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Strain-Specific Viral Properties of Variant Creutzfeldt–Jakob Disease (vCJD) Are Encoded by the Agent and Not by Host Prion Protein

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ABSTRACT

Human CJD, endemic sheep scrapie, epidemic bovine spongiform encephalopathy (BSE), and other transmissible spongiform encephalopathies (TSEs), are caused by a group of related but molecularly uncharacterized infectious agents. The UK-BSE agent infected many species, including humans where it causes variant CJD (vCJD). As in most viral infections, different TSE disease phenotypes are determined by both the agent strain and the host species. TSE strains are most reliably classified by incubation time and regional neuropathology in mice expressing wild-type (wt) prion protein (PrP). We compared vCJD to other human and animal derived TSE strains in both mice and neuronal cultures expressing wt murine PrP. Primary and serial passages of the human vCJD agent, as well as the highly selected mutant 263K sheep scrapie agent, revealed profound strain-specific characteristics were encoded by the agent, not by host PrP. Prion theory posits that PrP converts itself into the infectious agent, and thus short incubations require identical PrP sequences in the donor and recipient host. However, wt PrP mice injected with human vCJD brain homogenates showed dramatically shorter primary incubation times than mice expressing only human PrP, a finding not in accord with a PrP species barrier. All mouse passage brains showed the vCJD agent derived from a stable BSE strain. Additionally, both vCJD brain and monotypic neuronal cultures produced a diagnostic 19 kDa PrP fragment previously observed only in BSE and vCJD primate brains. Monotypic cultures can be used to identify the intrinsic, strain-determining molecules of TSE infectious particles. J. Cell. Biochem. 106: 220–231, 2009.

KEY WORDS: BSE; SPORADIC CJD; 263K SCRAPIE; NEURODEGENERATION; TOXICITY; PLAQUES; SPLEEN; PRP

Epidemic bovine spongiform encephalopathy (BSE), an infectious disease of cows originating in the UK, belongs to the group of transmissible spongiform encephalopathies (TSEs). TSEs are caused by infectious particles of unknown molecular composition. The UK-BSE strain of agent is distinct from sheep scrapie strains, and has only recently appeared. Starting in 1985, recognized cases of BSE in the UK rapidly rose to epidemic proportions, and by 2000 probably infected more than 1 million cows. Many additional species exposed to contaminated bovine products were also infected with this BSE agent [Bruce et al., 1994, 1997; Will et al., 1996; Manuelidis et al., 1997; Lasmézas et al., 2001], and during the peak of the outbreak, BSE materials and livestock from contaminated farms were shipped to many countries where infected animals were subsequently identified [Manuelidis, 2000]. By 1996, the first cases of a new variant of CJD (vCJD) was reported in humans, and linked to epidemic BSE. The BSE-linked vCJD agent infected young adults rather than older people, had a unusually prolonged clinical course, and provoked large PrP amyloid plaques in the cerebellum, features that clearly distinguished vCJD from typical sporadic CJD (sCJD); sCJD brains show neither plaques nor a strong cerebellar involvement. The original BSE epidemic is now largely under control, but has the potential to reappear. Because TSE infections have a remarkably prolonged and clinically inapparent stage, one that can last for >20 years in humans, the BSE-linked vCJD agent may continue to spread unrecognized through iatrogenic procedures, as well as to wild animals via contaminated waste products [Manuelidis, 1997; Lasmézas et al., 2001]. In the present study we sought to develop rapid and stable murine models of vCJD, and to more rigorously define this agent's unique (strain-specific) biological properties and cross-species virulence as compared to other CJD and scrapie agents. In developing these new murine vCJD models we also sought to test some of the proposed roles for PrP in infection and toxic neurodegeneration.

By definition, any infectious agent must be able to encode its variant strains. There are many distinct TSE agent strains, and these display their very individual properties when propagated in mice

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TABLE I. Comparison of Different TSE Agents

	Country: original species	Diagnosis	Agent name	Incubation in CD-1 mice >p3	Mo. brain pathology	Mo. brain PrP-res
1	USA: human (++)	sCJD	SY-CJD	380 days	Very restricted	Type 1
2	Italy: human	sCJD	LU-CJD	360 days	Very restricted	Type 1
3	UK: human	GSS	MA-CJD	380 days	Very restricted	Type 1
4	Japan: human (+)	GSS	FU-CJD	120 days	Diffuse	Type 1
5	Japan: human	GSS	YAM-CJD	130 days	Diffuse	Type 1
6	UK: human	vCJD	vCJD	170 days	BSE	Type 2
7	UK: cow	BSE	BSE	160 days*	BSE	Ty-2 (and primate)
8	UK: sheep	Scrapie	22L Sc	140 days	Scrapie variant	Type 1
9	UK: sheep	Scrapie	Ch (RML)-Sc	120 days	Scrapie variant	Type 1
10	UK: sheep (++++)	Scrapie	263 K-Sc	330 days	263K	Type 1

Key features of different TSE agents propagated in mice with wt PrP. The country of origin, original species and diagnosis are listed. One or more + signs indicates the agent was passaged in non-murine species before being transmitted to mice. Thus SY-CJD was passaged in guinea pigs and hamsters before being transmitted to mice [Manuelidis et al., 1978] whereas the other human CJD homogenates were transmitted directly to mice [Manuelidis et al., 1988]. Nevertheless, the prolonged incubation time, even after ≥3 passages in CD-1 mice, was diagnostic for the sCJD agent, as were the very restricted medial thalamic lesions. In contrast, the geographic isolates from Japan were markedly different, with rapid incubation times and widespread spongiform lesions, even though they showed the same Type I PrP-res banding pattern as sCJD [Manuelidis, 1998; Manuelidis et al., 2000]. FU-CJD was first passaged in rats before being transmitted to mice, but is indistinguishable from YAM-CJD, that is, shows comparable stability as the sCJD agent that is independent of species PrP. Only the vCJD and BSE agents produced Type 2 PrP-res (with the 19 kDa diagnostic marker) in brain. The mutant 263K scrapie agent, repeatedly cloned at limiting dilution, passaged in five different species and further selected for its low pathogenicity in mice where it gave inapparent infection for >600 days [Kimberlin et al., 1989]. Yet it still yielded the standard Type I PrP-res profile (see Fig. 3) even though its biological properties were changed profoundly. It has a much more prolonged mouse incubation time than the other scrapie agents, even after serial passage, and induces a different distribution of brain pathology than any of the other agents (data not shown). BSE was propagated from a sick UK cow brain by N. Nishida in Japan using ddY mice (*) and at 2nd passage had the same incubation time as vCJD. BSE brain also showed the same lesion distribution as seen in vCJD brain (see text and Fig. 2A). Incubation time comparisons are all based on mice inoculated with 1% brain homogenates and PrP-res band differences in GT1 cells infected with these different agents are shown in Figure 4.

with wt PrP. For easy reference, including findings in this report, Table I lists key agent features that do, and do not, distinguish a representative variety of TSE agent strains evaluated here. It has been argued that the host-encoded prion protein (PrP) can spontaneously misfold and convert itself into the TSE infectious agent [Prusiner et al., 1990; Prusiner, 1998, 1999]. According to this prion hypothesis, minor PrP sequence differences encoded by mammalian hosts create a strong species barrier against the spread of non-homologous PrP agents. However, the natural spread of the UK-BSE agent to many different species, including humans, undermines this concept. It is also difficult to reconcile the many distinctive TSE agent strains that have been clearly discriminated from each other in mice expressing only a single invariant wt PrP. On the other hand, it is often impossible to identify individual TSE strains by abnormal PrP misfolding patterns in wt PrP mice (Table I). Misfolded PrP is visualized in situ as amyloid fibrils in fixed brain, lymphoid tissues and infected neuronal cell cultures. These amyloid fibers show no strain specific features [Manuelidis et al., 1997, 2007]. Biochemically, misfolded PrP is evaluated by treating infected samples with proteinase K (PK) for a limited time in the presence of detergents, and then resolving the truncated PrP bands (PrP-res) on Western blots. PrP-res bands are not found in normal samples, and therefore PrP-res is a reliable maker for TSE agent-induced pathology. Whereas different TSE strains often induce identical PrP-res bands, major differences in PrP-res band profiles are typically cell-type specific, as shown by infection of monotypic cell lines [Arjona et al., 2004]. Despite these major cell-type dependent PrP-res changes, the cultured agent replicates its fundamentally unique incubation time and neuropathology when reinoculated into mice [Arjona et al., 2004]. Furthermore, PrP-res bands remain constant during serial passages in a foreign species, yet TSE agents progressively evolve and adapt while retaining their fundamental and species-independent identity [Zlotnik and Rennie, 1965; Manuelidis et al., 1997]. In contrast to PrP-res, viruses can modify their nucleic acid genomes for progressive and reversible adaptive changes.

Although an agent-specific viral nucleic acid has not yet been identified, biophysical and structural studies reproducibly show the TSE infectious particle is ~25 nm in diameter and has a viral density of 1.28 g/cc [reviewed in Manuelidis, 2007]. These particles can be separated from the majority of abnormal PrP without losses in infectivity, as shown in fractionation studies of both infected brain and neuronal cells in culture [Manuelidis, 2007; Sun et al., 2008]. In situ ultrastructural studies have further confirmed the presence of arrays of viruslike 25 nm particles in infected brain and cultured cells, but not in mock infected controls. These viruslike arrays are unlikely to represent a secondary pathological feature because they are found in two types of neuronal cell cultures, neither of which shows obvious neurodegenerative changes [Manuelidis et al., 2007]. Remarkably, different infectious titers in animals infected with different agent strains also do not correlate with PrP-res levels. The sCJD and Japanese CJD isolates (Table I) generate a 10,000-fold difference in brain infectivity yet only a 10-fold difference in PrP-res levels [Manuelidis, 1998]. Moreover, despite many attempts, misfolded recombinant PrP itself has failed to reproduce significant infectivity [Manuelidis, 2007]. The overall TSE data implicate a 25 nm viral particle that interacts with host PrP, a necessary host membrane receptor for viral entry and/or reproduction. Binding of the viral particle to cellular membranes probably induces host PrP to misfold. In rare instances a unique agent may dock on variant regions of PrP and leave its strain-specific imprint for PrP misfolding.

Culture models of vCJD have not been reported, and infected neuronal cultures can simplify the analysis of agent induced PrP and toxic neurodegenerative changes. To find if the new human vCJD agent propagated in wt PrP mice and/or in monotypic neuronal cultures could produce a diagnostic PrP fragment related to BSE, we first inoculated mice with a UK human brain sample that by both

clinical and pathological features was typical for BSE linked vCJD. We here report successful primary and serial passages of the human vCJD agent to three genotypes of wt PrP mice. Only one laboratory has reported serial transmission of vCJD to a mouse with wt PrP [Bruce et al., 2001]. Whereas wt inbred and outbred mice have been used to reproducibly discriminate many different agent strains, transgenic (Tg) mice with substituted PrP sequences from other species, including bovine, ovine and human PrPs, have been used to evaluate only a few TSE agents, and thus agent comparisons are not feasible in these Tg models. Moreover, different Tg lines of mice expressing the same human PrP, when inoculated with the identical vCJD brain sample, have given variable results. These humanized PrP mice have also shown poor susceptibility to vCJD infection [Asante et al., 2002; Korth et al., 2003; Béringue et al., 2008]. We here report significantly shorter primary incubation times and 100% susceptibility for vCJD agent transmission to Tga20 mice expressing 8× the normal levels of wt PrP. Furthermore, subsequent passages in Tga20 as well as normal outbred CD-1 and inbred NZW mice, showed incubation times and neuropathological lesions that were the same as found in murine adapted BSE propagated from a UK cow infected during the epidemic. Surprisingly, vCJD mouse brains also displayed a diagnostic 19 kDa PrP band previously observed only in humans with vCJD and in primates infected with vCJD and BSE [Lasmézas et al., 2001]. This 19 kDa band, as well as the brain pathology, verify infection by the bone fide vCJD agent strain, and show that it is clearly derived from the UK-BSE agent. Similar murine transmission studies of the mutant and cloned 263K hamster scrapie agent demonstrated the uniqueness of each agent, and further corroborated the conclusion that individual strain-specific properties are encoded by the agent, not host PrP.

Monotypic neuronal GT1 cell cultures have been previously infected with a variety of CJD and scrapie agents [Arjona et al., 2004; Nishida et al., 2005], and this has made the GT1 model useful not only for further comparison of TSE strains, but also for rapid infectivity assays and agent purification studies [Liu et al., 2008; Sun et al., 2008]. We are unaware of any transmissions of vCJD or 263K scrapie agents to monotypic cell cultures, and we successfully propagated these additional agents to GT1 cells. Remarkably, GT1 cells infected with the vCJD agent displayed the same vCJD linked PrP band of 19 kDa as seen in vCJD brain. This band was diagnostic for vCJD infection since it was not present in GT1 cells infected by any other CJD or scrapie agent. Although it is often assumed that abnormal misfolded PrP induces neurodegeneration, neither this abnormal PrP fragment, nor the presence of large PrP amyloid aggregates within GT1 cells, provoked a visible toxic or neurodegenerative response. As later discussed, fundamental agent properties that are relevant for human health can now be analyzed with this culture model.

MATERIALS AND METHODS

TSE AGENTS, BRAIN SAMPLES, MOUSE GENOTYPES AND TISSUE CULTURE LINES

A human vCJD brain sample (RU98) with all the verified clinical and pathological hallmarks of vCJD was provided by the NIBSC CJD Resource center in the UK. For reference and comparison we propagated a representative and relevant set of murine adapted human CJD agents (see Table I). With the exception of BSE, defined as a terrorist agent in the USA, the human CJD and scrapie agent strains were passaged here in mice by inoculating 1% brain homogenates intracerebrally (ic) as previously detailed [Manuelidis et al., 1978]. All mice used expressed wt PrP. NZW/LacJ mice were purchased from the Jackson Laboratory and both the NZW, and the Tga20 mice expressing 8× levels of murine PrP (gift of C. Weissmann, [Fischer et al., 1996]) were bred at Yale to provide sufficient numbers (~10 per test inocula) in parallel transmission experiments; outbred CD-1 mice were purchased as needed. Cultures of the GT1–7 hypothalamic cell line (hereafter called GT1 cells) were maintained and infected by a 3-day exposure to 0.2% normal control or infected brain homogenates as described [Arjona et al., 2004]. Stocks of stably infected cells carrying a variety of agent strains [Arjona et al., 2004; Nishida et al., 2005] were used for comparison to the new vCJD and 263K scrapie agent infections. Both primary and secondary passage mouse brains infected with the vCJD agent were used for culture infection and findings were replicated with brain homogenates from all the mouse genotypes, that is, Tga20, NZW, and CD-1. Because all samples gave the same positive result, they are not further discussed in the results.

INFECTION, TISSUE EVALUATION, CYTOCHEMISTRY AND WESTERN BLOTS

Inoculations, daily clinical evaluations and antigen detections were done by established and standard methods used for >30 years at Yale. Mice at 4–6 weeks of age were inoculated intracerebrally (ic) with 25–30 μl of a 1% brain homogenate (a 10^{-2} dilution) to reduce deaths from inoculation. Most other human vCJD to murine brain transmissions have been done with a higher 10% (10^{-1}) brain homogenates that should result in shorter incubation times. At clinical stages of disease, half the brain and representative other tissues were fixed with 4% paraformaldehyde in PBS, and processed for histology and immunocytochemistry as detailed [Manuelidis et al., 1997]. The unfixed half of each mouse brain was frozen at −70°C, and subsequently homogenized in PBS for Western blot and serial passage. For Western blots, 1 μl of an undigested 10% brain homogenate was typically loaded in the reference lane and 1×–12× this amount digested with 25 μg/ml proteinase K (PK) for 30 min to quantify the % PrP-res of total PrP by chemiluminesce [Liu et al., 2008]; PrP-res loads varied depending on both the mouse genotype and the specific agent strain to attain comparable PrP-res intensities (see Fig. 3). A reference ddY second passage mouse brain that had been infected with the BSE agent from a UK cow brain, and fixed and paraffin embedded in Japan (gift of N. Nishida), was also tested for regional PrP amyloid deposits in parallel.

RESULTS

The leftmost plot in Figure 1 graphs all inoculated mice in the primary transmissions of vCJD to outbred normal CD-1 mice, and to the Tga20 mice that express ~8 times more murine wt PrP. The same clinically and histologically verified human vCJD case was inoculated into all mice as a 1% human brain homogenate. According

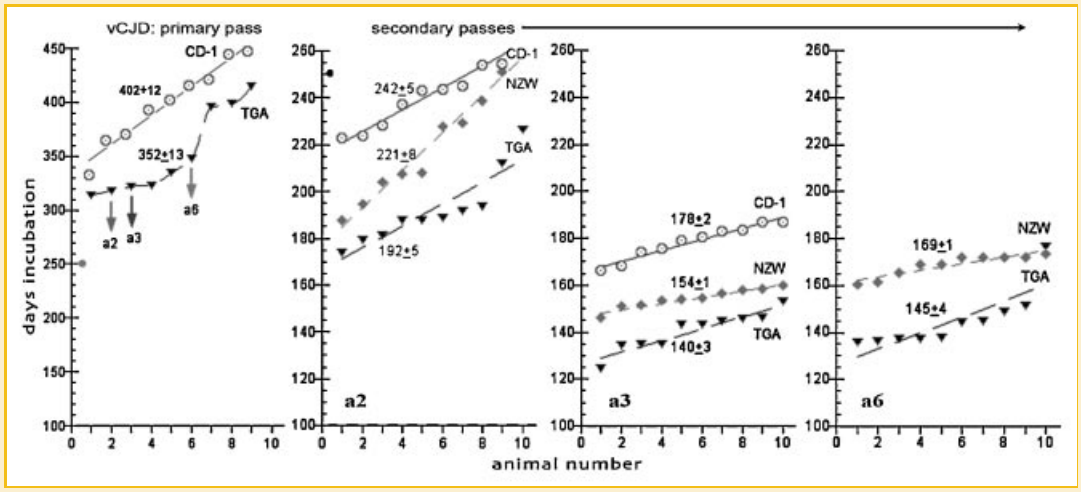

Fig. 1. Primary and secondary passages of vCJD in different genotypes of mice with wt PrP. Every mouse from each inoculated genotype group (9–10 mice each) is shown with group mean ± SEM. Three primary passage Tga20 mice (a2, a3, and a6) were used for a second murine passage as shown in the three adjacent panels to the right. Note the marked change in incubation time on 2° passages (2° incubation time scale reduced to ~½ as denoted by dots). Maximal non-toxic doses of brain homogenate (1% for mice) were inoculated in all mice shown here as well as in the other figures. By convention, maximal doses are inoculated ic for primary passage to overcome the species barrier.

to prion theory, high murine PrP expression, as in Tga20 mice, should create a very strong species barrier against the bovine and human TSE agents because each of these non-murine agents requires its own species-specific PrP sequence to induce a misfolded and infectious version of itself. Surprisingly, 100% of the Tga20 mice that express high levels of wt murine PrP (filled triangles) developed clear clinical symptoms at ~350 days, ~50 days earlier than the CD-1 mice with normal wt PrP levels (circles). The incubation times were significantly different between these two groups (paired *t*-test <0.0001). Thus high murine PrP expression resulted in greater susceptibility, rather than enhanced resistance, to the human vCJD agent. Moreover, all inoculated mice showed positive clinical neurological signs. These primary vCJD transmission to Tga20 and CD-1 mice were clearly more efficient than attempted transmissions of human vCJD to mice expressing only human PrP. For example, five patient samples (from France and the UK) inoculated intracerebrally (ic) in mice expressing a sixfold excess of human PrP encoding the 129MM sequence (the same sequence as in the vCJD patients), developed clinical signs only at far longer times (~500 days) than the mice here, and not all inoculated mice became sick [Béringue et al., 2008]. These inoculations were all done with higher equivalent homogenate concentrations than used here. Similarly, in another laboratory, a different group of Tg mice expressing only human PrP also showed a very long primary incubation time (>560 days) when inoculated with yet another UK verified vCJD human brain homogenate. Only a third humanized PrP Tg construct showed a Tga20-like incubation time [Korth et al., 2003] and serial passage did not reduce the initial incubation time. This indicated there was low vCJD agent adaptability in this Tg model. The continued long incubation time also made it an inefficient experimental model. Together, these Tg humanized PrP mouse results do not support the notion that a PrP species-specific sequence is required for effective infection of a foreign species. These data also undermine the concept that homologous PrP interactions facilitate the conversion of PrP into an "infectious form." On a biological level, the susceptibility of Tga20 mice to vCJD furthermore suggests that the human vCJD agent can be more virulent than the sporadic CJD (sCJD) agent. Sporadic CJD is very difficult to transmit to wt mice, with few positive takes and with primary incubation times of >500 days. Even after serial passage, the sCJD agent displays a very prolonged incubation time in wt PrP mice [Manuelidis et al., 1988, and see Table I]. Thus the BSE-linked vCJD agent is clearly different than the sCJD agent.

The 50-day shorter primary incubation time for vCJD in Tga20 versus CD-1 mice could be due simply to the higher PrP expression of Tga20 mice, that is, extra copies of the PrP host receptor for the infectious agent could facilitate agent uptake and reproduction. Inbred RIII mice with normal wt PrP levels also gave a Tga20-like primary incubation time of ~330 days when 10% vCJD brain homogenates were inoculated both intracerebrally (ic) and intraperitoneally [Bruce et al., 1997], and an incubation of ~355 days with two other 10% vCJD human brain homogenates inoculated only ic [Bruce et al., 2001]. However, when these homogenates were inoculated at a lower 1% concentration, as done here, the mean incubation was ~410 days, the same incubation time as found in our primary vCJD passage to outbred CD-1 mice (402 days). The longer incubations are consistent with the known lower infectious titers of more dilute samples. They confirm the similarity of all the different vCJD samples tested. Furthermore, in yet another laboratory, inbred normal FVB mice inoculated ic with two other human vCJD 1% brain homogenates also yielded a >400-day primary incubation time, and not all mice were affected [Asante et al., 2002]. Thus the Tga20 mice expressing high levels of murine wt PrP showed a consistent ~50-day incubation advantage over mice with normal levels of wt PrP, including inbred FVB and RIII mice, as well as outbred CD-1 mice. To find if host factors other than PrP might be involved in the enhanced susceptibility of

Tga20 mice to the vCJD agent, we serial passaged vCJD in Tga20, CD-1, and NZW mice. NZW mice also express normal levels of wt PrP. We chose NZW mice because several CJD and scrapie agents have yielded shorter incubation times, as well as a more extensive neuropathology as compared to other mouse genotypes including NZB (LM, unpublished data). NZW mice have high serum levels of retroviral gp70.

The relatively shorter Tga20 incubation time as compared to NZW and CD-1 mice was also found on subsequent serial murine passages. Figure 1 shows the secondary passages of vCJD in the three mouse genotypes, Tga20, CD-1, and NZW. Brain homogenates from three donor primary passage Tga20 mice, numbered a2, a3, and a6 in the graph were inoculated into these wt PrP mice. Secondary passage incubation times of each recipient mouse (from the a2, a3, and a6 donors) are shown in their respective panels. In all mice, the incubation time was markedly reduced during the second serial passage (note the large change in scale in graphs of the serial passages at dot). The a2 mouse was sacrificed when it showed only very early clinical signs. Nevertheless, it already produced secondary incubations that were dramatically shorter (by $>$100 days) than the primary passage mice. Additional primary passage donor mice (a3 and a6), sacrificed with more developed clinical signs when brains typically have even higher infectious titers, revealed an even larger $\sim$200-day reduction in incubation time on second passage. Thus the vCJD agent itself is able to adapt and evolve in a constant PrP background. Since the primary and secondary mouse passages have the same misfolded murine PrP-res, and no human PrP, it is difficult to explain this type of agent evolution with the prion hypothesis. Similar TSE changes on serial passage have been commonly observed with cross-species transmissions of other TSE agents [Manuelidis et al., 1997]. The three mouse genotypes also defined a smaller, but significantly different incubation time for each group ($t < 0.0001$ to <0.0003). This further indicated an influence of complex murine host factors, other than PrP, on susceptibility to infection. Tga20, NZW, and CD-1 mice yielded the shortest to longest incubations respectively regardless of the clinical stage and relative titer of the donor brain homogenates. Furthermore, mice receiving inocula from the two more developed vCJD samples (mouse a3 and a6) displayed identical second passage incubation times ($t > 0.6$ for tga20–Tga20 and NZW–NZW). Clinical signs that reflect involvement of major brain regions were present in all the recipient mice regardless of the donor sample or passage, and included initial hyperactivity with a unique type of spinning progressing to unsteady or slow gait, poor grooming, and hunching at later stages of disease.

Neuropathological evaluations and PrP molecular features substantiated the propagation of a distinct vCJD agent that was fundamentally faithful to its original source. Figure 2A shows a representative 2° passage CD-1 mouse brain with vCJD. Antibody binding regions are displayed in red in each panel and sections were treated to reveal only abnormal PrP as indicated. Adjacent slices are stained for glial fibrillary acidic protein (GFAP), a marker of astrocytic hypertrophy, as well as for Keratan sulfate (KS), a marker of microglia. All three antibodies delineate coincident regions of pathology, and as demonstrated many times before, these changes were not present in uninfected brains. The sections at low magnification show the heavy involvement of the hypothalamus (at arrow) as well as the thalamic nuclei in the PrP stained sections, with relative sparing of the cerebral cortex and hippocampus. The cortex shows only limited involvement, with PrP pathology restricted to a thin cortical layer. This vCJD picture contrasts with the one provoked by Japanese CJD agents that display a diffuse involvement of the cerebral cortex and widespread hippocampal lesions (Table I). The vCJD lesion profile is also completely different than found with sCJD agents. In mice infected with sCJD agents, PrP positive brain lesions are highly restricted to the medial thalamic region [Manuelidis et al., 1978; Manuelidis and Lu, 2000 and see Table I]. In contrast, there was a remarkably similar regional distribution of PrP amyloid deposition and spongiform change observed in mice infected with a UK-BSE brain sample from a diseased cow. Low power sections stained for pathological PrP in the BSE mouse brain are shown for comparison to vCJD at the bottom of Figure 2A as indicated. The coincident pattern of lesions in murine vCJD and BSE further solidifies a strong causal link between epidemic BSE and human vCJD. It also again shows that each TSE agent maintains its own distinct identity when passaged in a foreign species encoding a different species-specific PrP sequence.

Figure 2B–D shows representative higher power micrographs from the brains of CD-1 mice infected with the vCJD agent. The same pathology was found in all passages. An intense astroglial response in a region of the thalamus with spongiform change is shown in Figure 2B, and hypertrophic cell bodies packed with GFAP (g) in these regions are prominent. As shown in Figure 2C, PrP plaques (p) are abundant in the hypothalamus, as well as widespread vacuolization. In CD-1 mice, comparable PrP plaques to these were also found in the cerebellum, and notably, PrP plaques also mark this region in humans with vCJD. Figure 2D shows abundant activated microglia in the thalamus, with KS highlighting many arborizing processes (at m). KS positive microglia were abnormally increased in all regions with spongiform pathology. Furthermore, the obex was heavily involved in vCJD mouse brains, with strong staining of abnormal PrP (data not shown). The obex is also strongly affected in cows with BSE. Although the histological lesions showed slight variations in the different mouse genotypes, the distribution of severe spongiform change was similar, again emphasizing dominant characteristics are determined by the agent. PrP plaques and microglial activation were less prominent in the Tga20 mice, and in the NZW mice cerebellar plaques were not found. Thus although the major regional neuropathology is defined by the agent strain, complex murine host factors other than PrP can cause minor differences in plaque pathology as well as incubation times.

Lymphoid changes can also be used to discriminate some TSE strains. Figure 2E shows abnormal PrP deposits in spleen germinal centers that contain follicular dendritic cells. Previous confocal studies demonstrated that abnormal PrP accumulates at the surface of these cells in FU-CJD (Table I) with a comparable overall pattern to that seen here [Manuelidis et al., 2000]. This peripheral tissue feature does not distinguish the vCJD agent from Japanese CJD isolates but it does show again how vCJD is different from sCJD. The sCJD murine models do not show similar deposits of abnormal PrP in the spleen in any of our five independent sCJD transmissions to mice [Manuelidis et al., 1988]. Only small $\sim$1 μm abnormal PrP positive

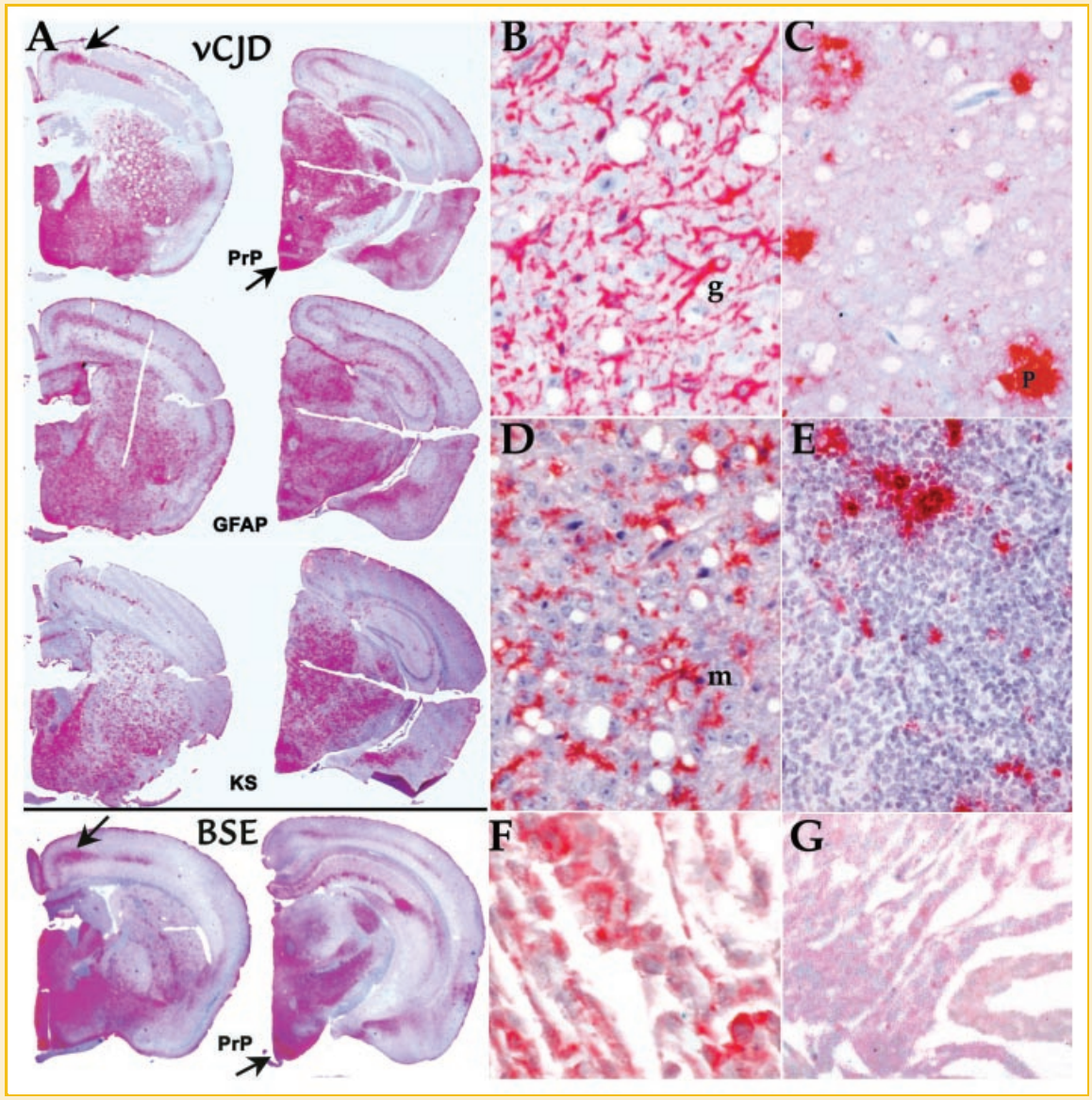

Fig. 2. Low power brain sections are shown in (A), with each section showing each antigen marker in red for pathological PrP, glial fibrillary acidic protein in astrocytes (GFAP) and Keratan sulfate positive microglia (KS). The top three sets show vCJD infected CD-1 mouse brains, and the bottom set shows comparable slices from BSE infected ddY mice. Note that both vCJD and BSE brains show a coincident pattern of PrP pathology. There is marked involvement of the hypothalamus (e.g., arrows by PrP) and thalamus, but few deposits of pathological PrP in the cerebral cortex and hippocampus. The cortex also displays a distinctive pattern of PrP pathology limited to a narrow layer of cortex (arrows). Higher power representative sections are shown in the micrographs B–G. B: Many abnormal astrocytic processes stained for GFAP as well as hypertrophied cell bodies (as at g); this hypothalamic region also shows the vacuoles typically found in TSEs. C: PrP plaques in the hypothalamus (as at p), and (D) shows microglial hypertrophy brought out by KS staining. The spleen is shown in E and demonstrates abundant PrP accumulations in germinal centers. Paraffin sections from GT1 hypothalamic cells in culture that were infected with the vCJD agent also displayed abnormal PrP accumulations intracellularly (F); uninfected control cells, prepared in parallel, show none (G).

bodies within macrophages could be found in the vicinity of some follicular dendritic cells in sCJD. Our vCJD and sCJD lymphoid tissues observations are also in accord with other recent results [Béringue et al., 2008]. However, universal conclusions about an absence of lymphoid targeting by a particular TSE agent can be misleading when based on transmissions only to a single species such as the mouse. This is important because lymphoid targeting has ramifications for the iatrogenic spread of these agents (see Discussion Section).

To further define if the incubation time, tissue distribution, and PrP molecular pathology induced by the vCJD agent was unique, we transmitted the hamster 263K scrapie strain to Tga20, CD-1, and NZW mice for comparison. This mutant 263K agent is very unusual, and hence should show very different transmission characteristics than vCJD. The 263K sheep derived scrapie strain was passaged in multiple species including goats, mice, hamsters and rats with repeated cloning by limiting dilution, and finally selected for its inapparent infection of mice [Kimberlin et al., 1989]. This commonly

used 263K strain also yields a uniquely short incubation time of ~60 days in hamsters, and produces unusually high levels of brain infectivity. The transmission of 263K to wt PrP mice also allowed us to compare its abnormal PrP profile on Western blots with that of vCJD and other representative, non-mutant CJD and scrapie strains (Table I). Although mouse brains typically show the same PrP-res pattern with different TSE agents, some CJD agents, such as vCJD, have been distinguished by minor PrP-res band differences in human brain [Asante et al., 2006]. In human vCJD the fastest migrating PrP-res band is 19 kDa, whereas in sCJD it is 21 kDa. We wanted to know if this distinctive vCJD brain band of 19 kDa PrP band could also be found in mice with vCJD. A diagnostic PrP band is of biological interest because it has been essential for demonstrating that two TSE agents can simultaneously infect in neuronal cells in culture [Nishida et al., 2005].

Figure 3A graphs the transmission of the 263K hamster scrapie agent to mice in four serial passages, and compares the progressive change in incubation times with those of serially passaged vCJD. The 263K scrapie agent showed markedly different properties than the vCJD agent. Firstly, whereas vCJD caused clinical disease in all mice, only 5/9 mice showed clinical signs of 263K scrapie in the primary passage. One mouse died of a lymphoma without spongiform change, and the three other mice died at an old age without clinical signs at the same very prolonged incubation time (>600 days). Because of autolysis we do not know if these three mice were infected, but since some scrapie agents as well as sCJD can latently infect mice until they die of old age [Dickinson et al., 1975; Manuelidis et al., 2000], they are included in the incubation time calculation. Secondly, unlike the vCJD serial transmissions that showed a marked reduction in incubation time from the 1st to the 2nd passages, the infected 263K scrapie mice showed only a minimally reduced incubation time on the second passage. This indicates a relative lack of adaptability intrinsic to the 263K scrapie agent. The incubation time remained very prolonged at >500 days. The 3rd passage of 263K scrapie continued to be very long (355 days in CD-1 mice) and was significantly more, by ~150 days, than the 3rd passage of vCJD in CD-1 mice; these 263K mice displayed well-developed clinical signs by 186 days. Thus wt PrP mice are strongly protected against infection by the 263K scrapie agent. The stringent 263K agent selection process probably isolated a rare mutant strain that was defective for utilizing murine PrP as a receptor. This 263K mutant also shows relatively little adaptability and still had remarkably long incubations even after four serial passages. Thirdly,

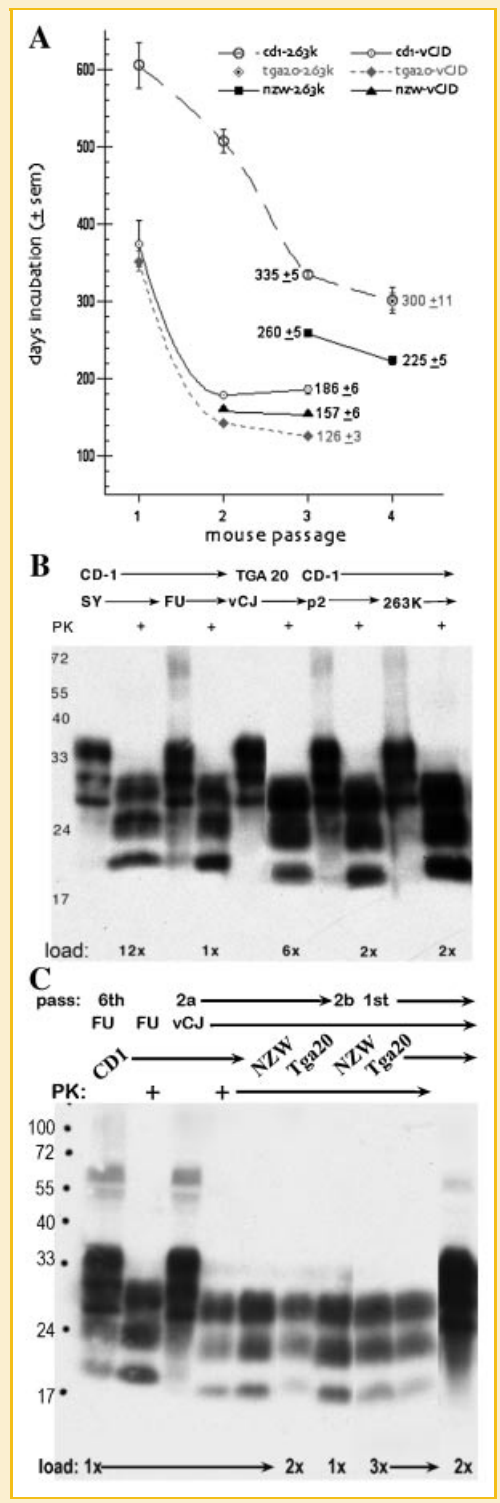

Fig. 3. A: Compares the human vCJD agent and the hamster 263K agent inoculated in mice of different genotypes at primary and progressive serial passages. All vCJD inoculated mice showed an incubation time that rapidly falls, and then levels off between passages 2 and 3; (the 2nd passage days are the mean of a3 and a6 mouse recipients). In contrast to the sudden drop in incubation time in the 2nd passage of vCJD, the 263K scrapie agent shows only a small decrease in the 2nd passage. Even after 4 passages, the 263K scrapie incubation time is still very prolonged at 300 days in both CD-1 and Tga20 mice in stark contrast to vCJD-Tga20 mice. Only the NZW mice infected with the 263K scrapie agent displayed significantly shorter incubation times than parallel inoculated CD-1 mice. These agent differences in the same host genotypes demonstrate host–agent interactions that cannot be determined by PrP sequence or amount. B,C: Western blot PrP bands from representative brain samples in the different mouse genotypes; the agent strain and serial passage are labeled. Lanes digested with PK (+) as well as relative loads in each lane are also indicated. Only the vCJD samples showed the lower 19 kDa band. The very different sCJD (SY), Japanese CJD (FU), and 263K scrapie agents all show the same PrP pattern in infected mouse brain, and could not be distinguished from each other on this basis.

NZW mice inoculated in parallel with the same 263K brain homogenates showed significantly shorter incubation times than the other mouse genotypes. At the 3rd passage NZW mice developed disease at 260 days versus 335 days for CD-1 mice. This NZW-263K incubation still remained 100 days longer than the NZW-vCJD incubations (260 vs. 157 days, $t < 0.0001$). This is a profound difference in the ~650-day lifespan of the mouse. Thus the NZW genotype, and not its constant murine wt PrP, significantly affected the susceptibility of these mice to the 263K scrapie agent. Finally, passage of 263K scrapie to Tga20 mice further distinguished the 263K and vCJD strains. 263K inoculated Tga20 mice gave the same long incubation time (300 days) as CD-1 mice at passage 4. Thus an 8× higher expression of murine wt PrP had no effect on the 263K scrapie incubation time whereas it enhanced vCJD infection. Thus the high levels of host PrP were insufficient to "proportionally" increase the susceptibility of mice to this 263K agent as proposed in the prion hypothesis [Prusiner et al., 1990].

Figure 3B shows a representative blot of PrP and PrP-res in mouse brains infected with four very different TSE agents that are summarized in Table I. The long incubation sCJD agent (SY-CJD) that induces very restricted anatomical lesions in the CNS, the fast incubation Japanese FU-CJD agent that produces widespread brain lesions, and the cloned 263K scrapie agent with its own unique brain lesion profile (data not shown), all display the same major three-band PrP-res pattern. On these blots the two highest Mr bands are glycosylated whereas the lowest band is not [Sklaviadis et al., 1986]; PK digestion attacks the more accessible regions of PrP with less β-amyloid folding. It also removes the N terminal region of PrP [Arjona et al., 2004] to give rise to the truncated PrP-res fragments. Because the migration and intensities of these three PrP-res bands are the same, these agents are indistinguishable in mouse brains on the basis of their molecular PrP-res profiles. In contrast, the vCJD brains showed that the third, slowest migrating PrP band was always ~19 kDa as compared to the 21 kDa band visualized with all the other agents. This vCJD PrP-res difference allowed it to be discriminated from the other infected brains, and was also apparent in the Tga20 and the CD-1 mouse genotypes as shown in Figure 3B. To further confirm the universality of this lower ~19 kDa PrP-res band in vCJD infected murine brains, we looked at many vCJD infected brain samples. These included brains derived from the three different primary passage animals shown in Figure 1, as well as the three mouse genotypes. Figure 3C representatively shows that all these vCJD infected mice have the faster migrating 19 kDa band that is diagnostic for infection by the vCJD agent. Control uninfected brain showed no PrP-res or any other bands as depicted in multiple previous experiments [e.g., Manuelidis, 1998, data not shown]. This consistent human vCJD-like 19 kDa fragment in both high and normal wt PrP murine brains was unexpected because it has not been previously demonstrated in wt mice, and can even be absent from PrP humanized mice inoculated with vCJD [Hill et al., 1997]. Human PrP is insufficient to convert itself into a vCJD strain with a unique PrP-res profile.

In mice and humans, PrP-res bands can be tissue type specific rather than agent-strain specific, and all the evidence to date shows TSE agents are not modified by their tissue source. We previously propagated CJD and scrapie agents in two monotypic neuronal cell lines. These infected cells showed very divergent PrP-res profiles from each other and from brain, but the fundamental agent biology was unaltered. The original mouse incubation times and agent-specific neuropathological features were reproduced on retransmission of infected cells to animals [Arjona et al., 2004]. This indicates that PrP pathology can be used to identify an agent strain even though it does not encode agent properties. Nevertheless, when a unique PrP-res band is diagnostically specific for a given agent, the PrP-res band becomes useful experimentally, as in the confirmation of infection with two different agents [Nishida et al., 2005]. The successful transmission of a variety of different agents in the established murine hypothalamic cell line (GT1) has also made its possible to simplify variable murine genotype factors, cell type differences, and the complexity of host responses that can be evoked by the agent in different tissues such as brain and spleen. Thus we attempted to transmit vCJD to GT1 cells for simpler agent strain comparisons. We also wanted to find if this single monotypic cell type alone could produce the diagnostic PrP-res band of 19 kDa. It would also be possible to determine if this 19 kDa PrP-res band induced any cellular pathology. We transmitted the 263K scrapie agent to GT1 cells at the same time as an additional parallel control.

To show major cell-type PrP-res band patterns that are not agent-strain specific we included infected N2a58 cells on gels as seen in the representative Western blot in Figure 4 as controls. Two N2a58 cultures infected with the FU-CJD and 22L scrapie agents are shown in the N2a58 lanes. GT1 cells infected with various CJD and scrapie agents are shown in other lanes with undigested or digested (PK+) samples. Sample loads are indicated, and mock infected GT1 cells (lanes labeled non) showed no PrP-res or any other bands even with high 13× sample loads. The N2a58 cells infected with the 22L scrapie and the FU-CJD agents (N_2 lanes) show very different PrP and PrP-res patterns from GT1 cells infected with the same two agents, that is, the PrP and PrP-res bands are cell type, but not agent type specific. Both the N2a58 and GT1 cell line PrP patterns are also different than the typical standard three-band pattern of brain as shown in Figure 3B,C. In GT1 cells, infection with the Japanese FU-CJD agent resulted in a unique 13 kDa PrP band not seen in brain or in N2a58 cells [Nishida et al., 2005]. This FU-CJD specific PrP band was also visualized here in the non-digested samples (at arrow) as well as in the PK sample. To confirm this Japanese isolate represented a unique and reproducible geographic strain of CJD, we passaged a second CJD agent from Japan in CD-1 mice. This agent (lane YA) provoked an identical band pattern in GT1 cells, including the extra 13 kDa band not seen in mouse brain. In contrast to this pattern, the undigested GT1 sample with vCJD (vCJ lane) showed a doublet of bands (dotted) at 19 kDa. The two bands of this doublet were probably resolved from each other, as compared to the digested 19 kDa PrP-res band, because it was more abundant in the digested sample. Four different vCJD brain homogenates from first and second passage mice, including all the genotypes used (Tga20, NZW, and CD-1), were tested for their ability to infect GT1 cells. All these vCJD samples induced the identical vCJD pattern in culture with the diagnostic 19 kDa PrP-res band. Only vCJD provoked the 19 kDa PrP-res doublet, whereas all the other agents in GT1 cells, including the 263K scrapie agent, yielded the standard 21 kDa PrP-res band.

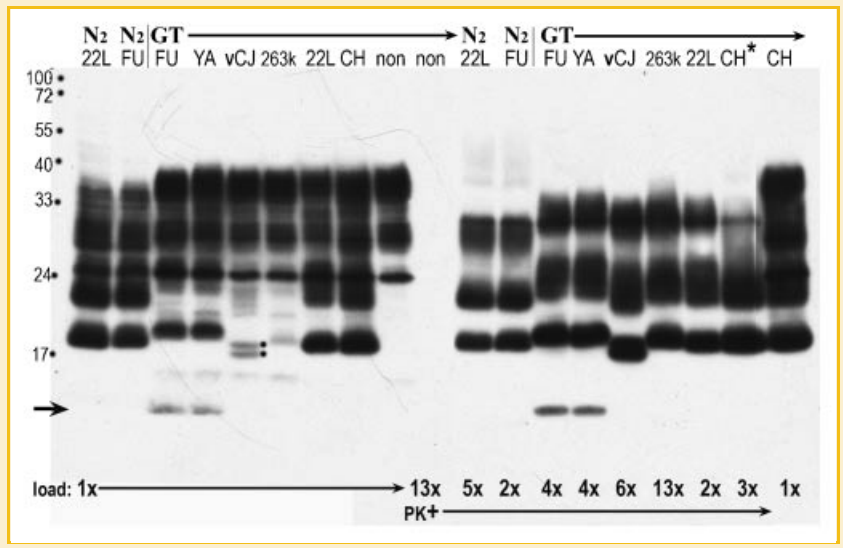

Fig. 4. Western blot of the PrP and PrP-res pattern of cultured cells infected with different TSE agents. N2a58 cells infected with 22L scrapie and Japanese FU-CJD agents (lanes labeled N_2) show a very different pattern than GT1 cells, infected with these same agents, as well as with a variety of other TSE agent strains. Despite these cell-type differences in PrP band pathology in cultured cells, neither the 22L nor the FU agents were modified. The FU and 22L agents, but not cell-type PrP-res patterns, bred true as shown by re-inoculation of mice [Arjona et al., 2004; Nishida et al., 2005; Liu et al., 2008]. PK + lanes for PrP-res are indicated. The Japanese CJD isolates FU and YAM (lane labeled YA) show the same 13 kDa band in both undigested and digested samples, and this band distinguishes these geographic isolates from all the other agents in GT1 cells. Since this band is not visible in infected brain, it is a cell type-specific band that can be induced only by Asiatic CJD agents. The undigested vCJD lane (labeled vCJ) also shows a doublet at 19 kDa that corresponds to the thicker 19 kDa band in the PK + lane, as well as to the 19 kDa band found in brain (see Fig. 3). This 19 kDa doublet was consistently observed after infection with donor brain samples from 1° and 2° passages of vCJD propagated in all the different host genotypes (Tga20, NZW, and CD-1). All the other agents induced the 21 kDa PrP band in GT1 cells. Note that PrP and PrP-res profiles are virtually the same after infection with the markedly different 263K and 22L scrapie agents. Chandler-RML scrapie infection resulted in a greater sensitivity of PrP to limited PK digestion.

There were negligible differences in the pattern or amount of the two higher molecular weight glycosylated PrP-res bands with five of the six agents. The Chandler-RML scrapie agent however induced abnormal PrP that had a greater PrP sensitivity to PK with a lower proportion of the slowest migrating ~34 kDa PrP-res band (lane CH*). These PrP and PrP-res patterns were all reproducible as determined by analysis of other passages as well as replicate GT1 infections with vCJD.

Finally, the production of abnormal intracellular PrP amyloid was apparent in vCJD cells, and comparable to its accumulation in other infected cultures [Arjona et al., 2004; Liu et al., 2008]. Figure 3F shows these intracellular PrP amyloid aggregates in infected cells. They were not present in control uninfected GT1 cells that were stained and processed in parallel (Fig. 3G). Both the infected and uninfected cells appeared healthy morphologically, and a causal role for the vCJD diagnostic doublet (as for all other TSE-specific PrP-res bands identified to date), show that they do not cause obvious toxicity or neurodegenerative changes in these neuronal cells. Indeed, non-neuronal brain cells such as microglia and astrocytes may instead elaborate the major toxic factors in TSEs [Manuelidis et al., 1997]. In summary, a 19 kDa PrP-res band corresponding to that found in human vCJD infected brain was also found in both wt PrP mouse brain and monotypic neuronal cells. In GT1 cultures this band appeared as a doublet and required no artificial proteinase digestion for visualization. It was produced by living cells, and generated no obvious toxicity. This 19 kDa band represents a feature that allows one to rapidly distinguish infection by the BSE-linked vCJD agent from other TSE agents in wt PrP murine, as well as primate brains.

DISCUSSION

The above studies with the human vCJD agent in wt PrP mice, the most reliable model for evaluating the many different TSE agent strains, clarified both the tissue and the cell type changes diagnostic for vCJD infection. Together the data demonstrate that PrP species-specific misfolding do not distinguish, and cannot encode, the profoundly different agent properties observed. Rather, some agents may leave their individual imprint when docking to host PrP for infection. In some cases this imprint is species-specific, whereas in others it is cell-type specific. In the case of the BSE-linked vCJD agent, the 19 kDa PrP fragment is diagnostic for this newly virulent agent, regardless of the species origin (cow or human) or species to which it is transmitted (primate and murine). The 19 kDa band was also found in monotypic neuronal cells in culture further indicating vCJD is a highly invariant agent. In addition, the distribution of lesions and brain pathology in murine vCJD and BSE were remarkably similar. Large plaques were also observed that were consistent with those seen in human vCJD brains. Other agents, such as sCJD, give a completely different picture, as previously demonstrated, and summarized here in Table I. Because PrP is identical in all regions of brain, the profoundly different regional distribution of lesions induced by different agent strains is difficult

to reconcile with any observed or even artificially mutated constructs of host PrP.

These studies also generated convenient and rapid mouse and culture models of vCJD as well additional details that are important for concepts of the infectious agent. We have demonstrated effective transmission and serial propagation of the infectious vCJD agent from human brain to mice not only with normal, but also with high copy levels of murine wt PrP. All the mice of each genotype (outbred CD-1, inbred NZW and Tga20) were highly susceptible to the human agent. The Tga20 mice with 8× levels of wt murine PrP were the most susceptible to vCJD, as shown by their shorter incubation times. This observation is inconsistent with the simple concept that PrP sequence homology determines the TSE species barrier to infection, as well as the proposal that high copies of a foreign PrP sequence will augment the species barrier. Thus prion theory predicts that there will be few positive animals and very prolonged incubation times on human to mouse vCJD transmission, and that high copy Tga20 mice will have the longest incubations and fewest positive animals. However, all the wt PrP mice inoculated here were completely susceptible to the human vCJD agent. The Tga20 mice showed the shortest primary incubation time, as well as the most rapid incubation times on serial passages. The higher level of murine PrP thus facilitated infection, as would be expected for a viral receptor. The wt PrP mice of all genotypes also displayed shorter incubation times than several models of Tg mice expressing high levels of only human PrP. Humanized PrP mice in different laboratories have repeatedly shown very prolonged primary incubation times that were 100–200 days longer than demonstrated here [Asante et al., 2002; Béringue et al., 2008]. Second passages have not significantly shortened these vCJD incubation times, making the humanized PrP models less attractive experimentally. Together, these data emphasize vCJD agent properties of cross-species virulence that are not determined by PrP sequence, or by some yet to be resolved special type of PrP tertiary conformation.

The unique cloned and mutated hamster 263K scrapie agent, unlike the vCJD agent, was much more difficult to transmit to the same mouse genotypes and adapted more slowly than the vCJD agent. The 263K scrapie agent maintained a very prolonged incubation time over four serial passages in the constant murine PrP background. In addition, unlike vCJD, Tga20 mice infected with this agent showed longer incubation times than mice with normal PrP levels on serial passage. Only propagation in the NZW genotype of mice (with the identical wt PrP) led to a significantly shorter, but still prolonged incubation time of >220 days. These differences again show that vCJD and 263K scrapie agents display dominant agent-encoded properties, including ones that may be sensitive to complex background mouse factors not related to PrP. Comparison of other agent strains in wt PrP mice, including sporadic CJD (sCJD) and Japanese CJD isolates, as detailed in Table I, further confirm the dramatic differences among agent strains, by incubation time after serial mouse passages, as well as by neuropathology. The mutant 263K scrapie agent also shows stable and reduced infectious capabilities in mice that are different from the parental scrapie strain. Thus it is a permanent change in this agent, not the species PrP, which determines the ability of these agents to infect a foreign species. Indeed, the remarkable precision of incubation times shown by different TSE agents in normal mice has been known for many years, and remains completely unexplained. How a host protein can thermodynamically determine the timing of its own conversion, and also encode such a diversity of incubation times (100–600 days) is, perhaps, even more of a mystery.

The 263K scrapie agent had a paradoxically long incubation time in high PrP copy mice, as compared to the vCJD and sCJD agents that display significantly faster incubation times in Tga20 mice than in other mouse genotypes with normal PrP levels. Thus for vCJD and sCJD agents, high levels of PrP itself can enhance infection, even when the PrP does not match the PrP of the original, natural host. These data are also consistent with our proposal that host PrP is a necessary receptor for TSE agents, and minor changes in PrP sequence may be less important for transmission than intrinsic agent properties [reviewed in Manuelidis, 2007]. Minor PrP sequence differences among species have been unable to predict the actual cross-species spread of agents [Manuelidis, 2000]. Intrinsic agent properties of cross-species spread and PrP receptor preferences, are apparent during vCJD transmissions to Tg mice expressing only bovine PrP. The human vCJD agent infected bovinized PrP mice much more rapidly and efficiently than humanized PrP mice [Scott et al., 1999; Asante et al., 2002; Béringue et al., 2008]. This again underscores a profound and stable agent identity that was not altered by its >5-year replication in human brain that contains no bovine PrP. The BSE-linked vCJD agent still clearly preferred cow PrP as a receptor. Similarly, murine passage did not alter the fundamental nature of a typical sheep scrapie agent as shown by retransmission experiments [Zlotnik and Rennie, 1965]. This underscores the stability of yet another TSE agent. This stability strongly indicates an independent agent structure with its own nucleic acid genome. As shown here, the vCJD and BSE agents are essentially identical as they display not only the same neuropathology in mice, but also the same diagnostic 19 kDa PrP band imprint.

Epidemiologically, infection of humans by the BSE agent appears to be very rare and much less effective than infection of other species. The conclusion that this agent may pose no real threat to human health, however, should be tempered by the potential for more efficient human-to-human spread of vCJD as it adapts to the human host; such spread can take place through iatrogenic routes, including transfusion [Manuelidis, 1997; Lasmézas et al., 2001]. The spleen of vCJD mice contained abundant abnormal PrP deposits that mimicked the lymphoreticular involvement described in people with vCJD [Hill et al., 1999]. This represents a change in tissue targeting from the one seen in cows. Cows with BSE show no infection of lymphoid tissues, whereas other primates infected with vCJD do [Lasmézas et al., 2001]. The sCJD agent can likewise show poor targeting of murine spleen but profound splenic involvement when transmitted to rats [Manuelidis et al., 1997]. The public health ramifications of splenic involvement in humans with vCJD are not yet clear. Because of the long inapparent clinical stage of vCJD in humans, it is important to have a convenient animal model to evaluate the spread of vCJD. The serially passaged vCJD mouse model, with its relatively short incubation time (125–150 days), provides a highly relevant and valid way to test the risks of transmitting vCJD by blood transfusion as well as by other tissues and routes. Strong lymphoreticular involvement gives a TSE agent more

avenues for spread, and the BSE agent may show unpredictable and greater virulence for different tissues as it adapts to the human host. Universal conclusions about a lack of lymphoid targeting, and the consequent improbability of blood and peripheral tissue infections by a given TSE agent, such as BSE, can be misleading when based on only to a single species. This also further complicates public health measures to contain the spread of BSE and vCJD to wild animals [Manuelidis, 1997].

Recently it has been reported that some human vCJD samples contain a minor sCJD-like variant [Asante et al., 2002; Béringue et al., 2008]. Animal models cannot clarify if this sCJD variant represents a mutant agent generated by a parental vCJD particle, or if it represents selection of an evolving variant brought out by growth in the more resistant human species. Because cells infected with a single agent can be selected by cloning, it should be possible to determine if the original vCJD agent can actually give rise to an attenuated mutant such as sCJD that does not induce the 19 kDa PrP band. One can assess the stability of the vCJD agent by following the 19 kDa PrP band. A third alternative for the finding of sCJD in vCJD human brains is that a vCJD brain may harbor an unrelated independent sCJD agent that is latent or clinically inapparent. Such latent agents may be brought out of hiding by neurodegenerative changes as well as other stresses [Manuelidis, 1994]. Double infections of mice as well as cultured cells by TSE agents have been demonstrated, and the rapid identification of infection by two different agents in GT1 cells is facilitated by agent-diagnostic PrP markers [Manuelidis and Lu, 2000; Nishida et al., 2005]. Moreover, superinfection experiments, where an infected cell is challenged with a second agent, can also clarify the relationship of vCJD to other TSE agents. In virology, such experiments are classically used to identify closely related agents from more distant ones. The 19 kDa PrP marker that is diagnostic for the vCJD agent can be used to find if sCJD and vCJD agents can independently infect the same cells in culture. Since the vCJD particle appears to have very stable properties, it would seem more likely that the vCJD agent may infect people who in some cases may already harbor the latent sCJD agent.

The study of TSE agents in monotypic cells added further insight into constant agent properties. Transmission of TSE agents to different types of neuronal cells in culture, such as the N2a58 neuroblastoma cell line and the GT1 hypothalamic cell line, has shown that PrP-res profiles are strongly cell-type rather than agent-type specific. Long term growth of an agent in these monotypic cells with markedly different PrP glycosylation and PrP-res fragment patterns from each other, as well as from brain, does not alter the agent phenotype [Arjona et al., 2004]. Nevertheless, some agents can induce pathological PrP bands that are diagnostic for that particular agent. Simplified cell culture models effectively uncovered one such diagnostic marker in GT1 cells infected with the Japanese FU-CJD agent. FU-CJD infected cells showed a 13 kDa PrP-res band that was not apparent in infected human or mouse brains, nor in N2a58 cells [Arjona et al., 2004; Nishida et al., 2005]. Results here from another Japanese CJD case (YAM-CJD), replicated the 13 kDa PrP-res band in GT1 cells. Thus this 13 kDa marker was not only specific for the geographic TSE agent, but was also cell-type dependent. By testing additional CJD and scrapie agents we further expanded these findings. The 22L and 263K scrapie agents that are widely divergent by both incubation times and neuropathology, showed the same PrP-res banding pattern as well as the common 21 kDa fragment found with many other TSE agents (Table I). In contrast, the 19 kDa PrP marker was specific only for vCJD. Remarkably, the same 19 kDa band was seen in vCJD mouse brain, as shown here, and also in both vCJD and BSE primate brains [Lasmézas et al., 2001]. Hence it was a diagnostic marker that was independent of cell type and species, and therefore can be used reliably to diagnose vCJD in widely divergent species as well as monotypic cell cultures. The cell culture model will also facilitate the fractionation and isolation of vCJD infectious particles using new accurate and rapid GT1 cell infectivity assays [Liu et al., 2008]. Additionally, these cells can be monitored to find if they contain the same 25 nm viruslike particles found in other TSE infected cells and preparations [Manuelidis et al., 2007].

Currently there is a great deal of interest in finding the PrP fragment or tertiary conformation that could cause neurodegeneration. Peptide fragments of PrP that form amyloid fibrils can be toxic when added to neuroblastoma cultures [Forloni et al., 1993]. However, we have yet to see any toxic effects of PrP amyloid deposits generated by infection of hypothalamic and N2a58 neuroblastoma cells [Arjona et al., 2004; Liu et al., 2008] or in the GT1 cells infected with the vCJD agent. Notably, both the Japanese CJD agent and the vCJD agent induced short abnormal PrP fragments that were present in living cells not artificially treated with PK and detergents. These abnormal PrP fragments provoked no direct toxic effects on either type of neuronal cells. It is likely that all of the various versions of abnormal PrP shown here, in situ, and by Western blot, are the result of infection by an exogenous and stable viral particle, and are a consequence of the neurodegenerative disease process rather than its cause. Based on protein array studies of progressive CJD brain infections [Lu et al., 2004], high levels of agent, as well as complex innate immune factors and cellular responses to the foreign infectious particle probably cause the severe neurodegenerative changes in animals. Non-neuronal cells can contribute to the spongiform pathology, as shown by early changes in rat brain [Manuelidis et al., 1997]. Although often discussed as an isolated molecule, it is worth remembering that PrP is a tethered membrane protein that does not work alone. PrP pathology is probably generated by a complex set of host molecular interactions that are induced by the invading infectious particle.

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