Transmission and characterization of bovine spongiform encephalopathy sources in two ovine transgenic mouse lines (TgOvPrP4 and TgOvPrP59)

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Transgenic mice expressing the prion protein (PrP) of species affected by transmissible spongiform encephalopathies (TSEs) have recently been produced to facilitate experimental transmission of these diseases by comparison with wild-type mice. However, whilst wild-type mice have largely been described for the discrimination of different TSE strains, including differentiation of agents involved in bovine spongiform encephalopathy (BSE) and scrapie, this has been only poorly described in transgenic mice. Here, two ovine transgenic mouse lines (TgOvPrP4 and TgOvPrP59), expressing the ovine PrP (A136 R154 Q171) under control of the neuron-specific enolase promoter, were studied; they were challenged with brainstem or spinal cord from experimentally BSE-infected sheep (AA136 RR154 QQ171 and AA136 RR154 RR171 genotypes) or brainstem from cattle BSE and natural sheep scrapie. The disease was transmitted successfully from all of these sources, with a mean of approximately 300 days survival following challenge with material from two ARQ-homozygous BSE-infected sheep in TgOvPrP4 mice, whereas the survival period in mice challenged with material from the ARR-homozygous BSE-infected sheep was 423 days on average. It was shown that, in the two ovine transgenic mouse lines, the Western blot characteristics of protease-resistant PrP (PrPres) were similar, whatever the BSE source, with a low apparent molecular mass of the unglycosylated glycoform, a poor labelling by P4 monoclonal antibody and high proportions of the diglycosylated form. With all BSE sources, but not with scrapie, florid plaques were observed in the brains of mice from both transgenic lines. These data reinforce the potential of this recently developed experimental model for the discrimination of BSE from scrapie agents.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are fatal, neurodegenerative diseases of the central nervous system (CNS) affecting both humans (Creutzfeldt–Jakob disease; CJD) and animals, mainly sheep and goats (scrapie), deer and elk (chronic wasting disease), and cattle (bovine spongiform encephalopathy; BSE). Incubation periods are long and ultimately lead to fatal neurological clinical signs. The disease is generally associated with the accumulation of an abnormal form of a host-encoded prion protein (PrP\textsuperscript{c}) in the CNS (Prusiner, 1982). This disease-associated PrP (PrP\textsuperscript{d}) differs from the cellular protein (PrP\textsuperscript{c}) in its biochemical properties, including partial resistance to protease K degradation (PrP\textsuperscript{res}) and insolubility in non-denaturing detergents.

Prion-disease transmission between different species, if it occurs, is generally characterized by prolonged incubation periods and low susceptibility at first passage in a new host species. On subsequent subpassages in the new host species, the infectious agent generally adapts to give shorter incubation times and higher susceptibility. This illustrates the existence of a ‘species barrier’ phenomenon. The transmission efficiency of the disease across species largely depends on differences in sequences of the PrP protein between the host and the donor. For experimental transmission, as wild-type mouse models are time-consuming and not totally efficient, the use of transgenic mice expressing the prion gene (Prnp) of the natural host of prion disease helps considerably in TSE transmission to mice, as...
was shown recently for both scrapie (Crozet et al., 2001b; Vilotte et al., 2001) and BSE (Buschmann et al., 2000; Castella et al., 2003; Scott et al., 1997, 1999) with ovine and bovine transgenic mice, respectively.

This opened new avenues for the discrimination of BSE from scrapie agents among natural TSE isolates (Baron et al., 2004), which is important for animal and human health, especially in light of the recent confirmation that small ruminants can be infected naturally by a TSE agent indistinguishable from the BSE agent (Elloit et al., 2005). Despite the possibility of identification by molecular approaches of some TSE isolates sharing similarities with BSE in small ruminants (Elloit et al., 2005; Hope et al., 1999; Jeffery et al., 2006; Lezmi et al., 2004), the final identification of the BSE agent still requires characterization of the infectious agent by transmission in murine models. The potential of recently developed transgenic models thus needs to be assessed and compared with that of wild-type mouse lines that have been largely used for characterization of scrapie strains and identification of BSE (Bruce et al., 2003).

Unlike scrapie, a unique BSE strain from cattle has indeed been isolated in wild-type mice, with specific biochemical and biological properties and which also appeared remarkably stable following transmission in other species (Bruce et al., 1997; Collinge et al., 1996).

As has been observed in the brain of BSE-infected ruminants and humans with variant CJD (vCJD), Western blot analyses of PrPres accumulated in the brain of BSE-infected mice have demonstrated specific molecular features compared with most other forms of prion disease. This molecular signature is essentially characterized by high proportions of diglycosylated PrPres and a low molecular mass of the unglycosylated PrPres moiety (Baron & Biacabe, 2001; Collinge et al., 1996; Kuczius & Groschup, 1999; Somerville et al., 1997). The latter is associated with a distinct cleavage of PrPres by proteinase K, as largely explored for the discrimination of BSE and scrapie infections in small ruminants (Baron et al., 2000; Hope et al., 1999; Lezmi et al., 2004; Stack et al., 2002; Thuring et al., 2004). Recently, the transmission of this specific molecular signature was also observed in ovine transgenic mice (TgOvPrP4) following experimental ovine BSE infection (Baron et al., 2004).

Whilst identification of BSE in defined inbred wild-type mice relied essentially on the demonstration of a specific distribution of spongiform lesions throughout the brain (Bruce et al., 1994; Green et al., 2005), the identification of typical florid plaques, as described previously in humans with vCJD or in BSE-infected macaques, was considered as a potential BSE signature in an ovine transgenic mouse model (TgOvPrP4) when inoculated with experimental ovine BSE (Crozet et al., 2001a).

In this study, we further investigated transmission of the BSE agent in two ovine transgenic mouse lines (TgOvPrP4 and TgOvPrP59) overexpressing the ovine PrP protein (A136 R154 Q171) under the control of the neuron-specific enolase promoter. Mice were inoculated with CNS tissues from sheep of both genetically susceptible (AA136 RR154 Q171) and resistant (AA136 RR154 RR171) genotypes infected experimentally with BSE, and from cattle. We demonstrate, after successful transmission of the disease from all of these sources, that whatever the source of BSE, identical and typical features emerge and clearly allow discrimination from scrapie. These data highlight the interest of these transgenic mouse models for the identification of the BSE agent in sheep.

**METHODS**

**TSE isolates.** TSE isolates used in this study are described in Table 1. This includes experimental ovine BSE sources from sheep with different genotypes (AA136 RR154 Q171 and AA136 RR154 RR171) inoculated with a French cattle BSE isolate (Baron et al., 2000) by either the intraperitoneal (i.p.) (SB1) or intrasplenic (i.s.) (SB3 and SB4) route. The two ARQ-homozygous sheep (SB1 and SB3) were sacrificed after manifestation of TSE-consistent clinical signs (Lezmi et al., 2004), whereas the ARR-homozygous sheep (SB4) was sacrificed in the absence of any clinical signs indicative of neurological disease. PrPres detection in tissues from the two ARQ-homozygous sheep and from cattle (Bruce et al., 1997; Collinge et al., 1996) was shown recently for both scrapie (Crozet et al., 2001b; Vilotte et al., 2001) and BSE (Buschmann et al., 2000; Castella et al., 2003; Scott et al., 1997, 1999) with ovine and bovine transgenic mice, respectively.

**Methods.** Samples from TSE isolates were inoculated into transgenic mice (TgOvPrP4 and TgOvPrP59), which were generated as described previously for the TgOvPrP4 mouse line (Crozet et al.,)

**Table 1. BSE and scrapie sources used for transgenic mouse inoculation**

<table>
<thead>
<tr>
<th>Identification</th>
<th>TSE source and infection route</th>
<th>Breed</th>
<th>PrP genotype</th>
<th>Survival period after inoculation (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB1</td>
<td>BSE (i.p.)</td>
<td>Lacaune</td>
<td>ARQ/ARQ</td>
<td>672</td>
</tr>
<tr>
<td>SB3</td>
<td>BSE (i.s.)</td>
<td>Lacaune</td>
<td>ARQ/ARQ</td>
<td>1444</td>
</tr>
<tr>
<td>SB4</td>
<td>BSE (i.s.)</td>
<td>Lacaune</td>
<td>ARR/ARR</td>
<td>2191</td>
</tr>
<tr>
<td>O256</td>
<td>Natural scrapie</td>
<td>Manech tête rousse</td>
<td>ARQ/VRQ</td>
<td>–</td>
</tr>
<tr>
<td>Cattle BSE</td>
<td>Natural BSE</td>
<td>Prim’Holstein</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

i.p., Intraperitoneal injection; i.s., intrasplenic injection.
Briefly, the ovine PrP (A136 R154 Q171) open reading frame was inserted into the pNSE-Ex4 vector (containing the neuron-specific enolase promoter). Transgenic founders were crossed with PrP-knockout mice to obtain mice homozygous both for the ovine Prnp transgene and for the deletion of the murine Prnp locus. Two transgenic mouse lines, TgOvPrP4 and TgOvPrP59, derived from two different founders, were used in this study. These two mouse lines express around as much PrP<sub>ARQ</sub> in the brain compared with sheep brain.

Animal inoculation and follow-up. Experimental groups (Table 2) of 4- to 6-week-old female transgenic mice were inoculated intracerebrally (i.c.), following anaesthesia with 80 µm ketamine/0.12 % xylazine; a few were found dead. The whole brain of every second mouse was frozen and stored at −80°C before Western blot analysis; the other brains were fixed in buffered 4 % paraformaldehyde. When one of these signs occurred, the animal was monitored daily and, upon exhibition of any signs of distress or confirmed evolution of clinical signs of prion disease, was sacrificed by an anaesthetic-solution overdose (200 µm ketamine/0.12 % xylazine).

Western blot analysis

Extraction of PrP<sup>Pes</sup>. Mouse-brain tissues were homogenized in a 5 % glucose solution (10 %, w/v). The homogenates were forced through a 0.4 mm diameter needle before incubation for 1 h at 37°C with proteinase K (10 µg per 100 mg brain tissue). Samples were then incubated for 15 min in Sarkosyl (Sigma; final concentration, 10 %) and centrifuged at 200 000 x g for 2 h over a 10 % sucrose cushion (Beckman TL100 ultracentrifuge). The pellets were resuspended and heated for 5 min at 100°C in 50 µl denaturing buffer (4 % SDS, 2 % β-mercaptoethanol, 192 mM glycine, 25 mM Tris, 5 % sucrose) and centrifuged at 12 000 g for 15 min. Finally, pellets were discarded and supernatants were run on an SDS/polyacrylamide gel.

Western blotting procedures. Proteins were separated by 15 % polyacrylamide-gel electrophoresis and transferred to nitrocellulose membranes (Amersham Biosciences) in a 25 mM Tris, 192 mM glycine, 10 % 2-propanol buffer at 400 mA constantly for 1 h. For immunoblotting, the membranes were blocked for 1 h with 3 % BSA in PBS/Tween 20 (0.1 %) (PBST), then incubated for 1 h at room temperature with monoclonal antibodies (mAbs) Bar233 (1:10 000 in PBST), directed against aa 145–156 of the ovine PrP sequence (FGNDYEDRYRE) (kindly provided by J. Grassi, Commissariat à l’Energie Atomique, France), or P4 (200 ng ml<sup>−1</sup> in PBST), directed against aa 93–99 of the ovine PrP sequence (WGQGGSH) (R-Biopharm). After washes in PBST, the membranes were incubated for 30 min at room temperature with peroxidase-labelled conjugates against mouse IgG (1:2500) in PBST. The membranes were finally washed three times in PBST and once in PBS, and bound antibodies were detected with enhanced chemiluminescence (Amersham Biosciences) or Supersignal (Pierce). PrP<sup>pes</sup> signals were visualized either on film (Bi maxX; Kodak) or directly in an image-analysis system (Versadoc; Bio-Rad).

Analysis. For quantitative studies of the glycoform ratios, chemiluminescent signals corresponding to the three glycoforms of the protein were quantified by using Quantity One software (Bio-Rad). Glycoform ratios were expressed as mean percentages (±SD) of the total signal for the three glycoforms (di-, mono- and unglycosylated forms), with at least six different runs from three or four different mice per experimental group. Molecular masses of the three PrP<sup>pes</sup> glycoforms were determined as the mean of the centre positions of the bands from at least six repeated runs from three or four different mice per experimental group, as measured by comparison with a biotinylated marker (B2787; Sigma) run on each gel. Immunological reactivities of the P4 and Bar233 antibodies were compared by visual examination from Western blot analysis run in parallel with the same samples using both antibodies.

Statistical analysis. The influence of the three factors of interest, i.e. the TSE isolate, the mouse line and the region of the CNS, on

Table 2. Sources of the samples inoculated into ovine transgenic mice, survival periods, detection of PrPd and florid plaques

<table>
<thead>
<tr>
<th>Mouse line/TSE source</th>
<th>CNS region</th>
<th>Mean survival period (SD) (days p.i.)</th>
<th>No. PrPd-positive mice by WB or IHC</th>
<th>Florid plaque</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TgOvPrP4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O256</td>
<td>Brainstem</td>
<td>317 (67)</td>
<td>3/3</td>
<td>–</td>
</tr>
<tr>
<td>SB1</td>
<td>Brainstem</td>
<td>296 (46)</td>
<td>19/19</td>
<td>+</td>
</tr>
<tr>
<td>SB3</td>
<td>Brainstem</td>
<td>297 (62)</td>
<td>5/6</td>
<td>+</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>436 (36)</td>
<td>10/10</td>
<td>9/9</td>
<td>+</td>
</tr>
<tr>
<td>SB4</td>
<td>Brainstem</td>
<td>423 (65)</td>
<td>10/10</td>
<td>+</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>474 (69)</td>
<td>10/10</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Cattle BSE</td>
<td>Brainstem</td>
<td>420 (48)</td>
<td>10/10</td>
<td>+</td>
</tr>
<tr>
<td><strong>TgOvPrP59</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O256</td>
<td>Brainstem</td>
<td>330 (13)</td>
<td>8/10</td>
<td>–</td>
</tr>
<tr>
<td>SB1</td>
<td>Brainstem</td>
<td>396 (63)</td>
<td>10/10</td>
<td>+</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>436 (41)</td>
<td>10/10</td>
<td>10/10</td>
<td>+</td>
</tr>
<tr>
<td>SB4</td>
<td>Spinal cord</td>
<td>538 (47)</td>
<td>10/10</td>
<td>+</td>
</tr>
<tr>
<td>Cattle BSE</td>
<td>Brainstem</td>
<td>511 (51)</td>
<td>9/10</td>
<td>+</td>
</tr>
</tbody>
</table>
RESULTS

Transmission and characterization of BSE and scrapie in TgOvPrP4 ovine transgenic mice

Susceptibility of TgOvPrP4 ovine transgenic mice to different ovine BSE was tested by i.c. inoculation of brainstem homogenates from three experimentally infected sheep (SB1, SB3 and SB4) (Table 1) and compared with TgOvPrP4 mice inoculated with a natural ARQ/VRQ sheep scrapie isolate and a cattle BSE isolate. The survival data observed in experiments performed in TgOvPrP4 mice infected with brainstem samples (Table 2) are illustrated in Fig. 1(a). Similar survival periods [means, 296 and 297 days post-inoculation (p.i.)] were observed with the two ARQ-homozygous sheep (SB1 and SB3), which were also comparable with that obtained with the scrapie isolate (mean, 317 days p.i.). The survival periods appeared to be significantly longer ($P<0.05$) with homogenates from the ARR-homozygous sheep and with the cattle BSE isolate (means, 423 and 420 days p.i., respectively).

As summarized in Table 2, in every experimental group, most mice developed clinical signs indicative of TSE disease and these were generally found to be positive for the presence of disease-associated PrP (PrPd) by Western blot analysis. Western blotting on the same gel using the Bar233 antibody, the same PrPres from every mouse brain first analysed by Western blot or by immunohistochemistry. In each experimental group, presence of disease-associated PrP (PrPd) by Western blot and these were generally found to be positive for the most mice developed clinical signs indicative of TSE disease. As summarized in Table 2, in every experimental group, presence of disease-associated PrP (PrPd) by Western blot and these were generally found to be positive for the most mice developed clinical signs indicative of TSE disease.

The apparent molecular masses of unglycosylated, mono-glycosylated and diglycosylated fragments of PrPres from TgOvPrP4 transgenic mice infected with material from SB1, SB3 or SB4 did not show any significant differences ($\text{mean}_{\text{un-}}, 18.8–19.0 \text{kDa}; \text{mean}_{\text{mono-}}, 23.5–23.6 \text{kDa}; \text{mean}_{\text{di-}}, 27.3–27.5 \text{kDa}$ [Fig. 1b (top panel), c]). These patterns were associated with a poor labelling of the three PrPres glycoforms by the P4 mAb (which recognizes an epitope close to the N-terminal protease-cleavage site) compared with that obtained with Bar233, directed against an epitope in the central region of the protein (Fig. 1b, lower panel). These PrPres features were significantly different (see Supplementary Table S1, available in JGV Online) from those observed in mice infected with the scrapie isolate ($\text{mean}_{\text{un-}}, 19.8 \text{kDa}; \text{mean}_{\text{mono-}}, 24.2 \text{kDa}; \text{mean}_{\text{di-}}, 28.1 \text{kDa}$) (Fig. 1b). Here, the P4 mAb also labelled PrPres more strongly in scrapie-infected mice. Glycoform ratios obtained from repeated runs of these samples are shown in Fig. 1(d). Transgenic mice infected with all BSE sources formed a relatively homogenous group with a significantly higher (5–10% higher) proportion of the diglycosylated form than in mice inoculated with the sheep scrapie isolate. In histopathological studies, the florid-plaque type of PrPd deposition was detected in TgOvPrP4 mice infected with all BSE sources.

Transmission and characteristics of BSE and scrapie sources in TgOvPrP59 ovine transgenic mice

We also assessed susceptibility to the same TSE sources of TgOvPrP59 mice, a similarly produced ovine transgenic mouse line, but obtained from a different founder. Brainstem homogenates from an ARQ-homozygous BSE-infected sheep (SB1), from cattle BSE and from the scrapie isolate, as well as spinal cord from the preclinical ARR-homozygous BSE-infected sheep (SB4), were inoculated i.c. into these mice. In comparison with TgOvPrP4 mice, the survival periods in TgOvPrP59 mice were increased significantly with the three BSE sources from ARQ-homozygous sheep or cattle ($P<0.001$ and $P=0.001$, respectively) (around 100 days longer), but was not significantly longer (13 days) for the sheep scrapie isolate (Fig. 2a). Similar prolongation of the incubation periods was also observed in TgOvPrP59 mice infected with BSE from the ARR-homozygous sheep (Table 2; Supplementary Fig. S2, available in JGV Online). Longer survival periods (115 and 181 days longer, respectively) were also observed in TgOvPrP59 transgenic mice inoculated with cattle BSE compared with those inoculated with the experimental ovine BSE isolate ($P<0.001$) or the scrapie isolate ($P<0.001$) (Table 2). According to the statistical analyses adjusted on the mouse line, PrPres molecular discrimination of BSE and scrapie sources is identical in these two transgenic mouse lines (Fig. 2b). As in TgOvPrP4 mice, PrPres from scrapie-infected mice had an apparent molecular mass significantly higher (about 1 kDa) than that from BSE sources (Fig. 2b) and the ratio of the diglycosylated band was lower than in mice infected with BSE sources (see Supplementary Fig. S1a, available in JGV Online). Furthermore, these TgOvPrP59 mice infected with all BSE sources also presented florid plaques.
Comparisons of ovine transgenic mouse infections with different CNS regions of BSE-infected sheep

Susceptibility of ovine transgenic mice (TgOvPrP4 and TgOvPrP59) to the ovine BSE agent from different CNS regions (brainstem or spinal cord) was evaluated by i.c. inoculation of spinal-cord homogenates, in comparison with previously described brainstem samples, from two experimentally BSE-infected sheep (SB3 and SB4). TgOvPrP4 mice inoculated with spinal-cord samples had longer survival periods (means, 139 and 51 days longer for SB3 and SB4, respectively) than TgOvPrP4 mice inoculated with brainstem samples from the same sheep (Fig. 3a; Table 2); nevertheless, the difference was significant only for sheep SB3 ($P < 0.001$). The same phenomenon was observed in TgOvPrP59 mice inoculated with SB1 spinal cord, which died on average 40 days later than TgOvPrP59 mice inoculated with SB1 brainstem (Table 2). Western blot analyses showed no differences in the PrPres pattern between mice inoculated with brainstem and those inoculated with spinal cord (Fig. 3b; Supplementary Fig. S1b, available in JGV Online).

**DISCUSSION**

We studied the transmission of the infectious agent associated with BSE in two ovine transgenic mouse lines expressing the ARQ ovine Prnp sequence under the control of the neuron-specific enolase promoter. Whilst BSE agent from both ARQ-homozygous sheep showed the same
features after transmission to TgOvPrP4 mice (Crozet et al., 2001a), we report the successful transmission of the disease in this mouse line from an ARR-homozygous BSE-infected sheep. Whilst such sheep are known to be strongly resistant to scrapie (Goldmann et al., 1994; Hunter et al., 1997), it was shown that they could nevertheless be infected with cattle BSE, at least by i.c. inoculation (González et al., 2005; Houston et al., 2003; Martin et al., 2005) and, in our case, by i.s. inoculation (F. Ronzon, A. Bencsik, S. Lezmi, J. Vulin, A. Kodjo & T. Baron, unpublished data). Until now, only one study has shown infectivity of PrPARR, but the current study concerns a recently identified atypical form of scrapie (Benestad et al., 2003; Buschmann et al., 2004). Infectivity of brain tissues from such animals has been demonstrated after experimental challenge of ovine transgenic mice expressing high levels of VRQ ovine PrP (Le Dur et al., 2005). To our knowledge, the TgOvPrP4 transgenic mouse line is the only transgenic mouse model in which transmission of ovine BSE has been described. In this study, we also assessed the use of the TgOvPrP59 mouse line, which is similar to TgOvPrP4, but derived from a founder obtained from another oocyte microinjection. Incubation periods of the disease were somewhat prolonged with BSE sources, but not with the natural scrapie isolate, suggesting that, although produced similarly, both mouse lines, which might differ by number or/and locations of the transgenes, could show different behaviours of TSE agents. As a single, natural scrapie isolate has been studied in TgOvPrP59 so far, further experiments are required with different scrapie sources in order to evaluate whether this prolongation of the incubation period is only observed for BSE. Comparisons of ovine BSE transmissions to other transgenic mouse models expressing different alleles of the ovine prion protein, such as those expressing the sheep VRQ allele (Vilotte et al., 2001), or with those expressing the bovine prion protein (Buschmann et al., 2000; Castilla et al., 2003; Scott et al., 1997, 1999) would be of utmost interest.

Regarding the survival periods of the disease observed here with ovine BSE in ovine transgenic mice, comparable survival periods were reported previously in other studies in wild-type RIII mice infected with brainstem samples from BSE-infected sheep (Bruce et al., 1994). Whilst among wild-type mouse lines, RIII mice provide one of the shortest survival times after transmission of the BSE agent, such results were, however, obtained after inoculation of mice with brain homogenates by both i.c. and i.p. routes (Bruce et al., 1994; Fraser et al., 1992; Green et al., 2005), using a sixfold higher load of BSE inoculum compared with the experiments described here. It would be of interest to evaluate the effect of such experimental challenge in our ovine transgenic mice as, despite the use of a promoter leading to neuron-targeted expression of PrP and, as a result, the absence of detectable PrP expression in peripheral tissues (Crozet et al., 2001b), TgOvPrP4 mice can also be infected successfully by i.p. challenge (C. Crozet, unpublished data).
Compared with the two ARQ-homozygous sheep infected with BSE, the survival period was prolonged with material from the ARR-homozygous sheep, i.e. from both brainstem and, to a lesser extent, spinal-cord samples. It should be emphasized that this ARR-homozygous sheep was sacrificed 6 years after experimental challenge in the absence of any clinical signs indicative of TSE. Abnormal PrP in the brain of the ARR-homozygous sheep SB4 was detected by both Western blot and immunohistochemistry, but lower levels of infectivity associated with the preclinical stage of the disease in this case could explain a longer survival period in ovine transgenic mice. Moreover, it was shown in other studies (González et al., 2005) that ARR-homozygous BSE-infected sheep displayed lower levels of PrPd in the brain than sheep of other genotypes. The small increase of the survival period in TgOvPrP4 mice inoculated with SB4 spinal cord versus brainstem, compared with those inoculated with SB3 samples, could also be explained by the earlier PrPres accumulation in the spinal cord compared with brainstem in sheep (Beekes et al., 1998; Glatzel & Aguzzi, 2000; Glatzel et al., 2001; McBride et al., 2001). Indeed, unlike SB1 and SB3, sheep SB4 was sacrificed in the absence of any clinical sign indicative of the disease, suggesting that neuroinvasion might have involved the spinal cord rather than the brainstem. Whilst the behaviour of BSE from ARR-homozygous sheep has not been described previously in wild-type mice, studies in RIII mice challenged similarly (i.c. route only) with the same ovine BSE sample (spinal cord) (see Supplementary Fig. S2, available in JGV Online) showed that the first PrPd-positive mouse was observed only 576 days p.i., in contrast with 390 days p.i. in TgOvPrP4 ovine transgenic mice. These data are thus of considerable interest, given the need to develop bioassays with the shortest survival period for the detection of the BSE agent.

Our study also confirms the unique signature regarding the molecular features of PrPres after ovine BSE transmission to ovine transgenic mice. In line with previous studies (Baron et al., 2004), Western blot analysis showed the same molecular features for PrPres not only from the ARQ-homozygous BSE-infected sheep, but also from the ARR-homozygous BSE-infected sheep, a signature that is clearly distinct from that observed following infection with a natural scrapie isolate (a higher apparent molecular mass of unglycosylated PrPres and strong labelling by mAb P4). Also, similar features and the same differences between ovine BSE and scrapie sources were observed in both TgOvPrP4 and TgOvPrP59 mouse lines and whatever the region of the CNS (brainstem or spinal cord) used for the experiments. Higher levels of diglycosylated PrPres were also observed in mice infected with ovine BSE samples. Overall, with the clear molecular discrimination between BSE and scrapie sources, our data further reinforce previous studies supporting the strain-specific transmission of particular molecular features in BSE, which might be due to a distinct conformation of PrPd.

It was proposed previously that the presence of florid plaques could help in the discrimination of ovine BSE and scrapie in the TgOvPrP4 mouse line (Crozet et al., 2001b). Similar observations were also reported recently following transmission of cattle BSE or human vCJD to bovine transgenic mice (Scott et al., 2005). Our data now confirm the presence of florid plaques in mice infected with the different ovine BSE samples tested, including the ARR-homozygous sheep case. These plaques were also observed following infection with different CNS regions (brainstem or spinal cord), as well as in both TgOvPrP4 and TgOvPrP59 mouse lines. Discrimination of BSE from scrapie has so far relied essentially on the examination of the distribution and intensity of vacuolation brain lesions or/and PrPd deposits detected by immunohistochemistry in wild-type mice, particularly RIII mice (Bruce, 1996). A major drawback of this approach is, however, the failure to transmit the disease from a number of TSE sources from small ruminants, including from some isolates that show the closest molecular similarities to ovine BSE, such as the CH1641 scrapie isolate (Baron et al., 2004; Foster & Dickinson, 1988; Hope et al., 1999). In this regard, ovine transgenic mice, such as TgOvPrP4, which allow the transmission of all TSE isolates from sheep and goats inoculated so far (Baron et al., 2004; Crozet et al., 2001a; unpublished data), with relatively short survival periods, are helpful.

In this study, we also examined the potential usefulness of ovine transgenic mice to study the transmission of cattle BSE. Following i.c. experimental infection with a cattle BSE isolate, the survival period of the disease was longer than those observed following the inoculation of homogenates from the two ARQ-homozygous BSE-infected sheep. This survival period was also longer than that reported in some published studies of cattle BSE transmitted to wild-type RIII mice, inoculated by combined i.c. and i.p. routes (Bruce et al., 1994). However, conflicting results have been reported after using these mice to detect the BSE agent from cattle, after a combined i.c. and i.p. inoculation, in other laboratories (Buschmann et al., 2000; Green et al., 2005), which reported survival periods comparable to those obtained in our model by the i.c. route only. It was hypothesized that different infectious titres of the bovine samples had been used in the different studies performed with RIII mice, possibly linked to sampling at different stages of the disease, which could explain such discrepancies. It should be emphasized that both situations (RIII and ovine transgenic mice) are characterized by the existence of a species barrier, despite higher similarities between bovine and ovine Prnp genes. In the present work, molecular studies of PrPres also demonstrated the characteristic BSE-associated features, with a low apparent molecular mass of unglycosylated PrPres associated with poor labelling by P4 antibody, as well as a high proportion of diglycosylated PrPres.

Our data thus reinforce the usefulness of ovine transgenic mice, such as the TgOvPrP4 or TgOvPrP59 mouse lines, for identification of the BSE agent in sheep and for its discrimination from scrapie. Most importantly, they show
that the same BSE-specific molecular and lesional features that had been described previously in this model were found in mice infected with samples from an ARR-homozygous BSE-infected sheep. Finally, preliminary findings observed following transmission of cattle BSE in this model also open new avenues for the characterization of the cattle BSE agent, especially in the context of recent findings suggesting that prion diseases in cattle might be less unusual than expected previously (Biacabe et al., 2004).

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