Evidence of subclinical prion disease in aged mice following exposure to bovine spongiform encephalopathy

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The occurrence of variant Creutzfeldt–Jakob (vCJD) disease in humans was almost certainly the result of consumption of food contaminated with bovine spongiform encephalopathy (BSE) prions. Despite probable widespread exposure of the UK population to BSE-contaminated food in the 1980s, vCJD has been identified predominantly in young individuals, and there have been fewer cases of clinical disease than anticipated. The reasons for this are uncertain. Following peripheral exposure, many prions replicate within the lymphoid tissues before infecting the central nervous system. We have shown that the effects of host age on the microarchitecture of the spleen significantly impair susceptibility to mouse-adapted prions after peripheral exposure. The transmission of prions between different mammalian species is considered to be limited by the ‘species barrier’, which is dependent on several factors, including an intact immune system. Thus, cross-species prion transmission may be much less efficient in aged individuals. To test this hypothesis, we compared prion pathogenesis in groups of young (6–8 weeks old) and aged (600 days old) mice injected with primary BSE brain homogenate. We showed that prion pathogenesis was impaired dramatically in aged mice when compared with young animals. Whereas most young mice succumbed to clinical prion disease, all aged mice failed to develop clinical disease during their lifespans. However, the demonstration that prion accumulation was detected in the lymphoid tissues of some aged mice after injection with primary BSE brain homogenate, in the absence of clinical signs of prion disease, has important implications for human health.

INTRODUCTION

Transmissible spongiform encephalopathies (prion diseases) are subacute neurodegenerative diseases that affect humans and animals. These diseases are defined by a number of characteristic pathological changes in the central nervous system (CNS) including vacuolation of the neuropil, gliosis and aggregations of PrPSc, an abnormally folded isoform of the cellular prion protein (PrPC). The precise nature of the infectious prion is uncertain, but PrPSc is considered to constitute the major, if not sole, component (Bolton et al., 1982; Legname et al., 2004).

In 1996 a ‘new variant’ of Creutzfeldt–Jakob disease (vCJD) was described in 10 individuals with a clinical and pathological phenotype distinct from that of sporadic (s)CJD (Will et al., 1996). Transmissions to mice have produced compelling evidence to show that brain material from vCJD-affected individuals produces a signature undistinguishable from that of bovine spongiform encephalopathy (BSE) (Bruce et al., 1997). The components of this signature include distinct incubation periods in specific mouse strains, distinct neuropathological characteristics in the brains of recipient mice and distinct PrPSc glycoforms (Somerville et al., 2005). One of the most striking features of vCJD is the young age of the affected individuals (median age at onset of disease ~26 years old) (Boe¨lle et al., 2004; Ghani et al., 2000) (Fig. S1, available in JGV Online). Of the 177 recorded definite and probable vCJD cases that had been reported in the UK at the time of writing (August 2013), the majority had occurred in young patients, with only five cases (2.8 %) observed in the elderly (≥60 years old). This contrasts starkly with those affected with sCJD, which typically affects individuals >60 years old. The reasons behind this apparent age-related susceptibility are not fully understood. A causal link between dietary preference (the consumption of foodstuffs considered to be at high risk of BSE contamination) and disease incidence in the young has not been substantiated (Boe¨lle et al., 2004), suggesting that other factors may significantly influence susceptibility.

Although the major pathology associated with prion infection appears to be restricted to the CNS, the peripheral immune system plays an essential role in the pathogenesis of many natural and experimental prion
the PrP species or polymorphisms and mutations in the PRNP gene (which encodes PrPC) having significant influence (Barron et al., 2001; Bishop et al., 2006; Houston et al., 2003; Prusiner et al., 1990). However, the precise molecular mechanism responsible for these effects is uncertain. Studies using immunodeficient mice have shown that a functional immune system is also essential for the efficient cross-species transmission of BSE prions (Brown et al., 1997). SCID (severe combined immunodeficiency) mice, which lack functional, mature FDCs in their lymphoid tissues, show dramatically reduced susceptibility to prion infection following injection with primary BSE prions. This suggests that the effects of ageing on FDC status and the splenic MZ may likewise impede the cross-species transmission of BSE. To test this hypothesis, we compared primary prion pathogenesis in young and aged mice injected with primary BSE brain homogenate. Our data showed that aged mice did not develop clinical disease after exposure to BSE prions. However, some mice had evidence of prion accumulation in their spleens. These data suggest that, while elderly individuals may be less susceptible to clinical prion disease after BSE exposure, they may accumulate significant levels of prions within their lymphoid tissues and may pose a potential risk for the horizontal spread of disease.

RESULTS

Effect of host age on susceptibility to BSE infection

The transmission of BSE to specific inbred strains of mice produces distinctive neuropathological characteristics and reproducible incubation periods (Bruce et al., 1997). Here, following primary BSE injection into groups of young (6–8 weeks old) RIII mice and young C57BL mice, clinical disease was confirmed in the majority of the recipients with incubation periods consistent with previously published data (RIII, 361 ± 8 days; C57BL, 505 ± 27 days; Table 1) (Bruce et al., 1997). Histopathological analysis of the brains from the clinically affected young mice revealed that the severity and distribution of the vacuolar (spongiform) pathology was typical of that associated with BSE transmission to mice (Fig. 1a, b) (Bruce et al., 1997). The absence of disease in a small number of the young mice (Table 1) was not unexpected, as cross-species transmission is often less efficient. Of the 17 aged RIII mice that were injected with BSE prions (aged RIII mice were 600 days old at the time of BSE injection), seven mice survived until after the first clinically positive case in the corresponding young mice (aged RIII mice were 928–1041 days old at the time of cull; Table 1). However, there was no evidence of clinical disease or positive vacuolar pathology in any of the brains from the BSE prion-injected aged RIII mice (Fig. 1a, c, Table 1). Unfortunately, the disease incubation period after primary BSE injection in C57BL mice (~500 days) exceeded the lifespan of the aged mice in this study (aged C57BL mice were 600 days old at time of BSE prion injection and survived to ~900 days old). However, histopathological analysis of all the brains from the BSE-injected aged C57BL mice, including nine mice that survived to over two-thirds of the incubation period in the corresponding young mice, found no evidence of vacuolar pathology (Fig. 1b, d, Table 1).

Effect of host age on PrPSc accumulation in the brain

In addition to vacuolar changes, CNS prion infection is accompanied by astrogliaisis, increased microglial activity and abnormal accumulations of disease-specific PrP. As
anticipated, all the brains from the BSE prion-injected, clinically affected young RIII mice and young C57BL mice with positive vacuolar pathology in their brains demonstrated high levels of astrogliosis and microgliosis consistent with the terminal stage of prion disease (Fig. 2). However, none of the histopathological characteristics of CNS prion disease were detected in any of the brains from the BSE prion-injected aged RIII mice and C57BL mice (Fig. 2). Together, these data showed that BSE disease susceptibility is reduced dramatically in aged mice.

Antigenic and biochemical differences between PrPSc and PrPd have been used to describe the disease-specific, abnormal accumulations of PrP that are characteristically found only in prion-affected tissues. Prion disease-specific PrP accumulations are relatively resistant to proteinase K (PK) digestion, whereas cellular PrPc is destroyed. Thus, prion-specific PK-resistant PrP (referred to as PrPSc) can often be used as a biochemical marker for the presence of prions (Bolton et al., 1982). On histological sections where PK treatment is not used because it destroys the tissue

Table 1. Effect of host age on susceptibility to primary BSE infection

| Mouse model* | Mean disease incubation period (days ± SEM) | Survival times (days) | Clinical disease§ | Positive vacuolar pathology in brain|| | PrP in brain§ | PrP in spleen§ |
|--------------|--------------------------------------------|-----------------------|-------------------|-------------------------------|-------------------|----------------|----------------|
| Young RIII   | 361 ± 8 | 342, 370, 401, 430 | 16/20 | 20/20 | 20/20 | 13/16# |
| Young C57BL  | 505 ± 27 | 446, 518 | 10/12 | 9/12 | 10/12 | 9/12 |
| Aged RIII    | No | No | No | No | No | No |
| Aged C57BL   | No | No | No | No | No | No |

ND, Not determined.

*Young mice were 6–8 weeks old at the time of primary BSE exposure; aged mice were ~600 days old at the time of primary BSE exposure.
†Mean time from time of injection with primary BSE prions to time of definite diagnosis of clinical prion disease, at which point the mice were culled.
§Mice that did not develop clinical signs of prion disease during their lifespans; individual times from time of injection with prions to cull are given.
¶Number of animals affected/number of animals tested.
||Prion disease-specific vacuolar (spongiform) pathology was scored on a 0–5 scale in nine distinct grey-matter areas as described previously (Bruce et al. 1997). A score of 1 (a small number of vacuoles) or greater in any brain region was considered positive.
¶¶Detection of PrPd in any region of the tissue section was considered positive.
§§Spleens from four animals were unavailable for analysis.
microarchitecture, we refer to these abnormal disease-specific PrP accumulations as PrPd. However, to confirm the presence of prion-specific PK-resistant PrPSc, adjacent brain sections were applied to nitrocellulose membranes, PK treated and analysed by paraffin-embedded tissue (PET) immunoblotting (Schulz-Schaeffer et al., 2000).

Our previous studies of the transmission of mouse-passaged prions to aged mice revealed the presence of prion-specific PrPSc deposition in the majority of the brains from aged mice after peripheral (intraperitoneal and oral) exposure, despite the absence of clinical signs of disease or vacuolar pathology (Brown et al., 2009). We therefore determined whether PrPSc was present in the brains of the clinically negative, BSE prion-injected aged mice. Large accumulations of PrPd were detected by immunohistochemistry (IHC) in the brains of all the clinically affected, BSE prion-infected, young mice (Fig. 2, Table 1). PET immunoblot analysis of adjacent sections confirmed that the PrPd detected by IHC was PK-resistant, prion-specific PrPSc (Fig. 2). In contrast, PrPSc was only detected in a small focus in the brain of one of the BSE-infected aged RIII mice (the vestibular nucleus) and was undetectable in any of the brains from the aged C57BL mice (Figs 2 and 3a, Table 1).

Effect of host age on the accumulation of PrPd in spleen

The accumulation of high levels of PrPSc upon FDCs in lymphoid tissues is a characteristic feature of many experimental and natural prion infections, and studies in mice show this is crucial for efficient neuroinvasion. Furthermore, SCID mice that lack mature FDCs are also refractory to prion infection after exposure to BSE prions (Brown et al., 1997). Here, high levels of PrPd consistent with association upon FDCs were detected in almost all the spleens from the prion-infected young RIII and C57BL mice (Fig. 3, Table 1). In aged mice, despite the absence of clinical and histopathological signs of disease in the CNS, heavy PrPd accumulations were also detected upon the few intact FDCs remaining in the spleens of most of the BSE-challenged RIII mice (nine of 16) and two of nine of the C57BL mice (Fig. 3, Table 1).
FDC status is adversely affected in the spleens of aged RIII mice

We have shown previously that the status of FDCs and their ability to trap and retain immune complexes are compromised in the spleens of aged C57BL mice (Brown et al., 2009, 2012). We therefore determined whether ageing also affected adversely FDC status in RIII mice. FDCs in the spleens of young mice express high levels of complement receptor (CR) 1 and CR2 and cellular PrP$^C$, and trap and retain large amounts of complement component C4 on their surfaces (McCulloch et al., 2013; Taylor et al., 2002; Zabel et al., 2007) (Fig. 4). The expression of CR2/CR1 by FDCs in the spleens of aged mice was similar to that observed in young mice (Fig. 4). However, the majority of FDC networks in the spleens of the aged RIII mice showed reduced expression of PrP$^C$ and impaired retention of complement component C4 (Fig.

Fig. 2. Histopathological analysis of the characteristic signs of prion disease in the brains of BSE prion-injected young and aged mice. (a) Cartoons illustrating the anatomical brain regions analysed. (b, d) In young RIII mice (b) and C57BL mice (d), pathology consistent with terminal BSE infection was observed in the brains of all the clinically affected mice. The typical neuropathological signs included heavy accumulations of disease-specific PrP (PrP$^d$, brown, second column), reactive astrocytes expressing high levels of glial fibrillary acidic protein (GFAP; brown, third column) and active microglia expressing Iba-1 (brown, fourth column). PET immunoblot analysis of adjacent sections confirmed the PrP$^d$ accumulations were PK-resistant PrP$^Sc$ (black, first column). (c, e) In contrast, none of the aged mice had detectable histopathological signs of prion disease in their brains. The exception was one clinically negative, BSE prion-injected aged RIII mouse that had evidence of PrP$^d$ accumulation only in the vestibular nucleus (see Fig. 3a). Bars, left-hand panels 500 μm, right-hand panels 200 μm.
expression of PrP^C by FDCs, and their ability to trap and retain complement-containing immune complexes, are each important for the efficient retention and replication of prions upon their surfaces (Klein et al., 2001; Mabbott et al., 2001; McCulloch et al., 2011; Michel et al., 2012; Zabel et al., 2007). Our data showed that these characteristics are dramatically affected in the spleens of aged RIII mice (Fig. 4a, b) and aged C57BL mice (Fig. 4c, d).

**Ageing affects adversely the distribution of MZ macrophages in the spleens of aged C57BL mice but not aged RIII mice**

The splenic MZ surrounds the white pulp and comprises a distinct channel of mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1)-expressing sinus lining cells through which blood percolates on its way to the red pulp. Specific populations of macrophages and B-cells are attached firmly to this network, enabling the continuous surveillance and clearance of pathogens, toxins and apoptotic cells from the bloodstream (Mebius & Kraal, 2005). MZ B-cells also capture blood-borne immune complexes and rapidly shuttle them to FDCs (Cinamon et al., 2008). We and others have reported previously that the MZ microarchitecture is disturbed in the spleens of aged C57BL mice, impeding the delivery of immune complexes and prions to FDCs (Birjandi et al., 2011; Brown et al., 2012). We therefore determined whether similar disturbances to the MZ microarchitecture occurred in the spleens of aged RIII mice. First, we graded the disruption to the network of MADCAM1-expressing sinus lining cells, and detected a significant disruption to this network in spleens from aged RIII and C57BL mice (Fig. 5a, b). In young mice, marginal metallophilic (MM) macrophages (CD169^+ cells) typically formed a continuous thin rim at the inner border of the MZ, but in aged mice this area was likewise thickened, distorted and broken (Fig. 5a). Morphometric analysis confirmed that the MM macrophage layer was also significantly disrupted (Fig. 5c) and significantly thicker (Fig. 5d) in the spleens of aged RIII and C57BL mice when compared with young mice.

The MZ also contains an outer ring of MZ macrophages expressing the C-type lectin SIGNR1. The distribution of SIGNR1^+ MZ macrophages was also reduced significantly in the spleens of aged RIII mice (as observed in aged C57BL mice), the number and size of the PrP^C-expressing FDC networks, and the level of trapped C4 on their surfaces, were reduced significantly. The
Fig. 4. Effect of host age on FDC status in the spleen. (a, c) Immunofluorescent analysis of FDC status in aged and young RIII mice (a) and C57BL mice (c). FDCs in the spleens of young mice typically express high levels of the complement receptors CR2/CR1 (red) and cellular PrP c (green), and trap complement component C4 (blue) on their surfaces (left-hand panels). In contrast, most of the FDC networks in the spleens of RIII mice and C57BL aged mice appeared to be disrupted, and the expression of PrP c and retention of complement component C4 was dramatically reduced (right-hand panels). Bar, 50 µm. (b, d) Morphometric analyses of the effect of host age on the size of the CR2/CR1-expressing FDC networks, number and size of PrP c-expressing FDCs, and area of trapped C4 on the surface of FDCs in the spleens of RIII mice (b) and C57BL mice (d). Differences between groups were analysed by a Mann–Whitney U test.
**Fig. 5.** Effect of host age on the microarchitecture of the splenic MZ. (a) IHC analysis of the distribution of MADCAM1-expressing sinus lining cells (upper row, brown) and CD169-expressing MM macrophages (lower row, brown) in the MZ of young and aged RIII and C57BL mice. RP, red pulp; WP, white pulp; FO, FDC-containing B-cell follicle. Bar, 20 μm. (b, c) Semi-quantitative assessment of the effects of ageing on the distribution to the layers of MADCAM1⁺ sinus lining cells (b) and CD169⁺ MM macrophages (c) in the spleen. Immunostained spleen sections were coded and four fields of view per mouse scored on a scale of 1 (normal) to 3 (severely disrupted). Representative images for each grade are shown. Differences between groups were analysed by Student’s t-test. (d) The effects of ageing on the thickness of the layer of CD169⁺ MM macrophages. Coded spleen sections from each mouse group were immunostained to detect CD169 (green) and the thickness of the cell layer measured at three distinct sites in four fields of view per mouse. Differences between groups were analysed by a Mann–Whitney U test.
DISCUSSION

We showed that the susceptibility of aged mice to prion disease after exposure to primary BSE is reduced dramatically. Furthermore, there were no histopathological signs of prion disease in any of the brains from the BSE-injected aged mice. In addition, PrPSc was undetectable in all the brains from the BSE-injected aged C57BL mice and only detected in a small focus (the vestibular nucleus) of the brain of one of the aged RIII mice. An intact immune system is important for cross-species transmission of BSE prions (Brown et al., 1997). Here, the effects of ageing on prion susceptibility after BSE exposure coincided with disturbances to FDCs and the microarchitecture of the MZ in the spleen. However, despite the absence of clinical and histopathological signs of prion disease in the CNS, heavy PrPd accumulations were detected upon the remaining intact FDCs in the spleens of many of the BSE-challenged aged RIII mice and some of the aged C57BL mice. The detection of PrPd in spleens of BSE-injected aged mice in the absence of neuropathological signs and clinical disease has important implications for human health and the potential for the horizontal spread of vCJD (e.g. by accidental iatrogenic transmission).

Following the emergence of BSE in the 1980s, it was estimated that over half a million infected cattle may have entered the UK food chain (Valleron et al., 2001; Wilesmith, 1993). Despite the probable widespread exposure of the UK population to the BSE agent via contaminated foodstuffs (Valleron et al., 2001), the number of confirmed clinical cases of vCJD remains relatively low (Bishop et al., 2013). Furthermore, analysis of the age ranges of the definite and probable clinical vCJD cases recorded in the UK reveals a striking age-related distribution (Fig. S1). Of the 177 probable and definite cases reported to date (August 2013), only five had occurred in elderly patients (2.8%; ≥60 years old) and 20 (11%) in middle-aged individuals (~45–60 years old). Therefore, in order to study the effects of ageing on prion pathogenesis in a laboratory mouse model, two age groups were used: young adult mice (6–8 weeks old) and immunosenescent aged (elderly) mice (~600 days old).

The splenic MZ is a specialized microenvironment that plays an important role in the capture and removal of blood-borne immune complexes, pathogens and their toxins (Mebius & Kraal, 2005). The continual shuttling of MZ B-cells between the MZ and follicles is also important for the efficient delivery of blood-borne, complement-opsonized immune complexes to FDCs (Cinamon et al., 2008). Prion replication upon PrPc-expressing FDCs is crucial for the efficient spread of infection to the CNS (Brown et al., 1999; Mabbott et al., 2000; McCulloch et al., 2013; Montrasio et al., 2000). Data suggest that an intact MZ is also required to facilitate the initial delivery of complement-opsonized prions to FDCs (Brown et al., 2012). We have shown previously that the effects of ageing on prion disease susceptibility are not
simply due to reduced Prnp mRNA (which encodes PrPC) expression levels in the CNS, or effects on the density or distribution of peripheral nerves in lymphoid tissues (Brown et al., 2009, 2012). Although ageing effects on other host factors cannot be excluded entirely, our data imply that the ageing-related changes to FDCs status and the MZ microarchitecture are likely to have had a major influence on prion disease susceptibility in the BSE-injected aged mice by impeding the initial delivery of prions into the B-cell follicles and reducing the availability of PrPC-expressing FDCs. Similarly, the significant changes to the thickness and distribution of the CD169+ MM macrophages in the MZ may also have impeded the delivery of prions to FDCs in aged mice by aiding their sequestration and clearance.

In the aged spleens from each mouse strain, there were highly significant reductions to the number and size of the PrPC-expressing FDCs networks, but small numbers of intact (PrPC-expressing) FDCs were evident. In the current study, heavy PrP accumulation were detected upon the remaining intact FDCs in the spleens of many of the BSE-challenged RIII mice but in a much smaller proportion of spleens from aged C57BL mice. The reasons for these apparent mouse strain-specific differences in splenic PrPd accumulation are uncertain. Both the RIII and C57BL mice share the same PrP genotype (Prnp+) (Lloyd et al., 2004). The effects of ageing on the number and size of the PrP-expressing FDC networks were similar for each mouse strain, as was the ageing-associated disruption to the network of MADCAM1-expressing sinus lining cells, and the distribution and thickness of the CD169+ MM macrophage layer in the MZ. However, the effects of ageing on the distribution of SIGNR1+ MZ macrophages differed between mouse strains: their distribution was significantly impaired in the MZ of aged C57BL mice, but appeared to be unaffected in aged RIII mice. C-type lectins such as SIGNR1 recognize specific carbohydrate structures that are present on cell wall components of pathogens (Lanoue et al., 2004). As the prion protein is highly glycosylated, it is plausible that SIGNR1 may play a similar role in the recognition and uptake of prions in the splenic MZ.

We have shown previously that whereas the susceptibility of aged mice to peripheral exposure with mouse-passaged scrapie prions was dramatically reduced, no effect on disease susceptibility was observed when prions were injected directly into the brain by intracerebral injection (Brown et al., 2009, 2012). However, in the current study aged mice were refractory to clinical prion disease after injection with primary BSE prions by combined intracerebral and intraperitoneal injection. After peripheral exposure FDCs are considered to amplify prions above the threshold required to achieve neuroinvasion (Mabbott, 2012; McCulloch et al., 2011). We have shown previously that young SCID mice which lack mature FDCs in their spleens are also refractory to prion disease after intracerebral injection with primary BSE prions (Brown et al., 1997). These data imply that the routing of prions through an intact lymphoreticular system is import for efficient neuroinvasion after cross-species transmission. These data also suggest that after interspecies prion exposure, the processing and replication of prions upon FDCs in the peripheral lymphoid tissues is important for their adaptation to the new host and their ability to subsequently infect the nervous system. Data in the current study showing that the effects of ageing on FDCs status and the marginal zone are coincident with reduced prion disease susceptibility are consistent with the reduced availability of replication sites (PrPC-expressing FDCs) in the spleen upon which the cattle BSE prions can adapt to the new host (mouse) environment, limiting dramatically their ability to replicate and subsequently spread to the brain.

Retrospective analyses of prion-specific PrPSc accumulation in archived appendix and tonsil samples (Clewley et al., 2009; Hilton et al., 2002, 2004) suggest that the prevalence of vCJD in the UK population may be much higher than the clinical case data alone, implying the potential existence of a subclinical carrier state (Clewley et al., 2009; Garske & Ghani, 2010). Similarly, vCJD accumulation was detected in the spleen of an asymptomatic individual who was heterozygous (MV) at codon 129 of the prion protein gene (PRNP in humans) (Bishop et al., 2013; Peden et al., 2004). These data show that significant levels of vCJD prions can accumulate in the lymphoid tissues of infected individuals in the absence of observable clinical signs, and suggest that the exposure of aged individuals to BSE agent may likewise have led to the accumulation of significant levels of vCJD prions within their lymphoid tissues in the absence of obvious clinical and histopathological signs of CNS involvement. The data presented here are consistent with data published elsewhere showing that low-dose prion exposure (Thackray et al., 2003), immunodeficiency (Frigg et al., 1999) and cross-species prion transmission (Hill et al., 2000), like ageing, can under some circumstances result in subclinical prion disease in affected individuals. However, in contrast to the above examples, we did not observe significant evidence of PrPSc accumulation or neuropathology in the brains of any of the primary BSE prion-injected aged mice. Our data suggest that the effects of ageing on FDC status and the MZ had impeded the replication of prions in the spleen, blocking or substantially delaying neuroinvasion.

Data in the current study raise important issues for human health and the potential for horizontal transmission of vCJD. We showed that aged mice did not succumb to clinical prion disease during their lifespans after cross-species BSE exposure. However, many BSE prion-injected aged mice had detectable levels of PrPd in their spleens. Thus, although the combined effects of ageing and the species barrier effect may represent a significant barrier to susceptibility to clinical disease after cross-species prion transmission, our data suggest that there may be significant levels of prion accumulation in the lymphoid tissues of...
aged individuals in the absence of CNS involvement. The potential for the existence of a subclinical carrier state suggests a plausible risk for the horizontal spread of disease (e.g. after accidental iatrogenic exposure).

METHODS

Mice. C57BL/Dk and RIII mice were aged to ~600 days under specific-pathogen-free conditions prior to use in subsequent studies. Young mice were 8–12 weeks old at the time of analysis or use in subsequent studies. All experimental procedures were approved by the University of Edinburgh’s ethical review process and conducted according to the strict regulations of the UK Home Office Animals (Scientific Procedures) Act 1986.

BSE agent exposure and disease monitoring. The BSE source used in this study was cattle brain homogenate from the brain of a clinically affected UK Friesian Holstein cow (PG63/87) collected on 1 October 1987. This material has been used in a range of primary transmissions to mice at this Institute (e.g. Bruce et al., 1997; Fraser et al., 1992) and produces a characteristic pattern in the mice consistent with classical BSE. Groups of C57BL and RIII (both of the Prnp\(^a\) genotype) (Lloyd et al., 2004) were injected with a 10 % (w/v) brain homogenate of cattle BSE by a combination of intracerebral (20 μl) and intraperitoneal (100 μl) routes (Brown et al., 1997; Bruce et al., 1997). These mouse strains were selected, as previous transmissions of BSE showed that they produce highly reproducible incubation periods of disease and distinct patterns of vacuolar degeneration in their brains (Bruce et al., 1997). Following BSE exposure, mice were coded and assessed weekly for signs of clinical disease. The clinical end point of disease was defined in one of the following ways: (1) the day on which a mouse received a second consecutive ‘definite’ rating, (2) the day on which a mouse received a third ‘definite’ rating within 4 consecutive weeks or (3) the day on which a mouse was culled in extremis.

The following criteria were used to help distinguish between the clinical signs of ageing (senility) in mice from those of prion disease (Brown et al., 2012). The fur of aged mice may lose colour and appear less sleek. Body shape may gradually change. Senile mice may have a ‘vacant stare’ whereby the face looks thinner and the eyes not as bright. Mice beginning to display clinical signs of prion are often less motile and become more conspicuous, whereas those displaying definite positive signs are immobile and less interactive with their cage mates. In contrast, senile mice still move around their cages and interact with their cage mates. Survival times were recorded for mice that did not develop clinical signs of disease and were culled when they showed signs of intercurrent disease. Prion diagnosis was confirmed by histopathological assessment of vacuolation in the brain. For the construction of lesion profiles, vacuolar changes were scored in nine grey matter areas of each brain as described previously (Bruce et al., 1997).

IHC. For the detection of disease-specific PrP (PrP\(^\beta\)), tissues were fixed in 10 % formal saline for 48 h and treated for 1 h in 98 % formic acid to reduce infectious BSE titre prior to ex vivo analysis. Sections (thickness 6 μm) were deparaaffinized, and pre-treated to enhance the detection of PrP\(^\beta\) by hydrated autoclaving (15 min, 121 °C, hydration) and subsequent immersion in formic acid (98 %) for 5 min (McBride et al., 1992). Sections were then immunostained with 1B3 PrP-specific polyclonal antiserum (Farquhar et al., 1989) or the PrP-specific mAb BH1 (Cancellotti et al., 2013). For the detection of astrocytes, brain sections were immunostained with anti-glia fibrillary acidic protein (Dako). For the detection of microglia, deparaaffinized brain sections were first pre-treated with Target Retrieval Solution (Dako) and subsequently immunostained with anti-ionized calcium-binding adaptor molecule 1 (Iba-1; Wako Chemicals). Immunolabelling was revealed using HRP conjugated to avidin–biotin complex (Novared kit; Vector Laboratories). PET immunoblot analysis of brain was used to confirm that PrP\(^\beta\) detected by IHC was PK-resistant PrP\(^\beta\). Membranes were subsequently immunostained with 1B3 PrP-specific polyclonal antiserum and developed as described previously (Schulz-Schaeffer et al., 2000).

To compare the status of FDCs and the MZ microarchitecture, spleens were snap frozen in liquid nitrogen and frozen sections (thickness 10 μm) were fixed in acetone. FDCs were visualized by staining with mAb FDC-M2 (212-MK-1FDCM2; ImmunoContact) to detect complement component C4 and mAb 7G6 to detect CR2/CR1 (CD21/CD35; BD Pharmingen). Cellular PrP\(^\beta\) was detected using 1B3 PrP-specific polyclonal antiserum. The following rat anti-mouse mAbs were used to define the MZ microarchitecture: mAb 2D1, which recognizes the C-type lectin SIGNR1 expressed on MZ macrophages (eBioscience); mAb MOMA-1 (AbD Serotec), which recognizes CD169 expressed on MM macrophages and mAb MEGA-367, which detects MADCAM1 (AbD Serotec) on the endothelial cells lining the MZ sinus. For immunofluorescent labelling, species-specific secondary antibodies coupled to Alexa Fluor 488 (green), Alexa Fluor 594 (red) or Alexa Fluor 647 (blue) dyes (Invitrogen Life Technologies) were used. For visualization at light level, the avidin–biotin complex technique (Novared kit) was used.

Semi-quantitative scoring of disturbances to the MZ architecture. Well-oriented white pulp areas of spleen were selected at random from sections from four mice from each mouse group. MZ disruption was measured using a semi-quantitative scoring method (Figs 5b–d and 6c) as described previously (Birjandli et al., 2011). Briefly, coded spleen sections were first immunostained to detect the marker of interest (CD169, MADCAM1 or SIGNR1). The relative distortion of the splenic MZ was then characterized by comparing the following criteria: thickness of the cell layer, advancement of cells of the MZ into the white pulp and presence of a discontinuous ring of immunolabelling indicating disruption to the MZ. Grading of each image was scored as: 1, no disruption/minimal; 2, moderate; 3, severe (Fig. 5b, c). To quantify the MZ thickness, the depth of the cell layer was measured at three separate points from four randomly selected white pulp areas from four mice from each mouse group. For the quantitation of SIGNR1 expression on MZ macrophages, four randomly selected fields of view were collected from each mouse from each group and the percentage area of SIGNR1\(^\beta\)-immunolabelling in each field was quantified using ImageJ software (http://rsbweb.nih.gov/ij/).

Image analysis and quantification of PrP\(^\beta\) expression on FDC networks. The area of PrP\(^\beta\) expression upon FDCs was measured using ImageJ software as described previously (Brown et al., 2009, 2012; McCulloch et al., 2011, 2013). From each spleen, three sections were studied and on each section data from three randomly selected fields of view containing individual FDC networks were collected. Therefore, from each group, data from a total of 36 individual FDCs were analysed.

Statistical analyses. Data are presented as means ± SEM. Unless otherwise indicated, differences between groups were statistically compared using Student’s t-test. In instances where there was evidence of non-normality, data were analysed by a Mann–Whitney U test. Values of P<0.05 were accepted as significant.
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