Prion infectivity in variant Creutzfeldt–Jakob disease rectum

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Background: Disease-related prion protein (PrP^{Sc}) is readily detectable in lymphoreticular tissues in variant Creutzfeldt–Jakob disease (vCJD), but not in other forms of human prion disease. This distinctive pathogenesis, with the unknown population prevalence of asymptomatic vCJD infection, has led to significant concerns that secondary transmission of vCJD prions will occur through a wide range of surgical procedures. To date PrP^{Sc}:prion infectivity ratios have not been determined in vCJD, and it is unknown whether vCJD prions are similar to experimental rodent prions, where PrP^{Sc} concentration typically reflects infectious prion titre.

Aim: To investigate prion infectivity in vCJD tissue containing barely detectable levels of PrP^{Sc}.

Methods: Transgenic mice expressing only human PrP (Tg(HuPrP129M^{+/+} Prnp^{0/0})-35 and Tg(HuPrP129M^{+/+} Prnp^{0/0})-45 mice) were inoculated with brain or rectal tissue from a previously characterised patient with vCJD. These tissues contain the maximum and minimum levels of detectable PrP^{Sc} that have been observed in vCJD.

Results: Efficient transmission of prion infection was observed in transgenic mice inoculated with vCJD rectal tissue containing PrP^{Sc} at a concentration of 10^{4.7}-fold lower than that in vCJD brain.

Conclusions: These data confirm the potential risks for secondary transmission of vCJD prions via gastrointestinal procedures and support the use of PrP^{Sc} as a quantitative marker of prion infectivity in vCJD tissues.

Most people in the UK might have been exposed to bovine spongiform encephalopathy (BSE) prions, the causative agent of the novel human prion disease, variant Creutzfeldt–Jakob disease (vCJD).¹ The unknown, but potentially high prevalence of clinically silent infection with BSE prions,^{1–3} allied with the distinctive pathogenesis of vCJD,^{4–8} has led to significant concerns that there may be substantial risks of iatrogenic transmission of vCJD prions via blood products, and contaminated surgical and medical instruments.^{1 5 8–10} Two cases of transfusion-associated vCJD prion infection have now been reported,^{11 12} with a third probable case announced by the UK Health Protection Agency in February 2006. Prions resist many conventional sterilisation procedures, and surgical stainless steel-bound prions transmit disease with remarkable efficiency when implanted into mice.^{10 13}

Although bioassay is generally regarded as the gold standard for prion detection in experimental studies, a substantial transmission barrier limits the sensitivity of such methods to assay vCJD prions in conventional mice.^{14–16} Risk assessment for secondary transmission of vCJD prions has thus relied on high-sensitivity immunoblot detection of disease-related prion protein (PrP^{Sc}) to inform on the potential levels of prion infectivity in vCJD tissues. These methods, with conventional immunohistochemistry, have shown that the tissue distribution of PrP^{Sc} in vCJD differs strikingly from that in classical CJD, with uniform and prominent involvement of lymphoreticular tissues.^{4–8 17} Depending on the density of lymphoid follicles, PrP^{Sc} concentrations in vCJD peripheral tissues can vary enormously, with levels relative to brain as high as 10% in tonsil^{4 5} or as low as 0.002% in rectum.⁵

According to the protein-only hypothesis¹⁸ of prion propagation, PrP^{Sc} is the principal component of the infectious prion.^{14 19 20} However, it is well recognised that the ratio of PrP^{Sc} to infectivity varies in different prion disease models and that prion diseases may occur without detectable PrP^{Sc}.^{21–23} However, as comparative end-point titration of vCJD prions in humans cannot be carried out, PrP^{Sc}:prion infectivity ratios have not been determined in vCJD. In this regard, it is unknown whether vCJD prions are similar to experimental rodent prions, where PrP^{Sc} concentration typically reflects infectious prion titre.²⁴

To investigate this, we inoculated transgenic mice expressing only human PrP (Tg(HuPrP129M^{+/+} Prnp^{0/0})-35 and Tg(HuPrP129M^{+/+} Prnp^{0/0})-45 mice referred to as 129MM Tg35 and Tg45 mice, respectively)^{15 25} with brain and rectal tissue from a previously characterised patient with vCJD.⁵ These tissues contain the maximum and minimum levels of detectable PrP^{Sc} that we have observed in vCJD, with the concentration of PrP^{Sc} in rectum being 10^{4.7}-fold lower than that in brain.⁵

METHODS

This study was carried out after approval from the Institute of Neurology/National Hospital for Neurology and Neurosurgery Local Research Ethics Committee.

Abbreviations: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jakob disease; PBS, phosphate-buffered saline; PrP, prion protein; PrP^{Sc}, disease-related prion protein; vCJD, variant Creutzfeldt–Jakob disease

All procedures were carried out in a microbiological containment level 3 facility, with strict adherence to safety protocols.

Transmission studies

Care of mice was according to institutional guidelines. Transgenic mice homozygous for a human PrP 129 methionine (M) transgene array and murine PrP null alleles (*Prnp^{0/0}*), designated Tg (HuPrP129M^{+/+} *Prnp^{0/0}*)-35 mice and Tg (HuPrP129M^{+/+} *Prnp^{0/0}*)-45 mice (129MM Tg35 and 129MM Tg45 mice), have been described previously.^{15 25} 129MM Tg35 and Tg45 mice have 9 and 17 copies of the transgene, respectively, resulting in respective expression of human PrP at levels two or fourfold higher than human brain.¹⁵ vCJD brain (frontal cortex) and vCJD rectum (full thickness tissue) were prepared as 10% w/v homogenates in Dulbecco's sterile phosphate-buffered saline (PBS) lacking Ca²⁺ and Mg²⁺ by either serial passage through needles of decreasing diameter (brain) or use of Duall tissue grinders (rectum). After intracerebral inoculation with tissue homogenate (30 µl 1% w/v tissue homogenate in PBS) as described previously,¹⁵ mice were examined daily and were killed if they exhibited signs of distress, or once a diagnosis of clinical prion disease was established.²⁶ Brains from inoculated mice were analysed by high-sensitivity immunoblotting and by neuropathological examination.

Immunohistochemistry

vCJD rectum or transgenic mouse brain was analysed with anti-PrP monoclonal antibody ICSM 35 (D-Gen Ltd, London, UK), using a Ventana automated immunohistochemical staining machine (Ventana Medical Systems, Tucson, Arizona, USA) as described previously.^{3 8 27} Briefly, tissue was fixed in 10% buffered formal saline followed by incubation in 98% formic acid for 1 h. After further washing for 24 h in 10% buffered formal saline, tissue samples were processed and embedded in paraffin wax. Sections were cut at a nominal thickness of 4 µm, treated with 98% formic acid for 5 min and then boiled in a low ionic strength buffer (2.1 mM TRIS, 1.3 mM EDTA, 1.1 mM sodium citrate, pH 7.8) for 20 min. Abnormal PrP accumulation

was examined using anti-PrP monoclonal ICSM 35, followed by a biotinylated anti-mouse IgG secondary antibody (iView Biotinylated Ig, Ventana Medical Systems) and an avidin-biotin horseradish peroxidase conjugate (iView SA-HRP, Ventana Medical Systems) before development with 3' 3 diaminobenzidine tetrachloride as the chromogen (iView DAB, Ventana Medical Systems). Haematoxylin was used as the counterstain. Haematoxylin and eosin staining of serial sections was performed using conventional methods. Appropriate controls were used throughout.

Immunoblotting

Human or mouse tissues prepared as 10% w/v homogenates in PBS were analysed by proteinase K digestion (50 or 100 µg/ml final protease concentration, 1 h, 37°C) and immunoblotting with anti-PrP monoclonal antibody 3F4²⁸ using high-sensitivity enhanced chemiluminescence as described previously.⁵ Sodium phosphotungstic acid precipitation of PrP^{Sc} from tissue homogenate²⁹ was carried out as described previously.⁵ This method facilitates highly efficient recovery and detection of PrP^{Sc} from vCJD tissue homogenate when present at levels 10⁴–10⁵-fold lower than that in vCJD brain.^{3 5} Densitometry of immunoblots was carried out using ImageMaster 1D software (GE Healthcare UK, Chalfont, St Giles, UK).

RESULTS

Immunoblot detection of PrP^{Sc} in vCJD rectal tissue

Previously, we reported the distribution of PrP^{Sc} in autopsy tissues from four patients with neuropathologically confirmed vCJD.⁵ In one of these patients we detected PrP^{Sc} in rectum at a

Figure 1 Immunoblot detection of disease-related prion protein (PrP^{Sc}) in variant Creutzfeldt-Jakob disease (vCJD) rectal tissue. Proteinase K digested sodium phosphotungstic acid pellets from 0.5 ml of 10% normal human brain homogenate (NB) or 0.5 ml of 10% normal human brain homogenate spiked with 50 ml of 10% vCJD brain homogenate (NB+spike) are compared with a proteinase K digested sodium phosphotungstic acid pellet from 0.5 ml of 10% rectum of homogenate from the same patient with vCJD. The immunoblot was analysed with anti-prion protein (PrP) monoclonal antibody 3F4 using high-sensitivity of chemiluminescence. Densitometry showed that the PrP^{Sc} signal from 0.5 ml of 10% rectum homogenate has an intensity equivalent to 20% of that derived from 50 ml of 10% vCJD brain homogenate. The PrP^{Sc} concentration in rectum homogenate is thus about 1/50 000 of that present in brain homogenate.

Figure 2 Immunohistochemical detection of abnormal prion protein (PrP) deposition in variant Creutzfeldt-Jakob disease (vCJD) rectum. (A) Haematoxylin and eosin staining of vCJD rectum. Muc, mucosa; LA, submucosal lymphoid aggregate; MM, muscularis mucosae; TM, tunica muscularis. (B) Abnormal PrP immunoreactivity in a submucosal lymphoid aggregate in vCJD rectum (anti-PrP monoclonal antibody ICSM 35). The immunostaining pattern is similar to that observed previously in vCJD terminal ileum.⁸ Scale bar: (A) 500 µm; (B) 40 µm.

concentration of 1/50 000 ($10^{-4.7}$) of that present in brain.⁵ To generate inocula for transmission studies, we prepared a new series of 10% w/v homogenates from this rectal tissue and analysed 0.5 ml of each homogenate for detectable PrP^{Sc} by sodium phosphotungstic acid precipitation, proteinase K digestion and high-sensitivity immunoblotting.⁵ Through this analysis, we again identified one 10% rectum homogenate containing PrP^{Sc} at a level of about 1/50 000 of that present in the brain (fig 1). Previously we reported that the glycoform ratio of protease-resistant PrP fragments in vCJD rectum might be distinct from type 4t PrP^{Sc} seen in vCJD tonsil.⁵ However, PrP^{Sc} fragments in the newly prepared rectum homogenate showed a clear predominance of diglycosylated PrP, consistent with the glycoform ratios of PrP^{Sc} seen in other vCJD lymphoreticular tissues.^{4, 5} We consider that the apparent difference in the glycoform ratio of rectal PrP^{Sc} observed between different tissue homogenates simply reflects the difficulty in carrying out such analysis at the limits of detection sensitivity.

Unfortunately, PrP^{Sc} positive vCJD rectum was available only as frozen tissue and consequently this precluded immunohistochemical examination. Recently, however, we have been able to examine both frozen and formalin-fixed rectal tissue from another patient with vCJD. Analysis of three 10% homogenates prepared from this tissue by high-sensitivity immunoblotting failed to show detectable PrP^{Sc}. However, immunohistochemical examination of rectal tissue at multiple levels showed abnormal PrP immunostaining in one of eight identified submucosal lymphoid aggregates (fig 2). No abnormal PrP immunostaining was found in the mucosa, in muscularis mucosae or in the nerves of the submucosal plexus. Although only a single sample such as this must be treated cautiously, these findings suggest that abnormal PrP immunoreactivity in vCJD rectum is probably associated mainly with lymphoid tissue, as observed in other areas of the intestine in vCJD.^{2, 7, 8, 27, 30} The relative paucity of lymphoid tissue in rectum (in comparison to terminal ileum, where PrP^{Sc} is uniformly

detected in vCJD⁸) seems likely to underlie the irregular detection of PrP^{Sc} in rectal tissue homogenates by immunoblotting.

Prion transmission from vCJD rectal tissue

Previously, we showed that challenge of transgenic mice expressing human PrP 129M (129MM Tg35 and 129MM Tg45 mice) with vCJD brain results in 100% transmission of prion infection, with uniform propagation of type 4 PrP^{Sc} accompanied by the key neuropathological hallmark of vCJD—the presence of abundant florid PrP plaques.¹⁵ However, most of the mice in these transmissions do not develop clinical prion disease; instead, they remain subclinically affected until they succumb to either intercurrent illness or senescence. This lack of clinical end point precludes conventional estimation of prion titre through serial dilution and incubation period methods,^{26, 31} and the demonstration of vCJD prion transmission in these mice therefore relies on detection of PrP^{Sc} in brain.^{15, 25}

Consistent with other vCJD brain isolates,¹⁵ brain inocula from the vCJD patients with PrP^{Sc} positive rectum produced a 100% attack rate of subclinical prion infection in both the 129MM Tg35 and Tg45 mice (table 1, fig 3). This was shown by typical neuropathological changes^{15, 25} (data not shown) and by immunoblot detection of PrP^{Sc} in the brain of all inoculated mice (table 1, fig 4). In most of these mice (9 of 11) PrP^{Sc} was readily detected by direct immunoblotting (table 1) and we were able to establish the propagation of type 4 PrP^{Sc} (fig 4).

Remarkably, prion infection was also clearly evident in 7 of 12 mice inoculated with vCJD rectal tissue containing a $10^{4.7}$ -fold lower concentration of PrP^{Sc} than brain (table 1, figs 3 and 4). However, in these transmissions, the level of PrP^{Sc} propagated in affected mouse brain was much lower than seen after inoculation with vCJD brain, and sodium phosphotungstic acid preconcentration was required for reliable detection of PrP^{Sc} (table 1, fig 4). Consistent with these low concentrations of PrP^{Sc}, PrP immunohistochemistry was negative in affected mice (data not shown). Importantly, despite the requirement for phosphotungstic acid preconcentration, the level of PrP^{Sc} in affected mouse brain was in all cases $>10^3$ -fold higher than that present in the vCJD rectum inoculum, which itself would

Table 1 Prion transmission from variant Creutzfeldt–Jakob disease (vCJD) brain and rectum to transgenic mice

	129MM Tg35 mice	129MM Tg45 mice
Inocula*	Attack rate†	Attack rate†
I3140 vCJD brain	3/3‡	8/8§
I3247 vCJD rectum	3/7¶	4/5**

*Mice were inoculated intracerebrally with 30 µl of 1% tissue homogenate. Disease-related prion protein (PrP^{Sc}) concentration in rectum homogenate was $10^{4.7}$ -fold lower than brain homogenate.

†Attack rate reports the number of mice propagating PrP^{Sc} in brain as a proportion of the number of inoculated mice. Brains scored negative for PrP^{Sc} after analysis of 20 µl of 10% brain homogenate were re-analysed by sodium phosphotungstic acid precipitation of PrP^{Sc} from 250 µl of 10% brain homogenate. All PrP^{Sc} positive mice were asymptomatic for clinical prion disease.

‡Affected mice died at 228, 272 and 418 days after inoculation. PrP^{Sc} was reliably detected in two brain homogenates by direct immunoblotting and in one brain homogenate only after sodium phosphotungstic acid precipitation.

§Affected mice died 161–606 days after inoculation. PrP^{Sc} was reliably detected in seven brain homogenates by direct immunoblotting and in one brain homogenate only after sodium phosphotungstic acid precipitation.

¶Affected mice died at 563, 575 and 585 days after inoculation. PrP^{Sc} was reliably detected in brain homogenates only after sodium phosphotungstic acid precipitation. Non-affected mice died at 377, 412, 462 and 563 days after inoculation.

**Affected mice died at 362, 404, 424 and 550 days after inoculation. PrP^{Sc} was reliably detected in brain homogenates only after sodium phosphotungstic acid precipitation. The single non-affected mouse died at 455 days after inoculation.

Figure 3 Prion transmission from variant Creutzfeldt–Jakob disease (vCJD) brain and rectum to transgenic mice. The total number of prion-affected mice is reported for each inoculated group: 129MM Tg35 mice (white) and 129MM Tg45 mice (grey). Transgenic mice were scored positive for prion infection by immunoblot detection of disease-related prion protein (PrP^{Sc}) in brain.

Figure 4 Disease-related prion protein (PrP^{Sc}) detection in variant Creutzfeldt-Jakob disease (vCJD) prion transmissions to transgenic mice. Immunoblots of proteinase-K-treated brain homogenates from vCJD and transgenic mice were analysed by enhanced chemiluminescence with anti-prion protein (PrP) monoclonal antibody 3F4. The identity of the brain sample is designated above each lane. * denotes PrP^{Sc} recovered by sodium phosphotungstic acid precipitation.

have been undetectable. Moreover, although sodium phosphotungstic acid precipitation precludes accurate PrP fragment size analysis used to distinguish prion strain types,⁵ the glycoform ratio of PrP^{Sc} propagated in the brain of vCJD rectum-inoculated mice was consistent with type 4 PrP^{Sc} (fig 4). Collectively, these data provide convincing evidence for prion transmission from vCJD rectal tissue. The relative difference in the concentration of PrP^{Sc} propagated in the brain of transgenic mice inoculated with vCJD rectum or vCJD brain is consistent with a tissue-specific difference in the prion titre of the inoculum.

DISCUSSION

In this study, we have shown the presence of prion infectivity in vCJD rectal tissue. As already stated, the levels of PrP^{Sc} detected in vCJD tissues cannot be directly correlated with units of human prion infectivity. However, if levels of prion infectivity in vCJD brain approach the highest levels seen in experimental rodent models (10^8 intracerebral infectious U/ml 10% brain homogenate^{29–31,32}), then vCJD rectal tissue with a $10^{4.7}$ -fold lower PrP^{Sc} concentration might contain $10^{3.3}$ intracerebral infectious U/ml 10% homogenate.⁵ Bioassay of 30 µl of 1% rectal homogenate would thus correspond to injection of only approximately 6 human intracerebral infectious units. The fact that efficient prion transmission has occurred in transgenic mice after inoculation with vCJD rectal tissue is consistent with PrP^{Sc}:prion infectivity ratios in vCJD being closely similar to experimental rodent prion strains. These findings strongly endorse the value of PrP^{Sc} analysis in vCJD for defining risk reduction strategies to limit secondary transmission of vCJD prions.

The ready demonstration of prion infectivity in vCJD rectum, despite barely detectable levels of PrP^{Sc}, clearly suggests even higher prion titres in other vCJD peripheral tissues in which greater levels of PrP^{Sc} have been consistently shown. In this regard, it is of note that vCJD tonsil has been shown to transmit prion infection to wild-type mice despite the presence of a transmission barrier.¹⁶ Our present findings with vCJD rectum, together with the demonstration of much greater levels of PrP^{Sc} in vCJD terminal ileum,⁸ reinforce concerns that iatrogenic transmission of vCJD prions might occur through contaminated intestinal endoscopes, biopsy forceps or surgical instruments.^{9,33}

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EDITOR'S QUIZ: GI SNAPSHOT

Chronic diarrhoea with thickening of the colonic wall

Robin Spiller, Editor

Clinical presentation

A 36-year-old woman who had a seven-year medical history of type 1 diabetes mellitus presented with diarrhoea of 1-month duration. She denied abdominal pain or rectal bleeding. On examination, she was afebrile and there was no tenderness on palpation in the abdomen, with increased bowel sounds. Laboratory examination was normal except for hyperglycaemia. Stool culture was negative. A colonoscopic picture of the sigmoid colon (fig 1), a barium enema study (fig 2) and a pelvic computed tomography scan (fig 3) were carried out.

Question

What is the diagnosis?

See page 114 for answer

This case is submitted by:

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Figure 1 Colonoscopy of the sigmoid colon.

Figure 2 Barium contrast study of the sigmoid colon.

Figure 3 Computed tomography of the sigmoid colon.