Detection of bovine spongiform encephalopathy, ovine scrapie prion-related protein (PrP^{Sc}) and normal PrP^C by monoclonal antibodies raised to copper-refolded prion protein

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Prion-related protein (PrP) is a glycosylphosphatidylinositol-linked cell-surface protein expressed by a wide variety of cells, including those of the nervous system and the immune system. Several functions of normal cellular PrP (PrP^C) have been proposed that may be associated with the capacity of this protein to bind copper. In the present study, we describe the generation of a panel of monoclonal antibodies raised to copper-refolded PrP, which may be used to analyse the normal and disease-associated forms of this protein. The anti-PrP monoclonal antibodies were reactive by Western blot and ELISA with recombinant murine PrP^C refolded in the presence or absence of either copper or manganese, and with the disease-susceptible allelic form V₁₃₆R₁₅₄Q₁₇₁ ('VRQ'; where single-letter amino-acid notation has been used) and disease-resistant allelic form

A₁₃₆R₁₅₄R₁₇₁ ('ARR') of recombinant ovine PrP^C. FACS analysis of lymphoid cells using these monoclonal antibodies showed that wild-type non-activated mouse lymphocytes expressed little, if any, PrP^C. These monoclonal antibodies were shown to react with the unglycosylated and monoglycosylated forms of PrP^{Sc} (abnormal disease-specific conformation of PrP) in prion-infected tissue samples from all of the different species tested by Western blot. In addition, this analysis allowed one to make a distinction between bovine spongiform encephalopathy ('BSE') and scrapie PrP^{Sc} isolates from experimentally infected sheep on the basis of their different electrophoretic mobilities.

Key words: conformational, epitope, metal ion, neurodegenerative.

INTRODUCTION

Prion diseases, or transmissible spongiform encephalopathies, are a group of central nervous system neurodegenerative disorders that affect both humans and animals. These diseases, which include Creutzfeldt–Jakob disease (CJD) of humans, bovine spongiform encephalopathy (BSE) of cattle and scrapie of sheep, are believed to comprise a common pathogenic mechanism that involves the conversion of a normal cellular prion-related protein (PrP^C) into a form that is infectious and partially protease resistant, termed PrP^{Sc} (the abnormal disease-specific conformation of PrP) [1,2]. The conversion of PrP^C into PrP^{Sc} is considered to involve a conformational change from the predominantly α -helical form of PrP^C to the predominantly β -sheet structure of PrP^{Sc} [3–6]. At present, the only established disease-specific marker of prion diseases is the accumulation of PrP^{Sc} in nervous tissue, and in some cases peripheral lymphoid tissue, of affected individuals [7–12]. The relationship between PrP^{Sc}, the identity of the infectious agent and the neurotoxic molecule responsible for pathology seen in these diseases remains to be clarified [13]. It is important therefore to generate reagents that identify PrP^{Sc} and other possible pathogenic forms of the PrP molecule that may act as intermediates in the disease process. Such reagents will be essential in the elucidation of the mechanism of disease pathogenesis and in the diagnosis of infected individuals, particularly during preclinical prion disease.

Although it is established that PrP is essential for the de-

velopment of prion diseases [14], the normal function of PrP^C remains an enigma. Several lines of evidence have indicated that the trace element copper may influence the biology of PrP^C. It has been shown that the N-terminal octapeptide repeat region of PrP^C binds copper ions with high affinity [15–20]. This interaction is dependent upon pH and induces a conformational change in the normally unstructured N-terminal region of PrP [17,21]. The binding of copper to PrP^C rapidly and reversibly stimulates the endocytosis of this protein from the cell surface, indicative of a role for PrP^C in the cellular transport of copper [22]. A disturbance in PrP-mediated copper metabolism or transport is implicated as a contributing factor in prion disease through the interaction of this metal ion with PrP^{Sc}. Copper facilitates the restoration of protease resistance and infectivity associated with guanidine-denatured PrP^{Sc} [23]. Copper has also been reported [24] to convert PrP^C into a protease resistant form that is distinct from PrP^{Sc}. In addition, the cleavage pattern of human CJD PrP^{Sc} is affected by its copper ion occupancy [25]. Collectively, these observations strongly suggest that the molecular nature of both PrP^C and PrP^{Sc} is likely to be influenced by copper and possibly other related metal ions.

The majority of existing monoclonal antibodies specific for PrP have been generated through immunization with either recombinant protein refolded in the absence of metal ions or short synthetic peptides. It is clear that a full understanding of the biology of PrP and the pathogenesis of prion diseases will require a diverse panel of PrP-specific reagents. These may allow

Abbreviations used: ARR, disease-resistant allelic form A₁₃₆R₁₅₄R₁₇₁; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jakob disease; ECL, enhanced chemiluminescence; PBS-T20, PBS containing 0.1 % Tween 20; PBS-T80, PBS containing 0.1 % Tween 80; PrP, prion-related protein; PrP^C, normal cellular PrP; PrP^{Sc}, abnormal disease-specific conformation of PrP; TBS, Tris-buffered saline; TBS-T, TBS containing 0.05 % Tween 20; VRQ, disease-susceptible allelic form V₁₃₆R₁₅₄Q₁₇₁.

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the identification of possible intermediates involved in the conversion of PrP^c into PrP^{sc}. In the present study, we have immunized PrP^{-/-} mice with murine recombinant PrP^c that has been refolded in the presence of copper to obtain a panel of monoclonal antibodies specific for PrP. These monoclonal antibodies react with PrP from a variety of mammals, including bovine, ovine and murine species forms. They are capable of detecting PrP^c on the surface of cells measured by FACS, and can identify PrP^{sc} by Western blot. In the present study, we report our initial characterization of these monoclonal antibodies in terms of their reactivity with normal and pathogenic forms of PrP.

MATERIALS AND METHODS

Mice

PrP^{-/-} [26] and tga20 mice [27] were obtained from Professor Charles Weissmann (MRC Prion Unit/Neurogenetics, Imperial College School of Medicine, London, U.K.). 129Sv × C57BL/6 F1 were bred in house from 129Sv and C57BL/6 wild-type mice purchased from Harlan UK (Bicester, Oxon., U.K.). All regulated procedures involving experimental animals were carried out under a project and personal licence authority issued in accordance with The Animals (Scientific Procedures) Act 1986.

Purification of recombinant PrP^c and truncated PrP peptides

Recombinant PrP^c was purified from BL21(DE3)pLysS bacteria transformed with the prokaryotic expression vector pET-23b, which contained the open reading frame coding sequence of mature murine PrP^c, ovine PrP^{c-VRQ} (where VRQ represents the disease-susceptible allelic form V₁₃₆R₁₅₄Q₁₇₁; single-letter amino-acid notation has been used) or ovine PrP^{c-ARR} (where ARR represents the disease-resistant allelic form A₁₃₆R₁₅₄R₁₇₁). For truncated peptides of murine PrP^c, BL21(DE3)pLysS cells were transformed with pET-23b that contained DNA encoding amino acids 23–90, 91–159 or 160–231 of the full-length protein (PrP23–90, PrP91–159 and PrP160–231 respectively). Transformed bacteria were grown at 37 °C in Luria–Bertani broth supplemented with 100 µg/ml ampicillin and induced with 1 mM isopropyl β-D-thiogalactoside. Bacteria were harvested by centrifugation at 4000 g for 15 min at 4 °C, resuspended in 20 mM Tris/HCl, 8 M urea and 20 mM imidazole (pH 7.5) and lysed by sonication. The soluble fraction was applied to a nickel–Sepharose column and PrP^c protein was eluted off the column with 20 mM Tris/HCl, 8 M urea and 500 mM imidazole (pH 7.5). Full-length murine PrP^c was refolded by rapid dilution into 20 mM Tris/HCl (pH 6.5) in the absence or presence of copper or manganese [28] to a final approximate concentration of 50 ng/µl. Residual traces of urea and imidazole were removed by dialysis in PBS. The samples were snap-frozen in liquid nitrogen and stored at –70 °C.

Generation of monoclonal antibodies

Monoclonal antibodies were prepared by conventional hybridoma technology [29]. Briefly, 6-week-old PrP^{-/-} mice were immunized by subcutaneous injection with 50 µg of copper-refolded recombinant PrP emulsified in complete Freund's adjuvant and boosted twice at monthly intervals with a similar amount of PrP in incomplete Freund's adjuvant. Mice were finally injected intravenously with antigen in PBS 3 days prior to the fusion. Spleens were removed and single cell suspensions were fused to the NS0 cell line and selected in hypoxanthine/aminopterin/thymidine ('HAT') medium. Supernatants were screened by ELISA using murine and ovine recombinant PrP as

antigens, and positive cell lines were cloned by limiting dilution. Antibody was purified from hybridoma tissue culture supernatant by affinity chromatography on Protein G columns. The isotype of the monoclonal antibodies was determined by capture ELISA with anti-mouse immunoglobulin-isotype-specific reagents (Sigma, Poole, Dorset, U.K.). For biotinylation, monoclonal antibodies were dialysed into 0.1 M NaHCO₃ (pH 8.3) and subsequently reacted with 75 µl of a 1 mg/ml solution of biotin-*o*-succinimide ester (Sigma) in DMSO per 1 mg of IgG for 4 h with shaking at 21 °C. Biotinylated antibodies were dialysed extensively against PBS and the concentration determined by A₂₈₀ prior to storage at 4 °C.

ELISA

Recombinant PrP protein at 1 or 5 µg/ml was coated on to 96-well flat-bottomed plates overnight at 4 °C. Excess protein was removed and wells blocked with PBS containing 5% non-fat milk for 60 min at 37 °C. Plates were washed three times with PBS containing 0.1% Tween 80 (PBS-T80). Appropriate dilutions of anti-PrP antibodies diluted in PBS were added and the plates incubated for 60 min at 37 °C, followed by three washes with PBS-T80. A biotin-conjugated anti-(mouse IgG) or anti-(rabbit IgG) antibody (Sigma), both at 1:1000 dilution, was added for 60 min at 37 °C, followed by three washes with PBS-T80. After washing, avidin–alkaline phosphatase conjugate (Sigma), at a 1:1000 dilution, was then added for 60 min at 37 °C. Plates were washed three times in PBS-T80 and once with ELISA buffer (0.05 M glycine, 0.03 M NaOH and 0.25 M each of ZnCl₂ and MgCl₂) before addition of the substrate *p*-nitrophenyl phosphate (Sigma) at 0.5 mg/ml for 30–60 min at 37 °C. ELISA plates were read at 405 nm on a MR5000 micro ELISA plate reader (Dynatech, Billingham, West Sussex, U.K.).

Isolation of spleen mononuclear cells for immunofluorescence staining

Spleens were isolated from mice, placed in FACS buffer [PBS containing 1% (v/v) heat-inactivated fetal-calf serum and 0.1% sodium azide] and processed through a nylon cell strainer (40 µm pore size). Single cell suspensions were layered on to NycoPrep™ Animal (density 1.077 g/ml; osmolarity 265 mOsm) and centrifuged at 600 g for 15 min at 21 °C. Mononuclear cells were recovered from the density medium interface and washed three times in FACS buffer prior to immunofluorescence staining. Cell-surface phenotype was assessed using samples of 1 × 10⁶ cells incubated with 2.5 µg of biotinylated monoclonal antibody for 20 min at 4 °C, followed by 0.25 µg of streptavidin–phycoerythrin for 20 min at 4 °C. Cells were washed three times with FACS buffer following each treatment. FACS analysis was performed using a FACSCalibur® (Becton Dickinson, Mount View, CA, U.S.A.). Cells (10000 per sample) were analysed, with dead cells excluded on the basis of forward- and side-light scatter.

Western-blot analysis for recombinant PrP and truncated peptides

For Western-blot analysis of recombinant PrP, proteins were subjected to SDS/PAGE under reducing conditions and were subsequently transferred on to nitrocellulose membranes by semi-dry blotting. Membranes were blocked with Tris-buffered saline [TBS; 10 mM Tris/HCl (pH 7.8) and 100 mM NaCl] and 0.05% Tween 20 (TBS-T) containing 5% (w/v) non-fat milk and subsequently incubated in 1% (w/v) non-fat milk in TBS-T with rabbit polyclonal anti-PrP serum XN [30] or mouse polyclonal or monoclonal antibodies for 60 min. These were followed by either biotin-conjugated goat anti-(rabbit IgG) (Sigma) or

biotin-conjugated goat anti-(mouse IgG) (Sigma) and finally extravidin-horseradish peroxidase (Sigma). All detection antibodies were diluted 1:1000 in 1% (w/v) non-fat milk in TBS-T. PrP bands were detected by enhanced chemiluminescence (ECL; Sigma).

Extraction of PrP^{Sc} and detection by Western blot

PrP^{Sc} was extracted from various sources, including the murine neuroblastoma cell line N2a58/22L, which are persistently infected with the 22L scrapie strain, and tissues from prion-infected animals. The animal tissues were from cases of natural scrapie in sheep or BSE in cattle identified by the French surveillance laboratories. Tissues from experimental BSE of sheep [31] or mice inoculated with the C506M3 prion strain [32] were obtained as reported previously [31,32]. PrP^{Sc} was extracted from N2a58/22L cells in a 25 cm² flask by lysis of the cell sheet in 1 ml of Triton X-100/deoxycholic acid lysis buffer [150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholic acid and 50 mM Tris/HCl (pH 7.5)] on ice for 15 min. The supernatant was collected and the total protein concentration was adjusted with lysis buffer to 1 mg/ml. The samples were digested with 20 µg/ml of proteinase K (Roche, Lewes, East Sussex, U.K.) at 37 °C for 30 min, and the digestion was stopped by the addition of 5 mM PMSF for 5 min on ice. The samples were centrifuged at 20000 *g* for 45 min at 4 °C and the pellet was resuspended in 30 µl of denaturing buffer [4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 192 mM glycine, 25 mM Tris/HCl (pH 7.5) and 5% (w/v) sucrose] and heated for 5 min at 100 °C before analysis by SDS/PAGE. For the extraction of PrP^{Sc} from tissues, samples were homogenized (10%, w/v) in a 5% (w/v) glucose solution. Homogenates were forced through a 0.4 mm diameter needle before treatment at 37 °C for 60 min with 10 µg of proteinase K/100 mg of tissue. After the addition of *N*-lauroylsarkosyl to produce a final concentration of 10% (w/v), samples were incubated for 15 min at 21 °C and then centrifuged at 465000 *g* for 2 h on a 10% (w/v) sucrose cushion (TL 100 ultracentrifuge; Beckman, High Wycombe, Bucks., U.K.). The pellets were resuspended in 100 µl of denaturing buffer, heated for 5 min at 100 °C and re-centrifuged at 12000 *g* for 15 min at 21 °C. Finally, the pellet was discarded and the supernatant analysed by SDS/PAGE.

PrP samples were separated on 15% (w/v) polyacrylamide gels and the proteins were transferred on to nitrocellulose membranes (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.) in 25 mM Tris/192 mM glycine/10% (v/v) isopropanol buffer at 400 mA constant for 60 min. For immunoblotting, the membranes were blocked with 5% (w/v) non-fat milk in PBS containing 0.1% Tween 20 (PBS-T20) and subsequently incubated for 60 min at 21 °C with the rabbit polyclonal anti-PrP serum XN or RB1 [33], or individual anti-PrP monoclonal antibodies. The membranes were subsequently washed twice in PBS-T20 and incubated in peroxidase conjugated anti-(rabbit Ig) or anti-(mouse IgG) antibody (1:2500 dilution; Southern Biotechnology Associates, Birmingham, AL, U.S.A.) in PBS-T20 for 30 min at 21 °C. Blots were finally washed three times in PBS-T20 and once in PBS before the bound antibodies were visualized on film (Biomax Light; Kodak) using ECL [ECL[®] (Amersham Biosciences) or Super Signal Ultra (Pierce)].

Deglycosylation of PrP^{Sc}

Pellets obtained after proteinase K treatment and ultracentrifugation of PrP^{Sc}-infected brain homogenates were denatured by heat treatment at 100 °C for 10 min in 50 µl of 0.5%

SDS in distilled water. After addition of 200 µl of 0.5% Triton X-100 in 50 mM Tris/HCl (pH 8.0) containing 5 mM PMSF, the samples were treated with peptide N-glycosidase F (Roche) (1 unit/sample) and incubated at 37 °C overnight. Proteins were subsequently precipitated in ethanol and resuspended in 50 µl of denaturing buffer before analysis by SDS/PAGE.

RESULTS

Characterization of monoclonal antibodies reactive with PrP

We have generated a panel of anti-PrP monoclonal antibodies by immunization of PrP^{-/-} mice with copper-refolded murine recombinant PrP^C. Positive hybridoma tissue culture supernatants were initially identified by ELISA using murine or ovine recombinant PrP^C as substrate. Hybridoma cells were subsequently cloned by limiting dilution and, from a total of 30 positive hybridomas, eight clones (numbered 241, 242, 244, 245, 246, 248, 249 and 251) were used for further investigation and the data reported in the present study. Monoclonal antibodies were purified from tissue culture supernatant by affinity chromatography on Protein G columns. Preliminary characterization of the monoclonal antibodies by SDS/PAGE showed the presence of light- and heavy-chain protein bands at approx. 55 kDa and 25 kDa respectively, indicative of an IgG antibody (results not shown). The purity of the Protein G-purified monoclonal antibodies was estimated to be > 99% as judged by SDS/PAGE

Figure 1 Rectivity of monoclonal antibodies with metal-ion-refolded PrP^C by Western-blot analysis

Western-blot analysis of representative monoclonal antibodies against copper-, manganese- or water-refolded PrP^C. Lanes 1, copper-refolded PrP^C; lanes 2, manganese-refolded PrP^C; and lane 3, water-refolded PrP^C. PrP^C (10–15 µg) was loaded in each lane. Monoclonal antibodies 249 and 251 were used at 5 µg/ml and are shown in comparison with rabbit polyclonal anti-PrP serum XN used at 1:1000 dilution as the positive control. The negative control panel (Control) shows an identical gel with the primary antibody omitted. Molecular-mass markers (in kDa) are indicated on the left.

Figure 2 Reactivity of anti-PrP monoclonal antibodies with metal-ion-refolded PrP^{Sc} by ELISA

The reactivity of the monoclonal antibodies with metal-ion-refolded PrP^{Sc} was investigated by ELISA. The seven positive monoclonal antibodies (at 10 µg/ml) were reacted against water- (no metal), manganese- and copper-refolded PrP^{Sc}, using 1 µg/ml in triplicate wells of the relevant protein to coat the plates. The rabbit polyclonal anti-PrP serum XN was used in comparison as a positive control at a 1:1000 dilution. Plates were read at an absorbance of 405 nm. Results are means ± S.E.M. from triplicate wells. The dotted line represents the level of background reactivity of monoclonal antibodies with BSA instead of PrP, or PBS instead of the monoclonal antibodies. O.D., optical density.

Figure 3 Reactivity of anti-PrP monoclonal antibodies with recombinant PrP^{Sc} by ELISA

The reactivity of the monoclonal antibodies with different species forms of PrP^{Sc} was investigated by ELISA. The eight monoclonal antibodies were tested over a range of dilutions as shown against water-refolded PrP^{Sc} used at 5 µg/ml in triplicate wells. (a) Murine PrP^{Sc}; (b) ovine PrP^{Sc}-VRQ; and (c) ovine PrP^{Sc}-ARR. Plates were read at an absorbance of 405 nm. The dotted line represents the level of background reactivity of monoclonal antibodies with BSA instead of PrP, or PBS instead of the monoclonal antibodies. O.D., optical density.

and Coomassie Blue staining. The monoclonal antibodies were all of the IgG₁ isotype except for 246, which was IgG₃.

Reactivity of monoclonal antibodies with metal-ion-refolded PrP^{Sc}

PrP^{Sc} has been shown to bind copper within its N-terminal octapeptide repeat region and, in addition, between these motifs and the C-terminal globular domain [15–20]. The binding of

copper to the N-terminal octapeptide repeat region induces a more ordered structure to this part of the molecule that is normally highly flexible. It has also been reported [28] that manganese may substitute for the binding of copper to PrP, although the conformational changes that result are not established. We investigated whether the monoclonal antibodies raised to copper-refolded PrP^{Sc} did indeed show a preference for this form of prion protein. Initially, representative monoclonal

Figure 4 Regional epitope mapping with truncated peptides of PrP

Western-blot analysis of monoclonal antibodies 249 and 251 against purified truncated forms of PrP^{Sc}. Lanes 1, amino acids 23–89; lanes 2, amino acids 90–159; and lanes 3, amino acids 160–231. Monoclonal antibodies 249 and 251 are shown in comparison with a mouse polyclonal anti-PrP serum as the positive control. The negative control panel shows an identical gel omitting the primary antibody. Molecular-mass markers (in kDa) are indicated on the left.

Figure 5 Detection of cell-surface PrP by FACS analysis

Single cell suspensions of spleen mononuclear cells were analysed by FACS for PrP expression. (a) Scatter profile of gated cells. R1 encompasses small cells, possibly representing newly divided progenitor mononuclear cells, and R2 represents mature resting lymphocytes and antigen-presenting cells. (b) Representative FACS profiles showing the reactivity of monoclonal antibody 251 with mouse spleen mononuclear cells isolated from PrP^{Sc}−/−, wild-type and tga20 mice. The rabbit polyclonal anti-PrP serum XN was used as a positive control.

antibodies were tested by Western-blot analysis for their reactivity with copper- or manganese-refolded murine PrP^{Sc} or PrP^{Sc} refolded in the absence of any metal ion. Figure 1 shows that the

monoclonal antibodies 249 and 251 both reacted with all three forms of PrP^{Sc} and in each case a single band of approx. 25 kDa, which corresponds to the molecular mass of murine recombinant

Figure 6 PrP^{Sc} detection by Western blot

Uninfected wild-type, PrP^{-/-} and tga20 mouse brain homogenates were analysed by Western blot using a representative monoclonal antibody 249 (a), rabbit polyclonal anti-PrP serum XN (b) and an anti-(β -actin) antibody (c). (d) Reactivity of monoclonal antibody 249 with non-proteinase K-treated (lane 1) and proteinase K-treated (lane 2) tga20 brain homogenate. The amount of sample loaded was 10 μ l for the non-proteinase K-treated samples and 15 μ l (equivalent to 40–50 μ g of total protein) for the proteinase K-treated samples. Molecular-mass markers (in kDa) are indicated on the right.

PrP, was detected. As a control, the rabbit polyclonal anti-PrP serum XN [30] was shown to react with a protein band of the same molecular mass for all three forms of PrP^{Sc} as for the monoclonal antibodies.

The reactivity of the monoclonal antibodies with metal-ion-refolded PrP^{Sc} was subsequently investigated by ELISA, where the conformation of the different forms of PrP^{Sc} may resemble the native shape of the molecule compared with the presumably denatured form that occurs in SDS/PAGE. Figure 2 shows that the monoclonal antibodies could be divided into two groups. The first, comprising 241, 244 and 248, which showed equal reactivity with all three forms of PrP, and a second group, comprising 242, 245, 249 and 251, which showed stronger reactivity with copper-refolded PrP^{Sc} compared with PrP^{Sc} with no metal ion. Within this second set there was differential reactivity for

manganese-refolded PrP^{Sc} exemplified by 245 and 251. Antibody 246 was not included as it only showed low reactivity (results not shown). The rabbit polyclonal anti-PrP serum XN showed the same reactivity profile with all three forms of PrP^{Sc}. These data suggest that the metal-ion occupancy of PrP^{Sc} does influence the binding of monoclonal antibodies to this protein.

Cross-reactivity of anti-PrP monoclonal antibodies with different species forms of recombinant PrP^{Sc}

Figure 3 shows the reactivity of the panel of monoclonal antibodies with either murine or ovine recombinant PrP^{Sc}, re-folded in the absence of any metal ion, measured by ELISA. Predictably, because of the high degree of amino acid sequence conservation between different species forms of PrP, all of the monoclonal antibodies reacted to some extent with either murine or ovine recombinant PrP^{Sc}. However, there was some variation in their apparent affinity for the different species forms of PrP^{Sc}. For example, monoclonal antibody 248 reacted well with ovine PrP^{Sc}, but bound weakly to murine PrP^{Sc}. A further difference between some of the monoclonal antibodies was seen with their reactivity with different allelic forms of ovine recombinant PrP^{Sc}. Expression in sheep of the VRQ and ARR allelic forms of ovine PrP^{Sc} is associated with high and low risk of scrapie disease respectively [34,35]. These variations in amino acid residues are located at positions 136, 154 and 171 of the mature ovine PrP^{Sc} protein, which may represent regions of the PrP molecule that are affected by metal-ion binding. Some of the monoclonal antibodies appeared to differ in their affinity for the different allelic forms of ovine PrP^{Sc}. For example, monoclonal antibody 245 reacted well with ovine PrP^{Sc}-VRQ, but bound weakly to ovine PrP^{Sc}-ARR.

Regional epitope mapping of anti-PrP monoclonal antibodies with truncated peptides of PrP

Truncated forms of murine PrP^{Sc} corresponding to amino acids 23–89, 90–159 and 160–231 were expressed from a prokaryotic expression vector in order to determine which region of the PrP molecule the monoclonal antibodies recognized. Each PrP peptide contained a C-terminal His₆ tag, which allowed its purification by affinity chromatography on a nickel-ion column. These purified truncated forms of PrP^{Sc} were subsequently used to localize the binding region of the different monoclonal antibodies. A representative Western blot with monoclonal antibody 249 and 251 is shown in Figure 4. Both monoclonal antibodies reacted with the 160–231 peptide of murine PrP^{Sc}, indicating that 249 and 251 bind to the C-terminal region of the PrP molecule. All other monoclonal antibodies from this panel reacted similarly with the C-terminal peptide of PrP. Mouse polyclonal anti-PrP serum (obtained from the mouse used for the original hybridoma fusion) was used as a positive control and this reacted with all three of the truncated PrP peptides.

Detection of cell-surface PrP by FACS

PrP^{Sc} is a membrane-linked glycoprotein and the copper-bound form of this protein is believed to represent a physiologically relevant form of PrP. It remains to be established if copper-loaded PrP^{Sc} is expressed on the surface of cells that possess this protein. Accordingly, we investigated whether our panel of copper-refolded anti-PrP^{Sc} monoclonal antibodies was capable of detecting cell-surface expression of PrP^{Sc}. Figure 5 shows the representative FACS profiles for the reactivity of monoclonal

antibody 251 with mouse spleen mononuclear cells isolated from PrP^{-/-}, wild-type and tga20 mice, which express zero, normal and 10-fold levels of PrP^c protein respectively. Cells within two electronic gates were assessed: R1, which encompasses small mononuclear cells that probably represent newly divided progenitor mononuclear cells; and R2, which encompasses mature resting lymphocytes and antigen-presenting cells.

When gate R1 was used to assess the fluorescence intensity, there was a significantly greater level of PrP expression by wild-type and tga20 cells above the background level seen with PrP^{-/-} cells. The level of PrP expression by tga20 cells was approx. 5-fold greater than that seen with wild-type and reflects the increased cellular level of PrP expression by this mouse strain. In contrast, when gate R2 was used to assess PrP expression, there was little, if any, increase in the percentage of cells stained positive for PrP^c compared with the background level on PrP^{-/-} cells. The profiles seen with monoclonal 251 were almost identical with those seen when similar cells were reacted with the rabbit polyclonal anti-PrP serum XN, as shown in Figure 5(b). These data show that monoclonal antibody 251 is capable of reacting with cell-surface PrP^c.

Despite the ability of these monoclonal antibodies to recognize PrP, as judged by several techniques including FACS, these antibodies do not appear to react with this protein by immunocytochemistry. The failure of monoclonal antibodies to work effectively by immunocytochemistry is not uncommon and is quite often the case when antibodies are selected using techniques such as ELISA and Western blotting, as occurred with these particular reagents reported in the present study.

Detection of PrP^c and PrP^{Sc}

Western-blot analysis was used to characterize the reactivity of this panel of anti-PrP monoclonal antibodies with normal and prion-disease associated forms of PrP. Figure 6(a) shows the detection of PrP^c in mouse brain tissue by monoclonal antibody 249, which reacted with proteins of 25 and 31 kDa that are characteristic molecular masses of unglycosylated and monoglycosylated forms of normal PrP. No reactivity was detected with brain homogenate from PrP^{-/-} mice. The same brain homogenate samples reacted with rabbit polyclonal anti-PrP serum XN as a positive control (Figure 6b), and all reacted with anti- β -actin to demonstrate the presence of sample in all three lanes (Figure 6c). Figure 6(d) demonstrates that the PrP^c-specific bands detected by monoclonal antibody 249 were sensitive to proteinase K digestion.

Figure 7(a) shows that the monoclonal antibodies detected the unglycosylated and monoglycosylated forms of PrP^{Sc} when tested against samples extracted from the prion-infected neuroblastoma cell line N2a58/22L or from scrapie-infected sheep brain material. None of the monoclonal antibodies appeared to react with the diglycosylated form of PrP^{Sc} when compared with the pattern obtained with polyclonal anti-PrP serum such as RB1. Figure 7(b) shows that a similar pattern of reactivity was obtained when PrP^{Sc} from bovine, murine or ovine origin or spleen from scrapie-infected sheep was tested. Importantly, this analysis revealed differences in the Western-blot mobility of PrP^{Sc} from sheep brain homogenates that are thought to be associated with strain specificities. It can be seen that BSE-induced PrP^{Sc} in sheep runs with an apparent lower molecular mass than scrapie-induced PrP^{Sc} in sheep. Figure 7(c) shows that the reactivity of the monoclonal antibodies correlated with the amount of PrP^{Sc} loaded per lane. In addition, the reactivity of the monoclonal antibodies with PrP^{Sc} was decreased after deglycosylation to a

Figure 7 PrP^{Sc} detection by Western blot

PrP^{Sc} was detected in ovine, bovine and murine samples by Western-blot analysis with monoclonal antibodies 249 and 251 (used at 5 μ g/ml) or rabbit polyclonal anti-PrP RB1 (1:2500). The data obtained with the two monoclonal antibodies appeared identical and only the results for monoclonal 249 are shown. (a) Reactivity of monoclonal antibody 249 (lanes 1 and 2) or RB1 anti-serum (lanes 3 and 4) with proteinase K-treated PrP^{Sc} extracted from the prion-infected neuroblastoma cell line N2a58/22L (lanes 1 and 3) or proteinase K-treated PrP^{Sc} extracted from the brain of a scrapie-infected sheep (lanes 2 and 4). A total of 12.5 μ g of protein was loaded in lanes 1 and 3 (N2a58/22L cells) and 5 mg of brain equivalent (BE) in lanes 2 and 4 (sheep material). (b) Reactivity of monoclonal antibody 249 with proteinase K-treated PrP^{Sc} extracted from brain (lanes 1–6) or spleen (lane 7) of the following species (the amount of BE loaded per lane): lane 1, BSE in cattle (5 mg); lane 2, scrapie in sheep (5 mg); lane 3, BSE in mouse (0.1 mg); lane 4, scrapie in mouse (0.1 mg); lane 5, scrapie in mouse (0.1 mg); lane 6, experimental BSE in sheep (5 mg); and lane 7, spleen from a scrapie-infected sheep (10 mg). (c) Serial dilutions of sheep brain PrP^{Sc} (5, 2.5, 1 and 0.5 mg of BE in lanes 1–4 respectively) and deglycosylated brain PrP^{Sc} from different species origin [lane 5, mouse (0.1 mg); lane 6, sheep (5 mg); and lane 7, cattle (5 mg)] reacting with rabbit polyclonal anti-serum RB1 (upper panel) or monoclonal antibody 249 (lower panel). Molecular-mass markers (in kDa) are indicated on the left.

single PrP^{Sc} band of approx. 19 kDa depending on its species origin. The monoclonal antibodies showed no reactivity against various tissues from prion-infected animals that were predicted to be negative for PrP^{Sc}, such as spleen from BSE-infected cattle (results not shown). These same samples were also negative for PrP^{Sc} when polyclonal anti-PrP serum RB1 was used for Western blotting (results not shown). The monoclonal antibodies showed no reactivity against proteinase K-treated brain homogenates from uninfected sheep or cattle (results not shown). In addition, no reactivity was seen with PrP^{Sc} samples from these species in the absence of the primary monoclonal antibody (results not shown).

DISCUSSION

The extensive conservation of DNA and protein sequence between different species forms of mammalian PrP^c implies a conserved function for this molecule. It has been proposed that PrP^c is involved in copper metabolism and a consensus view is that one of the principal sites for metal-ion binding is within the N-terminal octapeptide repeat region of the protein. The octapeptides are comprised of the sequence PHGGGWGQ (where single-letter amino-acid notation has been used) in human PrP and these sequences are extensively conserved between different mammalian forms of this molecule. MS, CD and electron paramagnetic studies, using peptides corresponding to these sequences or full-length PrP, have suggested that each octapeptide binds a single copper(II) ion via a nitrogen from the histidine residue imidazole side chain and deprotonated amide nitrogens from the second and third glycine residues [36,37]. These structural features of the copper-loaded octapeptide repeat region predict that the side chains of the glutamine residues in each octapeptide repeat unit facilitate intermolecular non-covalent bonding and possible dimerization between PrP molecules [38]. These observations support the view that copper is involved in PrP^c function and that metal-ion binding to this molecule modifies its conformational state.

In the present study, we have generated monoclonal antibodies that are specific for PrP by immunization of PrP^{-/-} mice with copper-refolded murine PrP^c. When tested by Western-blot analysis, these monoclonal antibodies reacted with similar intensity against recombinant PrP^c refolded in the absence or presence of different trace metal elements. However, when tested by ELISA against the same forms of PrP^c, when a more native conformation of PrP^c would be expected to be present, some of the monoclonal antibodies showed a clear preference for copper-refolded PrP^c. In addition, at least one of the monoclonal antibodies showed a preference for manganese-refolded PrP^c above that of PrP^c refolded in the absence of any metal ion. As each individual monoclonal antibody reacted with these various forms of PrP^c in a specific manner, these data support the view that different metal ions are capable of inducing different conformational shapes of the PrP molecule [25]. It is interesting to note that copper and manganese have been implicated in the induction of a conformational change in PrP^c associated with increased resistance to proteinase K [24,28]. In both cases, the proteinase K-resistant fragment resembles, in approximate peptide length, authentic PrP^{Sc}, as the N-terminal region was cleaved during enzymic digestion. Our present results do not reveal which epitope(s) of PrP^c are affected by the binding of either copper or manganese. Preliminary regional mapping to localize the epitopes recognized by monoclonal antibody 249 and 251, two antibodies that appear to show a preference for copper-refolded PrP^c, revealed their binding within the C-terminal amino acid sequence 160–231. This implies that the binding of copper to either the N-terminal octapeptide repeat region, or possibly some other site within the molecule, may be capable of inducing conformational changes within the C-terminal region of the polypeptide chain or allows greater accessibility of some monoclonal antibodies to their C-terminal epitope. This view is supported by other reports [39,40] that indicate perturbations in the N-terminal region of PrP influence conformational changes or binding events elsewhere in the molecule. Li et al. [39] have shown that the binding of N-terminus-specific anti-PrP monoclonal antibodies to PrP resulted in the failure of C-terminal-specific antibody to bind the same molecule. This effect appeared to be mediated specifically by the N-terminus portion of the molecule as the binding of monoclonal antibodies to other

regions of the protein did not induce the same effect. In addition, the binding of a monoclonal antibody specific to the central portion of PrP showed pH-dependent binding to truncated PrP90–231 [40]. Both of these effects may be attributed to structural changes in the more flexible regions of the PrP molecule, in particular the N-terminal region, which subsequently lead to alterations in epitope exposure in the C-terminal portion of the polypeptide chain. In this regard, it would seem quite likely that subtle changes in the conformational shape of PrP could result from the nature of the metal ion bound to specific sites within the molecule.

We have demonstrated that monoclonal antibodies 249 and 251 are capable of detecting PrP^{Sc} from bovine, ovine and murine origin. In all of these species, both monoclonal antibodies reacted with proteins of approx. 21 and 25 kDa characteristic of the unglycosylated and monoglycosylated forms of PrP^{Sc} [41,42]. The monoclonal antibodies described also reacted with the corresponding deglycosylated form of PrP^{Sc} from these three species. This latter result, together with the failure to see reactivity with the diglycosylated form of PrP^{Sc}, suggests that the epitope(s) recognized by these particular monoclonal antibodies may be sterically hindered by one or other of the carbohydrate moieties. Monoclonal antibodies with specificity for different glycosylated forms of PrP have been described by Zanusso et al. [43]. These workers have identified a panel of 12 monoclonal antibodies raised to recombinant mouse PrP that could be distinguished by their immunoreactivity with glycosylated and deglycosylated PrP^c on immunoblots. The majority of these monoclonal antibodies recognized the C-terminal portion of PrP that contains the two asparagine-linked carbohydrate moieties of PrP. The observations presented in the present study provide further evidence that the epitope(s) recognized by our monoclonal antibodies raised to copper-refolded PrP are located in the C-terminal region of the PrP molecule and may be close to one or other of the two glycosylation sites. Moreover, the apparent molecular masses of these two PrP^{Sc} bands varied according to the species origin of PrP^{Sc} within the infected tissue. Consistent data has been reported that describes differences in the band patterns of PrP^{Sc} following SDS/PAGE and Western blotting, which may be used as a molecular means for the distinction of various prion strains [32,44–47]. In the data presented in the current study, Western blot results of natural and experimental isolates of scrapie and BSE were obtained with two of the monoclonal antibodies that show a lower molecular mass of BSE-induced ovine PrP^{Sc} compared with scrapie-derived ovine PrP^{Sc}. These results support data described previously that suggest proteinase K digestion of BSE-derived ovine PrP^{Sc} occurs more distal to the N-terminus compared with that of scrapie-derived ovine PrP^{Sc} and results in the appearance of smaller fragments of the BSE associated form of PrP^{Sc} [31,48]. These molecular characteristics of PrP^{Sc} may be used in conjunction with other properties, such as incubation time and vacuolation patterns [49,50], for the characterization of different prion strains.

We have recently shown [51] that brain PrP^{Sc}, which accumulates during the preclinical phase of prion disease, is characterized by a decrease in copper content and an increase in the amount of bound manganese. It has also been reported [52] that PrP^{Sc} from CJD brain homogenates is elevated in manganese and depleted in copper compared with PrP^c from normal human brain. These data tentatively suggest that a change in the metal-ion content of PrP may be associated with acquisition of the abnormal form of this molecule. In support of this, it has been shown [25] that conversion of different strains of human CJD PrP^{Sc} can be affected by the variation in their metal-ion content. As the monoclonal antibodies generated in the present study are capable

of reacting with recombinant PrP refolded in the absence or presence of metal ions, these data do not allow us to distinguish whether the detectable PrP^{Sc} is loaded with copper, manganese or neither of these metals. Nevertheless, as the absence or presence of divalent metal cations is suggested to influence the cleavage pattern of the PrP^{Sc} molecules [25], further investigations on the reactivity of the monoclonal antibodies with PrP^{Sc} in the presence of various concentrations of divalent metal cations may reveal further insights into the relationship between proteinase K digestion of PrP^{Sc} and prion strain properties. It will also be relevant to study further the interaction of these monoclonal antibodies with PrP^{Sc} by ELISA, where it may be expected that the PrP molecule is in a more relevant conformational state and the effect of metal-ion binding more clearly shown.

It is a distinct possibility that perturbations in the level of essential trace metal elements are a contributing factor to the formation of PrP^{Sc} during prion disease [51,53]. We have shown [51] that the preclinical phase of Rocky Mountain Laboratory ('RML')-induced murine prion disease is associated with an increase in the blood level of manganese. This condition may provide an environment for the exchange of metal ions within PrP and the subsequent formation of disease-specific conformations of this molecule distinct from PrP^{Sc}. Since PrP is expressed on leucocytes [54,55], it is further possible that these conformations of the molecule may exist on the surface of such cells during prion disease and therefore represent potential novel diagnostic markers that can readily be identified from blood samples. Recently, it has been shown [56] that altered forms of PrP do reside in the blood of prion-infected individuals as infectivity can be transmitted from BSE-infected sheep to recipient animals by blood transfusion. It will therefore be of interest to determine whether leucocytes from prion-infected individuals do indeed express altered conformations of PrP. We have shown that the anti-PrP monoclonal antibodies generated in the present study are capable of recognizing cell-surface PrP by FACS analysis. These reagents may therefore be used to examine whether alterations occur in expression or conformation of cell-surface PrP on blood cells during the progression of prion disease. In addition, these antibodies also lend themselves to a diagnostic evaluation concerned with the modulation of epitopes within the PrP molecule that may be present in serum during the course of prion disease. We speculate that the panel of monoclonal antibodies generated in the present study may prove useful in elucidating the role of metal ions in the function of PrP.

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