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Protein Conformation Significantly Influences Immune Responses to Prion Protein

Azadeh Khalili-Shirazi,* Sonia Quaratio,† Marco Londei,† Linda Summers,* Mourad Tayebi,* Anthony R. Clarke,* Simon H. Hawke,* Graham S. Jackson,* and John Collinge2*

In prion diseases, such as variant Creutzfeldt-Jakob disease normal cellular prion protein (PrPC), a largely α-helical structure is converted to an abnormal conformational isoform (PrPSc) that shows an increase in β-sheet content. Similarly, the recombinant form of PrPSc (rα-PrP) can be converted to a conformation dominated by β-sheet (rβ-PrP) by reduction and mild acidification in vitro, a process that may mimic in vivo conversion following PrPSc internalization during recycling. Despite PrPSc accumulation and prion propagation in the lymphoreticular system before detectable neuroinvasion, no Ab response to PrP has been detected, probably due to immune tolerance. To investigate how the immune system may respond to α- and β-PrP, we immunized PrnpC0/0 mice that are not tolerant of PrP with rα-PrP and rβ-PrP. In this study, we show that although T cells stimulated by these differently folded conformers PrP recognize similar immunodominant epitopes (residues 111–130 and 191–210) the cytokine profile in response to rα- and rβ-PrP was different. Challenge with rα-PrP elicited a strong response of IL-5 and IL-10, whereas rβ-PrP led to an early increased production of IFN-γ. In addition, immunization with rα-PrP led to production of predominantly IgG1 isotype Ab in the sera, whereas after immunization with rβ-PrP, IgG2b was significantly produced. Thus, both humoral and cellular responses to these differently folded isoforms of the same protein are different, indicating a possible involvement of Th1 and Th2 pathway activation. These differences may be exploitable diagnostically and therapeutically for prion diseases, such as variant Creutzfeldt-Jakob disease. The Journal of Immunology, 2005, 174: 3256–3263.

The prion diseases or transmissible spongiform encephalopathies are a group of fatal, transmissible, neurodegenerative conditions, which include Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, and kuru in humans, and scrapie and bovine spongiform encephalopathy in animals. The transmissibility of human prion disease has become an intense public health issue because of evidence that bovine spongiform encephalopathy-like prions have infected humans (1–3). A common feature of all prion diseases is the posttranslational conversion of the normal cellular prion protein (PrPC),3 a host-encoded GPI-anchored sialoglycoprotein (4), α-helical in structure and expressed on most cells including B and T cells (5), into an abnormal isoform termed PrPSc (6), structurally dominated by β-sheet.

Neither amino acid sequencing nor systematic studies of known covalent posttranslational modifications have shown any consistent differences between PrPC and PrPSc (7). The abnormal isoform, PrPSc, is isolated from tissue as a highly aggre-gated and detergent insoluble polymer, which has markedly increased resistance to proteolysis. PrPC can be completely digested by the serine protease, proteinase K (PK), while in marked contrast, PK removes between 90 and 100 amino acids from the N terminus of abnormal isoform PrPSc, depending upon prion strain type, leaving a large protease-resistant C-terminal fragment (8). At low pH, following reduction of the native disulphide bond, the folded C-terminal domain of human prion protein is shown to exist as a soluble material with β-sheet structure (9). It is possible that the in vitro conversion of α-PrP to β-PrP, caused by reduction and mild acidification (10), might occur in vivo, following PrPSc internalization within the cell during recycling (11), and this could underlie prion propagation (9).

Accumulation of PrPSc has been found in the lymphoreticular system in human variant Creutzfeldt-Jakob disease (12, 13). In scrapie-affected mice, prions are associated with splenic B and T cells, but not (14) or very little (15, 16) with circulating PBMCs. In mice, infectious prions are detectable in the spleen and lymph nodes long before they can be detected in the brain (17) (A. Khalili-Shirazi and S. H. Hawke, unpublished observation), and PrPSc is accumulated in splenic follicular dendritic cells (FDC) (18–20). An intact immune system is essential for prion propagation. FDCs are required for neuroinvasion and the development of clinical scrapie, and in turn, B cells are required for maturation of FDCs and formation of germinal centers (21). In addition, PrPSc has also been reported to be associated with macrophages and dendritic cells (22–26).

Despite the involvement of the immune system in PrPSc replication and neuroinvasion, no Ab response to PrP has been detected in prion disease. The lack of detectable immunity in prion infection is thought to be largely due to immune tolerance because of the widespread expression of PrPSc in the hemopoietic system (27–29).

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2 Address correspondence and reprint requests to Dr. John Collinge, Medical Research Council Prion Unit, Department of Neurodegenerative Disease, Institute of Neurology, University College London, Queen Square, London WC1N 3BG, U.K. E-mail address: j.colllinge@prion.ucl.ac.uk
3 Abbreviations used in this paper: PrPC, cellular prion protein; PrPSc, abnormal isoform prion protein; PK, proteinase K; FDC, follicular dendritic cells; r, recombinant; RML, Rocky Mountain Laboratory isolate of Chandler mouse scrapie prion; SI, stimulation index; B, biotinylated; Con-A, concanavalin A.
For the production of mAbs, Prnp0/0 mice were immunized s.c. at the base of the tail with 100 μg of Ag (human rα- or rβ-PrP91–231) in CFA and boosted on days 21 and 42 with 100 μg of the similar Ag in IFA, then finally boosted i.p. on day 50 with 50 μg of the Ag in PBS. Three days after final boosting, single cell suspensions of spleenocytes were prepared and fused with the NS0 myeloma cell line, selecting hybridomas using conventional methodology. Detailed methods for screening of these mAbs have been described elsewhere.4

**Proliferation and cytokine assays**

For proliferation assays, cells derived from the lymph nodes of mice immunized with either rα- or rβ-PrP Ag and control mice were used. The study with rα- and rβ-PrP as Ag were repeated four times and with PBS twice. The T cells were derived from a pool of lymph nodes from three mice and tested in triplicate, presenting the results as stimulation index (SI) = ΔSE. The cells proliferated to ~9000 cpm and a SI of 2.5–4 was considered significant. For proliferation assays, cells were plated in round-bottom 96-well plates (Falcon), at 4 × 105 cells per 0.2-ml well, and for cytokine assays in 24-well plates, at 1–2 × 106 cells per 2-ml well. Human rα- and rβ-PrP were sterile filtered through a 0.2-μm filter (Nalgene), titrated in culture for T cell proliferation, and 50 μg/ml was found to be the optimal concentration. Human PrP peptides were also sterile filtered and used at 10 μg/ml. Where appropriate, OVA at 50 μg/ml was used as negative control Ag. Cells were maintained in a standard 72-hr proliferation assay and pulsed with [3H]thymidine (0.5 μCi/well) (Amersham Biosciences, Little Chalfont, UK) in the last 18 hr prior to harvesting (Tomtec cell harvester) and determining cpm, using a beta counter (Microbeta counter; PerkinElmer).

The production of cytokines from cells in lymph nodes were measured in vitro after 24, 48, and 72 h. IL-2, IL-4, IL-5, IL-10, TGF-β, and INF-γ were analyzed by sandwich ELISA, using standard protocols, including a standard curve for each plate (BD Biosciences, BD Pharmingen).

**Characterization of anti-PrP mAbs**

As previously described, mAbs ICSM4, -10, and -18 were obtained upon immunization with rα-PrP91–231 while ICSM35 and -37 with rβ-PrP91–231 were obtained using rβ-PrP91–231 as Ag, being a soluble form. For these studies, ELISA plates were coated with rα-PrP91–231, and rβ-PrP for detecting native PrP. In a Rocky Mountain Laboratory isolate of Chandler mouse scrapie prion (RML) (33)-infected neuroblastoma cell line (N2a) (33, 34), the cells were grown in culture on multichamber slides (Sigma-Aldrich) until semiconfluent. To label cell surface PrP, they were incubated at 4°C with anti-PrP mAbs, before fixation with paraformaldehyde. For detection, FITC-conjugated anti-mouse secondary Ab was used.

**ELISA**

Direct ELISA method was used to detect binding of ICSM18 and -35 to rα-PrP91–231 at 20 μg/ml, using anti-mouse IgG (Fab-specific) HRP (Sigma-Aldrich) as secondary Ab and o-phenylenediamine (Sigma-Aldrich) as chromogen. Direct ELISA for detection of native PrP proved difficult, despite using different brain homogenate concentrations, plate manufactures, coating-buffers (carbonic bicarbonate (pH 9.5), Tris (pH 8.5), and PBS (pH 7.4)), blocking solutions (normal goat serum, normal mouse serum, FCS, BSA, and soya milk), and temperatures (4°, 23°, and 37°C), so an indirect sandwich ELISA technique was devised. ICSM35 and ICSM35 were individually used to coat the ELISA plate and after washing, normal or scrapie-affected brain homogenate was added as Ag, and PrP was detected with biotinylated (B) mAb ICSM18B or ICSM35B, using a different mAbs for capture and detection. Finally streptavidin-HRP was added and o-phenylenediamine was used as chromogen. The brain homogenates were tested at 0.5–1%, the mAb used for PrP capture coating the ELISA plate was tested at IgG concentrations of 2–10 μg/ml, and the biotinylated mAb used for PrP detection was tested at IgG concentrations of 1 and 0.5 μg/ml. Both ICSM18 and 35 worked well for capturing and for detecting native PrP.

The isotypes of these mAbs were determined using a panel of anti-mouse Ig subclass-specific mAbs (Pierce) and their immunodominant epitopes were defined by direct ELISA, using 20-mer synthetic peptides (10–20 μg/ml), spanning the PrP sequence 91–231 overlapping by 10 residues. These epitopes were further refined by direct peptide ELISA, using 15-mer peptides overlapping by 13 residues. The accuracy of the immunodominant epitopes found was confirmed by competition and inhibition ELISA, where the peptides were used to inhibit binding to recombinant rα- and rβ-PrP. For these studies, ELISA plates were coated with rα- and rβ-PrP and the mAbs were added either by preincubating them with the peptides to inhibit binding to rα- and rβ-PrP, or the peptides were added simultaneously as the mAb to ELISA plates, to compete for the binding of the mAbs to rα- and rβ-PrP. Similar results were obtained, and the latter

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The overall proliferation of T cells from different H9251 Immunization with either rPrP (20–10 αPrP or βPrP) evoked clear proliferative T cell responses to both αPrP- or βPrP-Ags (Fig. 1), unlike T cells from immunizations with PBS-CFA (Fig. 1) or OVA-CFA (data not shown). There was no significant difference between the in vitro recognition of αPrP- and βPrP-immunized T cells, each derived from either PBS-CFA (Fig. 1) or OVA-CFA (data not shown). The viability and Ag-recognition pathway of all the T cells was tested by their response to stimulation with anti-CD3 Ab, where a mean SI of 7–12 was observed (data not shown).

T cell epitope recognition of αPrP- and βPrP-immunized T cells from αPrP- and βPrP-immunizations showed no significant difference in their recognition of PrP peptides (Fig. 2, A and B). T cells from lymph nodes of both αPrP- (Fig. 2A) and βPrP- (Fig. 2B) immunized mice recognized N-terminal residues within the sequence 111–130 and C-terminal residues within 191–210 (p = 0.005, ANOVA). In keeping with the previous result, T cells from PBS (or OVA)-immunized mice did not proliferate in response to PrP peptides (Fig. 2C and data not shown). PrP residues 111–130 are thought to be within the structured part of PrP while residues 191–220 are just C-terminal to the glycosylation sites of PrP.

The intracellular proteolytic degradation of Ag by APC might generate different peptide ligands for interaction with MHC class II molecules. Because for both αPrP- and βPrP-immunizations we used Prnp<sup>−/−</sup> mice of FVB/N background with identical MHC class II molecules, it is not surprising that we found a similar linear sequence of peptides being recognized by T cells from both groups.

The cytokines produced by αPrP- and βPrP-derived T cells are different The overall proliferation of T cells from Prnp<sup>−/−</sup> mice, and in particular proliferation to PrP peptides was weak. Therefore, we checked for differences in cytokine profiles of lymph node cells from αPrP- and βPrP-immunized mice. Measuring cytokines, in cultures of lymph nodes cells challenged in vitro with rPrP, was most suitable after 24 h and 48 h. Although there was some overlap of the cytokines produced, T cells from lymph nodes of βPrP-immunized mice were skewed more toward Th1 (producing more IFN-γ and sooner, and less IL-5 and IL-10), whereas those from αPrP-immunized mice were skewed more toward Th2 (producing more IL-5 and IL-10, and less or later IFN-γ) (Fig. 3).

The cytokine levels were clearly affected by the rPrP isoform used for in vitro challenge, as well as for immunization. After 24-h stimulation with αPrP in vitro, T cells from αPrP-immunized

**Figure 1.** In vitro T cell proliferation, by αPrP and βPrP challenge. The mean SI ± SEM of lymph nodes T cells from animals immunized with αPrP, βPrP, and PBS is shown, after in vitro challenge with αPrP (A) and βPrP (B). The data of α- and β-PrP are the means of four experiments (and those for PBS from two), each done in triplicate, using T cells derived from a pool of lymph nodes of three mice. The mean ± SEM of the absolute cpm of unstimulated control cultures from αPrP immunization = 1535 ± 346, βPrP immunization = 1419 ± 325, and from PBS immunization = 1093 ± 378.

**Figure 2.** Immunodominant epitope of α-PrP and β-PrP immunized T cells. The mean SI ± SEM of lymph nodes T cells, four experiments with three animals, immunized with A. α-PrP (□); B. β-PrP (■); and C. PBS (□) (one experiment), challenged in vitro with 20-mer synthetic peptides spanning PrP 91–231 overlapping by 10 residues. Each experimental mean is that of triplicate readings. The mean ± SEM of the absolute cpm of unstimulated control cultures from α-PrP immunization = 1535 ± 346, β-PrP immunization = 1419 ± 325, and from PBS immunization = 1093 ± 378.

**Figure 3.** Statistical analysis

Data were analyzed for statistical significance using ANOVA and two-tailed t test.

**Results**

*Both α- and β-PrP are recognized by T cells*

Immunization with either α- or β-PrP evoked clear proliferative T cell responses to both α- or β-PrP Ags (Fig. 1), unlike T cells from immunizations with PBS-CFA (Fig. 1) or OVA-CFA (data not shown). There was no significant difference between the in vitro recognition of α- and β-PrP by T cells derived from either α- or β-PrP immunization (p = 0.316, p = 0.154, two-tailed t test). The viability and Ag-recognition pathway of all the T cells was tested by their response to stimulation with anti-CD3 Ab, where a mean SI of 7–12 was observed (data not shown).

*T cell epitope recognition of α- and β-PrP is similar*

T cells from α- and β-PrP immunizations showed no significant difference in their recognition of PrP peptides (Fig. 2A and B). T cells from lymph nodes of both α-PrP (Fig. 2A) and β-PrP (Fig. 2B) immunized mice recognized N-terminal residues within the sequence 111–130 and C-terminal residues within 191–210 (p = 0.005, ANOVA). In keeping with the previous result, T cells from PBS (or OVA)-immunized mice did not proliferate in response to PrP peptides (Fig. 2C and data not shown). PrP residues 111–130 are thought to be within the structured part of PrP while residues 191–220 are just C-terminal to the glycosylation sites of PrP.

The intracellular proteolytic degradation of Ag by APC might generate different peptide ligands for interaction with MHC class II molecules. Because for both α- and β-PrP immunizations we used Prnp<sup>−/−</sup> mice of FVB/N background with identical MHC class II molecules, it is not surprising that we found a similar linear sequence of peptides being recognized by T cells from both groups.

The cytokines produced by α- and β-PrP-derived T cells are different

The overall proliferation of T cells from Prnp<sup>−/−</sup> mice, and in particular proliferation to PrP peptides was weak. Therefore, we checked for differences in cytokine profiles of lymph node cells from α- and β-PrP-immunized mice. Measuring cytokines, in cultures of lymph nodes cells challenged in vitro with rPrP, was most suitable after 24 h and 48 h. Although there was some overlap of the cytokines produced, T cells from lymph nodes of β-PrP-immunized mice were skewed more toward Th1 (producing more IFN-γ and sooner, and less IL-5 and IL-10), whereas those from α-PrP-immunized mice were skewed more toward Th2 (producing more IL-5 and IL-10, and less or later IFN-γ) (Fig. 3).

The cytokine levels were clearly affected by the rPrP isoform used for in vitro challenge, as well as for immunization. After 24-h stimulation with αPrP in vitro, T cells from α-PrP-immunized
mice produced only 340 pg/ml IFN-γ, whereas T cells from rβ-PrP-immunized mice produced >2000 pg/ml IFN-γ (Fig. 3A). After 48-h in vitro stimulation of cells, T cells from both α- and β-PrP immunizations produced a large amount of IFN-γ (>2000 pg/ml). A small amount of IFN-γ was also produced after 48 h, by unstimulated T cells from rβ-PrP immunization, and from PBS immunization stimulated in vitro with rβ-PrP (Fig. 3A).

Th2-induced cytokines like IL-5 and IL-10 were also investigated. IL-5 production was substantially higher after in vitro stimulation with re-PrP compared with rβ-PrP, by T cells from both α-PrP (p = 0.001, t test) and rβ-PrP (p = 0.0108, t test) immunizations. T cells from α-PrP-immunized mice produced 227 pg/ml IL-5 in response to α-PrP but only 90 pg/ml in response to rβ-PrP, and T cells from β-PrP-immunized mice produced 122 pg/ml in response to α-PrP and only 16 pg/ml in response to rβ-PrP (Fig. 3B). The T cells from α-PrP-immunized mice also produced significantly more IL-5 in response to in vitro stimulation with α-PrP, than those from rβ-PrP immunization (p = 0.0147, t test). No IL-5 was produced by T cells from PBS-immunized mice, regardless of using α- or rβ-PrP for the in vitro challenges (Fig. 3B). The IL-10 responses showed that it was produced, slightly more by T cells from α-PrP than rβ-PrP-immunized mice (Fig. 3C).

Not surprisingly, in view of the results shown in Figs. 1 and 2, there was no statistical difference in the production of IL-2 in response to either α- or rβ-PrP from both control groups (Fig. 3D), and small amounts of IL-2 was also produced by T cells from PBS-immunized mice, after in vitro challenge with α- or rβ-PrP (Fig. 3D). Production of IL-4 and TGF-β are not shown, as they could not be convincingly measured.

**PrP conformation influences the Ab Ig isotypes produced in the sera**

Total IgG Ab levels were similar in mice immunized with α- and rβ-PrP and their sera Abs were positive to α- and rβ-PrP Ags by ELISA, while no Ab to α- and rβ-PrP was present in the serum of the unimmunized mouse (Fig. 4A). However, a significant difference was observed in the Ig isotypes produced in the sera of mice immunized with re-PrP, as compared with those immunized with rβ-PrP. Immunization with re-PrP evoked predominantly Th2 isotype Abs of IgG1 isotype, whereas rβ-PrP evoked mainly Th1 IgG2b in the serum (Fig. 4B).

Significantly higher levels of IgG1 Abs were produced in the sera of α-PrP-immunized mice, compared with those produced in sera of mice immunized with rβ-PrP (p < 0.005, two-tailed t test). In contrast, IgG2b Ab was produced at higher levels in the sera of β-PrP-immunized mice, when compared with α-PrP-immunized mice (p < 0.005, t test). There was no significant difference between the levels of the other Ig isotypes in the sera of these two groups of mice. Because the mice were bred under specific pathogen-free conditions, it is not surprising that no Ab IgG isotypes and only very low levels of IgM were detected in the sera of the unimmunized mice (Fig. 4B). In addition, different anti-PrP Ab IgG isotypes were found in the sera of mice immunized with re-PrP and rβ-PrP and tested by ELISA against either rα-PrP (data not shown) or rβ-PrP (Fig. 4C). IgG1 anti-PrP Ab was produced in sera of mice immunized with re-PrP and IgG2b Ab produced after rβ-PrP immunization (Fig. 4C). These findings confirm that despite the weak T cell proliferation responses, the α- and β-isomers of PrP are recognized differently by the immune system.

**PrP conformation affects the IgG isotype and the specificity of the mAbs produced**

A significant difference was also observed in the specificity and IgG isotype of the mAbs raised against α- and β-isomers of PrP. The results obtained for the mAbs produced to α- and rβ-PrP further demonstrate that PrP in the different conformations (α or β) give rise to a different type of cellular response, skewed more toward Th1 for rβ-PrP and Th2 for rα-PrP. This reconfirms the

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**FIGURE 3.** In vitro Th1 and Th2 cytokine production of α-PrP- and β-PrP-derived T cells. Lymph nodes T cells from animals immunized with α-PrP, β-PrP, and PBS were challenged in vitro with α-PrP (■), β-PrP (□), and PBS (○), and their cytokine production was measured. Unless stated otherwise, data are for 48-h sampling time for IFN-γ (A), IL-5 (B), IL-10 (C), and IL-2 (D), by T cells derived from pools of lymph nodes of three mice immunized with α-PrP, β-PrP, and PBS, measured by ELISA. IFN-γ production for the in vitro stimulation of α-PrP immunized T cells with β-PrP was not tested, because enough cells were not available. Where there are interrupted bars in the figure, the amount of cytokines produced was off scale.
data already obtained for T cells and the sera Ab Ig isotypes. ICSM4, -10, -18, -35, and -37 mAbs, raised to r/H9251- and r/H9252-PrP, had different specificities and IgG isotypes. Those produced against r/H9251-PrP (ICSM4, -10, and -18) were of IgG1 isotype, whereas r/H9252-PrP-derived mAbs (ICSM35 and -37) were IgG2b and IgG2a. Furthermore, IgG isotypes were also different in the 40 mAbs we have produced to r/H9251-PrP and 41 mAbs to r/H9252-PrP. The isotypes of the mAbs produced from r/H9251-PrP immunizations were 92.5% IgG1 and 7.5% IgG2 (2.5% IgG2a and 5% IgG2b), whereas the isotypes of those produced to r/H9252-PrP immunizations were 53.6% IgG1, 41.5% IgG2 (12.2% IgG2a, 29.3% IgG2b), and 4.9% IgM (manuscript in preparation).

Both ICSM18 and ICSM35 (raised against rα- and rβ-PrP respectively), were equally suitable for capturing or detecting native PrP from normal and RML-infected scrapie-affected mouse brain homogenates by sandwich ELISA (Fig. 5A). As previously described ICSM4, -10, and -18 (raised to rα-PrP) and ICSM35 and
and ICSM35 were chosen, as strongly binding representatives of A (Fig. 6). ICSM18 has a high affinity for B than ICSM35 (Fig. 6). The Western blots of ICSM18 and ICSM35 showed that they both recognize all the PrP glycoforms (Fig. 5B). In contrast, ICSM4 only recognized unglycosylated PrPSc and PrPSc; while ICSM18 recognized di- and mono- and unglycosylated PrPSc and PrPSc, by Western blot using aliquots of the same normal and scrapie-affected mouse brain homogenates (Fig. 5B). ICSM4 and -10 also differentially immunoprecipitated PrP glycoforms from PrPSc, while anti-β-PrP mAbs ICSM35 and -37 did not. ICSM18 and ICSM35 were chosen, as strongly binding representatives of PrP-specific Th2 response for α-PrP and Th1 response for β-PrP. We have labeled PrP on RML-infected N2a cells with ICSM18 and ICSM35 by immunofluorescence (Fig. 6). Although both ICSM18 and ICSM35 specifically labeled PrP on these cells, staining of PrP with ICSM18 proved to be stronger and more uniform (Fig. 6A) than ICSM35 (Fig. 6B). ICSM18 has a high affinity for native PrPSc and a weak affinity for native PrPC, while ICSM35 has a higher affinity for native PrPSc than for native PrPC by immunoprecipitation. This may explain the strong immunofluorescence detected by ICSM18 and the weaker immunostaining detected by ICSM35. The weak staining of ICSM35 may also indicate that native PrPSc is mainly intracellular. No nonspecific binding of PrP was observed using negative control mAb (Fig. 6C), or no primary Ab, second layer alone (Fig. 6D).

Detailed epitope mapping, showed striking differences for the α- and β-PrP-generated mAbs. Approximately similar epitopes for α-PrP-derived mAbs ICSM35 and -37 were found that were different from the PrP-derived mAb ICSM18 (Fig. 7). ICSM18 was specific for the linear sequence 143–153 (Fig. 7A). ICSM35 and ICSM37 for sequences 93–105 and 97–105, respectively (Fig. 7B). ICSM4 and -10 were negative in the peptide ELISA and could not be epitope mapped. The immunodominant epitopes of ICSM18 (Fig. 7C) and ICSM35 (Fig. 7D) were reconfirmed by peptide competition ELISA, using the peptides as competitors for binding of the mAbs to α- and β-PrP Ags. These data show that α- and β-PrP generate diverse immune responses, cellular and humoral, and mAbs could be raised with high affinity for native PrPSc (like ICSM18) or for native PrPSc (like ICSM35).

**Discussion**

In this study, we have demonstrated that immunization of mice with either α- or β-PrP evokes a different cytokine milieu and Ab isotype profile. Usually CD4+ T cells recognize linear processed peptides presented in the groove of MHC class II molecules, whereas B cells recognize both conformational and/or linear epitopes of an Ag. When we immunized PrP-deficient mice (of FVB/N MHC H-2d haplotype) with either α- or β-PrP, comparable T cell proliferation against similar immunodominant epitopes was observed. Although α- and β-PrP do not differ in amino acid sequence, they elicit qualitatively different cellular and humoral response. Although T cells from α- and β-PrP immunizations recognized both α- and β-PrP and peptide residues 111–130 and 191–210; by contrast the mAbs reflecting the activity of B cells could partially discriminate between PrPSc and PrPC, and their immunodominant peptides were different: 93–105 for α-PrP immunizations (ICSM35 and -37) and 143–153 for α-PrP (ICSM18).

We have observed that the T cell proliferation, particularly against PrP peptides, was weak. One possible explanation for this could be the absence of PrP in the cells of Prnp<sup>0/0</sup> mice. Lack of PrP<sub>C</sub> on T lymphocytes could lead to generally low activation levels, as we found that even the response to soluble anti-CD3 Ab was very modest. Indeed, PrP<sub>C</sub> may have an important role in T cell activation. PrP<sub>C</sub> is significantly up-regulated on mouse T cells upon challenge with concanavalin A (Con-A), but in Prnp<sup>0/0</sup> mice, T cell response to Con-A is significantly reduced to 50–80%, suggesting that PrP<sub>C</sub> is a lymphocyte surface molecule that participates in T cell activation (35). In human T lymphocytes, PrP<sub>C</sub> expression is also increased upon challenge with Con-A and reduced by the addition of anti-PrP polyclonal Ab (36). PrP<sub>C</sub> expression on normal human T lymphocytes is also increased after challenge with either Con-A or anti-CD3 Ab and inhibited by addition of anti-PrP mAbs, and in addition, CD45RO<sup>+</sup> memory T cells consistently expressed more PrP<sup>C</sup> than CD45RA<sup>+</sup> naive T lymphocytes (37). A recent report suggests PrP<sub>C</sub> to be a component of the multimolecular signaling complex, within microdomains involved in T cell activation (38). The absence of PrP<sub>C</sub> on T lymphocytes of Prnp<sup>0/0</sup> mice may therefore be responsible for the weak proliferative response we have observed in this study, but

**FIGURE 6.** Immunofluorescence labeling of native PrP on RML-infected N2a cells by anti-α- and β-PrP mAbs. ICSM18 (A) and ICSM35 (B) successfully labeled PrP on RML-infected N2a cells. The cells were grown on multichamber glass slides, and when semi-confluent, they were directly incubated with the mAbs. An irrelevant mAb (C) and no primary Ab, second layer alone (D) were used as negative control.
apparently it does not affect their cytokine production. The proliferation response was not particularly weak after in vitro challenge with the peptides, but in a preliminary study we have found that splenocyte T cells from rPrP-immunized mice produce IL-5 in response to in vitro challenge with peptide sequence 191–210, and T cells from both rα- and rβ-PrP immunizations produce IFN-γ in response to this peptide (S. H. Hawke and Z. Sattar, unpublished data). Sera Abs were also positive to this epitope after immunization of Prnp<sup>−/−</sup> mice with rPrP (A. Khalili-Shirazi and L. Summers, unpublished observation).

The processing of different proteins by different APCs, such as dendritic cells and macrophages, leads to activation of different signaling pathways leading to the production of different cytokines (39). T cell activation leads to a CD4<sup>+</sup> or CD8<sup>+</sup> T cell response, CD4<sup>+</sup> initiating either a Th1 or Th2 immunity, each triggering production of a series of different cytokines and IgG isotypes (40–42). Th1 type response leads to activation of macrophages and is characterized by production of IFN-γ, whereas Th2 leads to activation of different APCs, and production of IL-4, IL-5, and IL-10 (43, 44). This dichotomy might be interpreted as the result of different T cell activation. Indeed, it has been reported that Th2 cells provide B cell help and stimulate the production of noncomplement fixing IgG1 Abs, while Th1 cells induce B cells to switch to IgG2 and IgG3 (45).

In this study, we have shown that despite some overlap, the immune response to rα-PrP was skewed more toward Th2, inducing T cells to produce increased amounts of IL-5 and IL-10, and B cells to produce polyclonal IgG1 Abs in the sera, and IgG1 mAbs such as ICSM4,-10, and -18. In contrast, the immune response to rβ-PrP was skewed more toward Th1, with a high and early production of IFN-γ, less IL-5 and IL-10 cytokines, and production of serum IgG2b isotype Abs and IgG2b and IgG2a mAbs, like ICSM35 and -37. These data indicate that different APCs may be involved in processing of rα- and rβ-PrP. When rα-PrP is used as the immunogen, its greater sensitivity to proteolytic cleavage might mean that it is more easily processed for presentation to B cells and may involve different APCs from those that process rβ-PrP. In comparison with rα-PrP, rβ-PrP is rich in β-sheet and partially PK-resistant (9). It is possible that rα-PrP is mainly processed by B cells and dendritic cells and much less by macrophages, whereas rβ-PrP is mainly processed by macrophages and less by B cells or dendritic cells. Immune tolerance to PrP in Prnp<sup>−/−</sup> mice can reportedly be overcome by improved immunization techniques, using mouse rPrP (46) and dimeric mouse rPrP (47), leading to production of anti-PrP polyclonal Abs. In the course of prion inoculation, activated T cells have been detected in the brain and spleen of mice affected with scrapie (48). Macrophages appear to have an important role in neutralizing infectivity (23) and an association between dendritic cells and PrP<sup>S<sub>c</sub></sup> has been reported (26, 49). Our findings may provide an insight into how PrP<sup>C</sup> and PrP<sup>S<sub>c</sub></sup> might be differentially processed by APCs, such as dendritic cells and macrophages. The data presented in this study on the immune responses to rα- and rβ-PrP might reflect those to PrP<sup>C</sup> and PrP<sup>S<sub>c</sub></sup>, if there was no tolerance to PrP or if it could be overcome by vaccination. A transient, but undetected, immune response may also occur early in the course of prion infection, when conversion of PrP<sup>E</sup> to PrP<sup>S<sub>c</sub></sup> has just begun and before formation of large PrP<sup>S<sub>c</sub></sup> deposits and onset of clinical signs. These findings may therefore be relevant to the understanding of the molecular mechanisms underlying prion diseases in humans and help in the design of an effective therapeutic immunization or vaccination approach.

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Disclosures

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