PrP\textsuperscript{TSE} in muscle-associated lymphatic tissue during the preclinical stage of mice infected orally with bovine spongiform encephalopathy

Franco Cardone,\textsuperscript{1} Achim Thomzig,\textsuperscript{2} Walter Schulz-Schaeffer,\textsuperscript{3} Angelina Valanzano,\textsuperscript{1} Marco Sbriccoli,\textsuperscript{1} Hanin Abdel-Haq,\textsuperscript{1} Silvia Graziano,\textsuperscript{1} Sandra Pritzkow,\textsuperscript{2} Maria Puopolo,\textsuperscript{1} Paul Brown,\textsuperscript{4} Michael Beekes\textsuperscript{2} and Maurizio Pocchiari\textsuperscript{1}

\textsuperscript{1}Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
\textsuperscript{2}Robert Koch-Institut (P24 – Transmissible Spongiform Encephalopathies), Nordufer 20, 13353 Berlin, Germany
\textsuperscript{3}Prion and Dementia Research Unit, Department of Neuropathology, University Medical Center, Georg-August University Goettingen, Robert-Koch-Str. 40, 37075 Goettingen, Germany
\textsuperscript{4}7815 Exeter Road, Bethesda, MD 20814, USA

The involvement of muscles in the pathogenesis of transmissible spongiform encephalopathies (TSEs) is irregular and unpredictable. We show that the TSE-specific protein (PrP\textsuperscript{TSE}) is present in muscles of mice fed with a mouse-adapted strain of bovine spongiform encephalopathy as early as 100 days post-infection, corresponding to about one-third of the incubation period. The proportion of mice with PrP\textsuperscript{TSE}-positive muscles and the number of muscles involved increased as infection progressed, but never attained more than a limited distribution, even at the clinical stage of disease. The appearance of PrP\textsuperscript{TSE} in muscles during the preclinical stage of disease was probably due to the haematogenous/lymphatic spread of infectivity from the gastrointestinal tract to lymphatic tissues associated with muscles, whereas in symptomatic animals, the presence of PrP\textsuperscript{TSE} in the nervous system, in neuromuscular junctions and in muscle fibres suggests a centrifugal spread from the central nervous system, as already observed in other TSE models.
neuromuscular junctions and muscle spindles in scrapie- or TME-infected hamsters (Mulcahy et al., 2004; Thomzig et al., 2004) and in scrapie-infected sheep (Andréolletti et al., 2004). In mice experimentally infected intracerebrally with BSE, the presence of PrP<sub>TSE</sub> in muscles is more restricted than in hamsters (Thomzig et al., 2003, 2006), but it is similar to that found in CJD patients (Glatzel et al., 2003; Peden et al., 2006) and in sheep with scrapie (Andréolletti et al., 2004). Because the oral route of infection can be important in the occurrence of some 'natural' TSEs, as well as variant CJD (Safař et al., 2008; Sigurdson & Aguzzi, 2007; Ward et al., 2006), we studied the chronological deposition of PrP<sub>TSE</sub> in muscles of mice fed with a mouse-adapted BSE strain.

To prepare the infectious BSE inoculum, brains from terminally ill C57BL/6 mice infected with the BSE isolate 6PB1 (Maignien et al., 1999; kindly provided by Dr Jean-Philippe Deslys, CEA, Fontenay-aux-Roses, France) were homogenized in 9 vols PBS. Clarified inoculum (100 μl) was then absorbed by food pellets and fed immediately to individually caged adult female C57BL/6 mice (n=26) previously subjected to 2 days starvation. After complete ingestion of BSE-infected pellets, the animals were housed (eight per cage) and observed daily for clinical signs of BSE. Groups of five animals were sedated and sacrificed by CO<sub>2</sub> asphyxia during the preclinical stage of the disease at 100, 200 and 300 days post-infection (p.i.). One mouse died of intercurrent disease after 290 days. The remaining animals were sacrificed at clinical onset. Control animals (n=9) were fed pellets soaked with normal mouse-brain homogenate and were sacrificed following the schedule described for BSE-infected mice. All animals were autopsied and brain, spleen and a set of nine different muscles were sampled and either fixed in formalin for histological examination or frozen at −70°C for PrP<sub>TSE</sub>-30 (the protease-resistant form of PrP<sub>TSE</sub>) purification, biochemical and paraffin-embedded tissue (PET)-blot analyses (Thomzig et al., 2003, 2004, 2006).

All mice allowed to survive became symptomatic, with a mean ± SD incubation period of 368.3 ± 13.7 days (n=10), confirming the efficiency of the oral route of infection in mouse BSE. As shown in Table 1 and Fig. 1, the earliest PrP<sub>TSE</sub>-positive immunoblots (one Musculus triceps brachii caput laterale and one M. trapezius) occurred in two different mice sacrificed at 100 days p.i. The proportion of PrP<sub>TSE</sub>-positive mice increased as the incubation period progressed and, at 300 days p.i., multiple muscle involvement began to occur. Heart and tongue samples, as well as muscles from mock-infected animals, were consistently PrP<sub>TSE</sub>-negative.

Among the entire group of infected animals, six of the nine sampled muscles were affected, with M. psoas major being involved most often, in both the preclinical and clinical stages. Immunoblot signals of PrP<sub>TSE</sub>-30 from 20–50 μg muscle were comparable to those of positive controls containing 10 μg equivalents of mouse BSE brain and to those of muscle from uninfected mice spiked with 50 μg equivalents of brain, suggesting that the level of PrP<sub>TSE</sub> in muscles is about three orders of magnitude lower than that in the brain. Analyses of muscles from mock-infected mice and some BSE-infected mice showed thin bands at about 30 kDa (e.g. Fig. 1a, lanes M2, M5–M9; Fig. 1e, lanes M1–M3), sometimes appearing as a doublet, also visible after omission of the primary anti-PrP antibody, that were clearly distinguishable from PrP<sub>TSE</sub>-30 triple bands and were therefore considered unrelated to TSE infection. Analysis of brains from infected mice showed PrP<sub>TSE</sub> in two of five asymptomatic mice sacrificed at 300 days p.i. (during the preclinical period) and in all symptomatic animals. The electrophoretic pattern of PrP<sub>TSE</sub>-30 from muscles and brain was indistinguishable from the typical BSE signature observed in intracerebrally infected mice.

Immunohistochemical studies in preclinical animals showed granular PrP<sub>TSE</sub> deposition in the brainstem and pontine reticular nuclei (Fig. 2a). PET-blots of M. triceps brachii and M. psoas major were performed in some mice (Table 1) and revealed PrP<sub>TSE</sub> positivity only in the lymphatic tissue associated with muscle of preclinical animals. In brains from clinically affected animals, granular deposition was associated with PrP<sub>TSE</sub> plaques in the thalamus, mesencephalon, cerebellar cortex, pons and brainstem, together with spongiosis in the brainstem and cerebellum (Fig. 2b–d). In these animals, PET-blots of M. triceps brachii and M. psoas major revealed PrP<sub>TSE</sub> in the muscle, single fibres of small nerves and neuromuscular junctions.

To evaluate the involvement of the lymphoreticular system, spleens were analysed by immunoblot and found to be PrP<sub>TSE</sub>-positive from 100 days p.i. onward (Table 1). All but one animal with PrP<sub>TSE</sub>-positive muscles had moderate to high PrP<sub>TSE</sub> load in the spleen (Fig. 1h).

The novelty of these data is that, in our rodent model for BSE in cattle, i.e. mice infected orally with a mouse-adapted BSE strain, there is an unexpected early preclinical deposition of PrP<sub>TSE</sub> in muscles, probably associated with the lymphatic tissues, which occurs as early as during the first one-third of the incubation period. This result is similar to what has been found in sheep infected orally with scrapie, where muscle PrP<sub>TSE</sub> was detected during the first one-quarter of the incubation period (Andréolletti et al., 2004). Interestingly, this phenomenon does not occur in all TSE models; for example, in hamsters fed with 263K scrapie, muscles only became PrP<sub>TSE</sub>-positive near clinical onset (Thomzig et al., 2004).

The early involvement of spleen and other lymphatic tissues found in this study is consistent with the observations reported by Maignien et al. (1999) in mice infected with the same BSE strain and by the same route. Interestingly, they also observed that in the later stages of the incubation period, PrP<sub>TSE</sub> appeared simultaneously in the thoracic spinal cord and brain, suggesting that after oral infection, the BSE agent spreads by blood or lymph to...
Table 1. Time-course deposition of PrP<sup>TSE</sup> in selected tissues of mice infected orally with BSE

IB, Immunoblot; PB, PET-blot; NT, not tested.

<table>
<thead>
<tr>
<th>Mouse ID no.</th>
<th>Biceps femoris</th>
<th>Tibialis cranialis</th>
<th>Triceps brachii caput laterale</th>
<th>Extensor carpi radialis</th>
<th>Trapezius</th>
<th>Masseter major</th>
<th>Psoas major</th>
<th>Tongue apex</th>
<th>Heart apex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IB PB&lt;sup&gt;*&lt;/sup&gt;</td>
<td>IB PB&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSE-infected – preclinical</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 days p.i.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>– +</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>200 days p.i.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+ NT</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+ NT</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>290 days p.i.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>300 days p.i.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+ NT</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
<td>–</td>
<td>+ NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+ NT</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BSE-infected – clinically ill</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>368.3 ± 13.7 days p.i.&lt;sup&gt;‡&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>18</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>19</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>21</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>22</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>NT</td>
<td>–</td>
</tr>
<tr>
<td>23</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+ NT</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>24</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+ NT</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>25</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>26</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>No. positive/no. analysed</td>
<td>1/24</td>
<td>0/24</td>
<td>5/24</td>
<td>3/8</td>
<td>1/24</td>
<td>4/24</td>
<td>3/24</td>
<td>8/24</td>
<td>2/7</td>
</tr>
<tr>
<td>Non-infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100–368 days p.i.&lt;sup&gt;§&lt;/sup&gt;</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
</tr>
</tbody>
</table>

* Nerves and lymphatic tissues were not present in mouse 5; lymphatic tissues were not present in mice 7, 13, 25 or 26. PrP<sup>TSE</sup> positivity was always associated with the lymphatic tissues, except in mouse 25 (positive in neuromuscular junction) and mouse 26 (positive in muscle, single fibres and small nerves).

† Lymphatic tissues were not present in mice 1, 6, 13 or 26. PrP<sup>TSE</sup> positivity was always associated with lymphatic tissues, except in mouse 26 (positive in neuromuscular junction).

‡ Mean ± SD.

§ Two mice per time point were sacrificed at 100, 200 and 300 days p.i. Three mice were sacrificed at 368 days p.i.
lymphatic tissues associated with muscles, and only at a later stage during the incubation period enters the CNS and is then projected into muscles via nerve fibres.

The late centrifugal spread of PrP\textsuperscript{TSE} from the CNS to muscle in orally BSE-infected mice is also consistent with observations in other experimental models (Thomzig \textit{et al}., 2004; Herzog \textit{et al}., 2005) or in scrapie-infected sheep (Andréolletti \textit{et al}., 2004), where the CNS plays a primary role in the dissemination of infectivity to muscles (Beekes & McBride, 2007).

In the light of these new data, there would seem to be a very low probability that BSE-infected cows harbour infectivity in muscle destined for human consumption, in view of the minimal involvement of the lymphoreticular system in cattle and the continued absence of evidence for infectivity in bovine muscles (Buschmann & Groschup, 2005; Espinosa \textit{et al}., 2007; WHO, 2006). It is nevertheless possible that limited muscle sampling and methodological insensitivity could fail to detect the irregular

---

**Fig. 1.** Detection of PrP\textsubscript{27–30} in tissues of BSE-infected mice. (a–e) Immunoblots using monoclonal anti-PrP antibody ICSM-18 (diluted 1:4000) of muscles from mice sacrificed at different time points: (a) 100 days p.i.; (b) 200 days p.i.; (c) 300 days p.i.; (d) terminal stage of disease; (e) mock-infected mice. Control lanes: 1, 1×10\textsuperscript{-5} g equivalents of proteinase K-digested brain homogenate from BSE-affected mice (‘digested mouse BSE brain’); 2, uninfected muscle spiked with 5×10\textsuperscript{-5} g equivalents of digested mouse BSE brain before extraction. Sample lanes: M1, M. biceps femoris; M2, M. tibialis cranialis; M3, M. triceps brachii caput laterale; M4, M. extensor carpi radialis; M5, M. trapezius; M6, M. masseter; M7, M. psoas major; M8, lingual muscle (tip of the tongue); M9, heart muscle (apex). Amount of muscle tissue analysed, 20–50 mg. (f) Plot reporting the proportion of immunoblot PrP\textsuperscript{TSE}-positive samples/analysed samples for each muscle during preclinical (grey line, n=15) and clinical (black line, n=8) phases. (g) Immunoblots of brains from mice sacrificed at different time points. Lanes: 1, positive control, 1×10\textsuperscript{-5} g equivalents of digested mouse BSE brain; 2, infected mouse at 100 days p.i.; 3, infected mouse at 200 days p.i.; 4–5, infected mice at 300 days p.i.; 6, infected mouse during the early clinical stage; 7, infected mouse during the late clinical stage. Lanes 1–6 contain 2×10\textsuperscript{-3} g and lane 7 contains 1×10\textsuperscript{-5} g equivalents of brain tissue. (h) Immunoblots of spleens from mice sacrificed at different time points. Lanes: 1, positive control, 2.5×10\textsuperscript{-5} g equivalents of digested mouse BSE brain; 2, infected mice at 100 days p.i.; 3, infected mice at 200 days p.i.; 4, infected mice at 300 days p.i.; 5, infected mice during the early clinical stage; 6, infected mouse during the late clinical stage. (i) Timescale displaying mean incubation period, preclinical phase, range of clinical onset in diseased animals, sampling time points and first PrP\textsuperscript{TSE} detection in tissues of mice infected orally with BSE.
distribution of low levels of PrP\textsuperscript{TSE} in muscle. Moreover, the absence of PrP\textsuperscript{TSE} does not necessarily imply absence of infectivity (Barron et al., 2007; Berardi et al., 2006; Lasme\'zas et al., 1997). A more thorough examination of entire muscle groups using the ultrasensitive protein-misfolding cyclic-amplification technique (Soto et al., 2005) might yield a positive result, but would need to be verified by infectivity bioassay before inferring a risk of disease transmission.

**Acknowledgements**

The skilful technical assistance of Patrizia Reckwald, Tatjana Pfander, Nicola Bellizzi, Maurizio Bonanno, Ivano Itro and El\'fino Laconi is gratefully acknowledged. We thank Dr Alessandra Garozzo for editorial assistance. Special thanks go to Giovanni Martino, Antonio Cardarelli, Petros Tsamatropoulos and Pierpaolo Peluso. This work was partially supported by the Istituto Superiore di Sanit\'a, the EU-funded Network of Excellence 'NeuroPrion' and the Deutsche Forschungsgemeinschaft (DFG, TH 1376/2-1).

**References**


**Fig. 2.** Neuropathology of BSE-infected mice. (a) Immunohistochemical staining with SAF84 monoclonal anti-PrP antibody (diluted to 1.5 µg ml\textsuperscript{-1}) demonstrated granular PrP\textsuperscript{TSE} deposits in the reticular nuclei of the brainstem from a mouse sacrificed at 300 days p.i. (b) Immunohistochemical staining with SAF84 antibody showed remarkable deposition of PrP\textsuperscript{TSE} in the brainstem (particularly in the cochlear nuclei) of an affected mouse. (c) PrP\textsuperscript{TSE} plaques in the brainstem of an affected mouse. (d) Haematoxylin–eosin staining revealed the presence of vacuoles localized mainly in the white matter of the cerebellum of an affected mouse. VCN, Ventral cochlear nuclei; Pr5, principal sensory trigeminal nuclei. Bars: (a, b, d) 100 µm; (a, inset) 10 µm; (c) 10 µm.


