Protease-sensitive prion species in neoplastic spleens of prion-infected mice with uncoupling of PrPSc and prion infectivity

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Prion diseases are fatal neurodegenerative disorders. An important step in disease pathophysiology is the conversion of cellular prion protein (PrP^C) to disease-associated misfolded conformers (PrP^Sc). These misfolded PrP variants are a common component of prion infectivity and are detectable in diseased brain and lymphoreticular organs such as spleen. In the latter, PrP^Sc is thought to replicate mainly in follicular dendritic cells within spleen follicles. Although the presence of PrP^Sc is a hallmark for prion disease and serves as a main diagnostic criterion, in certain instances the amount of PrP^Sc does not correlate well with neurotoxicity or prion infectivity. Therefore, it has been proposed that prions might be a mixture of different conformers and aggregates with differing properties. This study investigated the impact of disruption of spleen architecture by neoplasia on the abundance of different PrP species in spleens of prion-infected mice. Although follicular integrity was completely disturbed, titres of prion infectivity in neoplastic spleens were not significantly altered, yet no protease-resistant PrP^Sc was detectable. Instead, unique protease-sensitive prion species could be detected in neoplastic spleens. These results indicate the dissociation of PrP^Sc and prion infectivity and showed the presence of non-PrP^Sc PrP species in spleen with divergent biochemical properties that become apparent after tissue architecture disruption.

INTRODUCTION

Prion diseases are transmissible fatal neurodegenerative disorders. They include Creutzfeldt–Jakob disease in humans, bovine spongiform encephalopathy in cattle, scrapie in sheep and chronic wasting disease in elk and deer. Prion diseases represent the prototype of transmissible proteinopathies (Aguzzi & Rajendran, 2009; Geissen et al., 2007; Glatzel et al., 2005; Jeffrey & González, 2004; Jewell et al., 2006). The cellular prion protein, PrP^C, is highly abundant in central nervous system (CNS) tissue but also in other compartments such as muscle and the lymphoreticular system. In prion diseases, PrP^C is converted into a disease-associated isoform, PrP^Sc (Aguzzi, 2004; Prusiner, 1998). PrP^Sc seems to be an essential component of the transmissible entity and propagates by conversion of host PrP^C into PrP^Sc independently of a coding nucleic acid (Barria et al., 2009; Prusiner, 1982, 1998; Supattapone, 2004). This conversion is accompanied by a change in biochemical properties. It renders PrP^Sc highly prone to aggregation and more resistant to proteolytic digestion (Caughey et al., 1991; Horiuchi & Caughey, 1999). The controlled digestion of tissue homogenates from diseased organisms with proteinase K (PK) leads to N-terminally truncated PrP^Sc highly characteristic for the predominant prion isolate. Detection of these digestion products via Western blotting results in a typical PrP^Sc pattern and serves as the main diagnostic criterion to confirm a prion disease (Oesch et al., 1985; Schoch et al., 2006). Protease-sensitive prion species have been described. Here, we use the term ‘protease-sensitive prion species’ for PrP or cleavage products of PrP that are not present in uninfected tissue, are detectable in Western blots and are degradable with increasing amounts of PK (Gambetti et al., 2008). In contrast, prion infectivity is usually determined by the ability to elicit a prion disease after transmission to animals or susceptible cultured cells (Fischer et al., 1996; Klöhn et al., 2003; Mahal et al., 2007). In the former, the incubation time to clinical disease is a good measure for the titre of infectivity; in the latter, the number of infected cells with detectable PK-resistant PrP per dish is quantified. PrP^Sc amounts may correlate with prion infectivity (Büeler et al., 1992). However, in several models, an uncoupling of PrP^Sc amounts and titres of prion infectivity has been observed (Barron et al., 2007; Glatzel & Aguzzi, 2000; Hsiao et al., 1994; Lasmézas et al., 1997; Manson et al., 1999; Piccardo et al., 2007). Therefore, PrP^Sc
amount alone does not serve as a marker for tissue infectivity. Moreover, this disconnection indicates the existence of different prion conformers with distinct biological properties. Recently, Sandberg et al. (2011) showed that prion propagation in the brain occurs in two distinct phases. PrPSc accumulation plateaus and precedes a second phase with neuronal death and the onset of clinical signs. These findings imply the existence of at least two different types of PrPSc in the brain (Sandberg et al., 2011). Moreover, it is known from analyses of hamster prions that different prion aggregation states exist differing in their infectious potential (Silveira et al., 2005).

Following prion infection in rodents, PrPSc and prion infectivity are cleared and reappear in lymphoid organs such as the spleen (Glatzel & Aguzzi, 2001; Prinz et al., 2003a; Urayama et al., 2011). Follicular dendritic cells (FDCs), which are characterized by expression of milk fat globule protein-epidermal growth factor-8 (MFG-E8), play an important role in this process, and titres of prion infectivity in spleens rise to reach a plateau ~30–40 days following prion inoculation (Blättler et al., 1997; Kimberlin & Walker, 1989; Kranich et al., 2008; Zabel et al., 2007). Interestingly, peripheral prion replication occurs without obvious signs of tissue damage (Aguzzi, 1997, 2003). In addition, in peripheral tissue such as spleen, the dissociation of PrPSc amount and titre of prion infectivity has been shown (Mabbott et al., 2001; Race & Ernst, 1992; Sakaguchi et al., 1993). However, the prion species that constitute tissue infectivity in the absence of bona fide protease-resistant PrPSc have not been identified to date.

Here, we used disruption of spleen architecture by retrovirus-induced neoplasia in mice with established prion colonization in the spleen to investigate the kinetics of prion infection. We showed that this procedure effectively disrupted the composition of lymphoid follicles, reducing the number of MFG-E8-positive cells and levels of PrPSc. Interestingly, no PK-resistant PrPSc could be detected in the neoplastic spleens. However, titres of prion infectivity were almost unchanged. Our data showed an uncoupling of PrPSc and prion infectivity in the spleen. Interestingly, instead of protease-resistant bona fide PrPSc, we could detect protease-sensitive prion species in neoplastic tissue. Our results showed the presence of different PrP species in spleen that might both add to prion infectivity. Protease-sensitive prion species only became apparent upon disruption of the tissue architecture. The identification of these protease-sensitive species provides valuable insights into their generation.

RESULTS

Previously, we showed that persistent retroviral infection with Moloney murine leukemia retrovirus (MoMuLV) changed the neuropathological signature and phenotype of prion disease (Krasemann et al., 2012). In this study, a subset of mice were used that developed spleenomegaly (Fig. 1a) as a result of proliferation and infiltration of MoMuLV-transformed haematopoietic cells (Rosenberg & Jolicoeur, 1997). This provided the opportunity to investigate the impact of lymphoid tissue integrity on the presence of different prion species in spleens of prion-infected mice, the objective of this study. Mice with spleenomegaly were seen as early as 80 days after birth corresponding to 60 days after prion infection. At day 50 after birth, corresponding to 30 days after prion administration, no such mice were detected (0/12 mice for day 30, 1/12 mice for day 60, 2/12 mice for day 90 and 5/20 mice for day 150–170). In contrast, none of the mice that were inoculated with prions only developed leukaemia (0/36). Matched pairs of MoMuLV+prion-infected mice with and without spleenomegaly, taken at identical time points, were investigated further in this study. As controls, prion-only-infected mice, mock-infected mice and MoMuLV-only-infected mice were used. The latter were divided into groups with and without spleenomegaly (0/3 mice for day 30, 1/3 mice for day 60, 2/5 mice for day 90 and 4/14 mice for day 170). Histopathological examination of these latter two groups using formalin-fixed, paraffin-embedded or frozen tissue revealed complete disruption of the spleen architecture with no discernible follicular structure in mice with spleenomegaly (Fig. 1b). Examinations also included a mock-infected control spleen to rule out the effects of MoMuLV infection itself. No differences could be detected in non-neoplastic spleens after MoMuLV retroviral infection alone (Fig. 1b). In comparison with the normal-sized MoMuLV-only-infected spleens, the abundance of highly proliferating T-cells (high MIB-1 labelling index, CD3 positivity) could be demonstrated in neoplastic spleens (Fig. 1c).

As the presence of PrPC is a prerequisite for prion replication (Büeler et al., 1992), we determined the amounts of PrPC in normal and neoplastic spleens by immunohistochemistry. In normal spleens, we observed PrP immunoreactivity mainly in regions where FDCs reside (Fig. 1c) (Kranich et al., 2008). Weaker expression of PrP was detectable in other cell types surrounding follicular structures. The PrP distribution was completely changed and drastically reduced in neoplastic spleens, where no PrP-positive follicular structures were present. However, individual cells that were faintly immunopositive for PrP could also be detected in neoplastic spleens (Fig. 1c). To determine the abundance of MFG-E8-positive cells in spleens with or without neoplasia, we applied these antibodies to frozen sections. Unexpectedly, the number of MFG-E8-positive cells was not as drastically reduced as would have been expected from the PrPSc staining (Fig. 2a). Although the spleen architecture was disrupted, MFG-E8-positive cells still clustered in distinct areas of the spleen. The reduction in MFG-E8-positive cell numbers was quantified and could be shown to be about two- to fourfold decreased compared with the non-neoplastic spleens (Fig. 2b). To assess whether this cell population...
Fig. 1. Comparison of normal and neoplastic spleen architecture. (a) Macroscopic examination of spleens showing that the neoplastic spleens were remarkably enlarged (scale in cm). (b) Immunohistochemical examination of spleen tissue from mice infected with MoMuLV only showing neoplasia-induced disruption of the spleen microarchitecture with no apparent follicular structures in the neoplastic spleens [haematoxylin and eosin (H&E) staining]. In contrast, mock-infected or MoMuLV-only-infected spleens showed the proper architecture. Bars, 100 μm (top row), 10 μm (bottom row). (c) High level of proliferation of neoplastic cells (MIB-1 staining), diffuse growth pattern neoplastic T-cells (CD3 staining) and reduced abundance of PrP<sup>C</sup> in neoplastic tissue only. Bar, 10 μm. Immunohistochemical staining for PrP identified proper networks only in the normal and not in the neoplastic spleens.
was distinct from macrophages and could be defined as FDCs, we performed IBA-1 staining of spleens (Fig. 2a). In non-neoplastic spleens, macrophages were more abundant in the white pulp but could also be found in the red pulp, whereas in neoplastic spleens, the macrophages were dispersed evenly. Thus, in neoplastic tissue IBA-1-positive macrophages may be found at sites where MFG-E8-positive cells can be detected.

Fig. 2. Neoplasia disturbs the follicle architecture and drastically reduces the amount of PrP<sup>C</sup> and PrP<sup>Sc</sup>. (a) Staining with antibodies against MFG-E8 and IBA-1 in mice infected with MoMuLV without and with neoplasia revealed a disturbance of the follicular architecture in the neoplastic tissue. (b) Quantification of MFG-E8-positive staining showed a two- to fourfold reduction in the number of putative FDCs (n=3 for each group). a.u., Arbitrary units. (c) Western blots for PrP<sup>C</sup> of normal and neoplastic spleens showed a drastic reduction in PrP<sup>C</sup> in neoplastic tissue. Each lane was loaded with 0.5 mg tissue corresponding to 100 µg total protein. (d) Western blots for PrP<sup>Sc</sup> following PK digestion showed complete absence of PrP<sup>Sc</sup> in neoplastic spleens using both regular Western blots [0.5 mg tissue (100 µg total protein)<sup>−1</sup> per lane (upper panel)] and NaPTA enrichment prior to Western blotting [25 mg tissue per lane (lower panel)]. Note that the spleen harboured high levels of PrP<sup>Sc</sup> at 30 days after prion infection, at time points where we could not detect neoplasia. Therefore, it seems feasible to assume that prion colonization of the spleen was established before the onset of neoplasia and regressed subsequently. As a positive control for NaPTA, brain homogenate of a terminally prion-diseased mouse was spiked into normal spleen homogenate and PK digested or not. Molecular mass markers (kDa) are indicated on the right.
To confirm the findings from immunohistochemistry, we performed Western blotting to determine PrP^C\textsuperscript{Sc} amounts in normal and neoplastic spleens of mice infected with MoMuLV and prions (Fig. 2c). As expected, PrP^C\textsuperscript{Sc} amounts were drastically reduced in neoplastic spleens (Fig. 2c). To investigate whether and to what extent PrP^Sc loads in spleen are influenced by disturbed spleen architecture, spleen tissue of MoMuLV + prion-infected mice with and without spleenomegaly were examined by standard and sodium phosphotungstic acid (NaPTA)-enhanced Western blotting for PrP^Sc (Fig. 2d). For this, 0.5 mg (standard Western blot) or 25 mg (NaPTA-enhanced Western blot) of spleen was analysed using published methods (Glatzel et al., 2003; Wadsworth et al., 2001). All PK digestions were performed with 20 μg PK ml\textsuperscript{-1} for 1 h at 37 °C. Western blots were developed with anti-PrP POM-1 antibody (Polymenidou et al., 2008).

PrP^Sc was easily detectable at day 30 after prion infection in mice infected with prions only or with MoMuLV + prions, in the absence of neoplasia (Fig. 2d). Interestingly, PrP^Sc amounts in neoplastic spleens were below the detection limit, even for the NaPTA-enhanced Western blot (Fig. 2d). As FDCs within lymphoid follicles are thought to be the major replication site for PrP^Sc and an important source of PrP^Sc, it was not unexpected to see drastically decreased PrP^Sc loads in neoplastic spleens (Brown et al., 1999, 2000; Montrasio et al., 2000), although the degree of PrP^Sc reduction was not reflected by the only two- to fourfold decrease in MFG-E8-positive cell numbers. Accumulation of PrP^Sc in

![Fig. 3. PrP^Sc load and prion titre are uncoupled in neoplastic spleens. (a) PrP^Sc accumulation in spleen follicles of mice 90 days after prion infection is not changed following additional MoMuLV infection, but is undetectable in neoplastic spleens. Bar, 10 μm. (b) Determination of prion titres by bioassay in tga20 mice showing high prion titres ranging from 3.86 to 5.18 log LD\textsubscript{50} (ml homogenate)\textsuperscript{-1} irrespective of neoplasia at 30, 60 and 90 days after prion inoculation. Neoplastic spleens without detectable PrP^Sc had mean prion titres of 4.1 and 4.15 log LD\textsubscript{50}. Each dot represents one of four animals and the horizontal line is the mean of the four individual tga20 mice.](http://vir.sgmjournals.org)
spleenic follicles could be detected in tissue sections taken 90 days after prion infection (Fig. 3a). The amount of PrPSc deposition was independent of an additional MoMuLV infection. However, in the neoplastic spleen infected with MoMuLV+ prions, we were not able to detect PrPSc-positive staining.

To determine the titres of prion infectivity, 30 µl 1% spleen homogenate of mice at 30, 60 and 90 days after prion infection was inoculated intracerebrally into PrPC-over-expressing tga20 mice (four mice per investigated tissue) (Fischer et al., 1996). Animals were sacrificed when clinical signs of terminal prion disease were evident (summarized in Table 1) and prion titres were calculated according to published protocols (Fischer et al., 1996). High-titre prion infectivity could be detected in the cohorts by 30 days after prion infection and was independent of an additional retrovirus infection. Unexpectedly, there was no gross difference in incubation time to clinical prion disease and the respective titres of prion infectivity between spleens with or without neoplasia at the investigated time points (Fig. 3b; Table 1).

The uncoupling of PrPSc amount and tissue prion infectivity in neoplastic spleens led us to speculate that infectivity might be determined by partially PK-resistant or even protease-sensitive prion species. Here, PK resistance would be defined as producing a typical three-band pattern of

Fig. 4. Partial PK digestion reveals PK-sensitive prion species in neoplastic tissue. (a) Cold PK digestions of 100 µg total protein per sample with 200 µg PK µl−1 on ice did not reveal atypical PrP digestion patterns for MoMuLV+ prion-infected spleens. With additional neoplasia, no PrPSc at all could be detected with this method. (b) Spleen samples (100 µg total protein per lane) were partially digested using decreasing amounts of PK and probed with the anti-prion antibodies POM-1 for the classical detection pattern and 1E4 for low-abundance PrP fragments. Examinations with POM-1 did not reveal differences in PrP or PrPSc between spleens of mice that were infected with prions only or with MoMuLV+ prions. Accordingly, spleens of mice without prion or retroviral infection (mock control) or infected with MoMuLV only did not show differences in PrPSc abundance. Detection with antibody 1E4 revealed unique PK-sensitive small fragments only in the MoMuLV+ prion-infected mice with additional neoplasia (arrow).
protease-resistant PrP in Western blotting after digestion with high amounts of PK. 'Protease-sensitive prion species' was used here to describe PrP or cleavage products of PrP that are not present in uninfected tissue, are detectable in Western blots and are degradable with increasing amounts of PK. We performed cold PK digestion using enhanced amounts of protease to detect aberrant digestion patterns. However, no change in the digestion pattern could be detected for tissue without neoplasia after MoMuLV + prion infection (Fig. 4a). With additional neoplasia, no PrP<sup>Sc</sup> could be detected at all applying this technique (Fig. 4a). Therefore, we performed Western blot analyses of partially PK-digested tissue samples using decreasing amounts of protease (Fig. 4b). In addition to the use of the anti-prion antibody POM-1 for standard detection of the typical PrP<sup>Sc</sup> banding pattern, we used anti-prion antibody 1E4, which also enables the detection of low-abundance PK digestion fragments and PK-sensitive fragments (Fig. 4b) (Gambetti <i>et al.</i>, 2008). As a control, MoMuLV + prion-infected mouse tissue, prion-only-infected tissue, mock-infected tissue and MoMuLV-only-infected tissue (with and without neoplasia) corresponding to day 90 after prion infection were analysed (Fig. 4b). The PrP digestion pattern detected with POM-1 was as expected and, moreover, demonstrated no difference between prion-only-infected and MoMuLV + prion-infected tissues in general. With these analyses, we also showed that the abundance of PrP<sup>Sc</sup> was not altered following retroviral infection and was only altered with additional neoplasia. Remarkably, detection with antibody 1E4 showed small protease-sensitive PrP signals only in the tissue of mice infected with MoMuLV + prions and displaying additional neoplasia (Fig. 4b). As we could not detect the respective PrP fragments in any other cohort, it is conceivable that these protease-sensitive prions account, at least partially, for the high tissue infectivity in the absence of bona fide PrP<sup>Sc</sup>.

Here, we demonstrated the existence of at least two different PrP species in peripheral tissue: (i) PK-resistant bona fide PrP<sup>Sc</sup> representing the biochemically detectable prion species in Western blots, and (ii) a seemingly PK-sensitive PrP species that co-occurred with high tissue infectivity and might, at least in part, determine the titre of infectious prions. The protease-sensitive species only became apparent in our experimental setting when the spleen architecture was disturbed by neoplasia.

**DISCUSSION**

Prions may be defined as the infectious agent responsible for prion diseases (Barria <i>et al.</i>, 2009; Bolton <i>et al.</i>, 1982; Mahal <i>et al.</i>, 2007; Supattapone, 2004). A critical step in disease establishment is the conversion of host-derived PrP<sup>C</sup> into its misfolded counterpart PrP<sup>Sc</sup>, which is defined by its partial resistance to protease digestion. In principle, the presence and amount of PrP<sup>Sc</sup> correlate well with titres of prion infectivity, although this correlation is not linear (Büeler <i>et al.</i>, 1992; Gonzalez <i>et al.</i>, 2012; Lewis <i>et al.</i>, 2012). Recently, it has become clear that there are diverse PrP conformers, including oligomeric PrP species that do not fulfill the criteria of PrP<sup>Sc</sup>, yet contain considerable prion infectivity (Lewis <i>et al.</i>, 2012; Sandberg <i>et al.</i>, 2011; Silveira <i>et al.</i>, 2005).

In our experiments, bona fide PrP<sup>Sc</sup> was removed entirely from the neoplastic spleens. As neoplastic spleens showed a destroyed follicular structure with a significantly reduced number of MFG-E8-positive cells and a drastic decrease in PrP<sup>C</sup>, this is in line with data showing a strong correlation between the presence of MFG-E8-positive cells and PrP<sup>Sc</sup> (Brown <i>et al.</i>, 1999, 2000; McCulloch <i>et al.</i>, 2011; Montrasio <i>et al.</i>, 2000). In contrast to other studies, in our model, titres of prion infectivity were almost unaffected and were entirely dissociated from the amounts of PrP<sup>Sc</sup>. How can this be explained?

Recently, it has become obvious that other cell types in the spleen (natural killer cells and plasmacytoid dendritic cells) also harbour considerable amounts of prion infectivity and may account for high titres (Castro-Seoane <i>et al.</i>, 2012). How prion infectivity in these cell types is associated with PrP<sup>Sc</sup> remains to be investigated.

Another potential explanation for our findings relates to the contribution of PrP species to infectivity (Jeffrey <i>et al.</i>,

### Table 1. Incubation time in days of individual tga20 mice in the bioassay

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HDIC, High dosage inoculated intracerebrally; HDIP, high dosage inoculated intraperitoneally (see Methods).
It is conceivable that, in neoplastic spleens, extracellular PrPSc, but not intracellular PrP isoforms, are diluted out due to cell proliferation or are degraded due to the activity of neoplasia-associated proteases. In this case, intracellular PrP species, residing in intracellular subdomains protected from degradation, may make up the majority of prion infectivity, despite very low levels of PrPSc (Lewis et al., 2012; Sandberg et al., 2011).

Degradation of PrPSc due to upregulation of proteolytic processes in the extracellular microenvironment of neoplastic spleens might contribute to the absence of PrPSc (Ganor et al., 2009; Owen et al., 2008). Possibly, in the leukemic microenvironment, proteolytic processing of PrPSc that is composed of larger aggregates drastically reduces the PrPSc amount. Moreover, cleavage of PrPSc aggregates might generate smaller fragments that might be identical to infectious PK-sensitive PrP species. This would be in line with studies showing the presence of these species in the brains of patients with protease-sensitive prionopathy (Cronier et al., 2008; Gambetti et al., 2008). We showed that such small protease-sensitive prion species occurred in neoplastic spleens and hypothesize that these species might potentially account for prion infectivity in the absence of bona fide PrPSc. Moreover, it has already been shown that the most infectious prion is a small oligomer rather than a large aggregate (Silveira et al., 2005). The abundance of such species below the detection limit in our system cannot be ruled out. It has been shown that changes in pH lead to fragmentation of fibrillar PrPSc into smaller pieces in vitro (Qi et al., 2012). This scenario might also apply to neoplastic spleens: local changes in pH due to neoplastic processes might lead to the dissociation of PrPSc aggregates generating highly infectious PrP species.

The detection of protease-sensitive prion species in our system is in favour of the first theory, whereby the degradation of aggregates into smaller fragments either directly generates small infectious prion species or serves as a platform for conformational intermediates that form subsequently into infectious isoforms or small aggregates.

Protease-sensitive PrP species are still poorly defined and range from PK-sensitive patterns resembling PrPSc digestion patterns and different sized multimers to completely novel digestion patterns including small fragments (Cronier et al., 2008; Gambetti et al., 2008; Safar et al., 1998; Sajnani et al., 2012; Thackray et al., 2007; Tzaban et al., 2002). However, here, protease sensitivity was defined as a PK-degradable PrP protein signal that was detectable using defined anti-PrP antibodies after Western blotting. Answering the question on the molecular composition of infectious prions in the periphery will be essential to our understanding of the pathophysiology of this group of diseases and in adapting current protocols of prion detection in non-CNS tissues, which are based largely on the detection of bona fide PrPSc. Characterization of these PrP species will help to understand the differential roles of infectious and non-infectious PrP species in prion transmission and the execution of neurodegenerative pathways.

METHODS

Chemicals. Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich.

Virus production. The ecotropic retrovirus MoMuLV was used to induce leukaemia and subsequent spleenomegaly in this study. MoMuLV for our approach was generated by transduction of mouse fibroblast SC1 cells with the molecular clone Mov3 (Harbers et al., 1981). A virus titre of ~106 infectious units (IU) ml−1 in cleared supernatant of these cells was determined by an XC plaque assay.

Animals. All procedures involving animals were performed in accordance with the institutional guidelines from the animal facility of the University Medical Center Hamburg-Eppendorf and were in compliance with the Guide for the Care and Use of Laboratory Animals. Mice were inoculated with MoMuLV on post-natal day 5 with ~7 × 104 IU to achieve virus persistence (Krasemann et al., 2012; Rodenburg et al., 2007; Schwieger et al., 2002). For prion-only infection or infection with both pathogens (MoMuLV + prion), strain RML 5.0 prions (Prinz et al., 2003b) were injected on day 21. Mice were inoculated intraperitoneally with 100 μl 1% brain homogenate of RML 5.0 prions at high dosage (HDIP) (6 log LD50) or intracerebrally with 30 μl at high dosage (HDIC) (3 × 105 log LD50), respectively. Animals were analysed pre-clinical on days 30, 60 and 90 after prion infection. Control animals received mock injections (mock mice: 1% brain homogenate from uninfected CD-1 mice) or were infected with MoMuLV only (~7 × 104 IU) on post-natal day 5.

Western blot analysis. For Western blot analysis, samples were homogenized (FastPrep FP120; Qbiogene) at 10% (w/v) in buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS] and selected samples (100 μg total protein) were digested with PK (0.1–20 μg ml−1; Roche) for 1 h at 37°C. Cold PK digestions were performed with 200 μg PK ml−1 on ice for 1 h. Samples were analysed by SDS-PAGE, followed by transfer to PVDF membranes (Bio-Rad). As primary antibodies, anti-PrP antibody POM-1 (diluted 1:1000 in blocking buffer; Polymenidou et al., 2008) or IE4 (diluted 1:500 in blocking buffer; Sanquin) was used. Signals were detected using ECL Femto reagent (Thermo Scientific).

NaPTA precipitation. PrPSc was enriched by NaPTA according to published protocols adapted for spleen tissue (Glätzel et al., 2003; Wadsworth et al., 2001). Briefly, 50 mg tissue samples were dissociated in 900 μl buffer [25 mM HEPES (pH 7.2), 0.3 M sucrose and 53.6 μg Liberase Blendzyme 2 (Roche)], incubated for 30 min at 37°C and homogenized (FastPrep FP120). Five hundred microlitres of 10% (w/v) tissue homogenate corresponding to 25 mg tissue and 500 μl 4% (w/v) Sarkosyl in PBS was incubated for 10 min at 37°C with constant agitation. Benzonase (Novagen) and MgCl2 were added to 50 U ml−1 and 1 mM, respectively, and incubated at 37°C for 30 min with vigorous agitation. To this, 81.3 μl of a pre-warmed (to 37°C) freshly prepared 4% (w/v) NaPTA/170 mM MgCl2 solution (pH 7.4) was added, vortexed and incubated with vigorous agitation for 30 min at 37°C. Samples were then centrifuged at 25 000 g for 30 min and the pellets resuspended in 22.5 μl 0.1% Sarkosyl in PBS, followed by PK digestion (20 μg ml−1) for 45 min at 37°C.

Immunohistochemistry. Tissues were fixed with 4% buffered formalin, and prion-infected samples were additionally inactivated by 98% formic acid for 1 h, followed by processing for paraffin embedding. Sections (3 μm) were subjected to H&E staining according to a standard protocol (Glätzel et al., 2003). Immunohistochemistry was performed using marker proteins for proliferation (MIB-1), T-cell amount (CD3), macrophages (IBA-1) and PrP abundance (POM-1).
PrPSc was detected as described previously with slight modifications (Hoffmann et al., 2011). Inactivated paraffin-embedded tissue sections (3 μm) were deparaffinized, incubated in formic acid for 30 min, washed with H2O and autoclaved in citrate buffer (pH 6.0) for 5 min before the first antibody Saβ84 (Cayman) was applied (diluted 1:100). Detection with secondary antibody was carried out according to standard protocols.

MFG-E8-positive cell staining was performed on cryosections from spleen tissue according to the manufacturer’s protocol (Anti-Rat Ig HRP Detection kit; BD Pharmingen). Briefly, snap-frozen spleens were cut into 8 μm sections and fixed in cold acetone. Samples were incubated with primary antibody (anti-mouse FDC-M1 rat antibody, diluted 1:50, at 4 °C overnight) and secondary antibody (biotin-coupled anti-rat, diluted 1:50, for 30 min at room temperature) and developed with streptavidin–horseradish peroxidase and 3,3′-diaminobenzidine and counterstained with H&E. For quantification of LD50 (Fischer et al., 1981), the incubation time to terminal disease in days and y was LD50 (Fischer et al., 1996; Kaeser et al., 2001).

Determination of prion titres by mouse bioassay. To determine the titres of prion infectivity, 30 μl aliquots of 1 % spleen homogenate were inoculated intracerebrally into groups of four PrP-C-overexpressing tga20 transgenic mice per investigated tissue (Fischer et al., 1996). Animals were observed daily and sacrificed when clinical signs of prion disease were evident, such as reduced motor activity, weight loss, hunched posture, hind limb paresis and ataxia. Prion titres were calculated according to the equation y = 11.45 − 0.088x, where x was the incubation time to terminal disease in days and y was LD50 (Fischer et al., 1996; Kaeser et al., 2001).

ACKNOWLEDGEMENTS

S. K. was supported by a DLR grant (01 GZ 0712) and Pro Exzellenza (City of Hamburg). S. K. would especially like to thank Lucien van Keulen for helpful discussions at the Prion2012 conference in Amsterdam. The authors would like to thank the UKE core facility for Mouse Pathology, and Sandra Deutsch and Kendra Richter for technical assistance.

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