Chronic wasting disease prions are not transmissible to transgenic mice overexpressing human prion protein

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Chronic wasting disease (CWD) is a prion disease that affects free-ranging and captive cervids, including mule deer, white-tailed deer, Rocky Mountain elk and moose (Williams & Young, 1980, 1982; Williams, 2005; Baeten et al., 2007). Like all mammalian prion diseases, which include Creutzfeldt–Jakob disease (CJD), kuru and variant CJD (vCJD) in humans and bovine spongiform encephalopathy (BSE) in cattle, the central event in CWD infection is the post-translational conversion of the host-encoded, cellular prion protein (PrPC), to an abnormal isomorph, designated PrPSc (Prusiner, 1998; Collinge & Clarke, 2007). Progressive accumulation of PrPSc in the central nervous system (Guiroy et al., 1993) is associated with clinical signs of CWD which include weight loss, behavioural changes, excessive salivation, difficulty swallowing, polydipsia, polyuria, and ataxia prior to death (Williams & Young, 1980, 1982; Williams, 2005). International concern over CWD is growing as infected cervids have now been reported in 14 states in North America, two Canadian provinces and in South Korea (Kim et al., 2005; Williams, 2005; Sigurdson & Aguzzi, 2007; Sigurdson, 2008). To date, CWD has not been reported in Europe, although surveillance has been limited.

The prevalence of CWD infection can reach levels of up to 30% in free-ranging herds in North America and up to 90% in animals housed in CWD research facilities (Williams, 2005). Infectious prions in the saliva (Mathiason et al., 2006; Haley et al., 2009; Mathiason et al., 2009), urine (Haley et al., 2009) and faeces of CWD-infected animals (Tamguney et al., 2009) may underlie the highly efficient natural transmission of CWD among cervids through environmental contamination (Mathiason et al., 2009). Protease-resistant cervid prion protein has recently been demonstrated in an environmental water sample from a CWD endemic area (Nichols et al., 2009).

INTRODUCTION

Chronic wasting disease (CWD) is a prion disease affecting free-ranging and captive cervids, including mule deer, white-tailed deer, Rocky Mountain elk and moose (Williams & Young, 1980, 1982; Williams, 2005; Baeten et al., 2007). Like all mammalian prion diseases, which include Creutzfeldt–Jakob disease (CJD), kuru and variant CJD (vCJD) in humans and bovine spongiform encephalopathy (BSE) in cattle, the central event in CWD infection is the post-translational conversion of the host-encoded, cellular prion protein (PrPC), to an abnormal isomorph, designated PrPSc (Prusiner, 1998; Collinge & Clarke, 2007). Progressive accumulation of PrPSc in the central nervous system (Guiroy et al., 1993) is associated with clinical signs of CWD which include weight loss, behavioural changes, excessive salivation, difficulty swallowing, polydipsia, polyuria, and ataxia prior to death (Williams & Young, 1980, 1982; Williams, 2005). International concern over CWD is growing as infected cervids have now been reported in 14 states in North America, two Canadian provinces and in South Korea (Kim et al., 2005; Williams, 2005; Sigurdson & Aguzzi, 2007; Sigurdson, 2008). To date, CWD has not been reported in Europe, although surveillance has been limited.

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Despite efficient horizontal transmission of CWD prions among cervids, to date there is no clear evidence for natural disease transmission to other species. A recent survey for transmissible spongiform encephalopathy in scavengers of white-tailed deer carcasses in a CWD endemic area of Wisconsin found no evidence for cross-species transmission (Jennelle et al., 2009). Nevertheless, the zoonotic transmission of BSE prions (Collinge et al., 1996; Hill et al., 1997; Bruce et al., 1997; Asante et al., 2002; Wadsworth & Collinge, 2007) has dramatically highlighted the potential risk posed to humans from dietary exposure to CWD prions (Belay, 2004; Sigurdson, 2008). Infectious prions are present in the blood (Mathiason et al., 2006), skeletal muscle (Angers et al., 2006) and fat (Race et al., 2009a) of CWD-infected deer and CWD prions have been shown to be experimentally transmissible after oral inoculation of elk and deer and cervid PrP expressing transgenic mice (Hamir et al., 2006; Fox et al., 2006; Trifilo et al., 2007). Consumption of hunted deer and elk is widely practised in North America and a survey conducted by the American Red Cross and other blood banking establishments has reported that ~40% of USA blood donors have consumed venison obtained from the wild (Belay et al., 2001). To date, however, epidemiological surveillance has not indicated any link between human disease and CWD exposure (Belay, 2004; Mawhinney et al., 2006; Anderson et al., 2007). However, incubation periods in human prion disease even in the absence of a transmission barrier can exceed 50 years (Collinge et al., 2006, 2008). Accordingly, there has been intense research interest in establishing the host range of CWD prions through experimental transmission to laboratory animals (Tamgune et al., 2006; Raymond et al., 2007; Sigurdson et al., 2008; Heisey et al., 2010) and through the use of in vitro prion amplification systems (Raymond et al., 2000; Kurt et al., 2009).

Concern that CWD prions might be transmissible to humans was heightened in 2005 by the finding that squirrel monkeys can be infected by intracerebral inoculation with CWD mule deer brain homogenate (Marsh et al., 2005). However, a more recent study has shown that cynomolgus macaques (that are evolutionarily closer to humans) differ significantly from squirrel monkeys with respect to their susceptibility to infection with CWD prions, with no evidence for clinical disease in macaques at 70 months post-inoculation (Race et al., 2009b). Crucially however, because prion transmission barriers and prion strains are intimately related by conformational selection (Collinge, 1999; Collinge & Clarke, 2007) the ability of CWD prions to propagate in humans cannot be inferred by studying the interaction of CWD prions with distinct (albeit highly conserved) PrP sequences from other species. To date, two studies have reported that transgenic mice expressing human PrP with methionine at polymorphic residue 129 are resistant to intracerebral challenge with CWD prions. The first of these studies used two lines of transgenic mice expressing human PrP at either one or two times the endogenous level of mouse brain. After inoculation with CWD-infected elk brain homogenate, none of these transgenic mice showed clinical signs of prion disease or detectable accumulation of abnormal PrP by either immunohistochemistry or immunoblotting (Kong et al., 2005). Although these mice are susceptible to infection with atypical BSE prions, their susceptibility to classic BSE prions or vCJD prions has not been reported (Kong et al., 2008). The second study used hemizygous transgenic mice expressing human PrP at two times the endogenous level of murine PrP expression in mouse brain. No evidence of clinical prion disease was observed following intracerebral challenge with CWD-infected elk, mule deer or white-tailed deer brain homogenate; however, importantly subclinical infection was not excluded (Tamgune et al., 2006). Susceptibility of these mice to infection with BSE or vCJD prions has not been reported. Here, to investigate further the potential risks for transmission of cervid prions to humans, we have transmitted mule deer CWD prions to lines of transgenic mice overexpressing human PrP two- to sixfold with either methionine or valine at polymorphic residue 129 in which we have extensive experience of transmission of a wide range of human acquired, sporadic and inherited prion disease isolates, including kuru and multiple vCJD cases (Collinge et al., 1995a, b, 1996; Hill et al., 1997; Wadsworth et al., 2008a). Extensive comparative data are available on transmission of multiple cattle BSE isolates (Hill et al., 1997; Asante et al., 2002, 2006; Wadsworth et al., 2004) as well as BSE experimentally passaged or naturally transmitted to multiple mammalian species and these mice are therefore suitable for comparative assessment of the zoonotic potential of CWD prions.

RESULTS

Immunoblot analysis of CWD-infected brain and spinal cord

CWD-infected mule deer brain (from animal D10) and spinal cord (from animal D08) originated from captive animals housed at the Colorado Division of Wildlife, Wildlife Research Centre, Colorado, USA. Homogenates (10% w/v) of these tissues were prepared in PBS and examined for proteinase K (PK)-resistant PrP by immunoblotting. Both samples showed a high level of cervid PrPsc (Fig. 1) with a PrP glycoform ratio that showed a dominant diglycosylated conformer, typical of that associated with CWD prions (Race et al., 2002). In contrast, identical analysis of brain homogenates prepared from uninfected mule deer showed no detectable PK-resistant PrP (Fig. 1 and data not shown).

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PrPsc-positive CWD-infected brain and spinal cord homogenates were used to prepare inocula for transmission studies in transgenic mice overexpressing human PrP with...
either methionine or valine at polymorphic residue 129. 129MM Tg35, 129MM Tg45 and 129VV Tg152 transgenic mice overexpress human PrP in brain at levels of two, four and six times that of human brain, respectively (Collinge et al., 1995b, 1996; Hill et al., 1997; Asante et al., 2002). These lines of mice have been extensively used by us for over 15 years and have proved susceptibility to infection with human or BSE prions (Collinge et al., 1995b, 1996; Hill et al., 1997; Asante et al., 2002, 2006; Wadsworth et al., 2004, 2007, 2008a). Following intracerebral inoculation with CWD brain or spinal cord, groups of 10 transgenic mice were observed throughout their life time for clinical signs of prion disease. As reported in Table 1, we observed no clinical prion disease in any inoculated mouse, including those with post-inoculation intervals greater than 700 days (Table 1). Accordingly, brains from mice culled as a result of intercurrent illness or senescence were examined for subclinical prion transmission. In all cases examined, pathological PrP accumulation in brain was undetectable by either immunoblotting (Fig. 2, Table 1) or immunohistochemistry (Fig. 3, Table 1). Furthermore, neuropathological examination of CWD-inoculated transgenic mouse brain, showed no evidence of spongiform change or gliosis consistent with prion disease and their appearance was indistinguishable from the brain of age matched control mice inoculated with normal mule deer brain (Fig. 3 and data not shown). In summary, we conclude that intracerebral challenge of these transgenic mice with CWD prions caused no clinical or subclinical prion infection, indicating that both methionine and valine 129 polymorphs of human PrP are refractory to pathological conversion by CWD prions.

**DISCUSSION**

In this study, we have shown that transgenic mice overexpressing human PrP of both residue 129 polymorphic forms, known to be susceptible to a wide range of human and other prions, are highly resistant to infection with mule deer CWD prions. These findings agree with those of others who have previously reported an inability of CWD prions to transmit disease to transgenic mice expressing human PrP 129 methionine (Kong et al., 2005; Tamguney et al., 2006) or a poor ability of human PrP to act as a substrate for CWD prions in *in vitro* conversion assays (Raymond et al., 2000; Kurt et al., 2009). Importantly, the transgenic mice used in our study have proven susceptibility to infection with BSE prions [Hill et al., 1997; Asante et al., 2002, 2006; Wadsworth et al., 2004 (Table 1)]. The negative transmissions that we report here therefore strongly support the conclusion that the transmission barrier associated with the interaction of human PrP and these CWD prions is greater than that associated with interaction of human PrP and the prion strain causing epizootic BSE in cattle.

The failure to show propagation of CWD prions using human PrP as a substrate either *in vivo* in transgenic mice or *in vitro* in biochemical conversion assays suggests that potential zoonotic threat from CWD is low. However, an important caveat in this regard is that the number of prion strains propagated in CWD is currently unknown.

**Table 1. Primary transmission of CWD and BSE prions to transgenic mice**

Data for BSE transmissions have been published previously (Hill et al., 1997; Asante et al., 2002).

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>CWD brain</th>
<th>CWD spinal cord</th>
<th>BSE brain</th>
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<tr>
<td>Attack rate*</td>
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<tr>
<td>129VV Tg152</td>
<td>0/8†</td>
<td>0/6‡</td>
<td>10/26</td>
</tr>
<tr>
<td>129MM Tg45</td>
<td>0/7§</td>
<td>0/6¶</td>
<td>9/12</td>
</tr>
<tr>
<td>129MM Tg35</td>
<td>0/9¶</td>
<td>0/5#</td>
<td>14/49</td>
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*All mice were inoculated with 30 μl of 1% (w/v) tissue homogenate. Attack rate is defined as the total number of both clinically affected and subclinically infected mice as a proportion of the number of inoculated mice. Subclinical prion infection was assessed by sodium phosphotungstic acid precipitation of 250 μl 10% brain homogenate and analysis for PrPSc by immunoblotting and/or immunohistochemical examination of brain.

†Mice culled at 274, 316, 321, 436, 517, 517, 578 and 781 days post-inoculation.
‡Mice culled at 354, 364, 463, 541, 704 and 724 days post-inoculation.
§Mice culled at 322, 322, 395, 400, 529, 656 and 736 days post-inoculation.
¶Mice culled at 275, 345, 396, 462, 462 and 532 days post-inoculation.
#Mice culled at 341, 559, 662, 662, 680, 707, 707, 747 and 748 days post-inoculation.

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"http://vir.sgmjournals.org"
(Browning et al., 2004; Raymond et al., 2007; Green et al., 2008; Angers et al., 2010). Because prion strains can adapt and mutate on passage in new species (Collinge & Clarke, 2007; Beringue et al., 2008; Castilla et al., 2008; Collinge, 2010), and also within species as a result of PrP polymorphisms and other genetic factors (Asante et al., 2002; Lloyd et al., 2004; Wadsworth et al., 2004; Mead et al., 2009; Lloyd et al., 2009), the risk that each prion strain poses to public health must be evaluated directly. There is now growing evidence that polymorphisms of cervid PrP may dictate prion strain selection (O’Rourke et al., 2004; Meade-White et al., 2007; Green et al., 2008; Angers et al., 2010). Thus, while the available experimental data appear reassuring, further transmission studies will be of vital importance to evaluate the properties of distinct cervid prion strains as they are isolated.

**METHODS**

**Mule deer tissues.** Importation, storage and use of CWD-infected tissues was performed under licence granted by Defra under the terms of the Importation of Animal Pathogens Order 1980. CWD-infected mule deer brain (from animal D10) and spinal cord (from animal D08) originated from naturally infected captive animals from Colorado, USA that had clinical signs consistent with terminal stages of prion disease. CWD-infection in these animals was confirmed by the presence of histopathological lesions in the brain, including spongiform degeneration of the perikaryon, by immunohistochemical or immunoblot detection of disease-related PrP and by positive transmission of prion disease to transgenic mice expressing cervid PrP (Browning et al., 2004; Angers et al., 2006; Green et al., 2008). Brain from uninfected mule deer fawns (FPS 6.98 and FPS 3.98) was used as negative controls.

**Transgenic mice.** Transgenic mice homozygous for a human PrP 129V transgene array and murine PrP null alleles (Prnp<sup>−/−</sup>) designated Tg(HuPrP129V<sup>+/+</sup> Prnp<sup>−/−</sup>)-152 mice (129VV Tg152 mice) or homozygous for a human PrP 129M transgene array and murine PrP null alleles (Prnp<sup>−/−</sup>) designated Tg(HuPrP129M<sup>+/+</sup> Prnp<sup>−/−</sup>)-35 mice (129MM Tg35 mice) or Tg(HuPrP129M<sup>+/+</sup> Prnp<sup>−/−</sup>)-45 mice (129MM Tg45 mice) have been described previously (Collinge et al., 1995b, 1996; Hill et al., 1997; Asante et al., 2002, 2006; Wadsworth et al., 2004, 2007, 2008a).

![Fig. 2](image1.png)

**Fig. 2.** Failure to detect PrP<sub>Sc</sub> in the brain of CWD prion-inoculated transgenic mice. The high sensitivity immunoblot using anti-PrP monoclonal antibody 3F4 shows PK-digested sodium phosphotungstic acid pellets recovered from 10% (w/v) transgenic mouse brain homogenates. Lanes 1 and 2, positive controls showing efficient recovery of PrP<sub>Sc</sub> after spiking 2 μl 10% (w/v) BSE-inoculated 129MM Tg45 and 129MM Tg35 transgenic mouse brain homogenates (Asante et al., 2002) into 100 μl 10% (w/v) uninfected 129MM Tg45 and 129MM Tg35 mouse brain homogenates, respectively. Lane 3, PK-digested sodium phosphotungstic acid pellet from 250 μl 10% (w/v) brain homogenate from a 129MM Tg45 mouse inoculated with normal mule deer brain. Lanes 4–9, PK-digested sodium phosphotungstic acid pellets from 250 μl 10% (w/v) brain homogenates from 129MM Tg35, 129MM Tg45 and 129VV Tg152 mice inoculated with CWD-infected mule deer brain.

![Fig. 3](image2.png)

**Fig. 3.** Failure to detect abnormal PrP deposition in the brain of CWD prion-inoculated transgenic mice. Representative PrP immunohistochemistry using anti-PrP monoclonal antibody ICSM35. Panels (a–f) show no abnormal PrP deposition in either the hippocampus or thalamus of 129VV Tg152, 129MM Tg45 or 129MM Tg35 mice inoculated with CWD-infected brain homogenate. These mice were culled 517, 529 and 559 days post-inoculation, respectively. Panels (g) and (h) show hippocampus and thalamus from an age matched control 129MM Tg45 mouse brain inoculated with 10% (w/v) uninfected mule deer brain homogenate. In contrast, extensive deposition of abnormal PrP is seen in the hippocampus and thalamus of a BSE-infected 129MM Tg45 mouse with subclinical prion disease (panels i and j) (Asante et al., 2002). Bar, 500 μm.
Transmission studies. All procedures were carried out in a microbiological containment level 3 facility with strict adherence to safety protocols. Care of mice was according to institutional guidelines. Mule deer tissues were prepared as 10 % (w/v) homogenates in sterile PBS lacking Ca\(^{2+}\) and Mg\(^{2+}\) ions by serial passage through needles of decreasing diameter, and subsequently diluted to 1 % (w/v) in PBS. Following intracerebral inoculation with 30 μl of 1 % (w/v) tissue homogenate as described previously (Asante et al., 2002, 2006; Wadsworth et al., 2004), mice were examined daily and were killed if exhibiting signs of distress or once a diagnosis of clinical prion disease was established. Brains from inoculated mice were analysed by PrP immunoblotting or immunohistochemistry and by neuropathological examination.

Immunoblotting. All procedures were carried out in a microbiological containment level 3 facility with strict adherence to safety protocols. Tissue homogenates (10 % w/v) were prepared in PBS lacking Ca\(^{2+}\) or Mg\(^{2+}\) ions. PK digestion (50 or 100 μg ml\(^{-1}\) final protease concentration, 1 h, 37 °C), electrophoresis and immunoblotting was performed as described previously (Wadsworth et al., 2001, 2008b). Immunoblot detection was performed using anti-PrP monoclonal antibody ICSM35 (D-Gen) for cervid PrP or 3F4 (Kascak et al., 1987) for human PrP in transgenic mice. Brain homogenates scored negative for PrP\(^{Sc}\) after analysis of 10 μl 10 % (w/v) brain homogenate were re-analysed by sodium phosphotungstic acid precipitation of PrP\(^{Sc}\) (Safar et al., 1998) from 250 μl of 10 % (w/v) brain homogenate as described previously (Wadsworth et al., 2001).

Neuropathology and immunohistochemistry. All steps prior to prion decontamination with formic acid were performed within a microbiological containment level 3 facility with strict adherence to safety protocols. Brain was fixed in 10 % buffered formal saline and then immersed in 98 % formic acid for 1 h and paraffin wax embedded. Serial sections of 4 μm thickness were pre-treated by boiling for 10 min in a low ionic strength buffer (2.1 mM Tris, 1.3 mM EDTA, 1.1 mM sodium citrate, pH 7.8) before exposure to 98 % formic acid for 5 min. Abnormal PrP accumulation was examined using anti-PrP monoclonal antibody ICSM35 (D-Gen) on a Ventana automated immunohistochemical staining machine (Ventana Medical Systems) using proprietary secondary detection reagents (Ventana Medical Systems) before development with 3′-diaminobenzidine tetrachloride as the chromogen (Wadsworth et al., 2008b). Harris haematoxylin and eosin staining was done by the Colorado Division of Wildlife for access to the CWD-infected deer samples. This research was funded by the Medical Research Council (UK) and the European Union. Conflict of interest statement: J. C. is a Director and J. C. and J. D. F. W. are shareholders and consultants of D-Gen Limited, an academic spin-out company working in the field of prion disease diagnosis, decontamination and therapeutics. D-Gen markets the ICSM35 antibody used in this study.

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wasting disease to hamsters and transgenic mice: evidence for strains. 


