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The Murine B Cell Repertoire Is Severely Selected against Endogenous Cellular Prion Protein

Sylvie Grégoire,2* Anne Sophie Bergot,2* Cécile Féraudet,† Claude Carnaud,* Pierre Aucouturier,* and Martine Bruley Rosset3*

Abs to the prion protein (PrP) can protect against experimental prion infections, but efficient Ab responses are difficult to generate because PrP is expressed on many tissues and induces a strong tolerance. We previously showed that immunization of wild-type mice with PrP peptides and CpG oligodeoxynucleic acid overcomes tolerance and induces cellular and humoral responses to PrP. In this study, we compared Ab and T cell repertoires directed to PrP in wild-type and PrP knockout (Prnp−/−) C57BL/6 mice. Animals were immunized with mouse PrP-plasmid DNA or with 30-mer overlapping peptides either emulsified in CFA or CpG/IFA. In Prnp−/− mice, Abs raised by PrP-plasmid DNA immunization recognized only N-terminal PrP peptides; analyses of Ab responses after PrP peptide/CFA immunization allowed us to identify six distinct epitopes, five of which were also recognized by Abs raised by PrP peptides/CpG. By contrast, in wild-type mice, no Ab response was detected after PrP-plasmid DNA or peptide/CFA immunization. However, when using CpG, four C-terminal peptides induced Abs specific for distinct epitopes. Importantly, immune sera from Prnp−/− but not from wild-type mice bound cell surface PrP. Abs of IgG1 and IgG2b subclasses predominated in Prnp−/− mice while the strongest signals were for IgG2b in wild-type mice. Most anti-PrP Th cells were directed to a single epitope in both Prnp−/− and wild-type mice. We conclude that endogenous PrP expression profoundly affects the Ab repertoire as B cells reactive for epitopes exposed on native PrPC are strongly tolerated. Implications for immunotherapy against prion diseases are discussed. The Journal of Immunology, 2005, 175: 6443–6449.

T
ransmissible spongiform encephalopathies (TSE)4 are neurodegenerative disorders that are associated with the conversion of the normal host-encoded cellular prion protein (PrPC) into an abnormal conformer, PrPSc. TSE are transmitted through unconventional agents termed “prions,” the major component of which is PrPSc (1, 2). The immune system participates in the transport and replication of prions in peripheral lymphoid organs (3), but no acquired immune response can be evidenced in the course of TSE infections (4). In contrast, Abs to prion protein (PrP) were shown to inhibit PrPSc accumulation in stably infected neuroblastoma cells (5–8) and epithelial Rov cells (9) or to prolong survival when passively transferred into mice infected with scrapie (8, 10) and to protect transgenic mice producing anti-PrP Abs (11).

In a previous work, we demonstrated that PrP knockout (Prnp−/−) mice immunized with mouse PrP plasmid DNA (PrP-pDNA) encoding full-length PrP or with 30-mer PrP peptides in CFA generated PrP-specific T cells and Abs that recognized native PrPC (12). No immune response to PrP could be detected in wild-type (wt) mice using the same immunization protocols, likely due to natural tolerance since PrPC is expressed in the thymus and many peripheral tissues (13). The lack of a response in wt mice could relate to anergy or deletion of B cell and/or Th precursor cells expressing specific Ag receptors for PrP epitopes. However, self-proteins carrying a foreign T cell epitope often induce autoantibodies in normal mice, showing that B cell tolerance is generally incomplete and that autoreactive B cells can be activated when appropriate T cell help is provided (14). Heppner et al. (11) found that transgenic mice expressing an anti-PrP Ig H chain displayed high titers of Abs to PrP along with a normal level of PrPC, suggesting that tolerance to PrP is unlikely to be entirely confined to B cells, but could also be attributed to unresponsiveness or deletion of specific CD4+ T cell clones. Indeed, the development of an immune response is determined not only by exposure of immunogenic epitopes on the injected Ags but also by the presence of functional primary B and T cell repertoires.

Tolerance of Th cells may be overcome with the use of suitable adjuvants. Using three PrP peptides in combination with CpG oligodeoxynucleotides (referred to as CpG) (15), we generated T and B cell responses to PrP peptides in C57BL/6 wt mice (16). In the present study, we compared the anti-PrP T and B cell responses in Prnp−/− and wt mice with the same genetic background. Distinct Ab repertoires were generated after immunization of wt and Prnp−/− mice with PrP-pDNA or 30-mer PrP peptides in CFA or CpG. Notably, Abs that bound to native PrPC were not detected in immunized wt mice. The only anti-PrP Abs raised in peptide-immunized wt mice bound peptide segments that are probably not accessible on cellular PrPC. Most of the anti-PrP Th cell repertoire of Prnp−/− and wt mice was directed to a single epitope

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4 Abbreviations used in this paper: TSE, transmissible spongiform encephalopathy; PrP, prion protein; PrPC, normal cellular PrP; wt, wild type; pDNA, plasmid DNA; PrPSc, pathological conformer PrP; MFI, mean fluorescence intensity.

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located around residues 158–172 in the PrP molecule. These results have important implications for the design of immunotherapeutic strategies against TSE.

Materials and Methods

Mice

Six-week-old female C57BL/6 wt mice were purchased from Janvier. Prnp<sup>−/−</sup> mice (provided by C. Weissmann, Imperial College, London, U.K.) have been backcrossed 10 times into the C57BL/6 background. All mice were bred in specific pathogen-free conditions in accordance with European recommendations on animal ethics.

Peptides and adjuvants

The 30-mer PrP peptides with 15 aa overlaps on each side were synthesized by the Neosystem; some were shorter (especially P10) because of high hydrophobicity which rendered the synthesis difficult. They were purified (>80%) by HPLC on a C8 reverse-phase column and controlled by electrospray mass spectrometry (Table I). A library of 15-mer overlapping peptides covering PrP sequence 140–189 was synthesized under the same conditions (Table II). Oligonucleotide-CpG 1826 (18) was synthesized by Sigma-Aldrich.

Immunization protocol

pcDNA<sub>1</sub> plasmid (Invitrogen Life Technologies) mouse PrP DNA (PrP-pDNA) was prepared and purified as previously described (12). Control DNA consisted in an empty pcDNA<sub>1</sub> plasmid. Mice were injected three times at weekly intervals with 100 μg DNA divided between the two tibialis anterior muscles, treated 5 days before with 50 μl of cardiotonic (10 mM solution in PBS) from <i>Naja nigricollis</i> venom (Latoxan). PrP peptides (100 μg) were mixed with CpG (50 μg) and emulsified in IFA (v/v) and then injected s.c. at the base of the tail. A boost was performed 10–14 days later under identical conditions. Spleen and blood were collected 10–14 days after the last injection.

ELISPOT assay

The number of IFN-γ-producing cells from spleens of immunized mice was evaluated by ELISPOT as previously described (19). Briefly, nitrocellulose-based 96-well plates (Millipore) were coated with anti-mouse IFN-γ-Ab (1/500; BD Biosciences) for 2 h at 37°C followed by overnight incubation at 4°C. Plates were washed with PBS-0.05% Tween 20 (PBS-T) and blocked with RPMI 1640 containing 10% FCS for 2 h at room temperature. Plates were then coated anti-CD3 mAb (2-C11, 10 μg/ml) or with medium alone. Plates were incubated at 37°C in 5% CO2 during 24 h, washed with PBS-T, blocking with 1% nonfat milk in PBS-T and incubated for 2 h at 37°C with biotinylated anti-mouse IFN-γ (BD Biosciences). After washing with PBS-T, alkaline phosphatase-conjugated streptavidin (Roche) was added and left for 2 h; IFN-γ-secreting cells were revealed using nitroblue tetrazolium/5-bromo-4-chloro-indolyl phosphate/diaminobenzidine (Promega), and spots were counted using an ELISPOT plate reader (AID). Test wells were assayed in triplicates and the frequency of IFN-γ-producing cells from spleens of immunized mice was calculated by subtracting the mean number of spots obtained after incubation of medium without peptide. An experimental value was considered significantly positive when over the mean control value plus 3 SD.

Ab titration (ELISA)

Flat-bottom 96-well plates (Maxisorp; Nunc) were coated with 10 μg/ml PrP peptides in sodium carbonate buffer (0.05 M, pH 9.6) overnight at 4°C. Plates were washed with PBS and blocked with 1% nonfat milk in PBS-T for 2 h at 37°C. Serially diluted sera from immunized and control mice were added in duplicate and reacted for 2 h at room temperature. Plates were washed again and 200 μl of peroxidase-conjugated anti-mouse IgG (Roche) was added and left for 2 h at room temperature. Plates were then washed and 200 μl/well of freshly prepared H<sub>2</sub>O<sub>2</sub>-diaminobenzidine substrate solution (Sigma-Aldrich) were added. The reaction was stopped with 2 N sulfuric acid and OD was measured at 492 nm. An experimental value was considered significantly positive when exceeding the mean control value plus 3 SD.

IgG subclass determination of anti-PrP Abs

Sera were serially diluted and tested in duplicates in ELISA as described above. Detection of IgG1, IgG2a, and IgG2b isotypes was performed using specific peroxidase-conjugated goat anti-mouse Abs (Southern Biotechnology Associates).

Flow cytometry

EL-4 cells overexpressing murine PrPC (allotype s7) were obtained after transfection as described elsewhere (12). Cells were activated with plastic-coated anti-CD3 mAb (2-C11, 10 μg/ml) 24 h before testing immune sera for maximal expression of PrPC. The level of PrPC expression was checked on EL-4 cells using FITC-conjugated anti-PrP mAb 8A13 (from Dr. J. Grassi (Commissariat à l’Energie Atomique, Saclay, France). After blocking Fc receptors with Ab 2.4G2 for 20 min at 4°C in FACS buffer, cells were incubated with control or immune sera diluted 1/10 for 20 min at 4°C, washed, and analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences). A serum was considered significantly positive for native PrPC binding when mean fluorescence intensity (MFI) of peptide-immunized serum was over the MFI + 3 SD of sera from mice treated with CFA or CpG alone.

Results

Anti-PrP B cell repertoire in Prnp<sup>−/−</sup> mice and wt mice

To have an exhaustive view of Ab repertoires, Prnp<sup>−/−</sup> and wt mice were immunized with PrP-pDNA. Epitope mapping was performed using a library of overlapping PrP peptides covering the entire sequence of PrP (Table I). Abs generated in Prnp<sup>−/−</sup> are directed against linear epitopes located at the N-terminal part of the PrP molecule (shown by ELISA, Fig. 1A). Five peptides were most frequently recognized by individual immune sera: P1 (12 of 15, 80%), P2 (9 of 15, 60%), P3 (3 of 15, 21%), P4 (8 of 15, 53.3%), and P5 (3 of 15, 20%). Titration of individual sera on peptides indicated that Abs to P1, P2, and P4 dominated the response elicited by PrP-pDNA (Fig. 1B). Under the same immunizing conditions, no Abs against peptides were detected in wt mice (data not shown).

In a second set of experiments, Prnp<sup>−/−</sup> and wt mice were immunized twice with each of the overlapping PrP peptides emulsified in CFA and serum reactivities to the corresponding peptides were tested by ELISA. Results (Fig. 2A) indicated that 8 of 12 peptides elicited in Prnp<sup>−/−</sup> mice Ab responses with variable frequency and intensity. These data confirmed that P1, P2, P4, and P5 contain immunogenic epitopes as shown above with antisera raised against PrP-pDNA. In addition, P8, P9, P11, and P12 also generated Abs.

In contrast, no Ab response could be obtained by immunizing wt mice with peptides in CFA (Ref. 14, and data not shown). On the basis of our previous findings (16), wt mice were then immunized twice with each of the peptides mixed with CpG. Sera collected 10–12 days later were analyzed for the presence of Abs in peptide ELISA (Fig. 2B). Results failed to demonstrate a serum reactivity for the PrP portions P1–P4 (positions 23–112). As found in Prnp<sup>−/−</sup> mice Ab responses with variable frequency and intensity. These data confirmed that P1, P2, P4, and P5 contain immunogenic epitopes as shown above with antisera raised against PrP-pDNA. In addition, P8, P9, P11, and P12 also generated Abs.

Table 1. Sequence of 30-mer overlapping peptides covering the entire murine PrP

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>P1</td>
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<td>39–67</td>
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<td>P3</td>
<td>68–97</td>
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<td>P4</td>
<td>83–112</td>
</tr>
<tr>
<td>P5</td>
<td>98–127</td>
</tr>
<tr>
<td>P6</td>
<td>118–142</td>
</tr>
<tr>
<td>P7</td>
<td>128–157</td>
</tr>
<tr>
<td>P8</td>
<td>143–172</td>
</tr>
<tr>
<td>P9</td>
<td>158–187</td>
</tr>
<tr>
<td>P10</td>
<td>173–189</td>
</tr>
<tr>
<td>P11</td>
<td>193–218</td>
</tr>
<tr>
<td>P12</td>
<td>212–232</td>
</tr>
<tr>
<td>P13</td>
<td>218–240</td>
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</table>
mice, Abs could be raised against P5 and P11 but not P3, P6, P7, and P10. However, in contrast to Prnp<sup>−/−</sup> mice, wt mice poorly responded to P9 and not at all to P12; only two of eight mice were positive for P8. Because adjuvants may influence immune responses, peptide immunizations of Prnp<sup>−/−</sup> mice were also performed with CpG in the same experimental conditions as those done in wt mice. Fig. 2C shows that P1, P2, P5, P8, P9, and P12 could raise specific Abs with variable frequency and intensity. Some differences appeared when compared with Ab responses obtained with CFA: P4 and P11 did not induce Abs whereas P6 raised low but consistent Ab levels.

**Epitope mapping of PrP segments recognized by Abs elicited in Prnp<sup>−/−</sup> and wt mice**

Because most of the peptides used for specificity determination were 30-mer peptides with 15-aa overlaps, we attempted to determine the number and positions of PrP segments recognized by
Abs. We compared the ELISA reactivity of sera from Prnp<sup>−/−</sup> and wt mice tested against the immunizing peptide and against the upstream and downstream adjacent overlapping peptides.

In Prnp<sup>−/−</sup> mice (Fig. 3, A and B), several sera raised against one immunizing peptide recognized adjacent peptides. Anti-P1 and -P2 sera obtained with peptides/CFA (Fig. 3A) cross-reacted with P1 and P2, suggesting that they shared a common epitope (residues 29–57). Sera from mice immunized with P4 or P5 recognized P4 and P5 but not P3 or P6, suggesting the existence of an epitope shared by P4 and P5 (residues 98–112). Sera from mice immunized with P8 recognized two distinct PrP segments, one shared with P7 (residues 143–157) and another with P9 (residues 158–172). The latter segment was also recognized by sera from P9-immunized mice. Finally, Abs induced by P11 and P12 did not cross-react with each other nor with P10 and P13, and thus seemed to recognize two distinct epitopes. The results obtained with Prnp<sup>−/−</sup> mice after immunization with peptides/CpG indicated that Abs also recognized the epitopes shared by P1/P2, P4/P5, P7/P8, P8/P9, and P12 (Fig. 3B).

In wt mice immunized with peptides/CpG, no significant reactivity to P1, P2, and P4 was detected. P5 elicited Abs that did not cross-react with P4 or P6 and thus likely recognized a PrP segment around residues 112–118, different from the segment 98–112 recognized by P5-immunized Prnp<sup>−/−</sup> mice sera (Fig. 3C). P8-immunized sera cross-reacted with P9 and therefore contained Abs recognizing a segment located around residues 158–172. In contrast, Abs to P9 did not cross-react with adjacent peptides and were thus specific for a segment located around residues 173–187, different from the segment 158–172 recognized by P9-immunized Prnp<sup>−/−</sup> mice sera. P11 elicited Abs which did not bind to P10 and P12 (residues 193–212).

The PrP portion between residues 143 and 187 appeared to contain three distinct immunogenic segments which were recognized differently in Prnp<sup>−/−</sup> and wt mice after immunization with P8 and P9: one shared by P7/P8, another by P8/P9, and one on P9 not shared by P8. To localize more precisely these epitopes, we used a library of 15-mer peptides with 4 aa overlaps covering sequence shared by P8. To localize more precisely these epitopes, we used P9: one shared by P7/P8, another by P8/P9, and one on P9 not used instead of CFA (data not shown).

### Table II. Sequence of 15-mer overlapping peptides covering residues 140–189

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tr>
<td>D</td>
<td>140–154</td>
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<tr>
<td>E</td>
<td>144–158</td>
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<tr>
<td>F</td>
<td>148–162</td>
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<tr>
<td>G</td>
<td>152–166</td>
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<td>H</td>
<td>156–170</td>
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<td>I</td>
<td>160–174</td>
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<tr>
<td>J</td>
<td>164–178</td>
</tr>
<tr>
<td>K</td>
<td>168–182</td>
</tr>
<tr>
<td>L</td>
<td>172–186</td>
</tr>
<tr>
<td>M</td>
<td>176–189</td>
</tr>
<tr>
<td>N</td>
<td>180–194</td>
</tr>
<tr>
<td>O</td>
<td>184–198</td>
</tr>
</tbody>
</table>

### FIGURE 4. Mapping B cell epitopes: sera from individual Prnp<sup>−/−</sup> and wt mice immunized with the 30-mer overlapping P8 (A) or P9 (B) with CFA or CpG, respectively, were tested in ELISA on 15-mer overlapping peptides covering PrP sequence 140–189. Results represent the mean reactivity (±SD) of five sera from P8- or P9-immunized Prnp<sup>−/−</sup> mice and of two sera from P8- or six from P9-immunized wt mice.

**Binding capacity of immune sera to native PrPC**

All sera from Prnp<sup>−/−</sup> mice immunized three times with PrP-pDNA strongly bound native PrPC expressed at the surface of activated Prnp<sup>-/-</sup>-transfected EL-4 cells, with a MFI ranging from 34 to 72 (Fig. 5A).

Peptide-specific sera from Prnp<sup>−/−</sup> mice were also analyzed for their binding capacity to native PrPC (Fig. 5B). All sera from mice immunized with P5 bound to PrPC with a high MFI whether mice were immunized emulsified in CFA or in IFA/CpG. Two of six P1 immune sera obtained with CFA and one of four P1 immune sera obtained with CpG displayed low but significant binding to EL-4 cells. Surprisingly, four of eight positive sera from mice immunized with P8 and CFA significantly bound also to EL-4 cells while none of those obtained with CpG bound to EL-4 cells. Sera specific for P2, P4, P6, P7, P9, P11, and P12 did not bind native PrPC.

In contrast to Prnp<sup>−/−</sup> mice, none of the sera collected from wt mice, whether immunized with PrP-pDNA or peptides with CFA or CpG, significantly stained PrPC on EL-4 cells (Fig. 5C).

**IgG subclasses of anti-PrP Abs**

Because IgG subclasses display diverse effector properties, we compared the IgG isotype patterns of Abs raised in Prnp<sup>−/−</sup> and wt mice after immunization with P5, P8, or P9 emulsified in IFA/CpG. The strongest signal was found for IgG1 and IgG2b subclass Abs. We compared the IgG isotype patterns of Abs raised in Prnp<sup>−/−</sup> and wt mice. Identical iso-type patterns were observed in Prnp<sup>-/-</sup>-transfected EL-4 cells, with a MFI ranging from 34 to 72 (Fig. 5A).

**T cell repertoire in Prnp<sup>−/−</sup> and in wt mice**

The quality of Ab responses is also dependent on specific Th activity. In contrast to Prnp<sup>−/−</sup> mice, the T cell repertoires specific for PrP was not stimulated in wt mice after immunization with PrP-pDNA or PrP peptides mixed with CFA (data not shown and Refs.
12 and 16). However, we previously demonstrated that P9 and, to a lesser extent, P8 administered with CpG induced T cell responses in wt mice (16). Thus, we compared the T cell repertoire of Prnpo/o and wt mice generated after immunization with each peptide emulsified in IFA/CpG and we measured the frequency of specific IFN-γ-secreting T cells in ELISPOT. Fig. 7A shows that in Prnpo/o mice, P9 appears as the most immunogenic, yielding a frequency (mean ± SE) of 306 ± 64 IFN-γ-secreting T cells/10⁶ splenic cells. P8 also stimulated a significant but lower number of IFN-γ-secreting T cells (97 ± 32). No other tested peptide consistently generated a T cell response although a few mice yielded significant numbers of IFN-γ-secreting peptide-specific T cells (Fig. 7A). In wt mice, P9 was also the most immunogenic (201 ± 26 IFN-γ-secreting T cells/10⁶ spleen cells) but only one of five mice responded to P8 (Fig. 7B). P8 or P9 immune spleen cells cross-reacted with both P8 and P9 and thus were specific for a shared epitope located between residues 158–172. This was true for wt mice (16) and also for Prnpo/o mice immunized with PrP-pDNA as well as with peptides in CFA (12) or CpG (data not shown).

Discussion
Studies of Ab responses in wt and Prnpo/o mice with the same C57BL/6 genetic background indicates that the endogenous expression of PrP qualitatively affects the specific B cell repertoire: in wt mice, no Ab response was detected to N-terminal epitopes of PrP. In addition, Abs raised in wt mice recognized PrP segments not exposed on membrane-bound PrPC and were distinct from those recognized by Abs raised in Prnpo/o mice.

Immunization of Prnpo/o mice with PrP-pDNA activated an Ab repertoire preferentially directed to peptides P1–P5, which are located in the N-terminal region and may thus be considered as immunodominant epitopes (Fig. 8). In addition, such sera contained Abs that bound native PrPC on activated Prnp-transfected EL-4 cells. However, in a competitive assay, addition of peptides P1–P5 lowered the reactivity of PrP-pDNA sera for native PrPC only by 30–50% (data not shown), strongly suggesting the presence of

FIGURE 5. Ab binding to native PrPC: A, sera from Prnpo/o mice immunized with PrP-pDNA; B, with PrP peptides with CFA (○) or CpG (●); and C, sera from wt mice immunized with PrP peptides and CpG were incubated (1/10 diluted) with EL-4 cells overexpressing PrPC. Abs were revealed with PE-conjugated antimouse Ig and fluorescence was analyzed by FACS. Each point represents the MFI of an individual serum.

FIGURE 6. Ab IgG subclass determination: sera (1/50 diluted) from Prnpo/o and from wt mice immunized with peptides and CpG were tested in ELISA plates coated with the corresponding peptide and revealed with peroxidase-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b.

FIGURE 7. Frequency of IFN-γ-secreting peptide-specific T cells in Prnpo/o and wt mice measured in ELISPOT. Mice were immunized s.c. and boosted once with 100 µg of each PrP peptide with CpG. Spleenocytes from immunized mice were restimulated for 24 h in vitro with 10 µg of the corresponding peptide per ml. Each point represents the frequency of IFN-γ-secreting peptide-specific T cells per 10⁶ spleen cells for individual immunized mouse.
additional Abs specific for conformational epitopes. Other PrP segments (P8, P9, P11, and P12 emulsified in CFA) elicited Abs after immunization of Prnp<sup>−/−</sup> mice. Epitope mapping using adjacent 30-mer overlapping peptides and a panel of 15-mer overlapping peptides covering the 140–189 (P8/P9) PrP region revealed that Prnp<sup>−/−</sup> B cells produced Abs specific for at least six distinct immunogenic segments of PrP. Because the use of CpG instead of CFA might cause differences in immune responses, Prnp<sup>−/−</sup> mice were also immunized with peptides/CpG. Abs raised in these conditions recognized six distinct epitopes (Fig. 3, A and B), five of which seemed identical to those recognized by Abs induced by immunization with peptides/CFA (Fig. 8).

By contrast, injection of PrP-pDNA or peptides with CFA did not elicit detectable Ab or T cell responses in wt mice (12). CpG oligodeoxynucleotides, which are known to stimulate dendritic cells (20) and B cells via TLR9 (21), allowed us to generate immune responses in wt mice (16). When peptides were injected with CpG, wt mice produced Abs only against four segments located at the C-terminal part of the PrP molecule (Fig. 8). Characterization of epitope specificities further demonstrated that Abs to segments P5 and P9 recognized epitopes distinct from those recognized by Abs raised in Prnp<sup>−/−</sup> mice.

These findings suggest that the lower Ab responses from wt mice are not due to the inability of CpG to stimulate Ab production but rather to unresponsiveness of B cells specific for the N-terminal peptides. However, Abs to P6 could be stimulated in Prnp<sup>−/−</sup> mice immunized in the presence of CpG while Abs to P11 could not. These results may be attributed to the type of response elicited by CFA or CpG: although both adjuvants promote T cell responses toward Th1 (22), CpG stimulated a greater number of CD4<sup>+</sup> IFN-γ-secreting Th cells (23) and was able to break the tolerance (16–23).

Another interesting observation was that none of the Abs elicited in wt mice recognized membrane-bound PrPC, whereas several Abs elicited in Prnp<sup>−/−</sup> mice did (particularly those specific for the 98–112 segment). Yet, we previously showed that Abs to P5, P8, and P9 from both Prnp<sup>−/−</sup> or wt mice reacted with plastic-bound recombinant PrP (12, 16). Our observations are in accordance with those of Polymenidou et al. (24), who found that Abs produced by wt mice after immunization with recombinant PrP failed to recognize native membrane PrPC despite reactivity for recombinant PrP in ELISA. They postulated that membrane PrPC and recombinant PrP differed in some structural features or that the microenvironment of PrPC with associated proteins or lipids masked or distorted epitopes that were exposed on recombinant PrP.

That Abs to native PrPC were not detected in wt mice’s immune sera might relate to a lower sensitivity of the cyttofluorometric assay as compared with ELISA. However, Ab levels measured by ELISA in sera positive for P5 or P8 were similar in both Prnp<sup>−/−</sup> and wt mice. It is also possible that in PrP<sup>−/−</sup> mice, Abs would be produced but spontaneously form complexes with endogenous PrPC, thus preventing their detection in the serum (Ref. 25 and C. Féraudet, manuscript in preparation). Although no Ab-PrP complexes could be detected in tested wt sera (data not shown), Abs may also be adsorbed on cell surface PrPC, which is expressed on several hemopoietic cell lineages (13).

Alternatively, Abs to cellular PrPC might not be produced in wt mice because B cells reacting against segments that are exposed on PrPC are either tolerized or lack T cell help. Anti-P5 Abs raised in Prnp<sup>−/−</sup> mice recognized segment 98–112 and bound to native PrPC while in wt mice, they were specific for segment 112–118, and failed to bind native PrPC, strongly suggesting that B cells specific for 98–112 are anergized or deleted in wt mice. In P8-immunized Prnp<sup>−/−</sup> mice, four of eight sera recognized native PrPC and were specific for segment 143–157 while sera which did not bind native PrPC recognized segment 158–172. In accordance with these data, wt mice immunization by P8 generated Abs only to segment 158–172 and they did not bind to native PrPC. This dual reactivity might explain the lack of binding to native PrPC of sera obtained after immunization of Prnp<sup>−/−</sup> mice with P8 using CpG as adjuvant. These sera contained Abs specific for two distinct segments, some of them recognizing native PrPC (Abs to 143–157) and others that did not (Abs to 158–172); the proportion of these specificities might dictate the capacity of immune sera to bind native PrPC.

Difference in the location of the epitopes recognized by Abs from wt and Prnp<sup>−/−</sup> mice after immunization with the same peptide suggest that B cells specific for PrP epitopes expressed on native PrPC are strongly tolerized in wt mice. Only B cells potentially binding epitopes that are not exposed on PrPC would escape tolerance and be the main, if not exclusive, contributors to the autoantibody response. Self-proteins expressed as membrane-bound surface Ags result in the deletion of specific B cells during bone marrow ontogeny (26). In contrast, a substantial number of autoreactive B cells, specific for the unfolded conformation of a self-Ag, escape tolerance (27).

Abs raised by peptide immunization in Prnp<sup>−/−</sup> and wt mice displayed different patterns of IgG subclasses. Immunization of Prnp<sup>−/−</sup> mice, whatever the peptide and the adjuvant used, induced predominantly IgG1 and IgG2b subclasses, whereas in wt mice, the strongest signals were found for IgG2b. It was reported that T cells increase the Ig switching process with a hierarchy of IgG subclass Ab production which directly correlated with the 5′ to 3′ IgH-C gene order, i.e., IgG1>IgG2b>IgG2a (28, 29). Consequently, the predominance of IgG2b in wt mice might be related to the skewing toward a more Th1 cell-dependent response than in Prnp<sup>−/−</sup> mice, possibly because of the tolerant context.
We may exclude the possibility that the observed differences could be related to genetic disparities in antigenic background, since Prnp<sup>-/-</sup> mice had been backcrossed 10 times with C57BL/6 mice. Furthermore, no proliferation was found when splenocytes from Prnp<sup>-/-</sup> and wt mice were primed in vivo by each other (data not shown).

Besides a deletion in the B cell repertoire, the Th signal promoting Ab production may be insufficient in wt mice. In general, Ab responses to peptides are weak and highly dependent on T cell help. Only two PrP epitopes could induce a T cell proliferation from Prnp<sup>-/-</sup> but not from wt mice immunized with PrP-pDNA or peptides with CFA (12). We recently demonstrated that CpG allowed us to induce the generation of IFN-γ-secreting T cells specific for an epitope shared by PrP99 in wt mice (16). In this study, the T cell repertoires generated by immunization with PrP peptides/CpG were rather similar in Prnp<sup>-/-</sup> and in wt mice: P99 consistently triggered the strongest T cell responses against a P99/PrP99 shared epitope. Yet, the magnitude of T cell responses to peptides was generally lower in wt mice than in Prnp<sup>-/-</sup> mice, which is compatible with the possibility that the T cell repertoire is partially tolerized in wt mice.

If one assumes that only Abs that bind membrane PrPC are efficient in blocking prion pathogenesis (24), the very modest effects of active immunization of wt mice against PrP in protecting against prion infections are not surprising (30, 31). However, it is worth noting that mAbs that target both normal and pathological forms of PrP blocked PrPSc accumulation in vitro more efficiently than mAbs recognizing PrPC only (9). Abs that do not bind PrPC, such as those generated by peptide immunization with Cpg, might be efficient in blocking PrPSc replication in vivo by targeting epitopes only exposed on PrPSc. B cell repertoires producing such neutralizing Abs might escape central tolerance and reach the periphery. New perspectives in immunotherapeutic approaches in TSE could rely on the stimulation of such B cells: immunogens using appropriate PrP peptides conjugated with a potent, non-self Th epitope could enhance B cell maturation and sustain long-term and high-affinity Ab production in tolerant mice.

Disclosures

The authors have no financial conflict of interest.

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6. Gilch, S., F. Wopfner, I. Renner-Muller, E. Kremmer, C. Bauer, E. Wolf, G. Schmitt-Ulms, I. R. Mehlhorn, G. Legname, Wormald, P. M. Rudd, et al. 2004. Divergent therapeutic and immunologic effects of a deletion in the B cell repertoire, the Th signal promoting Ab production may be insufficient in wt mice. In general, Ab responses to peptides are weak and highly dependent on T cell help. Only two PrP epitopes could induce a T cell proliferation from Prnp<sup>-/-</sup> but not from wt mice immunized with PrP-pDNA or peptides with CFA (12). We recently demonstrated that CpG allowed us to induce the generation of IFN-γ-secreting T cells specific for an epitope shared by PrP99 in wt mice (16). In this study, the T cell repertoires generated by immunization with PrP peptides/CpG were rather similar in Prnp<sup>-/-</sup> and in wt mice: P99 consistently triggered the strongest T cell responses against a P99/PrP99 shared epitope. Yet, the magnitude of T cell responses to peptides was generally lower in wt mice than in Prnp<sup>-/-</sup> mice, which is compatible with the possibility that the T cell repertoire is partially tolerized in wt mice.

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