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NOTES

Scrapie Pathogenesis in Subclinically Infected B-Cell-Deficient Mice

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Prion infections can present without clinical manifestations. B-cell deficiency may be a model for subclinical transmissible spongiform encephalopathy, since it protects mice from disease upon intraperitoneal administration of scrapie prions; however, a proportion of B-cell-deficient mice accumulate protease-resistant prion protein in their brains. Here, we have characterized this subclinical disease. In addition, we have studied the possibility that a neurotoxic factor secreted by B cells may contribute to pathogenesis.

Prion diseases are transmissible neurodegenerative disorders of rodents, ruminants, and humans. According to the protein-only hypothesis (21) the infectious principle is a pathological isoform (termed PrP^{Sc}) of the normal, host-encoded prion protein (termed PrP^{C}): PrP^{Sc} is thought to replicate by converting PrP^{C} into further PrP^{Sc}. PrP^{C} is necessary for prion replication (7, 24), and differences in the amino acid sequences of PrP^{C} were shown to be the main determinants of interspecies transmission barriers (25). Upon intracerebral (i.c.) or peripheral exposure, replication of prion infectivity occurs in the lymphoreticular system (LRS) of experimental animals long before becoming detectable in the central nervous system (CNS) (10, 12).

LRS colonization is important for the preneural phase of scrapie pathogenesis. Peripheral inoculation of SCID (2), *RAG-1*$^{-/-}$ (18), and *RAG-2*$^{-/-}$ (26) mice which lack mature lymphocytes and follicular dendritic cells (FDCs), as well as μMT mice (15) which lack mature B cells and immunoglobulins, fails to establish prion replication in the LRS and does not lead to manifest scrapie for >600 days (16). However, upon i.c. challenge with a mouse-adapted scrapie strain, immunodeficient mice succumb to scrapie similarly to wild-type controls. SCID mice resist infection with bovine spongiform encephalitis (BSE) even upon i.c. challenge (5). Hence, lymphocytes and FDCs are involved in prion transfer from peripheral sites to the CNS and in trespassing species barriers.

The brains of B-cell-deficient animals were sampled randomly at late time points (222 to 504 days) after inoculation with scrapie prions and assayed for protease-resistant PrP^{Sc} and/or infectivity: 5 of 14 mice (13 to 65% within a confidence interval of 95%) tested positive by either Western blot analysis, infectivity bioassay, or both (16). However, clinically apparent scrapie was never detected in B-cell-deficient mice ($n = 27$), even 665 days after intraperitoncal (i.p.) inoculation with prions (16), suggesting that a neurotoxic or catalytic factor secreted by B cells may contribute to pathogenesis and lead to the manifestation of symptoms. If this were true, i.c. inoculation of μMT or *RAG-1*$^{-/-}$ mice with inoculum derived from μMT or *RAG-2*$^{-/-}$ brains would not contain such a hypothetical cofactor and therefore should not lead to scrapie in the recipient mice.

We therefore prepared prion inocula from brains of two *RAG-2*$^{-/-}$ and two μMT mice which had been scrapie inoculated i.p. (Table 1). Brain homogenates (1%) were prepared as described previously (8) and inoculated i.c. into μMT and *Rag-1*$^{-/-}$ mice, which were kept under pathogen-free conditions.

One *RAG-2*$^{-/-}$ mouse and one μMT mouse contained levels comparable to terminally-scrapie-sick wild-type mice, as assayed by titration of infectivity by incubation time (22) in PrP-overexpressing *tga20* indicator mice (11). The brain infectivity titer in the second *RAG-2*$^{-/-}$ mouse was approximately 2 log 50% lethal dose units lower. The second μMT mouse was not tested for infectivity, but showed PrP^{Sc} levels exceeding the ones of the infected wild-type control upon Western blotting (16). For the latter, 80 μg of total protein was electrophoresed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide), transferred to nitrocellulose membranes, probed with monoclonal antibody 6H4 (17) to mouse PrP, and developed by enhanced chemiluminescence (Amersham).

Sixteen mice were inoculated i.c. with prions derived from brains of asymptomatic μMT and *Rag-2*$^{-/-}$ mice which had been challenged i.p.: all mice developed weight loss, hind limb paresis, ataxia, apathy, and hunched posture, with incubation periods shown in Table 1, and were killed when terminally sick. To confirm the diagnosis of scrapie, we investigated the accumulation of PrP^{Sc} in one brain for each group of mice by Western blot and histoblot analyses, and assessed spongiosis and gliosis in paraffin-embedded brain sections of all mice. PrP^{Sc} (Fig. 1) and/or transmissible spongiform encephalopathy-characteristic histopathology (not shown) was demonstrated in all brains of clinically sick mice.

We noted a statistically significant tendency towards shorter incubation periods when compared with the incubation periods of *RAG-2*$^{-/-}$ and μMT mice primarily inoculated i.c. with standard Rocky Mountain Laboratory (RML) prions (Student's t test, $P < 0.001$). For example, the inoculum derived from a μMT mouse sacrificed 436 days after i.p. inoculation which did not display clinical symptoms provoked terminal stage scrapie with an average of 149 days, whereas μMT mice i.c. challenged with RML prions (derived from terminally sick

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TABLE 1. Origin of scrapie inoculum, occurrence of clinical scrapie, and latency of disease in immunodeficient mice inoculated with prions derived from asymptomatic immunodeficient mice[a]

Inoculum[b] and days of inoculation	Recipient (i.c.)	Average incubation period (days) ± SD
RAG-2$^{-/-}$		
Day 286	*RAG-1*$^{-/-}$	182 ± 35
Day 342		155 ± 2.2
μMT		
Day 375	μMT	161 ± 5.5
Day 432		149 ± 9.4

[a] Note that in each group, four of four mice developed scrapie.
[b] One percent brain homogenate.

CD1 wild-type animals) succumbed with an average of 181 days.

PrP^{Sc} was demonstrated in all brains tested by Western blotting (Fig. 1). Glycoform ratios were calculated by densitometric analysis with ImageQuaNT software, and evidenced a characteristic pattern of proteinase K-resistant un-, mono-, or diglycosylated PrP (9), which was similar to the patterns of RML-inoculated C57/BL6, *RAG-2*$^{-/-}$, and μMT mice (Fig. 1).

For histoblots (27), frozen sections (8 μm) were mounted on nitrocellulose and subjected to limited proteolysis (proteinase K at 100 μg/ml, 37°C, 4 h). Detection was accomplished with monoclonal antibody 6H4 to PrP (17) (1:2,000) as described previously (3). Histoblots of forebrain with cortex and basal ganglia, rostral hippocampus with thalamus and brain stem, and cerebellum did not reveal differences in the distribution of proteinase K-resistant PrP in infected yet clinically healthy immunodeficient animals compared to terminally-scrapie-sick μMT and *RAG* mice inoculated i.c. with RML prions, and with one RML-inoculated, terminally scrapie sick *TcR*α$^{-/-}$ mouse (20) lacking all T-cell receptor alpha/beta (TCR-α/β)-expressing lymphocytes (Fig. 2).

Upon formic acid inactivation (6) and paraffin embedding, brain sections were stained with hematoxylin-eosin and with antibodies to glial fibrillary acidic protein. Gliosis (a nonspecific but early indicator of brain damage) was visualized by the presence of large immunostained reactive astrocytes. All scrapie-sick mice displayed characteristic vacuolar changes and strong reactive gliosis with a regional lesion profile very similar to that of *RAG-2*$^{-/-}$, μMT, and C57/BL6 mice inoculated i.c. with RML prions (Fig. 3).

In a former study, we discovered that mice lacking differentiated B lymphocytes can harbor high titers of prion infectivity and PrP^{Sc} in their brains following peripheral challenge with an RML standard inoculum, yet do not develop clinical symptoms of scrapie. This surprising finding led us to hypothesize that once prions have reached the CNS, a neurotoxic or catalytic cofactor produced by B lymphocytes could be responsible for, or at least dramatically accelerate, the decline of CNS neurons

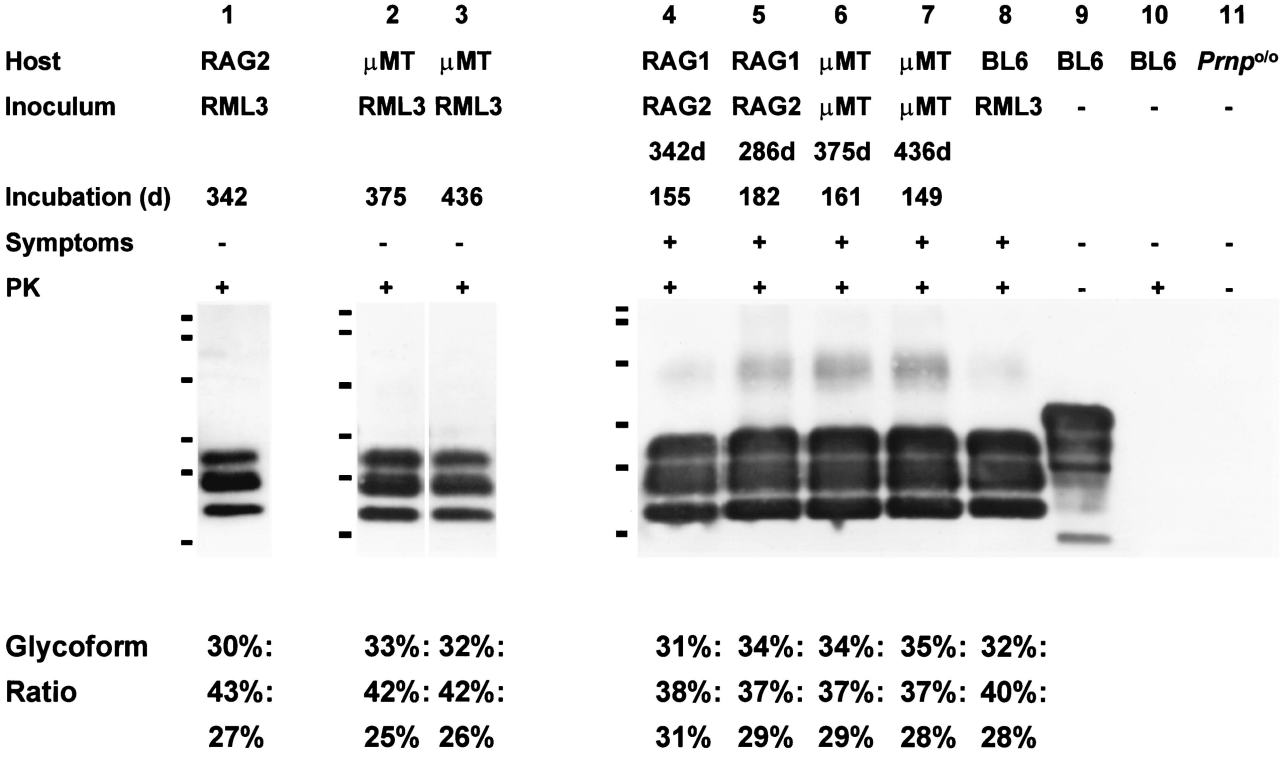

FIG. 1. Western blot analysis of brains of immunodeficient mice challenged i.p. with RML prions and not exhibiting clinical symptoms (lanes 1 to 3) and of immunodeficient mice challenged i.c. with inocula derived from asymptomatic *RAG-2*$^{-/-}$ and μMT mice (lanes 4 to 7). The latter mice were terminally sick. The lower tabulation indicates the relative percent contribution of un-, mono-, and diglycosylated prion protein bands after proteinase K (PK) digestion to the total PrP^{Sc} immunoreactivity present on the blot. The predominance of the monoglycosylated form in all mice, as well as the very similar glycoform ratio in all samples tested, indicates that no alteration of the strain properties detectable by glycotype analysis has taken place during the passage of CD-1 mouse-derived RML prions onto *RAG-2*$^{-/-}$ and μMT mice (genetic background, C57/BL6), and upon a second passage onto further *RAG-1*$^{-/-}$ and μMT mice. Lane 8, brain of a terminally scrapie sick C57/BL6 mouse inoculated with RML prions; lanes 9 and 10, brain of a noninfected C57/BL6 mouse, showing no anti-PrP immunoreactive bands upon treatment with proteinase K; lane 11, brain of a *Prnp*$^{0/0}$ mouse.

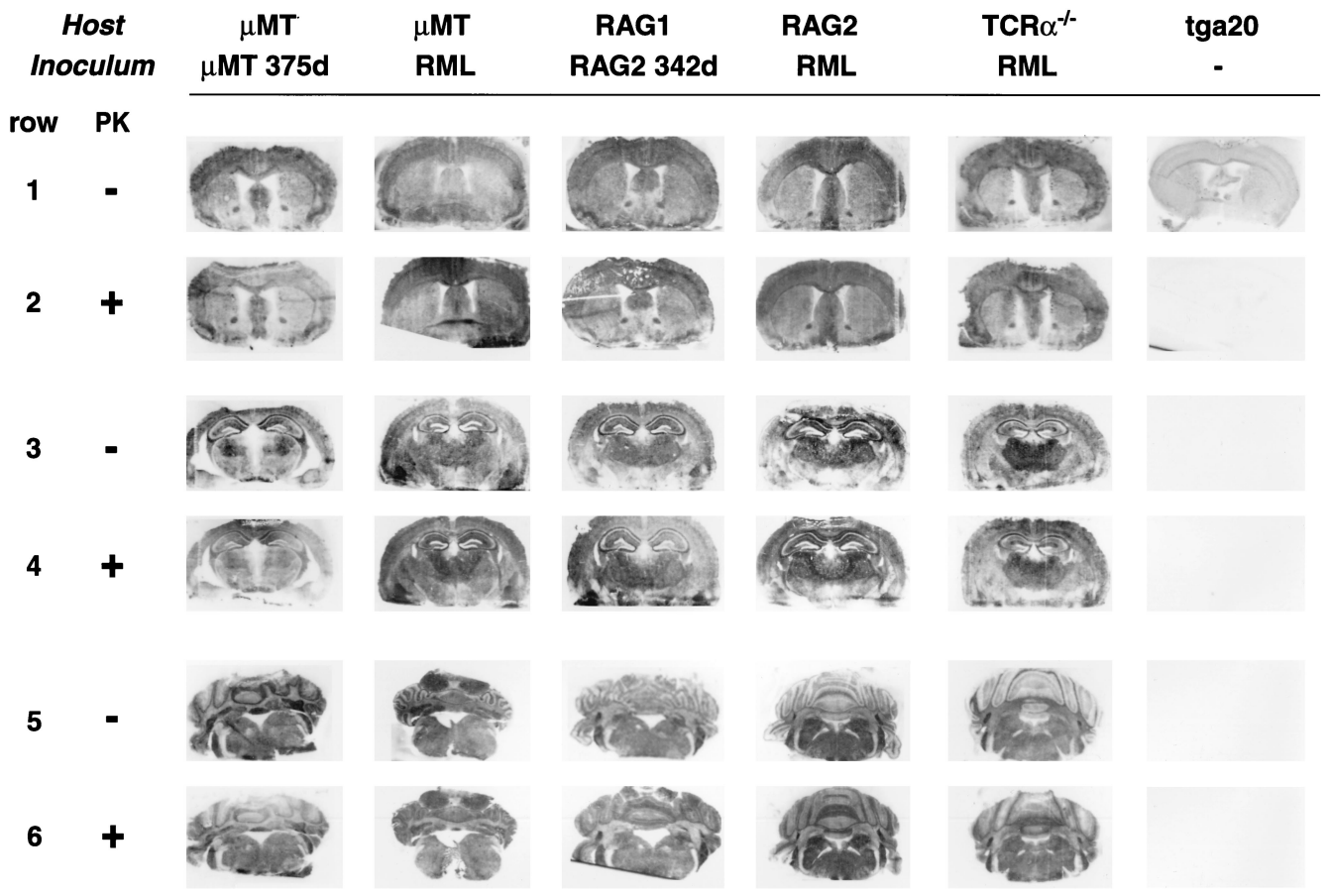

FIG. 2. Histoblot analysis of distribution patterns of proteinase K (PK)-resistant PrP^{Sc} in different brain regions of immunodeficient mice. Coronal sections through the forebrain including the basal ganglia (rows 1 and 2), through the rostral hippocampus and thalami (rows 3 and 4), and through the caudal regions including brain stem and cerebellum (rows 5 and 6) show indistinguishable patterns of PrP^{Sc} distributions in *RAG-2*$^{-/-}$ and μMT mice inoculated i.c. with RML prions (columns 1 and 3, respectively) and in *RAG-1*$^{-/-}$ and μMT mice inoculated i.c. with inocula derived from asymptomatic *RAG-2*$^{-/-}$ and μMT mice (columns 2 and 4, respectively), as well as in *TCR*α$^{-/-}$ mice succumbing from scrapie upon i.c. inoculation with RML prions (column 5). 375d and 342d, days of inoculation.

and lead to the manifestation of symptoms in concert with the infectious agent. There are examples for similar pathogenetic mechanisms: in human immunodeficiency virus infection of the CNS, for example, pathogenesis is mediated not only by viral products (28), but also by nitric oxide produced by infected microglial cells (13).

Because B-cell-deficient mice develop scrapie upon i.c. infection with a standard RML inoculum with the same latency and efficiency as wild-type control mice (16), a possible neurotoxic cofactor would have to be present in infectious brain homogenates derived from immunocompetent animals. If so, i.c. inoculation of B-cell-deficient mice with an inoculum derived from brains of subclinically infected B-cell-deficient mice should result in greatly (or indefinitely) prolonged incubation times, since the primary i.p. inoculation of B-cell-deficient mice would dilute the cofactor.

However, the results of the present study confirm that B lymphocytes and their secreted products do not influence scrapie pathogenesis if the agent is directly inoculated into the brain. This remains true even after prions have been passaged twice in B-cell-deficient mice. We can also exclude the involvement a cofactor produced by T lymphocytes, which are known to be able to invade CNS parenchyma when activated (4, 19), since *RAG-1*$^{-/-}$ and *RAG-2*$^{-/-}$ mice are devoid of both B and T cells.

We have considered the possibility that the agent may have undergone a "strain shift" changing its pathogenic properties (1), because it was passaged twice in immunodeficient hosts and it induced disease with shortened incubation times upon its second passage. However, this is very unlikely since (i) the glycoform distribution of PrP^{Sc}, (ii) the regional patterns of PrP^{Sc} deposition as assessed by histoblotting, and (iii) the lesion profiles in the brains of *RAG-1*$^{-/-}$ and μMT mice inoculated with prions from immunodeficient mice are indistinguishable from those of RML-inoculated *TcR*α$^{-/-}$, RML-inoculated *RAG-2*$^{-/-}$, μMT, and wild-type mice.

While it is remarkable that immunodeficient mice harboring brain infectivity titers equaling or even exceeding those of terminally sick wild-type controls can be perfectly healthy on clinical examination, there are further documented examples of chronic subclinical infections with prions. Mice heterozygous for a disrupted PrP gene show high prion and PrP^{Sc} levels, but a delayed onset of disease (7). Hamster prions can persist and perhaps even replicate in a clinically silent fashion when transmitted to mice (23). In the latter experiment, mice inoculated with hamster prions clinically resisted infection, yet when brain homogenates of these mice were transmitted to further animals, hamsters (but not mice) developed scrapie.

However, subclinical infection may also occur when prions are being passaged within the same species. Although many

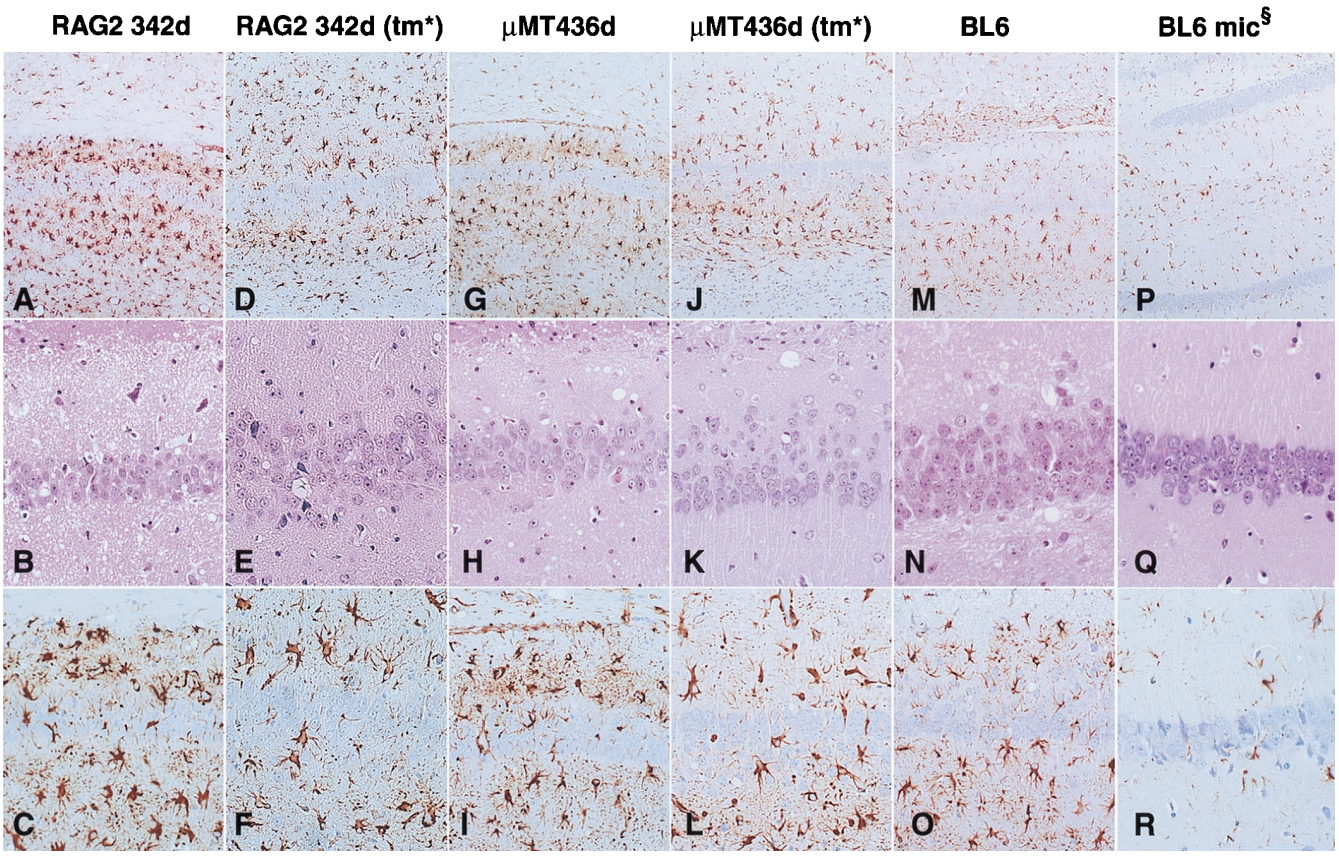

FIG. 3. Brain histopathology of asymptomatic *RAG-2*$^{-/-}$ (A to C) and μMT mice (G to I) inoculated i.p. with scrapie prions, and of *RAG-1*$^{-/-}$ (D to F) and μMT mice (J to L) inoculated i.c. with inocula derived from asymptomatic *RAG-2*$^{-/-}$ and μMT mice, respectively. The hippocampal formation shows the typical morphology of scrapie with vacuolar changes in both asymptomatic (B and H) and in terminally sick (E and K) immunodeficient mice. Despite some interindividual variations in the density of microvacuolation (middle row, hematoxylin & eosin), immunostaining for glial fibrillary acidic protein (top and bottom rows) reveals extensive, diffuse gliosis surrounding the pyramidal cell ribbon in all mice (original magnification and stain, upper row, ×100; middle and bottom rows, ×200). (M to O) RML-inoculated, terminally sick C57/BL6 mouse. (P to R) Mock-inoculated, age-matched C57/BL6 mouse. An asterisk indicates transmission (tm) of sample *RAG-2* 342d into *RAG-1*$^{-/-}$ and of sample μMT 436d (inoculated on days 342 and 436, respectively) into μMT mice. mic§, i.c. mock inoculation.

patients were presumably exposed to Creutzfeldt-Jakob disease-contaminated batches of growth hormone and gonadotropins, only about 100 (14) developed overt disease. This may be interpreted as a sign that the "take" of infection occurred only in few individuals, perhaps because the inoculum was small, or that incubation times vary vastly so that many individuals may have developed subclinical infection.

Also the fact that only single cows developed clinically overt BSE in exposed herds raises the possibility that subclinical infection of cattle with BSE may occur more frequently than suspected. Given the health hazards posed by BSE, the phenomenon of subclinical prion infections deserves in-depth investigations: B-cell-deficient mice may serve as a suitable model system. The results presented here argue against a direct effect of B lymphocytes or their secreted products on establishment of subclinical prion infection and raise the possibility that secondary events, such as maturation of cells induced by B lymphocytes—e.g., FDCs in lymphoid organs or immune cells resident in the CNS—may contribute to scrapie neurotoxicity.

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