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Immunopathology and Infectious Diseases

Detection of Type 1 Prion Protein in Variant Creutzfeldt-Jakob Disease

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Molecular typing of the abnormal form of the prion protein (PrP^{Sc}) has come to be regarded as a powerful tool in the investigation of the prion diseases. All evidence thus far presented indicates a single PrP^{Sc} molecular type in variant Creutzfeldt-Jakob disease (termed type 2B), presumably resulting from infection with a single strain of the agent (bovine spongiform encephalopathy). Here we show for the first time that the PrP^{Sc} that accumulates in the brain in variant Creutzfeldt-Jakob disease also contains a minority type 1 component. This minority type 1 PrP^{Sc} was found in all 21 cases of variant Creutzfeldt-Jakob disease tested, irrespective of brain region examined, and was also present in the variant Creutzfeldt-Jakob disease tonsil. The quantitative balance between PrP^{Sc} types was maintained when variant Creutzfeldt-Jakob disease was transmitted to wild-type mice and was also found in bovine spongiform encephalopathy cattle brain, indicating that the agent rather than the host specifies their relative representation. These results indicate that PrP^{Sc} molecular typing is based on quantitative rather than qualitative phenomena and point to a complex relationship between prion protein biochemistry, disease phenotype and agent strain. (*Am J Pathol* 2006, 168:151-157; DOI: 10.2353/ajpath.2006.050766)

In human prion diseases, the abnormal prion protein (PrP^{Sc}) is thought to exist in one of two major conformational states, termed type 1 and type 2, which are recognized by differences in the extent of their N-terminal truncation following proteolytic cleavage under defined conditions. Type 1 PrP^{Sc} yields a protein-

ase K-resistant core fragment with an N-terminus at glycine 82, and type 2 yields a core fragment with an N-terminus at serine 97.¹ The presence of either type 1 or type 2 PrP^{Sc} is a characteristic feature of the subtypes of sporadic Creutzfeldt-Jakob disease (sCJD) adding weight to the argument that PrP^{Sc} type, in part, underlies disease phenotype.^{2,3} Although microheterogeneity occurs within type 1 and type 2 N-termini,¹ which may be affected by the conditions of proteolytic degradation, no condition has been found that can convert type 1 to type 2 PrP^{Sc}.⁴ Recent reports show that certain cases of sCJD contain both type 1 and 2 in the same brain.⁵⁻⁸ Regional variation in PrP^{Sc} type has subsequently been reported in iatrogenic CJD⁹ and in familial CJD,¹⁰ suggesting that co-occurrence of different PrP^{Sc} types is perhaps the rule in CJD. In contrast, all of the available evidence so far has suggested that the variant CJD (vCJD) brain contains a single type, which has been interpreted to reflect infection of susceptible individuals by a single defined pathogen, namely bovine spongiform encephalopathy (BSE).^{3,6,11-13} Here we re-examine that proposition, by using a monoclonal antibody (12B2) that recognizes an epitope (WGQGG) found at position 89-93 of human PrP, between the type 1 and type 2 N-termini and which should therefore specifically detect type 1 PrP^{Sc} in proteinase K-treated samples. We compare these results with those found using the commonly used monoclonal antibody 3F4, that binds the epitope

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(MKHM) found at position 109-112 of human PrP and which recognizes both type 1 and type 2 PrP^{Sc} in proteinase K-treated samples.¹⁻¹¹

Materials and Methods

Human Tissue Specimens

The human tissue specimens used were collected at autopsy, with consent and ethical approval (Lothium Research Ethics Committee/2000/4/157) for retention and research use, from patients who received a final diagnosis of definite vCJD ($n = 21$) or definite sCJD ($n = 7$), over the period 1995-2004 in the United Kingdom. The specimens were stored at -80°C until used.

Bovine Spongiform Encephalopathy Tissue

Central nervous system tissue from a Friesian cow with terminal BSE from the Central Veterinary Laboratory (New Haw, UK) was obtained from Dr. R.M. Ridley (Division of Psychiatry, Clinical Research Centre, Harrow, UK).

Novel Monoclonal Antibodies

Mouse monoclonal antibody 94B4 has been described previously.¹⁴ Mouse monoclonal antibodies 9A2 and 12B2 were produced from PrP-knockout mice,¹⁵ generously provided by Charles Weissmann (Scripps Research Institute, Jupiter, FL), by immunization with a synthetic peptide corresponding to ovine PrP amino acids 89-107. Prior conjugation of the peptide to keyhole limpet hemocyanin was as previously described.¹⁶ To detect the linear epitope specificities of 94B4, 12B2 and 9A2, Pepscan analysis of solid-phase synthetic peptides was performed by Pepscan Systems BV (Lelystad, The Netherlands) in an enzyme-linked immunosorbent assay-like setup as previously described.¹⁴ This used a set of overlapping 15-mer peptides covering the entire amino acid sequence of ovine PrP (GenBank accession number AJ000739). Using the human PrP sequence numbering, the epitope for 94B4 was determined by Pepscan analysis to be ₁₈₇HTVTTTTK₁₉₄. The epitope for 9A2 was found to require the residues ₉₉WNK₁₀₁ and the epitope for 12B2 was found to require the residues ₈₉WGQGG₉₃ (both numbered according to the human PrP sequence). These sequences are conserved in the human, bovine, and murine species analyzed in these studies. Enzyme-linked immunosorbent assay blocking experiments using synthetic peptides and recombinant PrP confirmed the epitope mapping for 9A2 and 12B2. However the linear sequence 187-194 of PrP that was found to bind monoclonal antibody 94B4 must represent only part of the epitope because enzyme-linked immunosorbent assay antibody binding to peptide or recombinant PrP could only be partially blocked by recombinant PrP or peptide.

Sample Preparation and Western Blotting

Samples were extracted, digested with proteinase K and analyzed by Western blotting as described previously.⁶ A centrifugation step was included to concentrate the lower levels of PrP^{Sc} found in the tonsil samples.⁹ Ten percent bis-Tris NuPage gels, buffers and molecular weight markers (MagicMark; Invitrogen Life Technologies, Paisley, UK) were used, in accordance with the manufacturer's instructions. The proteinase K used throughout this study was supplied stabilized in solution at 20 mg/ml (minimum specific activity 600 mAnson U/ml) from a single batch manufactured by British Drug Houses, and obtained from VWR International Ltd. (Poole, UK). The standard proteinase K digestion conditions were 50 $\mu\text{g}/\text{ml}$ at 37°C for one hour. In some experiments samples were taken from a digestion reaction at specific time points throughout a three-hour digestion. In other experiments a standard one-hour digestion reaction was carried out, but the proteinase K concentration ranged from 5 to 500 $\mu\text{g}/\text{ml}$. The monoclonal antibody 3F4 was obtained from Dako (High Wycombe, UK) and used at 50 ng/ml. The monoclonal antibody 6H4 (Prionics AG, Schlieren, Switzerland) was used at 100 ng/ml. The monoclonal antibodies 12B2, 9A2 and 94B4 were used at 200 ng/ml. Blotting membranes (Hybond-P), horseradish peroxidase-conjugated anti-mouse secondary antibody, chemiluminescent reagent (ECL+) and X-ray film (Hyperfilm) were all obtained from Amersham Biotechnology (Bucks, UK).

Transmission to Mice

Ten percent (w/v) extracts of vCJD brain were used to inoculate C57BL, RIII and VM mice by the intracerebral and intraperitoneal routes as described previously.¹³ The mice were sacrificed after the appearance of signs of disease and one half of the brain was examined histologically to confirm the presence of a spongiform encephalopathy and the other half stored at -20°C for biochemical investigations. The transmissions were performed under an appropriate home office project license and had been approved by the Institute for Animal Health (Edinburgh, UK).

Results

The Western blot profile of PrP^{Sc} in sCJD (type 1) and vCJD (type 2B) standard brain samples detected by the newly developed monoclonal antibodies 12B2, 9A2, and 94B4 were compared with those detected by the well-characterized 3F4 and 6H4 antibodies (Figure 1). Antibodies reacting with epitopes C-terminal to serine 97 (9A2, 3F4, 6H4, and 94B4) detected both type 1 and type 2B PrP^{Sc}. Each antibody detected the triplet pattern corresponding to di-, mono-, and nonglycosylated PrP^{Sc} in both type 1 and type 2B standard samples. The fragment sizes and glycoform ratios were as expected for type 1 and type 2B PrP^{Sc} irrespective of antibody used. In addition to these fragments, a lower molecular weight fragment was also detected in the type 1 standard with the

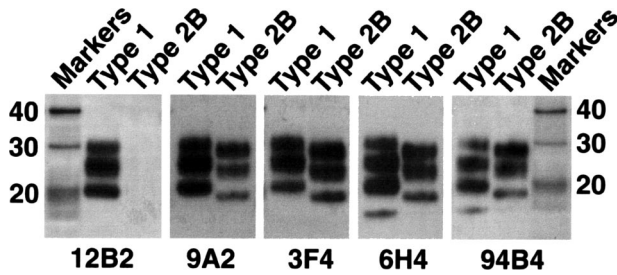


Figure 1. The monoclonal antibody 12B2 detects type 1 but not type 2 PrP^{Sc}. Western blot analysis of protease K-treated samples of cerebral cortex from standard cases of sporadic CJD MM1 (Type 1) and variant CJD (Type 2B) using the monoclonal antibodies 12B2, 9A2, 3F4, 6H4 and 94B4 is shown. Molecular weight markers (Markers) indicate weights in kd.

antibodies recognizing more C-terminal epitopes (6H4 and 94B4). In marked contrast to these antibodies, 12B2 detected the type 1 PrP^{Sc} standard but failed to detect the PrP^{Sc} in the type 2B vCJD standard sample. These data confirm that the monoclonal antibody 12B2 successfully targets the epitope₈₉WGQGG₉₃, which is intact in proteinase K-digested type 1 PrP^{Sc} but which is removed when type 2 PrP^{Sc} is digested with proteinase K. Hence, antibodies such as 3F4 detect both type 1 and type 2 PrP^{Sc}, but following proteinase K digestion the antibody 12B2 is, in effect, type 1 specific. The situation is summarized in diagrammatic form in Figure 2.

To determine the sensitivity with which 12B2 can detect type 1 PrP^{Sc} when it exists as part of a mixture of type 1 and type 2, we mixed a dilution series of proteinase K-treated type 1 standard with a uniform amount of proteinase K-treated type 2B standard. Duplicate Western blots were probed with 3F4 (to detect type 1 and 2) and 12B2 to detect type 1 specifically (Figure 3). Both the 20-kd (type 1) and 19-kd (type 2) nonglycosylated bands

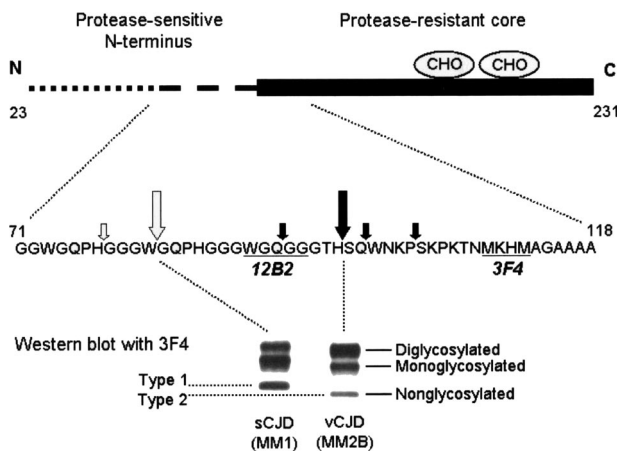


Figure 2. Schematic diagram of the prion protein (amino acids 23 to 231) showing the protease K-resistant core (filled box), the region of variable proteinase K resistance (dashed line), and the region of uniform proteinase K sensitivity (dotted line). The amino acid sequence of the region of variable proteinase K sensitivity is shown. Superimposed on to this is the position of major (large arrows) and minor (small arrows) N-termini of proteinase K-treated type 1 (open arrows) and type 2 (filled arrows).¹ The epitopes recognized by the monoclonal antibodies 3F4 and 12B2 are marked. The resultant 3F4 Western blot profiles when sporadic CJD MM1 (Type 1) and variant CJD MM2B (Type 2) samples are digested with proteinase K are shown below the sequence and the glycoforms (Di-, Mono-, and Nonglycosylated) indicated.

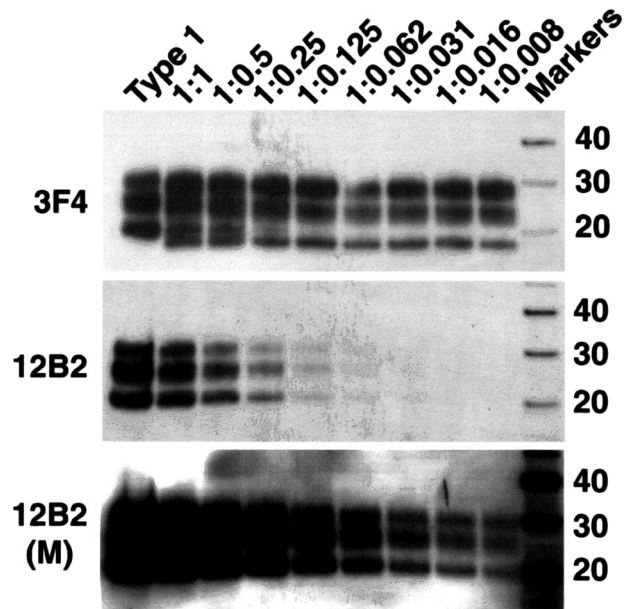


Figure 3. The monoclonal antibody 12B2 detects type 1 PrP^{Sc} with sensitivity and specificity in mixtures of type 1 and type 2B PrP^{Sc}. Western blot analysis of serially diluted proteinase K-treated sCJD brain homogenate mixed with a constant amount of proteinase K-treated variant CJD brain homogenate is shown. The type 1 sCJD: type 2B vCJD ratio is shown for each lane. Duplicate blots were probed with 3F4, which detects both type 1 and type 2 PrP^{Sc}, and with 12B2, which detects type 1. The blot probed with 12B2 was given a short exposure (12B2), comparable with that for the 3F4 blot, and maximal exposure (12B2 (M)). Both blots included samples of cerebral cortex from a case of sporadic CJD MM1 alone (Type 1) and molecular weight markers (Markers) indicate weights in kd.

were clearly visible when the two samples were equally mixed. Type 1 remained visible when the ratio was increased to 1:0.5 and 1:0.25, but type 1 was no longer readily distinguishable when the ratio was increased to 1:0.125 or greater. Extending the exposure time failed to make type 1 visible at any greater dilutions because of the increasingly saturating signal from the nearby type 2B nonglycosylated band (data not shown). When a duplicate blot was probed with 12B2, the type 2B component of the mixture was not detected and type 1 remained detectable when the ratio between type 2 and type 1 is as high as 1:0.125. Because of the absence of interfering co-detected type 2 signal, blots probed with 12B2 can be given extended exposures. A maximal one-hour exposure of the 12B2 blot showed type 1 PrP^{Sc} to be readily detectable even when present at two orders of magnitude lower than the type 2 PrP^{Sc} present in the same mixture. These data show that 12B2 is capable of detecting type 1 PrP^{Sc} specifically and that 12B2 increases the sensitivity of detecting type 1 PrP^{Sc} such that it may be detected when it comprises less than 1% of the material present in a mixture of type 1 and type 2. The possible presence of type 1 PrP^{Sc} at previously undetectable levels in samples from cases of vCJD previously classified as type 2 was conducted using the 12B2 antibody and extended Western blot exposure times.

Western blot analysis of PrP^{Sc} in samples of cerebral cortex from six cases of vCJD using the monoclonal antibody 3F4 showed three bands corresponding to di-, mono-, and nonglycosylated forms (Figure 4). The type 2

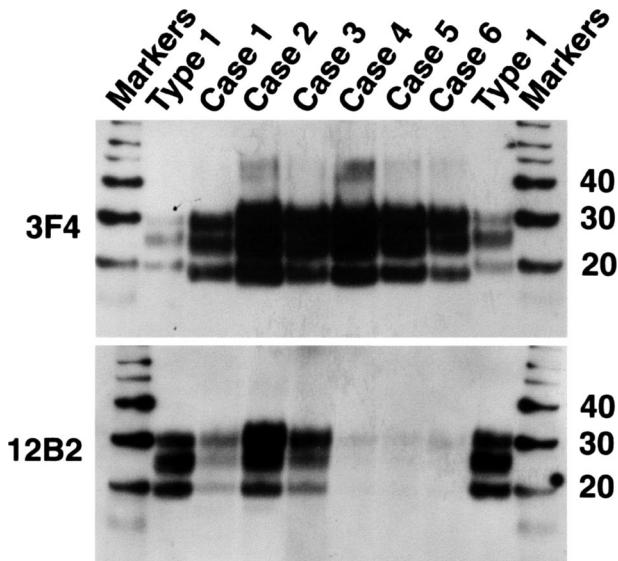


Figure 4. Type 1 PrP^{Sc} is detectable in the vCJD brain. Western blot analysis of PrP^{Sc} in the cerebral cortex from six cases of vCJD is shown. Brain homogenates were subjected to proteinase K digestion and duplicate blots were probed with 3F4, which detects both type 1 and type 2 PrP^{Sc}, and with 12B2, which detects type 1. Both blots included samples of cerebral cortex from a case of sporadic CJD MM1 (Type 1) and molecular weight markers (Markers) indicate weights in kd.

nonglycosylated band in vCJD was clearly distinct from the more slowly migrating nonglycosylated band found in a typical case of sCJD (type 1), which migrated with the 20-kd molecular weight marker. When a duplicate blot was probed with 12B2, the type 2 band was undetectable but a band was seen that is indistinguishable in mobility from the type 1 seen in the case of sCJD (Figure 4). The signal found in the vCJD brain using the 12B2 therefore had both the immunological and size characteristics expected of genuine type 1 PrP^{Sc}. The glycoform ratio of the type 1 PrP^{Sc} found in vCJD closely resembled the glycoform signature of vCJD type 2 PrP^{Sc}, with a predominance of the di-glycosylated band. The abundance of the

type 1 PrP^{Sc} detected by 12B2 varied between vCJD cases and was independent of the abundance of the type 2 PrP^{Sc} detected by 3F4. We examined cerebral cortex from 21 cases of vCJD, and in each sample we were able to detect type 1 PrP^{Sc} using 12B2. Sampling of 17 different regions of a single case of vCJD using the protocol described previously⁶ showed that the presence of type 1 PrP^{Sc} is not restricted to the cerebral cortex but is a feature of all regions where type 2 PrP^{Sc} is detectable by 3F4 (Figure 5).

One possible explanation for the presence of type 1 PrP^{Sc} in these samples is that it represents incompletely truncated type 2 PrP^{Sc}. We addressed this issue by performing a proteinase K-digestion time-course experiment (Figure 6). When probed with 3F4 the amount of PrP signal did not decline dramatically during the three-hour digestion, but minor increases in mobility were seen. A shorter exposure showed this progressive increase in mobility more clearly. In contrast there was a rapid initial decline in the signal detected with 12B2, which plateaued between 30 and 60 minutes and then remained constant over the next two hours. Semiquantitative densitometric analysis (data not shown) indicated that ~60% of the starting PrP was stably resistant to digestion and detectable by 3F4. In contrast, the 12B2 detectable proteinase K-resistant PrP was less than 10% of the starting material. The type 1 PrP^{Sc} found in the vCJD brain resisted conversion to type 2 even at proteinase K concentrations ten times higher (0.5 mg/ml) than the standard assay conditions (data not shown). These two experiments indicate that the type 1 PrP^{Sc} found in the vCJD brain is not a degradative intermediate, but rather that it results from a subpopulation of genuine type 1 PrP^{Sc} that is consistently present in the vCJD brain samples.

To test whether this phenomenon was tissue specific, we examined the lower levels of PrP^{Sc} that accumulated in the vCJD tonsil. Autopsy samples of tonsil from two cases of vCJD were tested, and both showed proteinase K-resistant PrP that was detectable by 12B2 and had a

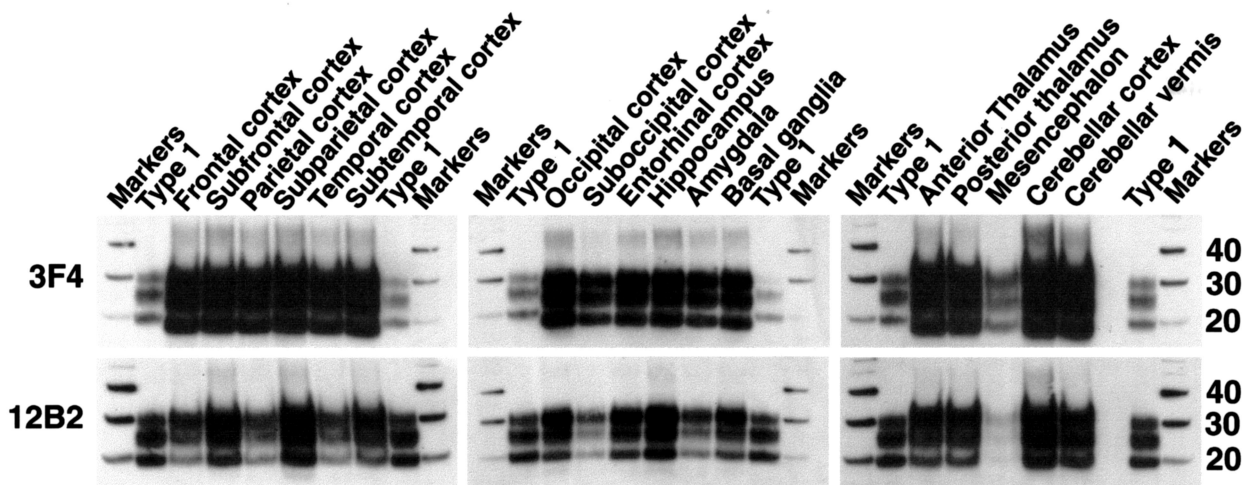


Figure 5. Type 1 PrP^{Sc} is found throughout the vCJD brain. Western blot analysis of PrP^{Sc} in 17 defined brain regions of a single case of vCJD is shown. Brain homogenates were subjected to proteinase K digestion and duplicate blots were probed with 3F4, which detects both type 1 and type 2 PrP^{Sc}, and with 12B2, which detects type 1. Both blots included samples of cerebral cortex from a case of sporadic CJD MM1 (Type 1) and molecular weight markers (Markers) indicate weights in kd.

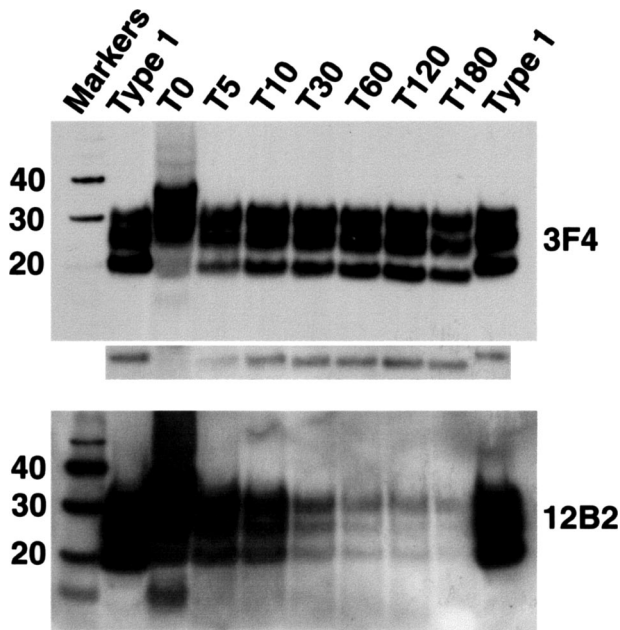


Figure 6. Type 1 PrP^{Sc} is a stable minority component of PrP^{Sc} from the vCJD brain. Western blot analysis of PrP in a sample of cerebral cortex from a case of vCJD during digestion with proteinase K is shown. Time points assayed are indicated in minutes (T0, 5, 10, 30, 60, 120, 180). Duplicate blots were probed with 3F4, which detects both type 1 and type 2 PrP^{Sc}, and with 12B2, which detects type 1. The insert shows a shorter exposure of the same time course study from a separate experiment also probed with 3F4. Both blots included samples of cerebral cortex from a case of sporadic CJD MM1 (Type 1) and molecular weight markers (Markers) indicate weights in kd.

lower mobility than the band detected by 3F4 (Figure 7). To test whether this mixture of mobility types was a feature of the agent (BSE) or the host (humans), we transmitted vCJD to wild-type mice (C57BL, VM and RIII inbred lines) and examined the PrP^{Sc} that accumulated in the brain. We also examined brain material from a cow with BSE. Neither bovine nor murine PrP contained the 3F4 epitope, so we used the 6H4 antibody. Individual C57BL mice from successful transmissions from three different cases of vCJD all showed a band detectable by 12B2 with a lower mobility than that detected by 6H4. An example is shown in Figure 7. The same phenomenon was observed in a vCJD transmission to the VM and RIII inbred lines (data not shown). The example shown in Figure 7 resulted from the transmission from vCJD case 5 (shown in Figure 4) to C57BL mice. BSE cow brain showed a similar result to that seen in vCJD, with 12B2 detecting a minority component that migrated more slowly than that detected by 6H4 (Figure 7).

Lastly we considered whether this same situation applied in cases of sporadic CJD that had hitherto been classified as containing type 2A PrP^{Sc} only. Examples from each of the three *PRNP* codon 129 genotypes (MM, MV, VV) were examined, and each showed the presence of type 1 PrP^{Sc} as detected by 12B2, in addition to type 2A PrP^{Sc} as detected by 3F4 (Figure 8).

Discussion

In the apparent absence of a foreign nucleic acid genome associated with the agents responsible for trans-

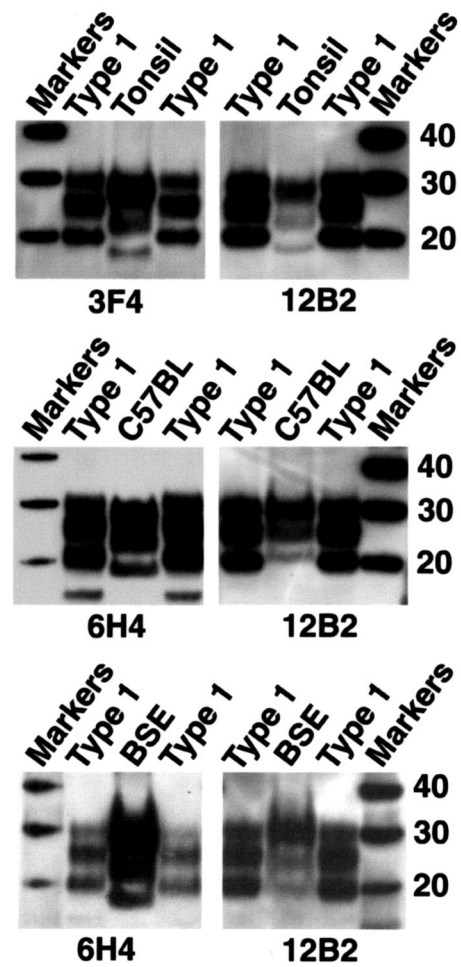


Figure 7. A minority type 1-like PrP^{Sc} is found in vCJD tonsil, vCJD transmitted to mice and in BSE. Western blot analysis of PrP^{Sc} in a concentrated sample of tonsil from a case of vCJD (Tonsil), in a concentrated brain sample of a wild-type mouse (C57BL) infected with vCJD and in a sample of cattle BSE brain (BSE) is shown. Tissue extracts were digested with proteinase K. Duplicate blots were probed with either 3F4 or 6H4, both of which detect type 1 and type 2 PrP^{Sc}, and with 12B2, which detects type 1. The blots included samples of cerebral cortex from a case of sporadic CJD MM1 (Type 1) and molecular weight markers (Markers) indicate weights in kd.

missible spongiform encephalopathies or prion diseases, efforts to provide a molecular definition of agent strain have focused on biochemical differences in the abnormal, disease-associated form of the prion protein, termed PrP^{Sc}. Differences in PrP^{Sc} conformation and glycosylation have been proposed to underlie disease phenotype and form the biochemical basis of agent strain. This proposal has found support in the observation that the major phenotypic subtypes of sCJD appear to correlate with the presence of either type 1 or type 2 PrP^{Sc} in combination with the presence of either methionine or valine at codon 129 of the prion protein gene.² Similarly, the PrP^{Sc} type associated with vCJD correlates with the presence of type 2 PrP^{Sc} and is distinct from that found in sCJD because of a characteristically high occupancy of both N-linked glycosylation sites (type 2B).^{6,11} The means by which such conformational difference is detected is somewhat indirect; relying on the action of proteases, primarily proteinase K, to degrade the normal

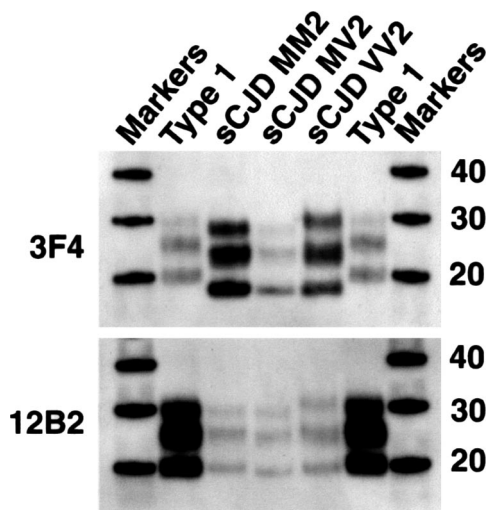


Figure 8. Type 1 PrP^{Sc} is a minority component in cases of sporadic CJD MM2, MV2, and VV2. Western blot analysis of PrP^{Sc} in the cerebral cortex from three cases of sporadic CJD (sCJD) of the subtypes MM2, MV2, and VV2 is shown. Duplicate blots of proteinase K-treated brain homogenates were probed with 3F4 which detects type 1 and type 2 PrP^{Sc}, and with 12B2, which detects type 1. Both blots included samples of cerebral cortex from a case of sporadic CJD MM1 (Type 1) and molecular weight markers (Markers) indicate weights in kd.

cellular form of PrP and produce a protease-resistant core fragment of PrP^{Sc} that differs in the extent of its N-terminal truncation according to the original conformation.

A complication has recently arisen with the finding that both type 1 and type 2 can co-exist in the brains of patients with sCJD.^{2,5-8} More recently this same phenomenon has been demonstrated in patients with iatrogenically acquired and familial forms of human prion disease.^{9,10} The existence of this phenomenon is now beyond doubt but its prevalence and its biological significance remain a matter of debate.

Conventional Western blot analysis using antibodies that detect type 1 and type 2 PrP^{Sc} has severe quantitative limitations for the co-detection of type 1 and type 2 PrP^{Sc} in individual samples, suggesting that the prevalence of co-occurrence of the two types might be underestimated. We have sought to circumvent this problem by using an antibody that is type 1-specific and applied this to the sole remaining human prion disease where the phenomenon of mixed PrP^{Sc} types has not yet been shown, namely vCJD.

These results show that even in vCJD where susceptible individuals have been infected supposedly by a single strain of agent, both PrP^{Sc} types co-exist: a situation reminiscent of that seen when similarly discriminant antibodies were used to analyze experimental BSE in sheep.^{14,17} In sporadic and familial CJD, individual brains can show a wide range of relative amounts of the two types in samples from different regions, but where brains have been thoroughly investigated a predominant type is usually evident.^{2,6,10} This differs from this report on vCJD, where type 1 is present in all samples investigated but always as a minor component that never reaches a level at which it is detectable without a type 1-specific antibody. It would appear that the relative bal-

ance between type 1 and type 2 is controlled within certain limits in the vCJD brain. A minority type-1-like band is also detected by 12B2 in vCJD tonsil, in BSE brain and in the brains of mice experimentally infected with vCJD, suggesting that this balance of types is agent, rather than host or tissue, specific. Interestingly the "glycoform signature" of the type 2 PrP^{Sc} found in vCJD (type 2B) is also seen in the type 1 PrP^{Sc} components, suggesting that it could legitimately be termed type 1B.

PrP^{Sc} isotype analysis has proven to be extremely useful in the differential diagnosis of CJD and is likely to continue to have a major role in the investigation of human prion diseases. However, it is clear, on the basis of these findings, that molecular typing has quantitative limitations and that any mechanistic explanation of prion replication and the molecular basis of agent strain variation must accommodate the co-existence of multiple prion protein conformers. Whether or not the different conformers we describe here correlate in a simple and direct way with agent strain remains to be determined. In principle two interpretations present themselves: either the two conformers can be produced by a single strain of agent or vCJD (and, therefore, presumably BSE) results from a mixture of strains, one of which generally predominates. Evidence for the isolation in mice of more than one strain from individual isolates of BSE has been presented previously.^{18,19}

One practical consequence of our findings is that the correct interpretation of transmission studies will depend on a full examination of the balance of molecular types present in the inoculum used to transmit disease, in addition to a thorough analysis of the molecular types that arise in the recipients. Another consequence relates to the diagnostic certainty of relying on PrP^{Sc} molecular type alone when considering the possibility of BSE infection or secondary transmission in humans who have a genotype other than methionine at codon 129 of the *PRNP* gene. In this context it is interesting to note that this minority type 1B component resembles the *type 5* PrP^{Sc} described previously to characterize vCJD transmission into certain humanized *PRNP*^{129VV} transgenic mouse models.^{12,20} This apparently abrupt change in molecular phenotype might represent a selection process imposed by this particular transgenic mouse model. Irrespective of whether this proves to be the case, the results shown here point to further complexities in the relationship between the physico-chemical properties of the prion protein, human disease phenotype, and prion agent strain.

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