Molecular Bases of Disease:

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Characterization of Variant Creutzfeldt-Jakob Disease Prions in Prion Protein-humanized Mice Carrying Distinct Codon 129 Genotypes*

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Background: Secondary vCJD infection may occur in all human *PRNP* genotypes, but its clinicopathological and biochemical phenotype is uncertain.
Results: The biochemical characteristics and transmission properties of the newly generated vCJD prions are not affected by the host *PRNP* genotypes.
Conclusion: Secondary vCJD infection can be adequately diagnosed by biochemical analysis and experimental transmission.
Significance: Effective means to identify secondary vCJD infection are presented.

To date, all clinical variant Creutzfeldt-Jakob disease (vCJD) patients are homozygous for methionine at polymorphic codon 129 (129M/M) of the prion protein (PrP) gene. However, the appearance of asymptomatic secondary vCJD infection in individuals with a *PRNP* codon 129 genotype other than M/M and transmission studies using animal models have raised the concern that all humans might be susceptible to vCJD prions, especially via secondary infection. To reevaluate this possibility and to analyze in detail the transmission properties of vCJD prions to transgenic animals carrying distinct codon 129 genotype, we performed intracerebral inoculation of vCJD prions to humanized knock-in mice carrying all possible codon 129 genotypes (129M/M, 129M/V, or 129V/V). All humanized knock-in mouse lines were susceptible to vCJD infection, although the attack rate gradually decreased from 129M/M to 129M/V and to 129V/V. The amount of PrP deposition including florid/amyloid plaques in the brain also gradually decreased from 129M/M to 129M/V and to 129V/V. The biochemical properties of protease-resistant abnormal PrP in the brain and transmissibility of these humanized mouse-passaged vCJD prions upon subpassage into knock-in mice expressing bovine PrP were not affected by the codon 129 genotype. These results indicate that individuals with the 129V/V genotype may be more susceptible to secondary vCJD infection than expected and may lack the neuropathological characteristics observed in vCJD patients with the 129M/M genotype. Besides the molecular typing of protease-resistant PrP in the brain, transmission studies using knock-in mice carrying bovine PrP may aid the differential diagnosis of secondary vCJD infection, especially in individuals with the 129V/V genotype.

Prion diseases are fatal transmissible neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD)[2] in human, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep and goats. Among human prion diseases, variant CJD (vCJD) is remarkable because of its causative link with dietary exposure to BSE prions and accumulation of infectivity in lymphoreticular tissues as well as the central nervous system (1). Indeed, secondary vCJD infection has occurred through iatrogenic routes such as blood transfusion (2–5). The pathogenesis of prion diseases is associated with the accumulation of the abnormal isoform (PrP^{Sc}) of prion protein (PrP), which is converted from the normal cellular isoform (PrP^{C}) (6). Susceptibility to vCJD is influenced by the normal polymorphism at codon 129 (methionine (M) or valine (V)) of the PrP gene (*PRNP*). To date, all patients with probable and definite vCJD are homozygous for methionine at codon 129 (129M/M) (7). However, asymptomatic peripheral involvement in vCJD infection has been reported in two 129M/V individuals (3, 5). One patient had received transfusion of red cells from a donor who subsequently died from vCJD, and the other patient had received treatment with plasma products from a donor who subsequently died from vCJD. In addition, a retrospective study on the prevalence of subclinical vCJD infection using appendectomy and tonsillectomy specimens in the United Kingdom found 3/12,648 positive cases, two of which were found to be the 129V/V genotype (8, 9). These studies indicate that individuals with the 129M/V or 129V/V genotype may be susceptible to vCJD, including secondary vCJD infection. Because definite

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[2] The abbreviations used are: CJD, Creutzfeldt-Jakob disease; vCJD, variant CJD; sCJD, sporadic CJD; dCJD, dura mater graft-associated CJD; PrP, prion protein; BSE, bovine spongiform encephalopathy; FDC, follicular dendritic cell.

TABLE 1
Transmission of vCJD prions to knock-in mice expressing human PrP

Inoculum[a]	Genotype	Positive/total[b]	Incubation	Incubation of positive transmission
vCJD96/02	Ki-Hu129M/M	5/6	540.2 ± 82.6	455, 458, 552, 595, 641
	Ki-Hu129M/V	2/5	576.5 ± 6.4	572, 581
	Ki-Hu129V/V	0/4		
	TK-Hu129V (2.1×)[c]	3/7	799.3 ± 139.7	638, 880, 880
vCJD05/02	Ki-Hu129M/M	11/11	668.0 ± 69.1	529, 607, 622, 635, 641, 666, 697, 720, 739, 740, 752
	Ki-Hu129M/V	5/5	793.0 ± 44.5	743, 761, 783, 834, 844
	Ki-Hu129V/V	5/8	765.8 ± 73.9	720, 728, 742, 742, 897
	TK-Hu129V (2.1×)	6/7	803.2 ± 85.6	683, 750, 761, 838, 890, 897

[a] Intracerebral inoculation with 20 μl of a 10% (w/v) brain homogenate from either of the vCJD patients.
[b] The number of mice positive for PrP accumulation in the immunohistochemical analysis/number of inoculated mice.
[c] TK-Hu129V express human PrP with 129V at a 2.1-fold level of wild-type mouse brain.

vCJD cases with the 129M/V or 129V/V genotype with PrP^{Sc} accumulation in the brain have not yet been identified, it is unknown whether the clinicopathological characteristics and biochemical properties of vCJD with the 129M/M genotype will appear in patients with the other genotypes. In 2009, one patient with the 129M/V genotype who presented atypical symptoms and the MRI pulvinar sign, one of the clinical characteristics of vCJD, was reported (10). However, no tonsil biopsy or autopsy was performed, and the diagnosis therefore remains uncertain.

To gain insights into clinicopathological phenotype of vCJD with a *PRNP* genotype other than 129M/M, transmission studies using humanized transgenic mice or knock-in mice have been performed (11–15). These studies raised the possibility that the neuropathological phenotypes of vCJD in individuals with a 129M/V or 129V/V genotype might be different from that of patients with the 129M/M genotype and questioned the current neuropathological diagnostic criteria for vCJD. To reevaluate these findings and to analyze in detail transmission properties of vCJD prions to animals carrying distinct *PRNP* codon 129 genotypes, we performed intracerebral inoculation of vCJD prions to PrP-humanized knock-in mice with the *PRNP* 129M/M, 129M/V, or 129V/V genotypes.

EXPERIMENTAL PROCEDURES

Production of Knock-in Mice and Transgenic Mice—The generation of the knock-in mice was reported previously (16, 17). The ORF of murine *Prnp* was replaced by the bovine *PRNP* (Ki-Bov/Bov), human *PRNP* with the 129M/M genotype (Ki-Hu129M/M), or human *PRNP* with the 129V/V genotype (Ki-Hu129V/V). We produced Ki-Hu129M/V by cross-breeding. To assess the effect of overexpression of human PrP with the 129V/V genotype, Ki-Hu129V/V were crossed with transgenic mice expressing human PrP with 129V as reported previously (TK-Hu129V) (18).

Sources of Prion Inocula and Transmission Experiments—Human brain tissues were obtained at autopsy from CJD patients after receiving informed consent for research use. Brain homogenates were prepared from patients with vCJD (MM2B, cases 96/02 and 05/02), sCJD (MM1, MM2C, MM2T, MV1, MV2, or VV2), or dura mater graft-associated CJD with or without PrP plaques (dCJD/PL or dCJD/SY) (19). The ORF of *PRNP* was analyzed by PCR direct sequencing (20). Human or mouse brain homogenates (10%) were prepared as described previously (21). Transmission studies were performed using 20 μl of the homogenates for intracerebral inoculation or 50 μl for intraperitoneal inoculation. Intraperitoneally inoculated mice were sacrificed at 75 days after inoculation for the follicular dendritic cell (FDC) assay. Our previous study showed that the level of PrP^{Sc} that accumulated in the FDC of the spleen of knock-in mice reached a plateau at 45 days after inoculation (16). Thus, we decided to perform the FDC assay at 75 days after inoculation (17). Half of the spleen was immediately frozen for Western blotting, and the remaining half was fixed in 10% buffered formalin for the immunohistochemistry. Intracerebrally inoculated mice were sacrificed after the onset of clinical disease or at death. One hemisphere of the brain was immediately frozen for Western blotting or subpassage, and the other hemisphere was fixed in 10% buffered formalin for immunohistochemistry.

Western Blotting—PrP^{Sc} was extracted from either spleen or brain with collagenase treatment as described previously (22) with modifications. For the protease-resistant core of PrP^{Sc} (PrP^{res}) analysis of the spleen, samples (corresponding to 7.5 mg of wet weight of spleen tissue at most) were subjected to 13.5% SDS-PAGE and transferred to a PVDF membrane. ChW antiserum (17) was used as the primary antibody, and anti-rabbit EnVision was used as the secondary antibody. For PrP^{res} analysis in the brain, the 3F4 antibody was used as the primary antibody, and anti-mouse EnVision was used as the secondary antibody. Enhanced chemiluminescence detection (GE Healthcare) was used to visualize Western blots. The signal intensities of the Western blots were quantified with the Quantity One software using an imaging device VersaDoc 5000 (Bio-Rad Laboratories).

Immunohistochemistry—Mouse tissues were treated with 60% formic acid before embedding in paraffin wax. Tissue sections were pretreated by hydrolytic autoclaving before PrP immunohistochemistry (23). The PrP-N antiserum (24) or rabbit anti-glial fibrillary acidic protein polyclonal antibody (Dako) was used as the primary antibody. A goat anti-rabbit immunoglobulin polyclonal antibody labeled with a peroxidase-conjugated dextran polymer, EnVision (Dako), was used as the secondary antibody.

Image Analysis—For morphometric analysis, digital microscopy images were analyzed using the ImageJ software (rsb.info.nih.gov/ij). Background intensity thresholds were first applied using an ImageJ macro, which measures pixel intensity across all immunostained and unstained areas of the images. The obtained pixel intensity threshold value was then applied in all subsequent analyses. Next, the number of positively immuno-

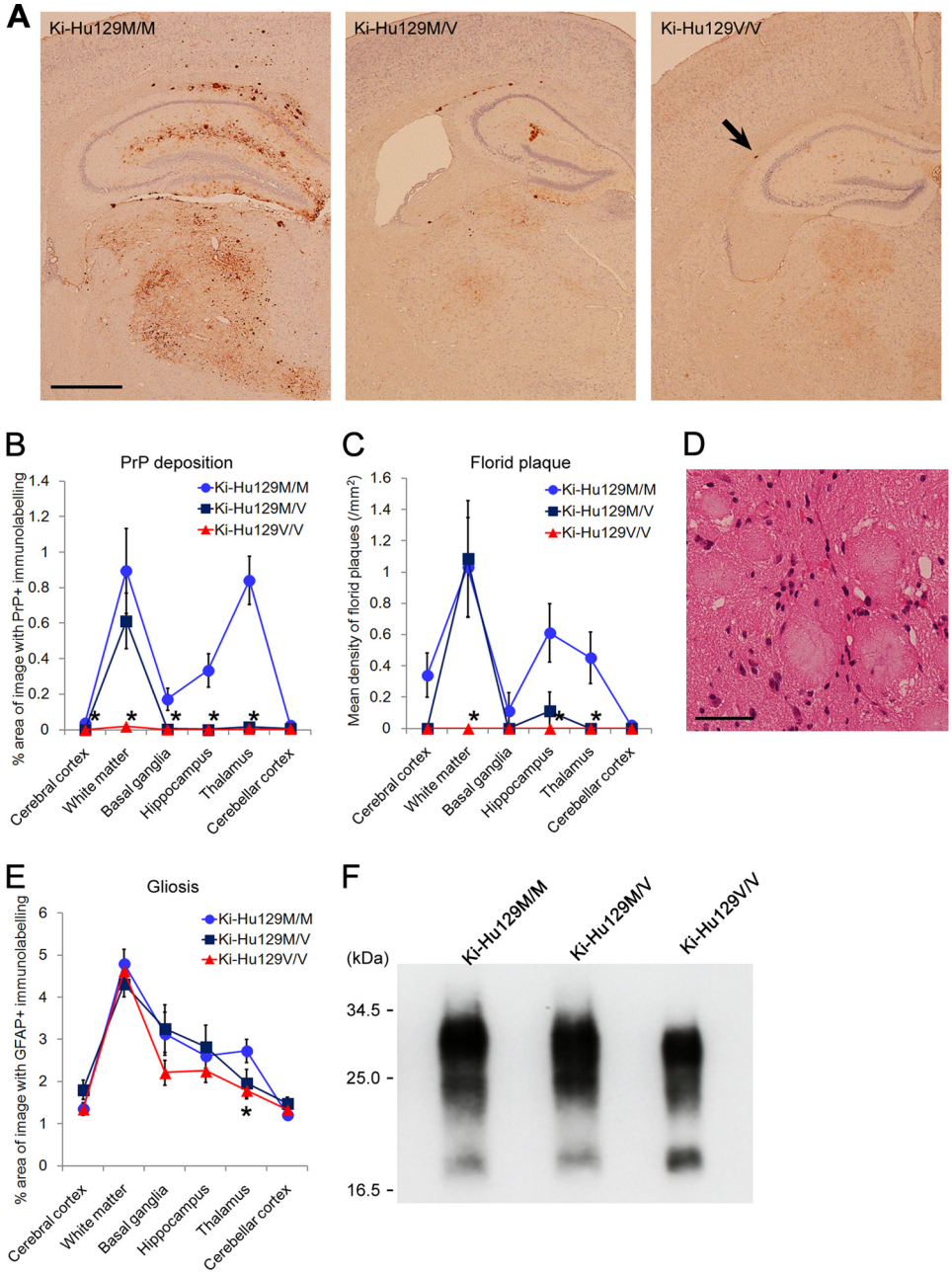

FIGURE 1. **vCJD transmission to humanized knock-in mice.** *A*, immunohistochemical analysis of abnormal PrP in the brain of humanized knock-in mice inoculated with vCJD prions (vCJD05/02). Ki-Hu129V/V had small plaques (*arrow*) and coarse PrP deposits mainly in the cerebral white matter and thalamus. *Scale bar*: 500 μm. *B* and *C*, regional distribution of PrP deposition (*B*) or florid plaques (*C*) in the brain. *D*, florid plaques found in the brain of Ki-Hu129V/V (hematoxylin and eosin stain). *Scale bar*: 50 μm. *E*, quantification of anti-glial fibrillary acidic protein-positive ($GFAP^+$) astrocytes in the brain. For morphometric analysis, at least four representative digital microscopy images of the cerebral cortices, white matter, basal ganglia, hippocampus, thalamus, and the cerebellar cortices were obtained from each mouse. These images were analyzed using the ImageJ software to quantify PrP deposition, florid plaques, and gliosis. Data were collected from all diseased vCJD05/02-inoculated mice. *, $p < 0.05$. *F*, Western blot analysis of PrP^{res} in the brain. Ki-Hu129V/V (the percentage of di-, mono-, and nonglycosylated PrP^{res} was 60.3, 25.8, and 13.2%, respectively) showed similar glycosylation patterns to those in Ki-Hu129M/M (di:mono:non = 65.9%:24.8%:9.3%) or those in Ki-Hu129M/V (di:mono:non = 53.9%:35.4%:10.6%).

stained pixels was automatically counted and presented as a proportion of the total number of pixels in each area under analysis. For the quantification of florid/amyloid plaques, the number of florid/amyloid plaques was manually counted on hematoxylin and eosin-stained sections. Data were collected from all diseased mice.

Statistical Analysis—Data are presented as mean ± S.D. Differences between groups were analyzed by one-way analysis of variance with Tukey-Kramer post test using the JMP Pro software version 10.0 (SAS Institute Inc.). Values $p < 0.05$ were considered as significant.

RESULTS

Transmission of vCJD Prions to Knock-in Mice Expressing Human PrP—The intracerebral transmission experiments of vCJD prions to humanized knock-in mice are summarized in Table 1. These knock-in mice (Ki-Hu129M/M, Ki-Hu129M/V, or Ki-Hu129V/V) express human PrP with the 129M/M,

TABLE 2
Intraperitoneal transmission of humanized mouse-passaged vCJD prions to knock-in mice expressing bovine PrP

Genotype	Inoculum[a]	Positive mice/total[a]	Positive FDC/follicles[c]	Lane in Fig. 2
129M/M	vCJD96/02	6/6	54/545 (9.90%)	
	vCJD96/07	4/5	24/367 (6.5%)	
	vCJD05/02	4/4	99/294 (33.67%)	vCJD05/02
	sCJD-MM1 (H3)	0/5	0/605	sCJD-MM1
	sCJD-MM2C (Kn)	0/4	0/448	sCJD-MM2C
	sCJD-MM2T (Ng)	0/4	0/406	sCJD-MM2T
	Ki-Hu129M/M[vCJD05/02]	6/6	209/738 (28.32%)	Ki-Hu129M/M[vCJD]
	dCJD (Gf, SY)	0/3	0/293	dCJD/SYa
	dCJD (Tk, SY)	0/4	0/296	dCJD/SYb
	dCJD (Tk, PL)	0/5	0/499	dCJD/PLa
	dCJD (Kh, PL)	0/5	0/338	dCJD/PLb
	dCJD (Kr, PL)	0/6	0/618	dCJD/PLc
129M/V	sCJD-MV1 (Sm)	0/5	0/416	sCJD-MV1
	sCJD-MV2 (Ph)	0/5	0/471	sCJD-MV2
	Ki-Hu129M/V[vCJD05/02]	6/6	129/741 (17.41%)	Ki-Hu129M/V[vCJD]
129V/V	sCJD-VV2 (Ak-1)	0/4	0/424	sCJD-VV2a
	sCJD-VV2 (Ak-2)	0/5	0/484	sCJD-VV2b
	Ki-Hu129V/V[vCJD05/02] (exp1)[d]	5/5	33/481 (6.86%)	Ki-Hu129V/V[vCJD]a
	Ki-Hu129V/V[vCJD05/02] (exp2)	6/6	85/619 (13.73%)	Ki-Hu129V/V[vCJD]b

[a] Intraperitoneal inoculation with 50 μl of a 10% (w/v) brain homogenate. Ki-Hu129M/M[vCJD05/02], Ki-Hu129M/V[vCJD05/02] Ki-Hu129V/V[vCJD05/02], or TK-Hu129V[vCJD05/02] represent the brain homogenate from Ki-Hu129M/M mice with vCJD05/02, Ki-Hu129M/V mice with vCJD05/02, Ki-Hu129V/V mice with vCJD05/02, or TK-Hu129V mice with vCJD05/02, respectively.
[b] The number of mice positive for PrP accumulation in immunohistochemical analysis/number of inoculated mice.
[c] The number of PrP^{Sc}-positive FDCs/number of follicles examined (with percentages in parentheses).
[d] Transmission experiments of Ki-Hu129V/V[vCJD05/02] were performed twice independently using different inocula.

129M/V, or 129V/V genotype at the same level. Because a high expression level of PrP in transgenic mice influences directly the prion disease incubation time regardless of the host PrP genotype, these knock-in mice have an advantage over transgenic mice for evaluating the susceptibility of each genotype (17). In addition, knock-in mice enable an equivalent expression from heterozygous genes. Ki-Hu129M/M and Ki-Hu129M/V showed high and moderate susceptibility, respectively, to vCJD prions. Furthermore, Ki-Hu129V/V unexpectedly showed moderate susceptibility to one of the two vCJD inocula (vCJD05/02). To confirm the susceptibility of 129V/V animals to vCJD prions, we also inoculated vCJD prions to TK-Hu129V carrying human PrP with 129V at a level 2.1-fold that of wild-type mouse brain. These mice showed moderate susceptibility to both vCJD inocula. Immunohistochemical analysis of PrP revealed that the amount of PrP deposition in the brain gradually decreased from Ki-Hu129M/M to Ki-Hu129M/V to Ki-Hu129V/V (Fig. 1, *A* and *B*). In Ki-Hu129M/M, coarse PrP deposits accompanied by large PrP plaques were distributed throughout most brain areas. By contrast, PrP deposits were much fewer and mainly restricted to within the cerebral white matter and thalamus in Ki-Hu129V/V. Moreover, the number of florid/amyloid plaques, one of the pathological hallmarks of vCJD, also gradually decreased from Ki-Hu129M/M to Ki-Hu129M/V to Ki-Hu129V/V (Fig. 1*C*). Among the five diseased Ki-Hu129V/V, only a single mouse had florid/amyloid plaques only in septum (Fig. 1*D*). Ki-Hu129V/V showed slightly mild gliosis when compared with Ki-Hu129M/M or Ki-Hu129M/V (Fig. 1*E*). Western blot analysis of PrP^{res} demonstrated that the biochemical properties of PrP^{res} in the brain were almost the same among the codon 129 genotypes (Fig. 1*F*). These PrP^{res} retained characteristics of vCJD PrP^{res} such as a nonglycosylated fragment located at 19 kDa and the predominance of the diglycosylated fragment.

Transmission of Humanized Mouse-passaged vCJD Prions to Knock-in Mice Expressing Bovine PrP—To further evaluate the characteristics of the prions generated in humanized mice infected with vCJD (hereafter these prions are denoted as Ki-Hu129M/M[vCJD05/02], Ki-Hu129M/V[vCJD05/02], Ki-Hu129V/V[vCJD05/02], or TK-Hu129V[vCJD05/02]: host mouse[original inoculum]), we then subpassaged these prions to knock-in mice expressing bovine PrP (Ki-Bov/Bov). We reported previously that Ki-Bov/Bov were highly susceptible to vCJD prions. vCJD represents human infection with BSE from cattle (17). This phenomenon has been designated as "traceback," and traceback studies have been proven to be a useful tool to identify the origin of prions (17, 25–27). Therefore, we examined whether the transmissibility to Ki-Bov/Bov was maintained among humanized mouse-passaged vCJD prions. As reported previously (17), Ki-Bov/Bov intraperitoneally inoculated with vCJD prions showed PrP deposition in the FDC of the spleen at 75 days after inoculation, whereas PrP deposition was not observed in Ki-Bov/Bov inoculated with sCJD prions or dCJD prions (Table 2). Furthermore, all Ki-Bov/Bov intraperitoneally inoculated with humanized mouse-passaged vCJD prions showed PrP deposition in the spleen. Similarly, Western blot analysis of PrP^{res} in the spleen revealed that Ki-Bov/Bov were susceptible to Ki-Hu129M/M[vCJD05/02], Ki-Hu129M/V[vCJD05/02], Ki-Hu129V/V[vCJD05/02], and TK-Hu129V-[vCJD05/02] as well as to the parental vCJD05/02 prions (Fig. 2).

To confirm the transmissibility of humanized mouse-passaged vCJD prions to Ki-Bov/Bov, we also subpassaged these prions by intracerebral inoculation. Similar to the results of intraperitoneal inoculation, Ki-Hu129M/M[vCJD05/02], Ki-Hu129M/V[vCJD05/02], and Ki-Hu129V/V[vCJD05/02] were successfully transmitted to Ki-Bov/Bov with a 100% attack rate (Table 3). The affected Ki-Bov/Bov showed coarse PrP deposits and PrP plaques in most brain areas (Fig. 3). The pattern of PrP deposition was almost the same regardless of the codon 129 genotype of the inocula. By contrast, Ki-Bov/Bov inoculated with sCJD-VV2 prions survived until an advanced age without either clinical signs or the accumulation of PrP in

the brain. These results clearly showed that the transmissibility of vCJD prions to Ki-Bov/Bov was maintained after a passage through each codon 129 genotype.

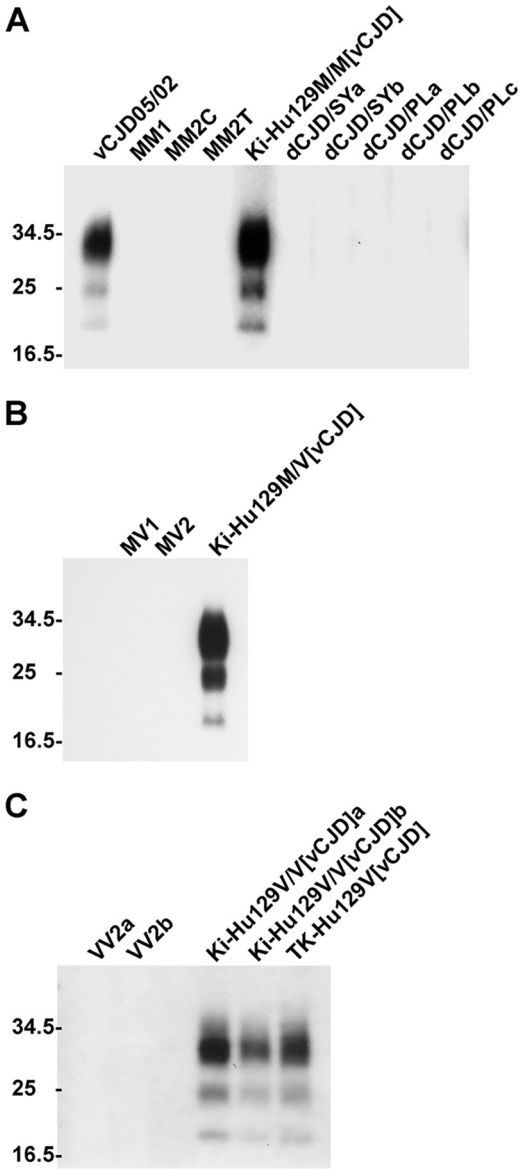

FIGURE 2. **Western blot analysis for PrP^{res} in proteinase K-treated spleen homogenates from Ki-Bov/Bov.** *A*, intraperitoneal inoculation of CJD prions with the 129M/M genotype. *B*, intraperitoneal inoculation of CJD prions with the 129M/V genotype. *C*, intraperitoneal inoculation of CJD prions with the 129V/V genotype. Positive immunoreactivities were observed only in Ki-Bov/Bov mice inoculated with vCJD05/02, Ki-Hu129M/M[vCJD05/02], Ki-Hu129M/V[vCJD05/02], Ki-Hu129V/V[vCJD05/02], or TK-Hu129V[vCJD05/02]. *Numbers* show the molecular size standards (kDa).

DISCUSSION

Here we report a detailed comparison of the transmission properties of vCJD prions among humanized knock-in mice carrying distinct *PRNP* codon 129 genotypes. All three humanized knock-in mouse lines were susceptible to vCJD infection, although the attack rate gradually decreased from 129M/M to 129M/V to 129V/V. The amount of PrP deposition including florid/amyloid plaques in the brain also gradually decreased from 129M/M to 129M/V to 129V/V. The biochemical properties of PrP^{res} in the brain and the transmissibility of these humanized mouse-passaged vCJD prions upon subpassage into Ki-Bov/Bov were not affected by the codon 129 genotype. These results indicate that individuals with the 129V/V genotype may be more susceptible to secondary vCJD infection than expected and may lack some neuropathological characteristics observed in vCJD patients with the 129M/M genotype.

These results have potential public health implications concerning the future occurrence of secondary vCJD transmission to individuals carrying the 129M/V or 129V/V genotype. We had expected that Ki-Hu129V/V were highly resistant to vCJD infection because these mice showed negative results when intraperitoneally challenged with vCJD prions (17, 29). In addition, Bishop *et al.* (15) reported a very low attack rate (1/16 (6.25%)) in humanized knock-in mice with the 129V/V genotype intracerebrally inoculated with vCJD prions. However, Ki-Hu129V/V showed moderate susceptibility to intracerebral transmission of vCJD in the present study (the sum total attack rate from two independent experiments using different inocula: 5/12 (41.7%)). This susceptibility is comparable with the reported attack rate in transgenic mice expressing human PrP with 129V (the sum total attack rate from six independent experiments using different inocula: 25/56 (44.6%)) (11, 13). Because the expression level of PrP directly affects the susceptibility to prion infection regardless of the codon 129 genotype, the susceptibility reported in the transgenic mice carrying human PrP with 129V to vCJD prions had been considered to be due to their high PrP expression level (15). However, the present study clearly shows that this is not solely due to the overexpression of PrP. The route of infection in the present study is not that expected for the human-to-human transmission of vCJD, *e.g.* blood transfusion contaminated with a lower dose of vCJD prions, suggesting that the possible secondary infection might be restricted. Meanwhile, intravenous transmission of BSE is as efficient as the intracerebral inoculation (29). We reported previously that knock-in mice expressing human PrP with heterozygosity for glutamine/lysine at another polymorphic codon 219 (219E/K) are susceptible to vCJD prions (28). Indeed, two vCJD patients with the 219E/K genotype

TABLE 3
Intracerebral transmission of humanized mouse-passaged vCJD prions to knock-in mice expressing bovine PrP

Inoculum[a]	Positive/total[b]	Incubation	Incubation of positive transmission
Ki-Hu129M/M[vCJD05/02]	5/5	605.2 ± 53.5	559, 573, 582, 620, 692
Ki-Hu129M/V[vCJD05/02]	5/5	598.2 ± 47.3	543, 553, 617, 648, 630
Ki-Hu129V/V[vCJD05/02]	4/4	772.8 ± 35.5	727, 770, 781, 813
sCJD-VV2 (Ak-2)	0/6		

[a] Intracerebral inoculation with 20 μl of a 10% (w/v) brain homogenate. Ki-Hu129M/M[vCJD05/02], Ki-Hu129M/V[vCJD05/02], or Ki-Hu129V/V[vCJD05/02] represent the brain homogenate from Ki-Hu129M/M mice with vCJD05/02, Ki-Hu129M/V mice with vCJD05/02, or Ki-Hu129V/V mice with vCJD05/02, respectively.
[b] The number of mice positive for PrP accumulation in the immunohistochemical analysis/number of inoculated mice.

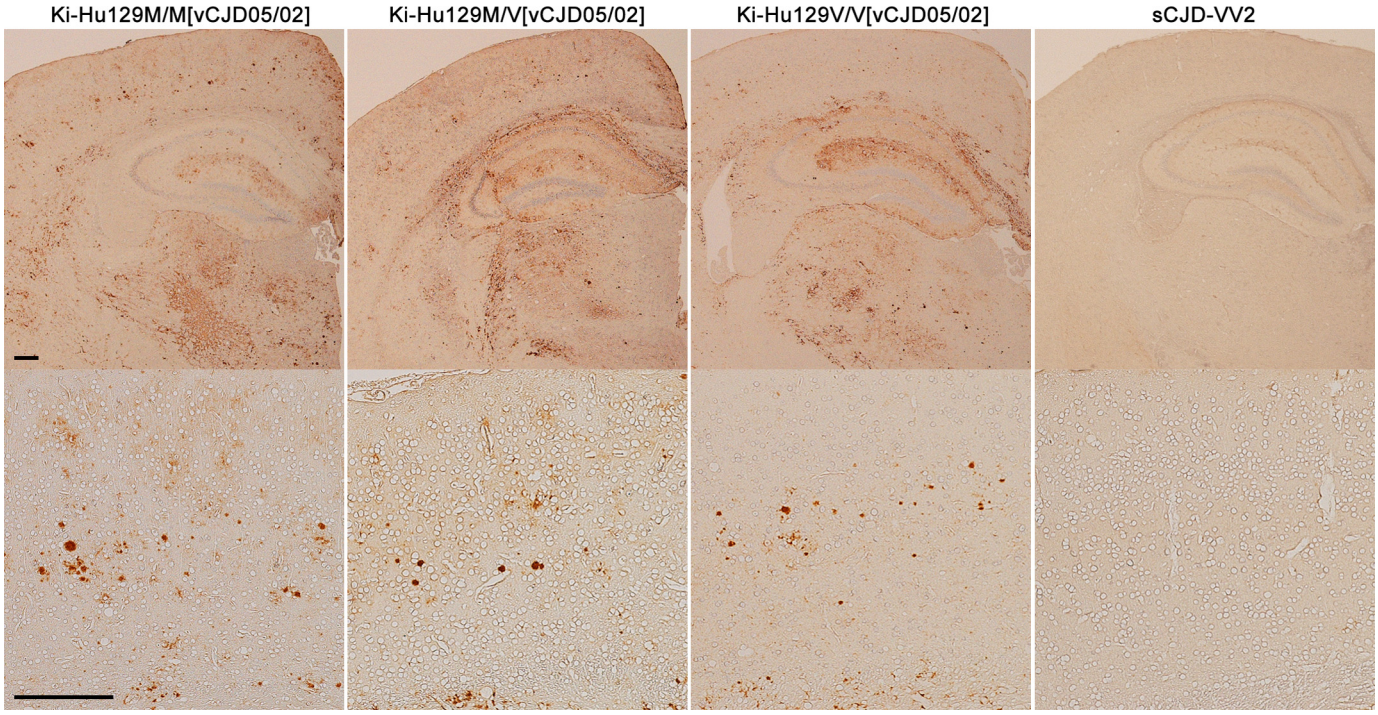

FIGURE 3. **Immunohistochemical analysis of abnormal PrP in the brain of Ki-Bov/Bov.** Coarse PrP deposits accompanied by PrP plaques were observed in Ki-Bov/Bov intracerebrally inoculated with Ki-Hu129M/M[vCJD05/02], Ki-Hu129M/V[vCJD05/02], or Ki-Hu129V/V[vCJD05/02], whereas Ki-Bov/Bov inoculated with sCJD-VV2 prions showed no positive staining. *Scale bars*: 200 μm.

were reported subsequently (30). Therefore, our transmission study using humanized knock-in mice could properly predict that individuals with the 219E/K genotype, which is rarely observed in the European population (31, 32), have a potential risk for vCJD infection. Taken together, the present intracerebral transmission data raise the concern that individuals with the 129V/V genotype are more susceptible to secondary vCJD infection than had been expected.

The reason for the fluctuating transmissibility of vCJD prions between the two inocula (vCJD96/02 and 05/02; attack rates in Ki-Hu129V/V were 0/4 (0%) and 5/8 (62.5%), respectively) is unclear. Similar fluctuation in transmissibility among vCJD inocula has been observed in transmission study using transgenic mice (from 0 to 80%) (13). These fluctuations might be due to differences in the prion titers in the inoculum. Western blot analysis of PrP^{res} in the brain of the patients showed that vCJD05/02 had greater amounts of PrP^{res} (about five times) than vCJD96/02, perhaps reflecting the clinical course of vCJD05/02 (43 months), which was much longer than that of vCJD96/02 (18 months). The low vCJD attack rate in humanized knock-in mice with the 129V/V genotype reported by another group (15) might also be explained by the prion titer in the inoculum as their inoculum was diluted to 10^{-2}, whereas our inoculum was a 10^{-1} dilution. Although typical vCJD cases were selected for this study, further extensive analysis with additional cases having various PrP^{res} concentrations should be carried out in the future.

We confirmed that the characteristic neuropathological features of vCJD can be modified through transmission to the 129M/V or 129V/V genotype as reported previously (11, 13–15). Particularly, the amount of PrP deposition and the number of florid/amyloid plaques in the brain, one of the most important clinicopathological hallmarks of vCJD, were markedly reduced in Ki-Hu129V/V in the present study. Four out of five (80%) diseased Ki-Hu129V/V lacked florid/amyloid plaques, despite extensive examination of the brain. Similarly, florid/amyloid plaques have never been observed in other mouse models of vCJD carrying the 129V/V (or 129M/V) genotype (11, 14, 15). Although the neuropathological features of humanized knock-in mice with vCJD may not be fully recapitulated in human brain tissue with vCJD, the present study, together with data from other groups, raises the concern that vCJD with the 129V/V genotype cannot be neuropathologically distinguished from sCJD patients with the 129V/V genotype (*e.g.* sCJD-VV2). In contrast to the neuropathological phenotype, the biochemical properties of PrP^{res} were not altered through transmission to the 129M/V or 129V/V genotype as reported in another knock-in mouse model (15). These results support the view that the molecular typing of PrP^{res} will remain a useful diagnostic feature of secondary vCJD infection irrespective of the codon 129 genotype (15).

The present study shows that transmission studies using Ki-Bov/Bov are also a useful means to detect vCJD prions, even in secondary infection. Not only Ki-Hu129M/M[vCJD05/02] but also Ki-Hu129M/V[vCJD05/02] and Ki-Hu129V/V[vCJD05/02] showed positive transmissibility to Ki-Bov/Bov, *i.e.* the traceback phenomenon, whereas all sCJD or dCJD prions examined failed to be transmitted. These results suggest that BSE prions retain their host preference after repeated passages through human PrP regardless of the codon 129 genotype. Intracerebral transmission generally shows higher sensitivity when compared with intraperitoneal transmission but requires a very long incubation period of over 2 years to obtain results. In contrast, the FDC assay after intraperitoneal inoculation

requires only 75 days to assess the transmissibility. In addition, the positive rate of the FDC assay was as high as that of intracerebral transmission in the present study. Therefore, the FDC assay after intraperitoneal inoculation of patient materials to Ki-Bov/Bov may help in the differential diagnosis of vCJD when atypical cases emerge in CJD surveillance (33, 34).

In conclusion, the present study underpins the importance of systematic assessment of all human prion disease patients based on the clinicopathological phenotype and molecular typing of PrP^{res} to monitor secondary vCJD infection (13–15). Traceback studies using Ki-Bov/Bov may facilitate the differential diagnosis of secondary vCJD infection, especially in individuals with the *PRNP* 129V/V genotype.

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REFERENCES

1. Wadsworth, J. D., Joiner, S., Hill, A. F., Campbell, T. A., Desbruslais, M., Luthert, P. J., and Collinge, J. (2001) Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. *Lancet* **358,** 171–180
2. Llewelyn, C. A., Hewitt, P. E., Knight, R. S., Amar, K., Cousens, S., Mackenzie, J., and Will, R. G. (2004) Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet* **363,** 417–421
3. Peden, A. H., Head, M. W., Ritchie, D. L., Bell, J. E., and Ironside, J. W. (2004) Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* **364,** 527–529
4. Wroe, S. J., Pal, S., Siddique, D., Hyare, H., Macfarlane, R., Joiner, S., Linehan, J. M., Brandner, S., Wadsworth, J. D., Hewitt, P., and Collinge, J. (2006) Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. *Lancet* **368,** 2061–2067
5. Peden, A., McCardle, L., Head, M. W., Love, S., Ward, H. J., Cousens, S. N., Keeling, D. M., Millar, C. M., Hill, F. G., and Ironside, J. W. (2010) Variant CJD infection in the spleen of a neurologically asymptomatic UK adult patient with haemophilia. *Haemophilia* **16,** 296–304
6. Prusiner, S. B., Scott, M. R., DeArmond, S. J., and Cohen, F. E. (1998) Prion protein biology. *Cell* **93,** 337–348
7. Will, R. G., Zeidler, M., Stewart, G. E., Macleod, M. A., Ironside, J. W., Cousens, S. N., Mackenzie, J., Estibeiro, K., Green, A. J., and Knight, R. S. (2000) Diagnosis of new variant Creutzfeldt-Jakob disease. *Ann. Neurol.* **47,** 575–582
8. Hilton, D. A., Ghani, A. C., Conyers, L., Edwards, P., McCardle, L., Ritchie, D., Penney, M., Hegazy, D., and Ironside, J. W. (2004) Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. *J. Pathol.* **203,** 733–739
9. Ironside, J. W., Bishop, M. T., Connolly, K., Hegazy, D., Lowrie, S., Le Grice, M., Ritchie, D. L., McCardle, L. M., and Hilton, D. A. (2006) Variant Creutzfeldt-Jakob disease: prion protein genotype analysis of positive appendix tissue samples from a retrospective prevalence study. *BMJ* **332,** 1186–1188
10. Kaski, D., Mead, S., Hyare, H., Cooper, S., Jampana, R., Overell, J., Knight, R., Collinge, J., and Rudge, P. (2009) Variant CJD in an individual heterozygous for PRNP codon 129. *Lancet* **374,** 2128
11. Hill, A. F., Desbruslais, M., Joiner, S., Sidle, K. C., Gowland, I., Collinge, J., Doey, L. J., and Lantos, P. (1997) The same prion strain causes vCJD and BSE. *Nature* **389,** 448–450, 526
12. Asante, E. A., Linehan, J. M., Desbruslais, M., Joiner, S., Gowland, I., Wood, A. L., Welch, J., Hill, A. F., Lloyd, S. E., Wadsworth, J. D., and Collinge, J. (2002) BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *EMBO J.* **21,** 6358–6366
13. Wadsworth, J. D., Asante, E. A., Desbruslais, M., Linehan, J. M., Joiner, S., Gowland, I., Welch, J., Stone, L., Lloyd, S. E., Hill, A. F., Brandner, S., and Collinge, J. (2004) Human prion protein with valine 129 prevents expression of variant CJD phenotype. *Science* **306,** 1793–1796
14. Asante, E. A., Linehan, J. M., Gowland, I., Joiner, S., Fox, K., Cooper, S., Osiguwa, O., Gorry, M., Welch, J., Houghton, R., Desbruslais, M., Brandner, S., Wadsworth, J. D., and Collinge, J. (2006) Dissociation of pathological and molecular phenotype of variant Creutzfeldt-Jakob disease in transgenic human prion protein 129 heterozygous mice. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 10759–10764
15. Bishop, M. T., Hart, P., Aitchison, L., Baybutt, H. N., Plinston, C., Thomson, V., Tuzi, N. L., Head, M. W., Ironside, J. W., Will, R. G., and Manson, J. C. (2006) Predicting susceptibility and incubation time of human-to-human transmission of vCJD. *Lancet Neurol.* **5,** 393–398
16. Kitamoto, T., Mohri, S., Ironside, J. W., Miyoshi, I., Tanaka, T., Kitamoto, N., Itohara, S., Kasai, N., Katsuki, M., Higuchi, J., Muramoto, T., and Shin, R. W. (2002) Follicular dendritic cell of the knock-in mouse provides a new bioassay for human prions. *Biochem. Biophys. Res. Commun.* **294,** 280–286
17. Asano, M., Mohri, S., Ironside, J. W., Ito, M., Tamaoki, N., and Kitamoto, T. (2006) vCJD prion acquires altered virulence through trans-species infection. *Biochem. Biophys. Res. Commun.* **342,** 293–299
18. Kobayashi, A., Asano, M., Mohri, S., and Kitamoto, T. (2007) Cross-sequence transmission of sporadic Creutzfeldt-Jakob disease creates a new prion strain. *J. Biol. Chem.* **282,** 30022–30028
19. Parchi, P., Giese, A., Capellari, S., Brown, P., Schulz-Schaeffer W., Windl, O., Zerr, I., Budka, H., Kopp, N., Piccardo, P., Poser, S., Rojiani, A., Streichemberger, N., Julien, J., Vital, C., Ghetti, B., Gambetti, P., and Kretzschmar, H. (1999) Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. *Ann. Neurol.* **46,** 224–233
20. Kitamoto, T., Ohta, M., Doh-ura, K., Hitoshi, S., Terao, Y., and Tateishi, J. (1993) Novel missense variants of prion protein in Creutzfeldt-Jakob disease or Gerstmann-Sträussler syndrome. *Biochem. Biophys. Res. Commun.* **191,** 709–714
21. Taguchi, Y., Mohri, S., Ironside, J. W., Muramoto, T., and Kitamoto, T. (2003) Humanized knock-in mice expressing chimeric prion protein showed varied susceptibility to different human prions. *Am. J. Pathol.* **163,** 2585–2593
22. Grathwohl, K. U., Horiuchi, M., Ishiguro, N., and Shinagawa, M. (1996) Improvement of PrP^{Sc}-detection in mouse spleen early at the preclinical stage of scrapie with collagenase-completed tissue homogenization and Sarkosyl-NaCl extraction of PrP^{Sc}. *Arch. Virol.* **141,** 1863–1874
23. Kitamoto, T., Muramoto, T., Mohri, S., Doh-Ura, K., and Tateishi, J. (1991) Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. *J. Virol.* **65,** 6292–6295
24. Kitamoto, T., Muramoto, T., Hilbich, C., Beyreuther, K., and Tateishi, J. (1991) N-terminal sequence of prion protein is also integrated into kuru plaques in patients with Gerstmann-Sträussler syndrome. *Brain. Res.* **545,** 319–321
25. Scott, M. R., Will, R., Ironside, J., Nguyen, H. O., Tremblay, P., DeArmond, S. J., and Prusiner, S. B. (1999) Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 15137–15142
26. Scott, M. R., Peretz, D., Nguyen, H. O., Dearmond, S. J., and Prusiner, S. B. (2005) Transmission barriers for bovine, ovine, and human prions in transgenic mice. *J. Virol.* **79,** 5259–5271
27. Kobayashi, A., Sakuma, N., Matsuura, Y., Mohri, S., Aguzzi, A., and Kitamoto, T. (2010) Experimental verification of a traceback phenomenon in prion infection. *J. Virol.* **84,** 3230–3238
28. Hizume, M., Kobayashi, A., Teruya, K., Ohashi, H., Ironside, J. W., Mohri, S., and Kitamoto, T. (2009) Human prion protein (PrP) 219K is converted to PrP^{Sc} but shows heterozygous inhibition in variant Creutzfeldt-Jakob disease infection. *J. Biol. Chem.* **284,** 3603–3609
29. Lasmézas C. I., Fournier J. G., Nouvel V., Boe H., Marcé D., Lamoury F., Kopp N., Hauw J. J., Ironside J., Bruce M., Dormont D., Deslys J. P. (2001)

Adaptation of the bovine spongiform encephalopathy agent to primates and comparison with Creutzfeldt-Jakob disease: implications for human health. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 4142–4147
30. Lukic, A., Beck, J., Joiner, S., Fearnley, J., Sturman, S., Brandner, S., Wadsworth, J. D., Collinge, J., and Mead, S. (2010) Heterozygosity at polymorphic codon 219 in variant Creutzfeldt-Jakob disease. *Arch. Neurol.* **67,** 1021–1023
31. Soldevila, M., Calafell, F., Andrés, A. M., Yagüe, J., Helgason, A., Stefánsson, K., and Bertranpetit, J. (2003) Prion susceptibility and protective alleles exhibit marked geographic differences. *Hum. Mutat.* **22,** 104–105
32. Beck, J. A., Poulter, M., Campbell, T. A., Adamson, G., Uphill, J. B., Guerreiro, R., Jackson, G. S., Stevens, J. C., Manji, H., Collinge, J., and Mead, S. (2010) PRNP allelic series from 19 years of prion protein gene sequencing at the MRC Prion Unit. *Hum. Mutat.* **31,** E1551–E1563
33. Head, M. W., Tissingh, G., Uitdehaag, B. M., Barkhof, F., Bunn, T. J., Ironside, J. W., Kamphorst, W., and Scheltens, P. (2001) Sporadic Creutzfeldt-Jakob disease in a young Dutch valine homozygote: atypical molecular phenotype. *Ann. Neurol.* **50,** 258–261
34. Mead, S., Joiner, S., Desbruslais, M., Beck, J. A., O'Donoghue, M., Lantos, P., Wadsworth, J. D., and Collinge, J. (2007) Creutzfeldt-Jakob disease, prion protein gene codon 129VV, and a novel PrP^{Sc} type in a young British woman. *Arch. Neurol.* **64,** 1780–1784