}_j(x'',x';E) = (\partial^2 W_j/\partial t^2)^{-1} \det(-\partial^2 W_j/\partial x'' \partial x')\big|_{t=t_0} \tag{21}$$

$S_j(x'',x';E)$ is the reduced action obtained by the Legendre transformation of the action W_j,

$$S_j(x'',x';E) = W(x'',x';t_0) + Et_0 = \int_{x'}^{x''} p(x,E)dx \tag{22}$$

Using the basic relations in the analytical mechanics,

$$\partial W/\partial t = -E, \partial S/\partial E = t, \partial^2 W/\partial t^2 = -\partial E/\partial t = -(\partial^2 S/\partial E^2)^{-1} \tag{23}$$

$$\hat{T}_j(x'',x';E) = (-1)^d (\partial^2 S_j/\partial E^2) \det(-\partial^2 W_j/\partial x'' \partial x')\big|_{t=t_0}$$

$$= \begin{vmatrix} \dfrac{\partial^2 S_j}{\partial x' \partial x''} & \dfrac{\partial^2 S_j}{\partial x' \partial E} \\ \dfrac{\partial^2 S_j}{\partial E \partial x''} & \dfrac{\partial^2 S_j}{\partial E^2} \end{vmatrix} , \tag{24}$$

which is the determinant of the (d+1) x (d+1) matrix.

$$TrG_{scl}(E) = 2\pi(2\pi i\hbar)^{-(d+1)/2}$$
$$\sum_j \int dx \sqrt{|\hat{T}_j|} \exp\{iS_j(x,x;E)/\hbar - i\mu_j\pi/2\} \tag{25}$$

In case of integrable system, the phase space is occupied by the torus and the action integral of the d closed trajectories is;

$$S_k = \oint_{\Gamma_k} ydx, (k = 1,2,\cdots d). \tag{26}$$

Thus effective action is described by the sum of the winding number l_k times S_k.

$$TrG_{scl}(E) \cong V \sum_{l_1=0}^{\infty} \cdots \sum_{l_d=0}^{\infty} \prod_{k=1}^{d} \exp[il_k(S_k/\hbar - \mu_k\pi/2)])$$
$$= V \prod_{k=1}^{d} (1 - \exp[i(S_k/\hbar - \mu_k\pi/2)])^{-1} \tag{27}$$

μ_k is the Morse-Maslov index number and V is the d-dimensional volume. By counting only the contributions from the poles,

$$\frac{1}{2\pi} \oint_{\Gamma_k} y(E)dx = (n_k + \mu_k/4)\hbar, (n_k = 0,1,2,\cdots). \tag{28}$$

This is the Einstein-Brillouin-Keller (EBK) quantization rule.

However, in case of non-integrable system, if saddle point approximation is applicable,

$$\partial S(x,x)/\partial x \equiv \partial S(x'',x')/\partial x'' + \partial S(x'',x')/\partial x'|_{x''=x'=x} = y''-y' = 0. \tag{29}$$

The conditions, i.e. y"=y' and x"=x'=x indicate that only the periodic orbital contribute to $TrG_{scl}(E)$. Although the measure of the periodic orbital is zero, its number is infinite. Therefore, $TrG_{scl}(E)$ is represented by the sum of the periodic orbital (semi-classical quantization). Jacobian is rewritten by the global characteristics of the periodic orbital.

Let us introduce the local coordinate with the component along the periodic orbital, $x_{//}$ and those perpendicular to it, $x_\perp$,

$$x = (x_{//}, x_{\perp 1}, x_{\perp 2}, \cdots, x_{\perp(d-1)}) = (x_{//}, x_\perp). \tag{30}$$

According to the energy conservation law in the Hamilton-Jacobi equation,

$$H(x,y) = H(x, \partial S/\partial x) = E, |\partial^2 S/\partial x' \partial x''| = 0. \tag{31}$$

Therefore,

$$\hat{T}_{po} = -\frac{\partial^2 S_{po}}{\partial E \partial x_{//}''} \frac{\partial^2 S_{po}}{\partial E \partial x_{//}'} \left| \frac{\partial^2 S_{po}}{\partial x_\perp' \partial x_\perp''} \right| = (-1)^d \frac{1}{\dot{x}_{//}' \dot{x}_{//}''} \det\left(\frac{\partial y_\perp'}{\partial x_\perp''}\right) \tag{32}$$

$$S(x'',x',E)\big|_{q''=q'=q} = S_{po}(E) + (1/2)\sum_{i,j=1}^{d-1} R_{ij}(x_{//})x_{\perp i}x_{\perp j} \tag{33}$$

$S_{po}(E)$ is the action on the periodic orbital,

$$S_{po}(E) = \oint_{po} y dx \tag{34}$$

$$R(x_{//}) = \left(\frac{\partial^2 S}{\partial x_\perp'' \partial x_\perp''} + \frac{\partial^2 S}{\partial x_\perp'' \partial x_\perp'} + \frac{\partial^2 S}{\partial x_\perp' \partial x_\perp''} + \frac{\partial^2 S}{\partial x_\perp' \partial x_\perp'} \right)_{x''_\perp = x'_\perp = 0} \tag{35}$$

The contribution of each periodic orbital onto the Green function is;

$$\delta Tr G_{scl}(E) \propto \exp\{-i(S_{po}/\hbar - \mu_{po}\pi/2)\}$$
$$\int \left| \hat{T}_{po}(x_{//}) \right|^{1/2} \left| \det R(x_{//}) \right|^{-1/2} dx_{//} \tag{36}$$

μ_{po} is the generalized Morse-Maslov index, which includes also negative eigenvalues of $R(x_{//})$.

By use of the linearized Poincare map, i.e. monodoromy matrix, we can rewrite the above integral. Using the relationship, $\partial S / \partial x'' = y'', \partial S / \partial x' = -y'$, $\det R(q_{//})$ can be rewritten,

$$\det R(x_{//}) = \det\left(\frac{\partial y_\perp''}{\partial x_\perp''} - \frac{\partial y_\perp'}{\partial x_\perp''} + \frac{\partial y_\perp''}{\partial x_\perp'} - \frac{\partial y_\perp'}{\partial x_\perp'} \right)_{x_\perp'' = x_\perp' = 0}$$
$$= -\det\left(\frac{\partial(y_\perp'' - y_\perp', x_\perp'' - x_\perp')}{\partial(x_\perp'' - x_\perp')} \right)_{x_\perp'' = x_\perp' = 0} \tag{37}$$

On the other hand,

$$\left| \hat{T}_{po}(x_{//}) \right| = \frac{1}{\dot{x}_{//}^{\,2}} \left| \frac{\partial y_\perp'}{\partial x_\perp''} \right|_0 = \frac{1}{\dot{x}_{//}^{\,2}} \left| \frac{\partial(y_\perp', x_\perp')}{\partial(x_\perp'', x_\perp')} \right|_0 \tag{38}$$

0 means $x_\perp' = x_\perp'' = 0$.

$$\left| \hat{T}_{po}(x_{//}) \right|^{-1/2} \left| \det R(x_{//}) \right|^{1/2} = \left| \dot{x}_{//} \right| \times \left| \frac{\partial(x_\perp'' - x_\perp', y_\perp'' - y_\perp')}{\partial(x_\perp', y_\perp')} \right|_0 \tag{39}$$

Here we introduce the 2(d-1) dimensional vector $\xi_\perp = (x_\perp, y_\perp)$, and the last term can be rewritten using the monodoromy matrix M_{po} of 2(d-1) x 2(d-1) dimensional periodic trajectory,

$$\left| \frac{\partial(x_\perp'' - x_\perp', y_\perp'' - y_\perp')}{\partial(x_\perp', y_\perp')} \right|_0 = \left| \frac{\partial(\xi_\perp'' - \xi_\perp')}{\partial \xi_\perp'} \right|_0 = \left| \det(M_{po} - I) \right| \tag{40}$$

Therefore, when if we take into account,

$$\int \frac{1}{\dot{x}_{//}} dx_{//} = \int_p dt = T_p \,, \tag{41}$$

$TrG_{scl}(E)$ can be represented by the global characteristics of each periodic trajectory. Individual periodic trajectory is composed of prime periodic orbital or arbitrary natural number of the prime orbital. T_p is the period of p. If we can sum up all the contribution from the periodic trajectory, we can calculate TrG_{scl}. Here we consider that the unstable periodic orbital can be decomposed into the prime periodic orbital p and the natural number l_p. Action on p and period are represented by $S_p(E)$ and $T_p(E)$, respectively. Then, we can obtain the following Gutzwiller's trace formula,

$$TrG_{scl}(E) - TrG_0(E) = (i\hbar)^{-1} \sum_p T_p \sum_{l_p=1}^{\infty} \{\det(M_p^{l_p} - I)\}^{-1/2} \tag{42}$$

$$\exp\{il_p(S_p/\hbar - \mu_p \pi/2)\} + O(\hbar^0)$$

$$TrG_0(E) = (\pi/i)(2\pi\hbar)^{-d} \iint dy dx \delta(H(x,y) - E) + O(\hbar^{-d+1}) \tag{43}$$

Monodoromy matrix describes the time evolution of the derivatives perpendicular to the trajectory $\delta x_{\perp}^{(n)}$. Eigenvalues of M_p, Λ_p depend on the type of fixed points. $\Lambda_p = \exp(\pm u_p)$, and $\exp(iu_p)$ for unstable and stable orbital, respectively. Especially, monodromy matrix for the homoclinic orbital with hyperbolic fixed points is, using Lyapunov index u_p (>0),

$$\det(M_p^l - I) = 4\sinh^2(lu_p/2) \tag{44}$$

If we modify the above equation using

$$[2\sinh(lu_p/2)]^{-1} = \sum_{k=0}^{\infty} \exp[-l(k+\frac{1}{2})u_p] \,. \tag{45}$$

$$TrG_{scl}(E) - TrG_0(E) \cong -\frac{d}{dE} \ln \zeta(E) \,, \tag{46}$$

where ζ (E) is the Ruelle dynamical zeta function.

$$\zeta(E) = \prod_p \prod_{k=0}^{\infty} (1 - t_p \Lambda_p^{-k})^{-1} \qquad \text{and} \qquad \text{weight}$$

$$t_p = |\Lambda_p|^{-1/2} \exp[i(S_p/\hbar - \mu_p \pi/2)] \,. \tag{47}$$

Dynamical susceptibility can be formulated using the trace formula [11],

$$\chi'' = -\frac{\omega}{2\pi}\int dt \int \frac{d^f x d^f y}{(2\pi\hbar)^f}\delta(E-H)A_W(x,y)[e^{(i\omega-\hat{L}_{cl})t}B_w](x,y) + 0(\hbar^{-f+1})$$

$$-\frac{\omega}{2\pi\hbar}\delta(E_F-H)\sum_{p,r}\frac{\cos(\frac{r}{\hbar}S_p - r\frac{\pi}{2}\upsilon_p)}{\left|\det(m_p^r - I)\right|^{1/2}}\int dt e^{i\omega t}\oint_p d\tau A_W(\tau)B_W(t+\tau) + 0(\hbar^0)$$

$$\cong A - \frac{d}{ds}\ln\varsigma(s)\int dt e^{i\omega t}\oint_p d\tau A_W(\tau)B_W(t+\tau) + 0(\hbar^0)$$

$$\cong A - B\frac{\varsigma'(s)}{\varsigma(s)}$$

, (48)

where f is the degrees of freedom, L_{cl} is the Liouvillian operator, S_p is the action, ν_p is the Maslov index, m_p is the monodromy matrix for the periodic orbit p, and r denotes the repetition number.

The equality of periodic orbital sum and the Riemann ζ function is proved as follows:

Riemann staircase function is defined as follows;

$$N(\omega) = \sum_{K=1}^{\infty}\Lambda(\omega - \omega_k). \tag{49}$$

Oscillating part of the Riemann staircase function is described as follows;

$$N_{osc}(\omega) = -\frac{1}{\pi}\lim_{\eta\to\infty}\mathrm{Im}\ln\zeta(\frac{1}{2} - i(\omega + i\eta))$$

$$= -\frac{1}{\pi}\mathrm{Im}\sum_p\sum_{m=1}^{\infty}\frac{1}{mp^{\frac{m}{2}}}e^{i\omega m\ln(p)} \tag{50}$$

Spacing between Riemann zeros is

$$\rho_{osc} = \frac{dN_{osc}}{d\omega} = \frac{d\ln\zeta}{d\omega} = -\frac{1}{\pi}\mathrm{Im}\,g(\omega) \tag{51}$$

where

$$g(\omega) = i\sum_p\sum_{m=1}^{\infty}\frac{\ln(p)}{p^{\frac{m}{2}}}e^{iwm\ln(p)}. \tag{52}$$

While Gutzwiller's periodic orbit sum is

$$\rho_{osc}(E) = -\frac{1}{\pi}\mathrm{Im}\sum_{po} A_{po} e^{iS_{po}} \tag{53}$$

By comparing eq. 52 and 53, we can speculate as follow,

$$A_{pm} = i\frac{\ln(p)}{p^{\frac{m}{2}}},\ S_{pm} = m\omega\ln(p). \tag{54}$$

Or by comparing eq.52 and 42,

$$T_p \sim \ln(p),\ \det(M_p^m - 1) \sim p^m,\ im(\frac{S_p}{\hbar} - \mu_p\frac{\pi}{2}) \sim i\omega m\ln(p) \tag{55}$$

According to E. C. Titchmarsh [12],

$$\frac{\varsigma'(s)}{\varsigma(s)} = b - \frac{1}{s-1} - \frac{1}{2}\frac{\Gamma'(1+s/2)}{\Gamma(1+s/2)} + \sum_{\mathrm{Im}\rho<0}(\frac{1}{\rho}+\frac{1}{\rho^*}+\frac{1}{s-\rho}+\frac{1}{s-\rho^*})$$

$$\cong -\frac{1}{1/4+\omega^2} + \frac{1}{2}\sum_{\omega_\rho>0}(\frac{1}{(1/4)^2+(\omega+\omega_\rho)^2}+\frac{1}{(1/4)^2+(\omega-\omega_\rho)^2}) \tag{56}$$

Dynamic susceptibility is usually represented using the transverse relaxation time:

$$\chi'' = \frac{\gamma M_0}{2V}\frac{T_2}{1+(\omega-\omega_0)^2T_2^2} \tag{57}$$

Thus, we can essentially evaluate the transverse relaxation times in terms of the periodical orbitals of the nuclei using the Ruelle's dynamical zeta function. Using known Riemann's zero points, we evaluated eq. 56, as shown in Fig. 1. Fig. 1 illustrates the behavior of the susceptibility as a function of frequency of periodical orbitals of nuclear spins, which is essentially independent of the nuclear magnetic resonance frequency.

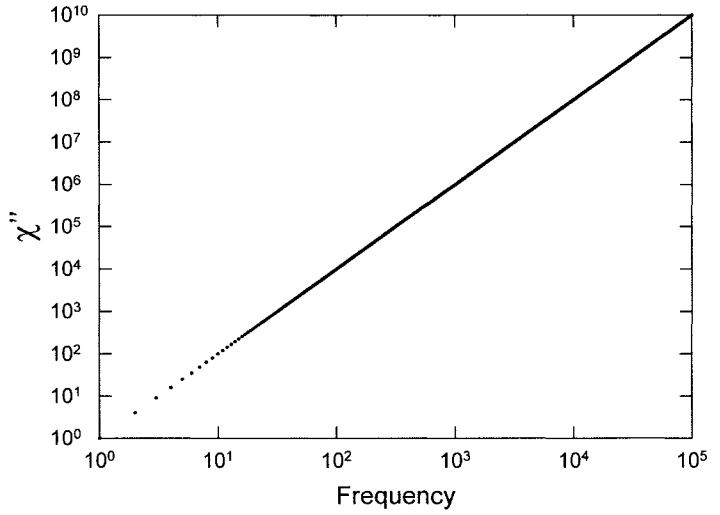

Fig. 1. A profile of susceptibility as a function of the frequency of periodical orbitals of nuclear spins, calculated using eq. 56 and known Riemann zeros.

Hecke algebraic representation of the Mori's continued fraction expansion [13]

Generalized Langevin equation (GLE) can be rewritten as follows;

$$\frac{dA(t)}{dt} = i\omega_0 A(t) - \int_0^t d\tau M(\tau) A(t-\tau) + f_1(t) \tag{58}$$

Using the following relations,

$$f_0(t) \equiv A(t), L_0 \equiv L, \Sigma_0 \equiv \Sigma(t) \equiv (f_0 \mid f_0)^{-1}(f_0 \mid f_0(t)) \tag{59}$$

$$\frac{d\Sigma_0(t)}{dt} = i\omega_0 \Sigma_0(t) - \delta_1 \int_0^t d\tau \Sigma_1(\tau)\Sigma_0(t-\tau), \tag{60}$$

where

$$\delta_1 \equiv (f_0 \mid f_0)^{-1}(f_1 \mid f_1), \Sigma_1(t) \equiv (f_1 \mid f_1)^{-1}(f_1 \mid f_1(t)). \tag{61}$$

Using the Laplace transformation, we can obtain

$$\Sigma_0(z) = [z - i\omega_0 + \delta_1 \Sigma_1(z)]^{-1} \tag{62}$$

$$\Sigma_j(z) = [z - i\omega_j + \delta_{j+1}\Sigma_{j+1}(z)]^{-1} \tag{63}$$

Thus

$$\Sigma_0(z) = \cfrac{1}{z - i\omega_0 + \cfrac{\delta_1}{z - i\omega_1 + \cfrac{\delta_2}{z - i\omega_2 + \cfrac{\delta_3}{z - i\omega_3 + \ldots}}}} = \tag{64}$$

$$\cfrac{1}{z - i\omega_0 + \cfrac{1}{\cfrac{z - i\omega_1}{\delta_1} + \cfrac{1}{\cfrac{z - i\omega_2}{\delta_1\delta_2} + \cfrac{\delta_3}{z - i\omega_3 + \ldots}}}}$$

Here we define

$$k_i = \frac{z - i\omega_i}{\delta_1\delta_2\cdots\delta_i} \tag{65}$$

$$p_n = [k_0, k_1, \cdots, k_{n-1}] \tag{66}$$

$$q_n = [k_1, k_2, \cdots, k_{n-1}], \tag{67}$$

where

$$[k_0] = k_0, [k_0, k_1] = k_0k_1 + 1, \cdots,$$
$$[k_0, k_1, \cdots, k_n] = [k_0, k_1, \cdots, k_{n-1}]k_{n-1}[k_0, k_1, \cdots, k_{n-2}]. \tag{68}$$

Then, Mori's equation forms modular group in this point of view, since

$$p_nq_{n-1} - p_{n-1}q_n = (-1)^n \tag{69}$$

In other words,

$$M(n) = \left\{ z \to \frac{az + b}{cz + d} : ad - bc = n; a, b, c, d \in Z \right\} \tag{70}$$

Also we can take

$$M(n) = \left\{ z \to \frac{az + b}{d} : ad = n; a, b, d \in Z \right\} \tag{71}$$

Then we can define Hecke operator,

$$(T(n)f)(z) = n^{-\frac{1}{2}} \sum_{ad=nb \bmod d} \sum f\left(\left(\frac{az + b}{d}\right)\right) \tag{72}$$

It forms the Hilbert Space and we can define the Hecke eigenvalue,

$$T(n)\psi_j = t_j(n)\Psi_j \tag{73}$$

and Hecke L-fucntion which is analogous to Riemann ζ function.

$$H_j(s) = \sum_{n=1}^{\infty} t_j(n) n^{-s} = \prod_p (1 - t_j(p) p^{-s} + p^{-2s})^{-1} \tag{74}$$

where t_j is the Hecke eigenvalue. According to the above procedures, we can deal with the Langevin modes similar to the quantum mechanics in a Hilbert space, using a number theoretical technique in a framework of Hecke algebra.

Experimental results

By nuclear magnetic relaxation measurements of proteins using high resolution NMR at various nuclear magnetic resonance frequencies, we may be able to verify the above theoretical results especially represented by eq. 56 and Fig. 1. We used $p8^{MTCP1}$ as an example of thermodynamically stable globular protein [14], since the standard R1, R2 and NOE at various nuclear magnetic resonance frequencies were reported in detail. They used the model-free system for the calculation of slow exchange rate constant, Rex [10]. Briefly, R1, R2 and NOE were measured. Correlation times of the fast side chain motion (picoseconds time scale) and that of the overall tumbling motion (nanoseconds time scale) were estimated from R1 and NOE data. The contributions of these fast modes are subtracted from the R2 data. Since R2 parameter essentially includes the contribution of the slow exchange dynamics, experimentary obtained R2 minus predected R2 using the above dynamical information could be interpreted as Rex. Therefore the obtained Rex is rather conventional parameter. However, as shown in Fig. 2, nuclear magnetic resonance frequency dependencies of Rex in $p8^{MTCP1}$ were rather uniform and similar for almost all residues.

This phenomenon is reasonable, because Rex must be proportional to the square of the nuclear magnetic resonance frequency deviation, i.e. amplitude of the chemical shift deviation. Although this behavior is not contradictory to the periodic orbit approximation, the above representation must be verified further.

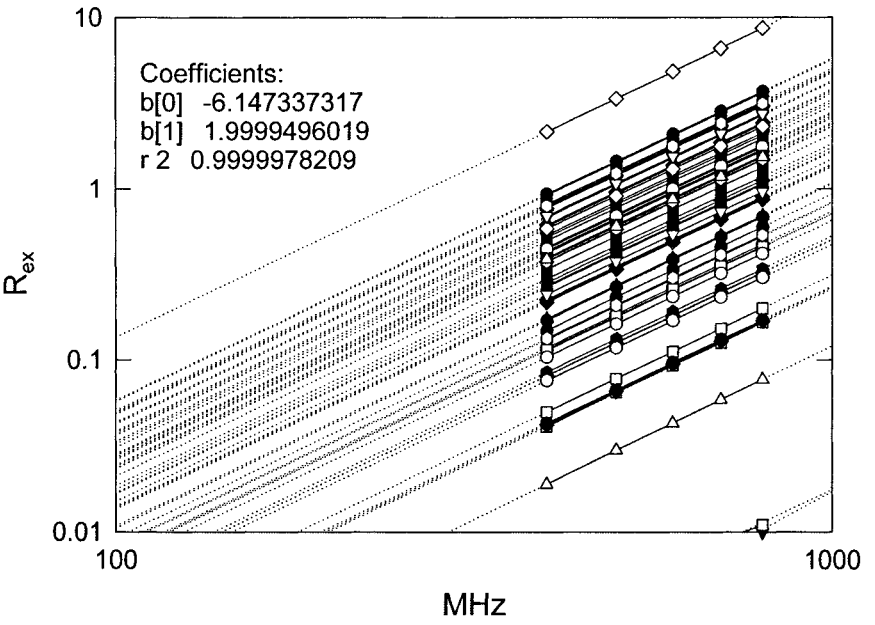

Fig. 2. Experimentally obtained Rex for $p8^{MTCP1}$ as a function of nuclear magnetic resonance frequency (^1H). All the amide nitrogen atoms showed quite similar behavior. Rex values were obtained by the model free analysis.

On the other hand, nuclear magnetic resonance frequency dependencies of Rex for prions obtained by CPMG relaxation dispersion methods [4,5] were somewhat different than those of $p8^{MTCP1}$, as shown in Fig. 3.

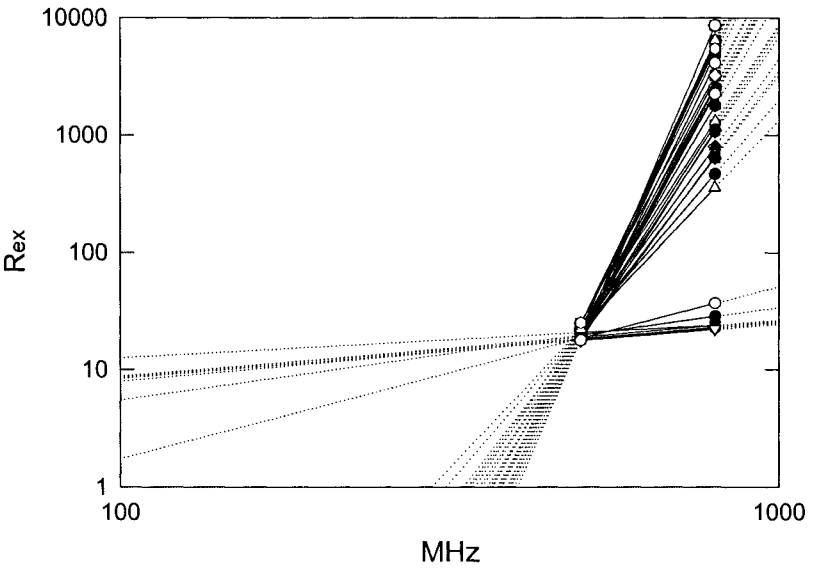

Fig. 3. Experimentally obtained Rex for hamster prion as a function of the nuclear magnetic resonance frequency (^{1}H). Amide nitrogen atoms exhibited quite heterogeneous behavior. Here Rex were estimated using CPMG relaxation dispersion methods, since model free analysis was not applicable to prion proteins [5].

They were essentially heterogeneous and seemed to be roughly categorized into two groups. Although physical details are still unknown, we may assume that the geometry of phase space around the native conformer of prion cannot be represented by a unique saddle point.

Dynamics Based Drug Design (DBDD)

We already found that the hamster prion has a folding intermediate, and residues with low stabilities undergo very slow fluctuations on a time scale of micro- to milliseconds [5]. Furthermore, distribution of residues with low stabilities corresponds to that of the causative mutations. Therefore in case of hamster prion, thermodynamical instabilities may be directly related to the initial conformational conversion process. Or thermodynamic fluctuation occurs along the trajectory between the normal to pathogenic conformation on a timescale of micro- to milliseconds.

Based on this assumption, we can design the anti-prion agents as the therapeutics quite logically using *in silico, ex vivo* and *in vivo* screening. We designated this strategy as "Dynamics Based Drug Design (DBDD)".

More than 1000 severe diseases including neurodegenerative diseases, cancer, collagen diseases, enzyme deficiency involve more or less protein structural abnormalities or thermo-dynamical instabilities. DBDD is the completely novel strategy to connect the number theory, the structural biology, and the drug discovery in the 21st century.

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Surveillance of chronic wasting disease (CWD) in Japan

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Abstract

Chronic wasting disease (CWD) in cervids including elk, mule deer, and white-tailed deer, is a member of the transmissible spongiform encephalopathies (TSEs). CWD is a serious problem in North America. The detection of abnormal isoforms of prion protein (PrP^{Sc}) is a key factor for the diagnosis of CWD, similar to other TSEs. The surveillance program for TSEs in animals is conducted by the Ministry of Agriculture, Forestry, and Fishery (MAFF) and is targeted to sheep, goats, and deer. In Japan, several different anti-prion protein (PrP) monoclonal antibodies (mAbs) are utilized for bovine spongiform encephalopathy (BSE) confirmation. Since CWD does not occur naturally in Japan, the immunoreactivity of the antibodies against PrP^{Sc} found in deer was not known. In this study, we examined the immunoreactivities of these antibodies against PrP^{Sc} found in CWD. The protocols that are used in Japan for confirmation of BSE cases are Western blot (WB) and immunohistochemistry (IHC). We used these same protocols to examine CWD positive brain samples which were provided by Dr. A. Davis of the National Veterinary Service Laboratory, USA. Mabs. T1, T2, 44B1, and 72-5, were used successfully to detect PrP^{Sc} in CWD affected mule deer brains by WB. In IHC, PrP^{Sc} was detected with mAbs T2, 44B1, and polyclonal antibody B103. These results determined that the antibodies used for BSE confirmation are also applicable to CWD, as for scrapie. These same antibodies could detect PrP^{C} from Japanese deer by WB without proteinase digestion. The amino acid sequence of PrP of Japanese deer was found to be the same as sequence as the one reported for mule deer. These antibodies were then

used for CWD surveillance in Japan. When 127 of hunter-killed deer from Hokkaido were examined, PrP^{Sc} was not detected in any of the animals.

Unexpected high incidence of visceral AA-amyloidosis in slaughtered cattle in Japan

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Abstract

Objectives

Systemic AA amyloidosis in aged cattle is a well-known disease entity. Recent experimental studies have shown that mouse AA amyloidosis can be transmissible by dietary ingestion of pathological agents including AA amyloid fibrils themselves. Bovine liver and gastrointestinal tract have been frequently used for Oriental foods. To clarify the safety of our foods the incidence of visceral amyloidosis in slaughtered cattle is evaluated.

Methods

Renal tissues obtained from 302 aged cattle were provided from a local abattoir. The ages of cattle examined ranged from 4 to 19 years old and the median age was 8 years old. Amyloid deposition was microscopically examined after phenol Congo red staining, and amyloid protein was determined by immunocytochemical and biochemical analyses of tissue amyloid.

Results

On routine veterinary examinations before slaughter, all cattle with no history of diseases looked healthy. Amyloid deposition on renal tissues was seen in 15 out of these 302 cattle and its incidence was 5%. Amyloid protein was identified to be AA. On gross examinations these 15 cases were commonly found to have some pathological findings on their visceral organs, including superficial small hemorrhages on liver, fatty liver, biliatry tract infection and extensive erosive lesions on gastrointestinal tract. Thus, all their visceral organs except for kidneys were discarded, although the meat had been consumed.

Conclusion

The present incidence of renal amyloidosis in slaughtered cattle (5%) is considerably high comparing with those reported from Japan (1.2 %) and from foreign countries (0.4 to 2.7%). AA-amyloidosis is a life-threatening complication in patients with rheumatoid arthritis and other chronic inflammatory diseases. If this amyloidosis can be transmissible by a prion-like mechanism, these patients at the risk of secondarily induced AA-amyloidosis need to avoid taking the foods that contain amyloid enhancing factor-like activity. There has been no evidence that foods made of visceral organs obtained from aged cattle give rise to harmful effects on human health, but diet and environment have been paid much attention as risk factors for the induction of systemic amyloidosis. Further investigation on bovine AA-amyloidosis is necessary in providing the guarantee for the safety of foods.

A Novel BSE screening kit with simplified preparation method for EIA

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Abstract

Introduction

In Japan, all slaughtered and deceased bovine materials are tested for BSE infection. The primary screening test is undertaken by the meat inspection office or live stock hygiene service center in each prefecture. In these circumstances, BSE kits adapted for relatively small number of samples are required. Here we describe the development of a new BSE screening system, which has simpler and safer protocol for sample preparation steps for EIA. This assay system has been named the NippIBL BSE Assay system.

Detection Method

1. The first step involves the homogenization of bovine brain and enzyme treatment. We developed a new apparatus for homogenization by passing the tissue samples through a porous rigid polypropylene filter named the Bio-Masher. In the enzyme treatment a purification step is not involved. This means that the frequency of opening the sample tubes is reduced (safer) and this also shortens the time of sample preparation. In the first process, bovine brain was homogenized using the Bio Masher, and homogenate was suspended in the buffer containing the enzyme, and incu-

bated at 56℃. After heat inactivation of the enzyme at 100℃, the sample is ready for EIA assay.

2. The second step is the detection of prion protein (PrP) by sandwich EIA. The dynamic range for detection of recombinant PrP with our kit was 31.3 -2,000 pg/ml, and the sensitivity was 2.8 pg/ml.

Application of the NipplBL BSE ASSAY KIT

We tested the detection sensitivity of our assay kit using confirmed and intentionally deteriorated BSE samples. BSE hypothalamus homogenates were incubated at 37℃ for 39hr, and the sensitivity of NippIBL kit was compared with the PLATERIA. The detection limit of the BSE samples by PLATERIA was reduced some 10-30 fold after the 37℃ incubation, while the sensitivity of NippIBL was unchanged. Thus our new assay system could be useful for detection of abnormal prion not only in fresh samples but also in decayed samples.

Conclusion

We have established a new BSE assay system. There are many advantages in our system : 1. The time needed for sample pretreatment is substantially shortened, and the risk of infection during sample processing is effectively reduced. 2. No extra equipment is required. 3. The system is very simple and inexpensive. 4. The test results with our kit have proven and guaranteed sensitivity and reproducibility.

Detection of recombinant bovine prion protein by fluorescence correlation spectroscopy

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Abstract

Fluorescence correlation spectroscopy (FCS) is a detection technique for molecular parameters, such as concentration, diffusion constant and interaction in solution with single molecule level. FCS consists of a confocal optics with a well focused laser beam and detects fluorescent fluctuation that is caused by the diffusion of fluorescently labeled molecules through a tiny open volume element (< one femtoliter). Measured fluctuation can be used to calculate the lateral diffusion coefficient and thus the size of the molecule. We propose FCS as a newly method for the detection of prion diseases.

Although FCS is well suited for development of homogeneous assays, an obstacle lies on the detection of antibody-antigen systems. The difference in molecular weight has to be at least a factor of four between unbound fluorescently-labeled antibodies and the immune complexes, because FCS sensitivity is based on the diffusion coefficients. Therefore, it was difficult to discriminate the immune complexes of prion (180 kDa) and labeled antibodies (150 kDa). To overcome this limitation, we have applied the fluorescent-labeled Fab' fragments (50 kDa) and IgG for the detection of monomeric prion proteins (30 kDa).

By using FCS, recombinant bovine prion proteins could be detected at about 5 nM concentrations. The value was about one order less sensitive than ELISA analysis. However, this study provides the basis for a rapid and specific assay method test for prion diseases using FCS.

PrPSc distribution of a natural case of bovine spongiform encephalopathy

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Abstract

Bovine spongiform encephalopathy (BSE) is a disease of cattle that causes progressive neurodegeneration of the central nervous system. Infectivity of BSE agent is accompanied with an abnormal isoform of prion protein (PrPSc).

The specified risk materials (SRM) are tissues potentially carrying BSE infectivity. The following tissues are designated as SRM in Japan: the skull including the brain and eyes but excluding the glossa and the masseter muscle, the vertebral column excluding the vertebrae of the tail, spinal cord, distal illeum. For a risk management step, the use of SRM in both animal feed or human food has been prohibited. However, detailed PrPSc distribution remains obscure in BSE cattle and it has caused controversies about definitions of SRM. Therefore we have examined PrPSc distribution in a BSE cattle by Western blotting to reassess definitions of SRM.

The 11th BSE case in Japan was detected in fallen stock surveillance. The carcass was stocked in the refrigerator. For the detection of PrPSc, 200 mg of tissue samples were homogenized. Following collagenase treatment, samples were digested with proteinase K. After digestion, PrPSc was precipitated by sodium phosphotungstate (PTA). The pellets were subjected to Western blotting using the standard procedure. Anti-prion protein monoclonal antibody (mAb) T2 conjugated horseradish peroxidase was used for the detection of PrPSc.

PrPSc was detected in brain, spinal cord, dorsal root ganglia, trigeminal ganglia, sublingual ganglion, retina. In addition, PrPSc was also detected in the peripheral nerves (sciatic nerve, tibial nerve, vagus nerve).

Our results suggest that the currently accepted definitions of SRM in BSE cattle may need to be reexamined.

Report of the first oral inoculation of BSE prion into cattle in Japan

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Abstract

A Prion Disease Research Center at the National Institute of Animal Health (NIAH) was established to conduct comprehensive research on BSE in response to the discovery of bovine spongiform encephalopathy (BSE) in Japan. A new research facility for the center has been constructed. It was designed as a biosafety level (BSL) 3 facility with the capacity to inoculate and hold experimental animals. Experiments have begun to infect cattle with BSE orally. This route of inoculation simulates the feeding of contaminated meat and bone meal that caused pandemic occurrence of BSE in the UK. An abnormal isoform of the prion protein (PrP^{Sc}) accumulates in BSE affected cattle. The purpose of this study is to examine the spread of the abnormal prion from digestive tract to the central nervous system and to describe the pathological changes in cattle during the course of infection. Atypical BSE and young BSE cases have been found in the abattoir surveillance program. As a result the cattle used in this experiment were imported from Australia, a country free of BSE, to exclude the possibility of prior BSE infection before inoculation. Each calf (Holstein heifer, 10-months old) was inoculated orally with 5g of brain stem from BSE infected cattle (courtesy provided by Veterinary Laboratory Agency, Weybridge, UK) into the rumen with a catheter. The cattle will be euthanized at intervals during the clinical stages of disease. Infectivity from different tissues at different stages of clinical disease as well as the deposition of PrP^{Sc} will be analyzed. Other factors that may be related to the pathogenesis of the BSE prion will be investigated.

The development of the intracerebral inoculation method and BSE experimental transmissions to calves

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Abstract

It is very difficult to obtain cattle infected with bovine spongiform encephalopathy (BSE) because there is no means of diagnosing BSE in live animals. This has been the obstacle of the BSE research. It is pressing need to make sure of enough cattle and bovine tissues infected with BSE for it. An intracerebral (i.c.) inoculation is the most efficient route of a prion disease transmission in each animal. However there are few reports about methods of i.c. inoculation to cattle. We developed the efficient and safe method of an i.c. inoculation to calves. In addition, we challenged BSE brain tissues to calves. It is the first BSE experimental transmission to cattle in Japan.

The inoculation site was decided using heads of carcass calves. A frontal bone was drilled by a pin-drill with a diameter of 2 mm on the spot of 1 cm nose side from the front edge of the bulge between horns and 2 cm right side from the median line. The tip of the disposable needle (70 mm, 18G) could penetrate a midbrain of calves. Four calves were injected intracerebrally with stained normal brain tissue homogenates to this site under mollification and local anesthesia. Clinical symptoms of these calves were observed for 3 or 4 hours and the distribution of the inoculated brain homogenate were investigated on the postmortem.

There were no calves presenting obvious neurological symptoms after challenges. The almost of all homogenate spread ventricles and cerebral aqueduct. Cerebrospinal fluid flowed over from the hole of inoculation site by intracranial pressure, which leads to the leakage of infectious factors and the secondary infection for calves. Using a 3 x 8 mm stainless screw has settled this problem.

Up to the present, we have challenged BSE brain tissue to 18 Holstein-Friesian calves. These calves are under observation of clinical and neurological signs, and blood and urine samples are collected for BSE biochemical studies.

Comparative analyses of three mouse-adapted scrapie strains G1, Obihiro, and I3/I5 and pathogenesis of G1 strain-induced polyuria in ICR mice

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Abstract

The causative agent of transmissible spongiform encephalopathies, prion, is thought to be composed mainly of abnormal isoform of prion protein (PrP^{Sc}). Although prion is devoid of agent-specific nucleic acid, there exist prion strains that are characterized biologically. Distribution of neuropathological lesions is one of the phenotyes of prion strains, however, there are only a few reports that addressed the linkage of the clinical manifestations to neuropathological lesions. In this study, we compared the biological, biochemical, and neuropathological differences among three mouse adapted-scrapie strains, G1, I3/I5, and Obihiro. G1 exhibited longer incubation periods (~330 days) than others in mice carrying $PrP^{A/A}$ allotype. Eighty percent of G1 strain-infected ICR mice showed severe obesity and polyuria. Diffuse deposition of PrP^{Sc} was widespread in cerebral cortex, hippocampus of I3/I5 and Obihiro strain-infected mice, while in G1 strain-infected mice, deposition of PrP^{Sc} was rather restricted in the thalamus and hypothalamus and large PrP amyloid plaques were observed in cerebral cortex. PrP^{Sc} of three strains could also be distinguished in combination of relative proteinase K resistance and glycoform patterns. Serum concentration insulin and leptin levels were remarkable high in G1-infected ICR mice with obesity, suggesting endocrinopathy and/or carbohydrate metabolism failure. PrP^{Sc}-deposition and neuronal vacuolation were observed the regions in hypothalamus including suprachiasmatic nucleus, supraoptic nucleus and paraventricular nucleus.

Kinetic analysis indicated that vasopressin-positive cells in these nuclei decreased along with the progression of the neuronal degeneration. In contrast, vasopressin-positive cells in these nuclei were detected even at the terminal stage of G1-infected C57BL/J6 mice that did not exhibit polyuria. These results suggest that G1-affected ICR mice developed hypothalamic diabetes insipidus. The particular characteristics of G1 strain would be useful for further analyzing the target cell specificity of prion and pathogenesis of prion diseases.

Dose effect on detection of PrP^{Sc} in follicular dendritic cells of knock-in mice for rapid bioassay

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Abstract

We established a rapid bioassay method for transmissibility of human prions to knock-in mouse expressing human/mouse chimeric prion protein (Ki-ChM mouse). In Ki-ChM mice, we detected accumulation of abnormal isoform (PrP^{Sc}) of prion protein in follicular dendritic cells (FDCs) in lymphoid organ within 14 days following intraperitoneal administration of human prions. Intraperitoneal administration of the inoculum provides an advantage over intracerebral injection, considering only a limited volume of 20μl can be inoculated with the intracerebral route. To overcome this limitation and to confirm dose dependent effect on PrP^{Sc} accumulation with intraperitoneal injection, we made an attempt to inoculate brain homogenate of human sporadic CJD (sCJD) with large dose and repetitive injection of a volume up to ~5000μl. Fifty micro liter of 10^{-2}, 10^{-3}, and 10^{-4} diluted sCJD were inoculated respective groups intraperitoneally. Five hundred μl of 10^{-3} and 10^{-4} dilution of sCJD inoculated with one shot were shown as equivalent infectivity to 50μl of 10^{-2} and 10^{-3} dilution of them respectively. Five hundred micro liter of 10^{-4} dilution of sCJD inoculated with once a day during 10 days were shown as an equivalent infectivity to 50μl of 10^{-2} dilution of it. These results indicate that the detection of PrP^{Sc} in FDCs has increased in proportion to the amount of prion inoculated. It is conceivable that the prion of a low density piles up the intake frequency and the pathogenicity rises as well as a high density prion.

Cell surface retention of PrP^C by anti-PrP antibody prevents protease-resistant PrP formation

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Abstract

Because of the emergence of variant CJD and accumulation of iatrogenic CJD cases, the establishment of therapeutics for prion disease is urgently needed. One possible strategy for therapeutics is an inhibition of an abnormal isoform of prion protein (PrP^{Sc}) formation in the infected host. The C-terminal portion of prion protein (PrP), corresponding to a protease-resistant core fragment of PrP^{Sc}, is essential for prion propagation. Antibodies to the C-terminal portion of PrP are known to inhibit PrP^{Sc} accumulation in prion-persistently-infected cells. Here we show that, in addition to monoclonal antibodies (mAbs) to the C-terminal portion of PrP, a mAb recognizing the octapeptide repeat region in the N-terminal portion of PrP that is dispensable for PrP^{Sc} formation, reduced PrP^{Sc} accumulation in cells persistently infected with prion; mAb 110 and 31C6, recognizing linear epitope aa 59-90 and aa 143-149, respectively, and mAbs 44B1 and 72, recognizing discontinuous epitope located within aa 155-231 and aa 89-231, respectively, inhibited the PrP^{Sc} formation when the cells were cultured with medium containing these mAbs. The 50% effective doses were as low as ~1 nM, and, regardless of their epitope specificity, the inhibitory mAbs shared the ability to bind cellular prion protein (PrP^C) expressed on the cell surface. Flow cytometric analysis revealed that mAbs which bound to the cell surface during cell culture were not internalized even after their withdrawal from the growth medium. Retention of the mAb-PrP^C complex on the cell surface was also con-

firmed by the fact that internalization was enhanced by treatment of cells with dextran sulfate. These results suggest that anti-PrP mAb antagonized PrP^{Sc} formation by interfering with the regular PrP^{C} degradation pathway.

Prion-conformation-specific human antibodies established from phage display library

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Abstract

The pathogenesis of prion disease involves a structural change of prion protein (PrP). A series of antibodies recognizing a distinct conformational change of prion is useful for not only understanding the mechanism of molecular conversion but also for diagnostic and therapeutic reagents. Prions with various conformations can be prepared in vitro under varying physicochemical condition. Antibody-displaying phage library enables us to isolate such antibodies by simple biopanning procedure. This feature is superior to conventional animal-based approach. Recently, Prusiner et al. reported that the in vitro-refolded recombinant prion could be infectious in mouse model (Science 305: 673-676, 2004).

In this study, we used human prion protein (#90-231: In Pro, #23-231: kindly provided from Dr. S. Katamine, Nagasaki Univ.) and bovine prion protein (#102-241: In Pro, #23-231, Nagasaki Univ.) and prepared two kinds of refolded recombinant prions according to the Collinge's protocol (Science 283: 1935-1937, 1999). The α or β form was defined by circular dichroism (CD) spectropolarimetry. We successfully isolated five clones specific to α form and one clone specific to β form. In the case of α-specific clones, four clones showed the binding activity to human prion equal to bovine prion while one clone bound to human prion stronger than bovine prion. The β-specific clone showed no binding activity to monomeric nor fibril amyloid β peptide. We further search for α or β-specific clones and characterize the immunochemical features using ELISA and BIAcore.

Partial characterization of monoclonal antibodies which bind to disease-associated prion protein in immunoprecipitaion assay

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Abstract

Conformational conversion of cellular prion protein (PrP^C) to scrapie-form prion protein (PrP^{Sc}) is thought to be the central event in prion pathogenesis. Several studies have shown that their distinct conformation should cause different immunogenic properties, however, almost all mAbs generated against PrP are unable to recognize each ones separately. Such characters of mAbs make it diffcult to study conformational difference between PrP^C and PrP^{Sc} or mechanism of prion replication. Therefore the purpose of this study is to generate such mAbs that would react with PrP^{Sc} but not with PrP^C.

PrP knockout mouse was immunized with purified PrP^{Sc} without proteinase K (PK) treatment and hybridomas were prepared with common procedure. Supernatants from hybridomas were screened with ELISA, which was designed to detect native PrP^{Sc} preferably from scrapie-infected mouse brain homogenate. We obtained 9 mAbs that could react PrP^{Sc} with relatively high affinity. Among these mAbs, we selected 4 mAbs that failed to react with guanidine-treated PrP^{Sc}, whose intrinsic conformation should be lost. As a result of immunoprecipitation assay, these mAbs could precipitate PrP^{Sc} from scrapie-infected mouse brain homogenate, but they brought no precipitation from normal mouse one. These mAbs were able to precipitate PrP^{Sc} in PK-treated homogenate similarly. Immunoblot and pepspot analysis of these mAbs showed no signals.

Here, we obtained the new mAbs which distinguish scrapie-infected mouse brain homogenate from normal mouse one. As these mAbs could not recognize linear sequence of PrP, it is possible that they recognize

conformational epitope. Therefore these mAbs may be valuable tools for conformational analysis of PrP^{C} and PrP^{Sc}.

Production and Characterization of Monoclonal Antibodies Specific for Prion Protein

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Abstract

Creutzfeldt-Jacob disease (CJD) is a neurodegenerative disorder caused by piron, which mainly consists of abnormal prion protein (PrP^{Sc}). As PrP^{Sc} was detected recently in two patients who received transfusion from preclinical variant CJD (vCJD), concerns about the theoretical risk of transmission of vCJD through blood products increased. In order to contribute the blood products-safety against prion, we aimed at obtaining monoclonal antibodies (mAbs) eligible for screening procedures.

By immunizing prion protein (PrP) gene ablated (Prnp-/-) or normal (Prnp+/+) mice with insoluble form of recombinant human PrP, expressed in E. coli, we obtained a panel of hybridoma. Using purified mAbs we studied their immunoreactivity against PrP^{Sc} and recombinant PrP by Western blotting or by immunohistochemistry. Epitope mapping was performed using forty 15mer-peptides representing human PrP sequence, and using fusion proteins between glutathione-S-transferase and PrP-deletion mutants.

All mAbs recognized both the recombinant PrP and the proteinase K-resistant PrP (PrP^{res}). mAb 5A1, which bound oligopeptides corresponding amino acid residues 138-157, recognized two fragmented PrP^{res} bands around 16.5 kDa. mAb 12A1, which bound oligopeptides of 158-172 residues, recognized one fragmented PrP^{res} band around 12 kDa, but poorly bound to di-glycoform of PrP^{res}. mAb 4A1, which bound deleted PrP fu-

sion proteins but not any of oligopeptides, recognized three fragmented PrP^{res} bands around 12, and 16.5 kDa.

These mAb will help to establish the screening tests for plasma or blood products as well as to investigate PrP^{Sc}. We thank Prof. T. Kitamoto, assistant Prof. T. Muramoto (Tohoku Univ.), and Prof. S. Mohri (Kyushu Univ.) for cooperation and advices.

In vitro selection of anti-mouse prion protein RNA aptamers

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Abstract

Since the in vitro selection method, which chooses nucleic acid molecules of demands from huge scale clot of randomized nucleic acid molecules, has been developed (Tuerk, et al., Science.; 249 (4968):505-10, 1990 etc.), various species of functional nucleic acid molecules have been developed. Notably, one of those molecules called "Aptamer" can specifically recognizes and binds to its target from small molecule such as amino acid to large molecules such as proteins or virus particles. As its property is almost same as antibody, aptamer has been applied in basic study of nucleic acid/protein interaction and clinical or diagnostic drugs. (Allen, et al., Virology, 209, 327-336, 1995 etc.)

Recently, screening of anti-prion RNA aptamers have been carried on, expecting for the application to the prion diseases diagnostics or therapeutics, and novel tools for studying PrP^{sc} generation mechanism. In order to apply for diagnostics, we aimed to isolate anti-mouse prion protein (anti-mPrPc) aptamer.

From artificial RNA library including 30 mer random region, we carried out in vitro selection cycles and concentrated the candidate RNA molecules. After 10 cycles of selection, we obtained three types of anti-mPrPc RNA aptamers. These aptamers have high affinity to mPrPc (Kd= 10 nM), and 2'-fluoro modification for RNase resistance did not interrupt their binding to mPrPc (Kd= 20 nM). These aptamers mainly bind to N-terminus region of mPrPc including octa-repeat (amino acid 23-230), but also bind to C-terminus region (amino acid 89-230) weakly. Fur-

thermore, these aptamers could specifically detect mPrPc in mouse brain extract on Western blotting format.

Detection of the Prion Protein in a Liquid Phase Capture Assay Using Magnetic Beads Coupled to Protein A

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Abstract

Detection of the abnormal prion protein in blood and cerebral spinal fluid of transmissible spongiform encephalophathy (TSE) infected individuals has not been possible by Western blot, immunohistochemistry or the present ELISA tests. We used an analytical approach in conjugation with a fluorescence immunoassay to develop methods to measure the abnormal prion in blood and cerebral spinal fluid. Monoclonal antibodies (mabs) and the corresponding fluorescein-labeled peptides and magnetic beads coupled to Protein A (PA) were used. The mabs, 12F10 and 3B5 reacted with the prion protein or the fluorescein-labeled peptides very rapidly in a liquid phase assay using a 0.15M TAPS buffer pH 8.8 containing 0.1% bovine serum albumin (BSA) and 0.1% N-Octyl-glucoside. The magnetic beads coupled to PA were mixed with the mab and recombinant prion protein (rPrP) or a sample and incubated for 30 min at 25 C. After washing the beads, the appropriate fluorescein labeled peptide was added and incubated for 15 min at 25 C. An aliquot of the supernatant fraction was removed and analyzed by capillary electrophoresis using laser-induced detection. Blood samples from sheep exposed to sheep scrapie were collected in 10 ml tubes containing 17.55 mg of EDTA. Buffy coats were prepared from the blood samples in the usual manner and were washed twice with 0.04 M Tris, pH 8.35 containing 0.135 M NH_4Cl. The prepared buffy coats were frozen and thawed twice, incubated with 50 μg/ml of DNase and then incubated with Proteinase K (50μg /ml). The samples were then incubated with an equal volume of hexafluoro-2-propanol (HFIP) at 56 C for 5 min. The HFIP layer was dried in a vacuum centrifuge. The sample was re-suspended and used in

temperature and pH. The optimal pH and temperature for the reaction of the antibody to the fluorescein labeled peptide was 8.8 and 25 C respectively. When a standard curve was made with the rPrP, 10 pg of rPrP could be easily detected. The blood samples correlated with the scrapie status of the sheep as was determined by postmortem examination of the brain by western blot. Further development of this assay and validation will make possible a rapid test for early detection of the TSEs.

New pretreatment method for immunohistochemistry for abnormal prion protein

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Abstract

Immunohistochemistry for prion protein (PrP) is essential for pathological diagnosis of prion diseases, however, certain pretreatment procedures are required for antigen retrieval of formalin-fixed paraffin-embedded sections. Although hydrolytic autoclaving and formic-acid treatment are usually employed, these methods tend to result in nonspecific high background staining and poorly preserved sections. Thus, we tried autoclaving of the sections with nonionic detergent as follows: autoclave sections with 0.1% Triton X-100 in 50mM Tris-HCl buffer pH7.6, 121°C 20 minutes. Both monoclonal antibody (clone 3F4) and polyclonal anti-PrP-C-terminal antibody were used for human cases with Creutzfeldt-Jakob disease and mouse models of transmissible spongiform encephalopathies. This detergent autoclaving method resulted in significantly higher signal/noise ratio for accumulated PrP in the follicular dendritic cells compared to conventional pretreatment methods. Although synaptic staining in the brain was obtained rather weakly than with conventional methods, pretreatment with detergent autoclaving contributed to lower background and well preserved sections so that we could recognize PrP deposition well. Normal-form cellular PrP was not immunostained in control cases. These results were observed for both monoclonal and polyclonal antibodies, and both human and murine materials. The lower background staining, obtained with detergent autoclaving, enabled double immunofluorescence efficiently on the same sections. We, thus, propose the detergent autoclaving method as a useful choice of the pretreatment for PrP immunohistochemistry

Cellular prion protein suppresses the apoptosis in a neuronal cell line established from type-1 prion protein gene-deficient mice

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Abstract

Previous studies have reported a neuroprotective role for cellular prion protein (PrP^C) against apoptosis induced by serum deprivation in an immortalized prion protein gene deficient neuronal cell line. These cell lines were derived from Rikn-type prion protein gene-deficient ($Prnp^{-/-}$) (type-2) mice, which produce a prion protein (PrP)-like glycoprotein named as doppel protein (Dpl). To conclude that PrP^C inhibits apoptosis without Dpl, several immortalized cell lines were established from the brain of type-1 $Prnp^{-/-}$ mice (Zrch I). The data showed that PrP^C had anti-apoptotic function in both neuronal and glial cells under serum free condition. On the other hand, the $Prnp^{-/-}$ cells expressed Dpl showed apoptosis as with $Prnp^{-/-}$ cells. The results suggest that the functions of PrP involve anti-apoptotic effect without reference to ectopic production of Dpl. Also to investigate whether PrP production effect neurite outgrowth of neuronal cell in vitro, the extension of the neurite was measured with time after passage. The PrP^c expressed cells showed longer neurite outgrowth than that of $Prnp^{-/-}$ cells. The data suggest that PrP^c affects the subsequent growth of neurite.

Cellular prion protein suppressess the apoptotic cell death by mediating the intracellular H_2O_2 in primary culture and immortalized neuronal cells

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Abstract

To know the mechanisms in which cellular isoform of prion protein (PrP^C) is involved for the neuroprotection, we compared death signals in $Prnp^{-/-}$ primary cultivated neurons to those in wild-type (WT) primary cultivated neurons. These results are also confirmed by immortarized neuronal cells. When copper was exposed to these neurons, the both of type-1 and type-2 $Prnp^{-/-}$ neurons were more sensitive and underwent apoptotic cell death more readily than WT neurons. The cell death in $Prnp^{-/-}$ neurons was effectively inhibited by anti-oxidants or some caspase-inhibitors as seen in wild-type neurons. In $Prnp^{-/-}$ neurons, copper toxicity was enhanced more significantly by deprivation of anti-oxidants, and then induced more increasing apoptotic features. To know the level of the reactive oxygen species, the level of the intracellular hydrogen peroxide (H_2O_2) were measured. Intracellular H_2O_2 in $Prnp^{-/-}$ cells decreased more rapidly than that in WT cells. These data demonstrate that in $Prnp^{-/-}$ neurons, copper may be a strong mediator to convert H_2O_2 to more toxic free radicals in a fenton-like reaction, which induces more caspase-mediated apoptosis. These results suggest that PrP^C may moderate the intracellular H_2O_2 level as a copper binding protein or an anti-oxidant to protect neuronal cells from apoptotic death.

Targeting of cytosolic PrP^C via a novel 14-3-3-Tom 70-mitochondrial BCL-2 pathway induces mitochondrial apoptosis

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Abstract

Transgenic mice harboring a high-copy-number of wild-type mouse (Mo) cellular prion protein (PrP^C) are known to develop a spontaneous neurological dysfunction in an age-dependent manner, even without inoculation of the scrapie isoform of prion protein (PrP^{Sc}). Here we first demonstrate mitochondria-mediated apoptosis in aged transgenic mice overexpressing wild-type $MoPrP^C$. These mice remained healthy, and no neuropathological abnormality was observed, however, exhibited an aberrant mitochondrial localization of PrP^C concomitant with decreased proteasome activity, while younger littermates did not. Such aberrant mitochondrial localization of PrP^C was accompanied by cytochrome c release into the cytosol, caspase-3 activation, and DNA fragmentation, most predominantly in hippocampal pyramidal cells. Next, we reconstituted these results using cell culture system. When neuronal (N2a) culture cells expressing wild-type PrP^C were used in combination with proteasome inhibitors, PrP^C targeted to mitochondria and several apoptotic phenomenon was observed. Furthermore, using the assay system, we revealed a novel mitochondrial PrP-related apoptotic pathway as follows. First, we found that PrP residues 122-139 required for the mitochondrial targeting and 14-3-3 protein worked as a transporter of PrP^C to Tom70, a mitochondrial outer membrane receptor for mitochondrial precursor proteins. Second, we show for the first time that Tom70 is involved in mitochondrial apoptosis, and thus plays a pivotal role in cell survival and death. Third, PrP^C thereafter binds BCL-2 on the mitochondrial outer membrane and subsequently induces mitochondrial apoptosis. Our data indicate that

cell survival and death signals share, at least in part, a common pathway in the mitochondrial protein transport system.

Microtubule-dependent intracellular trafficking of cellular prion protein

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Abstract

To investigate intracellular trafficking of cellular prion protein (PrP^C), we performed a real-time imaging of fluorescent PrP^C (GFP-PrP^C) in living cells. Localization of GFP-PrP^C correlated with endogenous PrP^C, however, its localization was congregated in the cytosol after the treatment with a microtubule depolymerizer, nocodazole, suggesting that the microtubule-dependent transport of PrP^C. This microtubule-associated intracellular trafficking of PrP^C exhibited an anterograde movement towards the direction of plasma membranes at a speed of 140–180 nm/s, and a retrograde movement inwardly at a speed of 1.0–1.2 μm/s. The anterograde and retrograde movements of GFP-PrP^C were blocked by a kinesin family inhibitor (AMP-PNP) and a dynein family inhibitor (vanadate), respectively. Anti-kinesin antibody (α-kinesin) blocked its anterograde motility, whereas anti-dynein antibody (α-dynein) blocked its retrograde motility. These data showed that the kinesin family-driven anterograde and the dynein-driven retrograde movements of GFP-PrP^C. Mapping of the interacting domains of PrP^C identified amino acid residues indispensable for interactions with kinesin family: NH2-terminal mouse (Mo) residues 53–91 and dynein: NH2-terminal Mo residues 23–33, respectively. Our findings argue that the discrete N-terminal amino acid residues are indispensable for the anterograde and retrograde intracellular movements of PrP^C. Furthermore, by utilizing double-labeled fluorescent cellular PrP^C, we revealed that the NH2-terminal and COOH-terminal PrP^C fragments exhibit distinct distribution patterns in mouse neuroblastoma neuro2a (N2a) cells. The double-labeled fluorescent cellular PrP^C was successfully processed in the cell and the processing was clearly inhibited by metalloprotease inhibitors, such as EDTA, and calpain inhibitor, calpastain.

Hsp90 modifies the conformation of recombinant mouse prion protein in vitro

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Abstract

In mammals, prions reproduce by recruiting the normal cellular isoform of the prion protein (PrP^{C}) and by stimulating its conversion into the pathological conformational isoform (PrP^{Sc}). Since the conversion process is thought to require unfolding of PrP^{C} to cause prion infection, to identify such activities, we have tried to construct an in vitro protein-unfolding assay using Trypsin-digestion susceptibility of E. coli-expressed recombinant mouse prion protein (recPrP). The highly purified recPrP was refolded as a soluble monomeric protein in 1 M arginine and a glutathione redox system-containing solution. We explored protein-unfolding activities of a mouse neuroblastoma cell line, Neuro-2a, which was widely used for prion infection experiments, and found that the protein unfolding activities existed in the cell extracts, particularly, in the Cytosolic fraction (100,000g sup). From the Cytosolic fraction we purified the activity to homogeneity by three steps of chromatography. The active polypeptide has a molecular weight of 90 kDa in denature condition (SDS-PAGE) and 200 kDa in native condition (Native-PAGE). The MALDI-TOF-MS/MS analysis revealed that the purified polypeptide was identical to Hsp90. The protein unfolding activity of the purified Hsp90 was inhibited by treatment of ATP-hydrolyzing enzyme, Apyrase. These results suggested that Hsp90 can modify the recombinant prion protein's conformation in the presence of ATP and might be required for conformation change of PrP^{C} to PrP^{Sc} in prion propagation.

Purification and characterization of a novel ATP-dependent robust protein-unfoldase, Unfoldin

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Abstract

We have isolated a novel ATP-dependent robust protein-unfolding activity from S. cerevisiae and designated Unfoldin. ATP, but not its hydrolysis, promoted binding of Unfoldin to substrates and unfolded their conformation. Protein sequencing revealed that Unfoldin was identical to YDL178w identified as an actin interacting protein 2 (AIP2), the function of which is poorly understood. Gel-filtration and low angle shadowing electron microscopy revealed that Unfoldin formed a homo-oligomeric complex consisting of 10~12 subunits arranged in an grapple-like structure with a ~2 nm central cavity. Removal of the C-terminal coiled-coil region of Unfoldin led to dissociation of the oligomer concomitant with the loss of both substrate binding and protein-unfolding activity. Unfoldin bound to all substrates so far examined in vitro, and modified their conformation as determined by the trypsin susceptibility assay. It is worth noting that the robust protein-unfolding activity of Unfoldin modulated the conformation of several pathogenic, highly aggregated proteins such as prion protein in β-sheet form associated with prion disease, amyloid β(1–42) peptide with Alzheimer's disease and α-synuclein with Parkinson's disease, in the presence of ATP. Protein-unfolding activity of Unfoldin depends on the growth stage of yeast and the most significant activity was observed at the log phase, suggesting the presence of a cofactor/s. From the in vivo and in vitro experimental data, Unfoldin might have important roles in a development of new treatments for the neurodegenerative disorders.

Nucleic acid and Prion protein interaction produces spherical amyloids which in vivo can function as coats of Spongiform Encephalopathy agent

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Abstract

The infectious agent of Transmissible Spongiform Encephalopathies (TSE) has been considered to be PrP^{SC}, a structural isoform of cellular prion protein PrP^{C}. PrP^{SC} can exist as oligomers and/or as amyloid polymers. Nucleic-acids induce structural conversion of recombinant prion protein PrP and PrP^{C} to PrP^{SC} form in solution and in vitro. Here we report that nucleic acids by interacting with PrP in solution produce amyloid fibril and fibres of different morphologies similar to those identified in the prion diseased brains. In addition, the same interaction produces polymer lattices and spherical amyloids of different dimensions (15-150 nm in diameters). The polymer lattices show apparent morphological similarity to the two-dimensional amyloid crystals obtained from linear amyloids isolated in vivo. The spherical amyloids structurally resemble 'spherical particles' observed in natural spongiform encephalopathy (SE) and in scrapie infected brains (TSE). We suggest that spherical amyloids, PrP^{SC}-amylospheroids, are probable constituents of the coat of the spherical particles found in vivo and the latter can act as protective coats of the SE and TSE agents in vivo.

Species barrier in yeast [PSI$^+$] prion transmission

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Abstract

The Sup35 protein of Saccharomyces cerevisiae is a eukaryotic polypeptide release factor (eRF3), which is necessary for terminating protein synthesis at stop codons. The aggregated form of Sup35, which is referred to as the [PSI$^+$] element, self-propagates and is transmitted cytoplasmically in the manner of the "protein-only" transmission of mammalian prion diseases. In [PSI$^+$] cells, most of the Sup35 is converted from a soluble, active state into an insoluble, inactive state similar to the mammalian prion amyloid. As with mammalian prions, a species barrier prevents prion transmission between yeast species. The N-terminal of Sup35 of Saccharomyces cerevisiae, necessary for [PSI$^+$], contains two species-signature elements – a Gln/Asn-rich region (residues 1-41; designated NQ) that is followed by oligopeptide repeats (designated NR).

In this study, we show that S. cerevisiae [PSI$^+$] is transmissible through plasmid shuffling and cytoplasmic transfer to heterotypic Sup35s whose NQ is replaced with the S. cerevisiae NQ. In addition to homology, the N-terminal location is essential for NQ mediated susceptibility to [PSI$^+$] transmission amongst heterotypic Sup35s. Only heterotypic Sup35s with the NQ region swapped to the S. cerevisiae sequence could effectively cross-seed in vitro fiber formation reactions.

These findings suggest that NQ discriminates self from non-self, and is sufficient to initiate [PSI$^+$] transmission irrespective of whether NR is heterotypic. NR as well as NQ alone can coalesce into existing [PSI$^+$] aggregates, showing their independent potentials to interact with the identical sequence in the [PSI$^+$] aggregate. The role of NQ and NR in [PSI$^+$] aggregation is discussed.

Molecular memory of [PSI$^+$] prion strains in S. cerevisiae is dependent on the primary structure of the Sup35 prion domain

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Abstract

How prions generate and propagate variation in both mammals and yeast is a topic of great interest and increasing evidence indicates that the tertiary or quaternary structure of prion conformations determines the strain phenotype. Whether or not the conformational information of specific prion 'strains' is transmissible to heterologous prion domains at the molecular level and whether this corresponds to a phenotypic change at the 'strain' level was investigated using the [PSI$^+$] prion model of S. cerevisiae.

The prion domain (PrD) of the yeast translation termination factor Sup35 governs a heritable conformational change, analogous to PrP^{Sc}, leading to a nonsense suppressor phenotype termed [PSI$^+$]. Since the oligopeptide repeat region of the PrD is known to influence [PSI$^+$] stability and conformational conversion efficiency, targeted mutations were constructed within this region to probe its role in stably propagating [PSI$^+$] strains. These variant Sup35s replaced wild-type Sup35 in existing weak and strong [PSI$^+$] strains and were investigated for their influence on stability, conformational conversion frequency, and nonsense suppression efficiency.

Variant Sup35s containing a glycine to tyrosine change flanking the 3' end of the oligopeptide repeats increased nonsense suppression efficiency and stability of [PSI$^+$] strains by increasing the efficiency of conformational conversion to [PSI$^+$]. Nevertheless, the structural information governing the original [PSI$^+$] strains was templated to the variant Sup35s ac-

curately and therefore the original [PSI$^+$] strains were remembered upon return to wild-type Sup35.

Prions have the remarkable capacity to generate phenotypic diversity and propagate this diversity faithfully. These results indicate that the primary structure of the PrD is a major determinant of faithful propagation of prion strains. A mechanism of 'molecular memory' to remember original [PSI$^+$] strains during transmission amongst heterologous PrDs is proposed.

Expression of a splice variant of prion protein during hypoxia in human glioblastoma cell line T98G

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Abstract

Human prion diseases occur in sporadic, genetic, and transmitted forms. It is associated with the conversion of normal cellular prion protein (PrP^{C}) into a protease-resistant isoform (PrP^{res}). We have shown previously that the human glioblastoma cell line T98G had no coding-region mutation of the prion protein gene (PRNP) which was of the 129 M/V genotype and produced a form of proteinase K-resistant prion protein (PrP) fragment following long-term culture and high passage number (Kikuchi et. al., 2004, J. Gen. Virol. 85, 3449-3457). In this study, we identified an unusual alternative splicing occurring within the exon 2, which resulted in the generation of mRNA laking a C-terminal glycosylphosphatidylinositol (GPI) anchor signal sequence. Only a low level of an alternative spliced form of PRNP was identified in T98G cells under normoxia. Under hypoxia, however, expression levels of the alternatively spliced mRNA were increased. Treatment with cobalt chloride, which mimics anoxia, also increased its expression levels in T98G cells. These results indicate that decrease in oxygen pressure may modulate the PRNP gene expression. The alternatively spliced mRNA encoded the 231 amino acids of polypeptide, which consisted of the amino-terminal portion of PrP and additional residues at its carboxy terminus.

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PRNP promoter region polymorphism in Creutzfeldt-Jakob disease

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Abstract

Prion diseases appear as sporadic, inherited or acquired by an infection[1-2]. In the inherited (familial) prion diseases over 20 point and insertional mutations were described, whereas in sporadic and acquired forms codon 129 homozygosity of the PRNP gene acts as a susceptibility factor. The etiology of sporadic CJD (sCJD) cases remains obscure. It is supposed that the expression level of prion protein gene may modulate susceptibility to sCJD. This idea comes from the results of the experiments with transgenic mice with additional Prnp copies. These mice show shorter incubation period after challenge with scrapie in comparison with that of with wild type mice. The level of prion protein expression depends on the PRNP gene regulatory elements; thus we suppose that polymorphisms in the regulatory region of the PRNP gene may be a risk factor for sCJD.

In presented study, we subjected 45 sCJD cases and 117 healthy individuals to the sequencing of the PRNP promoter region polymorphisms. The C to G transversion at position -101 was found among sCJD and control group with -101G allele frequency 0.14 and 0.16, respectively. We found that -101G polymorphism is overrepresented among sCJD 129MV heterozygous cases compared with the control group (0.53 vs. 0.27; P = 0.0452). We observed no difference in the -101G allele frequency between 129MM homozygous sCJD and control groups. We conclude that the presence of -101G allele significantly reduces the protective effect of 129MV heterozygosity. This data suggests that polymorphism in the regulatory region of PRNP gene may influence the susceptibility to sCJD.

This study is supported by EC NeuroPrion Network of Excellence.

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Tubulovesicular structures are consistently found in prion diseases including vCJD and FFI

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Abstract

Tubulovesicular structures (TVS) are the only disease-specific structures found by electron microscopy in naturally occurring and experimentally induced prion diseases or transmissible spongiform encephalopathies (TSEs)[1-2,4-5]. We reported here the largest series of brain biopsy specimens of Creutzfeldt-Jakob disease (CJD), variant CJD and fatal familial insomnia (FFI) studied by thin-section electron microscopy. Our material includes two series of brain biopsies. The first, comprising 9 biopsy specimens collected at the Laboratoire de R. Escourolle, Hopital de la Salpetriere, Paris, France were coded by one of us (J-J.H) and examined blindly. This series has been completed between 1982 and 1986 as a part of epidemiology study of CJD in France. The second series consisted of brain (right prefrontal cortex) biopsies obtained between 1995–1999 by open surgery from 1 FFI case in a new French family, 1 vCJD case, 9 cases of sporadic CJD, and 2 cases of iatrogenic (human growth hormone) CJD. TVS were found in all specimens including cases of FFI and vCJD. They appeared as spheres approximately 30 nm in diameter or short tubules of helical appearance. They were of higher electron density than synaptic vesicles; occasionally they clustered as paracrystalline arrays. Their composition is unknown but they are not composed of PrP^3. Their constant presence in all TSEs studied so far, strongly suggests thair role in pathogenesis of these diseases. This study is supported by EC Neuro-Prion Network of Excellence.

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Autophagy is a common ultrastructural feature of neuropathology of prion diseases

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Abstract

Autophagy is a degradative mechanism involved in recycling and turnover of cytoplasmic components, including organelles. Eukaryotic cells have two major protein degradation systems; one is the ubiqutin-proteasome system, the other is lysosomal mechanism. Proteins can be transported to lysosome following at least four pathways; endocytosis, macroautophagy, microautophagy and chaperone mediated-autophagy. Autophagy (macroautophagy) is mediated by an autophagosome, a double membrane organelle containing undigested proteins and damaged organelles. Autophagy is associated with several pathological conditions including neurodegenerating diseases. One of the main pathological hallmarks of neurodegeneration is loss of neurons. Apoptosis was reported in several neurodegeneration diseases to be not high enough to account for the loss of neurons so autophagy was thought to be involved in neurodegenerative processes. We studied the ultrastructural signs of autophagy in prion diseases: in brain biopsies from Creutzfeldt-Jakob disease and fatal familial insomnia patients and in experimental scrapie in hamsters. Our biopsy material consisted of brain biopsies obtained by open surgery from 1 fatal familial insomnia case, 1 case of variant Creutzfeldt-Jakob disease, 7 cases of sporadic Creutzfeldt-Jakob disease and 1 case of iatrogenic (human growth hormone) Creutzfeldt-Jakob disease as well as brains of hamsters inoculated with the 263 K or 22C-H strains of scrapie. For electron microscopy , approximately 2 cubic mm samples were immersion fixed in 2.5% glutaraldehyde for less than 24 hours, embedded in Epon and routinely processed. Grids were examined in a transmition electron micro-

scope. In many cells a large area of the cytoplasm was transformed into a collection of autophagic vacuoles of different sizes. Autophagic vacuoles formed not only in neuronal perikarya but also in neurites and synapses of all categories of studied human transmissible encephalopathies and in experimental animals. Autophagic vesicles developed also within dystrophic axons. On a basis of ultrastructural studies, we suggest that autophagy plays a major role in transmissible spongiform encephalopathies and may even participate in a formation of spongiform change.

Type 1 and type 2 human PrP^{Sc} have different aggregation sizes in methionine homozygotes with sporadic, iatrogenic and variant Creutzfeldt-Jakob disease

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Abstract

In Creutzfeldt-Jakob disease (CJD), the type (type 1 and type 2) of abnormal isoform of prion protein (PrP^{Sc}) in the brain and the genotype at codon 129 of the PrP gene are major determinants of the clinicopathological phenotype. Type 1 and type 2 PrP^{Sc} are distinguished by the size of proteinase K (PK) resistant core (21 and 19 kDa), reflecting differences in the PK-cleavage site. Moreover, type 2 PrP^{Sc} can be subclassified into type 2A and type 2B by the difference in ratio of glycoforms. However, little is known about the difference in biochemical properties between the two types of PrP^{Sc}, exept for the different PK-cleavage sites. On the basis of these findings, we hypothesized that (1) type 1 and type 2 PrP^{Sc} may have distinct aggregation sizes and (2) PrP^{Sc} from a patient with PrP amyloid plaques in the brain has a larger aggregation size than PrP^{Sc} from a patient with synaptic non-amyloid type PrP deposits in the brain.

To clarify the difference in aggregation size, we filtered brain homogenates from various cases of CJD with the same genotype (homozygous for methionine at codon 129; type 1, type 2A, type 2B and both type 1 and

type 2 PrP^{Sc} coexisting; with or without amyloid plaques) through virus removal filters of mean pore size 72 ± 4 nm.

Type 2 PrP^{Sc} was efficiently removed from the filtrates by the filters in contrast to type 1. Even type 2 PrP^{Sc} from a patient without amyloid plaques was removed more efficiently than type 1 from patients with amyloid plaques. In the brain samples both types of PrP^{Sc} coexisting, type 2 was removed by filtration more efficiently than type 1 under the same conditions.

These results suggest that type 1 PrP^{Sc} aggregates are generally of small size, while type 2 PrP^{Sc} aggregates are all of a larger size and do not contain a fraction of small-sized aggregates. The present study indicate that type 2 PrP^{Sc} has a larger aggregation size than type 1, irrespective of the existence of amyloid plaques.

A pitfall in diagnosis of human prion diseases using detection of protease-resistant prion protein in urine: contamination with bacterial outer membrane proteins

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Abstract

To evaluate diagnostic usefulness and reliability of the detection of protease-resistant prion protein in urine, we extensively analyzed proteinase K (PK)-resistant protein in patients affected with prion diseases and the control subjects by Western blot, a coupled liquid chromatography and mass spectrometry analysis, and N-terminal sequence analysis. The PK-resistant signal migrating around 32 kDa previously reported by Shaked et al. was not observed in this study. Instead, discrete protein bands with an apparent molecular mass of approximately 37 kDa were detected in the urine of many patients affected with prion diseases and two diseased controls. Although these proteins also gave strong signals in the Western blot using a variety of anti-PrP antibodies as a primary antibody, we found that the signals were still detectable by the incubation of secondary antibodies alone, i.e., in the absence of the primary anti-PrP antibodies. Mass spectrometry and N-terminal protein sequencing analysis revealed that majority of the PK-resistant 37 kDa proteins in patients' urine

were outer membrane proteins (OMPs) of Enterobacterial species. OMPs isolated from these bacteria were resistant to PK and the PK-resistant OMPs from Enterobacterial species migrated around 37 kDa on SDS-PAGE. Furthermore, non-specific binding of OMPs to antibodies could be mistaken for PrP^{Sc}. These findings caution that bacterial contamination can affect the immunological detection of prion protein. Therefore, the presence of Enterobacterial species should be excluded in the immunological tests for PrP^{Sc} in clinical samples, in particular, urine.

Comparative analysis of chronological data of total tau protein in CSF, serial studies of MRI (DWI, and FLAIR) and staging of clinical features in patients with sporadic CJD

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Abstract

Total tau protein (t-tau) in CSF is now considered as a diagnostic biochemical marker of CJD. Especially, analysis of t-tau protein level in CSF is semi-quantitative, compared to western blot analysis of 14-3-3 protein in CSF. Also, MRI is diagnostic procedure of CJD. Diffusion-weighted MRI (DWI) is more diagnostic in early phase of CJD.

We report here the comparative analysis of t-tau protein and MR images, which have been serially performed in the clinical course of sporadic CJD patients.

Materials and Methods

Five patients (four males and one female) were diagnosed as "classical" CJD by clinical features, radiological studies including MRI, biochemical markers (14-3-3 protein and t-tau protein) in CSF and gene analysis of prion protein gene. The age of onset ranged 64-69 year-old. These patients were admitted in Nagasaki University Hospital, and were performed serial studies of lumbar puncture to obtain CSF and MRI, under the informed consent and agreement with patients and/or family member according to the permission of Ethical committee of Nagasaki University Hospital. T-tau protein, 14-3-3 protein, S-100B protein and NSE were serially measured. Also monthly MRI studies were performed. These data were compared to clinical staging to evaluate chronological progression of disease by biochemical and radiological markers.

Results

Chronological analysis of t-tau protein in CSF showed peaks at the middle stage of clinical course in all cases. T-tau protein level at first lumbar puncture ranges 2655±423.9pg/ml. Peak levels were observed around 10-16 weeks after the onset (14675±1240 pg/ml). After this peak, titers of t-tau decreased. At the last analysis of CSF, titer of t-tau was 8786±2975pg/ml. The other marker including S-100 B and NSE showed resemble time course. Comparison of these biochemical markers and serial study of MRI may contribute to staging of sporadic CJD. These data were also compared to clinical staging. So t-tau protein of CSF would have the possibility as some kind of hallmarks during the clinical time course.

Duration between initial manifestation of CJD and detection of PSD, specific findings on MRI, CSF 14-3-3 protein, or CSF high NSE

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Abstract

To evaluate the usefulness of clinical examinations in the diagnosis of CJD, we examined duration between initial manifestation of CJD and detection of PSD, specific findings on MRI, 14-3-3 protein, or high NSE. We analyzed data collected by Japanese CJD surveillance committee statistically. Patients consisted of 405 sporadic CJD and 52 infectious CJD. Average duration between the onset and detection of PSD, specific findings on MRI, 14-3-3 protein in the CSF, or high NSE in the CSF in the total cases was 3.6, 4.4, 4.8, or 3.6 months, respectively. There was no significant difference among 4 examinations (P=0.12). Peak of duration until positive result on MRI, 14-3-3 protein, or NSE was 1 month while that on EEG was 2 months. Similar result was obtained when we analyzed the subgroup of sporadic CJD. The sensitivity of EEG, MRI, 14-3-3 protein, or NSE in the definite cases was 72.4, 77.1, 74.2, 50.0 %, and the performed rate in the total cases was 99.1, 75.9, 36.5, 44.0 %, respectively as we showed in the previous report partially. The sensitivity and performed rate of EEG were high and duration until positive result was short while the number of detection of PSD reached its peak 2 months after the onset in contrast with 1 month as in the other examinations. Conventionality in diagnosis of CJD and inclusion in the criteria fot CJD might influence on high performed rate and short average duration. Although some researches reported very early detection of specific findings

on MRI, the average duration until the detection of specific MRI findings was not significantly shorter than those on the other examinations in Japanese CJD surveillance. This might be due to lower recognition rate of usefulness of DWI in diagnosis of CJD. The sensitivity of NSE in the definite cases was lowest although the difference of the sensitivity was not significant among 4 examination ($p=0.063$). Low performed rate of 14-3-3 protein or NSE was remarkable. In conclusion, analysis of data from Japanese CJD surveillance revealed no significant difference of duration between the onset and detection of positive results among 4 examinations. Further spread of recognition of usefulness of DWI in the diagnosis of CJD might shorten duration between the onset and detection of positive result on MRI.

Biochemical analysis of peripheral tissue involvement in transmissible spongiform encephalopathies

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Abstract

Transmissible spongiform encephalopathies (TSE) are transmissible, neurodegenerative disorders affecting humans and animals. Although the etiological agent of TSE has yet to be conclusively identified, a unifying feature is the accumulation of an abnormal protease-resistant isomer (PrP^{Sc}) of the host encoded prion protein (PrP^{C}). PrP^{C} expression is required for the transmission and pathogenesis of TSE disease. Despite the ubiquitous organ distribution of PrP^{C}, TSE pathogenesis and infectivity is principally restricted to the central nervous and lymphoreticular systems. However, recent studies have highlighted the presence of low levels of PrP^{Sc} and TSE infectivity in some peripheral tissues including muscle and the possible transmission of variant Creutzfeldt Jakob disease through blood transfusion. This has raised concerns regarding the risk of iatrogenic transmission of TSE disease through surgical procedures and organ or blood donation.

Several assays have been developed to study the conformational change that accompanies the conversion of PrP^{C} to PrP^{Sc}. These include a protein misfolding cyclic amplification assay, which mimics the conversion process in vitro. We have used a modified version of this assay to investigate the susceptibility of PrP^{C} expressed in the peripheral tissue of inbred mice to conversion to a protease resistant form, designated PrP^{res}, in the presence of a seed of PrP^{Sc} derived from a mouse adapted model of a human TSE. This study identifies tissues that have the potential to act as reservoirs of infectivity and identifies routes of potential peripheral transmission of TSE disease.

Involvement of the peripheral nervous system in human prion diseases including dural graft-associated Creutzfeldt-Jakob disease

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Abstract

Objective

To investigate abnormal prion protein (PrP) deposition in the peripheral nervous system (PNS) in human prion diseases.

Methods

We examined eight patients with prion diseases; patients included three with sporadic Creutzfeldt-Jakob disease (sCJD), two with dural graft-associated CJD (dCJD), one with Gerstmann-Sträussler-Scheinker disease (GSS) with a PrP P102L mutation (GSS102), and two with a P105L mutation (GSS105). An atypical case of sCJD with PrP plaques in the brain presented clinically with peripheral neuropathy. The PNS was investigated by immunohistochemical and western blotting analyses of PrP.

Results

In immunohistochemical studies, granular PrP deposits were detected in some neurons of dorsal root ganglia (DRG) and a few fibers of peripheral nerves and of spinal posterior roots in one sCJD and two dCJD patients, but not in GSS102 or GSS105 patients. The atypical case of sCJD with

peripheral neuropathy showed no obvious PrP deposition in the nerves. Western blotting analysis of the PNS from the dCJD patients revealed a small amount of protease K-resistant PrP in the DRG and the peripheral nerves.

Conclusions

Our results indicate that abnormal PrP deposition occurs in the DRG and peripheral nerves in sCJD and dCJD. This is the first report that western blot analysis disclosed protease K-resistant PrP in the DRG and the peripheral nerves from dCJD. Our results also imply that the PrP deposits in the PNS are not correlated with clinical manifestation of peripheral neuropathy in CJD.

MM2 type sporadic Creutzfeldt-Jakob disease: clinicoradiologic features and clinical diagnosis

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Abstract

Objective

To identify diagnostic markers for MM2 type sporadic Creutzfeldt-Jakob disease (sCJD).

Methods

Investigations of neuropsychiatric manifestations, cerebrospinal fluid (CSF), electroencephalography (EEG), and neuroimaging studies were performed in eight patients with MM2 type sCJD confirmed by neuropathologic, genetic, and Western blot analyses.

Results

The eight patients were pathologically classified into the cortical (n=2), thalamic (n=5), and combined (cortico-thalamic) forms (n=1). The cortical form was characterized by late onset, slowly progressive dementia, cortical hyperintensity signals on diffusion-weighted imaging (DWI) of brain magnetic resonance imaging (MRI), and elevated levels of CSF 14-3-3 protein. The thalamic form showed various neurologic manifestations including dementia, ataxia, and pyramidal and extrapyramidal signs with onset at various ages, and relatively long disease duration. Characteristic EEG and MRI abnormalities were almost absent. However, all four patients examined with cerebral blood flow (CBF) study using single-photon emission computed tomography (CBF-SPECT) showed reduction of CBF in the thalamus as well as the cerebral cortex. The combined form had features of both the cortical and thalamic forms, showing cortical hyperintensity signals on DWI of MRI and hypometabolism of the thalamus on 18F-2-fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET).

Conclusion

MM2 type sCJD patients present with the characteristic clinicoradiologic features; for the clinical diagnosis of MM2 sCJD, cortical hyperintensity signals on DWI of MRI would be characteristic features for cortical forms,

and thalamic hypoperfusion or hypometabolism on CBF-SPECT or FDG-PET for thalamic forms.

Early clinical and radiological diagnosis of sporadic Creuzfeldt-Jakob's disease (sCJD) – a case study of pathologically-proved sCJD MV2 –

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Abstract

Background

Early clinical and radiological features of sCJD with long clinical illness have not been clarified yet.

Subject

An exceptioal case of pathlogically-proved sCJD MV2 exhibiting severe status spongiosus in the entire cerebral cortex and kuru-plaque with only mild degeneration in the thalami and basal ganglia. Total clinical illness was about four years.

Method

We compared clinical and radiological features chronologically.

Results

At the initial radiological study, about half a year after the initial symptom (forgetfulness), diffusion weighted magnetic resonance images (DW-MRI) revealed abnormal high signal along the occipito-parietal lobe cortex with

left-side predominancy. Single photon emission computed tomography (SPECT) images indicated fronto-temporal lobe dysfunction predominant at the left side. Clinical features of the patient were attention deficit, frontal lobe dysfunction and semantic memory impairment, which correlated well to the SPECT findings rather than DW-MRI. Assay of 14-3-3 protein was negative at this stage. Half a year after the initial study, assay of 14-3-3 protein was positive. The patient showed myoclonus in the right upper limb, but electric encephalography did no show periodic sharp wave complexes. At the later stage, DW-MRI showed abnormal high signals along the entire cerebral cortex with the spared thalami and basal ganglia. SPECT images showed almost symmetrical hypoperfusion in the fronto-temporo-parietal cortex. Bilateral thalami or basal ganglia did not show hypoperfusion. The patient died after the clinical illness of about four years. Time interval from the initial symptom to the stage of akinetic mutism was almost three and half a year.

Conclusion

In our patient, DW-MRI was useful in the diagnosis of sCJD at the early stage, but clinical-radiological relationship was evident in the SPECT images. Both DW-MRI and SPECT images correlated well to the pathological findings.

Familial Creutzfeldt-Jakob disease with a point mutation (Met to Arg) at codon 232: two different phenotypes

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Abstract

Objectives

10 to 15% of Creutzfeldt-Jakob disease (CJD) cases are estimated to be familial. CJD with a causative point mutation of methionine to arginine at codon 232 (M232R) is a type of familial CJD (CJD232). We report here two cases of CJD232 with different clinical features.

Case report

Patient 1

A 55-year-old woman who had no family history of dementia developed insomnia followed by somnolence, intellectual deterioration, unsteady gait, psychiatric symptoms and myoclonus. Two months after the onset, she was admitted to our hospital because of severe dementia. EEG showed periodic sharp and wave complexes (PSWC) and diffusion-weighted MRI (DWI) demonstrated a high intensity lesion in the bilateral striatum. 14-3-3 protein immunoassay in CSF was positive. Prion protein gene (PRNP) analysis revealed M232R and methionine homozygosity at codon 129 (MM129). Three months after the onset she became akinetic and mute.

Case 2

A 70-year-old woman who had no family history of dementia developed memory disturbance followed by paranoia and insomnia. Four months after the onset she was admitted to our hospital because of progressing dementia. EEG showed no PSWC and DWI demonstrated a high intensity lesion in the wide range of bilateral cerebral cortex, thalamus, and striatum. 14-3-3 protein immunoassay was positive. PRNP analysis revealed M232R and MM129. Nine monthes after the onset, she was in a state of severe dementia but did not become akinetic and mute. EEG showed no PSWC.

Discussion

Many cases of familial CJD caused by PRNP abnormalities have been reported and some of the clinical features are different from those of sporadic CJD. However, the clinical features of familial CJD are regulated by the genotype of PRNP. The clinical features of our patients were quite different from each other; one showed rapidly progressing dementia, generalized myoclonus, and PSWC in EEG, whereas the other showed relatively slowly progressing dementia, no myoclonus, and no PSWC. However, the genotype of PRNP was the same including the polymorphism of codon 129. To date, nine rapid patients and six slowly patients with the same genotype have been recognized. There are two phenotypes in familial CJD with M232R and some factors apart from the genotype influence the phenotype.

Familial Creutzfeldt-Jakob disease with five octapeptide repeat insert

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Abstract

A pedigree of familial CJD with five octapeptide repeat insert (5ORI) is reported. In this family seven out of nine members from one generation had dementia. Three showed slowly progressive course, two with typical clinical features of CJD, and two without details. The probaband is a 62 year old man, who initially presented with abnormal behavior at age 52. The symptoms gradually deteriorated into a bed-ridden state with no verbal expression at age 59. There was no 14-3-3- protein in CSF or PSD in EEG. MRI showed cerebral atrophy with T2 high intensity of the white matter. At age 62, he still responds to a call for his name. The proband's oldest brother showed similar clinical features, starting difficulty in word recall around age 64, followed by progressive mental deterioration but was ambulatory even just prior to death. He died unexpectedly and no autopsy was done. The other two brothers showed typical clinical features of CJD, with onset at age 41 and 51, respectively. Their clinical course was about one year, accompanying myoclonus, PSD and akinetic mutism. The brain weight was 1,320g and 1,260g respectively. In the

cerebral cortex, severe neuronal loss and gliosis with spongiformic changes were present. Hippocampus was relatively spared. The cerebellum showed severe depletion of granular cells. Immunocytochemically the deposition of prion protein was of synaptic pattern in the cerebral cortex and confluent granular one in the cerebellar cortex. In addition, degeneration of the cerebral white matter was diffuse in one case but focal in another. In the latter case, 5ORI was detected. So far, the results of the sequence analysis differ in two other reported families with 5ORI. A current family may provide a direct evidence that two distinct phenotypes of rapidly or slowly progressive clinical course may arise from the same 5ORI sequence abnormality.

A case of Creutzfeldt-Jakob disease with a novel insertion mutation and codon 219 Lysine/Lysine polymorphism in the prion protein gene

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Abstract

Introduction

A prion disease patient with codon 219 Lysine (Lys) homozygote has not been reported. We present a patient with Creutzfeldt-Jakob disease (CJD) carrying two outstanding genetic features in the prion protein gene (PRNP): a novel mutation of three-time octapeptide insertion and a codon 219 Lys/Lys polymorphism.

Patient report

A 68-year-old Japanese man developed gait disturbance and vertigo. Three months after the onset, cognitive functions were severely impaired with diffuse hyperreflexia, rigidity, akinesia and parkinsonian gait. The levels of 14-3-3 and tau proteins in cerebrospinal fluid (CSF) were normal. EEG showed periodic synchronous discharges with generalized slowing. MRI on diffusion weighted sequences revealed widespread hyperintensities in the cerebral cortices and striatum. He became apallic 13 months after the onset. He died of pneumonia three years after the onset. There was no myoclonus throughout his clinical course. In PRNP, there was a 72-basepair insertion between codons 51 and 91, consisting of a single repeat of 24 nucleotides (R3g) and double repeats of another normal 24 nucleotides (R2). The R3g had an A-to-G transition at the last position of

the seventh codon, which did not lead to any change of amino acid. The genotypes of codon 129 polymorphisms were methionine (Met)/Met, and those of codon 219 were Lys/Lys.

Discussion

Our patient has a three-time octapeptide insertion in PRNP. Most patients with extra insertion of four-time repeats or less reported to show the typical clinical course of sporadic CJD. The slow progression without myoclonus of our patient and the normal 14-3-3 protein level in CSF are unusual as sporadic CJD. It suggests that codon 219 Lys allele may play a protective role for the disease progression, though the protective effect of codon 219 Lys homozygote on familial CJD may not be perfect.

Conclusion

Codon 219 polymorphism may have protective effect on disease progression in CJD.

Inhibition of Prion Propagation in Scrapie-infected Cell Lines using Mouse Monoclonal Antibodies against Prion Protein

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Abstract

Introduction

Monoclonal antibodies (mAbs) against prion protein (PrP) are useful for diagnosis of prion diseases as well as for research into the conformational change from the cellular isoform (PrP^{C}) to the abnormal isoform (PrP^{Sc}). Recent reports indicate that some PrP-specific mAbs are able to inhibit prion propagation and exclude PrP^{Sc} both in vitro and in vivo. However, no effective therapies against prion diseases currently exist and the mechanisms of prevention by PrP-specific antibodies are uncertain. Therefore, production and identification of PrP-specific mAbs for analysis of the PrP conformational change are important. In this study, we attempted to select mouse mAbs that inhibit PrP conversion and present here two such inhibitory mAbs.

Materials and Methods

In this study, five mouse mAbs specific for PrP generated by cell fusion using $PrP^{0/0}$ mice immunized with recombinant PrP were used and their specificity was characterized by ELISA and Western blotting analyses.

To identify mAbs that inhibit conversion from PrP^{C} to PrP^{Sc}, an inhibition assay of prion propagation was performed using two scrapie-infected neuroblastoma cell lines, N2a/22L and N2a/Chandler. Cells treated with 10 μg/ml of mAbs for 4 to 16 days were further cultured in the absence of mAbs for 4 to 16 days. After culture, PrP^{Sc} in the cells was analyzed by Western blotting.

Results and Discussion

Of the five mAbs used, 3S9 and 22L/2H9 (2H9), which recognize both PrP^{C} and PrP^{Sc}, were identified as inhibitory mAbs. 3S9 recognized mouse and sheep PrPs, while the 2H9 recognized mouse and hamster PrPs. Both mAbs inhibited the conversion of PrP^{C} to PrP^{Sc} in the two prion-infected cell lines used. These mAbs were also able to exclude PrP^{Sc} in these cells and inhibited accumulation of PrP^{Sc} in PrP^{Sc}-expressing cells. Interestingly, these mAbs recognized different PrP epitopes; 3S9 recognized residues 141-161, which includes the helix 1 region of PrP, while 2H9 recognized residues 151-221. These results indicate that different site(s) from the helix 1 region may be also important for the PrP conformational change and that the two mAbs selected may be useful for analyzing the prevention mechanisms of prion propagation.

The inhibitory effect of the ScFv of an anti-prion protein antibody secreted from N2a58 cells on abnormal prion protein accumulation in scrapie-infected cells, ScN2a

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Abstract

The central event in molecular prion pathogenesis is the conformational change of the cellular prion protein (PrP^{C}) to an abnormal prion protein (PrP^{Sc}), and the subsequent accumulation of PrP^{Sc} in infected human and animals. Recently, reports have shown that the exposure of scrapie-infected cells (ScN2a) to an anti-prion protein (PrP) antibody inhibited this conformational change and accumulation of PrP^{Sc}. This immunological approach has some problems for in vivo applications because of the difficulty of the blood brain barrier and the resulting lack of accessibility of the antibody to brain tissue. We developed an alternative intervention system using gene expression. In this study, we demonstrated that PrP^{Sc} accumulation in ScN2a cells could be prevented by this expression system in vitro. We cloned a cDNA of the single chain antibody variable region fragment (ScFv) of an anti-PrP monoclonal antibody T2, and transfected it into a mouse neuroblastoma cell, N2a58 (N2a58-T2ScFv). The T2-ScFv was expressed stably in the N2a58-T2ScFv cells and gave no effects for the PrP^{C} expression. The cells were co-cultivated with ScN2a cells for 4days or 7days, and the quantity of PrP^{Sc} was assayed by western blotting and enzyme-linked immunosorbent assay (ELISA). PrP^{Sc} in ScN2a cells reduced significantly with co-cultivation. This suggests that insertion of a PrP-specific antibody gene into a neuronal cell will be a potential therapeutic tool for prion diseases.

Mucosal immunogenicity of prion protein fused with heat-labile enterotoxin B subunit

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Abstract

The new variant of human Creutzfeldt-Jakob disease is transmitted orally via food contaminated with prions of bovine spongiform encephalopathy from cattle, a fact that demands the development of vaccines, in particular those enhancing mucosal immunity, to prevent the transmission of prions between species. Prions are thought to consist mainly of the proteinase K-resistant isoform of prion protein (PrP) encoded on the host genome. There have been reports showing that the mucosal immunogenicity of a peptide could be enhanced by fusion with the B-subunit of E. coli heat-labile enterotoxin (LTB) or cholera toxin. In the present study, to determine whether PrP can be also immunogenically enhanced by fusion with LTB, we compared the mucosal immunogenicity of LTB-fused mouse (mo) and bovine (bo) PrP (LTB-mo or boPrP) with that of non-fused mo and boPrP by intranasally immunizing C57BL/6 and Balb/c mice three times every two weeks in the presence of recombinant LT as an adjuvant. In the fusion protein, the C-terminal half of mo and boPrP was linked to the C-terminus of LTB by hinge sequence of Gly-Pro-Gly-Pro. The biochemical properties of these fusion proteins were similar to those of LTB, including pentameric protein structure and GM1 ganglioside-binding competence. Immune sera were collected one week after the final immunization and subjected to ELISA to detect the PrP-specific antibodies. MoPrP induced only a weak immune response in both mouse lines. No immunogenic enhancement could be detected in the mice immunized with LTB-moPrP. This poor immune response against moPrP and LTB-moPrP is attributable to the self-tolerance of mice to self-antigen. In contrast, boPrP was highly immunogenic in Balb/c mice

but not in C57BL/6 mice. This indicates that the antibody response against PrP can differ widely in individuals. However, the fusion with LTB markedly enhanced immonogenicity of boPrP in both mouse lines, producing more than 100-fold IgG and IgA capable of cross-reacting with human and sheep PrPs but not with mo or hamster PrPs. This striking enhancement of the mucosal immunogenicity of boPrP in mice by fusion with LTB points to LTB-fused PrPs as a possible mucosal vaccine to elicit antibodies prophylactic for the oral transmission of prions from one species to another.

Identification of drugs that enhance the stimulatory effect of PrP on the fibrinolytic system

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Abstract

Introduction

Both, the disease-associated isoform of the prion protein (PrP^{Sc}), and the cellular form (PrP^{c}) bind to plasminogen. PrP^{c} and the NH_2-terminal part spanning amino acids 23-110 (PrP23-110) can stimulate tissue-type plasminogen activator (t-PA) mediated plasmin generation. We have shown that the lysine dependent binding of PrP23-110 to the kringle 2-domain of t-PA is essential for this activation. We have also shown that the stimulatory activity of PrP23-110 can be enhanced by proteoglycans, which alter the self-binding of the PrP-fragment. The goal of our current study was to find additional drugs exhibiting the same effect.

Methods

Plasminogen activation was measured in a chromogenic assay in vitro and binding studies were carried out using surface plasmon resonance technology.

Results

We measured the t-PA mediated plasminogen activation by PrP and the selfbinding of PrP23-110 in the presence of different potential anti-prion drugs. The strongest stimulatory effect was observed for Pentosan polysulfate which has already been used as an experimental drug for the treatment of vCJD.

sulfate which has already been used as an experimental drug for the treatment of vCJD.

Conclusions

The specific stimulation of t-PA mediated plasminogen activation by PrP suggests a biological function of PrP as a cofactor in the regulation of plasmin activity in the central nervous system. Stimulation of the fibrinolytic system is a possible new approach for the therapy of prion-diseases. The in-vitro plasminogen activation assay can be used to screen for compounds exhibiting such an effect.

This work was supported by the German Ministry of Education and Research (BMBF).

Screening study of prion binding agents and their inhibitory effect on the conversion of prion protein

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Abstract

Prion is a host-encoded protein and folds into at least two conformations, physiological PrP^{C} and pathogenic PrP^{Sc}. PrP^{Sc} mediates the conversion of PrP^{C} into PrP^{Sc}. Since prion-binding agents are expected to show inhibitory or stimulating effects on the conversion of prion protein, the binding compounds are promising candidates for the anti-prion disease agents. We established an ELISA assay system using the prion peptide PrP(106-126) and an anti-prion monoclonal antibody 3F4 to detect the prion-binding activity, and tested the activity of the extracts of medical plants and microbial culture broth samples. Some of the samples that showed prion-binding activity in this ELISA assay were advanced to the next assay to detect the inhibition on conversion of PrP^{C} into PrP^{Sc} using ScN2a cells. Finally we found that some compounds derived from chlorophylls showed clear inhibition for the prion protein conversion. The mechanism of inhibition by these chemicals may stand on the binding to the 3F4 epitope area of prion protein and interrupting the PrP^{C}-PrP^{Sc} interaction. The safety of these chemicals are so sufficient, since they have already used as food additives (coloring agents). Furthermore, one of, them, sodium copper chlorophyllin, has managed as a drug to cure bad breath. They are expected to be good lead compounds to develop the new anti-prion drugs.

Can Forage Grasses inhibit Prion Replication ?

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Abstract

Prion diseases are a group of fatal neurodegenerative disorders, and thought to be caused by the accumulation of an infectious protease-resistant isoform of prion protein (PrP^{Sc}). Mysteriously, the cattle exposed to infectious feed do not necessarily catch bovine spongiform encephalopathy (BSE). For one reason, we assumed that some constituents in forage grasses inhibit the infection of BSE. We therefore investigated whether some forage grasses show inhibitory effects on the formation of PrP^{Sc}.

We collected six common forage grasses, Italian ryegrass (Lolium multiflorum), Eastern gamagrass (Tripsacum dactyloides), Reed canarygrass (Phalaris arundinacea), Kikuyugrass (Pennisetum dandestinum), Bahia grass (Paspalum notatum), and Bermuda grass (Cynodon dactylon), and then prepared their EtOH extract.

First, we examined whether each extract show the affinity for endogenous cellular prion protein (PrP^{c}) with the competitive enzyme-linked immunosorbent assay (ELISA). In consequence, Italian ryegrass extract showed the potent affinity for PrP^{c}.

We then investigated with prion-infected neuroblastoma cells (ScN2a and F3) whether Italian ryegrass extract inhibits the formation of PrP^{Sc}. In a preliminary examination, we found that EtOH extract of Italian ryegrass inhibited the PrP^{Sc} formation in a dose-dependent manner. These findings suggest that some constituents in forage grasses might be useful for the prevention of prion diseases.

Inhibition of abnormal PrP formation by amyloid-imaging probes in vitro

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Abstract

Amyloid deposition in the brain is assumed to correlate with pathogenesis of human neurodegenerative disorders, including transmissible spongiform encephalopathies (TSE). To date, many researchers have devoted themselves to early detection of pathological amyloids, and PET neuroimagings of beta-amyloid aggregates in Alzheimer's disease have been developed to be under clinical trials.

Two probes, 2-(1-{6-[(2-fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene)malononitrile (FDDNP) and 6-iodo-2-(4'-dimethylamino-)phenyl-imidazo[1,2-a]pyridine (IMPY) are currently representative candidates for amyloid imaging (Ref. 1 and 2). In this study, both compounds were investigated for application to TSE treatment, since we have already confirmed some imaging probes had anti-scrapie activities in vitro and in vivo (Ref. 3).

Using mouse neuroblastoma cells persistently infected with RML prion (ScN2a), we examined the therapeutic ability of FDDNP and IMPY dissolved in culture medium. Doses needed for 50% inhibition of PrPres formation by these compounds were in the nanomolar order, and therapeutic dose windows were found wide. Moreover, expression of normal PrP was not interfered in non-infected cells.

Considering FDDNP and IMPY showed high binding affinity towards beta-amyloids, and the former fluorescently labeled PrP plaques of pathological sections in the previous reports (Ref. 1, 2 and 4), this study suggests that both were potent by binding directly to PrP-amyloid molecules. So far, one of the most promising strategies for TSE treatment is a small-molecule drug that can enter the brain and inhibit abnormal PrP

formation. Both FDDNP and IMPY could lead to new approaches to prophylactic and/or therapeutic drugs for TSE.

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Treatment with Anti-malaria Agents, Quinacrine and Quinine, for Creutzfeldt - Jakob disease patients

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Abstract

Objects

To assess effects and safety of treatment with anti-malaria agents as treatment for Creutzfeldt-Jakob disease (CJD).

Background

There have been no effective treatments for CJD. Several chemicals inhibit an accumulation or a conformational change of prion protein in vitro. Anti-malaria agents such as quinacrine and quinine are known to have anti-prion effects.

Materials and Methods

The study was approved by the institutional ethics committee, and patients' relatives gave consent for the procedure. Thirty-one cases were treated with quinacrine including 22 with sporadic CJD, 5 cases with iatrogenic CJD, and 4 cases with familial CJD. Quinacrine 300 mg/day was administered enterally for three months. Six cases with sporadic CJD treated with quinine. Quinine 1.5 g/day was administrated enterally for three months. Motor and cognitive functions were monitored.

Results

Anti-malaria agents were well tolerated except for abnormal liver function tests, dermatitis or thrombocytopenia. Increased arousal levels and responses to visual and auditory stimulation were seen in 12 patients with quinacrine treatment. Out of 22 sporadic CJD, only one case improved from akinetic mutism. The other 8 patients, who had positive responses as eye contact to verbal and/or visual stimuli before treatment, showed clinical improvement. Out of 5 patients treated with quinine, two cases showed improved response to auditory and visual stimuli. Clinical improvement was transient and lasted for one to two months during treatment.

Conclusion

This is the first observation of moderate reversibility of cognitive function in a large series of patients with CJD treated with anti-malaria agents. Liver dysfunction is the major complication and sometimes requires discontinuing this medication. It is also suggested that quinacrine/quinine could be the treatment of choice for patients with CJD. However, these agents are not able to prevent the clinical declines or to improve these features. Optimal treatment regimen and further clinical trial will be required.

Effect of oral administration of pentosan polysulfate for patients with Creutzfeldt-Jakob disease (CJD) and new design for low molecular-weight of pentosan polysulfate

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Abstract

Although numbers of promising agents to control abnormal prion protein in vitro or in vivo, none of the sufficiently effective and safe agent has been discovered for patients with Creutzfeldt-Jakob disease (CJD). Quinacrine and pentosan polysulfate (PPS) have been reported as possible useful agents for treatment of CJD. We administrated PPS oral treatment for eight patients with CJD, then evaluated clinical effects of oral PPS. Oral PPS were not effective in seven cases except "response to person" and "frequency of myoclonus" in some patients. One patient was evaluated to be effective in "30m walking time" and in improvement of "preservation of words". In this case, these effects were transient.

So, more sufficient delivery system of PPS into brain is needed. Also, intraventricular administration of PPS therapies in animal model and human have been reported. But, it would be difficult to perform these operations for all CJD patients, because patients need to have surgical procedures. So we designed to produce the low molecular-weight of pentosan polysulfate (LMW-PPS), because we assumed that LMW-PPS could be pass through blood-brain-barrier (BBB). We collected some

fractions of LMW-PPS using gel chromatography column and membrane dialysis. Then we checked some fractions of LMW-PPS used by PrPSc-infected neuroblastoma cells to screen for inhibition of nascent PrPSc as well as the clearance of pre-existing PrPSc. We obtained useful fraction of LMW-PPS to be able to decrease the PrPSc. We also found that some of these fractions were able to penetrate in vitro BBB model. We hoped to be able to use this LMW-PPS for treatment of CJD patients.

Key Word Index