Transmissions of variant Creutzfeldt–Jakob disease from brain and lymphoreticular tissue show uniform and conserved bovine spongiform encephalopathy-related phenotypic properties on primary and secondary passage in wild-type mice

Diane L. Ritchie,1 Aileen Boyle,2 Irene McConnell,2 Mark W. Head,1 James W. Ironside1 and Moira E. Bruce2

1National CJD Surveillance Unit, School of Molecular and Clinical Medicine (Pathology), University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK
2Neuropathogenesis Division, The Roslin Institute and Royal (Dick) School of Veterinary Studies, The Roslin Institute, Roslin Biocentre, Roslin EH25 9PS, Midlothian, UK

Prion strains are defined by their biological properties after transmission to wild-type mice, specifically by their incubation periods and patterns of vacuolar pathology (‘lesion profiles’). Preliminary results from transmissions of variant Creutzfeldt–Jakob disease (vCJD) to wild-type mice provided the first compelling evidence for the close similarity of the vCJD agent to the agent causing bovine spongiform encephalopathy (BSE). Complete results from this investigation, including the transmission characteristics of vCJD from brain and peripheral tissues of 10 cases (after primary transmission and subsequent mouse-to-mouse passage), have now been analysed. All 10 vCJD sources resulted in consistent incubation periods and lesion profiles, suggesting that all 10 patients were infected with the same strain of agent. Incubation periods suggested that infectious titres may be subject to regional variation within the brain. Comparison of incubation periods and lesion profiles from transmission of brain and peripheral tissues showed no evidence of tissue-specific modification in the biological properties of the agent. Analysis of the protease-resistant prion protein (PrPres) by Western blotting from primary and subsequent passages in mice showed a glycosylation pattern closely resembling that of vCJD in humans, the so-called BSE ‘glycoform signature’. Minor variations in PrPres fragment size were evident between mouse strains carrying different alleles of the gene encoding PrP both in primary transmissions and on further passages of vCJD brain. Overall, the results closely resembled those of previously reported transmissions of BSE in the same mouse strains, consistent with BSE being the origin of all of these vCJD cases.

INTRODUCTION

It is now over 20 years since bovine spongiform encephalopathy (BSE) was first reported in cattle in the UK (Wells et al., 1987). BSE is a transmissible spongiform encephalopathy (TSE) or prion disease, a member of a unique group of fatal neurodegenerative disorders that occur in humans as well as in a number of animal species. In 1996, a new and distinct human prion disease was identified in the UK, known as variant Creutzfeldt–Jakob disease (vCJD) (Will et al., 1996). Experimental transmission of vCJD and BSE in animal models provided early evidence supporting the initial hypothesis that vCJD emerged as a result of human infection with the BSE agent, most probably through the consumption of contaminated meat products (Collinge et al., 1996; Lasmezas et al., 1996; Bruce et al., 1997; Hill et al., 1997; Scott et al., 1999).

A key characteristic of prion disorders is the deposition in the brain of an abnormal, protease-resistant form of a normal host-encoded protein, the prion protein (PrP). This abnormal protein (PrP Sc) has been proposed to be the major, if not sole, component of the infectious agent (Prusiner, 1982). The existence of individual prion strains continues to raise the question of how a single misfolded protein can encode all of the information required for the demonstrable phenotypic diversity of prion diseases (Farquhar et al., 1998). Prion strains are defined by their
biological properties after transmission to inbred lines of wild-type mice, specifically by the incubation period and the distribution of vacular pathology in the brain (referred to as the lesion profile), which together provide a ‘signature’ of the biological properties of the agent (Fraser & Dickinson, 1968; Dickinson & Meikle, 1971; Bruce et al., 1991). It has been proposed that the strain-specific biological properties may be encoded in different conformations and glycosylation states of PrPSc (Telling et al., 1996). These different conformers and glycootypes are usually distinguished by the analysis of the protease-resistant core of PrPSc (referred to as PrPres) after limited digestion with proteinase K and analysis by Western blotting; this is an approach commonly referred to as ‘molecular strain typing’ (Collinge et al., 1996; Parchi et al., 1996, 1999). A single unique PrP\textsuperscript{RES} type is associated with vCJD cases, defined by a predominance of the diglycosylated form of PrP\textsuperscript{RES} (Collinge et al., 1996). This same unique biochemical glycootype is found with cases of natural BSE and other BSE-related conditions. This BSE ‘glycoform signature’ further supports the hypothesis that these diseases are linked by a common agent (Collinge et al., 1996).

To date, all tested clinical cases of definite vCJD are homozygous for methionine at codon 129 on the human PrP gene (PRNP). The susceptibility of the other PRNP codon 129 subgroups to infection with the BSE agent and the development of clinical vCJD remain uncertain, although it appears that MV and VV individuals may also be susceptible to infection (Peden et al., 2004; Hilton et al., 2004b; Ironside et al., 2006; Bishop et al., 2006). In mice, three alleles of the mouse prion protein gene (Prn-p) have been described (Westaway et al., 1987; Lloyd et al., 2004). The vast majority of mouse strains analysed are either from the Prn-p\textsuperscript{R} (Leu-108, Thr-189) or Prn-p\textsuperscript{S} (Phe-108, Val-189) genotype (Westaway et al., 1987). These two alleles exert a major influence on the incubation period of the disease, a major determinant of agent strain, with each prion strain having a distinct and highly reproducible pattern of incubation periods in the three possible Prn-p genotypes (the two homozygotes and the heterozygote F\textsubscript{1} cross) (Dickinson & Meikle, 1971; Bruce et al., 1991). Different prion strains are often isolated from a single source by serial passage through mice of different Prn-p genotypes, so a full characterization of a source includes strain typing on both primary transmission to mice and subsequent mouse-to-mouse transmission of the isolate.

In vCJD, the detection of PrP\textsuperscript{Sc} in peripheral tissues has led to concerns over the possible secondary transmission of vCJD through blood transfusion and other iatrogenic routes. The existence of more than one BSE prion strain, which may have already infected humans, and which has a biochemical phenotype indistinguishable from that of sporadic CJD (sCJD), was suggested in a publication examining the transmission of BSE and vCJD in transgenic mice (Asante et al., 2002). This study raised concerns that patients with a phenotype consistent with sCJD may actually have acquired the disease from infection with the BSE agent (Asante et al., 2002; Beringue et al., 2008). These and other such concerns have prompted us to complete, collate and conduct a careful reappraisal of all the available data relating to transmission of vCJD to wild-type mice, including transmission from brain and peripheral tissues, pathological and biochemical assessment and analysis of selected subpassages.

**METHODS**

**Creutzfeldt–Jakob disease (CJD) inocula.** Frozen central nervous system (CNS) and peripheral tissue samples from 10 neuropathologically confirmed cases of vCJD were investigated. Consent and ethical approval for the retention and research use of tissues from these CJD cases had been obtained (LREC reference number LREC/2000/4/157). Grey-matter-enriched brain samples (approx. 2 g) from each vCJD case were taken from either the cerebellar hemisphere \((n=3)\) or the frontal cortex \((n=7)\). A 10 % (w/v) brain homogenate was prepared in physiological saline as described previously (Bruce et al., 1997). For transmission of vCJD from lymphoreticular tissues, samples \((\text{approx. } 2 \text{ g})\) of spleen \((n=1 \text{ vCJD case})\) and tonsil \((n=2 \text{ vCJD cases})\) were prepared as for brain samples. Subpassage of isolates derived from cerebellar samples of two different vCJD cases were carried out with a 1 % (w/v) brain homogenate prepared from the brain of a single RIII, VM and C57BL mouse which showed unequivocal clinical and histological signs of a prion disease.

**CJD transmissions.** Three inbred mouse strains (RIII, VM and C57BL) and one cross (C57BL × VM) were challenged. RIII and C57BL strains are both of the Prn-p\textsuperscript{S} genotype, whereas VM is of the Prn-p\textsuperscript{R} genotype. In an initial study, anaesthetised mice of each strain were inoculated with brain homogenates by a combination of intracerebral (20 \(\mu\)l) and intraperitoneal (100 \(\mu\)l) routes. In a further study, RIII and VM mice were injected with brain, spleen or tonsil homogenates by the intracerebral route (20 \(\mu\)l) only. For subpassages, all three inbred mouse lines and the C57BL × VM cross were inoculated via a single intracerebral injection. Mice were scored weekly for signs of clinical neurological disease as described previously (Fraser & Dickinson, 1968). Brains were removed at a defined clinical end point with one lateral half of each mouse brain frozen at \(-20 \text{ °C}\) for further passage in mice or for biochemical analysis; the other half of the brain was fixed in 10 % formal saline for histology and PrP immunohistochemistry. Experimental transmissions from two cattle BSE sources (10 % brain homogenates) were performed in the same mouse lines by a combination of the intracerebral and intraperitoneal routes as described for vCJD transmissions.

**Incubation period and histological assessment.** Incubation periods were calculated as the interval between injection and a defined clinical end point, when mice showed unequivocal signs of neurological disease with confirmed positive vascular pathology (Dickinson & Meikle, 1971; Bruce et al., 1997). Haematoxylin and eosin (H&E) staining was carried out on 5 \(\mu\)m paraffin sections from all mice. Vacuolar changes were scored in nine standard grey matter regions and three white matter regions using established criteria, and lesion profiles were constructed (Fraser & Dickinson, 1968).

**PrP immunohistochemistry.** Immunohistochemistry for PrP in 5 \(\mu\)m sections of formalin-fixed, paraffin-embedded mouse brain was carried out as described previously (Hilton et al., 2004a). PrP was detected with the monoclonal antibody 6H4 (Prionics) and PrP was visualized using the CSA amplification system (Salbattini et al., 1998).
Biochemical analysis of PrP<sup>Sc</sup> by Western blotting. Western blot typing of PrP<sup>Sc</sup> was carried out as described previously (Head et al., 2004), except that the monoclonal antibody 6H4 was used.

RESULTS

All vCJD brain isolates transmitted successfully to all three mouse strains and to the C57BL × VM cross, with the appearance of clinical and/or pathological signs associated with prion disease (Supplementary Table S1, available in JGV Online).

Incubation periods and lesion profiles

All transmissions of vCJD CNS tissue showed the same order of appearance of clinical symptoms in the mouse strains (Fig. 1a). The shortest incubation periods were observed in RIII mice and the longest were found in the C57BL × VM cross. Incubation periods were broadly similar irrespective of the route of injection or CNS tissue (frontal cortex or cerebellum) used for inoculation. However, the exact timing of the appearance of clinical symptoms showed some variability between individual vCJD cases, most noticeably with inoculum prepared from the frontal cortex of the brain. Inoculum prepared from the cerebellar hemisphere consistently resulted in shorter incubation periods when compared with inoculum prepared from the frontal cortex, suggesting a higher titre of infectivity. Incubation periods closely resembled those observed in transmission of BSE from two unrelated cattle, using the same protocol (Fig. 1b). In general, lesion profiling showed a similar pattern for each vCJD isolate regardless of brain region or route of inoculation (Fig. 2a). There was some variability in the intensity of vacuolation between individual vCJD sources, most noticeably in the C57BL × VM cross; however, the general pattern of vacuolation was similar. Lesion profiles did differ between mouse strains of different PrP genotypes, with the Prn-<sup>p<sub>a</sub></sup> genotype (RIII and C57BL mice) showing a pattern distinct from that of the Prn-<sup>p<sub>b</sub></sup> genotype (VM) and the Prn-<sup>p<sub>ab</sub></sup> cross (C57BL × VM). Lesion profiles resembled those of primary BSE transmissions (Fig. 2a).

Immunohistochemical localization of PrP in primary transmissions

Immunohistochemical detection of PrP showed a widespread accumulation of PrP<sup>Sc</sup> throughout the brains of infected mice, most prominently in the medulla, thalamus and around areas of intense vacuolation (Fig. 3a, b). Subtle differences in the distribution of PrP within the hippocampus region were observed between the different Prn-<sup>p</sup> genotypes. Amyloid plaques, a common feature of some forms of CJD including vCJD, were not a prominent feature in the mice and were observed only in the VM and C57BL × VM cross, mainly restricted to the corpus callosum (Fig. 3b). Levels of PrP immunostaining appeared higher in the Prn-<sup>p<sub>b</sub></sup> mice and the C57BL × VM cross compared to Prn-<sup>p<sub>a</sub></sup> mice.
Biochemical analysis of PrPres

Primary vCJD brain transmissions produced a glycosylation pattern closely resembling that of vCJD in human brain tissue, which was characterized by the predominance of the diglycosylated form of the protein (Fig. 4a). A feature of the experimentally infected mice that was not observed in vCJD in humans was the appearance of the unglycosylated fragment as a doublet, composed of distinct upper and lower bands. The relative amounts of these two bands varied and this correlated to a large extent with the mouse Prn-p genotype; the lower band predominated in Prn-<sup>p<sub>a</sub></sup> mice, whereas the upper band predominated in Prn-<sup>p<sub>b</sub></sup> mice. Both bands were readily discernible in the genetic cross (Fig. 4a).

Mouse-to-mouse passage

Both subpassages provided very similar results for all strain typing parameters examined, with the incidence of clinical and pathological features of disease being 100% in most groups and close to 100% in the others (Supplementary Table S2).

On further mouse-to-mouse passage, incubation periods shortened dramatically, indicative of the removal of a species barrier. Subpassage in Prn-<sup>p<sub>a</sub></sup> mice (C57BL or RIII) resulted in the incubation periods in the mouse genotypes (Fig. 5b) being identical to those seen in primary transmissions (Fig. 5a), whereas subpassage in Prn-<sup>p<sub>b</sub></sup> mouse (VM) changed that order, with very short incubation periods in Prn-<sup>p<sub>b</sub></sup> mice (Fig. 5c). These distinct incubation period rankings indicate that serial mouse passage resulted in the isolation of two distinct agent strains, determined by host genetics and recognized by incubation period. These results closely resembled those of serial passage of cattle BSE in the same mouse strains (Fig. 5). The isolation of two different agent strains was not clearly reflected in the lesion profile results, except in the case of C57BL × VM mice, in which the VM-passaged profile was lower. Lesion profiling from the serial passages (Supplementary Fig. S1a) showed a modified pattern compared with the primary
transmissions (Fig. 2a), but a similar pattern to that found for mouse-passaged BSE (Supplementary Fig. S1b). No significant differences were found between first and second mouse passages. Slight but consistent differences did occur between the recipient mouse strains, again showing the influence of host genetic factors on lesion profiles (Supplementary Fig. S1a).

PrP deposition in the brain was more intense on serial passage compared with the primary transmissions (Fig. 3c, d). The general distribution of PrP was similar to that observed on primary transmission, with the subtle differences observed in the PrP deposition within the hippocampus between the different Prn-p genotypes conserved on serial passage. In contrast with the primary transmissions, all four mouse strains passaged in Prn-pa and Prn-pb mice showed the presence of amyloid plaques, mainly restricted to the corpus callosum. No significant differences in patterns of PrP accumulation were observed between first and second passage or between Prn-pa- and Prn-pb-passaged isolates (Fig. 3c, d). Western blot analysis of PrPres in mice produced a glycosylation pattern similar to that of vCJD in human tissue and in vCJD primary mouse passages. No changes were observed between first and second passage, although variation in the relative abundance of the upper and lower unglycosylated doublet was seen. Unlike the primary transmission results, there was no simple direct correlation between Prn-p genotype and the composition of the doublet (Fig. 4b).

**Transmission of lymphoreticular tissue**

Clinical and pathological signs associated with prion disease were observed in RIII and VM mice challenged with vCJD spleen and tonsil tissue (Supplementary Table S3). Overall, transmission of vCJD from spleen and tonsil resulted in extended incubation periods compared with vCJD brain – this was most obvious with spleen tissue – indicating that infectious titres in these lymphoid tissues may be lower than those in the corresponding vCJD brain.
tissue, as has been shown previously (Fig. 1a, c; Bruce et al., 2001). Lesion profiling produced a consistent pattern irrespective of whether the tissue used for transmission was from the CNS or the lymphoreticular system (Fig. 2b). PrP deposition within the brains of mice challenged with vCJD lymphoid tissue homogenates was virtually indistinguishable from those found in mice challenged with vCJD brain homogenates (Fig. 3e, f). Transmission of vCJD from tonsil and spleen tissue into VM and RIII mice resulted in glycoform patterns closely resembling those found following transmission from vCJD brain tissue (Fig. 4c); however, the single RIII mouse inoculated with vCJD spleen tissue that was available for analysis had a substantially modified glycoform ratio (Fig. 4c). The apparent correlation between mouse Prn-p genotype and the relative abundance of the upper and lower bands comprising the unglycosylated doublet did not hold for transmissions from peripheral tissues.

DISCUSSION

In this paper, we confirm the original observations of Bruce et al. (1997) and extend them in a number of important respects. Firstly, we have increased the numbers of transmitted cases to a total of 10, providing complete incubation period and lesion profile data in a larger panel of mouse strains. Secondly, we have extended the pathological description to include the biochemical and immunohistochemical analysis of abnormal PrP accumulation in the mice. Thirdly, we have analysed the transmission characteristics of the isolates after a further two passages in mice and finally, we have analysed transmissions from vCJD tissues other than tissues from the brain.

We consistently observed shorter incubation periods with inocula prepared from the cerebellar cortex compared with inocula from the frontal cortex. Although sampled from different vCJD cases, this suggests that titres of infectivity are higher in the cerebellar cortex than in the frontal cortex. Pathological changes in these two brain regions in vCJD patients are qualitatively similar, but accumulation of PrP is often more pronounced in the cerebellum with more frequent florid plaques (Ironside et al., 2000), perhaps reflecting the apparent relatively high titre.

The difference in incubation period observed between the RIII and C57BL mice (both Prn-p^a), which is characteristic
of the BSE agent, was replicated in all seven transmissions from vCJD cases in which both mouse strains were included. In addition, subpassage of the vCJD agent in Prn-p<sup>p</sup> and Prn-p<sup>b</sup> mice resulted in the isolation of two distinct mouse-passaged strains, closely similar to the 301V and 301C strains which are derived by serial passage of BSE in VM and C57BL mice, respectively (Bruce et al., 2002). Subpassage of vCJD through VM mice resulted in incubation periods of around 110 days in VM recipients, one of the shortest incubation periods for a mouse-passaged TSE strain in non-transgenic mice, mirroring the behaviour of the BSE agent in the same mouse strain (Bruce et al., 2002). Interestingly, the biological properties of these two subpassaged vCJD-related strains were not reflected in differences in the PrP<sup>res</sup> type found in the brains of the mice replicating these two strains of agent. Although no obvious changes were found in the glycosylation pattern, certain differences were observed in the unglycosylated fragment, which was seen as a doublet. The upper and lower bands of this doublet appeared to be associated with the different Prn-p genotypes in that the lower band was predominant in the Prn-p<sup>p</sup> whereas the upper band predominated in Prn-p<sup>b</sup> mice on primary transmission. These apparent differences in the biochemical profile of PrP<sup>res</sup> might be attributed to differences in the polymorphic residues between Prn-p<sup>p</sup> and Prn-p<sup>b</sup> that result in different conformation or aggregation states of the misfolded form of the protein. However, the relationship did not hold when the subpassages were analysed and so the biochemical basis of this phenomenon and its biological significance remain unclear. Nevertheless, it resembles a phenomenon previously reported to occur in the BSE-derived 301V and 301C strains (Somerville et al., 2005).

Previous transmission experiments have shown that titres of infectivity in vCJD spleen and tonsil are lower than in brain, resulting in extended incubation periods (Bruce et al., 2001). In this study, we have found that although incubation periods are often longer in transmissions from lymphoid tissue, lesion profiles and patterns of PrP accumulation in the recipient mice are very similar to those found in vCJD brain transmissions (Figs 2 and 3), confirming previous findings that the strain properties of the agent are not substantially altered by the tissue in which it replicates (Beringue et al., 2008). This is interesting because minor differences in PrP<sup>res</sup> glycosylation and fragment size have been have been described in vCJD lymphoreticular tissues compared with the brain (Head et al., 2004). These alterations in glycoform pattern are not conserved on transmission to mice, implying that the differences seen in PrP<sup>res</sup> type between brain and lymphoreticular tissues in vCJD patients are tissue-specific epiphenomena and not relevant to the fundamental properties of the agent.

Bioassay in mice remains the definitive method for strain typing of prion disease isolates, but such experiments are often long and expensive. A number of new biochemical approaches to strain typing in prion diseases have been described (Caughey et al., 1998; Thomzig et al., 2004; Spassov et al., 2006). It will be interesting to see whether the biological strain typing properties of vCJD described in this study can be reproduced by these biochemical approaches.

In conclusion, in this extensive series of vCJD transmissions to wild-type mice, we confirm, using incubation period, lesion profile and glycoform analysis, that a BSE-related strain of agent is involved in vCJD, irrespective of the patient, the brain region or tissue type analysed.

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