Short Communication

Progression of prion infectivity in asymptomatic cattle after oral bovine spongiform encephalopathy challenge

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The presence of BSE prion infectivity in asymptomatic cattle and its tissue distribution are important concerns for both human and veterinary health and food safety. In this work, a collection of tissues from asymptomatic cattle challenged orally with BSE and culled at 20, 24, 27, 30 and 33 months have been used to inoculate intracerebrally BoPrP-Tg110 mice expressing bovine PrP to assess their infectivity. Results demonstrate that BSE infectivity in asymptomatic cattle is essentially restricted to the nervous system, Peyer’s patches and tonsils, as reported previously for terminally BSE-diseased cattle. BSE infectivity was detectable in Peyer’s patches and tonsils at all time points analysed, but infectivity in nervous tissues (brainstem and sciatic nerve) was only detectable after 27 months from inoculation. Infectivity in brainstem increased markedly at 33 months after inoculation. All other investigated tissues or fluids (spleen, skeletal muscle, blood and urine) revealed no detectable infectivity throughout the time course studied.

Transmissible spongiform encephalopathies (TSEs) or prion diseases are associated with the accumulation of abnormal PrPSc conformer in the brain and subsequent neurodegeneration (Prusiner, 2004). The mechanism of conversion of PrPSc to PrPSc is not well understood, but it may occur by direct interaction of PrPSc with PrPC, promoting refolding of the latter to produce additional PrPSc. The PrPSc conformer can be recognized by its partial resistance to proteinase K treatment.

Most TSEs are transmitted naturally by peripheral routes, either orally or transcutaneously. The mechanism(s) of spread from the periphery to the central nervous system (CNS) is an important issue. It is not clear how prions pass through the intestinal mucosa after oral uptake. M cells, which are portals for antigens and pathogens (Hathaway & Kraehenbuhl, 2000), may be involved in the transepithelial transport of prions (Heppner et al., 2001). Thus, the infectious agent may penetrate the mucosa through M cells and reach the Peyer’s patches. Although prion diseases are neurological disorders, critical events in their pathogenesis take place in restricted sites outside the nervous system, especially in peripheral lymph organs (Aucouturier et al., 2000).

Bovine spongiform encephalopathy (BSE) was recognized as a cattle prion disease during the 1980s (Wilesmith et al., 1988) in the UK. Ingestion of foods contaminated with BSE is the likely cause of the new variant Creutzfeldt–Jakob disease in humans (Bruce et al., 1997; Hill et al., 1997). Several studies indicate that, to date, the BSE agent has been found only in the brain, spinal cord and retinal (eye) tissue of BSE-diseased cattle. Infectivity assessment in several tissues from orally inoculated cattle, using bioassays based on RIII mice (Wells et al., 1994, 1998), revealed BSE infectivity in the CNS, all brain regions, the spinal cord, the optic nerve, the retina (neuronal cells) and the facial and sciatic nerves, as well as in distal ileum and bone marrow. The skeletal muscles, spleen and other lymphatic tissues were shown to be free of detectable infectivity. More recently, Wells et al. (2005) showed infectivity in tonsil tissue from cattle killed 10 months after oral BSE challenge by intracerebral inoculation in cattle. These finding are in contrast to the spreading of the scrapie agent in infected sheep, mice and hamsters in tissues such as spleen, other lymphatic tissues, muscles etc., even during the preclinical stage (Bosque et al., 2002; Heggebo et al., 2003; Thomzig et al., 2003, 2004). In addition, PrPSc can be found in the lymphoreticular system and is not restricted to the nervous system following oral inoculation of sheep and primates with the BSE agent (Bons et al., 1999; Jeffrey et al., 2001; Herzog et al., 2004; Andreoletti et al., 2006). Recently, experiments in transgenic mice overexpressing bovine PrP confirmed the essential restriction of infectivity to the nervous system in terminally BSE-diseased cattle (Buschmann & Groschup, 2005).

The distribution of BSE infectivity in asymptomatic cattle incubating the disease and its progression through the silent period from inoculation to the appearance of clinical
signs is of particular interest in relation to food safety. In the present work, we have used a highly sensitive bioassay based on transgenic mice overexpressing bovine PrP (BoPrP-Tg110 mice) (Castilla et al., 2003) to assess the infectivity in a panel of tissues from asymptomatic cattle at different times (20–33 months) after oral challenge.

Tissues from asymptomatic cattle after oral BSE challenge were prepared and kindly provided by the Veterinary Laboratory Agency (VLA), New Haw, Addlestone, Surrey, UK, as part of the VLA Project SE1736. Cattle (4–6 months of age) were inoculated orally with 100 g doses of BSE-infected brainstem material derived from a homogenate of about 150 clinically sick and pathologically confirmed cases of BSE. Control animals were maintained under the same conditions, but not infected. Clinical signs were assessed monthly by veterinarian experts from the VLA (New Haw, Addlestone, Surrey, UK). At different times post-inoculation, three infected and one control animal were culled and a panel of tissues and fluids were sampled aseptically. The tissue and fluids were stored at −70 °C. In this study, tissues from animals culled at 20, 24, 27, 30 and 33 months post-infection were investigated. Homogenates (10% in PBS) of each tissue or fluid from asymptomatic cattle sampled at the indicated times were used for infectivity assessment in BoPrP-Tg110 mice. Samples from the three inoculated cows at each time point were used as pools.

All pools, containing each tissue sampled from three different cattle at the same time after challenge, were tested for the presence of PrPSc before inoculation of BoPrP-Tg110 mice. PrPSc was analysed by Western blotting in brain tissues collected and homogenized in PBS. One hundred microlitres of 10% (w/v) brain homogenate was pre-cleared by centrifugation at 2000 g for 5 min in 5% sarcosyl. Samples were treated with 20 µg proteinase K ml⁻¹ (Roche) at 37 °C for 60 min and insoluble fractions were obtained by centrifugation at 25 000 g for 30 min. SDS sample loading buffer was added to all samples, boiled for 10 min and loaded on an SDS/12% polyacrylamide gel. For the immunoblotting experiments, mAbs 2A11 (Brun et al., 2004) and Sha31 (Feraudet et al., 2003) to assess the presence of PrPSc were used. Western blot was the least sensitive. Most of the positive mouse brains were positive for all three tests, except for those indicated in Table 2, which were negative by Western blot, but positive by ELISA and immunohistochemistry.

BSE infectivity was first detected in Peyer’s patches and tonsils as early as 20 months after oral inoculation (the first time point analysed) and maintained during the time course. Infectivity in nervous tissues (brainstem and sciatic nerve) was detectable only at 27–30 months after inoculation, but infectivity in brainstem increased remarkably at 33 months after inoculation. All other tissues or fluids examined, including the spleen, skeletal muscle, blood and urine, revealed no detectable infectivity throughout the time course studied (Table 2). All tissues sampled from control non-infected cattle were negative for infectivity when used to inoculate BoPrP-Tg110 mice (data not shown).

### Table 1. Detection of PrPSc in the brainstem of asymptomatic cattle sampled at the indicated times after oral challenge with BSE

<table>
<thead>
<tr>
<th>Period after oral challenge (months)</th>
<th>Bio-Rad ELISA TeSeE (absorbance)*</th>
<th>Western blot (mAb 2A11)</th>
</tr>
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<tbody>
<tr>
<td>20</td>
<td>0.026</td>
<td>Negative</td>
</tr>
<tr>
<td>24</td>
<td>0.028</td>
<td>Negative</td>
</tr>
<tr>
<td>27</td>
<td>0.037</td>
<td>Negative</td>
</tr>
<tr>
<td>30</td>
<td>0.027</td>
<td>Negative</td>
</tr>
<tr>
<td>33</td>
<td>0.957</td>
<td>Negative</td>
</tr>
</tbody>
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*Absorbance was measured at 450–620 nm; cut-off was 0.245.

Under the control of the murine Prnp gene promoter from the MoPrP.Xho vector (Borchelt et al., 1996) and are highly susceptible to BSE infection (Castilla et al., 2003, 2004). Groups of six mice (6–7 weeks old, weighing around 20 g) were inoculated with 20 µl of the appropriate tissue pool in the right parietal lobe by using a 25-gauge disposable hypodermic needle. After inoculation, the mice were observed daily and their neurological status was assessed twice weekly. Mice showing at least three of ten signs of neurological dysfunction (Scott et al., 1989, 1993) over several consecutive days were sacrificed and samples were collected for diagnostic evaluation. Survival times were calculated as the time between inoculation and death. All mice in an experiment were tested for PrPSc accumulation in their brains, and only those positive for PrPSc were included in the calculation of survival times.

PrPSc accumulation in mouse brains was tested by Western blotting and ELISA (as described above) and immunohistochemistry. Immunohistochemistry was accomplished by using mAb 2A11 (Brun et al., 2004) as described previously (Castilla et al., 2003). For negative controls, the primary specific antibody was replaced by non-immune mouse serum in tissue sections used as negative controls. The results are summarized in Table 2. Animals were scored as PrPSc-positive when PrPSc was detected by at least one of the three tests, the Western blot being the least sensitive. Most of the positive mouse brains were positive for all three tests, except for those indicated in Table 2, which were negative by Western blot, but positive by ELISA and immunohistochemistry.

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The PrPSc electrophoretic profiles found in the brain of BoPrP-Tg110 mice inoculated with the different tissues showed no apparent differences when positive tissues were compared with each other or with PrPSc from the brain of a natural BSE case (Fig. 1).

In contrast to the well-documented transmission and spread of scrapie in small ruminants, BSE in cattle is far less well understood. It is well known that the lymphatic system is involved in TSE pathogenesis in sheep, as demonstrated by the detection of infectivity during early preclinical disease stages (van Keulen et al., 1996; Race et al., 1998; Andreoletti et al., 2000; Madec et al., 2000; Heggebo et al., 2003). To date, all previous investigations to detect BSE infectivity in bovine lymphatic tissue have shown negative results, with the exception of the Peyer’s patches of the distal ileum from BSE-diseased cattle (Wells et al., 1998).

In the present study, spleen (which is one of the most important organs of the lymphoreticular system) was found to be free of detectable infectivity in asymptomatic cattle throughout the time course studied (Table 2), supporting the hypothesis that BSE infectivity cannot be found in this organ in cattle during the preclinical disease stages. Peyer’s patches and tonsils showed a low, but early, detectable infectivity in asymptomatic cattle after oral challenge that was maintained throughout the time course studied (Table 2). These results are consistent with the proposed model for prion spreading from lymphoid tissues to the CNS. We can speculate that the poor and limited BSE replication detected in lymphatic tissues could be sufficient to allow its spread to the CNS, but we cannot rule out the participation of other, untested lymphatic tissues.

In brainstem, the detection of infectivity was only achieved 27 months after oral challenge. The infectivity found in brainstem, the detection of infectivity was only achieved 27 months after oral challenge. The infectivity found in

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**Table 2.** Challenge of BoPrP-Tg110 mice with tissues from asymptomatic cattle infected orally with BSE

PsPSc (+) data are no. animals scored positive for PrPSc/no. inoculated animals; survival time is given in days (±SEM).

<table>
<thead>
<tr>
<th>Period after oral challenge</th>
<th>Sample used for inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brainstem</td>
</tr>
<tr>
<td>20 months</td>
<td>PrPSc (+)</td>
</tr>
<tr>
<td>Survival time</td>
<td>&gt;650</td>
</tr>
<tr>
<td>24 months</td>
<td>PrPSc (+)</td>
</tr>
<tr>
<td>Survival time</td>
<td>&gt;650</td>
</tr>
<tr>
<td>27 months</td>
<td>PrPSc (+)</td>
</tr>
<tr>
<td>Survival time</td>
<td>&gt;650†</td>
</tr>
<tr>
<td>30 months</td>
<td>PrPSc (+)</td>
</tr>
<tr>
<td>Survival time</td>
<td>&gt;650</td>
</tr>
<tr>
<td>33 months</td>
<td>PrPSc (+)</td>
</tr>
<tr>
<td>Survival time</td>
<td>580 (±83)</td>
</tr>
</tbody>
</table>

*Removed from the distal ileum.
†Where indicated, PrPSc in the mouse brains was scored negative for Western blot, but positive for ELISA and immunohistochemistry.

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**Fig. 1.** Western blot of brain homogenates from selected BoPrP-Tg110 mice inoculated with a pool of tissues from BSE-infected asymptomatic cattle (33 months after oral challenge). Bovine tissues used for the inoculation of BoPrP-Tg110 mice are indicated above each line, with the exception of BSE 2, which is a BoPrP-Tg110 mouse inoculated with BSE 2 derived from a natural case of BSE in cattle (Castilla et al., 2003). mAb 2A11 was used as indicated in the text. Molecular mass is given on the right in kDa.
brainstem at 27 or 30 months after challenge was low, as only one-third of the BoPrP-Tg110 mice inoculated were scored positive and with long incubation times. Previous work using the RIII mouse bioassay failed to detect infectivity in the CNS before 32 months after inoculation (Wells et al., 1998). In contrast, the pool of brainstem material from cattle sampled 33 months after oral challenge contained a high titre of infectivity, as shown by the maximum percentage of infected BoPrP-Tg110 mice found. This indicates a marked accumulation of the infectious agent in at least one of the three pooled animals during the last few months (from 30 up to 33 months) after challenge. This increased infectivity is confirmed by the detection of PrPSc by the Bio-Rad ELISA TeSeE test in this brainstem pool. As only one of the three animals used for each pool needs to be positive to transmit the disease to the BoPrP-Tg110 mice, we cannot be sure that the differences detected in the infectivity levels were not due to differences between individual cattle. A low level of infectivity was also detected in the sciatic nerve from animals sampled at 30 and 33 months after challenge. All other tissues or fluids investigated, including skeletal muscle, blood and urine, revealed no detectable infectivity throughout the time course studied (Table 1). Tissues with no detectable infectivity in the highly sensitive bioassay based on transgenic mice (without a transmission barrier for BSE prions) may be considered as being of very low risk for oral infection in humans, where a strong transmission barrier is present (Lasmezas et al., 2005).

In conclusion, our results confirm that BSE infectivity in asymptomatic cattle is essentially restricted to the nervous system, as reported previously for terminally BSE-diseased cattle (Buschmann & Groschup, 2005), and is consistent with the idea that BSE infectivity, after oral uptake, propagates only poorly in some intestinal lymphatic tissues (mainly Peyer’s patches) and from there spreads centrifugally to the CNS, probably by intraneural spread via the peripheral nervous system.

Acknowledgements

We thank the VLA (New Haw, Addlestone, Surrey, UK) for kindly providing tissues of experimentally infected cows at different time points. The authors wish to thank Dr J. Grassi from CEA (Commissariat à l’Energie Atomique), France, for providing the Sha31 antibody. Thanks are also due to Bio-Rad for supplying the ELISA TeSeE kits. This work was supported by grants INIA-CAL01-018, UE-FAIR-CT97-3306 and INIA-RTA-2006-0091.

References


