PrP-specific camel antibodies with the ability to immunodetect intracellular prion protein

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Although there is currently no effective treatment for prion diseases, significant advances have been made in suppressing its progress, using antibodies that block the conversion of PrP\textsubscript{C} into PrP\textsubscript{Sc}. In order to be effective in treating individuals that have prion diseases, antibodies must be capable of arresting disease in its late stages. This requires the development of antibodies with higher affinity for PrP\textsubscript{Sc} and systems for effective translocation of antibodies across the blood–brain barrier in order to achieve high concentrations of inhibitor at the site of protein replication. An additional advantage is the ability of these antibodies to access the cytosol of affected cells. To this end, we have generated PrP-specific antibodies (known as PrioV) by immunization of camels with murine scrapie material adsorbed to immunomagnetic beads. The PrioV antibodies display a range of specificities with some recognizing the PrP\textsubscript{27–30} proteinase K-resistant fragment, others specific for PrP\textsubscript{C} and a number with dual binding specificity. Independent of their PrP conformation specificity, one of the PrioV antibodies (PrioV3) was shown to bind PrP\textsubscript{C} in the cytosol of neuroblastoma cells. In marked contrast, conventional anti-PrP antibodies produced in mouse against similar target antigen were unable to cross the neuronal plasma membrane and instead formed a ring around the cells. The PrioV anti-PrP antibodies could prove to be a valuable tool for the neutralization/clearance of PrP\textsubscript{Sc} in intracellular compartments of affected neurons and could potentially have wider applicability for the treatment of so-called protein-misfolding diseases.

INTRODUCTION

Monoclonal antibody (mAb)-mediated therapy has shown potential application following successful treatment of scrapie-susceptible neuroblastoma (N2a) cells in vitro (Beringue et al., 2004; Enari et al., 2001; Peretz et al., 2001). An exciting report from White et al. (2003) was the first clear evidence that passive immunization of prion-infected animals inhibits prion replication and delays development of prion disease. Following that report, a number of groups (Campana et al., 2009; Pilon et al., 2007; Song et al., 2008; Wuertz et al., 2008) have demonstrated the effectiveness of immunization using anti-PrP antibody in delaying the onset of disease in vivo.

In more recent work (Tayebi et al., 2009), we have demonstrated that active immunization with non-denatured PrP\textsubscript{Sc} results in considerable prolongation of the incubation period and delayed onset of clinical prion disease in mice. Importantly, this study demonstrated that immunization with non-denatured PrP\textsubscript{Sc} predominantly elicits serum IgM. Consistent with this observation, all PrP\textsubscript{Sc}-specific antibodies raised to date are of the IgM isotype (Curin Serbec et al., 2004; Korth et al., 1999; Paramithiotis et al., 2003).

Both IgM and IgG antibodies are relatively large molecules and therefore cannot cross the blood–brain barrier (BBB). Furthermore, they are incapable of entering the cell and crossing the plasma membrane and accessing the cytosol. Hence, there is a need to develop prion-binding agents capable of crossing the BBB and, more crucially, accessing the cytosol of affected neurons.

In this report, we describe for the first time the development and characterization of camel anti-PrP antibodies (known as PrioV antibodies) capable of entering the cell cytosol in vitro. Of note, camels generate functional antibodies consisting of only two heavy chains. These differ from those of conventional antibodies in that they lack the CH1 domain (Hamers-Casterman et al., 1993). Biophysical studies have revealed that camel antibodies have a number of unique features when compared with those of conventional antibody molecules, notably their smaller size, greater solubility and higher stability (Dumoulin et al., 2002).
These unique features may allow PrioV antibodies to recognize unique epitopes that are poorly immunogenic for conventional anti-PrP antibodies.

We compared the capacities of prion antibodies raised in camel (PrioV3) and mouse (ICSMs) to access the cell, by cross-linking them to PrP\(^{C}\) and examining their cell distribution and binding patterns. We show that camel anti-PrP antibodies efficiently target the cytosol and are widely diffuse, while ICSM mAbs, raised in mice against recombinant prion protein (rPrP), remain associated with the plasma membrane, forming a ring around the cell body.

These results potentially form the basis for targeting intracellular misfolded proteins in neurodegenerative diseases, including prion, Alzheimer’s and Parkinson’s disease.

**RESULTS**

**Reactivity of immune sera against synthetic peptides**

Initially, sera from three camels immunized with PrP\(^{Sc}\)-Dynabeads were used to assess their specificity and differences in binding patterns using peptide ELISA (Fig. 1).

The peptide ELISA based on overlapping 20-mers by 5 aa spanning the mouse PrP sequence starting from codon 23, demonstrated that the camel response to PrP\(^{Sc}\)-Dynabeads is polyspecific, with all PrP regions being recognized (Fig. 1).

This is comparable with responses of Prn-p\(^{-/-}\) mice to PrP\(^{Sc}\)-Dynabeads and monomeric recombinant x-PrP (Tayebi et al., 2004). However, the polyclonal response in camels contrasted with the response of Prn-p\(^{-/-}\) mice to PrP\(^{Sc}\)-Dynabeads, which was highly focused with most of the antibodies recognizing the PrP 91–110 and 101–120 regions (Tayebi et al., 2004). This suggests a strong host species influence on the immunological outcome, but could also reflect the unusual immune system of camelids (Muyldermans, 2001).

**Reactivity of mAbs (PrioV) against synthetic peptides and rPrP**

We then used the PrioV antibodies (1 µg ml\(^{-1}\)) to determine their binding motifs on the prion protein by using overlapping 20-mer peptides spanning the mouse PrP sequence 23–230 (Fig. 2). Both PrioV1 and PrioV2 recognized N-terminal epitopes between residues 91–110 and 141–160, respectively (Fig. 2). PrioV3 appeared to bind to a more C-terminal region of the protein sequence that lies between 171 and 190 (Fig. 2), which contains the YYR motif used by Paramithiotis et al. (2003) to generate their PrP\(^{Sc}\)-specific mAbs. Nevertheless, the PrioV antibodies bound similar epitopes in peptide ELISA whether the peptides were human or mouse sequences (data not shown). Most previously characterized mAbs exhibit species cross-reactivity with only few exhibiting species selectivity, e.g. 3F4 (Kascak et al., 1987).

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**Fig. 1.** Pep-scan of sera generated with bead-bound PrP\(^{Sc}\). Sera from three camels immunized with PrP\(^{Sc}\)-Dynabeads were investigated using mapping ELISA coated with 20-mer peptides spanning the 23–230 region of the mouse PrP. A conventional IgG anti-PrP antibody (ICSM18) that binds to a region between 141 and 160 was used as positive control and normal camel serum (NCS) was used as negative control. Anti-PrP responses were measured in peptide ELISA. Values represent the mean ± SD of three independent experiments.
The PrioV antibodies (5, 0.5 and 0.05 μg ml⁻¹) were evaluated for reactivity with the truncated human and mouse α or β rPrP (rPrP91–231; Fig. 3). PrioV1 and PrioV2 reacted strongly with both α and β conformations of rPrP by ELISA (Fig. 3a–d). In contrast, PrioV3 displayed relatively strong binding against the truncated mouse α rPrP (Fig. 3a), but only weak recognition of all other recombinant forms of PrP (Fig. 3b–d). PrioV antibodies were shown to display stronger binding compared with ICSM mAbs using rPrP ELISA and were also assessed for the dominant isotypic response (data not shown).

PrioV3 mAb binds non-denatured PrPC/Sc

We selected PrioV3 mAb to investigate binding to non-denatured prion proteins on the basis that this particular antibody binds to a more C-terminal motif of the protein known to display preferential and specific binding for PrPSc-specific mAbs (Paramithiotis et al., 2003).

Binding to surface PrPC on N2a cells

Surface expression of PrPSc on N2a cells was shown to be high using ICSM18 or ICSM35 mAbs by immunofluorescence microscopy (Beringue et al., 2004; Khalili-Shirazi et al., 2005). PrioV antibodies were evaluated in the same way in order to demonstrate whether binding to surface PrPSc occurred (Fig. 4).

PrioV3 antibody (1 μg ml⁻¹) bound strongly to the surface of N2a cells when compared with ICSM18 or ICSM35 (1 μg ml⁻¹) antibodies, reflecting their high affinity for PrPC. Moreover, PrioV3 antibody displayed unexpected binding to intracellular PrPC following incubation of PrioV3 antibody with intact, non-permeabilized N2a cells (Fig. 4a). In contrast, ICSM18 and ICSM35 failed to target intracellular PrPC in intact cells. Disrupting the cell membrane with Triton X-100 slightly increased intracellular binding of PrioV3 to PrPC (Fig. 4b) and the negative control normal camel serum (NCS) as well binding to a Prn-pKO glial cell line from hippocampus (Nishimura et al., 2008) showed no binding (Fig. 4c, d). It should be noted that N2a cells express murine PrPC and PrioV antibodies were raised against murine PrP.

PrioV3 antibody binds non-denatured PrPSc

Scrapie-infected (RML) or PrnP–/– (KO) brain homogenate was treated with proteinase K (PK) (Qiagen) to evaluate the capacity of PrioV3 antibody to recognize PrPSc (Fig. 5). Treatment with PK cleaves approximately 67 aa from the N terminus of PrPSc and completely digests PrPC. PrioV3 and ICSM18 antibodies, which both detect all glycoforms and fragments of PrPSc, were used to immunocapture PrPSc from the homogenate and ICSM35 antibody conjugated to biotin was used for immunodetection in a sandwich ELISA (Fig. 5). NCS was used as negative control. It was clear that PrioV3 immunodetected both isoforms of PrP from mouse brain homogenates and displayed dual recognition for both PrPC and PrPSc.
Immunoprecipitation studies with PrioV3 antibody revealed a similar pattern banding profile with ICSM18 (Fig. 6). PrioV3 antibody did not react with Prn-p\textsubscript{2}/\textsubscript{2} brain homogenates (Fig. 6) but immunoprecipitated several PrPC bands from normal mice comprising one band at 27 kDa, two bands at 29–32 and 33–35 kDa, and one at 21–22 kDa. Upon PK treatment, normal PrP was completely digested, whereas the 33–35 kDa form of PrP\textsuperscript{Sc} was shortened to 27–30 kDa, probably as a result of degradation of the N-terminal segment of residues 23–90 analogous to hamster PrP\textsuperscript{Sc} (Oesch et al., 1985). We then sought to evaluate strain specificity of the PrioV3 antibody by immunoprecipitation (Fig. 6), using scrapie-infected brain homogenates, including RML, ME7 and 87V scrapie-adapted strains or isolates. The homogenates were PK-treated (or not) before incubation with PrioV3 antibody. PrioV3 antibody bound to both RML- and 87V-infected brain homogenates (Fig. 6) whether PK-treated or not, but failed to bind ME7-infected brain homogenates, albeit weakly to its normal isoform (Fig. 6).

Taken together, the results indicate that PrioV3 antibody led to specific strain recognition, confirming its conformation specificity for the prion motif it binds to.

**PrioV3 antibody binds membrane and cytoplasmic PrP\textsuperscript{C} with N2a cells**

During its synthesis, PrP\textsuperscript{C} transits through the secretory pathway. The conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc} is thought to occur after the former reaches the plasma membrane or is reinternalized for degradation (Borchelt et al., 1992). We sought to demonstrate the ability of PrioV3 antibody to enter the cell membrane and target cytoplasmic PrP\textsuperscript{C} by immunofluorescence labelling, pre- (Fig. 7) and post-permeabilization (Fig. 8). N2a cells were seeded on glass coverslips and grown to 50% confluence for staining of both external and internal PrP. Prior to immunofluorescence staining and imaging, a group of N2a cells were first lightly paraformaldehyde fixed and permeabilized with Triton X-100 (Fig. 8). Another group of N2a cells were fixed but not permeabilized prior to immunofluorescence staining and imaging (Fig. 7); this allowed us to assess whether PrioV3 antibody is able to target intracellular as well as surface PrP\textsuperscript{C} (Fig. 7c, d) and compare with the conventional ICSM mAbs which anti-PrP antibodies would target surface PrP\textsuperscript{C} exclusively and would only enter the cell cytoplasm following cell permeabilization (Fig. 8a, b).

N2a cells were stained with 100 \( \mu \)g PrioV3 ml\textsuperscript{-1} and 1 \( \mu \)g ICSM ml\textsuperscript{-1} mAb. One hundred microlitres of 5 \( \mu \)g NCS ml\textsuperscript{-1} was also used as negative control (Fig. 4c and data not shown).

Surprisingly, with non-permeabilized cells, PrioV3 staining of intracellular PrP\textsuperscript{C} was widespread in the cytosol and showed a diffuse and scattered binding pattern (Fig. 7c, d); this allowed us to assess whether PrioV3 antibody is able to target intracellular as well as surface PrP\textsuperscript{C} (Fig. 7c, d) and compare with the conventional ICSM mAbs which anti-PrP antibodies would target surface PrP\textsuperscript{C} exclusively and would only enter the cell cytoplasm following cell permeabilization (Fig. 8a, b).

Following cell permeabilization (Fig. 8), binding of PrioV3 to cytosolic PrP\textsuperscript{C} increased slightly and cell membrane breakage with Triton X-100 clearly led to intracellular staining with ICSM18 and ICSM35 (Fig. 8), albeit binding here remained limited and showed a less diffuse staining...
pattern as compared with PrioV3 staining pre- and post-permeabilization. Of note, ICSM18 and ICSM35 Fab fragments failed to stain intracellular PrPC (data not shown). In conclusion, staining of both groups of cells (i.e. permeabilized and non-permeabilized) did show considerable differences of binding between PrioV3 and ICSM mAbs.

**DISCUSSION**

We sought to investigate whether camel antibodies could bind intracellular PrP\(^C\) following entry into the cytosol of neuroblastoma cells by keeping the integrity of the cell membrane intact. An effective therapeutic strategy for prion diseases would crucially rely on the development of PrP\(^C\)/PrP\(^Sc\)-specific binders capable of entering the cell cytosol and neutralize the disease-associated agent, PrP\(^Sc\), *in vivo*.

Here, we have shown that antibodies with high-binding affinity for non-denatured PrP\(^C\) and/or PrP\(^Sc\) can be produced by immunization of camels with partially purified PrP\(^Sc\) adsorbed to immunomagnetic particles (Tayebi *et al.*, 2004).

Previously, we have demonstrated that Dynabeads coated with non-denatured PrP\(^Sc\) elicit an exclusive IgM response in mice (Tayebi *et al.*, 2004). The same methodology applied for immunization of camels with Dynabeads coated with non-denatured PrP\(^Sc\) led to an IgG isotypic response. This is similar to our previously described results, where immunization of *Pm^-/-* mice with Dynabeads coated with PrP\(^C\) or rPrP led to an exclusive IgG isotype response, even though the IgG subtypes were different depending on whether \(\alpha\) or \(\beta\) rPrP was used (Tayebi *et al.*, 2004). The species-specific differential outcome of the isotype response indicates a strong host influence on the protein itself, although a small number of camels were used.

**Fig. 4.** Immunofluorescence staining of PrP\(^C\) with PrioV3 antibody. PrioV3 antibody staining of both surface and intracellular PrP\(^C\). PrioV3 (1 \(\mu\)g) extensively stained intracellular PrP\(^C\) in fixed (post-treatment), but not permeabilized N2a cells (a). A slight increase of intracellular PrP\(^C\) binding by PrioV3 was seen following cell membrane permeabilization with Triton X-100 (b). NCS was used as negative control (c). PrioV3 was also used to stain a *Prn-p^-/-* glial cell line isolated from the hippocampus (d).

**Fig. 5.** Immunodetection of disease-associated PrP\(^Sc\) with PrioV3 antibody by sandwich ELISA. RML-infected and *Prn-p^-/-* (KO) brain homogenates were used to assess specific binding of PrioV3 antibody to PrP\(^Sc\) following PK digestion. Fifty microlitres of 5 \(\mu\)g PrioV3 antibody ml\(^{-1}\) was used to coat the ELISA plate in coating buffer. RML-infected brain homogenate was added to the wells followed by a biotinylated ICSM35 mAb raised in mouse. The sandwich format of the assay established the specificity of PrioV3 antibody for post-PK PrP\(^Sc\). NCS was used as negative control in the same way as PrioV3 and ICSM18 raised in mouse was used as positive control. Error bars represent the mean antibody level derived from \(n=4\) wells.
in this study. Of note, the polyclonal response generated against PrPSc-Dynabeads in camels led to binding of all regions of the prion protein as shown by the use of 20-mer murine or human prion peptides on ELISA. The identity of the epitope(s) recognized in PrPSc-Dynabeads was relatively invariable in sera from all three immunized animals. Since they lack the light chains of conventional antibodies, the antigen-binding site of camel antibodies is limited to a single domain. These antibody fragments display long and flexible surface loops, are often larger than in murine and human antibodies, and can penetrate cavities in target antigens, such as enzyme active sites and canyons in viral particles (Decanniere et al., 1999).

In other reports, a variety of polyclonal sera have been epitope mapped using synthetic peptides and results indicate that binding is directed to the N-terminal region of the PrP protein in the majority of cases (Barry et al., 1988; Harmeyer et al., 1998; Kascak et al., 1987; Krasemann et al., 1996, 1999; Serban et al., 1990; Tayebi et al., 2004; Williamson et al., 1996). Our results suggest a different outcome in camels relative to mouse, since the

**Fig. 6.** Immunoprecipitation of different mouse-adapted scrapie strains or isolates with PrioV3 antibody. PrioV3 typically bound both PrPC in wild-type (WT) brain homogenates of mice. PrioV3 was incubated with brain homogenates prepared from normal and Prnp−/− (KO) brain tissue with and without PK digestion. Bound PrioV3 antibody was immunoprecipitated with protein G agarose. RML-, ME7- and 87V-scrapie strains digested with PK were incubated with PrioV3 antibody to assess its strain specificity. Bound PrioV3 antibody was immunoprecipitated with protein G agarose. PrioV3 strongly bound both RML and 87V isolates pre- and post-PK. ME7 strain was poorly recognized with no PK digestion and showed no detection with PK using PrioV3 antibody. For comparison, ICSM18, raised in mouse (positive), was incubated with brain homogenates prepared from RML-infected and normal brain tissue with and without PK digestion. ICSM18 was used for subsequent detection.

**Fig. 7.** Immunofluorescent localization of PrPC in intact N2a cells. N2a cells were cultured for 24 h prior to co-treatment with 1 μg PrioV3 antibody and two different conventional IgG anti-PrP antibodies raised in mouse (ICSM18 and ICSM35). N2a cells were fixed but not subjected to Triton X-100 treatment to avoid cell permeabilization. Fluorescence microscopy was performed and images from each source [FITC (450–490 nm), Texas red (510–560 nm) and DAPI (330–380 nm)] were collected. As control, N2a cells were stained with the secondary anti-llama IgG (green) and anti-mouse IgG antibody (red), omitting the primary antibodies (a). Co-labelling of N2a cells with PrioV3 antibody and ICSM18 has shown distinct staining patterns, where PrioV3 immunodetected both cell membrane and intracellular PrPC and ICSM18 strictly bound cell surface PrPC as demonstrated by a compact ring around the cell body (b). Similar staining with PrioV3 and ICSM35 was observed (c), and with higher magnification in (d). Anti-llama IgG (green) and anti-mouse IgG antibody (red) were used for the co-localization studies.
latter has led to a similar binding pattern but only after PrP^Sc has been denatured prior to adsorption to Dynabeads. This suggests that perhaps the camel immune system is able to disaggregate PrP^Sc conferring the protein a more PrP^C-like conformation.

The isotype response for PrioV antibodies was of IgG_1 subtype. We have shown previously that Prn^-/- mice give rise to an exclusive IgM response when immunized with PrP^Sc-Dynabeads (Tayebi et al., 2004), but to an IgG response (either IgG_1 or IgG_2a/b depending on whether α or β rPrP is used) (Beringue et al., 2003). Prion protein conformation clearly has a major influence on the responding Ig subclass, but the isotypic response in camels against PrP^Sc-Dynabeads suggests that the host plays an important role in defining the outcome of the antibody isotypic response.

Three PrioV antibodies were characterized by ELISA, immunofluorescence, Western blotting and immunoprecipitation. Two of these antibodies bound both mouse and human α- and β-rPrP isoforms and one, namely PrioV3, recognized only mouse α-rPrP. All three PrioV antibodies tested seem to recognize a linear epitope as shown by peptide ELISA scan, although the results do not necessarily indicate that PrioV antibodies strictly recognize a linear motif on the prion protein, but could bind a conformational epitope as indicated by the rPrP ELISA where PrioV3 bound poorly to both isoforms of rPrP, which lacks the secondary and tertiary structure of the native PrP fold. Following SDS treatment, PrioV3 antibody did not detect denatured prion proteins by Western blotting, and displayed strong binding to non-denatured PrP^Sc by immunoprecipitation, leading to the conclusion that it recognizes a conformational epitope. This is in agreement with the work of Korth et al. (1999) where mAb 15B3 recognized three polypeptides on the prion protein that were not linear or sequential but were revealed to be contiguous with NMR studies. Williamson et al. (1998) have also shown that their antibodies mapped ‘discontinuous epitopes’ but bound denatured prion protein. We have previously generated antibodies that displayed a similar binding pattern (Beringue et al., 2003; Khalili-Shirazi et al., 2005). ICSM35 raised against human β rPrP (Beringue et al., 2003) also binds both normal and disease-associated prion protein isoforms from all species tested, whether the protein is in non-denatured conformation or denatured and was shown to be useful in delaying disease onset (White et al., 2003)

Molecular classification of human prion diseases has led to the recognition of distinct isolates, or strains (Hill et al., 2006; Wadsworth et al., 2003) that yield different fragment sizes in Western blots following PK treatment, suggesting that there are several different human PrP^Sc conformations. This has also been observed in mink and hamsters (Bessen & Marsh, 1992a, b). Prion strains can be classified by the ratio of the three PrP bands seen after protease cleavage, corresponding to N-terminal truncated products generated from dimeric, mono or non-glycosylated PrP^Sc. PrioV3 antibody immunodetected mouse-adapted scrapie strain RML and 87V, but failed to bind the ME7 strain by immunoprecipitation, suggesting a strain-specific-binding pattern. Of importance, PrioV3 discriminated between RML and 87V strains on the basis of their glycoform ratio. PrP^Sc from 87V strain was characterized by a higher level of diglycosylated glycoform, whereas PrP^Sc from RML strain was characterized by a predominance of the monoglycosylated form (Thackray et al., 2007; Vorberg & Priola, 2002).

Failure to recognize sheep scrapie (data not shown) suggests that PrioV3 antibody displays species-specific binding. Of note, 3F4, an IgG2a κ chain mAb (Kascak et al., 1987) was produced by immunizing C57BL/6j mice with SAF derived from the hamster 263K prion strain and reacted with hamster but not mouse SAF-derived 263K PrP, indicating that even PrP^Sc from its own species is seen as self protein just based on primary structure and not on conformational differences between PrP^C and PrP^Sc.

An effective therapy for prion and other protein-misfolding diseases would require the development of binders of sufficient affinity for the misfolded protein that can cross the BBB. More importantly, these binders should also be
able to target the cytosol of infected neurons and neutralize the disease causing agent. Binders that recognize PrP are readily available (Beringue et al., 2003; Curin Serbec et al., 2004; Korth et al., 1999; Paramithiotis et al., 2003; Polymenidou et al., 2008) and strategies to deliver these binders across the BBB are currently being pursued (Cardinale et al., 2005; Donofrio et al., 2005; Genoud et al., 2008; Polymenidou et al., 2008; Vetrugno et al., 2005; Wuerzter et al., 2008; Zuber et al., 2008a). These are based on the use of single chain variable fragments or scFv; these are antibody-like fragments comprising a single fusion polypeptide of the variable regions of the Ig light and heavy chains.

The use of anti-PrP scFv to treat prion-infected cells has been shown to be effective (Cardinale et al., 2005; Donofrio et al., 2005). Furthermore, scrapie-infected mice treated with anti-PrP scFv did not develop clinical signs or pathology (Vetrugno et al., 2005). Moreover, anti-laminin receptor scFvs delivered by passive immunization (Zuber et al., 2008a) or by adeno-associated virus (AAV)-mediated gene transfer (Zuber et al., 2008b) led to significant inhibition of peripheral prion replication. In more recent work, intracerebral delivery of prion-specific scFv by AAV transfer (Wuerzter et al., 2008) or the use of a soluble prion antagonist by lentivirus transfer (Genoud et al., 2008) were shown to delay the onset of the disease in infected mice, although all animals succumbed to disease.

In the current study, we show that ICSM mAbs can bind PrP on N2a cells that were first fixed and then permeabilized. These ICSM antibodies bound to both surface and intracellular PrP in N2a cells with diffuse but limited distribution in the cytosol; in contrast with binding of cell surface PrP, where binding to the plasma membrane was very strong, forming a ring around the cells.

An important finding of this study was the binding pattern shown by one of the PrioV antibodies. The PrioV3 antibody displayed extensive and diffuse binding of PrP\(^{EC}\) in the cytosol of N2a cells without a requirement for permeabilization. Moreover, co-localization studies using PrioV3 and ICSM mAbs demonstrated distinct binding patterns with PrioV3 targeting intracellular PrP\(^{EC}\), but ICSMs were limited to cell surface binding only. Moreover, a Fab fragment of ICSM18 also failed to enter the cell and target intracytoplasmic PrP\(^{EC}\).

For the first time, this work has led to the development of PrP binders able to target intracellular PrP\(^{EC}\) of intact cells without the use of gene-based technology. The camel antibodies developed here can potentially target all isoforms of misfolded proteins, including pathological conformations, and could form a valuable therapeutic tool for the treatment of protein-misfolding diseases.

**METHODS**

**Preparation of Dynabeads-adsorbed antigen for immunization.** Brains from terminally ill scrapie-infected wild-type (WT) FVB/N mice were homogenized in PBS (10% w/v) using an Ultra Turrax tissue homogenizer (SIS) as described previously (Tayebi et al., 2009).

**Immunization of camels with PrP\(^{SC}\)-Dynabeads.** All procedures involving animals were carried out under a project and personal licence authority issued in accordance with The Animals (Scientific Procedures) Act 1986 and approval by the Institutional Ethics Committee. Three adult male dromedaries (*Camelus dromedarius*) used for immunization received six subcutaneous injections at weekly intervals (days 0, 7, 14, 21, 28 and 35) of 5 \(\times\) 10\(^3\) PrP\(^{Dynabeads}\). For the first injection, the antigen was mixed with an equal volume of Freund's Complete Adjuvant, and all subsequent boosts were with Freund's Incomplete Adjuvant. Forty-five days after the first injection, 50 ml anti-coagulated blood was collected to evaluate the immune response raised against the injected antigens and for isolation of lymphocytes used subsequently to produce single-domain anti-PrP mAbs (Conrath et al., 2001). Library construction and selection of PrioV antibody fragment was performed as described previously (Conrath et al., 2001). Briefly, mRNA was extracted from the peripheral blood lymphocytes, followed by cDNA preparation and the final PCR fragments were ligated into a phagemid vector, then ligated material was transformed in *Escherichia coli* cells.

Briefly, total RNA then cDNA was obtained as described previously (Conrath et al., 2001). DNA fragments encoding antibody domains were amplified by PCR using CH2FORTA4 and VHBACKA6 primers (Sigma). The amplified product underwent a second round of PCR using primers VHBACKA4 and VHFOR36 specific for the long hinge homodimeric antibody. The primers were complementary to the 5' and 3' ends of the amplified product and incorporated SfiI and NotI restriction sites at the ends of the genes. The PCR products were digested and ligated into phage vector pHEN1. Specific PrioVs against prion proteins were enriched by three consecutive rounds of *in vitro* selection on a microtitre plate. Three antibodies, known as PrioV antibodies were used in these studies.

**\(\alpha\)- and \(\beta\)-rPrP production.** \(\alpha\) and \(\beta\) rPrPs were produced as described previously (Jackson et al., 1999). Large amounts of human or mouse PrP\(^{91-231}\) can be expressed in *E. coli* and purified as a highly soluble, monomeric protein with a single intact disulphide bridge.

**Murine synthetic peptides.** Peptides were made by automated solid-phase step-wise synthesis using the Fmoc N-terminal protection chemistry. Peptides were cleaved from the solid phase and fully side-chain deprotected using trifluoroacetic acid with water and triisopropylsilane as scavengers. Cleaved peptides were precipitated and washed in ice-cold methyl tertiary butyl ether, dried, dissolved in suitable aqueous solvents and analysed by reverse-phase HPLC and MALDI-TOF mass spectrometry. Purified fractions were freeze-dried and then reconstituted in either water or PBS prior to use (Tayebi et al., 2004).

**ELISA.**

**Epitope mapping ELISA with solid-phase synthetic peptides.** High binding, 96-well plates (Greiner) were coated with 50 \(\mu\)l per well of a 10 \(\mu\)g ml\(^{-1}\) peptide solution in coating buffer (35 mM NaHCO\(_3\), 15 mM Na\(_2\)CO\(_3\), pH 9.6). The plates were incubated for 1 h at 37 °C then washed three times with PBS/0.05 % Tween 20, and then blocked with SuperBlock (SB; Pierce) for 1 h at room temperature. After decanting the SB, 1 \(\mu\)g ml\(^{-1}\) of the relevant PrioV antibody diluted in PBS/0.05 % Tween 20 was added and incubated for 1 h at 37 °C. The plates were then washed three times with PBS/0.05 % Tween 20 and a 1/1000 dilution of horseradish-peroxidase (HRP)-conjugated goat anti-IgG (Bethyl Laboratories) was added for 25 min at 37 °C and the plates were again washed four times with PBS/0.05 % Tween 20. Finally, the plates were developed with OPD buffer (Sigma) until
optimum development occurred, then the reaction was stopped with 3 M sulphuric acid prior to spectrophotometric reading at 490 nm.

**ELISA for the detection of PrP-specific antibodies using truncated rPrP forms.** This assay was performed as described for the peptide ELISA, except that medium binding, 96-well plates (Greiner) were used and coated with 50 µl per well of a 10 µg ml⁻¹ recombinant protein solution in coating buffer.

A titration (5, 0.5 and 0.05 µg ml⁻¹) of the relevant PrioV antibody diluted in PBS/0.05% Tween 20 was added.

**Sandwich ELISA for the detection of intact PrPSc in brain homogenates.** Medium binding, 96-well plates (Greiner) were coated with 50 µl per well of a 5 µg ml⁻¹ PrioV antibody solution in coating buffer. The plates were incubated for 1 h at 37 °C then washed three times with PBS/0.05% Tween 20, and then blocked with SB for 1 h at room temperature. After decanting the SB, brain homogenate diluted to 0.5% in PBS/0.05% Tween 20 (w/v) with protease inhibitors (Roche Biochemicals) was added and incubated for 1 h at 37 °C. The plates were then washed three times with PBS/0.05% Tween 20 and a 1 µg ml⁻¹ of biotinylated ICSM18 or ICSM35 antibody was added for 1 h at 37 °C and the plates were again washed three times with PBS/0.05% Tween 20 before the addition of a 1/1000 dilution of HRP-conjugated anti-mouse IgG (Sigma) for 25 min at 37 °C and the plates were again washed four times with PBS/0.05% Tween 20. Finally, the plates were developed with OPD buffer until optimum development occurred when the reaction was stopped with 3 M sulphuric acid prior to spectrophotometric reading at 490 nm.

**Immunoprecipitation.** Brain tissues from scrapie-infected WT FVB/N mice were homogenized (10% w/v in PBS) using an Ultra Turrax tissue homogenizer (SIS), and centrifuged for 15 min at 1000 g. Mice were homogenized (10% w/v in PBS) using an Ultra Turrax tissue homogenizer (SIS), and centrifuged for 15 min at 1000 g. Brain tissues from scrapie-infected WT FVB/N mice were homogenized (10% w/v in PBS) using an Ultra Turrax tissue homogenizer (SIS), and centrifuged for 15 min at 1000 g. Mouse N2a neuroblastoma cultures were plated at 2–4 × 10⁶ in Opti-MEM medium [0.5% (w/v) glucose supplemented with 5% fetal bovine serum (FBS), 50 U penicillin ml⁻¹, 50 µg streptomycin ml⁻¹ and 200 mM L-glutamine]. Cultures were maintained at 37 °C in 5% CO₂ with a change of medium every 48–72 h. For subsequent treatment with anti-PrP antibodies (PrioV3 and ICSM3), N2a cells were first seeded on glass coverslips in 35 mm dishes and grown to 50% confluence at 37 °C in a humidified atmosphere of 5% CO₂/95% air.

For immunofluorescence staining, coverslips were rinsed three times in TBS (1 M Tris/HCl, 1.5 M NaCl, pH 7.5).

Intact, live cells were first treated with antibodies (5 µg PrioV3 or ICSM18/35) that were subsequently fixed in 300–500 µl cold (4 °C) 3:5:4% (w/v) paraformaldehyde (Fisher Scientific) in TBS for 20 min at room temperature prior to washing and blocking. Groups of cells underwent or not permeabilization with 0.2% (v/v) Triton X-100 (Roche) in TBS for 5 min at room temperature. After washing the coverslips with TBS, 100 µl blocking buffer [1% (v/v) FBS, 1% BSA (w/v) in TBS] was added. The coverslips were incubated with 100 µl 1 µg PrioV3 or ICSM IgG anti-PrP antibodies for 1 h at room temperature followed by the secondary antisera diluted in TBS [anti-llama IgG FITC-conjugate (Bethyl), anti-mouse IgG Texas red-conjugate (Sigma)] for 1 h at room temperature. After the final wash in TBS, the coverslips were mounted in fluorescence anti-fade solution (Invitrogen) and sealed with clear nail polish to prevent dehydration. DAPI (Sigma) was diluted to 2 µg ml⁻¹ in fluorescence anti-fade solution for nuclear staining and added to coverslips.

Florescence microscopy was performed with a Leica DM4000B microscope. Images from each source [FITC (450–490 nm), Texas red (510–560 nm) and DAPI (330–380 nm)] were collected by a high resolution DC500 colour camera attached. All images are saved digitally using Leica’s IM500 Image Manager Database software from the same field-of-view. Images were merged using Photoshop 6.0 (Adobe).

Confocal laser scanning microscopy was performed with a Zeiss LSM510 confocal system on an inverted Zeiss Axio100M. Z-series and snapshot images were collected. Dual scans were merged using Photoshop 6.0 (Adobe).

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