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Protein Conformation Significantly Influences Immune Responses to Prion Protein

Azadeh Khalili-Shirazi,* Sonia Quaratino,† 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 PrPC (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 Prnp0/0 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.

T he 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 aggregated 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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*Medical Research Council Prion Unit, Department of Neurodegenerative Disease, Institute of Neurology, and 1Institute of Child Health, University College London, London, United Kingdom; and 2Cancer Research United Kingdom Oncology Unit, Cancer Sciences Division, School of Medicine, University of Southampton, Southampton General Hospital, Southampton, United Kingdom

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1 This work was funded by the United Kingdom Medical Research Council.

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.collinge@prion.ucl.ac.uk

3 Abbreviations used in this paper: PrPSc, 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.
Furthermore, attempts to produce PrP\textsuperscript{Sc}-specific Abs by immunization of Prnp\textsuperscript{0/0} mice (that are not tolerant to PrP) with PrP\textsuperscript{Sc} have been uniformly unsuccessful, although it has recently been reported that Abs raised to the repeat motif of PrP, tyrosine-tyrosine-arginine, can specifically recognize the pathological isoform of the PrP (30). We had previously immunized Prnp\textsuperscript{0/0} mice with highly purified human recombinant (rα- and rβ-PrP (31) to obtain a panel of mAbs that strongly immunoprecipitate native PrP\textsuperscript{C} and PrP\textsuperscript{Sc}. In this study, we have characterized the T cell-mediated immune responses to rα- and rβ-PrP in Prnp\textsuperscript{0/0} mice and show that the different conformations of the same protein are able to induce a qualitatively different type of response. Such information may provide insights into the biology of prion diseases and the potential use of PrP conformers in therapeutic vaccination strategies.

Materials and Methods

**Animals**

We used Prnp\textsuperscript{0/0} mice (27) backcrossed onto an FVB/N mouse background to study the difference between the immune responses of the two different conformers of rα-PrP and rβ-PrP. These mice were housed under specific pathogen-free conditions. Animal care conformed to national and institutional guidelines.

**Ag-rα-PrP, rβ-PrP, and -PrP peptides**

Human and mouse rα-PrP\textsuperscript{91–231} and rβ-PrP\textsuperscript{91–231} used as Ag were synthesized, as already described (9, 31), rβ-PrP\textsuperscript{91–231} being a soluble α-helical monomer and PK-sensitive, whereas rα-PrP\textsuperscript{91–231} is a soluble monomer, rich in β-sheet, and relatively PK-resistant. We used 91–231 rPrP, rather than full-length protein because it more readily forms a stable helix. Also, native PrP\textsuperscript{C} is completely digested by PK, while depending upon prion strain type, between 90 and 100 amino acids from the N terminus of abnormal isoform PrP\textsuperscript{Sc} are removed leaving a protease-resistant core (8). Such protease-resistant fractions retain infectivity and transgenic mice expressing N-terminally truncated PrP remain able to support prion propagation (32), arguing that the 91–231 construct is the most appropriate study object.

For epitope mapping we used 20-mer peptides of human PrP sequence 91–231, with 10 residues overlap and 15-mer peptides with 13 residues overlap. Peptides were made by solid phase stepwise synthesis using the Fmoc N-terminal protection strategy, using the Applied Biosystems 433 model automated synthesizer (Advanced Biotechnology Centre, Imperial College London). After synthesis, peptides were cleaved from the solid phase and fully deprotected by standard methods. Purification was performed by reverse phase HPLC. Products were analyzed by reverse phase HPLC and MALDI mass spectrometry.

**Immunization**

For each arm of the study for T cell experiments, three 4- to 6-wk-old Prnp\textsuperscript{0/0} mice (27) backcrossed onto FVB/N background were used. These mice have been found to respond well to PrP Ag. The mice were immunized s.c./intradermally at the base of the tail, with 100 µg of Ag (human rα- or rβ-PrP\textsuperscript{91–231} 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 mouse splenocytes 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, s.c. cells 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) = \( \frac{cpm_{Ag}}{cpm_{EB}} \). 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 × 10\(^5\) cells per 0.2-ml well, and for cytokine assays in 24-well plates, at 1–2 × 10\(^5\) 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-h proliferation assay and pulsed with \(^{3}H\)thymidine (0.5 µCi/well) (Amersham Biosciences). In the last 18 h 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 IFN-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-PrP\textsuperscript{91–231} while ICSM35 and -37 with rβ-PrP\textsuperscript{91–231}. ICSM18 and -35 were tested for staining 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 rβ-PrP\textsuperscript{91–231} at 20–100 µg/ml, using anti-mouse IgG (Fab-specific) HRP (Sigma-Aldrich) as secondary Ab and α-phenylendiamine (Sigma-Aldrich) as chromogen. Direct ELISA for detection of native PrP proved difficult, despite using different brain homogenate concentrations, plate manufactures, coating-buffers (carbonate 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°C, 23°C, and 37°C), so an indirect sandwich ELISA technique was devised. ICSM18 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 different mAbs for capture and detection. Finally streptavidin-HRP was added and α-phenylendiamine 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 epitope 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 all the mAbs were tested at both 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

method was mainly conducted. ELISA plates were coated with αr- or βr-PrP (20–10 μg/ml) in carbonate/bicarbonate buffer (pH 9.5), to which each mAb IgG (1 μg/ml) and peptide (10–50 μg/ml) were added. The ELISA was then conducted by conventional methods as described above.

**Statistical analysis**

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

**Results**

**Both αr- and βr-PrP are recognized by T cells**

Immunization with either αr- or βr-PrP evoked clear proliferative T cell responses to both αr- or βr-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 αr- and βr-PrP by T cells derived from either αr- or βr-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 αr- and βr-PrP is similar**

T cells from αr- and βr-PrP immunizations showed no significant difference in their recognition of PrP peptides (Fig. 2, A and B). T cells from lymph nodes of both αr-PrP (Fig. 2A) and βr-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 αr- and βr-PrP immunizations we used Prnp0/0 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 αr- and βr-PrP-derived T cells are different**

The overall proliferation of T cells from Prnp0/0 mice, and in particular proliferation to PrP peptides was weak. Therefore, we checked for differences in cytokine profiles of lymph node cells from αr- and βr-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 βr-PrP-immunized mice were skewed more toward Th1 (producing more IFN-γ and sooner, and less IL-5 and IL-10), whereas those from αr-PrP-immunized 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 αr-PrP in vitro, T cells from αr-PrP-immunized

![FIGURE 1.](http://www.jimmunol.org/) In vitro T cell proliferation, by αr- and βr-PrP challenge. The mean SI ± SEM of lymph nodes T cells from animals immunized with αr-PrP, βr-PrP, and PBS is shown, after in vitro challenge with αr-PrP and βr-PrP. The data of αr- and βr-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 αr-PrP immunization = 1535 ± 346, βr-PrP immunization = 1419 ± 325, and from PBS immunization = 1093 ± 378.

![FIGURE 2.](http://www.jimmunol.org/) Immunodominant epitope of αr-PrP and βr-PrP immunized T cells. The mean SI ± SEM of lymph node T cells, four experiments with three animals, immunized with A, αr-PrP; B, βr-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 αr-PrP immunization = 1535 ± 346, βr-PrP immunization = 1419 ± 325, and from PBS immunization = 1093 ± 378.

![FIGURE 3.](http://www.jimmunol.org/)
immunizations produced a large amount of IFN-γ, and PBS (\( \alpha \)PrP/H9251 was off scale. A small amount of IFN-γ/H9253 mice produced only 340 pg/ml IFN-γ, whereas T cells from rβ-PrP immunization produced >2000 pg/ml IFN-γ (Fig. 3A). After 48-h in vitro stimulation of cells, T cells from both rα- and rβ-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 un-stimulated 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 rα-PrP compared with rβ-PrP, by T cells from both rα-PrP (\( p = 0.001, t \) test) and rβ-PrP (\( p = 0.0108, t \) test) immunizations. T cells from rα-PrP-immunized mice produced 227 pg/ml IL-5 in response to rα-PrP but only 90 pg/ml in response to rβ-PrP, and T cells from rβ-PrP-immunized mice produced 122 pg/ml in response to rα-PrP and only 16 pg/ml in response to rβ-PrP (Fig. 3B). The T cells from rα-PrP-immunized mice also produced significantly more IL-5 in response to in vitro stimulation with rα-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 rα- 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 rα-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 rα- 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 rα- 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 rα- and rβ-PrP and their sera Abs were positive to rα- and rβ-PrP Ags by ELISA, while no Ab to rα- 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 rα-PrP, as compared with those immunized with rβ-PrP. Immunization with rα-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 rα-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 rβ-PrP-immunized mice, when compared with rα-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 these unimmunized mice (Fig. 4B). In addition, different anti-PrP Ab IgG isotypes were found in the sera of mice immunized with rα-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 rα-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-isofoms 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-isofoms of PrP. The results obtained for the mAbs produced to rα- 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
data already obtained for T cells and the sera Ab Ig isotypes. ICSM4, -10, -18, -35, and -37 mAbs, raised to rH9251- and rH9252-PrP, had different specificities and IgG isotypes. Those produced against rH9251-PrP (ICSM4, -10, and -18) were of IgG1 isotype, whereas rH9252-PrP-derived mAbs (ICSM35 and -37) were IgG2b and IgG2a.4 Furthermore, IgG isotypes were also different in the 40 mAbs we have produced to rH9251-PrP and 41 mAbs to rH9252-PrP. The isotypes of the mAbs produced from rH9251-PrP immunizations were 92.5% IgG1 and 7.5% IgG2 (2.5% IgG2a and 5% IgG2b), whereas the isotypes of those produced to rH9252-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 α- and β-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 α-PrP) and ICSM35 and

FIGURE 4. A, Serum Abs are produced in mice immunized with α-PrP and β-PrP. Sera from Prnp0/0 mice immunized with α-PrP ( ), rβ-PrP ( ), or not immunized ( ) were tested at 1/100 dilution against α-PrP or β-PrP (20 μg/ml) by ELISA. Anti-mouse Ig (Fab ‐specific)‐HRP (Sigma‐Aldrich) was used as secondary Ab. B, Different serum Ab Ig isotypes are produced after immunization with α-PrP and β-PrP. The ELISA results of the total Ig isotypes, as the mean OD ± SEM, are shown for sera of mice immunized with α-PrP ( ), rβ-PrP ( ), and not immunized ( ). The sera from three mice immunized with α-PrP, three mice immunized with β-PrP, and three unimmunized mice were tested in duplicate at 1/100 dilution. C, Different serum anti-PrP Ab Ig isotypes are produced after immunization with α-PrP and β-PrP. Sera from three mice immunized with α-PrP ( ) and three mice immunized with β-PrP ( ) were tested in duplicates at 1/100 dilution in a β-PrP ELISA, using different anti-mouse IgG isotype‐specific HRP and IgM HRP (DakoCytomation) secondary Abs. The rβ-PrP ELISA data are shown as mean OD ± SEM.

FIGURE 5. Detection of PrP by ELISA (A) and Western blotting (B), using α-PrP- and β-PrP-derived mAbs. A, ICSM18 and -35 were tested for their ability to capture (at IgG 10 μg/ml) and detect (at IgG 0.5 μg/ml) PrP in an indirect sandwich ELISA, using normal wild-type (Wt) mouse brain homogenate, RML-infected scrapie‐affected (Sc) brain homogenate and PBS as Ag. For the ELISA, either ICSM18 was used for PrP capture and ICSM35B for detection ( ) or ICSM35 was used for PrP capture and ICSM18B for detection ( ). B, ICSM18 and ICSM35 recognize all three PrP bands by Western blot, representing unglycosylated (29 kDa), monoglycosylated (33 kDa), and diglycosylated PrP (36 kDa), in murine PrP C and PrPSc. ICSM4 labeled the full-length unglycosylated PrP band (~29 kDa), but not the mono- and diglycosylated bands in normal (N) brain homogenate. After PK treatment (+PK), all the truncated and the PrP C bands were digested from scrapie‐affected (Scrapie) brain homogenate and only PrPSc bands were recognized at reduced molecular weights of diglycosylated (~33 kDa), monoglycosylated (~33 kDa), and unglycosylated (~28 kDa) PrP. No binding to PrP was observed, when isotype control-negative mAbs were used and the above mAbs were negative, when tested against Prnp0/0 mouse brain (data not shown).
37 (raised to rβ-PrP) were all positive to denatured PrP and PK-treated PrPSc in a Western blot, but they recognized different PrP glycoforms. 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-, 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 and ICSM35 were chosen, as strongly binding representatives of PrP glycoforms. ICSM18 has a high affinity for PrPSc, while ICSM35 has a higher affinity 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 ro- and rβ-PrP-generated mAbs. Approximately similar epitopes for rβ-PrP-derived mAbs ICSM35 and -37 were found that were different from the ro-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 ro- and rβ-PrP Ags.

These data show that ro- and rβ-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-2k haplotype) with either ro- or rβ-PrP, comparable T cell proliferation against similar immunodominant epitopes was observed. Although α- and β-PrP do not differ in amino acid sequence, they elicited qualitatively different cellular and humoral response. Although T cells from ro- and rβ-PrP immunizations recognized both ro- and rβ-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 PrPSc, and their immunodominant peptides were different: 93–105 for rβ-PrP immunizations (ICSM35 and -37) and 143–153 for ro-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 Prnp0/0 mice. Lack of PrPSc 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, PrPSc may have an important role in T cell activation. PrPSc is significantly up-regulated on mouse T cells upon challenge with concanavalin A (Con-A), but in Prnp0/0 mice, T cell response to Con-A is significantly reduced to 50–80%, suggesting that PrPSc is a lymphocyte surface molecule that participates in T cell activation. PrPSc may have an important role in T cell activation. PrPSc is significantly up-regulated on mouse T cells upon challenge with Concavalain A (Con-A), but in Prnp0/0 mice, T cell response to Con-A is significantly reduced to 50–80%, suggesting that PrPSc is a lymphocyte surface molecule that participates in T cell activation.

In human T lymphocytes, PrPSc expression is also increased upon challenge with Con-A and reduced by the addition of anti-PrP polyclonal Ab (36). PrPSc 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+ memory T cells consistently expressed more PrPSc than CD45RA+ naive T lymphocytes (37). A recent report suggests PrPSc to be a component of the multimolecular signaling complex, within microdomains involved in T cell activation (38). The absence of PrPSc on T lymphocytes of Prnp0/0 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.
FIGURE 7. Immunodominant epitope of anti-α-PrP and β-PrP mAbs determined by ELISA. The mAbs were epitope mapped by ELISA, using 15-mer peptides spanning the relevant PrP sequence, overlapping by 13 residues. Data are shown for (A) anti-α-PrP mAb ICSM 18 (○), and isotype control (□); and (B) anti-β-PrP mAbs ICSM 35 (■) and 37 (△), using ICSM18 (△) as negative control for these peptide sequences. Their epitopes had originally been located with the 20-mer peptides used in Fig. 2. The finer specificities are further confirmed by competition with the peptides, in inhibiting the binding of (C) ICSM18 and (D) ICSM35 to α-PrP (○) or β-PrP (■). The mAbs were added together with the competitor peptides.

apparently it does not affect their cytokine production. The proliferation response was particularly weak after in vitro challenge with the peptides, but in a preliminary study we have found that splenocyte T cells from rr-PrP-immunized mice produce IL-5 in response to in vitro challenge with peptide sequence 191–210, and T cells from both rr- and rrβ-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−/− mice with rrPrP (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+ or CD8+ T cell response, CD4+ 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 rr-PrP was skew 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 rrβ-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 α- and β-PrP. When rr-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 rrβ-PrP. In comparison with rr-PrP, rrβ-PrP is rich in β-sheet and partially PK-resistant (9). It is possible that α-PrP is mainly processed by B cells and dendritic cells and much less by macrophages, whereas β-PrP is mainly processed by macrophages and less by B cells or dendritic cells. Immune tolerance to PrP in Prnp<sup>−<sup>−</sup></sup> mice can reportedly be overcome by improved immunization techniques, using mouse rrPrP (46) and dimeric mouse rrPrP (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>sc</sup> has been reported (26, 49). Our findings may provide an insight into how PrP<sup>sc</sup> and PrP<sup>sc</sup> might be differentially processed by APCs, such as dendritic cells and macrophages. The data presented in this study on the immune responses to α- and rrβ-PrP might reflect those to PrP<sup>sc</sup> and PrP<sup>sc</sup>, if there was no tolerance to PrP<sup>sc</sup> 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>sc</sup> to PrP<sup>sc</sup> has just begun and before formation of large PrP<sup>sc</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
J. Collinge and G. S. Jackson are consultants and shareholders in D-Gen Ltd., and J. Collinge is also a Director.

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