Prions and the lymphoreticular system

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Following intracerebral or peripheral inoculation of mice with scrapie prions, infectivity accumulates first in the spleen and only later in the brain. In the spleen of scrapie-infected mice, prions were found in association with T and B lymphocytes and to a somewhat lesser degree with the stroma, which contains the follicular dendritic cells (FDCs) but not with non-B, non-T cells; strikingly, no infectivity was found in lymphocytes from blood of the same mice. Transgenic PrP knockout mice expressing PrP restricted to either B or T lymphocytes show no prion replication in the lymphoreticular system. Therefore, splenic lymphocytes either acquire prions from another source or replicate them in dependency on other PrP-expressing cells. The essential role of FDCs in prion replication in spleen was shown by treating mice with soluble lymphotoxin- β receptor, which led to disappearance of mature FDCs from the spleen and concomitantly abolished splenic prion accumulation and retarded neuroinvasion following intraperitoneal scrapie inoculation.

Keywords: ectopic expression; PrP; follicular dendritic cells; lymphotoxin; B lymphocytes

1. INTRODUCTION

Prion diseases manifest themselves mainly by pathological changes in the central nervous system (CNS) and the ensuing clinical symptoms. Experimentally, the infectious agent is most efficiently transmitted by intracerebral (IC) inoculation. However, under field conditions prion diseases such as sheep scrapie, kuru and bovine spongiform encephalopathy (BSE) are believed to be mainly acquired through the gastrointestinal tract (Anderson *et al.* 1996; Wilesmith 1994; Will *et al.* 1996); the role of maternal or other modes of transmission is still not clear. Intraperitoneal (IP) inoculation of mice probably mimics the natural infection process more closely than IC inoculation.

In many animals, in particular the mouse, initial events following scrapie infection, whether IC or IP, take place in the lymphoreticular system (LRS) and precede neuroinvasion and replication of the agent in the brain (Büeler *et al.* 1993; Eklund *et al.* 1967; Fraser & Dickinson 1970). The host fails to mount a classical immune response to the scrapie agent (Kasper *et al.* 1982) presumably because it consists of a host-derived protein.

Studies involving splenectomy (Fraser & Dickinson 1978), whole body irradiation (Fraser & Farquhar 1987) and spleen fractionation (Clarke & Kimberlin 1984) suggested that mitotically quiescent cells located in the stromal fraction of the spleen are involved in propagation of the scrapie agent in this organ. Immunohistochemical studies showed that the protease-resistant isoform of PrP co-localizes with follicular dendritic cells (FDCs), a non-

lymphoid component, in the spleen of prion-infected mice (Kitamoto *et al.* 1991; Klein *et al.* 1998; McBride *et al.* 1992). As recently shown, irradiation of wild-type mice and reconstitution with PrP knockout (*Prnp*^{0/0}) bone marrow, but not irradiation of knockout mice and reconstitution with wild-type bone marrow, allowed prion accumulation in the spleen (Brown *et al.* 1999). These findings all implicate FDCs in the formation of prions in spleen.

Efficient transport of prions from the periphery to the CNS depends on elements of the LRS, as evidenced by the fact that not only accumulation of prions in the spleen but also development of CNS disease after IP inoculation with scrapie is impaired in mice with various forms of immunodeficiency, such as in SCID, RAG^{-/-} or μ MT mice, while IC inoculation continues to be fully effective (Brown *et al.* 1997; Fraser *et al.* 1996; Kitamoto *et al.* 1991; Klein *et al.* 1997; Lasmezas *et al.* 1996). Although these studies strongly suggest an important role for lymphoid cells, be it direct or indirect, there is also evidence for an LRS-independent, albeit less efficient, neural spread of the disease from the periphery to the CNS in the spread of the disease from peripheral sites to the brain (Beekes *et al.* 1996, 1998; Kimberlin & Walker 1986, 1989a,b; Lasmezas *et al.* 1996).

2. LOCALIZATION OF PRIONS IN THE SPLEEN

Little is known about the relationship between prion replication and lymphocytes. Early work on splenocytes from mice infected with Creutzfeldt-Jakob disease (CJD) prions suggested low-density lymphocytes as preferred targets for agent replication. *In vitro* stimulation of B and T cells with mitogens increased susceptibility to CJD

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Figure 1. Schematic representation of PrP transgenes driven by heterologous promoters. The genomic mouse *Prnp* locus is shown on top. Construction of the 'half-genomic' PrP vector (phgPrP) lacking the 12 kb intron 2 has been described (Fischer *et al.* 1996). Promoter cassettes were inserted into the promoterless vector pPrP-5'HG to yield plck-PrP-5'HG and pEμ/IRF1-PrP-5'HG (Raeber *et al.* 1999b). The construct for B-cell-specific expression, pIg-PrP, contained a PrP cDNA driven by the immunoglobulin κ (Igκ) light chain promoter–enhancer (Montrasio 1999).

Table 1. *Infectivity of spleen fractions and peripheral blood lymphocytes*

(Mice were inoculated intraperitoneally and culled after 120 days. Spleen fractions and peripheral blood lymphocytes were prepared and assayed as described (Raeber *et al.* 1999a). Tg94 is a line of PrP knockout mice overexpressing PrP transgenes under the control of a spleen-specific promoter (Raeber *et al.* 1999b).)

tissue	titre (log LD ₅₀ units spleen ⁻¹)	
	wild-type	tg94
spleen		
total splenocytes	6.0	5.5
splenic B lymphocytes	5.6	5.0
splenic T lymphocytes	5.2	4.0
splenic non-B, non-T cells	< 2	< 2
stroma	4.3	3.6
peripheral blood lymphocytes	< 2	< 2

infection (Kuroda *et al.* 1983). Similarly, mitogenic activation of the host enhanced susceptibility to scrapie infection, while immunosuppressive treatment of mice reduced susceptibility to scrapie infection (Dickinson *et al.* 1978).

To determine which cells in the spleen carry prions, fractionated splenocytes from scrapie-infected mice were assayed for infectivity. B and T cells were purified from the pulp fraction by magnetic-activated cell sorting (MACS), followed by complement lysis of B cells in the T-cell fraction and vice versa. Finally, intact cells were isolated by density gradient centrifugation. This procedure led consistently to more than 99% pure T- and B-lymphocyte preparations in a yield of 5–10%. In addition, a non-B-, non-T-cell population was obtained by depleting splenocytes of B and T lymphocytes by MACS; this fraction contained ≤2% T, but no detectable B, lymphocytes (Raeber *et al.* 1999a). The cell preparations and the stroma fraction were analysed for infectivity by end-point titration (Brandner *et al.* 1996; Fischer *et al.* 1996) (table 1). Total splenocytes had *ca.* 3.5 log LD₅₀ units per 10⁶ cells and both B and T cells showed essentially the same titres. Strikingly, the non-B-, non-T-cell population contained only *ca.* 1 log LD₅₀ unit per 10⁶ cells, probably due to the ≤2% contamination by T lymphocytes. Inasmuch as the purified B and T cells were representative of their class as regards infectivity, *ca.* 6 log LD₅₀ units of infectivity were associated with the pulp and *ca.* 4.3 log LD₅₀ units with the stroma of one spleen (Raeber *et al.* 1999a). Clarke & Kimberlin (1984)

Figure 2. Analysis of PrP expression by cytofluorometry (FACS). (a) Splenocytes, (b) thymocytes and (c) peripheral blood leucocytes (PBL) from *Prnp*^{+/+}, *Prnp*^{0/0}, Tg94/IRF and Tg33/lck mice were gated for lymphocytes. Cells were stained with anti-PrP polyclonal antiserum R340 and phycoerythrin-conjugated anti-rabbit IgG and analysed by FACS gated for lymphocytes. For two-colour FACS analysis (a) PrP staining was followed by B-cell staining with fluorescein isothiocyanate (FITC-conjugated anti-B220 antibodies or T-cell staining with FITC-conjugated anti-CD3 antibodies. (From Raeber *et al.* 1999a.)

reported about equal distribution of infectivity between pulp and stroma; however, their data were obtained with different mouse and scrapie strains than ours.

These findings suggested that lymphocytes might be responsible for spreading prions through the organism. We therefore determined the infectivity of peripheral blood leucocytes (PBLs) from the same animals whose spleens had been analysed. Surprisingly, despite the relatively high infectivity associated with splenic B and T cells, intracerebral inoculation of 10^6 PBLs from buffy coat did not cause disease in any of four indicator mice (Raeber *et al.* 1999a). Because *ca.* 80% of murine PBLs are lymphocytes (Binder *et al.* 1997), less than 1 LD₅₀ unit of infectivity was associated with 8×10^5 lymphocytes; the specific infectivity of peripheral lymphocytes was therefore at least 2000–3000 times less than that of splenic lymphocytes.

Why is infectivity associated with splenic but not with circulating lymphocytes? Perhaps only distinct subsets of splenic lymphocytes carry infectivity and these do not enter the bloodstream, or perhaps prion-carrying splenic lymphocytes have a short half-life.

3. SUSCEPTIBILITY TO PRIONS OF PrP KNOCKOUT MICE OVEREXPRESSING PrP IN VARIOUS COMPARTMENTS OF THE LYMPHORETICULAR SYSTEM

Are the prions associated with splenic lymphocytes synthesized *de novo* by these cells or are they acquired from other sources?

To investigate this issue, we targeted PrP expression to three different cell types or tissues in PrP null mice: the LRS (Raeber *et al.* 1999a), T lymphocytes (Raeber *et al.* 1999a) and B lymphocytes (Montrasio 1999) (figure 1).

(a) PrP knockout mice overexpressing PrP in spleen

To express PrP ectopically in the LRS we placed the PrP coding sequence under the control of the IRF1-promoter/immunoglobulin heavy chain enhancer (E_{μ}), which had been shown to lead to overexpression of a linked cDNA in thymus, spleen and bone marrow (Yamada *et al.* 1991). As shown by Western analysis, a transgenic *Prnp*^{0/0} mouse line carrying this construct (Tg94/IRF) expressed PrP in the spleen at a 1000-fold higher level than wild-type. PrP expression in Tg94/IRF brain was 20 times less than in spleen, in contrast to wild-type mice, where PrP levels in brain exceed those in spleen *ca.* 100-fold (Raeber *et al.* 1999b). PrP on the surface of peripheral blood leucocytes, as determined by cytofluorometry (FACS), was around tenfold higher in Tg94/IRF mice than in wild-type mice (figure 2c). High levels of PrP were also observed on B and T splenic lymphocytes of Tg94/IRF mice (figure 2a) (Raeber *et al.* 1999b).

In wild-type spleens, PrP was mainly present in germinal centres, while in Tg94/IRF spleens it was uniformly distributed over white and red pulp (figure 3a(ii,vii)). In figure 3b consecutive spleen sections were labelled with the FDC-specific antibody M1 (green) and PrP antiserum (red), again revealing a striking overlap of FDC and PrP staining within germinal centres in wild-type spleens (figure 3b(x,xi)). In Tg94/IRF spleens, FDCs were stained in the germinal centres, whereas PrP-specific fluorescence was uniform over the whole section (figure 3b(i,ii)), which is compatible with the FACS analysis showing that B and T lymphocytes expressed PrP and with the assumption that FDCs also expressed PrP (Raeber *et al.* 1999b).

Wild-type and Tg94/IRF mice hemizygous for the transgene cluster were inoculated with Rocky Mountain Laboratory prions by the IP route. Two weeks after inoculation, spleen extracts from Tg94/IRF mice and wild-type animals had the same titre, *ca.* 7 log LD₅₀ units ml⁻¹ 10% homogenate, and no infectivity was detected in brain. Six months after inoculation, the titres of Tg94/IRF spleen extracts were essentially unchanged, somewhat higher than the value of 6.5 for wild-type spleen, and no infectivity was detected in Tg94/IRF brains, as compared with

Figure 3. Analysis of PrP expression by immunohistochemistry. (a) Double immunofluorescence analysis of splenic germinal centres in non-inoculated Tg94/IRF (i–iv), wild-type mice (v–viii), and *Prnp*^{0/0} mice (ix–xii). Sections were stained with haemalaun (i, v, ix), with peanut agglutinin (PNA) (green; ii, vi, x), and with antiserum R340 to PrP (red; iii, vii, xi). The majority of PNA-labelled germinal centre B cells were PrP positive in Tg94/IRF mice (iv; yellow signal in superimposed images) and in wild-type mice (viii), but PrP negative in *Prnp*^{0/0} mice (xii). Original magnification $\times 250$. (b) Immunofluorescence labelling of FDCs and PrP on consecutive sections of spleen from non-inoculated Tg94/IRF (i–iv), Tg33/lck (v–viii), wild-type mice (ix–xii) and *Prnp*^{0/0} mice (xiii–xvi). Sections were stained with haemalaun (i, v, ix, xiii), antibody FDC-M1 to FDCs (green; ii, vi, x, xiv), antiserum R340 to PrP (red; iii, vii, xi, xv) and rabbit pre-immune serum (PIS) (iv, viii, xii, xvi). (*Cont.*)

8 log LD₅₀ units ml⁻¹ 10% homogenate for wild-type (table 1).

It is remarkable that despite the more than 1000-fold overexpression of PrP in the spleen of Tg94/IRF mice, the prion titre in this organ was not higher than that in wild-type mice. This apparent paradox can be explained if the high PrP levels in Tg94/IRF spleen reflected PrP in B and T lymphocytes but not in other spleen cells such as FDCs, and prion replication were dependent on the latter. Alternatively, it is possible that the level of PrP is not the limiting factor for the formation of prions. Similarly, at the terminal stage of scrapie, the prion titre in brains of *tga20* mice, which overexpress PrP five- to eightfold, is about the same as in wild-type mice, although the incubation times for the transgenic and wild-type mice are 60 and 160 days, respectively.

(b) *PrP* knockout mice overexpressing *PrP* in T cells

Having shown that prions are efficiently replicated in the spleens of PrP knockout mice transgenic for a construct directing expression of PrP in the spleen, we targeted PrP expression to B and T lymphocytes, respectively, and asked whether infectivity is synthesized in the spleen and thymus of such transgenic mice.

Transgenic mouse lines with PrP expression restricted to T lymphocytes were generated with the T-lymphocyte-specific Lck promoter (Chaffin *et al.* 1990). Thymus and spleen of Tg33/lck mice had PrP levels at least 100-fold and 40-fold higher, respectively, than wild-type mice, as determined by Southern analysis. PrP was undetectable in Tg33/lck brain (Raeber *et al.* 1999b). PrP expression on T lymphocytes as estimated by FACS analysis (figure 2b) was 50-fold higher than in wild-type. No PrP expression was detected in Tg33/lck splenic B lymphocytes (figure 2a). Immunohistochemical analysis of Tg33/lck spleens (figure 3b(vii)) showed that PrP expression (red) was predominantly in the perifollicular T-cell area, while the germinal centres, where the FDCs (green) were located, showed little red fluorescence over background (Raeber *et al.* 1999b). To determine whether the cellular form of prion protein (PrP^C) expression in T lymphocytes of Tg33/lck mice enabled prion replication in thymus and spleen, we assayed tissue extracts pooled from two animals sacrificed at two weeks and six months after IP inoculation. No infectivity was detected in Tg33/lck spleen or thymus six months after inoculation. Six months after IP inoculation, wild-type mice had titres of *ca.* 3.5 and 4.5 log LD₅₀ units ml⁻¹ 10% homogenate in thymus, respectively (Raeber *et al.* 1999b). Thus, even vast overexpression of PrP^C on T lymphocytes, comparable with levels found in wild-type brain, is not sufficient to allow prion replication in thymus or spleen of *Prnp*^{0/0} mice.

(c) *PrP* knockout mice overexpressing *PrP* in B cells

B cells are crucial for neuroinvasion by prions and for replication of the infectious agent in the LRS (Klein *et al.* 1997). However, it remained unclear whether the B cells themselves replicated prions or whether they play an

Figure 4. The march of prions from the periphery to the CNS. Prions probably survive in the gastrointestinal tract because of their protease resistance. They may traverse the mucosa through M cells (not proven) and make their way to Peyer's patches, where they have been demonstrated early on after feeding experiments and where they may replicate in FDCs. Transfer to other lymphoreticular tissues, in particular the spleen, is probably mediated by mobile cells of the lymphohaemopoietic system, such as macrophages, dendritic cells or lymphocytes; however, there are no relevant data. Prions then make their way through the vegetative nervous system to the spinal cord or medulla and thence to the brain.

auxiliary role. In order to determine the capability of PrP-expressing B lymphocytes to replicate prions, we generated transgenic mice with PrP expression restricted to B lymphocytes.

The regulatory elements from an immunoglobulin kappa (Igκ) variable region gene (*V_κ*) were used to drive the transcription of a PrP cDNA cloned with its own initiation codon and leader sequence behind the *V_κ* promoter. Nine transgenic founders were established; the founder with the highest PrP expression (Tg306/Igκ) showed PrP levels on B cells between 50 and 100% of wild-type. PrP expression was restricted to B cells, with no expression detectable on T cells or other tissues. Tg306/Igκ mice homozygous for the transgene cluster had PrP levels on B cells higher than wild-type and were inoculated intraperitoneally with prions. Spleen extracts from animals sacrificed two and eight weeks after IP inoculation showed no infectivity, whereas prion titres of *ca.* 10⁶ LD₅₀ units ml⁻¹ 10% homogenate were found in spleen from scrapie-infected *Prnp*^{0/+} mice sacrificed at two and eight weeks after inoculation (Montrasio 1999).

Thus, PrP-expressing lymphocytes by themselves are not competent to replicate prions.

4. WHY DO PrP-EXPRESSING LYMPHOCYTES IN A PrP KNOCKOUT MOUSE FAIL TO ACCUMULATE PRIONS?

Our experiments show that expression of PrP^C on B or T lymphocytes alone does not suffice for prion accumulation in thymus or spleen of *Prnp*^{0/0} mice, although in wild-type mice splenic lymphocytes carry prions. Maybe lymphocytes are inherently incapable of replicating prions because they are devoid of a conjectural receptor

Figure 3. (Cont.) In wild-type spleens (x,xi), PrP was evenly distributed over the entire section, including the region also stained by FDC-M1. In Tg33/lck spleens, PrP was visualized mainly in the T cell areas, but some cells were stained in the region also stained by FDC-M1. A few cells staining red with either anti-PrP (xv) or unspecific (xvi) antibodies are attributed to tingible body macrophages. Original magnification × 250. (From Raeber *et al.* 1999b.)

required for prion uptake or lack cellular factor(s) required for prion replication. Indirect evidence has led to the suggestion that a species-specific factor X is required for prion replication (Kaneko *et al.* 1997; Telling *et al.* 1995). Perhaps in wild-type mice, splenic lymphocytes can pick up prions from other sources, perhaps FDCs. Alternatively, lymphocytes are able to replicate prions, but when administered intraperitoneally in a PrP knockout environment, prions are not transported to the spleen or not transferred to lymphocytes because some infected PrP-expressing intermediary cell is lacking.

5. INVOLVEMENT OF FOLLICULAR DENDRITIC CELLS IN PRION REPLICATION IN THE LYMPHORETICULAR SYSTEM

Prion infectivity in the stromal component of peripheral lymphoid organs most likely resides in FDCs (Brown *et al.* 1999; Fraser & Farquhar 1987; Fraser *et al.* 1989; Kitamoto *et al.* 1991; Klein *et al.* 1998; McBride *et al.* 1992). We have made use of the observation that formation and/or maintenance of mature FDCs requires the presence of B cells expressing membrane-bound lymphotoxin- α/β , and that treatment of mice with soluble lymphotoxin- β receptor leads to disappearance of mature FDCs from the spleen (Mackay & Browning 1998). We have found that this treatment abolishes splenic prion accumulation and retards neuroinvasion following IP scrapie inoculation (Montrasio *et al.* 2000). Thus, the most likely interpretation of the findings described above is that splenic lymphocytes acquire prions from FDCs.

6. THE MARCH OF PRIONS

We have shown earlier that peripheral inoculation of PrP knockout mice with scrapie prions does not lead to infection of a PrP-expressing brain graft, even after adoptive transfer of PrP-expressing lymphohaemopoietic stem cells (Blättler *et al.* 1997). This observation suggested that prions are not simply transferred from the periphery to the CNS through blood or lymph, but that a further PrP-expressing compartment is required, most likely the peripheral nervous system (PNS) (Baldauf *et al.* 1997; Beekes *et al.* 1996, 1998; Beekes & McBride 2000; Groschup *et al.* 1999; Kimberlin & Walker 1989a; Race *et al.* 2000).

Figure 4 summarizes likely routes from the periphery to the CNS. Natural infections are mostly due to oral uptake of prions. Although it is not known how prions pass the gastrointestinal mucosa, M cells—follicle-associated cells that are specialized for the uptake and transcytosis of macromolecules and micro-organisms (Hathaway & Kraehenbuhl 2000)—may provide the entry portal. Soon after experimental oral infection prions are first found in Peyer's patches (Kimberlin & Walker 1989a) and the prion protein 'scrapie form' (PrP^{Sc}) accumulates in follicular dendritic cells (Maignien *et al.* 1999; Muramoto *et al.* 1993). It is not clear how prions make their way from the Peyer's patches to enteric lymph nodes and the spleen, but dendritic cells are possible carriers. Lymphoreticular organs, in particular the spleen, serve as amplification sites and FDCs are doubtless the site of prion replication. B and T lymphocytes recovered from spleen carry infectivity, while circulating lymphocytes do

not. It would seem that prions are not synthesized by lymphocytes but rather acquired from other sources, probably FDCs, with which they are in close contact in the germinal centre network. Because maintenance of mature FDCs depends on direct stimulation by mature B cells (Gonzalez *et al.* 1998), be they PrP-expressing or not (Klein *et al.* 1998), immunodeficiencies leading to absence of functional B cells impair accumulation of prions in spleen and efficient neuroinvasion. Following prion uptake and replication in the LRS, prions invade the CNS. The question whether lymphocytes play a role in the spread of prions from the periphery to the CNS is of more than academic interest. The finding of PrP^{Sc} in tonsils (Hill *et al.* 1997), and in the appendix of variant CJD (vCJD) sufferers even before outbreak of clinical disease (Hilton *et al.* 1998), raises new public-health concerns and demands reassessment of the risk of transmitting vCJD through blood or blood products. In our mouse model circulating lymphocytes appear not to be involved in transporting prions to the brain; however an extrapolation to the vCJD problem is not necessarily appropriate.

Several lines of indirect evidence point to the PNS as the crucial compartment by which prions access the CNS. Following peripheral infection, prions appear in the PNS prior to onset of replication in brain (Beekes *et al.* 1996; Kimberlin 1979), and PrP expression in the PNS is required for neuroinvasion (Race *et al.* 2000). Neuroinvasion originating from the LRS is thought to commence via nerve endings of the vegetative nervous system. Scrapie infectivity and PrP^{Sc} were found in the PNS of a scrapie-sick sheep (Groschup *et al.* 1996), but it is not clear whether prions can replicate in the peripheral nervous system or whether they are simply transported along nerve fibres. Interestingly, the innervation of lymphoid tissue is at least in part controlled by lymphocytes themselves, as both T and B cells secrete nerve growth factors and, on the other hand, nerve terminals secrete a variety of molecules to stimulate the immune system (Straub *et al.* 1998).

While the LRS plays a striking role in amplifying prions and mediating neuroinvasion, it would seem not to be essential. Intraneural infection of the sciatic nerve leads to direct infection of the CNS, bypassing the need for prion replication in the LRS (Kimberlin *et al.* 1983). Moreover, even in immunodeficient mice, prions ultimately make their way to the CNS, probably also via the PNS, from peripheral sites where prions are accumulated (Lasmézas *et al.* 1996). From a prophylactic–therapeutic point of view, interference with amplification of prions in the LRS may significantly retard progression of disease but probably not prevent it. Abrogating the transfer to and movement of prions through the PNS would seem to be a more effective, albeit quite ambitious, goal.

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Discussion

C. M. Dobson (*Oxford Centre for Molecular Sciences, New Chemistry Laboratory, University of Oxford, UK*). In the experiment with knockout mice, there is an incubation period between the infection and the development of the disease. Is that period very similar for different mice? Do all the mice have 170 days with little variation, or does it differ from animal to animal? Does the incubation period depend on the age of the animal?

C. Weissmann. It makes a difference if you inoculate them at birth or at one or two months, but the incubation

period in general is characteristic of the mouse strain. Different strains have very different responses.

C. M. Dobson. Is it known why the incubation period can be 40 years in humans but a much shorter time in mice?

C. Weissmann. It might be related to the size of the brain. The agent is not distributed by the blood or by lymph—it seems to pass from cell to cell. I have data that show that the agent spreads by accretion. So in the small mouse brain the agent might have to move only a few millimetres to reach a vital centre, but a lot further in the human. You can lose a lot of your brain but still survive.

M. B. Pepys (*Department of Medicine, Royal Free and University College Medical School, London, UK*). *Pro rata* to the mouse life span, 200 days is similar to 40 years out of the human life span.

M. Karplus (*New Chemistry Laboratory, University of Oxford, UK*). When you say there is one infectious unit out of 100 000, how is that measured? Have the 20 mutants you mentioned been studied for their effects on the protein?

C. Weissmann. Infectivity is measured by end-point dilution. The brain homogenate is diluted until half the mice get sick and half do not. From the Poisson relationship, you can then determine the number of infectious units. The total number of particles is determined by protein chemistry. All the mutant proteins were expressed in *Escherichia coli* and have been studied by R. Glockshuber. He finds that many of the mutant PrP proteins show a small reduction in stability.

M. B. Pepys. How does the one infectious PrP scrapie particle differ from the other 100 000 or so particles?

C. Weissmann. I believe that the infectious agent is not necessarily the protease-resistant form. I say this because there are some experimental forms of the disease where you cannot detect any protease-resistant material. You can always argue in these cases that the level is too small to be seen, but it may be that PrP^C changes into the infectious form but that this form is not very stable and collapses into PrP^{Sc}.

P. H. Byers (*Departments of Pathology and Medicine, University of Washington, Seattle, WA, USA*). Is there any constitutive splicing to the downstream *Doppel* gene?

C. Weissmann. Yes, but very little, only visible by PCR. We are conducting experiments to knock out the *Doppel* gene to see if the animals are equally susceptible.

C. M. Dobson. Is it clear that the prion diseases are the only infectious diseases of amyloidosis?

M. Pepys. We have exposed ourselves for decades to amyloid fibril preparations and they do not appear to be infectious as yet. On the other hand the amount of amyloid enhancing factor required to trigger rapid onset of amyloid deposition is very small, which is reminiscent of the scrapie situation.

C. Weissmann. An infectologist would say that the disease is transmissible but not infectious, because infectious means that the disease spreads spontaneously, while transmissible means that you have to do something before the disease will spread.