Human and mouse single-nucleus transcriptomics reveal TREM2-dependent and TREM2-independent cellular responses in Alzheimer's disease

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SPECIAL ISSUE REVIEW

Prion diseases of humans and farm animals: epidemiology, genetics, and pathogenesis

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Abstract
Neuronal vacuolation (spongiosis), neuronal death, and pronounced glial reactions are the hallmarks of transmissible spongiform encephalopathies (TSEs), or prion diseases. A wealth of physical, biochemical, and immunological evidence indicates that the TSE agent, termed prion, does not contain agent-specific nucleic acid encoding its own constituents, as is the case for all other infectious pathogens. Also, no adaptive immune responses are elicited upon infection. A defining feature of TSEs is the deposition, mainly in the brain and lymphoreticular tissues, of an aggregated and structurally abnormal protein, designated PrP^{Sc} or PrP-res, which represents a conformational isomer of the ubiquitous surface protein PrP^{C}. Biochemical and genetic evidence link PrP and its gene to the disease. Although TSEs are by definition transmissible, a growing number of *Prnp*-associated non-infectious neurodegenerative proteinopathies are now being recognized.
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Prion epidemiology

The most common human transmissible spongiform encephalopathy (TSE) is Creutzfeldt-Jakob disease (CJD). From an epidemiological viewpoint, CJD can be classified as sporadic (sCJD), familial (fCJD), iatrogenic (iCJD), and variant (vCJD). Even the most frequent form, sporadic CJD, is very rare, and appears to be evenly distributed worldwide: those countries that carry out surveillance of CJD report, quite uniformly, an incidence of approximately 0.6–1.2×10^{-6} per year (Ladogana *et al*. 2005). The etiology of sCJD is unknown: no exogenous or endogenous causes have been identified yet. A endemic form of CJD, designated Kuru, occurred among the aborigines in Papua New Guinea throughout the 1950s and 1960s. Kuru was horizontally transmitted by cannibalistic rituals, and has not been observed in individuals born after cannibalism was abandoned (Gajdusek 1977).

Familial forms of CJD are transmitted as autosomal dominant traits and invariably cosegregate with mutations in *Prnp*, the gene that encodes the prion protein (Hsiao *et al*. 1989). Experimental evidence from the mouse implies that additional factors play a role (Stephenson *et al*. 2000; Lloyd *et al*. 2001; Manolakou *et al*. 2001; Moreno *et al*. 2003). Some genetic loci other than *Prnp* have been implicated in the pathogenesis of human prion diseases (Jackson *et al*. 2001), but have been disputed by others (Pepys *et al*. 2003).

Iatrogenic CJD amounts to one of the largest catastrophes in the history of medicine (Brown *et al*. 1992). Several hundred cases of iCJD have been reported in the past decades, most of which have been attributed to transplantation of tissues or administration of pituitary hormones derived from deceased individuals suffering from unrecognized TSEs, or – to a lesser extent – to the use of contaminated instruments in neurosurgical interventions. Infection by contaminated hormones was effectively eliminated by the replacement of natural by recombinant pituitary hormones, and yet individual patients are developing the disease even now – owing to the long incubation times involved.

Biochemical and histopathological evidence suggests that vCJD represents transmission of bovine spongiform encephalopathy (BSE) prions to humans (Aguzzi and

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Abbreviations used: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; fCJD, familial Creutzfeldt-Jakob disease; iCJD, iatrogenic Creutzfeldt-Jakob disease; MVV, Maedi-Visna virus; PET, paraffin-embedded tissue; PK, proteinase K; sCJD, sporadic Creutzfeldt-Jakob disease; TSE, transmissable spongiform encephalopathy; vCJD, variant Creutzfeldt-Jakob disease.

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Weissmann 1996; Bruce *et al*. 1997; Hill *et al*. 1997). The incidence of vCJD in the United Kingdom rose each year from 1996 to 2001 (http://www.doh.gov.uk/cjd/cjd_stat.htm), evoking fears of a large upcoming epidemic. Since then, however, the incidence of vCJD in the UK appears to be stabilizing and may actually be even falling. Hence there is substantial hope that the total number of vCJD victims will be relatively small (Valleron *et al*. 2001).

In Switzerland, CJD has been a statutory notifiable disease since December 1987. A National Reference Center for Prion Diseases was established in 1995. Between 1996 and 2000, the incidence of CJD oscillated between 1.3×10^{-6} and 1.4×10^{-6} per year. However, in 2001 and 2002 the incidence was 2.6×10^{-6} per year (Glatzel *et al*. 2002), and this level appears to have been maintained through 2003 (Glatzel *et al*. 2003b). The cause of this apparent surge in incidence is unknown: beside statistical fluctuations, TSEs of iatrogenic or zoonotic origin have been discussed. It is also plausible that an 'awareness bias' may be contributing, at least in part, to the increased CJD reporting.

Transmission of CJD by presymptomatic carriers

It must be assumed that a number of asymptomatic carriers of the disease exist in human populations that have been exposed to BSE. The existence of such a chronic carrier state is a logical consequence of the long incubation time of prion diseases; this is typically in the order of several years and, in the case of oral exposure to prions, can reach several decades. Hence anybody who has contracted the infection, but has not (yet) developed clinical signs and symptoms, may be considered a carrier. Some of these carriers may be 'preclinical' and will , in due course, develop the disease. It is also conceivable that the carrier state may persist for an indefinite period of time, in which case infected individuals could be regarded as 'permanent asymptomatic carriers'. Studies performed in rodents suggest that the permanent subclinical carrier state may be a common phenomenon, e.g. when immune deficient mice are exposed to prions (Frigg *et al*. 1999). Anonymous screens for hallmarks of prion infectivity in archival tissues have suggested that the prevalence of individuals suffering from subclinical vCJD may be higher than previously anticipated, and may possibly reach 237 cases per million individuals (Hilton *et al*. 2004).

The recent discovery of transmission of vCJD via blood in two individuals has raised concerns that blood-borne prion transmission, in conjunction with an unknown prevalence of vCJD-infected carriers, may lead to secondary transmission of host-adapted prions (Peden *et al*. 2004). This may result in a prolongation of the vCJD epidemic or, in the worst-case scenario, may render vCJD endemic and self-sustained. Here we review how prions might act as blood-borne infectious agents, and consider strategies to restrict secondary transmission of prion diseases.

Diagnosis of CJD

Clinically, patients suffering from CJD typically present with rapidly progressive cognitive decline, which may be fulminant and progress to akinetic mutism within weeks. Cerebellar signs are also very frequent and electroencephalographic recordings often visualize periodic sharp wave complexes. The definitive diagnosis of sporadic CJD, however, must usually await the analysis of central nervous tissue, bioptically or *post mortem*. 'Probable CJD' cases are diagnosed mainly on the basis of clinical symptoms when no histopathological or biochemical confirmation is available. Such 'probable CJD' cases may contaminate mortality statistics in countries that register CJD cases based on surrogate markers, including elevation of protein 14.3.3 in the cerebrospinal fluid (Hsich *et al*. 1996; Zerr *et al*. 2000).

In the case of vCJD disease, a firm diagnosis can often be obtained by the biopsy of tonsils, which have been shown to harbor significant amounts of PrP^{Sc} in germinal centers (Hill *et al*. 1999). Highly sensitive methods have revealed that at least one-third of patients with sCJD have deposits of PrP^{Sc} in skeletal muscle and/or spleen (Glatzel *et al*. 2003a). While the sensitivity of 30% is insufficient for routine diagnostics, these data open the possibility of minimally invasive diagnostics for sCJD, perhaps in combination with more sensitive methods in the future.

Magnetic resonance imaging has provided evidence of the frequent presence of hyperintensity in the posterior thalamus of vCJD patients (Zeidler *et al*. 2000). This 'pulvinar sign' was originally thought to discriminate reliably between sCJD and vCJD, but cases of sCJD with the same type of neuroradiological changes have been described (Haik *et al*. 2003; Rossetti *et al*. 2004).

Determination of the molecular weight and glycosylation patterns of PrP^{Sc} upon protease digest have established themselves as proxies for determining strains of human prions, and for differentiating vCJD from sporadic forms of the disease (Parchi *et al*. 1996; Hill *et al*. 1997). However, sophisticated analyses with state-of-the art antibodies discriminating the fragment length of protease-digested PrP^{Sc} have suggested a much more complex reality, and are questioning the current classification of human prion diseases (Polymenidou *et al*. 2005).

Genetics and the incidence of CJD

While all fCJD cases cosegregate with *Prnp* mutations, it is possible that some *Prnp* mutations cause neurodegenerative disease that is not transmissible and therefore represents a proteinopathy rather than a prion disease; many such instances have been described in the mouse (Weissmann and Flechsig 2003) and are exemplified by the 'octapeptide repeat expansion' mutants of both mouse (Chiesa *et al*.

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2003) and man (Goldfarb *et al*. 1991; Tateishi and Kitamoto 1995).

Beside disease-causing mutations, *Prnp* may also comprise polymorphisms that have a profound effect on susceptibility to prion disease. Thus, all cases of vCJD have the met/met, rather than the val/val or met/val configuration at position 129 (Collinge *et al*. 1996a; Zeidler *et al*. 1997). Moreover, humans heterozygous at this site are largely protected from CJD: this effect is so important that it may have exercised selective evolutionary pressure (Mead *et al*. 2003). A lys, rather than a glu residue at position 219 is thought to be protective against sCJD (Shibuya *et al*. 1998).

However, it is becoming increasingly apparent that genetic susceptibility markers and modifiers are not limited to polymorphisms in the PrP-encoding reading frame, as revealed by the identification of several quantitative trait loci affecting incubation time in the mouse (Stephenson *et al*. 2000; Lloyd *et al*. 2001; Manolakou *et al*. 2001; Moreno *et al*. 2003). It is presently not clear what these modifiers might be. The possible protective effect against vCJD of a certain major histocompatibility complex class-II constellation (Jackson *et al*. 2001) has been disputed (Pepys *et al*. 2003). Nonetheless, on the basis of what is known about the critical role of the immune system in peripheral prion infection (Aguzzi 2003), immunity-controlling genes are likely to feature among endogenous modifiers.

A large proportion of the British population may have been exposed to BSE infection. Further, animal experiments indicate that the infectious dose (ID_{50}) for oral cross-species transmission of BSE is as low as 500 mg of brain tissue (Foster *et al*. 1996). Considering that only approximately 150 humans have contracted vCJD, it is likely that vCJD susceptibility is controlled by endogenous and/or exogenous factors other than the amount of infectious agent ingested (Bons *et al*. 1999).

A century of TSE research

Scrapie, the prototypic prion disease affecting sheep and goat, had been a concern since the 19th century (Table 1). A crucial breakthrough was achieved in the 1930s by the experimental transmission of scrapie to goats (Cuille and Chelle 1939). Little happened in the two following decades, until Carleton Gajdusek showed that Kuru, that was decimating the aborigines of Papua New Guinea (Gajdusek and Zigas 1957), was a transmissible spongiform encephalopathy. The first attempts at transmitting Kuru to primates failed for the same reason as experimental transmission of scrapie among sheep had failed for decades: the incubation time of the disease was longer than the patience of the investigators (Schwartz 2003). Following the suggestion by William Hadlow that Kuru resembled scrapie, and hence might exhibit a very long incubation time (Hadlow 1959), Gajdusek achieved transmission of Kuru (Gajdusek *et al*. 1966, 1967) and, shortly thereafter, transmission of Creutzfeldt-Jakob disease (CJD) to chimps (Gibbs *et al*. 1968).

It is remarkable and somewhat sobering to note that some of the questions that had already been formulated in the 19th century are still unanswered. For example, is sheep scrapie a predominantly genetic or infectious disease? If the latter, how does it spread among flocks? The wildfire-like epizootic of chronic wasting disease in North American cervids (Williams and Young 1980), as well as the 'scrapie eradication plan' of the European Union (which aims at selective breeding of purportedly scrapie-resistant sheep genotypes), bears the most recent witness to the general importance of these issues.

Nature of the prion

The available data and the failure to identify a disease-specific nucleic acid support the 'protein-only' hypothesis. As stated by S. Prusiner, this hypothesis proposes that the infectious agent consists of PrP^{Sc}, that it is devoid of nucleic acid, and that its 'replication' comes about by PrP^{Sc}-mediated, autocatalytic conversion of PrP^{C} to PrP^{Sc} (Prusiner 1989). However, it is not clear that the infectious entity is PrP^{Sc}, operationally defined as a protease-resistant, aggregated form of PrP, rather than some other conformer, generically designated as PrP* (Weissmann 1991), nor has the requirement for other components been excluded. Conversion of PrP^{C} into PrP^{Sc} has been achieved (Kocisko *et al*. 1994; Caughey and Chesebro 2001; Saborio *et al*. 2001; Deleault *et al*. 2003), and two reports appear to suggest that prion infectivity has also been produced *de novo* in the test tube (Castilla *et al*. 2005a; Legname *et al*. 2005). The propagation of conformationally changed yeast proteins (yeast prions) *in vitro* and *in vivo* offers proof-in-principle of the 'protein-only' hypothesis (Wickner 1994; Aguzzi 2004; King and Diaz-Avalos 2004; Tanaka *et al*. 2004).

Digestion with 50 μg/mL of proteinase K at 37°C for two hours does not degrade the carboxy proximal domain of PrP^{Sc} (McKinley *et al*. 1983), nor decrease the infectious titer of the prion preparation – although PrP^{Sc} can be digested by more vigorous enzymatic treatment, in which case prion infectivity titers also decline. This remarkable discovery identified PrP^{Sc} as the first reliable surrogate marker of prion infection. Even now, 20 years after it was first described, the detection of proteinase K-resistant prion protein (termed PrP^{27-30} because of its molecular weight after hydrolysis of its proteinase K-sensitive amino terminal domain) remains the gold standard for biochemical diagnosis of prion diseases, and forms the basis for all of the currently marketed BSE tests.

If PrP^{Sc} multiplies by imparting its conformation onto host-borne PrP^{C}, organisms devoid of PrP^{C} should be resistant to prion infection (Büeler *et al*. 1993). *Prnp*0/0 mice are alive and well (Büeler *et al*. 1992), notwithstanding some minor abnormalities (Collinge *et al*. 1994; Tobler *et al*.

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Table 1 Essential chronology of prion research

Mid 18th century	Earliest description of scrapie recorded
1898	Neuronal vacuolation discovered in brains of scrapie-sick sheep
1918	Contagious spread of scrapie suspected under natural conditions
1920	First possible cases of CJD described (Creutzfeldt 1920; Jakob 1921).
1937	Scrapie epidemic in Scotland following administration of formalin-treated louping ill vaccine prepared from sheep brain
1939	Experimental transmission of scrapie reported (Cuille and Chelle 1939)
1955–57	Kuru discovered among Fore people of Papua New Guinea (Gajdusek and Zigas 1957)
1959	Similarities between Kuru and scrapie noted (Hadlow 1959)
1961	Multiple strains of scrapie agent described (Pattison and Millson 1961)
1961	Scrapie transmitted to mice (Chandler 1961)
1963	Transmission of Kuru to chimpanzees reported (Gajdusek *et al.* 1966)
1966	Scrapie agent found to be highly resistant to ionizing radiation and ultraviolet light (Alper *et al.* 1966; Alper *et al.* 1967)
1967	First statement of the protein-only hypothesis (Griffith 1967)
1968	CJD transmitted to chimpanzees (Gibbs *et al.* 1968)
	Description of *Sinc* gene affecting scrapie incubation period in mice (Dickinson *et al.* 1968)
1974	First documented iatrogenic prion transmission (corneal graft) (Duffy *et al.* 1974)
1980	Protease resistant, highly hydrophobic protein discovered in hamster brain fractions highly enriched for scrapie infectivity (Prusiner *et al.* 1980)
1982	Prion concept enunciated (Prusiner 1982)
1985	Gene encoding PrP^C cloned (Chesebro *et al.* 1985; Oesch *et al.* 1985)
1986	PrP^C and PrP^{Sc} isoforms shown to be encoded by same host gene (Basler *et al.* 1986)
1987	Linkage between *Prnp* and scrapie incubation period in mice (Westaway *et al.* 1987)
	First report of BSE in cattle (Wells *et al.* 1987)
1989	Mutation in PrP linked to Gerstmann–Sträussler syndrome (Hsiao *et al.* 1989)
	Importance of isologous PrP^C/PrP^{Sc} interactions established (Scott *et al.* 1989)
1992	Ablation of *Prnp* by gene targeting in mice (Büeler *et al.* 1992)
1993	*Prnp*0/0 mice are resistant to scrapie inoculation (Büeler *et al.* 1993; Sailer *et al.* 1994)
	Structural differences between PrP^C and PrP^{Sc} isoforms noted (Pan *et al.* 1993)
1994	Cell-free conversion of PrP^C to protease-resistant PrP (Kocisko *et al.* 1994)
1996	New variant of CJD identified (Will *et al.* 1996)
	BSE prion strain carries a distinct glycotype signature (Collinge *et al.* 1996b)
	First NMR structure of core murine PrP^C solved (Riek *et al.* 1996)
1997	Evidence that nvCJD is caused by the BSE agent (Bruce *et al.* 1997; Hill *et al.* 1997)
	B-lymphocytes necessary for peripheral prion pathogenesis (Klein *et al.* 1997)
1998	Genes controlling incubation period are congruent with *Prnp* (Moore *et al.* 1998)
1999	Discovery of the PrP^C homolog (Moore *et al.* 1999)
2000	Temporary depletion of lymphoid follicular dendritic cells impairs prion replication (Montrasio *et al.* 2000)
	Experimental transmission of BSE in sheep by blood transfusion (Houston *et al.* 2000)
2001	Complement involved in prion pathogenesis (Klein *et al.* 2001; Mabbott *et al.* 2001)
2003	Transgenic expression of soluble PrP inhibits prion replication (Meier *et al.* 2003)
2005	Inflammation recognized as a modified of pathogenesis (Heikenwalder *et al.* 2005; Ligios *et al.* 2005; Seeger *et al.* 2005)
	Prion detection in blood by cyclic amplification (Castilla *et al.* 2005b)

1996; Watarai *et al.* 2003), some of which may not even be causally related to the prion gene (Aguzzi and Hardt 2003).

Inoculation of *Prnp*0/0 mice with brain homogenate from scrapie-sick mice does not induce disease of any kind (Büeler *et al.* 1993), and does not lead to any subclinical replication of the agent (Sailer *et al.* 1994). The latter findings provide considerable support in favor of the protein-only hypothesis. However, they do not completely exclude that PrP^C may be a receptor for a hitherto unidentified virus, whose ablation would confer antiviral resistance. *In vitro* modifications of bacterially expressed PrP followed by intracerebral injection into transgenic mice overexpressing a mutant form of PrP has resulted in transmissible disease (Legname *et al.* 2004). The latter findings may come as close as thinkable to positive proof of the prion hypothesis, although it is not entirely clear whether the host transgenic mice may develop a spontaneous

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proteinopathy which might complicate interpretation of the data. Finally, when brain homogenate spiked with a small amount of PrP^{Sc} was subjected to repeated cycles of sonication and 'recovery', the total amount of PrP^{Sc} increased and could be used for priming another sample of brain homogenate, effectively leading to cyclic amplification of PrP^{Sc} and of prion infectivity (Castilla *et al.* 2005a, 2005b).

The availability of *Prnp*0/0 mice has triggered a cascade of technological and conceptual advances. For example, it emerged that PrP^{C}, besides controlling prion replication, is necessary for neuronal damage: *Prnp*0/0 neurons adjacent to infected *Prnp*+/+ brain grafts do not incur damage (Brandner *et al.* 1996a). PrP^{C} is also involved in the transport of the infectious agent from peripheral sites to the central nervous system: its expression appears to be needed in a sessile compartment (Blättler *et al.* 1997), which is likely to be congruent with stromal components of the lymphoreticular tissue (Montrasio *et al.* 2000) and of the peripheral nervous system (Glatzel *et al.* 2001). The microenvironment of lymphoid organs appears to control the velocity of neuroinvasion (Prinz *et al.* 2003c).

PrP^{C} is not only produced by neurons: its expression is in fact quite ubiquitous, notably including lymphocytes (Cashman *et al.* 1990) and stromal cells of lymphoid organs (Kitamoto *et al.* 1991). As a result, wild-type mice enjoy an extremely tight immunological tolerance to PrP^{C}, which rendered the production of high-affinity immunoreagents very difficult. Instead, the immunization of *Prnp*0/0 mice yielded large numbers of very high affinity antibodies, some of which form the basis for the current crop of BSE tests.

It has still proved difficult to generate conformational antibodies that discriminate between PrP^{C} and PrP^{Sc}. This is surprising in view of the dramatic structural differences between these two isoforms and their differential binding to serum proteins (Fischer *et al.* 2000). Perhaps the failure of the immune system to generate antibodies specific for PrP^{Sc} indicates that all relevant neoepitopes of PrP^{Sc} newly exposed by the conversion of the protein to its disease-associated state are inaccessible. An antibody was raised against a tripeptide (YYR) reported to be exposed in PrP^{Sc} but not in PrP^{C} (Paramithiotis *et al.* 2003). However, the YYR motif is certainly not specific to PrP^{Sc}, and others have reported that a YYR epitope may be exposed also in PrP^{C} (reported by Dr J. Grassi at the Prion international conference in Dusseldorf in 2005).

A different approach consists of detecting epitopes which undergo masking upon conversion to PrP^{Sc}, rather than attempting to discover neoepitopes. Given that a sizeable proportion of PrP^{Sc} molecules appear to exist as aggregates, it is plausible to expect that at least some epitopes will be buried in the aggregates. This principle underlies the 'conformation-dependent immunoassay' (Safar *et al.* 1998), which highlights enhanced PrP immunoreactivity upon deaggregation and denaturation of PrP^{Sc}.

Extraneural PrP^{Sc}

Refinements in the technologies for detection of PrP^{Sc} have prompted a renaissance of studies of the distribution of the disease-associated prion protein in extracerebral organs of patients. These studies revealed that extraneural PrP^{Sc} is more widespread than previously thought. Zanusso and colleagues found that PrP^{Sc} is readily detectable in the olfactory mucosa of sCJD victims (Zanusso *et al.* 2003). Glatzel and colleagues have found that approximately one-third of the Swiss sCJD patients display PrP^{Sc} in their skeletal muscle and another third (partially overlapping) had PrP^{Sc} in lymphoid organs (Glatzel *et al.* 2003a). Further investigations are underway to determine whether these findings are universally valid for CJD patients, or are a specific characteristic of the Swiss CJD collective. If the latter were true, one might speculate that the abnormal peripheral pathogenesis of CJD in Swiss patients points to a specific etiology.

The UK vCJD cases are likely to be primary transmissions from cattle BSE. However, experimental transmission studies show that TSE strain characteristics can change upon serial passages after the original primary transmission (Asante *et al.* 2002). Therefore, horizontal vCJD transmission amongst humans could result in a different phenotype than vCJD. This scenario calls for innovative studies aimed at developing and validating classical and emerging, up-to-date prion strain typing tools.

Pathogenic mechanisms in prion diseases

The damage wrought by prions is mainly evident in the central nervous system, although pathological changes in the spleen of non-human primates have also been noted (C. Lasmezas, personal communication). Because PrP^{Sc} accumulates in the central nervous system and in some instances is deposited as an amyloid, it is has been indicted as the toxic entity causing neuronal apoptosis and eliciting disease. The finding that peptides derived from the PrP region 106–126 form aggregates and are toxic to cultured neuronal cells (Forloni *et al.* 1993; Brown *et al.* 1996) has been adduced in support of this contention, although there has been some dispute as to the reproducibility of the phenomenon (Kunz *et al.* 1999). It is not, however, evident that the pathogenicity of the oligomerized peptides on cultured cells mimic the properties of PrP^{Sc} accumulating in the CNS.

PrP^{Sc} produced by a prion-infected, PrP-expressing neuronal graft in the brain of PrP knockout mouse did not cause disease or result in damage to neighboring neuronal tissue devoid of PrP (Brandner *et al.* 1996a). In addition, prion-infected mice carrying only a single PrP allele and producing

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half the wild-type level of PrP do not exhibit disease until about 450 days after i.c. inoculation, in contrast to 150 days in wild-type mice, although they have accumulated levels of PrP^{Sc} similar to those of wild-type animals 150 days after infection (Bueler *et al.* 1994). Therefore, PrP^{Sc} is likely to be responsible for CNS pathology only in neurons that express PrP^{C}.

A toxic function gain by a PrP moiety that is different from PrP^{Sc} is a distinct possibility. Over several years, a lively debate has unfolded on the role of abnormal PrP^{C} topologies. Targeting of PrP to the cytosol was reported to result in rapidly lethal neurodegeneration (albeit without accumulation of PrP^{Sc}), and proteasome inhibition induces a slightly protease-resistant, cytoplasmic PrP species in cultured cells (Ma and Lindquist 2002; Ma *et al.* 2002). Therefore, prion toxicity was proposed to start with retrotranslocation of PrP^{C} from the endoplasmic reticulum to the cytosol, in conjunction with impaired proteasomal function. However, others have found that cytosolic PrP retains its secretory leader peptide and does not contain a glycosyl phosphatidyl inositol anchor, suggesting that it never enters the endoplasmic reticulum (Drisaldi *et al.* 2003). Moreover, the toxicity of cytosolic PrP has been contested (Heller *et al.* 2003; Roucou *et al.* 2003). Lingappa found that PrP^{C} assumes a transmembrane topology (^{Ctm}PrP), whose concentration correlates with neurotoxicity (Hegde *et al.* 1998,1999). These data have been taken to suggest that ^{Ctm}PrP represents a major toxic moiety.

From the above it becomes apparent that further work is needed to understand the role of alternative PrP topologies in prion neurotoxicity. Moreover, the biochemical pathways leading to pathogenicity, whether triggered by PrP^{Sc}, cytoplasmic PrP or ^{Ctm}PrP, are still obscure.

Spread of prions

Prion pathogenesis can be broken down into spatially and temporally distinct phases: (i) infection and peripheral replication; (ii) migration from the periphery to the CNS (neuroinvasion), and (iii) neurodegeneration. The resistance to prions of mice that lack PrP^{C} expression is amply documented (Büeler *et al.* 1993; Prusiner *et al.* 1993; Manson *et al.* 1994; Brandner *et al.* 1996a. PrP^{C} expression is required for transporting the infectious agent from the peripheral sites to the CNS (as monitored by PrP^{C}-expressing neurografts) (Blättler *et al.* 1997) and within the CNS (Brandner *et al.* 1996b). However, reconstitution of *Prnp*0/0 mice with wild-type bone marrow is insufficient to restore neuroinvasion in engrafted *Prnp*0/0 mice (Blättler *et al.* 1997), although the capacity of the spleen to accumulate prions of the RML strain is reconstituted (Blättler *et al.* 1997; Kaeser *et al.* 2001). This suggests that hematopoietic cells transport prions from the entry site to the lymphoreticular system, which accumulates and replicates prions, but that PrP^{C} expression is required in an additional compartment, presumably the peripheral nervous system. B-lymphocytes (not necessarily expressing PrP^{C}) are crucial for peripheral prion spread and neuroinvasion (Klein *et al.* 1997, 1998).

The dependence on lymphotoxin-mediated signaling by B-cells may explain, at least in part, the requirement for B-cells in peripheral pathogenesis. Follicular dendritic cells accumulate PrP^{Sc} following scrapie infection (Kitamoto *et al.* 1991), and maturation of the cells requires signaling by B cells expressing lymphotoxin α/lymphotoxin β trimers on their surface. Indeed, blockade of lymphotoxin-β signaling via administration of soluble lymphotoxin βR-Ig ablates mature follicular dendritic cells and significantly impairs neuroinvasion and accumulation of peripheral PrP^{Sc} and infectivity (Montrasio *et al.* 2000; Mabbott *et al.* 2001). Follicular dendritic cells are crucial to disease progression after oral scrapie challenge, but only within a short time window (Mabbott *et al.* 2002, 2003).

Follicular dendritic cells play a role in antigen trapping and in binding opsonized antigens to the CD21/CD35 complement receptors. Two studies have demonstrated that the complement system is relevant to prion pathogenesis. Mice genetically engineered to lack complement factors (Klein *et al.* 2001) or mice depleted of the C3 complement component (Mabbott *et al.* 2001) exhibited enhanced resistance to peripheral prion inoculation. Because follicular dendritic cells are probably immobile cells, they are unlikely to be responsible for prion transport into the CNS.

But just which cell types are involved in neuroinvasion? The innervation pattern of lymphoid organs is primarily sympathetic (Felten and Felten 1988). Sympathectomy delays the onset of scrapie, while sympathetic hyperinnervation enhances splenic prion replication and neuroinvasion, suggesting that innervation of secondary lymphoid organs is the rate-limiting step to neuroinvasion (Glatzel *et al.* 2001). Although there is no physical contact between follicular dendritic cells and sympathetic nerve endings (Heinen *et al.* 1995), the distance between the cells and splenic nerves affects the velocity of neuroinvasion (Prinz *et al.* 2003c). It remains to be determined whether this results from passive diffusion of prions or whether mobile cells (e.g. germinal center B-cells) are involved in an active transport process.

Under normal circumstances, B-lymphocytes are mainly localized to the B-cell areas of lymphoid follicles in spleen, lymph nodes, tonsils, Peyer's patches, and other lymphoreticular organs. However, in chronic inflammatory conditions B-lymphocytes can extravasate and colonize the site of inflammation. It was therefore of interest to ask what would happen to the host if a prion infection is superimposed to a chronic inflammatory condition. The latter scenario was studied in five distinct models of inflammation and in three organ systems: liver, pancreas, and kidney. In all cases PrP^{Sc} and prions were found to progressively accumulate at the site of inflammation, often reaching levels similar to those found in lymphoreticular organs (Heikenwalder *et al.* 2005).

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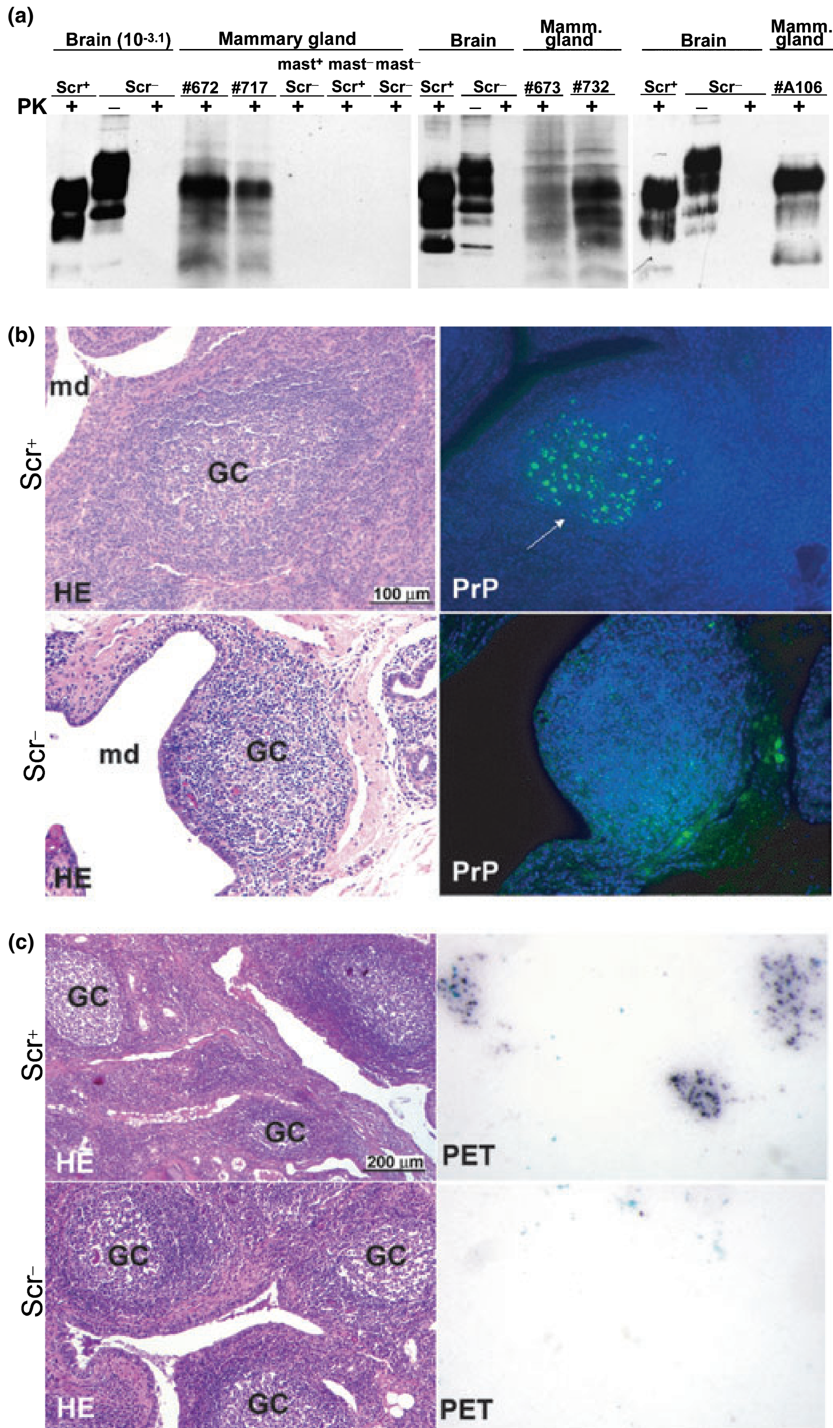

Fig. 1 Inflamed mammary glands of scrapie-infected sheep accumulate PrP^{Sc}. (a) Western blots with a PrP-specific antibody. Lanes 1–3, 9–11, 14–16 from left: native and proteinase K (PK)-digested brain homogenates (diluted 1400-fold) from a scrapie-infected (Scr^+) and a scrapie-free sheep (Scr^-). Lanes 6–8: mammary glands from a scrapie-free sheep with follicular mastitis (Scr^-, $mast^+$), a scrapie-positive sheep from a flock with neither MVV seropositivity nor mastitis (Scr^+, $mast^-$), and a sheep with neither mastitis nor scrapie (Scr^-, $mast^-$). Each one of five scrapie-infected sheep with mastitis displayed mammary PrP^{Sc} (Lanes 4, 5, 12, 13, and 17). Non-scrapie-infected brain and mammary gland extracts showed no PrP^{Sc} upon proteinase K digestion (Lanes 3, 6–8, 11, and 16). (b) Mammary gland micrographs from MVV-seropositive sheep with mastitis and coincident scrapie (Scr^+), or with mastitis but no scrapie (Scr^-). Lymphoid follicles are adjacent to milk ducts (md). Immunofluorescence stains reveal abundant PrP deposits within mammary lymphoid follicles (arrow) from scrapie-positive, but not from scrapie-free, sheep. GC, germinal center (the area including follicular dendritic cells). Scale bars: 100 µm. (c) Proteinase K-treated paraffin-embedded tissue (PET) blots of mammary gland sections reveal punctate PrP^{Sc} deposits colocalizing with lymphoid follicles in scrapie-infected (Scr^+), but not in scrapie-free (Scr^-) sheep with mastitis. Scale bars: 200 µm.

The above considerations suggest that the spectrum of affected organs in prion diseases may be modulated by chronic inflammation. If this were true for farm animals, one might have to revise the current concept of 'specified ruminant offals' (specified risk material), i.e. those organs that carry a high prion burden. For the past 15 years it was thought that such organs would encompass, in the cow, essentially only the central nervous and (to some extent) the lymphoreticular system. Given the findings explained above, I deem it reasonable to contemplate the possibility that superimposed pathologies may expand the specified risk material concept to sites of inflammation.

We investigated this question in a flock of sheep held in the Sassari region of Sardinia, Italy. Of 818 sheep, seven

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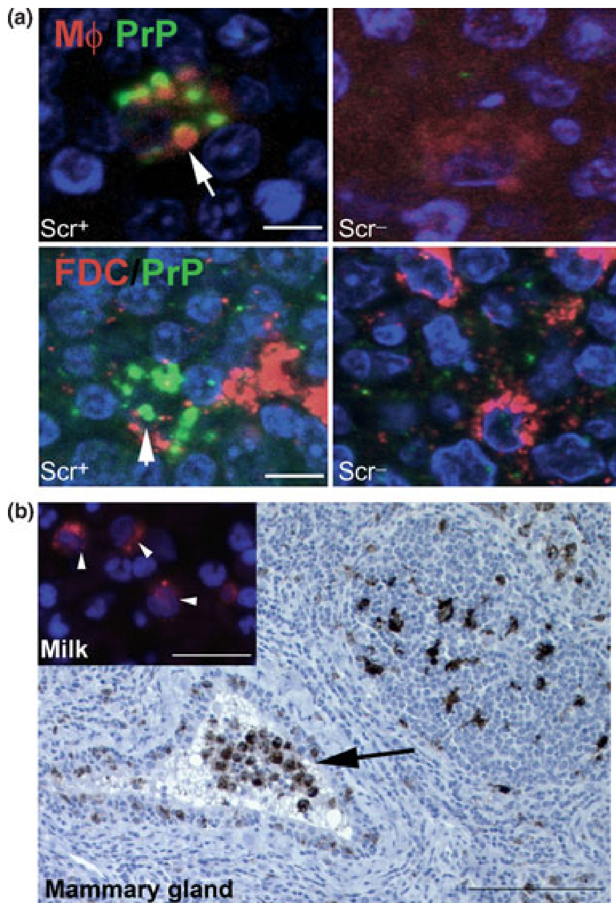

Fig. 2 Mammary PrP^{Sc} localizes to macrophages and follicular dendritic cells. (a) Mammary gland from a sheep with coincident mastitis, MVV seropositivity, and scrapie (#732). Confocal laser scanning micrographs of lymphoid follicles immunostained for PrP (green), macrophages (M, red) or follicular dendritic cells (red), and nuclear DNA (blue). PrP^{Sc} associates with $CD68^+$ M and follicular dendritic cells in scrapie-positive (Scr^+, arrows) but not in scrapie-free sheep (Scr^-). Scale bars: 6.3 μm (top) or 7.5 μm (bottom). (b) $CD68^+$ macrophages (arrow) and degenerating leukocytes within milk ducts and in adjacent lymphoid follicles of an inflamed mammary gland, as well as in milk sediment (inset, arrowhead). Scale bar: 100 μm (Mammary gland) or 20 μm (Milk cells).

had clinically overt scrapie with PrP^{Sc} in brain, lymph nodes, and tonsil. All scrapie-sick sheep and 100 randomly chosen healthy sheep were killed, and mammary glands were analyzed histologically. Ten sheep had lymphocytic mastitis, and four had coincident mastitis and scrapie. Using western blots, immunohistochemistry, and histoblots, we detected PrP^{Sc} in mammary glands of all four clinically scrapie-sick sheep with mastitis (Figs 1a,b), but neither in non-inflamed mammary glands from presymptomatic or scrapie-sick sheep from the same or a different flock, nor in inflamed mammary glands of scrapie-uninfected sheep. Within the inflammatory mammary lesions, PrP^{Sc} was found to be associated with lymphoid follicles by immunofluorescent labeling and by paraffin-embedded tissue blotting (Fig. 1c). PrP^{Sc} colocalized predominantly with $CD68^+$ macrophages and follicular dendritic cells within inflamed mammary glands (Fig. 2a).

We then surveyed a second Sarda flock (272 sheep) located 30 km away from the flock described above. One sheep was found to be scrapie-sick and was killed: necropsy revealed lymphofollicular mastitis and PrP^{Sc} in the brain and tonsil. Again, PrP^{Sc} was present in the mammary gland (Fig. 1a). These results indicate that coincidence of natural chronic inflammatory conditions and natural scrapie can expand the deposition of PrP^{Sc} to unexpected tissues of sheep (Ligios *et al*. 2005).

Common causes of lymphofollicular mastitis in sheep include Maedi-Visna virus (MVV) and *Mycoplasma* (Pepin *et al*. 1998). No *Mycoplasma* could be cultured from mastitic glands, whereas four of the five sheep with scrapie and mastitis were found to be MVV-seropositive, and the three scrapie-sick sheep without mastitis were MVV-seronegative. In the clinically healthy group, seven of 10 sheep with mastitis, but only 32 of 90 sheep without mastitis, were MVV-seropositive. Hence, MVV seropositivity correlated with lymphoid follicular mastitis (Fisher's exact test, $p = 0.01$) as reported previously (Peterhans *et al*. 2004; de Andres *et al*. 2005).

MVV and related small-ruminant lentiviruses are endemic in most, if not all, European small ruminant populations (Peterhans *et al*. 2004). The above data suggest that common viral infections of small ruminants may enhance the spread of prions. MVV is found within mammary epithelial cells and macrophages (Carrozza *et al*. 2003), and has been experimentally passed to lambs via milk (Preziuso *et al*. 2004). Milk is believed to represent a major route of transmission for the natural spread of MVV. The PrP deposits in $CD68^+$ cells of mammary lymphoid follicles, in concert with the copious shedding of macrophages into milk of mastitic sheep (Fig. 2b) (Lerondelle and Ouzrout 1990; Preziuso *et al*. 2004), raises the question whether coexistence of prion infection and inflammation in secretory organs may lead to prion contamination of secretions, and may represent a cofactor for horizontal prion spread within flocks.

As the kidney is an excretory organ, it was inevitable to that the question whether nephritis would lead to excretion of prions into the urine ('prionuria') would be raised. This was indeed found to be the case (Seeger *et al*. 2005) in mice suffering from lymphocytic nephritis (Fig. 3). Interestingly, isolated glomerulonephritis without lymphofollicular involvement, as in mice deficient for the milk fat globule-epidermal growth factor-like protein 8, did not lead to prionuria. These accrued data suggest that prion shedding by inflamed secretory and excretory organs may represent a relevant exogenous cofactor that modifies the spread of prions in populations.

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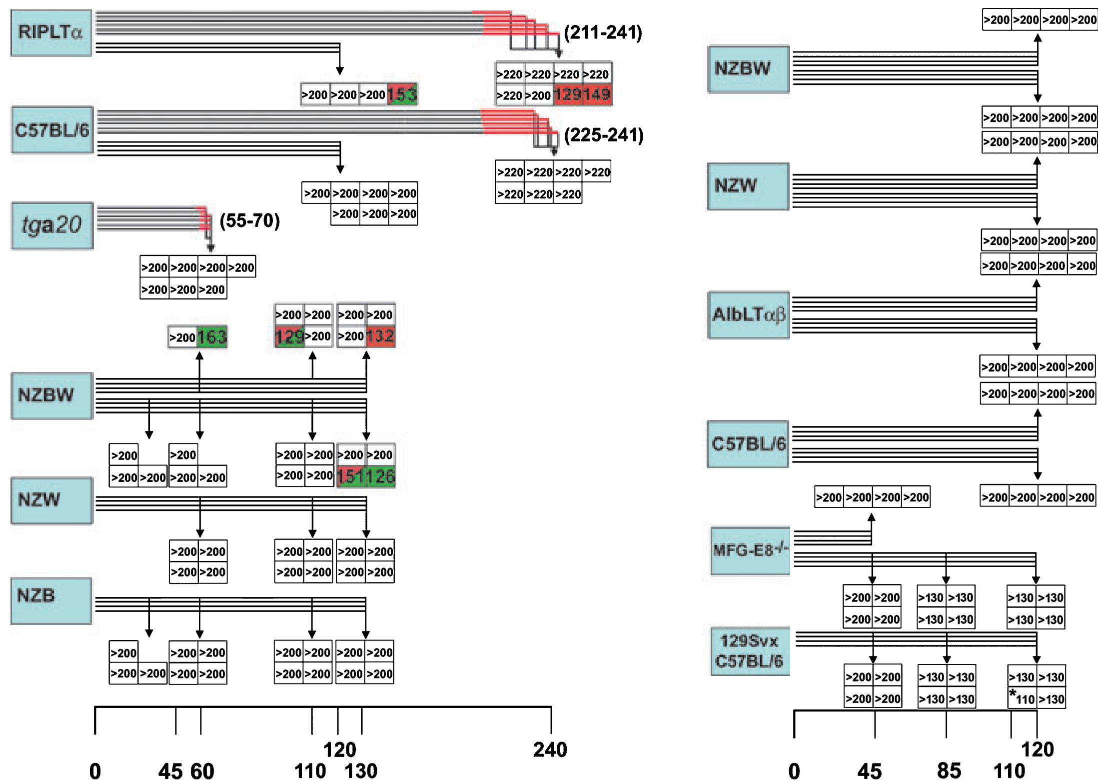

Fig. 3 Transmission of prions through urine. Urine samples were collected from individual donors (horizontal lines) at time points denoted by vertical lines, and pooled (intersections between lines, arrows). Squares represent individual *tga20* indicator mice inoculated i.c. with urinary proteins. White squares: no scrapie symptoms; red squares: histopathologically confirmed scrapie; green squares: positive PrP^{Sc} immunoblot. Numbers within squares: days to terminal disease. Clinical disease: red line. Prion incubation time is expressed in days. Asterisk: intercurrent death without clinical scrapie signs. RIPLT and NZBW mice, which develop progressive lymphofollicular nephritis, shed prions in their urine. Instead, the urine of MFGE8 knockout mice, which suffer from glomerulonephritis without lymphocytic inflammation, is devoid of prions. Overexpression of PrP^C does not suffice to induce prionuria, as prion-infected tga20 mice do not shed prions in their urine.

Active and passive vaccination

It was reported early that anti-PrP antiserum reduces the titer of infectious hamster brain homogenates some hundred-fold (Gabizon *et al.* 1988). Anti-PrP antibodies were found to inhibit formation of protease-resistant PrP in a cell-free system (Horiuchi and Caughey 1999). Also, antibodies (Enari *et al.* 2001; Perrier *et al.* 2004) and antigen binding fragments directed against PrP (Peretz *et al.* 2001) can suppress prion replication in cultured cells.

These data suggest the feasibility of antiprion immunoprophylaxis, which could be implemented as passive immunization (transfer of antibodies) or active immunization (administration of antigens as vaccines). Active immunization is generally more effective, but it proved exceedingly difficult to elicit humoral immune responses because the mammalian immune system is largely tolerant to PrP of the same species. Mice devoid of PrP (Büeler *et al.* 1992) show no tolerance and are highly susceptible to immunization with recombinant PrP (Prusiner *et al.* 1993) or PrP^C-expressing cells (Brandner *et al.* 1996b).

Tolerance is typically brought about by activation-induced cell death, which is incurred by B- or T-lymphocytes undergoing very strong cross-linking of their antigen receptors. To determine whether the resilience of wild-type mice to antiprion immunization is attributable to the T- or B-cell compartment, transgenic mice were generated that expressed an immunoglobulin/B-cell receptor μ chain containing the epitope-interacting region of 6H4, a high-affinity anti-PrP monoclonal antibody (Korth *et al.* 1997). The transgenic μ chain associated with endogenous κ and λ chains; some pairings lead to reactive moieties and, consequently, to anti-PrP^C titers in *Prnp*0/0 and *Prnp*+/+ mice. The buildup of anti-PrP^C titers, however, was more sluggish in the presence of endogenous PrP^C, suggesting that clonal deletion was actually occurring. B-cell clones with the highest affinity to PrP^C are probably eliminated by tolerance, whereas clones with medium affinity are retained. The latter sufficed to block prion pathogenesis upon intraperitoneal prion inoculation (Heppner *et al.* 2001). Hence, B cells are not intrinsically tolerant to PrP^C, and can – in principle – mount a protective humoral response against prions. These results were later

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confirmed by others in a passive-immunization study of mice (White *et al*. 2003).

The challenges to practical antiprion immunization, however, are enormous. While providing an encouraging proof of principle, transgenic immunization cannot easily be reduced to practice. Passive immunization failed to confer protection if treatment was started after the onset of clinical symptoms, suggesting that it might be a better candidate for prophylaxis rather than for therapy of TSEs. Active immunization, as with most antiviral vaccines, may be more effective, but is rendered exceedingly difficult by the stringent tolerance to PrP^C (Souan *et al*. 2001; Polymenidou *et al*. 2004).

A recent report has outlined a potentially serious obstacle to prion immunotherapy. Intracerebral injection of anti-PrP antibodies specific to certain epitopes at high concentrations provoked degeneration of hippocampal and cerebellar neurons (Solforosi *et al*. 2004). Since monovalent antigen binding fragments did not elicit these responses, it is likely that crosslinking of PrP^C by bivalent IgG antibodies is neurotoxic *in vivo* – perhaps by eliciting some deleterious signaling event. While these results are a cautionary note on the prospect of using antibodies against clinically overt prion diseases, it is possible that anti-PrP antigen binding fragments are capable of reducing infectious titers (Peretz *et al*. 2001) without exerting a toxic effect (Solforosi *et al*. 2004). Moreover, extraneural antibody administration may be useful for immunoprophylaxis of prion infections at early stages, before the agent reaches the brain.

Immunostimulation and antiprion prophylaxis

Cytidyl-guanyl oligodeoxynucleotides, which bind Toll-like receptor 9 and stimulate innate immune responses, were reported to delay disease upon chronic administration to scrapie-infected mice (Sethi *et al*. 2002). The contention that immune stimulation might protect against prions is difficult to reconcile with the observation that immune deficiencies of all kinds inhibit prion spread (Klein *et al*. 1997, 1998, 2001; Frigg *et al*. 1999; Prinz *et al*. 2003b). Besides, MyD88–/– mice undergo normal prion pathogenesis despite abrogation of Toll-like receptor 9 signaling (Prinz *et al*. 2003a). Hence, more detailed studies will be needed to understand the basis of the antiprion effect of cytidyl-guanyl oligodeoxynucleotides. The realization that repeated administration of cytidyl-guanyl oligodeoxynucleotides can derange the architecture of lymphoid germinal centers, which are sites of prion replication, suggests that the antiprion effect of these compounds may rely on their immunosuppressive rather than their immunostimulatory properties (Heikenwalder *et al*. 2004).

Concluding remarks

For the past 10 years, prions have been in the public limelight as the causative agent of 'mad cow disease'. This tremendous publicity has influenced political agendas, attracted large amounts of research funds, and motivated many researchers to enter the prion field. In the past 2 years, however, prions have all but disappeared from the public perception, mainly due to a – possibly premature – perception that BSE has been defeated. From a scientific viewpoint, however, the prion problem is enigmatic as ever, despite all the progress summarized in this review article. The precise physicochemical nature of the agent is unknown, the process of prion replication is essentially a black box, the phenomena underlying the various strains of prions are not understood, and the function of the normal prion protein is utterly unclear. Although some of these questions may be resolved in the near future, others – including the most basic characteristics of prions – may need to await the development of novel technologies in imaging and in structural biology for their resolution. Exciting times lie ahead for scientists wishing to enter the prion field!

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