Conformational variation between allelic variants of cell-surface ovine prion protein

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The distribution of prion infectivity and PrP^{Sc} between peripheral lymphoid tissues suggests their possible haematogenic spread during the progression of natural scrapie in susceptible sheep. Since ovine PBMCs (peripheral blood mononuclear cells) express PrP^C, they have the potential to carry or harbour disease-associated forms of PrP. To detect the possible presence of disease-associated PrP on the surface of blood cells, an understanding is required of the conformations that normal ovine cell-surface PrP^C may adopt. In the present study, we have used monoclonal antibodies that recognize epitopes in either the N- or C-terminal portions of PrP to probe the conformations of PrP^C on ovine PBMCs by flow cytometry. Although PBMCs from scrapie-susceptible and -resistant genotypes of sheep expressed similar levels of cell-surface PrP^C, as judged by their reactivity with N-terminal-specific anti-PrP monoclonal antibodies, there was considerable genotypic heterogeneity in the region between helix-

1 and residue 171. Cells from PrP-VRQ (V¹³⁶R¹⁵⁴Q¹⁷¹) sheep showed uniform reactivity with monoclonal antibodies that bound to epitopes around helix-1, whereas cells from PrP-ARQ (A¹³⁶R¹⁵⁴Q¹⁷¹) and PrP-ARR (A¹³⁶R¹⁵⁴R¹⁷¹) sheep showed variable binding. The region between β -strand-2 and residue 171, which includes a YYR motif, was buried or obscured in cell-surface PrP^C on PBMCs from scrapie-susceptible and -resistant sheep. However, an epitope of PrP^C that is influenced by residue 171 was more exposed on PBMCs from PrP-VRQ sheep than on PBMCs from the PrP-ARQ genotype. Our results highlight conformational variation between scrapie-susceptible and -resistant forms of cell-surface PrP^C and also between allelic variants of susceptible genotypes.

Key words: epitope, polymorphism, PrP^C, ruminant, secondary structure, transmissible spongiform encephalopathy.

INTRODUCTION

Prion diseases, such as scrapie in sheep, bovine spongiform encephalopathy in cattle and Creutzfeldt–Jakob disease in humans, are transmissible chronic neurodegenerative disorders characterized by the accumulation of PrP^{Sc} [the abnormal disease-specific conformation of prion-related protein (PrP)], an abnormal isomer of the host protein PrP^C (normal cellular PrP). The protein-only hypothesis postulates that the transmissible prion agent consists solely of proteinaceous material [1]. Consequently, it is proposed that PrP^{Sc} forms a part, or all, of the infectious prion agent, and this abnormal isomer is responsible for the modification of the normal cellular form PrP^C. The normal form of PrP is predominantly α -helical (42 %) with a low β -sheet content (3 %), whereas PrP^{Sc} has considerably more β -sheet content (43 %) and a similar α -helical content (30 %) [2]. These observations indicate that during the conversion of PrP^C into PrP^{Sc}, a major refolding event occurs that results in a more extensive β -sheet conformation. The highest level of PrP^C protein expression occurs within the central nervous system and, to a lesser extent, within the peripheral lymphoid system. Prion infectivity and PrP^{Sc} may accumulate at both these sites during the progression of prion disease.

Scrapie in sheep is the archetypal prion disease, and polymorphisms in ovine PrP at amino acid residues 136, 154 and 171 are associated with variation in susceptibility to natural scrapie. V¹³⁶R¹⁵⁴Q¹⁷¹ (PrP-VRQ) or A¹³⁶R¹⁵⁴Q¹⁷¹ (PrP-ARQ) animals show susceptibility to scrapie, whereas those that express A¹³⁶R¹⁵⁴R¹⁷¹ (PrP-ARR) show resistance [3,4]. The conformation of PrP^C has been modelled through NMR studies of full-length [5–8] and truncated recombinant PrP [5,9] from several species, and predicts

a predominantly α -helical structure comprised of a globular C-terminal domain and a flexible N-terminal region. The globular domain contains three α -helices, with helix-2 and -3 joined by a single disulphide bond, and two short anti-parallel β -sheet regions flanking helix-1. The overall structure of the sheep prion protein is predicted to share a similar secondary structural framework because of the high degree of amino acid sequence homology between ovine and other mammalian forms of PrP. A critical site within ovine PrP is the amino acid residue 171, which is glutamine in some scrapie-susceptible genotypes and arginine in scrapie-resistant sheep. The molecular mechanism that accounts for the variation in natural scrapie susceptibility is unknown. Clearly, critical polymorphic amino acid residues will influence the extent or stability of structural changes within ovine PrP or its interaction with potential cofactors such as Protein X [10,11] as it converts from the normal to disease-associated form of PrP.

During the preclinical phase of natural scrapie disease in sheep, prion infectivity and PrP^{Sc} are usually detected first in gut-associated lymphoid tissue. The subsequent appearance of prion infectivity and PrP^{Sc} within different peripheral lymph nodes during the disease process is suggestive of haematogenic distribution. It has been shown that circulating PBMCs (peripheral blood mononuclear cells) of sheep [12,13] and other species [14] express PrP^C on their cell surface. Consequently, peripheral blood is considered to be a possible reservoir of prion infectivity in scrapie-infected sheep and other TSE (transmissible spongiform encephalopathy)-affected individuals. In recent studies [15–17], it has been shown that prion disease can be transmitted through transfusion of whole blood or buffy coat from natural scrapie-infected sheep or experimentally bovine

Abbreviations used: PBMC, peripheral blood mononuclear cell; *Prnp*^{0/0}, *Prnp*-knockout; PrP, prion-related protein; PrP^C, normal cellular PrP; PrP^{Sc}, abnormal disease-specific conformation of PrP.

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spongiform encephalopathy-infected sheep into recipient sheep. This supports the view that blood cells may harbour or carry prion infectivity, and disease-associated PrP may be present on the surface of these cells. To determine whether disease-associated PrP can be detected on blood cells from TSE-affected sheep, it is first necessary to investigate the range of conformations that normal cell-surface PrP can acquire. In the present study, we have probed the cell-surface PrP^C conformations of ovine PBMCs using anti-PrP monoclonal antibodies and flow cytometry. Our results indicate that cell-surface PrP^C displays considerable conformational variation between scrapie-susceptible and -resistant genotypes and also between susceptible allelic variants. This analysis will provide a basis for investigating the presence of disease-associated PrP on the surface of blood cells during prion disease.

MATERIALS AND METHODS

Purification of recombinant and truncated PrP peptides

Recombinant PrP was purified from BL21(DE3)pLysS bacteria transformed with the prokaryotic expression vector pET-23b (Novagen, VWR International, Lutterworth, U.K.) that contained the open reading frame coding sequence of full-length ovine PrP-ARR, PrP-ARQ or PrP-VRQ (residues 25–232) or truncated ovine PrP-ARR or ovine PrP-VRQ (residues 89–233) by a method adapted from Hornemann et al. [18]. Briefly, transformed bacteria were grown at 37 °C in Luria–Bertani medium supplemented with 100 µg/ml ampicillin and 30 µg/ml chloramphenicol, and induced overnight 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, 50 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 10 µg/ml DNase, 1 mg/ml lysozyme and 1 mg/ml deoxycholic acid and incubated at 21 °C for 2 h before further lysis by sonication. Samples were centrifuged at 13 000 g for 20 min and resuspended in a buffer consisting of 8 M urea and 20 mM Tris/HCl (pH 8.0). The soluble fraction, collected after centrifugation at 13 000 g for 20 min at 21 °C, was applied to a nickel-ion-charged Sepharose column (Amersham Biosciences). PrP protein was eluted with 20 mM Tris/HCl, 8 M urea (pH 4.5) and reduced with 100 µM dithiothreitol. PrP was further purified by application to a cation-exchange column (sulphopropyl-Sephadex; Amersham Biosciences) and eluted with 50 mM Hepes buffer (pH 8.0) containing 200 mM NaCl and 8 M urea. Eluted PrP was oxidized using copper sulphate (five times molar concentration of PrP) and refolded by dialysis into three changes of 50 mM sodium acetate buffer (pH 5.5) containing 100 mM EDTA, followed by extensive dialysis into the same buffer without EDTA. Oxidized and refolded recombinant PrP was stored at –70 °C. Recombinant PrP proteins were verified by MS to confirm the correct protein sequence and the presence of a disulphide bond.

Generation of monoclonal antibodies

Anti-PrP monoclonal antibodies were prepared by conventional hybridoma technology. Briefly, 6-week-old *Prnp*^{0/0} (*Prnp*-knock-out) mice were immunized by subcutaneous injection with 50 µg of either refolded full-length murine recombinant PrP (residues 23–231) for the monoclonal antibody 245 [19] or a peptide of murine PrP (residues 161–231) for monoclonal antibodies 683, 884 and 968 and then emulsified in Complete Freund's adjuvant and boosted twice at monthly intervals with a similar amount of protein in incomplete Freund's adjuvant. To generate monoclonal antibodies A516 and V468, *Prnp*^{0/0} mice were im-

munized with truncated PrP-ARR or truncated PrP-VRQ (residues 89–233) respectively (A. M. Thackray and R. Bujdoso, unpublished work). Monoclonal antibodies T188 and T325 were produced from *Prnp*^{0/0} mice immunized with murine brain homogenate (A. M. Thackray and R. Bujdoso, unpublished work). Finally, mice were injected intravenously with antigen in PBS, 3 days before the fusion. Spleens were removed and single-cell suspensions were fused to the NS0 cell line and selected in HAT medium (hypoxanthine/aminopterin/thymidine). Supernatants were screened by ELISA using murine and ovine recombinant PrPs as antigens, and positive cell lines were cloned by limiting dilution. An 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, cat. no. ISO-2).

Epitope mapping

The epitope specificity of anti-PrP monoclonal antibodies was determined by the PEPSCAN method described previously [20]. Briefly, synthetic 15-mer peptides, overlapped by 14 amino acids and covering residues 25–232 of ovine PrP (190 peptides in total, including allelic variants), were covalently linked to polyethylene mini-PEPSCAN cards and screened by PEPSCAN-based ELISA. The PrP peptide-coated mini-PEPSCAN cards were reacted for 16 h at 4 °C with individual anti-PrP monoclonal antibodies routinely at 10 µg/ml in a blocking solution, containing 5 % (v/v) horse serum and 5 % (w/v) ovalbumin. After washing, anti-PrP monoclonal antibodies were detected by rabbit anti-mouse peroxidase using 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) and H₂O₂ as substrates. Colour development of the ELISA was quantified with a charge-coupled-device camera and processing system using the Optimas version 6.5 image-processing software package.

Western-blot analysis of recombinant PrP

For Western-blot analysis of recombinant PrP, proteins were subjected to SDS/PAGE under reducing conditions (100 ng of protein/track) and subsequently transferred on to nitrocellulose membranes by semi-dry blotting. Membranes were blocked with TBS-T (10 mM Tris/HCl, pH 7.8/100 mM NaCl/0.05 % Tween 20) containing 5 % (w/v) non-fat milk and then incubated with purified T325, 683 or 884 (3–5 µg/ml) or neat 968 culture supernatant for 1 h at 21 °C. This was followed by incubation with goat anti-mouse IgG–biotin (Sigma, cat. no. B-7264) at 1:1000 dilution and, finally, ExtrAvidin–horseradish peroxidase (Sigma, cat. no. E-2886) at 1:1000. All the antibody dilutions were in 1 % non-fat milk in TBS-T. PrP bands were detected by enhanced chemiluminescence (ECL[®]; Amersham Biosciences).

Direct ELISA

Recombinant PrP protein at the desired concentration was coated on to 96-well flat-bottomed plates for 16 h at 4 °C. Excess protein was removed and wells were blocked with PBS containing 5 % non-fat milk for 1 h at 21 °C. Plates were washed three times with PBS-T (PBS containing 0.1 % Tween 80). Purified anti-PrP antibodies [T325, 683 or commercially available 6H4 (Prionics, Schlieren, Switzerland)] or 968 cell culture supernatant were diluted in PBS, added to the plates and incubated for 1 h at 21 °C, followed by three washes with PBS-T. Goat anti-mouse IgG–biotin (Sigma, cat. no. B-7264) at 1:2000 or goat anti-mouse IgM–biotin (Caltag MedSystems, Silverstone, U.K.; cat. no. M31515) at 1:4000 was added for 1 h at 21 °C, followed by three washes

with PBS-T. Avidin–alkaline phosphatase (Sigma, cat. no. A-7294) at 1:3000 was added for 1 h at 21 °C. Plates were washed three times with PBS-T and once with ELISA buffer (0.05 M glycine, 0.03 M NaOH and 0.25 mM each of ZnCl₂ and MgCl₂) before adding the substrate *p*-nitrophenyl phosphate (Sigma, cat. no. N-2765) at 0.5 mg/ml in ELISA buffer for up to 1 h at 21 °C. ELISA plates were read at 405 nm on a Dynatech MR5000 micro ELISA plate reader.

Capture-detector ELISA

Capture antibody (T325 or A516) was coated at 1 µg/well in 96-well flat-bottomed plates for 16 h at 4 °C. Excess antibody was removed and wells were blocked with PBS containing 5 % non-fat milk for 1 h 30 min at 21 °C. Plates were washed three times with PBS-T, and appropriate dilutions of recombinant ovine PrP in PBS were captured for 1 h at 21 °C. Plates were washed three times with PBS-T, and recombinant PrP was detected by 50 ng/well biotinylated monoclonal antibody (245 or 683) for 1 h at 21 °C. Plates were washed three times with PBS-T, and avidin–alkaline phosphatase at 1:3000 was added for 1 h at 21 °C. Plates were washed twice with PBS-T and once with ELISA buffer before adding the substrate *p*-nitrophenyl phosphate at 0.5 mg/ml at 21 °C. ELISA plates were read at 405 nm on a Dynatech MR5000 micro ELISA plate reader.

Isolation of sheep PBMCs for immunofluorescence staining

Peripheral blood from 3.5–4-year-old female Cheviot sheep (New Zealand scrapie-free flock; ADAS, Mepal, Cambs., U.K.) was collected in EDTA tubes by venepuncture from live animals, transported on ice and routinely stored at 4 °C overnight. A buffy coat was prepared by centrifugation at 733 *g* for 20 min at 21 °C; the harvested cells 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 with FACS buffer (PBS containing 1 % heat-inactivated foetal calf serum, supplemented with 0.1 % sodium azide) before immunofluorescence staining. To assess the cell-surface phenotype, we used aliquots of 1 × 10⁶ cells incubated with monoclonal antibody culture supernatant or normal mouse serum at 1:1000 (as control) for 20 min at 4 °C, followed by three washes with FACS buffer and incubation with the following for 20 min at 4 °C: goat anti-mouse IgG–biotin (Sigma, cat. no. B-7264) at 1:1000 or goat anti-mouse IgG1–biotin (Caltag MedSystems, cat. no. M32115) or anti-mouse IgG2a–biotin (Caltag MedSystems, cat. no. M32215) or anti-mouse IgM–biotin (Caltag MedSystems, cat. no. M31515), all at 1:500 dilution. Cells were washed three times with FACS buffer and subsequently incubated with 0.25 µg of streptavidin–phycoerythrin (Pharmingen, BD UK, London, U.K.; cat. no. 554061) for 20 min at 4 °C. Finally, cells were washed three times with FACS buffer and analysed for cell-surface fluorescence using an FACSCalibur® (Becton Dickinson, Mount View, CA, U.S.A.). Cells (1 × 10⁴/sample) were analysed with dead cells excluded on the basis of forward and side light scatter.

Statistical analysis

Statistical analysis of the data was performed by one-way ANOVA together with Tukey HSD (honestly significant difference) for post hoc analysis.

Nomenclature

Amino acid residue numbers refer to the ovine PrP sequence.

RESULTS

Generation and epitope specificity of anti-PrP monoclonal antibodies

We have generated monoclonal antibodies that react with critical regions of ovine PrP that are believed to be involved in the conversion of PrP^C into PrP^{Sc}. These regions include the amino acid sequence around residue 171, which is involved in the determination of susceptibility to natural scrapie. Antibodies reactive with this region of PrP were generated by hybridoma fusion of spleen cells isolated from *Prnp*^{0/0} mice immunized with a C-terminal peptide of murine PrP (residues 161–231). PrP-reactive hybridoma culture supernatants were identified by ELISA using murine or ovine recombinant PrP as the substrate (results not shown). From approx. 20 positive hybridomas, three monoclonal antibodies numbered 683, 884 and 968 were selected for further investigation. The specificity of the anti-PrP monoclonal antibodies was determined by epitope mapping using a panel of overlapping 15-mer peptides that spanned the entire length of mature ovine PrP. The monoclonal antibody 968 (IgM isotype) reacted with the peptide sequence YYRPVD (residues 165–170). Monoclonal antibodies 683 and 884 (both IgG2a isotype) reacted with peptides that contained the core sequence PVDQY (residues 168–172). Both 683 and 884 failed to react with the corresponding allelic variant peptides of ovine PrP that contained the core sequence PVDRY. In addition to these monoclonal antibodies, more antibodies were generated (A. M. Thackray and R. Bujdoso, unpublished work) that reacted with other regions of ovine PrP. The monoclonal antibody A516 (IgG1 isotype), raised against truncated PrP-ARR (residues 89–233), reacted most strongly during epitope mapping with a peptide of the sequence DRYRENMYRYPNQV (residues 150–164). The monoclonal antibody V468 (IgG1 isotype), raised against truncated PrP-VRQ (residues 89–233), reacted with recombinant PrP-VRQ and PrP-ARQ, but not with PrP-ARR. The monoclonal antibody V468 did not react in the peptide mapping study, implying that its epitope is conformational. Monoclonal antibodies T188 and T325 (both IgG1 isotype) were N-terminal-specific since they reacted with an N-terminal peptide of murine PrP (residues 23–89) and full-length ovine PrP (residues 25–232), but did not react with truncated ovine PrP (residues 89–233) (A. M. Thackray and R. Bujdoso, unpublished work). Antibodies were purified from hybridoma tissue culture supernatant by affinity chromatography on Protein G columns and determined to be > 95 % pure by Coomassie Blue-stained SDS/PAGE. In some cases, purified antibodies were biotinylated for use with streptavidin conjugates.

Western-blot analysis for reactivity of anti-PrP monoclonal antibodies with ovine PrP

The reactivity of the selected monoclonal antibodies with different allelic forms of ovine PrP was first investigated by Western blotting against recombinant protein. Full-length ovine recombinant PrP protein (residues 25–232), either PrP-ARR, PrP-ARQ or PrP-VRQ, was expressed from a prokaryotic vector system and purified by nickel ion and ion-exchange chromatography, and it was subsequently oxidatively refolded. After SDS/PAGE, each allelic form of ovine PrP displayed the predicted molecular mass of 23 kDa (results not shown). Figure 1 shows that the N-terminal-specific monoclonal antibody T325 reacted equally well with all three allelic forms of ovine recombinant PrP protein. Similar results were seen with the monoclonal antibody T188 (results not shown). The monoclonal antibody 968 showed a similar reactivity profile to that seen for the N-terminal-specific reagents, and reacted equally well with all three allelic variants of ovine

Figure 1 Western-blot analysis for reactivity of monoclonal antibodies with ovine recombinant PrP

Full-length ovine recombinant PrP-ARR, PrP-ARQ or PrP-VRQ (100 ng/lane) was analysed by Western blotting using the following monoclonal antibodies: T325 (5 μ g/ml), 968 (culture supernatant), 683 (3 μ g/ml) and 884 (3 μ g/ml). Molecular-mass standards (kDa) are shown on the left-hand side.

PrP. In contrast, the monoclonal antibodies 683 and 884 reacted equally well with ovine PrP-ARQ and PrP-VRQ, but not with PrP-ARR. Collectively, these results indicate that the monoclonal antibodies T325, T188 and 968 react with epitopes common to all three allelic variants of ovine PrP, whereas 683 and 884 react with an epitope restricted to PrP-ARQ and PrP-VRQ. Since the only difference between ovine PrP-ARR and PrP-ARQ proteins is the single-amino-acid substitution Arg $\rightarrow$ Gln at residue 171, these results strongly support the epitope mapping results that indicate that 683 and 884 bind around residue 171. Furthermore, the binding of 683 and 884 to ovine PrP was allele-specific and required a glutamine residue at position 171. A516 failed to react with ovine recombinant PrP by Western blotting (results not shown), suggesting that this monoclonal antibody recognized a conformational epitope in the region of helix-1.

Direct ELISA for reactivity of anti-PrP monoclonal antibodies with ovine PrP

ELISA using ovine recombinant PrP attached directly to the plastic plates was performed to investigate the reactivity of the anti-PrP monoclonal antibodies with PrP in a less denatured form than that which occurs during SDS/PAGE. Figure 2(A) shows that the monoclonal antibody T325 reacted with full-length ovine PrP (residues 25–232), but not with the truncated ovine recombinant PrP (residues 89–233). T188 reacted in a similar manner (results not shown), confirming the N-terminus specificity of these monoclonal antibodies. Figure 2(B) shows that the monoclonal antibody 968 reacted equally well with all three forms of ovine recombinant PrP by ELISA. Figure 2(C) shows that the monoclonal antibody 683 displayed equal reactivity with PrP-ARQ and PrP-VRQ, but not with PrP-ARR. The monoclonal antibody 884 reacted in a similar manner (results not shown). These results confirm that monoclonal antibodies 683 and 884 do not react with PrP-ARR, the scrapie-resistant allelic form of the ovine prion protein, which correlates with their linear core epitope PVDQY (residues 168–172). In support of this, monoclonal antibodies 683 and 884 reacted with mouse and bovine recombinant PrPs, which also contain a glutamine residue at the equivalent amino acid position to ovine residue 171 (results not shown). The monoclonal antibody V468 reacted in a similar

Figure 2 Immunological detection of ovine recombinant PrP by direct ELISA

Full-length ovine recombinant PrP-ARR (■), PrP-ARQ (▲) or PrP-VRQ (●) (residues 25–232) or truncated PrP-ARR (□) or PrP-VRQ (○) (residues 89–233) at 1 μ g/ml was coated directly on to ELISA plates as described in the Materials and methods section. Recombinant PrP was detected by various concentrations of purified monoclonal antibody or, for 968, by dilutions of culture supernatant. (A) T325, (B) 968, (C) 683 and (D) 6H4. Results shown are the means $\pm$ S.D. for triplicate wells. O.D., absorbance.

manner to monoclonal antibodies 683 and 884, with a preference for PrP-ARQ and PrP-VRQ and little reactivity with PrP-ARR (results not shown). Figure 2(D) shows that the control antibody 6H4 reacted equally well with all three genotypes of ovine recombinant PrP.

Capture-detector ELISA for reactivity of monoclonal antibodies with ovine recombinant PrP

The attachment of ovine PrP directly to the ELISA plate may result in partial denaturation of the protein and increased accessibility to epitopes not normally exposed in a more native conformation of the molecule [21]. To avoid this issue, we probed the conformation of recombinant PrP by a capture-detector ELISA system to investigate whether true structural differences existed between the various allelic forms of ovine recombinant PrP. Figure 3(A) shows that PrP-ARQ was recognized more efficiently than PrP-VRQ when the monoclonal antibody 683 was used as the detector together with the N-terminal-specific monoclonal antibody T325 for capture. Figure 3(B) shows that a similar trend was seen when 683 was used as the detector together with the C-terminal-specific monoclonal antibody A516 for capture. These results suggest that the linear core epitope for monoclonal antibody 683, PVDQY (residues 168–172), is differentially exposed in these proteins. Less of a difference was seen when the monoclonal antibody 245, which binds around the start of helix-2 in ovine PrP [19], was used as the detector with either T325 or A516 as the capture, as shown by the results of Figures 3(C) and 3(D) respectively. Collectively, these results suggest that the accessibility of the peptide sequence in the loop structure around residue 171 in PrP-ARQ and PrP-VRQ is quite distinct. This difference is probably influenced by the N-terminal region. The monoclonal antibody

Figure 3 Capture-detector ELISA reactivity of monoclonal antibodies with recombinant PrP

ELISA plates were coated with capture antibody T325 (A, C) or A516 (B, D) as described in the Materials and methods section. Full-length ovine recombinant PrP-ARR (■), PrP-ARQ (▲) or PrP-VRQ (●) were detected by biotinylated 683 (A, B) or 245 (C, D). Results shown are the means $\pm$ S.D. for triplicate wells. O.D., absorbance.

683 failed to detect PrP-ARR when either T325 or A516 was used as a capture antibody (Figures 3A and 3B). This confirms that the reactivity of the monoclonal antibody 683 with ovine PrP is strongly influenced by the glutamine residue at position 171. PrP-ARR was found to be captured efficiently by monoclonal antibodies T325 and A516 when the monoclonal antibody 245 was used for detection (Figures 3C and 3D).

Structural differences between allelic forms of cell-surface ovine PrP^C

Our results suggest that critical regions of PrP associated with the conversion of PrP^C into PrP^{Sc}, such as the region around helix-1

Figure 5 Quantification of N-terminal PrP^C FACS analysis

(a) Percentage of positive cells (mean $\pm$ S.D.) and (b) mean channel number (mean $\pm$ S.D.) are shown for PBMCs from homozygous ARR (open bar); ARQ (grey bar) and VRQ (black bar) sheep stained with either T325 or T188 ($n \geq 4$). Statistical analysis of the data in (a): * $P < 0.05$, comparison with PrP-ARR and PrP-ARQ; ** $P < 0.01$, comparison with T188 PrP-ARQ; *** $P < 0.001$, comparison with T188 PrP-ARR; † $P < 0.001$, comparison with T188 PrP-VRQ.

and that around amino acid residue 171, together with the N-terminal region, are accessible in different allelic variants of ovine recombinant PrP. Accessibility to these regions therefore provides a means of probing the normal conformation of allelic variants of cell-surface ovine PrP^C. To do so, ovine PBMCs from homozygous ARR, ARQ or VRQ scrapie-free sheep were analysed by FACS using selected anti-PrP monoclonal antibodies.

Figure 4 shows the FACS profiles for PBMCs from homozygous ARR, ARQ or VRQ sheep reacted with the N-terminal-specific monoclonal antibodies T325 and T188. With T325, cells from each genotype showed a similar monophasic FACS profile. Figures 5(a) and 5(b) show that a similar percentage of PBMCs from PrP-ARR and PrP-ARQ sheep was detected by

Figure 4 N-terminal PrP^C staining of ovine PBMCs detected by FACS

Ovine PBMCs from PrP-ARR, -ARQ or -VRQ homozygous sheep were tested by FACS analysis as described in the Materials and methods section. Profiles shown are representative of 5 out of 5 sheep for each genotype. Top row, monoclonal antibody T325; bottom row, monoclonal antibody T188. Shaded peak represents control fluorescence; black line represents T325 or T188 fluorescence.

Figure 6 C-terminal PrP^{Sc} staining of ovine PBMCs detected by FACS

Ovine PBMCs from PrP-ARR, -ARQ or -VRQ homozygous sheep were tested by FACS analysis as described in the Materials and methods section. Profiles shown are representative of 5 out of 5 sheep for each genotype. Top row, monoclonal antibody A516; middle row, monoclonal antibody 968; bottom row, monoclonal antibody V468. Shaded peak represents control fluorescence; black line represents A516, 968 or V468 fluorescence.

the monoclonal antibody T325 and, in each case, with similar fluorescence intensity. Although no significant differences were seen between these two genotypes when reacted with T325, PBMCs from PrP-VRQ homozygous sheep routinely showed a small but significantly higher percentage of cells reactive with this monoclonal antibody ($P < 0.05$). These results indicate that all the three genotypes of sheep expressed similar levels of cell-surface PrP^C on peripheral blood cells. However, other N-terminal-specific monoclonal antibodies detected differences between allelic variants of cell-surface ovine PrP^C. Figure 4 shows that PBMCs from ARR and ARQ homozygous sheep displayed a biphasic FACS profile with the monoclonal antibody T188, whereas cells from VRQ homozygous sheep displayed a monophasic profile. Despite this difference, all three genotypes showed a similar percentage of positive cells and similar fluorescence intensity (Figures 5a and 5b). The level of cellular reactivity with T188 was significantly less for each genotype compared with that seen for T325 ($P < 0.001$ for PrP-ARR and PrP-VRQ, $P < 0.01$ for PrP-ARQ; Figure 5a).

Considerable heterogeneity between the three allelic variants of ovine PrP^C was seen when sheep PBMCs were reacted with the monoclonal antibody A516. Figure 6 shows that this monoclonal antibody reacted with all PBMCs from PrP-VRQ homozygous sheep and with a reactivity profile that was typically a single peak. In contrast, cells from PrP-ARR and PrP-ARQ homozygous sheep typically showed two populations of cells; one population displayed relatively low fluorescence intensity with A516, and the other expressed the A516 epitope with a similar intensity to that seen with cells from PrP-VRQ sheep. Despite the fact that ovine PBMCs expressed significant amounts of cell-surface PrP^C, the region between β -strand-2 and residue 171 was not accessible on cells from homozygous ARR, ARQ or VRQ sheep. The monoclonal antibody 968, which reacts with all three genotypes of ovine recombinant PrP, failed to show any significant reactivity with PBMCs from scrapie-free sheep (Figure 6). Similarly,

monoclonal antibodies 683 and 884, which show selectivity for ovine PrP with Gln-171, showed little reactivity with PBMCs from either homozygous ARQ or VRQ sheep (results not shown). These results indicate that the epitopes recognized by 968, 683 and 884 were either buried or obscured in native cell-surface PrP^C. However, not all of the epitopes around residue 171 were inaccessible in the native molecule. Figure 6 shows that the monoclonal antibody V468, which binds to an epitope that is influenced by Gln-171, reacted more effectively with PBMCs from PrP-VRQ homozygous sheep than with those from PrP-ARQ animals. Figure 7 shows quantification of the C-terminal FACS analysis of PBMCs from each of the three ovine PrP genotypes. PBMCs from PrP-VRQ homozygous sheep showed a significantly higher percentage of cells reactive with A516 compared with both PrP-ARR and PrP-ARQ cells ($P < 0.001$). PBMCs from PrP-VRQ homozygous sheep also showed a significantly higher percentage of cells reactive with V468 when compared with PrP-ARR ($P < 0.001$) and PrP-ARQ cells ($P < 0.01$). PBMCs from PrP-ARQ homozygous sheep showed a significantly higher percentage of cells reactive with V468 when compared with PrP-ARR cells ($P < 0.001$). Collectively, these results indicate that heterogeneity exists between allelic variants of cell-surface ovine PrP^C within the N-terminal region and the central portion of the protein, while particular epitopes around residue 171 are normally buried or obscured in the native molecule.

DISCUSSION

The structure of ovine PrP, based on sequence comparison, is predicted to be similar to that of other species; it comprises a relatively unstructured N-terminal region and a predominantly globular C-terminal region containing three α -helices, inter-dispersed by a short anti-parallel β -sheet region. The crystal structure of residues 123–230 of the globular C-terminal domain

Figure 7 Quantification of C-terminal PrP^C FACS analysis

(a) Percentage of positive cells (mean $\pm$ S.D.) and (b) mean channel number (mean $\pm$ S.D.) are shown for PBMCs from homozygous PrP-ARR (open bar), -ARQ (grey bar) and -VRQ (black bar) sheep stained with monoclonal antibodies A516, 968 or V468 ($n=5$). Statistical analysis of the data in (a): * $P < 0.01$, comparison with V468 PrP-ARQ; ** $P < 0.001$, comparison with A516 PrP-ARR and A516 PrP-ARQ; *** $P < 0.001$, comparison with V468 PrP-ARR; † $P < 0.001$, comparison with V468 PrP-ARR.

of the ARQ allele of ovine PrP has been described recently [22]. The globular domain reveals a close association between helix-1, the C-terminal region of helix-2 and the N-terminal region of helix-3. This central core is bound by an intramolecular disulphide bond between residues 179 and 214. Allelic variants of ovine PrP correlate with incidence, pathogenesis, incubation and survival time of scrapie in sheep [23]. These differences are attributed to different structural forms of PrP, which arise as a consequence of the polymorphisms in the protein. The major polymorphisms associated with differences in susceptibility to natural scrapie in sheep occur in the C-terminal portion of the molecule at amino acid residues 136, 171 and, to a lesser extent, 154. These polymorphic residues are located within, or close to, that region of PrP that undergoes the major conformational change associated with conversion of PrP^C into PrP^{Sc} during prion disease [24]. The molecular mechanism that accounts for the allelic difference in natural scrapie susceptibility has not been established, although dominant-negative inhibition of the binding of PrP to accessory proteins such as Protein X has been proposed [10,11]. Our results show that there is a distinct conformational variation between different allelic variants of cell-surface ovine PrP, and critical regions of the molecule are buried or obscured, and differentially exposed.

PBMCs from ARR, ARQ and VRQ homozygous sheep displayed similar levels of PrP^C, since cells from all the three genotypes showed similar reactivity with the N-terminal-specific monoclonal antibody T325. The N-terminal region of PrP^C is well conserved among mammalian species, consistent with its proposed role in binding copper; as a consequence, structural consistency within this region may be expected. Both T188 and T325 reacted with full-length ovine recombinant PrP (residues 25–232) and bound to a peptide of residues 23–89 of murine PrP, but did not react with truncated PrP (residues 89–233). This clearly locates the reactivity of these monoclonal antibodies to the N-terminal region of PrP; however, so far, mapping with overlapping 15-mers of this protein has not defined their specific epitopes. The variation in reactivity of the two monoclonal antibodies

with PBMCs from different ovine genotypes indicates that their epitopes are distinct. One reason for the decreased reactivity of T188 with PBMCs from ARQ and ARR homozygous sheep is that the epitope for this monoclonal antibody is probably more N-terminal than that of T325, and truncation of cell-surface PrP results in loss of this portion of the molecule. Truncated PrP molecules have been described on the surface of neuroblastoma cells [25]. Alternatively, the conformation of the intact N-terminal region of cell-surface PrP^C may be different on PBMCs from ARQ and ARR sheep compared with that from VRQ animals. Whatever the case, the N-terminal region of cell-surface PrP^C of ARQ and ARR allelic variants appears to be structurally distinct from that of the VRQ genotypic form.

Antibody reactivity in the central region of cell-surface ovine PrP^C revealed a greater extent of heterogeneity between the three ovine genotypes. The monoclonal antibody A516 reacted with most of the PBMCs from PrP-VRQ homozygous sheep and did so with a uniform profile. PBMCs from PrP-ARR and PrP-ARQ sheep routinely showed two populations of cells reactive with A516; one population reacted either weakly or not at all with this monoclonal antibody, and the other reacted with a similar intensity to that seen with cells from PrP-VRQ sheep. This phenomenon was not unique to A516, since several other monoclonal antibodies, including 6H4, reacted in a similar manner (results not shown). The monoclonal antibody A516 appears to bind to an epitope located wholly, or in part, within amino acid residues 150–164 of ovine PrP, which incorporates helix-1. We predict that A516 binds C-terminal to helix-1, since this monoclonal antibody does not bind significantly to PrP^C on bovine PBMCs. The only difference between the amino acid sequences of ovine and bovine PrP^C in this particular region of the PrP protein is a Tyr $\rightarrow$ His substitution at residue 148, implying that binding of the monoclonal antibody A516 requires a hydrophobic rather than a charged residue at this position. The difference in accessibility of A516 to cell-surface PrP-VRQ compared with PrP-ARR and PrP-ARQ implies a different conformation of the native PrP^C in this particular region of the molecule, since all three genotypes express similar

levels of cell-surface PrP. Since the major polymorphic residues of ovine PrP are situated in this central portion of the PrP molecule, it is probable that these amino acids influence the binding of A516 to its epitope. Computational molecular analysis of ovine PrP shows that the V¹³⁶ of PrP-VRQ is in close proximity to the proposed A516 epitope and, hence, seems to influence the conformation of PrP in the C-terminal portion of helix-1 (results not shown). It will be important to determine which subsets of ovine PrP-ARR peripheral blood cells show reduced reactivity with the monoclonal antibody A516, since these cells may play a role in determining the resistance to prion disease in these sheep.

The sequence between β -strand-2 and helix-2 is an important region of ovine PrP, since it includes residue 171. This residue plays a critical role in determining the susceptibility to natural scrapie, since the only difference in PrP primary structure between scrapie-resistant PrP-ARR and scrapie-susceptible PrP-ARQ sheep is the Arg $\rightarrow$ Gln substitution at this site. The susceptibility to natural scrapie does not appear to be due to a difference in accessibility to this region within susceptible and resistant forms of ovine PrP. PBMCs from scrapie-resistant PrP-ARR and scrapie-susceptible PrP-VRQ sheep both failed to show any significant reactivity with the monoclonal antibody 968. This monoclonal antibody binds to an epitope with the core peptide sequence YYRPVD, which represents residues 165–170 of ovine PrP and includes the majority of β -strand-2. Within this core peptide is the motif YYR, which has recently been shown to be exposed in brain PrP^{Sc} but cryptic in PrP^C [26]. Similarly, monoclonal antibodies 683 and 884, both of which have a core peptide sequence PVDQY, representing residues 168–172 of ovine PrP, did not react with cell-surface ovine PrP^C of the scrapie-susceptible genotypes PrP-ARQ or PrP-VRQ. Although the linear epitopes recognized by these monoclonal antibodies are not available in the native form of cell-surface ovine PrP, they are present in the recombinant form of this protein. The monoclonal antibody 968 reacted equally well with ovine recombinant PrP-ARR, PrP-ARQ and PrP-VRQ, and the monoclonal antibody 683 recognized recombinant PrP-ARQ and PrP-VRQ. These results support the view that the region around β -strand-2 is buried or obscured in native PrP^C; in addition, these results indicate that the conformation of this protein is the same both on PBMCs and on cells within the central nervous system. The fact that the sequence around residue 171 is buried or obscured may be due to homo- or hetero-dimeric structures of PrP or other molecules [27]. However, not all epitopes that include residue 171 are cryptic in the native form of cell-surface PrP^C. The monoclonal antibody V468, which has a preference for the scrapie-susceptible allelic variants of ovine PrP, showed a greater reactivity with PrP-VRQ PBMCs compared with PrP-ARQ cells. This suggests that variation in exposure of residue 171 may, in part, influence the susceptibility of some genotypes to natural scrapie.

Our results indicate that structural differences exist between the different allelic forms of cell-surface ovine PrP^C in the vicinity of helix-1 and residue 171. These regions appear to be quite flexible within the ovine PrP protein and represent a part of the central portion of PrP that undergoes the major conformational change during the conversion of PrP^C into PrP^{Sc} in scrapie disease. During this conversion, the β -sheet content of PrP is increased. The region spanning residues 120–140 of PrP is generally unstructured, but has the propensity to form a β -sheet [28–30]. The presence of valine, a β -sheet former, at residue 136 probably influences the formation of more prominent β -strand secondary structures in this area compared with the presence of alanine, an α -helix former, at the same amino acid position. The consequence of an increased β -sheet content within this central portion of PrP-VRQ, compared with PrP-ARR and PrP-ARQ, is probably reflected in other, more

distant, conformational differences, including an effect around the loop structure that includes residues 168–172. This may appear to be the case, as the monoclonal antibody V468, with an epitope influenced by Q171, shows more pronounced binding to cell-surface PrP-VRQ compared with PrP-ARQ. These predictions of structural differences between allelic variants of ovine PrP are supported by our *in vitro* studies with ovine recombinant PrP. We have recently shown that PrP-VRQ forms more β -sheet structures after binding copper when compared with PrP-ARR, indicating that events at the N-terminal region of the molecule stimulate different responses in the C-terminal portion of each allelic variant [31]. In addition, Haire et al. [22] have shown that the loop between β -strand-2 and helix-2 is relatively well structured in the ovine PrP crystal, in contrast with a similar region in human PrP. These observations reveal that genetic differences between different forms of PrP can have significant effects on the structure of this protein. Results of the present study show that there is allelic variation in accessibility to specific epitopes within cell-surface ovine PrP^C. This variation is seen with epitopes located in a critical region of the protein that is proposed to undergo unfolding as PrP^C converts into PrP^{Sc}. These observations begin to address the structural variation that may account for the difference in susceptibility to natural scrapie seen between different genotypes of ovine PrP. It also begins to shed light on the different structures that ovine PrP^C may adopt on PBMCs, which may allow us to distinguish disease-associated PrP on blood cells during scrapie disease.

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