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The absence of a detectable immune response during transmissible spongiform encephalopathies is likely due to the fact that the essential component of infectious agents, the prion protein (PrP), is a self Ag expressed on the surface of many cells of the host. To overcome self-tolerance to PrP, we used 30-mer PrP peptides previously shown to be immunogenic in Prnp<sup>−/−</sup> mice, together with CpG-oligodeoxynucleotides (CpG) in IFA. Generation of anti-PrP T and B cell responses was analyzed in the spleen, lymph nodes, and serum of immunized C57BL/6 wild-type mice. Immunization with PrP peptides emulsified in CFA did not trigger an immune response to PrP. When CpG were used, vaccination with peptides P143–172 and P158–187 generated IFN-γ-secreting splenic T cells, and only P158–187 significantly stimulated IL-4-secreting T cells. Both peptides induced few Ab-producing B cells, and low and variable serum Ab titers. In contrast, immunization with peptide P98–127 did not induce significant levels of T cell responses but elicited specific peptide Abs. T cell epitope mapping, performed using 15-mer peptides covering PrP segment 142–182, revealed that an immunogenic motif lies between positions 156 and 172. These results demonstrate that T and B cell repertoires against PrP can be stimulated in C57BL/6 when adjuvant of the innate immunity such as CpG, but not CFA, is added to PrP peptides, and that the pattern of immune responses varies according to the epitope. The Journal of Immunology, 2004, 172: 5168–5174.

Transmissible spongiform encephalopathies (TSE) are neurodegenerative disorders that are associated with the conversion of the normal host encoded prion protein (PrPc) to an abnormal protease-resistant form, PrP<sub>Scrapie</sub> (PrPSc). TSE are believed to be transmitted through unconventional agents termed “prions”, the major component of which is PrPSc (1, 2). Recent work pointed out the importance of the immune system in the transport and replication of prions in peripheral lymphoid organs (3). However, the immune system appears not to respond to prion infection in a classical way, because no production of anti-prion protein (PrP) Abs can be evidenced in the course of TSE infection (4). Recent studies reported that humoral response against PrP can be induced not only in PrP knockout (Prnp<sup>−/−</sup>) mice (5, 6, 7) but also in wild-type (PrP<sup>+</sup>) mice when tolerance to PrP was overcome by strong immunization procedures (9, 10, 11, 12). Moreover, protective immunity against scrapie agent propagation was obtained with anti-PrP Abs when induced by active immunization (10, 12) or injected passively (13). Similarly, in a mouse model of Alzheimer’s disease, immunization with fibrillar A<sub>β</sub>1–42-induced Abs that impaired the accumulation of amyloid plaques in the brain of mice and prevented the onset of neurological disease (14). However, clinical trials in Alzheimer’s disease patients treated with amyloid peptide provoked encephalitis in certain patients (15). Thus, because breaking tolerance to self Ag may be deleterious for the host, a characterization of the T cell response to different immunization protocols is required to design appropriate immunotherapeutic approaches. Few studies have focused on anti-PrP T cell responses, which remain poorly characterized (9, 16, 17). The present work was designed to identify immunogenic T cell epitopes and Th phenotypes triggered by PrP immunization of C57BL/6 wild-type mice. Indeed, it is not clear at present whether Th1 or Th2 polarization of the anti-PrP responses would be beneficial in controlling prion disease progression or harmful by inducing possible autoimmune complications. Furthermore, we investigated the influence of breaking Th cell tolerance to PrP on Ab response.

We previously found that immunizing Prnp<sup>−/−</sup> mice with plasmid DNA encoding mouse PrP could generate both T and B cell responses (18). Out of a library of 13 overlapping 30-mers encompassing the entire PrP sequence, three peptides only were able to stimulate in vitro CD4 T cell proliferation after in vivo priming of Prnp<sup>−/−</sup> mice with PrP-expressing plasmid DNA or PrP peptides in CFA. However, preliminary results indicated that this approach was not successful in eliciting an immune response in C57BL/6 wild-type mice. This absence of reactivity is likely due to natural tolerance to PrP. Expression of PrP on the surface of many cells (3, 19) may result in deletion of autoreactive T cells exhibiting high affinity for PrP. However, T cells expressing low affinity TCR specific for PrP may escape thymic deletion and become anergic at the periphery similar to what has already been described for myelin Ag involved in experimental autoimmune encephalomyelitis (20). An effective strategy to overcome this anergic state includes the use of both an appropriate immunogen and a potent adjuvant. We selected CpG-oligodeoxynucleotides (CpG) as adjuvants and

Abbreviations used in this paper: TSE, transmissible spongiform encephalopathy; PrP, prion protein; PrPc, normal host encoded PrP; CpG, CpG-oligodeoxynucleotide; PrPSc, PrP<sub>Scrapie</sub>.
mixed them with 30-mer PrP peptides in IFA for C57BL/6 wild-type mice immunization. Bacterial DNA containing CpG motifs are potent inducers of innate immunity (21). They stimulate Toll-like receptor-9-expressing cells, i.e., dendritic cells, monocytes, and macrophages, to secrete Th1-like cytokines (22, 23) and promote the generation of adaptive immune responses (24, 25, 26, 27). CpG also directly activate B cells to proliferate, and secrete IL-6 and Ig (28–29). Interestingly, the effect of CpG on various effector cells varies depending on CpG motifs (29). In this study, we used CpG-1826 which are potent activators of B cells and induce production of Th1-type cytokines (29, 30). Other CpG motifs (1585) were reported to strongly induce IFN-α and to indirectly enhance NK lytic activity with little effect on B cells (31).

We found that immunization with PrP peptides leads to a specific immune response when mixed with CpG but not with CFA. Vaccination with overlapping peptides P143–172 and P158–187 generated IFN-γ-secreting splenic T cells while only P158–187 significantly stimulated IL-4-secreting T cells. Both peptides induced few Ab-producing B cells and low and variable serum Ab titers. In contrast, P98–127 did not induce a significant number of IFN-γ and IL-4-secreting T cells, but lead to Ab production.

Materials and Methods

Mice

Six-week-old female C57BL/6 mice were purchased from Janvier (Le Genest-St-Isle, France) and kept in a specific pathogen-free animal facility.

Peptides and adjuvants

Overlapping 30-mer PrP peptides were synthesized by NeoSystem (Strasbourg, France) (see Table I), purified by HPLC on a C8 reverse-phase column, and identified by electrospray mass spectrometry (>98% purity). In addition, we designed another peptide library consisting of 10 15-aa long overlapping 30-mer PrP peptides were synthesized by NeoSystem (Strasbourg, France) and kept in a specific pathogen-free animal facility.

Immunization protocol

At day 0, peptides (100 μg) were either emulsified in CFA (v/v) or mixed with CpG (50 μg) emulsified in IFA (v/v), then injected s.c. at the base of the tail. A boost was performed 10–14 days postimmunization and spleen, axillary lymph nodes, and blood were collected 10–14 days after the last injection for analysis.

ELISPOT assay

The number of IFN-γ- and IL-4-producing cells from spleens of immunized mice was evaluated by ELISPOT assay as previously described (25). Briefly, nitrocellulose-based 96-well plates (Millipore, Fontenay-sous-Bois, France) were coated with anti-mouse IFN-γ Abs (1/500) or anti-IL-4 Abs (1/160) (BD Biosciences, Pont de Claix, France). CFA and IFA were purchased from Difco (Detroit, MI).

To determine the isotypes of Abs to PrP peptides, sera from experimental mice were serially diluted and tested in duplicates as described above. Detection of IgG1, IgG2a, and IgG2b isotypes was performed using specific peroxidase-conjugated goat anti-mouse Abs (Southern Biotechnology Associates, Birmingham, AL).

Ab subclasse determination

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B cells frequency analyses

To evaluate Ag-specific B cells, a limiting dilution assay was performed using cells from spleen and lymph nodes of immunized mice; 30 wells of flat-bottom 96-well plates were seeded at 50,000 cells/well, 30 at 10,000 cells/well, and 30 at 5,000 cells/well in RPMI with 10% FCS, IL-2 (1,000 U/ml) plus CpG-1826 (2.5 μg/ml). At the same time, the percentage of CD19+ cells was determined by FACS analysis of the plated cells. After 10 days of culture, supernatants (100 μl from each well) were harvested and tested by ELISA for the presence of anti-PrP peptide Abs. In the ELISA test, positive wells were defined as those with an OD value greater than the mean OD value from control mice cells supernatants plus 3 SD. The frequency of B cells producing Abs was calculated from the 37% interpolation of the regression line which, according to the Poisson distribution, represents the number of B cells containing a single precursor (32). The total cell number was corrected according to the percentage of B cells.

Statistical analyses

ELISPOT data are expressed as means ± SE. Statistical significance between different immunization groups were performed using the nonparametric Mann-Whitney U test and two-tailed p values.

Results

Generation of T cell responses to PrP peptides

Frequency of IFN-γ-secreting PrP peptide-specific splenic T cells.

C57BL/6 wild-type mice were immunized twice at 10- to 15-day intervals with P39–67, P98–127, P143–172, and P158–187 (Table I) together with either CFA or CpG in IFA. These four peptides were selected from 13 overlapping 30-mers because of their ability to stimulate a T cell response in PrpPγ–/− mice (18); all other peptides did not induce a detectable T cell proliferation. P39–67 was used as a negative control in further studies. Spleen cells from individual mice were collected 10–14 days after the final boost; frequency of IFN-γ-secreting T cells upon in vitro restimulation with the corresponding peptide was measured by ELISPOT assay. No response was detected in spleens from mice immunized with the four peptides in CFA except for P158–187, which stimulated few IFN-γ-secreting T cells (frequency = 28 spots/105 splenocytes) (Fig. 1A). In contrast, when peptides were injected with CpG, the percentage of mice that displayed a significant number of IFN-γ-secreting T cells varied according to the immunizing peptide (Fig. 1A): 11 of 11 mice immunized with P158–187, 5 of 6 (83%) with P143–172, 1 of 5

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>39–67</td>
<td>YPGGSGPPGDRYPGQGGST</td>
</tr>
<tr>
<td>98–127</td>
<td>WNFKSPKTNKLKVHAVAAAAGAVVGGLL</td>
</tr>
<tr>
<td>143–172</td>
<td>DWDHYRYPNYRYPQVYYPVPDVQYSN</td>
</tr>
<tr>
<td>158–187</td>
<td>NQVYYPVDQYSNQNFVHDVCNITKQHT</td>
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</tbody>
</table>

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To identify more precisely the epitope recognized by T cells, we restimulated in vitro splenocytes from mice immunized with P143–172 or P158–187 with 15-mer overlapping peptides covering the sequence from positions 143 to 186 (Table II). Fig. 2B shows that 15-mer P156–170 induced IFN-γ-secreting specific T cells both in mice immunized with P143–172 and with P158–187; the adjacent upstream peptide P160–174 stimulated only mice immunized with P158–187. This strongly suggests that the immunogenic epitope is located in the overlapping portion of P143–172 and P158–187. These results were confirmed by showing that immunization of B6 mice with P156–170 induced IFN-γ-secreting specific T cells (data not shown).

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**T cell epitope mapping.** Restimulation with overlapping 30-mer adjacent peptides showed that mice immunized with P143–172 responded best to the same peptide, somewhat less to P158–187 (p = 0.11; upstream overlapping peptide), but not to P128–157 (downstream overlapping peptide) (Fig. 2A). Similarly, mice immunized with P158–187 were restimulated in vitro with P143–172, although significantly (p < 0.005) less efficiently than by P158–187 itself, but never by P173–189. These results suggest that P143–172 and P158–187 share at least one epitope between positions 158 and 172.

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cyte cultures (n measured after 48 h of in vitro restimulation of individual mouse spleno-performed as described in immunized with PrP peptides mixed with CpG/IFA. Immunization was medium. Results are shown as mean numbers of peptide-specific spots per 10^6 cells ± SE calculated as described in Materials and Methods.

P158–187 (n = 10) mixed with CFA or CpG were tested by ELISA for the presence of Abs reacting with peptide-coated plates. Fig. 4 shows the reactivity of individual serum: immunization with peptides together with CFA clearly did not induce detectable signals, except for one mouse that received P98–127. All the mice immunized with P98–127 displayed significant levels of serum Abs. P143–172 with CpG yielded only occasional Ab responses with only 2 of 8 positive sera. P158–187 immunization elicited Ab production in 50% of the mice (5 of 10) and the titers were always low. To find out whether peptide immunization elicited Abs to full-length PrP, individual sera were assayed by ELISA on recombinant PrP. As shown in Fig. 5, 5 of 8 mice immunized with P98–127 plus CpG, as well as 3 of 7 mice treated with P143–172 plus CpG, and 4 of 8 mice treated with P158–187 plus CpG exhibited low but significant reactivities for the entire recombinant mouse PrP molecule. In each group, two mice had received a second peptide-boost displayed higher serum Ab titers (Fig. 5, •, •, •).

B cell epitope mapping. ELISA performed on adjacent overlapping peptides showed that sera from P98–127-immunized mice did not recognize P82–113 or P117–141, suggesting that the B cell epitope involves positions 113–117 on PrP (data not shown). We then tested whether Abs produced after P143–172 immunization also recognized P158–187 and vice versa as observed for the T cell response. Sera from P143–172-immunized mice reacted with P143–172 and also, but less intensely with P158–187; sera from P158–187-immunized mice displayed moderate reactivity for P158–187, but did not recognize P143–172 (Fig. 6).

Ab IgG subclasses. IgG2b were consistently found in all sera irrespective of the peptide used for immunization (Fig. 7). IgG1 and IgG2a isotypes were detected in four of nine and six of nine sera from P97–127-immunized mice, respectively, whereas these isotypes were only occasionally found in P143–172-immunized mice. No IgG1 and IgG2a were detected after P158–187 immunization.

Because PrPc is expressed in lymphoid organs, we examined by FACS analyses whether raising anti-PrP immune responses could affect immune cell populations. No significant differences in the cell number and composition (T/B and CD4/CD8 ratio or percentage of dendritic cells) of the spleen and LN were observed irrespective of the immunizing protocol. PrP expression was not modified on all cell types (data not shown).

Table III. Frequency of IgG-producing B lymphocytes in PrP peptide-immunized mice

<table>
<thead>
<tr>
<th>Immunization with</th>
<th>Lymph Nodes</th>
<th>Spleen</th>
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<tbody>
<tr>
<td>P98–127*</td>
<td>ND</td>
<td>1/60,000</td>
</tr>
<tr>
<td></td>
<td>1/2,000</td>
<td>1/99,000</td>
</tr>
<tr>
<td></td>
<td>1/15,000</td>
<td>1/45,000</td>
</tr>
<tr>
<td></td>
<td>1/12,000</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1/20,000</td>
<td>ND</td>
</tr>
<tr>
<td>P143–172</td>
<td>1/50,000</td>
<td>1/211,000</td>
</tr>
<tr>
<td></td>
<td>1/50,000</td>
<td>1/50,000</td>
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<tr>
<td></td>
<td>1/275,000</td>
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<tr>
<td></td>
<td>1/30,000</td>
<td>ND</td>
</tr>
<tr>
<td>P158–187</td>
<td>ND</td>
<td>1/50,000</td>
</tr>
<tr>
<td></td>
<td>1/50,000</td>
<td>1/390,000</td>
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<tr>
<td></td>
<td>1/35,000</td>
<td>1/81,000</td>
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<td></td>
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<td>ND</td>
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<td></td>
<td>1/50,000</td>
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</tr>
</tbody>
</table>

* Differences in frequency were tested by a non-parametrical not-paired Mann-Whitney U test, p value for B cell frequency in peptide 98-127-immunized mice was p = 0.02, 0.97 < R^2 < 0.99.
187 were tested on P158–172 coated ELISA plates. CFA, can stimulate tolerized myelin-reactive T cells in an IL-12-pressed from birth (34), a recent paper revealed that CpG, but not CFA, claimed that CpG failed to induce immune responses to Ags ex-
increases cell surface costimulatory molecules (33), and causes B cells to secrete Th1-like cytokines (IL-12 and TNF-
Engagement of Toll-like receptor-9 with CpG-1826 (22, 23, 30) on tumor cells by stimulation with a mixture of Ag and CpG. 27) is the induction of an immune response to autoantigens located in C57BL/6 mice and can be triggered when immunostimulatory CpG are used together with PrP Ag presented as peptides. Similar protocols using CFA instead of CpG in IFA proved inefficient. One Th epitope between positions 156 and 172 in the PrP sequence, and at least two B cell epitopes were found to be immunogenic.

The three PrP peptides selected in the present study are naturally processed and presented by APC as shown after immunization of Prnp−/− mice with a plasmid encoding the entire PrP sequence (18). However, peptide immunization with CFA as adjuvant could not induce a detectable PrP-specific response in PrP+ mice. A strategy that has been applied successfully by others (24, 25, 26, 27) is the induction of an immune response to autoantigens located on tumor cells by stimulation with a mixture of Ag and CpG. Engagement of Toll-like receptor-9 with CpG-1826 (22, 23, 30) activates APC to secrete Th1-like cytokines (IL-12 and TNF-α), increases cell surface costimulatory molecules (33), and causes B cells to proliferate and secrete IL-6 (28). Yet, although it was claimed that CpG failed to induce immune responses to Ags expressed from birth (34), a recent paper revealed that CpG, but not CFA, can stimulate tolerized myelin-reactive T cells in an IL-12-

**FIGURE 6.** Mapping B cell epitope. A. Sera from individual mice immunized with P143–172 and on P158–187-coated ELISA plates. B. Sera from individual mice immunized with P158–187 were tested on P158–187- and on P143–172-coated ELISA plates.

**Discussion**

The present results indicate that T and B cell repertoires against PrP exist in C57BL/6 mice and can be triggered when immunostimulatory CpG are used together with PrP Ag presented as peptides. Similar protocols using CFA instead of CpG in IFA proved inefficient. One Th epitope between positions 156 and 172 in the PrP sequence, and at least two B cell epitopes were found to be immunogenic.

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**FIGURE 7.** IgG subclass determination. Sera from peptide-immunized mice were tested on ELISA plates coated with the corresponding peptide and revealed with peroxidase-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b. Serum from individual mice immunized with peptides in CpG (—), P98–127 (n = 9), P143–172 (n = 4), P158–187 (n = 4), or CpG alone (----).
production against peptides was detected after immunization with PrP98–187 and PrP143–172, respectively. When tested individually on full-length recombinant PrP-coated plates, some sera exhibited a low but significant reactivity. It is worth noting that when mice received a second peptide boost, serum Ab titers were higher, likely due to B cell affinity maturation. Differences in serum reactivity to peptide vs whole PrP protein is probably due to poor exposure of the epitopes on PrP, and/or to lower total amount of available epitope when recombinant PrP was used instead of the target peptide. The relatively low Ab levels detected in the sera might relate to low affinity responses in wild-type B6 mice and/or to possible binding to endogenous PrPc. Abs raised against PrP143–172 reacted with both PrP143–172 and PrP98–187 although less strongly with the latter, whereas Abs against PrP158–187 recognized only the same peptide and not PrP143–172. Further studies are required to characterize B cell epitope(s) of anti-PrP Abs and to elucidate whether qualitative (affinity) or quantitative differences are responsible for these observations.

Regarding the peptides studied here, there was no correlation between the strength of the humoral response and the existence of T and B cell epitopes carried by the same peptide. Other T and B cell epitopes may exist on other PrP regions than those targeted by the four tested peptides. Many studies were recently dedicated to the development of strategies to trigger an anti-PrP immune response: Ab responses with variable titers were obtained after immunization with peptide conjugated with keyhole limpet hemocyanin (12) or emulsified in CFA (9, 16), recombinant full-length (10), truncated (12), or dimeric PrP (11) in CFA. Some of these treatments (10, 12) resulted in a prolongation of survival of mice inoculated with a scrapie strain. However, no effect on disease development was observed after immunization with PrP90–230 while treatment with P105–125 prolonged the incubation period by an average of 23 days (12). Interestingly, Ab reactivities after PrP90–230 immunization were predominantly directed to PrP regions from residues 159 to 211, and Abs against regions 105–125 and 144–152 were undetectable (12). This pointed out the importance of the PrPc region bound by a given Ab for its inhibitory capacity in PrPSc replication. In the present study, Abs to P98–127 and P143–172 appeared to target the PrPc–PrPSc interaction sites (P90–127 and P132–156) (36) and therefore, may be susceptible to interfere with PrPSc replication.

Because IgG subclasses display different effector functions, it might be useful to determine the isoatypes of anti-PrP Abs. Indeed, anti-β-amyloid peptide Abs of IgG1 and IgG2b isootypes were clearly more efficient than IgG2a Abs in clearing amyloid plaques in a mouse model of Alzheimer’s disease (37). Few data are available so far concerning the isoatypes of anti-PrP Abs generated in PrP+ mice after immunization with different sources of PrP, and the differential efficiency of Ab isoatypes in inhibiting PrPSc replication remains to be evaluated. In our hands, immunization with P98–127 and P143–172 elicited anti-PrP Abs of IgG1, IgG2a, and IgG2b subclasses while P158–187 induced essentially IgG2b Abs. All results on active (10, 11, 12) and passive immunizations (13) strongly support a protective role for anti-PrP Abs in either inhibiting in vitro PrPSc replication in scrape-infected cells (12, 38, 39) or blocking the propagation of experimental scrapie in vivo (10, 11, 40). A strong involvement of T cell or innate immunity might be required also for a prophylactic or therapeutic effect of immunization against PrP. Repeated injections of CpG (41) or CFA (42) without specific PrP Ag into mice delayed the onset of the clinical disease suggesting that effectors of innate immunity might protect against TSE development. However, a possible role of specific anti-PrP T cell responses were not explored in those studies; a strong T cell help may be necessary to enhance and sustain Ab production, but might also induce autoimmune side-effects. So far, no evidence of autoimmunity has been observed in transgenic mice (40) or in mice receiving passive transfer of a mAb (13). Severe skin lesions and hair loss that seemed related to inflammation were observed 8–12 mo after immunization of Lewis rats with an MHC class II-restricted PrP peptide (16).

Our demonstration that P98–127 and P158–187 are able to direct anti-PrP immune responses predominantly toward production of Abs or stimulation of Th cells, respectively, should allow evaluation of which types of responses would benefit in controlling prion disease progression, or result in the induction of autoimmune inflammatory reactions. Such studies should help in designing new immunotherapeutic approaches for TSE in as much as CpG may be a suitable adjuvant for future vaccinal approaches because of their safety and efficiency in human trials (43).

References


