Short Communication

Limited amplification of chronic wasting disease prions in the peripheral tissues of intracerebrally inoculated cattle

Nicholas J. Haley,1,2† Christopher Siepker,1 Justin J. Greenlee3 and Jürgen A. Richt1

1Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA
2Department of Basic Sciences, Midwestern University, Glendale, AZ, USA
3Virus and Prion Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames, Iowa, USA

Chronic wasting disease (CWD) is a fatal neurodegenerative disease, classified as a prion disease or transmissible spongiform encephalopathy (TSE) similar to bovine spongiform encephalopathy (BSE). Cervids affected by CWD accumulate an abnormal protease-resistant prion protein throughout the central nervous system (CNS), as well as in both lymphatic and excretory tissues—an aspect of prion disease pathogenesis not observed in cattle with BSE. Using seeded amplification through real-time quaking-induced conversion, we investigated whether the bovine host or prion agent was responsible for this aspect of TSE pathogenesis. We blindly examined numerous central and peripheral tissues from cattle inoculated with CWD for prion seeding activity. Seeded amplification was readily detected in the CNS, though rarely observed in peripheral tissues, with a limited distribution similar to that of BSE prions in cattle. This seems to indicate that prion peripheralization in cattle is a host-driven characteristic of TSE infection.

The transmissible spongiform encephalopathies (TSEs), or prion diseases, are infectious, progressive and uniformly fatal neurodegenerative diseases of humans and animals (Bolton et al., 1982; Prusiner, 1998). The designation ‘prion disease’ is based on the association of these diseases with aggregates of a conformationally altered and post-translationally modified isoform of the normal cellular prion protein (PrP^C) (Williams, 2005). The misfolded isoform has been designated variously, including PrP^Sc (scrapie-associated abnormal prion protein), PrP^Pres (protease-resistant abnormal prion protein) and PrP^d (disease-associated prion protein). In this report, the more general term ‘PrP^d’ will be used to denote the infectious prion protein associated with TSEs. While most TSEs are transmissible experimentally between susceptible species, distinct TSE strains, including chronic wasting disease (CWD) and bovine spongiform encephalopathy (BSE), show markedly different proclivities in their pathogenesis and transmissibility (Greenlee & Greenlee, 2015; Haley & Hoover, 2015).

Although the clinical endpoint in both CWD and BSE is the same—the pathological accumulation of the PrP^d in the central nervous system (CNS) leading to progressive neurologic dysfunction and death, the routes the agents take both prior and subsequent to CNS invasion vary significantly. In vitro amplification assays, including serial protein misfolding cyclic amplification (PMCA), have been employed to more sensitively evaluate both central and peripheral tissues to better elucidate TSE pathogenesis in both species (Franz et al., 2012; Haley et al., 2011). In cervids, the accumulation of the abnormal prion protein in the CNS is preceded by lymphoid amplification and transit through the sympathetic nervous system (Fox et al., 2006; Sigurdson et al., 2002; Spraker et al., 1997). Subsequent to or concurrent with CNS replication, the prion agent is thought to make its way to a range of peripheral organs where it may be shed in excreta (Haley et al., 2011). It is not known whether PrP^d is simply accumulating in these tissues as it spills from CNS and lymphoid systems or if it may be amplifying in situ. In cattle, BSE may be found in peripheral lymphoid centres prior to CNS appearance (Balkema-Buschmann et al., 2010; Hoffmann et al., 2011), though there is only limited dissemination to select peripheral tissues after CNS invasion, including select components of the...
autonomic nervous system, with no evidence for shedding in bodily fluids (Balkema-Buschmann et al., 2010; Buschmann & Groschup, 2005; Franz et al., 2012).

At present, no research has been directed at identifying the underlying pathological basis for these discrepancies, and it is unknown whether peripheralization is a trait conferred by either the host or prion strain. By sensitively evaluating tissue from CWD-challenged cattle using real-time quaking-induced conversion (RT-QuIC) (Atarashi et al., 2007), an in vitro amplification assay which can semi-quantitatively measure PrP\textsuperscript{d} in a sample through progressively monitored conversion of recombinant PrP\textsuperscript{C} (Henderson et al., 2014), we sought to further evaluate the significance of PrP\textsuperscript{d} peripheral pathogenesis in these interspecies transmission experiments.

In previous studies, cattle were inoculated intracerebrally with various brain-derived CWD isolates from cervids, including white-tailed deer (CWD\textsuperscript{WTD}), mule deer (CWD\textsuperscript{MD}), and elk (CWD\textsuperscript{Elk}) (Greenlee et al., 2012; Hamir et al., 2005, 2007, 2011). Attack rates (and mean incubation periods ±SD), by group, were 12/14 (645±59 days), 5/13 (1182±596 days) and 2/14 (495±15 days), respectively (Fig. 1). In total, 19 of 41 cattle inoculated developed both clinical and immunohistopathological evidence of a TSE affecting the CNS. In a subsequent study, TSE-positive brain material from one animal inoculated with CWD\textsuperscript{MD} was sub-passaged into a second group of cattle, resulting in a significantly higher attack rate (6/6, Fisher’s exact test \(P=0.017\)) and shorter incubation periods (mean 462±24 days, log-rank test; \(z=3.2, P=0.0014\)) (Hamir et al., 2006). Negative control groups were included in each of these studies, and included a total of 12 cattle inoculated intracerebrally with known CWD-negative brain tissue. A number of tissues were collected cleanly at necropsy from experimental cattle in all groups when possible, totalling 451 unique samples from 59 TSE-positive and -negative cattle and including brain, central and peripheral lymphoid tissues (e.g. mesenteric and retropharyngeal, and tonsillar, respectively), ileum (without Peyer’s patches grossly identified), kidney, tongue, salivary gland and nasal turbinate, and frozen at −80 °C prior to analyses.

Central and peripheral tissues were homogenized individually in RT-QuIC reaction buffer (0.02 %, w/v) and evaluated blindly, in triplicate, in two separate experiments, in the RT-QuIC assay using a truncated form of the Syrian hamster prion protein (SHrPrP) as a substrate as described previously (Haley et al., 2014). Preparations were subjected to 96 cycles of shaking and incubation (~24 h), with fluorescent readings taken every 15 min, allowing for realtime evaluation of seeded conversion. Control samples were represented in triplicate in each experimental plate, and consisted of a positive control – pooled homogenate (0.02 %, w/v) of CWD-positive brain from six experimentally inoculated white-tailed deer (CBP6), three confirmed negative-control tissue homogenates (0.02 %) from sham-inoculated animals, as well as an unspiked negative control.

Positive replicate wells were identified as those which amplified above a predetermined fluorescent threshold, five standard deviations greater than the mean fluorescence for all samples across cycles 2–8. The mean rate of amplification (\(\bar{r}\)) for a sample was defined as the inverse of meantime (in minutes) required for each of six total replicates to reach this threshold. Positive controls amplified in an expectedly narrow range of time (~330–390 min, plate dependent, e.g. \(\bar{r}=3.03–2.56 \times 10^{3} \text{ min}^{-1}\)), and all amplification rates of blinded samples were referenced back to this rate to produce a relative rate, or the ‘\(R\) score’. As an example, brain from animal 615, inoculated with CWD\textsuperscript{WTD}, had an \(\bar{r}=2.13 \times 10^{3} \text{ min}^{-1}\) in experiments with a positive control \(\bar{r}=2.67 \times 10^{3} \text{ min}^{-1}\), yielding an \(R\) score of 0.800 (2.13 \times 10^{3} \text{ min}^{-1}/2.67 \times 10^{3} \text{ min}^{-1}) for this sample (Fig. 2). Preconfirmed negative controls demonstrated no amplification throughout the study, and had \(R\) scores of 0.

At the completion of the amplification experiments, \(R\) scores were calculated and samples subsequently unblinded. At that time, a low level of background positivity was identified in blinded negative control samples, with 8 of 79 blind negative tissues showing amplification in no more than 1/6 replicates (e.g. 8 of 474 total blinded negative replicates), with an mean \(R\) score of 0.082 in these samples. From the blinded negative control data, an \(R\) score threshold of 0.14 (five standard deviations greater than the mean \(R\) score among all negative control tissues) was set to identify true
positive samples. The rare finding of false positives in blinded negative controls was not surprising given the imperfect specificity reported with RT-QuIC in the past (Haley et al., 2013), and highlights the importance of appropriate and blinded controls in addition to a strategy for careful sample collection, processing and analytical techniques developed a priori.

Brain samples from all but one TSE-positive cow were positive by RT-QuIC, with R scores ranging from 0.273 to 0.970 (mean=0.700, averaging 5.5 positive replicates). The brain sample from the TSE-positive, RT-QuIC-negative animal showed no evidence of seeded amplification, with an R score of 0, which could indicate either reduced sensitivity, a non-representative sample or an incorrect determination of TSE status in this animal. Unequivocally positive tissues amongst inoculated animals with histopathological and RT-QuIC confirmation of a CNS prion disease were rare, and included turbinate (n=4/33, mean R score 0.215 averaging 2.25 positive replicates), mesenteric lymph node (n=1/38, R score 0.273, 2 positive replicates) and tonsil (n=1/45, R score 0.390, 4 positive replicates) from TSE-positive cattle (Table 1). Intriguingly, each of these tissues was negative by conventional immunohistochemistry (IHC; Hamir et al., 2011), asserting that amplification assays may be more sensitive than IHC in interspecies transmission studies. Twelve additional tissues from positive animals had rates well below the threshold and within the range of scores observed among false positive tissues from negative control cattle. The remainder showed no evidence of seeded amplification. These findings confirm that the limited peripheralization of CWD prions in cattle, like that of BSE prions, may be a host-dependent phenomenon in this species. Nasal turbinates, which have been shown to convey BSE infectivity in cattle in the absence of detectable PrP<sup>d</sup>, have likewise shown evidence of PrP<sup>d</sup> or seeded amplification in other species (Balkema-Buschmann et al., 2010). The same holds true for tonsillar tissue, while mesenteric lymph nodes have only rarely been positive in BSE amplification assays (Franz et al., 2012). Importantly, it should be noted that no compelling evidence was found to support a hypothesis that peripheral adaptation had occurred following the second passage of CWD<sub>MD</sub>, despite clinical evidence of CNS adaptation (e.g. significantly higher attack rates and significantly shorter incubation periods). No positive peripheral tissues were found in animals inoculated with primary passage CWD<sub>MD</sub>, while just a single turbinate was considered positive in one animal inoculated with second passage CWD<sub>MD</sub> (Fisher’s exact test P=0.545).

These findings are contrasted by those seen in deer inoculated experimentally with CWD, importantly including the intracerebral route of exposure. Serial PMCA of central and peripheral tissues in intracerebrally inoculated white-tailed deer has demonstrated intense and widespread accumulation of amplifiable PrP<sup>d</sup>, targeting peripheral lymph nodes, salivary glands and tissues of the urinary and gastrointestinal tract (Haley et al., 2011). Distribution was significantly associated with CNS deposition and genotypic background, with broader patterns of CWD peripheralization observed in animals exhibiting higher levels of CNS amplification and those with more susceptible genotypes. The limited peripheralization of amplifiable prions in cattle inoculated with CWD could not be explained by low levels of PrP<sup>d</sup> deposition in the CNS, as all TSE-positive cattle demonstrated high levels of PrP<sup>d</sup> by IHC throughout the brainstem and midbrain. Likewise, cattle with brain tissue R scores greater than the mean of 0.700 were no more likely to have a positive peripheral tissue than those with lower R scores in brain (Fisher’s exact test P=0.35). Without an apparent influence of CNS burden in cattle, there remain two plausible, host-dependent explanations for our findings: peripheral distribution of PrP<sup>d</sup> may be dependent on (1) host PrP genotype (e.g. primary structure of PrP<sup>d</sup>) or, alternatively, (2) the peripheral expression levels of the cellular prion protein. A recent study investigating the oral transmissibility of BSE to European red deer reported immunohistochemical localization of PrP<sup>d</sup> in nervous tissues including brainstem, cerebellum and spinal cord, cranial nerves, sensory retina and throughout the enteric nervous system, though also in a limited number of enteric lymphoid aggregates. This finding, though limited in scope, offers no clearer explanation for prion peripheralization, though perhaps a more detailed examination of the peripheral tissues from these animals using amplification methods.
may provide additional insight into the role of host physiology (Dagleish et al., 2008, 2015), and further define the roles of host and agent in PrP\(^\text{Sc}\) peripheralization and, ultimately, horizontal transmissibility.

In summary, using seeded amplification via RT-QuIC, we have re-examined central and peripheral tissues from cattle inoculated with CWD prions and found no further evidence that the agent is peripherally distributed as it is in cervids, despite sub-passage and apparent CNS adaptation. Conversely, the tissue distribution mirrored that of BSE prions — reconfirming that bovine host factors may dictate TSE peripheralization and horizontal transmissibility. Parallel studies sensitively examining the peripheral tissues in other interspecies transmission experiments may help shed light on the role of the host and agent in TSE pathogenesis.

References


Table 1. RT-QuIC-positive samples amongst those from each group evaluated

Numbers represent the number of positive samples with the total number evaluated in parentheses. WTD, cattle inoculated with white-tailed deer CWD; MD1, primary passage of mule deer CWD in cattle; MD2, sub-passage of MD1 brain into cattle; RLN, retropharyngeal lymph node; MesLN, mesenteric lymph node; NA, no sample available.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>WTD</th>
<th>MD1</th>
<th>MD2</th>
<th>Elk</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>12 (14)</td>
<td>3 (13)</td>
<td>6 (6)</td>
<td>2 (14)</td>
<td>0 (10)</td>
</tr>
<tr>
<td>RLN</td>
<td>0 (14)</td>
<td>0 (13)</td>
<td>0 (6)</td>
<td>0 (12)</td>
<td>0 (10)</td>
</tr>
<tr>
<td>MesLN</td>
<td>1 (11)</td>
<td>0 (10)</td>
<td>0 (6)</td>
<td>0 (11)</td>
<td>0 (10)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0 (14)</td>
<td>0 (6)</td>
<td>0 (1)</td>
<td>0 (13)</td>
<td>0 (5)</td>
</tr>
<tr>
<td>Tonsil</td>
<td>0 (14)</td>
<td>0 (12)</td>
<td>0 (5)</td>
<td>1 (14)</td>
<td>0 (10)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0 (14)</td>
<td>0 (9)</td>
<td>0 (5)</td>
<td>0 (13)</td>
<td>0 (8)</td>
</tr>
<tr>
<td>Turinate</td>
<td>2 (13)</td>
<td>0 (0)</td>
<td>1 (6)</td>
<td>1 (14)</td>
<td>0 (7)</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>0 (1)</td>
<td>0 (2)</td>
<td></td>
<td>NA</td>
<td>0 (13)</td>
</tr>
<tr>
<td>Tongue</td>
<td>0 (14)</td>
<td>0 (0)</td>
<td>0 (6)</td>
<td>0 (13)</td>
<td>0 (6)</td>
</tr>
<tr>
<td>Ileum</td>
<td>0 (12)</td>
<td>0 (8)</td>
<td>0 (6)</td>
<td>0 (14)</td>
<td>0 (8)</td>
</tr>
<tr>
<td>Total</td>
<td>121</td>
<td>73</td>
<td>47</td>
<td>131</td>
<td>79</td>
</tr>
</tbody>
</table>

http://jgv.microbiologyresearch.org


