Humoral immune response to native eukaryotic prion protein correlates with anti-prion protection

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Abstract

Prion diseases are characterized by the deposition of an abnormal form (termed PrP(Sc)) of the cellular prion protein (PrP(C)). Because antibodies to PrP(C) can antagonize deposition of PrP(Sc) in cultured cells and mice, they may be useful for anti-prion therapy. However, induction of protective anti-prion immune responses in WT animals may be hindered by host tolerance. Here, we studied the cellular and molecular basis of tolerance to PrP(C). Immunization of Prnp(o/o) mice with bacterially expressed PrP (PrP(REC)) resulted in vigorous humoral immune responses to PrP(REC) and native cell-surface PrP(C). Instead, WT mice yielded antibodies that failed to recognize native PrP(C) despite immunoreactivity with PrP(REC), even after immunization with PrP-PrP polyprotein and/or upon administration of anti-OX40 antibodies. Consequently, immunized WT mice experienced insignificantly delayed prion pathogenesis upon peripheral prion challenge. Anti-PrP immune responses in Prnp(o/o) mice were completely abrogated by transgenic expression of PrP(C) in B cells, T cells, neurons, or hepatocytes, but only moderately reduced by expression in myelinating cells, despite additional thymic Prnp transcription in each case. We conclude that tolerance to PrP(C) can coexist with immunoreactivity to PrP(REC) and does not depend on thymic PrP(C) expression. Its circumvention might represent an important step toward the development of effective anti-prion immunotherapy.
Humoral immune response to native eukaryotic prion protein correlates with anti-prion protection

Magdalini Polymenidou*†, Frank L. Heppner*‡, Erica C. Pellicioli‡*, Eduard Urich§, Gino Miele*, Nathalie Braun*, Franziska Wopfner‡, Hermann M. Schätzli‡, Burkhard Becher‡, and Adriano Aguzzi*†

Prion diseases are characterized by the deposition of an abnormal form (termed PrPSc) of the cellular prion protein (PrPC). Because antibodies to PrPC can antagonize deposition of PrPSc in cultured cells and mice, they may be useful for anti-prion therapy. However, induction of protective anti-prion immune responses in WT animals may be hindered by host tolerance. Here, we studied the cellular and molecular basis of tolerance to PrPC. Immunization of Prnp0/0 mice with bacterially expressed PrP (PrPRec) resulted in vigorous humoral immune responses to PrPRec and native cell-surface PrPSc. Instead, WT mice yielded antibodies that failed to recognize native PrPC despite immunoreactivity with PrPRec, even after immunization with PrP-PrP polyprotein and/or upon administration of anti-OX40 antibodies. Consequently, immunized WT mice experienced insignificantly delayed prion pathogenesis upon peripheral prion challenge. Anti-PrP immune responses in Prnp0/0 mice were completely abrogated by transgenic expression of PrPC in B cells, T cells, neurons, or hepatocytes, but only moderately reduced by expression in myelinating cells, despite additional thymic Prnp transcription in each case. We conclude that tolerance to PrPC can coexist with immunoreactivity to PrPRec and does not depend on thymic PrP expression. Its circumvention might represent an important step toward the development of effective anti-prion immunotherapy.

Prion diseases or transmissible spongiform encephalopathies are lethal neurodegenerative disorders affecting many animal species. They include bovine spongiform encephalopathy of cattle, chronic wasting disease of deer and elk, and Creutzfeldt–Jakob disease in humans. The causative agent is termed prion (1) and was proposed to be identical with PrPSc, a pathological conformer of the cellular prion protein (PrPC) encoded by the Prnp gene (2). PrPC is expressed on the surface of almost all cells in the body, but at particularly high levels on neurons in the peripheral and central nervous systems. PrPC is essential for the development of prion disease, and Prnp0/0 mice, which lack PrPC, are resistant to scrapie (3).

Bovine spongiform encephalopathy (BSE) (4) is caused by prions amplified through the bovine food chain (5). Transmisison of bovine prions to humans has given rise to variant Creutzfeldt–Jakob disease (vCJD) (6). Although the incidence of BSE and vCJD may be stabilizing or even declining, the steep rise of chronic wasting disease (7) underlines the fact that prion diseases of farm and wild animals still represent a major threat. Thus, there is an urgent and growing need for efficient prophylactic and/or therapeutic measures against prion diseases (8).

Although prions use immune and lymphoreticular cells to gain access to the brain (9), several reports indicate that humoral immune responses to the PrP can antagonize prion infection. This is true even when such responses are directed primarily against PrPSc and do not selectively target PrPSc, mAbs and F(ab) fragments recognizing PrP were shown to prevent de novo scrapie infection and to abolish PrPSc as well as prion infectivity in chronically scrapie-infected neuroblastoma cells (10). Further, transgenic expression of anti-PrP antibodies in mice arrested peripheral scrapie pathogenesis (11). In line with our results, White and colleagues (12) confirmed the efficiency of anti-PrP antibodies in preventing prion disease by injecting such antibodies into WT mice upon peripheral prion challenge.

The prionostatic efficacy of anti-PrP antibodies is highest in extraneural compartments: transgenic 6H3µ mice expressing anti-PrP-specific IgM molecules were not protected when prions were administered intracerebrally (F.L.H. and A.A., unpublished observations), and passive transfer of PrP-specific IgGs (12) was inefficient when started after onset of clinical signs. This finding may be caused by the limited influx of Igs into the CNS and the high prion load of clinically symptomatic animals. By providing stable, sustained titers, active immunization may obviate some of the problems listed above. However, host tolerance to endogenous PrPSc remains a major obstacle to devising active immunization regimens. Nevertheless, several recent studies suggest that the induction of anti-PrP antibodies in WT mice is in principle feasible (13–18). Although anti-PrP Ig titers could be measured in most of these studies, the titers were rather low. Accordingly, the biological efficacy of these immunization series, if evaluated at all, was limited, emphasizing the need for alternative strategies.

The current study explores the efficacy of active immunization strategies against PrP. We found that none of these strategies leads to antibody titers to native cell-bound PrPSc as displayed on the cell surface of PrPSc-overexpressing ts33 splenocytes (19). This finding suggests that host tolerance to endogenous PrPSc is nonpermissive to generating high-affinity anti-PrP B cell clones or leads to deletion or anergy of the cognate T cell clones. To gain further insight into the mechanism of tolerance to PrP, a key to defining successful immunization strategies against prions in the future, we investigated the immune responses of transgenic mice expressing expression of PrPSc restricted to specific cell types. Expression of PrPSc within the thymus did not completely prevent humoral immune responses to PrPRec (19). However, extravascular and extraneural PrPSc, even if expressed in very small amounts, blocked all immune responses to both PrPSc and PrPRec. While antibodies recognizing cell-surface PrPSc interfered with prion pathogenesis in two independent paradigms, we...
found that humoral immune responses were not protective if their affinity was restricted to PrPREC.

Methods

Mice. All mice were maintained under specific pathogen-free conditions. WT mice F1 progeny of mixed background C57BL/6 × 129Sv were purchased from Harlan (Horst, The Netherlands) to match the genetic background of Prnp ko and all of the transgenic mice used: CD-19-PrP [Tg431 or Prnptm1-Tg(CD19-Prnp)431Zbz] (20), lck-PrP [Tg33 or Prnptm1-Tg(lck-Prnp)191Zbz], albumin (Alb)-PrP [Tg01 or Prnptm1-Tg(Alb-Prnp)431Zbz] (19), and neuron-specific enolase (NSE)-PrP [Tg1/52 or Prnptm1-Tg(NSE-Prnp)115Zbz] (O. Giger, M. Glatzel, B. Navarro, and A.A., unpublished data). Myelin basic protein (MBP)-PrP [Tg640 or Prnptm1-Tg(MBP-Prnp)640Zbz] transgenic mice express the full PrP ORF (21) under the control of the MBP promoter. Transgenic founders were mated to Prnp ko mice, and one transgenic line, designated tg640, was established from the F1 progeny on a Prnp ko mixed background C57BL/6 × 129Sv. Further breeding yielded the homozygous line tg640/+. (22).

Recombinant Proteins. Generation of recombinant PrP-PrP polyprotein has been described (23). PrP-PrP contains a tandem duplication of amino acids 23–231 of the murine PrP lacking the N- and C-terminal signal peptides (amino acids 1–22 and 232–254, respectively). The PrP-PrP protein includes an N-terminal polyhistidine tag that allows purification using a Ni²⁺/H₅. After washing, plates were incubated with 30 μl of 200 μg/ml, 0.1% (vol/vol) Tween 20 (PBST), and blocked with 5% BSA. After washing, plates were incubated with 30 μl of 2-fold serially diluted serum (1:20 prediluted) in PBST containing 1% BSA and then probed with horseradish peroxidase-conjugated rabbit anti-mouse (IgG1, M.P., Steinmetz, and A.A., unpublished data) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (Zymed). All sera were diluted 1:100 in blocking buffer. For detection of PrP Sc, brain homogenates of prP-inoculated mice were prepared in PBS, 0.05% sodium deoxycholate, and 0.05% Nonidet P-40. One part of each homogenate was removed and digested with proteinase K (25 μg/ml) to digest PrP Sc. Samples corresponding to 40 μg of total proteins (+/− proteinase K digestion) were loaded on an SDS/PAGE and transferred onto a nitrocellulose membrane incubated with monoclonal anti-PrP antibody POM-1 (M.P., Steinmetz, and A.A., unpublished data) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (Zymed). Blots were finally incubated with HRP substrate (ECL, Pierce) and exposed on photosensitive film (Kodak).

Recall Assay. Carboxyfluorescein (CFSE) Staining. Mice were immunized with PrPREC or keyhole limpet hemocyanin (KLH; Sigma) by s.c. injection of 200 μg of the respective protein emulsified in CFA. Seven days later, draining lymph nodes (inguinal and axillary) were isolated and homogenized to a single cell suspension. To perform proliferation recall assays, cells were plated in a 96-well plate at a density of 2 × 10⁶ cells per well, which had been coated with KLH or PrPREC, respectively, at a concentration of 50 μg/ml before seeding cells. Twenty four hours later, 5 μCi/ml radioactive [3H]thymidine was added. Twenty four hours later, cells were harvested, and radioactivity was measured with a β-counter system (1450 MicroBeta, PerkinElmer). CFSE staining was accomplished by adding CFSE at a final concentration of 10 μM to lymph node cells that had been adjusted to 1.5 cells × 10⁶/ml. Cells were incubated with CFSE for 10 min at room temperature, avoiding bright light, washed once, and, thereafter, cultured in 6-well plates (4 × 10⁶ per well) in the presence or absence of KLH or PrPREC respectively at a concentration of 50 μg/ml. Four days later, cells were stained with allophycocyanin (APC)-labeled anti-B220 (B cells), APC-labeled anti-CD4 (T helper cells), or APC-labeled anti-CD8 (cytotoxic T cells) antibodies after one further washing step, and FACS analysis was performed, as described above. All antibodies were obtained from Becton Dickinson.

Isolation of Total RNA and Quantitative Real-Time RT-PCR. Thymi of sex-matched 6- to 8-week-old mice were dissected, immediately frozen in liquid nitrogen, and stored at −80°C until further use. Total RNA was isolated by using a variable speed polytron homogenizer and TriZol (Life Technologies) according to the manufacturer’s instructions and stored as an ethanolic precipitate at −20°C. Approximately 20 μg of total RNA was processed for...
removal of contaminating genomic DNA (DNA-Free, Ambion, Austin, TX). First-strand cDNA was synthesized by using 5 μg of DNase-treated RNA and NotI-oligo(dT) as primer in a 15-μl reaction volume according to the manufacturer’s instructions (Amersham Pharmacia Biotech). In parallel, identical reactions were prepared omitting the reverse transcriptase mix to allow validation of complete removal of potentially contaminating genomic DNA (β-actin PCR, 40 cycles).

Assuming a 1:1 RNA/cDNA conversion, 33.3 ng of first-strand cDNA was used as template in real-time quantitative PCR measurement of PrP RNA expression levels (Applied Biosystems ABI Prism Sequence Detection System 7700) using SybrGreen technology (PerkinElmer). Three mice of each genotype were analyzed; each sample was measured in triplicate. PrP expression levels were normalized to β-actin values and expressed as arbitrary values. All transgenic mice were on the Zrch I Prnp o/o background (25), which express a PrP-neomycin fusion transcript. Thus, the following primers were designed to allow exclusive measurement of transgenic PrP RNA levels:

Prnp-F, 5’-gctggccctctttgtgacta-3’;
Prnp-R, 5’-ctgggcttgttccactgatt-3’;
actin-F, 5’-gacggccaggtcatcactat-3’; and actin-R, 5’-acatctgctggaaggtggac-3’.

Fig. 1. Immunization of Prnp+/- mice induces anti-PrPβEIC but not anti-PrPα responses and is not protective. (A) Mice were immunized three times with PrP-PrP in 2-week intervals. Mice were then inoculated with prions i.p. (black arrow) and boosted with PrP-PrP every month until the first clinical signs of scrapie were recorded. (B) Antibody titers to PrPβEIC were investigated by ELISA. Upon PrP-PrP immunization, Prnp+/- mice developed early and high anti-PrPβEIC titers, whereas Prnp+/- mice showed much lower titers at days 26–32 after immunization. (C) Specificity of measured titers. ELISA plates were coated with recombinant mouse PrP (mPrP23-231), a truncated variant of it (mPrP21-231), or an unrelated recombinant protein produced by a similar method in E. coli. The results indicate that antibodies were indeed directed to PrP rather than residual bacterial contaminants. MIF, Macrophage inhibitory factor. (D) Western blot using preimmune and immune serum of Prnp+/- and Prnp+/- mice and monoclonal anti–PrP antibody 6H4 for control. All strips were incubated with the same α-mouse IgG secondary antibody to allow comparison. Immune serum of both immunized mice specifically recognized PrPβEIC, but not BSA, which was loaded in the same amount as a negative control. Preimmune serum of the same Prnp+/- mouse showed no reactivity to PrPβEIC. (E) Survival plot visualizing incubation times until development of terminal disease of immunized Prnp+/- mice after i.p. challenge with prions. Two immunized mice that received anti-OX-40 did not develop scrapie symptoms until 585 days postinoculation, when they were killed. (F) Western blot analysis of brain homogenates of prion-inoculated immunized mice. The two mice that displayed no signs of disease after >580 days postinoculation showed no PrPβ accumulation in the brain. (G) Flow cytometric analysis of sera from immunized mice on tg33 transgenic splenocytes with PrP overexpression restricted to T cells. Cells were gated for live splenocytes and subsequently for CD3+ T cells. Histograms represent the intensity of the binding of anti-PrP antibodies present in sera of immunized mice. Serum of Prnp+/- mice at days 26 or 31 after immunization showed specific recognition of native PrPα (Left). Instead, sera of immunized WT mice and preimmune sera did not recognize native PrPα (Right).
Results

We used several immunization strategies to overcome tolerance to PrP\textsubscript{C}. In a first set of experiments, mice were immunized with bacterially expressed recombinant full-length PrP (PrP\textsubscript{REC}) emulsified in CFA and IFA, respectively in combination with anti-OX40 antibody (anti-CD134). This is an agonistic antibody against the signaling molecule CD134, which recently has been shown to break T cell tolerance (26). However, in contrast to other reports (14), immunization using PrP\textsubscript{REC} in CFA/IFA did not result in measurable anti-PrP\textsubscript{REC} titers in WT mice in our hands as assessed by ELISA. Additional administration of anti-OX40 antibodies resulted in an insignificant increase of Ab titers (data not shown).

A further immunization approach used a recombinant PrP-PrP polyprotein emulsified in CFA/IFA in combination with anti-OX40 antibodies (Fig. 1A), which had been previously shown to elicit antibody responses in WT mice that cured scrapie-infected neuroblastoma cells in vitro (23). Upon PrP-PrP/CFA immunization, WT mice displayed anti-PrP\textsubscript{REC} titers as assessed by ELISA (Fig. 1B) and Western blot (Fig. 1D). Titers were considerably lower than those of PrP-PrP/CFA-immunized Prnp\textsuperscript{-/-} mice and were not augmented by anti-OX40 (Fig. 1B). Usage of an irrelevant *Escherichia coli*-derived protein as ELISA substrate confirmed the specificity to PrP\textsubscript{REC} of the respective sera (Fig. 1C).

To determine whether antibodies recognized native PrP\textsubscript{C}, sera were added to splenocytes of tg33 mice, which transgenically overexpress PrP\textsubscript{C} on T cells (19). Binding of serum Ig to native PrP\textsubscript{C} was assessed by FACS as described (11). Whereas sera of PrP-PrP/CFA-immunized Prnp\textsuperscript{-/-} mice strongly stained tg33 splenocytes, none of the WT sera contained antibodies recognizing native PrP\textsubscript{C} regardless of the immunization method or time point of analysis (Fig. 1G).

To determine whether the anti-PrP Abs produced upon immunization were of any therapeutic value, we challenged PrP-PrP/CFA-immunized WT mice with scrapie prions by i.p. injection of the Rocky Mountain Laboratory strain. Despite the presence of substantial anti-PrP\textsubscript{REC} titers, immunized WT mice did not exhibit significant delays in the onset of prion disease (Fig. 1E), except for two of eight PrP-PrP/CFA-immunized WT mice that did not develop scrapie and, accordingly, did not display measurable cerebral PrP\textsubscript{SC} at 585 days postinoculation (Fig. 1F). This finding suggests that antibodies to PrP\textsubscript{REC} can be induced in WT mice, yet these antibodies do not recognize native PrP, do not interfere with scrapie pathogenesis, and may not be of therapeutic value.

To test the latter hypothesis, we asked whether those anti-PrP antibodies that were previously shown to interfere with prion disease would recognize native PrP\textsubscript{C} on the surface of tg33 T cells. We found that sera from 6H4tg33 transgenic mice (carrying the Prnp\textsuperscript{-/-}, Prnp\textsuperscript{+/-}, or Prnp\textsuperscript{+/-} genetic background) showed unambiguous binding to tg33, but not to Prnp\textsuperscript{-/-} T cells (Fig. 2). In addition, mAb ICSM18, which was shown to exert anti-prion activity in vivo (12), reacted strongly with tg33 T cells (Fig. 1G). These results add strength to the conjecture that binding to native PrP\textsubscript{C} is crucial for anti-prion protection.

The above results point toward host tolerance as a crucial barrier to the induction of functional anti-PrP immune responses. To understand the cellular requirements of tolerance to PrP\textsubscript{C}, we investigated whether the tissue-specific distribution of PrP determines the outcome of anti-PrP immunization. PrP\textsuperscript{-/-} (Prnp\textsuperscript{-/-}) mice transgenically expressing PrP\textsubscript{C} exclusively on B cells (20) or T cells (19) were immunized with mouse PrP\textsubscript{REC}/CFA. Expression of PrP\textsubscript{C} in either B or T cell compartment sufficed to prevent generation of anti-PrP\textsubscript{C} titers independent of the amount of PrP\textsubscript{C} expressed (Table 1). Even small amounts of PrP\textsubscript{C} expressed solely on B cells in transgenic tg306 mice (20) completely inhibited the induction of anti-PrP titers (Table 1).

To determine whether PrP\textsubscript{C} expression restricted to nonimmune cells would influence anti-PrP titers upon immunization with PrP\textsubscript{REC}/CFA, we used Prnp\textsuperscript{-/-} mice transgenically expressing PrP\textsubscript{C} under the Alb promoter, which directs PrP\textsubscript{C} expression to hepatocytes (Alb-PrP) (19). In addition, we immunized Prnp\textsuperscript{-/-} mice transgenically expressing PrP\textsubscript{C} in neurons under the control of the NSE promoter (NSE-PrP; O. Giger, M. Glatzel, B. Navarro, and A.A., unpublished data) or in oligodendrocytes and Schwann cells driven by the MBP promoter (MBP-PrP) (22).

Whereas PrP\textsubscript{REC} immunization of MBP-PrP mice mounted significant anti-PrP\textsubscript{REC} antibody titers, NSE-PrP and Alb-PrP mice were unresponsive to immunizations similarly to WT mice (Fig. 3). Further analysis revealed that anti-PrP antibodies of immunized MBP-PrP mouse consistently recognized native PrP\textsubscript{C} displayed on tg33 T cells 28 days after immunization (Fig. 3B and Table 1). In contrast, none of five PrP\textsubscript{REC} and five Alb-PrP mice developed detectable anti-PrP\textsubscript{C} antibodies. Anti-PrP titers in PrP\textsubscript{REC}-immunized MBP-PrP mice displayed delayed kinetics and were reduced compared to Prnp\textsuperscript{-/-} mice, suggesting that some tolerogenic mechanisms were still operative.

To better understand these surprising differences in the immune response of MBP-PrP, NSE-PrP, Alb-PrP, and control mice upon PrP\textsubscript{REC}/CFA immunization, a recall assay was performed. Lymph node cells of PrP\textsubscript{REC}/CFA-immunized transgenic and control mice were rechallenged with PrP\textsubscript{REC} in vitro, and the proliferative capacity of T and B cells was assessed. Whereas Prnp\textsuperscript{-/-} lymphocytes exhibited vigorous proliferation, lymphocytes of WT, Alb-PrP, and NSE-PrP mice did not proliferate, in agreement with their inability to mount anti-PrP\textsubscript{C} titers in vivo. Instead, MBP-PrP mice displayed moderate lymphocyte proliferation (Fig. 4D). These differences were fully attributable to PrP\textsubscript{REC}-specific responses, because a control antigen used in the same experiment (KLH) induced similar lymphocyte proliferation in all investigated mice (data not shown).

Next, CFSE-labeled B220\textsuperscript{+} B cell, CD4\textsuperscript{+} T helper, and CD8\textsuperscript{+} cytotoxic T cell subsets were analyzed individually for their proliferative capacity upon re-encountering PrP\textsubscript{REC}. B and CD4\textsuperscript{+} T helper lymphocyte subsets derived from MBP-PrP mice displayed proliferative responses comparable to Prnp\textsuperscript{-/-} lymphocytes, whereas CD8\textsuperscript{+} cytotoxic T cells were only moderately proliferative (Fig. 4B and C). In contrast, Prnp\textsuperscript{+/-} lymphocytes showed poor or no proliferative response of B220\textsuperscript{+} B cells and CD8\textsuperscript{+} cytotoxic T cells. Prnp\textsuperscript{+/-} CD4\textsuperscript{+} T helper cells were consistently nonresponsive (Fig. 4B and C). The fact that few B220\textsuperscript{+} B cell displayed some proliferative capacity might ac-
count for the transient induction of scanty anti-PrP IgM antibodies in a minority of PrPrREC-immunized WT mice (data not shown). Such anti-PrP IgM responses are short-lived and do not mature to IgG antibodies (Fig. 3B and Table 1) most likely because of the lack of T helper support. Thus, tolerance to PrP C in WT mice appears to be mainly caused by an inadequate T helper cell activation, as proposed (27).

To better understand why T helper cells of WT, NSE-PrP, Alb-PrP, and, in part, MBP-PrP are tolerized to PrP C, we investigated the level of thymic PrP C expression. Expression of antigens within the thymus induces negative selection, i.e., deletion of self-reactive cells during T cell development, a process known as central tolerance. PrP C protein was undetectable in the thymus of Alb-PrP and MBP-PrP mice (data not shown). Prnp mRNA was detected by real-time RT-PCR in WT and all transgenic lines, but mRNA levels were found to be 62-fold lower in Alb-PrP mice, 17-fold lower in MBP-PrP mice, and all transgenic lines, but mRNA levels were found to be

**Table 1. Transgenes, PrP C expression patterns, and anti-PrP C responses in mice immunized with PrPREC**

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Construct</th>
<th>Main site of PrP expression</th>
<th>Thymic transcription*</th>
<th>Anti-native PrP C response, log10 at day 28</th>
<th>T cell response, proliferation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZH-I</td>
<td>Prnp+/+</td>
<td>None</td>
<td>Undetectable</td>
<td>3–4</td>
<td>3.2 ± 1.68</td>
</tr>
<tr>
<td>WT</td>
<td>Prnp+/+</td>
<td>Ubiquitous</td>
<td>100 ± 15.9</td>
<td>0–1</td>
<td>1 ± 0.12</td>
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<td>Tg33</td>
<td>Lck-PrP</td>
<td>T cells</td>
<td>7,705 ± 428</td>
<td>0–1</td>
<td>0.42†</td>
</tr>
<tr>
<td>Tg306</td>
<td>CD19-PrP</td>
<td>B cells</td>
<td>Not done</td>
<td>0–1</td>
<td>0.35†</td>
</tr>
<tr>
<td>Tg01</td>
<td>Alb-PrP</td>
<td>Hepatocytes</td>
<td>1.5 ± 0.1</td>
<td>0–1</td>
<td>1.23†</td>
</tr>
<tr>
<td>Tg1152</td>
<td>NSE-PrP</td>
<td>Neurons</td>
<td>28.9 ± 6</td>
<td>0–1</td>
<td>0.87†</td>
</tr>
<tr>
<td>Tg640</td>
<td>MBP-PrP</td>
<td>Oligodendrocytes and Schwann cells</td>
<td>5.5 ± 1.3</td>
<td>2–3</td>
<td>1.3 ± 0.58</td>
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</tbody>
</table>

*Relative to WT transcription (arbitrarily set to 100). †n = 2.

**Discussion**

The original goal of this study was to assess the efficacy of PrP C vaccination as an anti-prion strategy. Disappointingly, active immunization of WT mice with various forms of bacterially expressed PrP, even in the presence of a variety of adjuvants, did not significantly interfere with prion disease despite the generation of vigorous humoral immune responses to bacterially expressed PrPREC. This result is in stark contrast to the encouraging results obtained by transgenic B cell reprogramming (11) and passive transfer (12) of anti-PrP C antibodies. We then attempted to elucidate the characteristics discriminating protective vs. nonprotective immune responses. We reasoned that a likely minimal prerequisite might be that protective antibodies recognize the native eukaryotic form of PrP C as expressed on the surface of cells. This hypothesis was analyzed by establishing an assay in which transgenic tg33 T cells, which overexpress PrP C under transcriptional control of the lck promoter, were exposed to sera of immunized mice. Binding was then assessed by quantitative cytofluorimetric analysis. Using this assay, we found that sera of PrPREC-immunized WT mice did not react with PrP C despite responses to PrPREC. In contrast, sera from immunized Prnp–/– mice as well as sera from 6H4μ transgenic mice (Fig. 2) and mAb ICSM18 (Fig. 1G), which was shown to exert anti-prion activity in vivo (12), reacted strongly with tg33 T cells. Therefore, the presence of anti-native PrP C titers appears to correlate with the capability to interfere with prion pathogenesis, and anti-native PrP C antibodies may represent an indispensable precondition for preventing prion diseases. These findings may explain the very modest effects of active PrP immunization attempts on prion pathogenesis that have been reported by others (14) and should lead to a critical reassessment of the value of immunization protocols, leading to immune responses whose affinity to native PrP C has not been investigated (13–18, 28).

What might be the molecular difference between antibodies that only react to PrPREC and those that recognize PrP C? Because bacterially expressed PrP lacks all glycosylation, the
Fig. 4. Cellular responses to PrP<sup>REC</sup> correlate with humoral responses. (A) Thymidine incorporation into lymph node cells 48 h after in vitro restimulation with PrP<sup>REC</sup>. Lymph node cells were taken from mice that had been immunized with PrP<sup>REC</sup> in C57BL/6 7 days earlier and were cultured for 48 h in the presence or absence of PrP<sup>REC</sup>. The proliferation index represents the ratio of thymidine incorporation of antigen-stimulated to unstimulated cells. Each symbol summarizes triplicate cultures from one individual mouse (results from three independent experiments). The dotted line represents a ratio of 1 and corresponds to the thymidine incorporation of unstimulated cells. (B) CFSE stains from lymph node cells of immunized Prnpp<sup>o/o</sup>, Prnpp<sup>1/2</sup>, and MBA-PrP mice 4 days after in vitro restimulation with PrP<sup>REC</sup>. Prnpp<sup>1/2</sup>-immunized mice showed poor or no proliferation of B220<sup>+</sup> (B cells) or CD8<sup>+</sup> (cytotoxic T cells) and totally unresponsive CD4<sup>+</sup> (T helper cells), whereas Prnpp<sup>o/o</sup> and MBA-PrP mice showed comparable proliferation in B and CD4<sup>+</sup> T cells. CD8<sup>+</sup> T cells were only moderately proliferative on some of the MBA-PrP mice. Proliferation index represents the ratio of the percentage of proliferating PrP<sup>REC</sup>-stimulated cells (lower CFSE intensity) to that of unstimulated cells. Results are of two independent experiments (Prnpp<sup>o/o</sup> and Prnpp<sup>1/2</sup>; n = 5, MBA-PrP; n = 6). (C) CFSE stains from lymph node cells of immunized Prnpp<sup>o/o</sup>, Prnpp<sup>1/2</sup>, and MBA-PrP mice 4 days after in vitro restimulation with the antigen (PrP<sup>REC</sup> or KLH). One representative mouse of each genotype is depicted.

Fig. 5. Thymic PrP does not correlate with anti-PrP<sub>REC</sub> immune responses. Thymic PrP mRNA levels of transgenic mice expressing PrP<sub>C</sub> under cell-specific promoters, assessed by RT-PCR. Alb-PrP mice showed only traces of PrP<sub>C</sub> mRNA, whereas MBA-PrP showed moderate and NSE-PrP showed relatively high levels of PrP<sub>C</sub> mRNA.

be more useful, at least in the case of ICSM18 and 6H4 µ; yet our attempts at such immune responses in WT mice were thwarted by their strict tolerance to PrP<sub>C</sub>.

It follows from the above that understanding the tolerogenic mechanisms preventing the generation of anti-native PrP<sub>C</sub> antibodies in WT mice is an essential prerequisite to successfully translating anti-PrP<sub>C</sub> vaccination into practice. Tolerance is unlikely to be entirely caused by specific B cell tolerance: B cells can be easily programmed to express anti-PrP<sub>C</sub> specificities in mice expressing PrP<sub>B</sub> by transgenesis and are therefore permissively expressive for expression of autoreactive B cell receptors to PrP<sub>B</sub>, unless PrP<sub>B</sub> is overexpressed at vastly supraphysiological levels (11). Tolerance to PrP<sub>C</sub> appeared to be tightly regulated and could not be overcome by the administration of stimulatory anti-CD134 antibodies, which were shown to break existing T cell tolerance in another model (26). Whereas two of eight anti-CD134-treated, PrP<sub>C</sub> polyprotein-immunized mice did not develop scrapie upon prion inoculation, the remaining six CD134-treated mice did not show a significant delay in the onset of prion disease. While far from spectacular, these results suggest that the combination of specific immunogens with CD134 stimulation might marginally influence prion pathogenesis.

Because T helper cell assistance is crucial for mediating adequate B cell responses, unresponsiveness or deletion of the appropriate CD4<sup>+</sup> T cell clones is likely to contribute to the lack of protective anti-PrP<sub>C</sub> immune responses in immunized WT mice. T cell tolerance to self-antigens is mediated by deletion of autoreactive T cells in the thymus (central tolerance) as well as by a regulatory control network active in the peripheral immune compartment (peripheral tolerance).

The role of T cell tolerance was probed by immunizing Prnpp<sup>o/o</sup> mice that do not express any PrP<sub>C</sub>, as well as Prnpp<sup>o/o</sup> mice bearing transgenes that direct PrP<sub>C</sub> expression predominately to B cells (CD19-PrP and Igk-PrP) (20). T cells (Ick-PrP) (19), hepatocytes (Alb-PrP) (19), neurons (NSE-PrP), or oligodendrocytes (MBP-PrP). In vitro recall challenges of lymphocytes after immunization showed that B and T lymphocytes from Prnpp<sup>o/o</sup> mice were fully responsive to immunization, whereas expression of PrP<sub>B</sub> by any of the transgenes studied prevented proliferative responsiveness, except for MBP-PrP × Prnpp<sup>o/o</sup> mice, which generated a measurable PrP<sup>REC</sup>-specific lymphocyte response. These data closely correlate with the generation of anti-PrP<sub>C</sub>-specific antibodies, as only Prnpp<sup>o/o</sup> mice, and to a lesser extent MBP-PrP mice, developed measurable anti-PrP<sub>C</sub> humoral immune responses.

Expression of PrP<sub>B</sub> on B and T lymphocytes was obviously expected to be tolerogenic, similar to what had been found in the

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hen-egg lysozyme transgenic model (29). Less surprising, minute PrPC expression levels in any extracerebral nonimmune compartment but myelinating cells prevented humoral anti-PrPC immunity as well. To improve our understanding of the above phenomena, we determined the thymic transcription levels of Prnp in all PrP transgenic mice. Thymic PrP expression did not appear to correlate well with the extent of tolerance, as MBP-PrP thymi yielded stronger signals than Alb-PrP thymi.

Persistent self-antigen in extrathymic compartments can also induce tolerance, and this is one of the mechanisms by which peripheral tolerance is mediated. A compartment that has been shown to be very efficient in conferring peripheral tolerance is the liver (30), which is in line with the complete lack of anti-PrP responses in PrP-immunized Alb-PrP mice despite minimal thymic PrP transcription. Although it is widely held that expression of tissue-specific antigens (TSAs) is excluded from the responses in PrP-immunized Alb-PrP mice despite minimal PrPC expression levels in any extracerebral nonimmune compartment that has been intrinsically tolerant to PrPC (11), and thymic expression of PrPC did not suffice to quench anti-PrP antibody generation, neither B cells nor thymic T helper deletion can fully account for host tolerance to PrP. Therefore, we infer that control by peripheral T helper cells may constitute one of the limiting steps when attempting to break tolerance in Prnp+/− mice.

A recent study illustrated that anti-PrP antibodies upon direct injection into the brain crosslink PrPC and provoke neuronal death (33). This may theoretically represent a serious caveat to successfully and safely implement active vaccination as a viable anti-prion regimen.

Although the development of anti-PrP antibodies recognizing native PrPC in immunized MBP-PrP mice is encouraging, the prionostatic potential of such antibodies cannot be tested directly because oligodendrocytes are intrinsically resistant to prion infection (22). As B cells transgenically expressing an anti-PrP μ chain (6H4μ) in the presence of PrPC are not intrinsically tolerant to PrPC (11), and thymic expression of PrPC did not suffice to quench anti-PrP antibody generation, neither B cells nor thymic T helper deletion can fully account for host tolerance to PrP. Therefore, we infer that control by peripheral T helper cells may constitute one of the limiting steps when attempting to break tolerance in Prnp+/− mice.

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