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Defining sporadic Creutzfeldt-Jakob disease strains and their transmission properties

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The biological determinants of the phenotypic variation in sporadic Creutzfeldt-Jakob disease (sCJD) are unknown. To categorize sCJD cases, the prion protein (PrP) codon 129 genotype and the biochemical characteristics of the disease-associated form of PrP (PrPSc) can be combined to form six subgroups (MM1, MM2, MV1, MV2, VV1, and VV2). This classification largely correlates with the known variation in the clinical and pathological features of sCJD, with the MM1 and MV1 cases representing the “classic” phenotype of sCJD. To address how this classification relates to different strains of sCJD we have inoculated each subgroup of sCJD to a panel of mice expressing different forms of the human PRNP gene (129MM, 129VV, and 129MV). We have established that all subtypes are transmissible to at least one genotype of mouse, and both agent and host factors determine transmission efficiency and the form of PrPSc deposited in the brain. Moreover, we have identified four distinct strains of sCJD using our in vivo strain typing panel.

Transmissible spongiform encephalopathies (TSE) or prion diseases are a group of fatal neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease in deer and elk. These diseases can be sporadic, familial, or acquired by infection, and the common hallmark is a distinct pathology in the central nervous system characterized by neuronal loss, spongiform degeneration, and gliosis (1). Sporadic CJD (sCJD) is the idiopathic form of CJD. There is much that remains to be understood about how and why this disease occurs and the speciation of sCJD strains and the influence of codon 129 genotype in the inserted human prion gene. The inserted human PRNP gene is under the direct control of the normal expression modifiers for the equivalent mouse Prnp gene and, after inoculation with human prions, there will be homologous human PrPSc–PrP interaction. These lines have been inbred on a 129Ola background, thus the only genetic variation (between the different mouse lines) is that of the codon 129 genotype in the inserted human prion gene. Thus, the direct effect of an M-to-V substitution in the mature prion protein can be studied in both the homozygous (HuMM and HuVV) and heterozygous (HuMV) lines. Six sCJD cases were selected for transmission to the transgenic mice, each of which showed the typical characteristics of that subgroup: MM1, MM2, MV1, MV2, VV1, and VV2. Our aim was to define the diversity of sCJD strains and the influence of codon 129 genotype on the transmission properties of sCJD.

Results

Incubation Times on First Passage Indicate Four Strains of sCJD. Incubation time data for mice showing clinical TSE symptoms are shown in Table 1. The presence/absence and time of onset of clinical manifestation of TSE disease were dependent on both the PRNP genotype of the host and the inoculum. These data suggest that there exist four discrete sCJD strains. The first strain comprises the subgroups sCJD(MM1) and sCJD(MV1) that produced similar incubation times in each of the lines of mice, thus, the direct effect of an M-to-V substitution in the mature prion protein can be studied in both the homozygous (HuMM and HuVV) and heterozygous (HuMV) lines. Six sCJD cases were selected for transmission to the transgenic mice, each of which showed the typical characteristics of that subgroup: MM1, MM2, MV1, MV2, VV1, and VV2. Our aim was to define the diversity of sCJD strains and the influence of codon 129 genotype on the transmission properties of sCJD.

electrophoresis of the PrPSc protease-resistant core can distinguish between two isoforms of the protein commonly referred to as type 1 (21 kDa) and type 2 (19 kDa), from the mobility of the unglycosylated component (17). The prion protein codon 129 genotype and the biochemical characteristics of the disease-associated form (PrPSc) can be combined to form six subgroups (MM1, MM2, MV1, MV2, VV1, and VV2). Further studies have been carried out to assess whether disease characteristics, according to PrPSc type, are maintained after passage to hosts with different PRNP genotypes (18, 19).

In vivo transmission studies have been undertaken to identify sCJD strains. Transmission of sCJD to wild-type mice does not often result in clinical disease (20); however, transmissions to bank voles have proved more productive in terms of a clinical outcome, with sCJD isolates classified as MM1 and MV1 behaving as a single strain, but VV2 and MV2 failed to cause disease after inoculation (21). A number of different lines of transgenic mice have been produced that express full-length or chimeric human and mouse PrP genes to facilitate transmission of CJD (22–24). When challenged with some, but not all, CJD isolates, transgenic mice have shorter incubation times than wild-type mice, and the data demonstrate that identity between host and agent codon 129 genotype often, but not always, facilitates transmission. Previously we reported the use of gene targeting methodology to produce mice expressing physiological levels of the human prion protein gene (25). The inserted human PRNP gene is under the direct control of the normal expression modifiers for the equivalent mouse Prnp gene and, after inoculation with human prions, there will be homologous human PrPSc–PrP interaction. These lines have been inbred on a 129Ola background, thus the only genetic variation (between the different mouse lines) is that of the codon 129 genotype in the inserted human prion gene.

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with the shortest in the HuMM and HuMV lines (446–475 d), whereas incubation times in HuVV mice were more than 100 d longer. The second strain comprises sCJD(MV2) and sCJD(VV2) inocula that produced clinical disease with relatively short incubation times (≈280 d) in the HuVV mice but much longer incubation times in HuMM and HuMV mice (450–582 d). For these inoculations, only a few mice in the HuMM and HuMV lines displayed clinical signs [sCJD(MV2): 3 of 13 HuMM and 2 of 16 HuMV; sCJD(VV2): 4 of 18 HuMM and 1 of 15 HuMV] compared with the high numbers of HuVV mice displaying clinical signs [sCJD(MV2): 16 of 17; sCJD(VV2): 13 of 16]. Although the two groups of HuMV mice produced different incubation periods after inoculation of sCJD(MV2) and sCJD(VV2), these data were limited to too few mice to compare statistically. The third and fourth strains comprise sCJD(VV1) and sCJD(MM2), which had transmission characteristics that were different from each other and from the other agents. No clinical disease was observed in the HuMM mice inoculated with sCJD(VV1), and only two cases were observed in each of the HuMV and HuVV lines between 546 and 568 d. Sporadic CJD (MM2) inoculation showed no clinical disease in any of the three lines of mice.

### Vacularization Profiles Demonstrate Strain Diversity of sCJD.

Examination of the vacuolar pathology of sCJD-infected mice allowed further assessment of the similarities and differences in transmission properties of each inoculum and provided further evidence for four discrete sCJD strains (Table 1). Inoculation with sCJD(MM1) and sCJD(MV1) produced similar levels of vacuolation, with 80–100% of HuMM and HuMV mice and ≈80% of HuVV mice scored positive for vacuolation. Inoculation with sCJD(MV2) and sCJD(VV2) produced 100% positive HuVV mice and ≈33% positive HuMV mice, but there was variation in the HuMM mice, with 69% positive with sCJD(MV2) inoculum compared with 33% with sCJD(VV2). The sCJD(VV1) inoculum showed no evidence of vacuolation in the HuMM mice, and ≈50% of mice were positive in the other two transgenic lines. The sCJD(MM2) inoculum showed no evidence of TSE vacuolation in any of the three mouse lines. Fig. 1 shows the lesion profiles generated from assessment of TSE vacuolation distribution in the brain. Data for sCJD(MM2) inoculation (all genotypes) and HuMM mice inoculated with sCJD(VV1) are absent because no mice were positive for vacuolation. There were similarities in lesion profiles for all genotype mice infected with both sCJD(MM1) and sCJD(MV1). The distribution of lesions is characterized by three peaks in the gray matter scores relative to the superior colliculus (GMS), hippocampus (GM6), and cingulate and adjacent motor cortex (GM9). Similarities were also seen between sCJD(MV2) and sCJD(VV2) inocula; however, unlike sCJD(MM1) and sCJD(MV1), these profiles differed between the mouse lines. The HuMM mouse profiles had peak scores for the hippocampus (GM6) and the cingulate and adjacent motor cortex (GM9) and appeared similar but not identical to those of sCJD(MM1) and sCJD(MV1). The HuVV mouse lesion profiles for these two inocula had peaks in the profiles again at GM3, GM6, and GM9; however, region GM9 had a much higher mean score, and additionally a high score was also recorded for the cortex region GM8 (retroplenial and adjacent motor cortex). The heterozygous HuMV mice inoculated with sCJD(MV2) and sCJD(VV2) produced lesion profiles that were dissimilar to both homozgyous lines of mice and also to the sCJD(MM1) and sCJD(MV1) inocula. Lesion profiles for HuMV and HuVV mice inoculated with sCJD(VV1) were different from each other and from all other profiles generated.

### Detection of PrPSc by Immunocytochemistry.

Analysis of PrPSc deposition was carried out to determine whether distinctive patterns exist for the sCJD subgroups (Fig. 2 (hippocampal/thalamic targeting), Table 1, and Fig. S1 (cerebellar targeting)). Brain sections from all mice in each inoculated group were examined by immunohistochemistry to assess the specific targeting of PrPSc deposition. The degree of within-group variation seemed to depend on the incubation time of the mice, with an increase in deposition over time. The regions targeted did not change over this time period. Both sCJD(MM1)- and sCJD(MV1)-inoculated mice of each genotype showed similar overall patterns of staining: in the thalamic region with discrete, punctate, deposits; hippocampal targeting of the stratum lacunosum moleculare; and occasional larger deposits in deep layers of the cerebral cortex. The cerebellum was spared with only very little evidence of PrPSc deposition. The sCJD(MV2)- and sCJD(VV2)-inoculated mice showed very similar patterns of PrPSc deposition, which were different from that found with sCJD(MM1) and sCJD(MV1). The same thalamic regions were targeted as for sCJD(MM1), but as well as the punctuate deposits, there was an increase in more diffuse staining. The HuMM and HuVV lines showed a similar degree of staining concentrated in the ventral posterial and posterolateral thalamic nuclei. For the HuVV line this area extended to include the lateral dorsal nucleus, the posterior thalamic nuclear group, and the medial dorsal and ventromedial thalamic nuclei. Outside the thalamic region the HuVV mice again showed differences from the other lines. The cerebral cortex was heavily stained with distinctive banding associated with the cell type layers present. Band 6 showed the densest deposits. Intensely stained aggregates were seen in the white matter of the corpus callosum in both HuMM and HuVV mice and not in the HuMV mice. The cerebellum showed similar staining patterns, targeted to the granular layer, for all mouse lines and both inocula. Deposition in the brains of all three genotype mice inoculated with sCJD(VV1) showed similar overall patterns of staining, and this was different from that found for sCJD(MM1) and sCJD(MM2). The region GM3, GM6, and GM9 had a much higher mean score, and additionally a high score was also recorded for the cortex region GM8 (retroplenial and adjacent motor cortex).
deposition in the thalamic region. The cerebellum showed no evidence of PrPSc deposition. These differences observed with each subtype confirm evidence of four major sCJD strains: sCJD(MM1 and MV1); sCJD(MV2 and VV2); sCJD(VV1); and sCJD(MM2).

Detection of PrPSc by Western Blot. Western blot detection of PrPSc was performed on brain material recovered during the postmortem of each mouse (Fig. S2). The appearance of type 2 PrPSc was restricted to the HuVV mice inoculated with either sCJD(MV2) or sCJD(VV2) on primary and secondary passage. These mice also showed a dominance of the diglycosylated form, a Western blot profile similar to variant CJD (see type 2 standard lanes in Fig. S2). There was no evidence for the appearance of type 2 PrPSc in mice homozygous or heterozygous for methionine at codon 129. All of the other mice/inoculum combinations in which PrPSc has been detected showed the typical sCJD type 1, monoglycosylated dominant profile even if the inoculum contained type 2 PrPSc. Very low levels of PrPSc were seen in the sCJD(VV1) experiment. With regards to understanding the strain nature of the inocula, the Western blot data have only limited capacity to confirm the previous findings. It is clear that sCJD(MV2) and sCJD(VV2) share similar properties that are different from the other inocula. Because all other inoculum/host combinations produce type 1 monoglycosylation dominant PrPSc (where this is present), there can be no further distinction between those sCJD subgroups.

Secondary Passage in Transgenic Mice. Secondary passage of brain material from each of the three transgenic mouse lines was set up for the three commonest subgroups of sCJD (MM1, MV2, and VV2) to examine the influence of host PrP in modifying the strain of agent. Table 2 lists the incubation periods found for the appearance of clinical TSE signs in the second passage, together with the scores for mice found with TSE vacuolation. Sporadic CJD(MM1) showed remarkably close similarities in onset of clinical TSE signs and number of affected mice between first and second passage regardless of the incubation period or genotype of the mice that donated the inoculum brain material. For example, primary inoculation of HuMM mice produced clinical disease at a mean time of 446 d, and secondary passage from HuMM-, HuMV-, and HuVV-sourced inocula gave mean incubation periods of 451, 446, and 451 d, respectively. Additionally, the extended incubation periods in HuVV mice (~550 d) were similar in both primary and all secondary passage experiments. These data indicate that sCJD(MM1) strain properties are being propagated efficiently and that this can occur independently of the genotype of the host, even if brain material is sourced from an HuVV mouse. Sporadic CJD(MV2) and sCJD(VV2) showed similar primary passage incubation times, but differences were observed with these inocula after secondary passage. Incubation times in HuVV mice were not affected by primary passage--sourced inocula from either HuMM or HuMV mice and gave incubation periods of 262–288 d. Second passage from HuVV-sourced brains resulted in reduction of incubation periods to 235–239 d, suggesting that codon 129 methionine may hinder propagation of this otherwise efficient genotype/agent combination. Further differences between primary and secondary passage were restricted to HuMV and HuMM mice with inocula originating from primary sCJD (MV2)-infected HuMV mice and sCJD(VV2)-infected HuMM mice. Reduction in incubation periods (by ~100 d) and an increase in clinically positive mice compared with primary passage suggest that the agent may be adapting toward having properties more similar to sCJD(MM1) and sCJD(MV1).
The number of mice positive for the presence of TSE-associated vacuolation closely followed the similarities and differences that were seen for clinical signs. In addition, the HuMM and HuMV mice (discussed above) that showed reductions in incubation periods were >90% positive, unlike 33–50% for primary transmission (see asterisked data in Table 1 and Table 2). In contrast to the primary transmission data, these results for incubation period and the appearance of TSE vacuolation may indicate a divergence of the strain properties of sCJD(MV2) and sCJD(VV2). Visual comparison of the lesion profile data (Fig. 3) showed that with each genotype host there were many instances in which the second passage profile was similar to the first passage. This was seen even when passage occurred from an alternative genotype host. For sCJD(MM1) the lesion profiles showed clear uniformity across all mice lines and with passage from the three genotype primary inoculations. Second passage data showed a general lowering of the scores in regions other than those peak scores seen in primary passage [superior colliculus (GM3), hippocampus (GM6), and cingulate and adjacent motor cortex (GM9)], suggesting adaptation to a more focused pathological response. This effect was not seen in the sCJD(MV2) and sCJD(VV2) passage data. Overall, passage of sCJD(MV2) and sCJD(VV2) gave similar data sets. The HuVV hosts with passage from each different genotype mice had profiles that most closely mirrored the primary data, whereas although the HuMM vacuolation scores gave a similar overall pattern there was more variation between the passage experiments. The HuMV response to the second passage inocula was similar to primary passage in that the profiles were variable and had wide SE ranges. Scores for the hippocampus (GM6) region in HuMM and HuVV mice may highlight a potential adaptation of the sCJD (MV2) and sCJD(VV2) agents, as shown by a more pronounced peak in the lesion profile at this point.

Sporadic CJD(MM1) seems to have dominance over the genotype of the host, with near identical lesion profile patterns for all primary and secondary passage mice. The patterns seen for both sCJD(MV2) and sCJD(VV2) are similar only within the HuMM and HuVV host mice groups. The heterozygous HuMV host shows the most variation between primary and secondary passage, with no specific pattern apparent. These data confirm the primary transmission findings that sCJD(MM1) has strain properties different from sCJD(MV2) and sCJD(VV2) and that the latter pair could be grouped together as one strain. Second-passage Western blot analysis was undertaken for mice inoculated with the sCJD(MV2) agent from an HuVV genotype host and showed that the same PrPSc types occurred in second passage as was seen in primary passage (Fig. S2); type 1 was seen in the HuMM and HuVV mice brains and type 2 in the HuVV mice.

**Discussion**

The similarities and differences that have emerged during this study indicate that six subgroups of sCJD, defined here by PrPSc type and PRNP codon 129 genotype, behave as four different strains of agent. Sporadic CJD(MM1) and sCJD(MV1) isolates have identical transmission properties for all three genotypes of mice. The sCJD(MV2) and sCJD(VV2) isolates have very similar transmission properties, and both the sCJD(MM2) and sCJD(VV1) strains behave differently from each other and from the other isolates. To facilitate discussion of this grouping and for future reference we propose to name these major strains “M1,CJD,” “V2,CJD,” “M2,CJD,” and “V1,CJD,” respectively. Our
studies, based on incubation time, lesion profile, and PrPSc Western blot profile and deposition in the brain using a panel of mice, are based on the “gold standard” strain typing of isolates originally established by Collinge et al. (26). This protocol is that our transgenic mice carrying the human PRNP gene have been substituted for the original wild-type panel, but the inbred nature and gene targeting of these mice ensure that they are suitable for such a panel, because there is only a single PrP amino acid difference between each of the lines. Previous sCJD transmission studies have provided additional evidence for sCJD (MM1) and sCJD(MV1) having similar transmission properties and some evidence for similarities between sCJD(MV2) and sCJD(VV2), but insufficient evidence to make a comparison between sCJD(VV1) and sCJD(MM2) (21, 24, 27). This study is a complete analysis of all major sCJD subgroups in inbred mouse models and is thus capable of a true comparison for distinguishing the codon 129 homozygote and heterozygote response. However, the present study examines only single cases of sCJD, with the assumption that transmission characteristics of a single case will be representative of the particular subgroup. Although typical cases of each subgroup were carefully selected for this study, there is phenotypic variation within sCJD subgroups, and it is therefore essential that the findings in this study are replicated in additional cases. Through a grant from the European Union (NeuroPrion: HUMTRANS) the panel of mice used in this study have been provided to a number of other groups, and transmission studies including a significant number of additional sCJD cases are underway. This is of particular importance for the sCJD(MM2) subgroup because our evidence for a unique strain derives partly from the inefficiency in transmission. Inoculum prepared from this specific patient has been used in parallel wild-type and transgenic studies (28) and shows clear evidence of successful transmission to those mice, confirming that infectivity titer was unlikely to be implicated in our findings.

Evidence in support of these four major strains of sCJD has recently been reported through a very different approach. Using in vivo assays, Uro-Coste et al. (29) examined the protease sensitivity and conformational stability of PrPSc found in 41 patients with sCJD and found groupings identical to those outlined in this study (i.e., MM1 and MV1; MV2 and VV2; MM2; VV1). Because this study was based entirely on in vitro analysis of PrPSc, this suggests that the four strains of agent identified in our study have different conformations of PrPSc. What is perhaps surprising is that only four discrete strains of sCJD have been identified. If each PrPSc protein carries a different affinity for the urogeneic isomers in a single host, why then do only four different strains of sCJD result in humans? Because the assumption is that each sCJD case arises spontaneously, this would require strong selection factors to be operating for these four strains and against others that may be produced.

There are diverse suggestions as to the origin of sCJD, including proposals that somatic mutations lead to protein misfolding and disease (30) or that sCJD has arisen through infection from an agent (31). The second common characteristic among the data was that HuMM and HuMV mice had similar levels of clinical disease, and mean incubation periods, for four of the six inocula (excluding sCJD(MM2) and sCJD(VV1)). This suggests that the methionine allele of PrPSc in the heterozygous HuMV mice may have had a dominant effect over the valine allele PrPSc with regard to the transmission properties. This study identifies two areas of risk in terms of developing sCJD. The first is that the highest risk of developing sCJD after iatrogenic infection is from strain MM1 (sCJD(MM1) or sCJD(MV1)) and the VV genotype confers the highest risk of acquiring infection. The epidemiological findings in sCJD demonstrate that approximately 80% of patients are diagnosed with “classic CJD” types MM1 and MV1, which might intriguingly suggest an infectious rather than genetic origin for the majority of sCJD cases.

PrPSc type has typically been used as a diagnostic indicator of the strain of agent infecting an individual. This study, however, clearly demonstrates that the PrPSc type is a result of the interaction between strain and host. Type 2 PrPSc was seen only in HuVV mice that had short incubation periods, after inoculation with primary or secondary sources of strain VV2 (sCJD(MV2) and sCJD(VV2)), indicating a specific pathological response from the valine homozygous mouse host and inocula with valine allele type 2 PrPSc. All other mice genotype/inoculum combinations produced type 1 PrPSc. Strain MM1 (sCJD(MM1), sCJD(MV2), and sCJD(VV2) in the transgenic mice has allowed us to examine the strain-genotype correlation and to assess the potential risk of transmission of these agents within the human population. The sCJD(MM1) strain transmission properties were unaffected by secondary passage; the strain could propagate within non-MM hosts and could transmit from non-MM hosts to all genotypes with the same properties as primary passage. Because of these facts, there may be difficulty in distinguishing sCJD from iatrogenic CJD caused by infection from sCJD(MM1) or the M1*CD3 strain. However, these data also indicate that passage of the commonest form of sCJD does not cause adaptation to a more highly transmissible form of human TSE. Conversely, the other two sCJD subgroups analyzed by secondary passage seemed to undergo modification of properties dependent on the codon 129 genotype of the source and recipient mouse. The sCJD(MV2) strain transmission properties were unaffected by secondary passage from non-MM hosts to all genotypes with the same properties as primary passage. Inoculation of HuVV mice with sCJD(MV2) from an HuMV mouse produced a reduction in incubation period, suggesting adaptation to a strain with more efficient transmission properties. An overabundance of iCJD cases in VV genotype recipients of contaminated human growth hormone in the United Kingdom supports these data (34), although this is not the case in France (8). A form of adaptation was also observed for HuMM and HuMV mice that received sCJD(MV2) inoculum from an HuMV mouse. Reduction in incubation periods and an increased number of mice positive for vacuolation suggest that a more efficient transmitting strain has been formed. The sCJD(VV2) strain transmission properties showed a similar response as for sCJD(MV2).
mission properties were unaffected by secondary passage from an HuMV mouse, and inoculation of HuVV mice with sCJD(VV2) from an HuVV mouse produced a reduction in incubation period. Strain adaptation was observed for HuMM and HuMV mice that received inoculum from sCJD(VV2) passed through an HuMM mouse. Analysis of incubation periods and lesion profile data suggests that the adaptation seen may be producing a host response more similar to that seen for sCJD(MM1) transmission. Therefore if sCJD(MV2) and sCJD(VV2) were to become iatrogenic sources of human infection, the host response may be indistinguishable from sCJD(MM1) and more transmissible with respect to further infection.

The results from this study can be used as a standard against which atypical or novel forms of human TSE can be compared. We have identified four discrete transmission strains, including M1\textsuperscript{CD}, sCJD(MM1 and MV1), V2\textsuperscript{CD}, sCJD(MV2 and VV2); V1\textsuperscript{CD}, sCJD(VV1); and M2\textsuperscript{CD}, sCJD(MM2). We hypothesize that iatrogenic spread of sCJD will depend on sCJD subgroup source and host codon 129 genotype and could produce more transmissible adapted forms of human TSE, highlighting the continued need for CJD surveillance and iatrogenic transmission risk evaluation. The sporadic CJD transmission strain characterization described in this study will now allow the full range of sCJD strains to be examined and atypical strains of human TSE to be readily identified so that the public health threats from potential new forms of infectious prion disease can be investigated.

Materials and Methods

Groups of 18 mice were inoculated intracerebrally with brain homogenate prepared from sCJD patients, and from primary inoculation mice for serial passage. SSEA1-based methodologies were used throughout for scoring of clinical TSE signs, for assessing the distribution of TSE-associated vacuolation (35), and for detection (immunocytochemistry) and characterization [Western blots (36)] of the disease-associated form of the prion protein. (See SI Materials and Methods.)

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