B lymphocyte-restricted expression of prion protein does not enable prion replication in prion protein knockout mice

Fabio Montrasio*†, Antonio Cozzio*‡, Eckhard Flechsig*§, Daniela Rossi*§, Michael A. Klein†, Thomas Rülicke¶, Alex J. Raeber*‖, Christian A. J. Vosshenrich††, Juliane Proft†, Adriano Aguzzi†, and Charles Weissmann*§‡‡**

*Institute of Molecular Biology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland; †Department of Pathology, Institute of Neuropathology, University of Zurich, Schmelzbergstrasse 12, CH-8091 Zurich, Switzerland; ¶Biologisches Zentrallabor, Sternwartstrasse 6, CH-8091 Zurich, Switzerland; and **Institute for Genetics, Weyertalstrasse 121, D-50931 Cologne, Germany

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Prion replication in spleen and neuroinvasion after i.p. inoculation of mice is impaired in forms of immunodeficiency where mature B lymphocytes are lacking. In spleens of wild-type mice, infectivity is associated with B and T lymphocytes and stroma but not with circulating lymphocytes. We generated transgenic prion protein knockout mice overexpressing prion protein in B lymphocytes and found that they failed to accumulate prions in spleen after i.p. inoculation. We conclude that splenic B lymphocytes are not prion-replication competent and that they acquire prions from other cells, most likely follicular dendritic cells with which they closely associate and whose maturation depends on them.

Ablation of the prion protein (PrP) gene renders mice ($Prnp^{0/0}$) resistant to experimental scrapie and precludes replication of prions (2–4). Introduction of PrP transgenes under the control of the PrP promoter into $Prnp^{0/0}$ mice restores susceptibility to scrapie (5).

I.p. inoculation of mice with the scrapie agent results first in prion replication in spleen and subsequently in brain. Transfer of prions from periphery to the central nervous system (CNS), i.e., neuroinvasion, requires interposed PrP^C-expressing tissues (6). B cells were found to be essential for facilitating neuroinvasion after peripheral infection (1). However, because B cells devoid of PrP also enabled neuroinvasion, it seemed that they might not replicate and transport prions but rather play an indirect role in this process (7). Nonetheless, splenic (but not circulating) B lymphocytes are associated with prion infectivity (8). This finding raised the question whether B cells could replicate prions or acquire them from other cells. In this paper, we report that PrP knockout mice that overexpress PrP specifically on B cells do not accumulate prions in the spleen after i.p. infection, showing that in wild-type mice they must be acquiring prions from other cells. Because follicular dendritic cells (FDCs) are essential for the propagation of prions in the spleen (9, 10), we propose that splenic B cells acquire prions from FDCs, with which they are closely associated. Thus, at least in the mouse, B cells are not instrumental in carrying infectivity to the CNS.

Materials and Methods

Construction of PrP Expression Vectors. To construct the pHT4-PrP.cDNA/*Eco*RI vector used to generate TgN(Igκ Prnp) mice, the 12.4-kb *Sac*I fragment, harboring the known control sequences (Igκ promoter and enhancer) and the plasmid backbone, was isolated from pHT4-YK 12 (11), blunted with T4 DNA polymerase, and dephosphorylated. The 1.37-kb *Bam*HI-*Eco*RI fragment, containing *Prnp* exons 1 and 2 and part of 3, was excised from pPrP/cDNA/*Eco*RI (A. Sailer, unpublished data) and blunted with T4 DNA polymerase. The two fragments were ligated.

pCD19-PrP.HG/Sal, used to generate TgN(CD19 Prnp) mice, was constructed from four fragments. (*i*) A fragment containing the entire hCD19 transgene was excised with *Hin*dIII from pSP65V7.J5 (C.A.J.V., unpublished data) and inserted between the *Pac*I and *Asc*I restriction sites of a modified pSP65 vector (pSP65AP, F.M., unpublished data) to generate pSP65AP-hCD19. A 15.9-kb fragment (harboring the plasmid backbone), 3.7 kb of hCD19 5′ noncoding sequences, and a 9.5-kb segment of hCD19 (comprising part of exon 4, exons 5–15, and the 3′ noncoding sequence), were isolated from pSP65AP-hCD19 by digestion with *Eco*RI and *Kpn*I. (*ii*) A 1.1-kb *Eco*RI-*Bam*HI fragment, harboring the hCD19 proximal 5′ noncoding sequence and exon 1 up to the *Bam*HI site within the multiple cloning site, was excised from phCD19MCS (C.A.J.V., unpublished data). (*iii*) A 1.3-kb *Sal*I-*Kpn*I fragment containing part of exon 1 (starting from the *Sal*I site within the multiple cloning site), exons 2, 3, and part of 4 was excised from phCD19MCS. (*iv*) The 5.3-kb *Prnp* expression cassette was excised from pPrP-5′HG Sal (12) with *Bam*HI and *Sal*I. The four fragments were joined by ligation.

Generation of Transgenic Mice. pHT4-PrP.cDNA/*Eco*RI was digested with *Sal*I and *Bgl*II, and pCD19.PrPHG/Sal was digested with *Asc*I and *Pac*I to remove plasmid backbone. Pronuclear injections into fertilized oocytes from homozygous $Prnp^{0/0}$ mice were done as described (5). Founders were identified by Southern analysis by using a 760-bp *Bst*EII-*Eco*RI DNA fragment from *Prnp* exon 3 (Probe A) that detects the $Prnp^0$ and PrP transgene alleles. $Prnp^0$ alleles and $Prnp^+$ transgenes were detected by PCR by using the primers P3 (specific for the $Prnp^0$ allele), P10 (specific for the $Prnp^+$ allele), and prPrP3′nc (specific for the $Prnp^0$ and the $Prnp^+$ alleles) (5). Of 10 mouse lines transgenic for the Igκ construct, the line with the highest transgene mRNA expression in the spleen (TgN306) was examined further. Of four mouse lines transgenic for the human CD19 construct, the one with the highest PrP^C expression on peripheral blood lymphocytes (TgN431) was used for further experiments.

Fluorescence-Activated Cell-Sorting (FACS) Analysis. A few drops of whole blood were collected in PBS with 2% (vol/vol) FCS/10

Abbreviations: CNS, central nervous system; FACS, fluorescence-activated cell sorting; FDC, follicular dendritic cell; PE, phycoerythrin; PrP, prion protein.

‡Present address: Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305.

§Present address: MRC Prion Unit/Neurogenetics, Imperial College School of Medicine at St. Mary's, Norfolk Place, London W2 1PG, United Kingdom.

‖Present address: Cytos Biotechnology AG, Wagistrasse 21, CH-8952 Zurich-Schlieren, Switzerland.

††Present address: Departement d'Immunologie, Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris, France.

‡‡To whom reprint requests should be addressed. E-mail: c.weissmann@ic.ac.uk.

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mM EDTA (pH 8)/0.1% sodium azide (FACS buffer). Leukocytes were pelleted for 10 min at 200 × *g*. Erythrocytes were lysed with FACS lysing solution (Becton Dickinson) according to the manufacturer's protocol. Single staining for PrP was achieved by incubation of peripheral blood leucocytes for 30 min at 4°C with 100 μl of monoclonal 6H4 antibody (13) (1:100; Prionics AG, Zürich). Cells were washed in cold FACS buffer, stained for 30 min at 4°C with 100 μl of goat anti-mouse IgG1-FITC (1:100; Southern Biotechnology Associates), and washed again before cytofluorometry (Becton Dickinson FACScan). For two-color FACS analyses, PrP staining was followed by B cell staining with phycoerythrin (PE)-conjugated anti-B220 antibodies (1:100; PE anti-mouse CD45R/B220, PharMingen) or T cell staining with PE-conjugated anti-CD3e antibodies (1:100; PE anti-mouse CD3e, PharMingen) for 15 min at 4°C. Before cytofluorometry, erythrocytes were lysed with FACS lysing solution (Becton Dickinson) by following the manufacturer's protocol. The lymphocyte population was gated for analysis on the basis of forward scatter and side scatter.

Immunoprecipitation. Homogenates (10% wt/vol) from fresh or frozen tissues were prepared in 1 × SaB buffer (Prionics AG, Zürich). The homogenates were centrifuged for 15 min at 400 × *g*. Supernatants were transferred to Eppendorf tubes, diluted to 2% with PBS, and either 500- or 250-μl aliquots (corresponding to 10 and 5 mg of spleen, respectively) were preadsorbed with 50 μl of blocked Sepharose 4B beads (Amersham Pharmacia Biotech) by incubation at 4°C for 30 min on a rocking platform. To the preadsorbed samples, 100 μl of Sepharose 4B beads linked to monoclonal antibody 6H4 (13) were added, and samples were incubated overnight at 4°C on a rocking platform. Sepharose beads were washed twice with 50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% (vol/vol) Nonidet P-40/0.5% sodium deoxycholate/1 mM EDTA, pH 7.5/1 mM PMSF; twice with 50 mM Tris·HCl, pH 7.5/500 mM NaCl/0.1% Nonidet P-40/0.05% sodium deoxycholate; and finally once with 50 mM Tris·HCl, pH 7.5/0.1% Nonidet P-40/0.05% sodium deoxycholate. Pellets were boiled in SDS sample buffer and analyzed by immunoblotting.

Western Blot Analysis. One hundred twenty micrograms of total protein of each brain homogenate and the equivalent of either 5 or 10 mg of spleen after immunoprecipitation were electrophoresed through a 12.5% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose membranes by wet blotting. Membranes were blocked with Tris-buffered saline + Tween 20 (0.05%)/5% nonfat dry milk, incubated with 1B3 rabbit polyclonal anti-PrP serum (14), and bands were visualized by enhanced chemiluminescence (Amersham Pharmacia). Quantification was by laser densitometry (Molecular Dynamics) of appropriately exposed films.

Inoculation of Transgenic and Control Mice. Mice were inoculated i.p. with 100 μl of brain homogenate containing either 1×10^5 (RML 4.0, Rocky Mountain Laboratories, Hamilton, MT) or 7×10^5 (RML 4.1) ID_{50} units of scrapie prions prepared as described (2).

Infectivity Bioassay. Spleen homogenates [10% (vol/vol) in 0.32 M sucrose] were prepared from infected animals as described (15). Thirty microliters of 1% vol/vol homogenates [in PBS/5% BSA] were administered intracerebrally to groups of four *tga20* mice for each sample. Incubation time to terminal scrapie sickness was determined and infectivity titers were calculated by using either the relationship $y = 14.37 - 0.11x$ (for RML 4.0) or $y = 11.45 - 0.088x$ (for RML 4.1), where y is the ID_{50}, and x is the incubation time (in days) to terminal disease.

Results

PrP Knockout Mice Expressing PrP^C on B Lymphocytes. To investigate whether expression of PrP in B lymphocytes is sufficient to enable scrapie replication in $Prnp^{0/0}$ mice, we generated two types of transgenic mice in which the PrP-encoding sequence was expressed in B cells. TgN(Igκ Prnp) transgenic mice were generated by placing the murine PrP ORF under the control of the Igκ light chain (Igκ) enhancer/promoter, which had been shown previously to drive expression exclusively in lymphoid cells (16). In TgN(CD19 Prnp) transgenic mice, a PrP cassette comprising the three PrP exons and the first intron, but lacking the second intron, was under the control of the human CD19 regulatory elements, known to mediate B lineage-specific expression in mice (17).

Transgene-positive founder mice were bred to yield $Prnp^{0/0}$ lines hemizygous or homozygous for the PrP-encoding transgene arrays. For each of the two transgenic mouse types, the line expressing the highest levels of PrP protein was chosen for further analysis, namely $Prnp^{0/0}$-TgN(Igk Prnp)306Zbz (TgN306) and $Prnp^{0/0}$-TgN(CD19 Prnp)431 Zbz (TgN431).

Expression of PrP^C on peripheral blood leukocytes (Fig. 1*A*) and splenocytes (data not shown) of hemizygous transgenic mice was monitored by FACS analysis. PrP^C expression levels in TgN306 and TgN431 were estimated to be 0.4- to 0.5-fold and 10- to 20-fold, respectively, of those observed in $Prnp^{+/+}$ mice. PrP^C expression was detected in B lymphocytes (B220 positive) but not in T lymphocytes (CD3 positive) of both transgenic lines (Fig. 1*B*). There was a population of PrP-expressing B220 negative lymphocytes in TgN306 mice that we did not characterize further because, as described below, there was no propagation of prions in these mice. PrP^C expression in spleens of homozygous TgN306 and hemizygous TgN431 mice was investigated further by immunoblot analysis and compared with that in $Prnp^{+/+}$, $Prnp^{0/+}$, and $Prnp^{0/0}$ mice (Fig. 2). $Prnp^{0/+}$ mice showed about half the wild-type levels of PrP^C. TgN306 mice had levels close to wild type (0.8 times), whereas TgN431 mice overexpressed PrP^C at least 50- to 100-fold (Table 1). The general architecture of the spleen was normal as was the localization of B lymphocytes in splenic follicles and germinal centers, as shown by B220 and PNA staining, respectively (data not shown).

PrP Knockout Mice Expressing PrP on B Cells Do Not Replicate Prions. To determine whether PrP^C expression restricted to B lymphocytes enabled prion replication in the spleen, TgN306 and TgN431 mice (hemizygous or homozygous for the PrP transgene), along with $Prnp^{+/+}$, $Prnp^{0/+}$, and $Prnp^{0/0}$ mice, were challenged i.p. with RML scrapie prions (Table 2). Prion levels in the spleens of animals killed at different times after inoculation were determined by intracerebral inoculation into *tga20* indicator mice. In spleens of both $Prnp^{+/+}$ and $Prnp^{0/+}$ mice, the titers were about the same 2, 4, and 8 weeks after inoculation, around 6.2 logarithm (log) ID_{50} units/ml 10% homogenate. Thus, in spleens of $Prnp^{+/+}$ (as previously shown) and $Prnp^{0/+}$ mice, maximal infectivity was reached around 2 weeks after inoculation and maintained for the remaining observation period.

In contrast, no prion infectivity was detected in the spleens of TgN306 transgenic mice at any time tested after i.p. challenge [<1.5 logarithm (log) ID_{50} units/ml of 10% (vol/vol) homogenate]. In the case of TgN431 mice, low prion titers [2.7 log ID_{50} units/ml of 10% (vol/vol) homogenate] were detected 2 weeks after infection, whereas samples taken after 4 and 8 weeks caused disease in only 1 of 8 indicator mice. These traces of infectivity are caused in all likelihood by residual inoculum; similar findings have been reported for PrP knockout mice (1, 3, 6, 9). Persistence of residual infectivity after 2 weeks in the

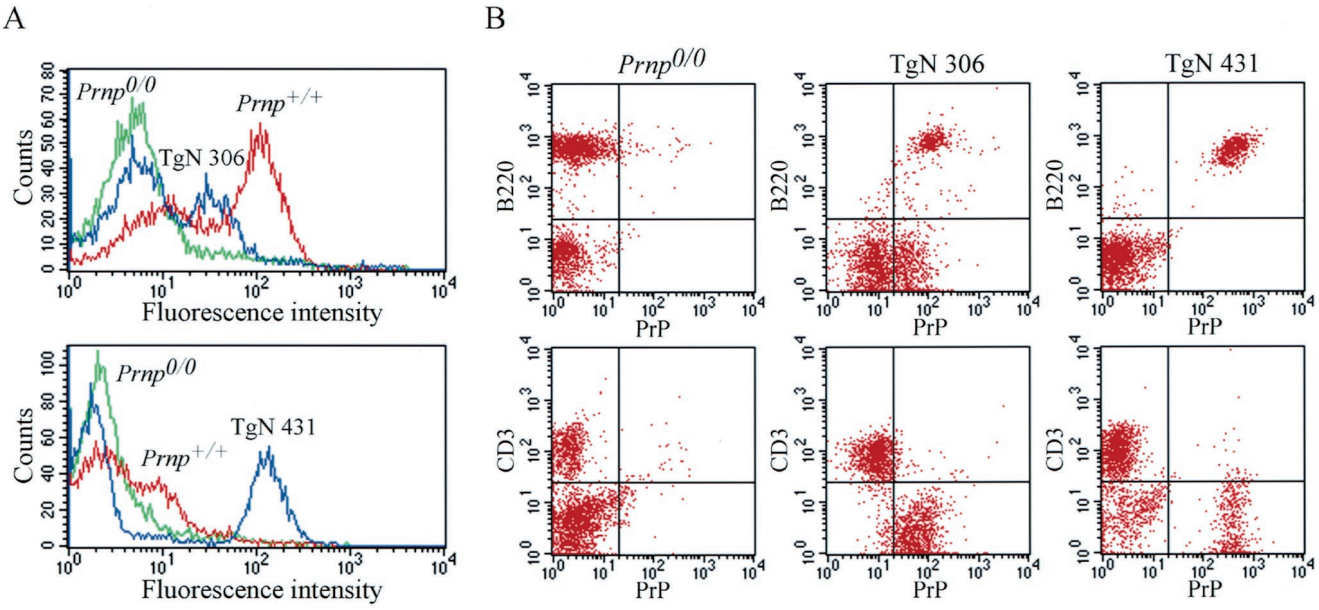

Fig. 1. Analysis of PrP^C expression on peripheral blood lymphocytes (PBLs) of TgN(Igκ Prnp)306 and TgN(CD19 Prnp)431 mice. (*A*) Single-stain FACS analysis for PrP^C was as described in *Materials and Methods*; gating was for lymphocytes. PrP^C expression on PBLs of TgN306 (*Upper*) and TgN431 mice (*Lower*) was compared with negative ($Prnp^{0/0}$) and positive ($Prnp^{+/+}$) controls. The two samples were analyzed on different occasions. (*B*) Double-color FACS analysis for PrP and B220 (*Upper*) and PrP and CD3 (*Lower*). PrP staining was followed by B lymphocyte staining with PE-conjugated anti-B220 antibodies or T lymphocyte staining with PE-conjugated anti-CD3e antibodies. PrP^C expression was detected in B lymphocytes but not in T lymphocytes of both transgenic lines.

spleens of TgN431 but not of TgN306 mice is likely because of the fact that the TgN431 mice were inoculated with an almost 10-fold higher dose of prions (see *Materials and Methods*).

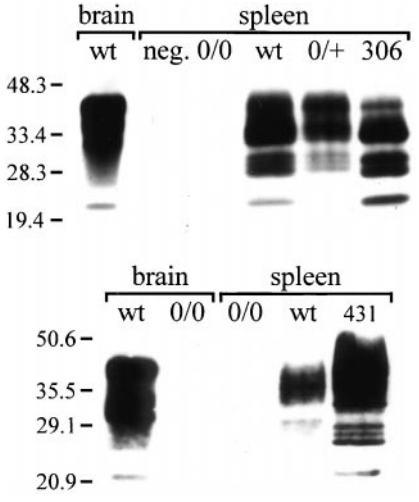

Fig. 2. Western blot analysis for PrP^C in spleens of TgN(Igκ Prnp)306 and TgN(CD19 Prnp)431 mice. PrP^C was enriched by immunoprecipitation with 6H4-linked Sepharose beads and was detected by using rabbit antiserum 1B3 against murine PrP (14). Crude wild-type and $Prnp^{0/0}$ brain homogenates (120 μg total protein) were included in the experiment as controls for PrP immunodetection. Positions of the molecular weight standards (in kilodaltons) are indicated on the left side of the fluorogram. (*Top*) Expression of PrP^C in the spleens of homozygous TgN306 mice. The equivalent of 10 mg of spleen was loaded per lane. A wild-type spleen sample immunoprecipitated with only Sepharose beads was included as a negative control (neg.). Genotypes of the mice analyzed: $Prnp^{0/0}$,0/0; $Prnp^{+/+}$, wt; $Prnp^{0/+}$, 0/+; homozygous TgN306, 306. (*Bottom*) Expression of PrP^C in the spleens of hemizygous TgN431 mice. Samples equivalent to 5 mg of spleen were loaded per lane. Genotypes of the mice analyzed: $Prnp^{0/0}$, 0/0; $Prnp^{+/+}$, wt; hemizygous TgN431, 431.

Discussion

Mice transgenic for the PrP ORF under the control of an interferon regulatory factor-1 promoter/Eμ enhancer (18), which expresses PrP in the lymphoreticular system, accumulated prions in spleen after i.p. inoculation (12). However, $Prnp^{0/0}$ mice expressing PrP transgenes under the control of T lymphocyte- or liver-specific regulatory elements failed to replicate prions, suggesting that expression of PrP^C is not sufficient to enable prion replication (12).

Our current experiments show that CD19-directed expression of PrP^C on B lymphocytes fails to sustain prion accumulation in the spleens of PrP knockout mice. Could it be that in wild type, spleen prion replication occurs in immature B cells that, in CD19-PrP transgenic animals, do not yet express PrP? This is unlikely, because CD19 transgene expression starts at the stage of pro- and preB cell development in the bone marrow (17, 19), whereas the spleen is populated by naive, CD19-expressing mature B cells. Therefore, the result of our experiment must be attributed either to the inability of splenic B cells to replicate

Table 1. Characteristics of transgenic mouse lines

Mouse line	Gene copy number*	PrP RNA† (spleen)	PrP‡ (spleen)	PrP§ (leukocytes)
$Prnp^{+/+}$	2	1	1	1
$Prnp^{+/0}$	1	ND	0.5	ND
TgN306 hemizygous	5	0.55	ND	0.4
TgN306 homozygous	10	ND	0.8	ND
TgN431 hemizygous	15	ND	50–100	10–20

ND, not determined.
*Relative to wild type; determined by Southern blot analysis.
†Relative to wild type; determination of PrP mRNA by quantitative Northern blot analysis.
‡Relative to wild type; determination of PrP^C by densitometry of Western blots.
§Relative to wild type; determined by FACS analysis.

Table 2. Prion titers in spleens of transgenic and control mice

Mouse line	Weeks after inoculation	Indicator mice succumbing to scrapie (unrelated causes)*	Days to terminal disease	Titer‡
Prnp$^{+/+}$	2	4/4	74 ± 2	6.2
	4	4/4	73 ± 2	6.3
	8	4/4	66 ± 1	7.1
Prnp$^{+/0}$	2	8/8	76 ± 3	6.0
	4	8/8	75 ± 2	6.1
	8	7 (1)/8	75 ± 2	6.1
Prnp$^{0/0}$	2	1/8	129, >200	<1.5
	4	0/8	>200	<1.5
	8	0/8	>200	<1.5
TgN306 hemizygous	2	0/4	>200	<1.5
	4	0/4	>200	<1.5
	8	0/4	>200	<1.5
TgN306 homozygous	2	0 (1)/8	>200	<1.5
	4	0/8	>200	<1.5
	8	0/8	>200	<1.5
TgN431 hemizygous	2	6 (2)/8†	99 ± 9	2.7§
	4	1/8	121, >200	<1.5
	8	1/8	185, >200	<1.5

*Tissue homogenates pooled from two animals were transmitted to four indicator mice.

†Individual spleen homogenates of TgN431 mice were transmitted to four indicator mice.

‡Wherever all indicator animals of a group contracted scrapie, titers [logarithm ID_{50} units/10% (vol/vol) homogenate] were calculated by the incubation time method (30) by using the relation $y = 14.37 - 0.11x$, where y is the ID_{50} and x is the incubation time (in days) to terminal disease.

§Titers in spleens of TgN431 mice were calculated by using the relation $y = 11.45 - 0.088x$.

prions or to a failure of prions to reach the spleen in a PrP knockout mouse. However, because spleens of TgN431 mice contained prions 2 weeks after i.p. inoculation, we conclude that B lymphocytes are inherently incapable of replicating prions.

Several lines of evidence show that follicular dendritic cells are the principal sites for synthesis and accumulation of scrapie-specific PrP (PrP^{Sc}) and infectivity in the spleen, and that they contribute, directly or indirectly, to neuroinvasion (9, 10, 20–23). Formation and maintenance of mature FDCs require the presence of B cells expressing membrane-bound lymphotoxin-α/β (24–26). Therefore, the requirement for B cells for prion replication in the spleen and efficient neuroinvasion (7, 27) may be explained readily by their essential role in the maturation of FDCs. This explanation is strengthened by the finding that B lymphocytes which lack PrP^{C} (and are therefore inherently unable to replicate prions) can restore prion accumulation in spleen and neuroinvasion in severe combined immunodeficient (SCID), RAG-1$^{0/0}$, and μMT mice (7). Also, mice expressing PrP in the peripheral nervous system but not in leukocytes were competent in neuroinvasion from the periphery (28).

Although mature B lymphocytes are unable to replicate prions, even when overexpressing PrP, they carry prions when isolated from spleens of wild-type mice inoculated i.p. (8), and they accumulate scrapie-specific PrP (PrP^{Sc}) (A.J.R, R.F., M.A.K., A.A., and C.W., unpublished results). We therefore propose that in wild-type mice, prions associated with B cells originate from other cells, most likely FDCs. Immunoelectron microscopy reveals ubiquitous accumulation of extracellular PrP in complex glomerular dendrites of FDCs (29). Because B cells interdigitate with FDCs, mechanical separation of B cells from the stroma-bound FDCs may result in their sequestering FDC membrane fragments with associated prions. During physiological detachment of B cells from FDCs, this would not occur, explaining why circulating B lymphocytes in scrapie-infected mice are devoid of scrapie infectivity.

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