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Potential candidate camelid antibodies for the treatment of protein-misfolding diseases

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ABSTRACT

Protein-misfolding diseases (PMDs), including Alzheimer's disease would potentially reach epidemic proportion if effective ways to diagnose and treat them were not developed. The quest for effective therapy for PMDs has been ongoing for decades and some of the technologies developed so far show great promise. We report here the development of antibodies by immunization of camelids with prion (PrioV3) and Alzheimer's (PrioAD12, 13 & 120) disease-derived brain material. We show that anti-PrP antibody transmigration across the blood–brain barrier (BBB) was inhibited with phosphatidylinositol-specific phospholipase C (PIPLC). Our camelid anti-prion antibody was also shown to permanently abrogate prion replication in a prion-permissive cell line after crossing the artificial BBB. Furthermore, anti-Aβ/tau antibodies were able to bind their specific immunogens with ELISA and immunohistochemistry. Finally, both PrioV3 and PrioAD12 were shown to co-localize with Lamp-1, a marker of late endosomal/lysosomal compartments.
These antibodies could prove to be a valuable tool for the neutralization/clearance of PrP^{Sc}, Aβ and tau proteins in cellular compartments of affected neurons and could potentially have wider applicability for the treatment of PMDs.

1. Introduction

Neurodegenerative disorders, most of which are protein-misfolding diseases (PMDs) such as Alzheimer's (AD) and prion diseases, already represent a significant health burden today, affecting the quality of life of many people. Currently, there is no effective treatment for PMDs, but significant advances have been made in suppressing their progress with agents able to block aggregation of misfolded proteins (Schenk et al., 1999; White et al., 2003).

The immune system is the preferred tool to produce specific binders against many agents and proteins, but the number of conventional antibodies able to bind non-linear epitopes, cross the blood–brain barrier (BBB) and enter plasma membrane of cells remains low. Camelid single-domain antibodies or VHH are tools of increasing interest for diagnostic and therapeutic applications in central nervous system (CNS) disorders (Muruganandam et al., 2002; Dumoulin & Dobson, 2004; Abulrob et al., 2005). These monovalent antibodies are considered to be the most promising candidates for the development of passive immunization approaches for the treatment of PMDs (Hamers-Casterman et al., 1993; Muyldermans, 2001; Nguyen et al., 2001; Dumoulin & Dobson, 2004) because of the combination of efficient BBB penetration, low potential neurotoxicity and poor host immunogenicity (Hamers-Casterman et al., 1993; Nguyen et al., 2001; Muyldermans, 2001; Conrath et al., 2001; Els Conrath et al., 2001; Desmyter et al., 2001; Cortez-Retamozo et al., 2002; Dumoulin et al., 2002; Dumoulin & Dobson, 2004).

In this report, we describe further characterization of camelid monoclonal antibody fragments raised against various misfolded proteins, including prions, amyloid β and tau proteins.

2. Materials and methods

2.1. Animal ethics

All procedures involving animals were carried out under a UK home office project and personal license authority issued in accordance with The Animals (Scientific Procedures) Act 1986 and approval by the Institutional Ethics Committee (approval ID: MT: Towards Immunotherapy for prion diseases, PIL 70/14970).

2.2. Immunization of camelids

Brains from terminally ill scrapie-infected wild type FVB/N mice or from an AD patient were homogenized in PBS (10% w/v) using an

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Ultra Turrax tissue homogenizer (SIS) as described previously (Tayebi et al., 2009). We immunized three adult male dromedaries (*Camelus dromedarius*) with six subcutaneous injections at weekly intervals of 5×10^6 immunomagnetic particles (Dynabeads M450) adsorbed with Rocky Mountain Laboratory (RML)-infected brain homogenates (PrP^{Sc}-Dynabeads) to produce VHH anti-prion monoclonal antibodies (called **PrioV**). We also immunized three young alpacas with brain from an AD patient that was homogenized (AD-Dynabeads) in PBS (10%w/v) as described previously (Tayebi et al., 2009) to produce VHH anti-$A\beta_{1-40}$ (**PrioAD12**), anti-$A\beta_{1-42}$ (**PrioAD13**) and anti-tau_{1-16} (**PrioAD120**) monoclonal antibody fragments. Library construction and selection of PrioV fragments were performed as described. 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. Specific PrioV antibody fragments against prion proteins were enriched by three consecutive rounds of *in vitro* selection on a microtiter plate (Conrath et al., 2001).

2.3. Enzyme-linked immunosorbent assay (ELISA)

2.3.1. Direct ELISA

Medium binding, 96 well plates (Greiner) were coated with 50 μl/well of a 1/10 dilution of serum or brain homogenate derived from mice injected with PrioV3 antibody or 200 μl supernatant taken from BBB transmigration studies with coating buffer (35 mM NaHCO3, 15 mM Na2HCO3, pH 9.6). The plates were incubated for 1 h at 37 °C then washed 3 times with PBS-0.05% Tween, and then blocked with SuperBlock (Pierce) for 1 h at room temperature. SuperBlock was then decanted and 50 μl of a 1/1000 horseradish-peroxidase (HRP) conjugated goat anti-llama IgG (Bethyl laboratories) was added and incubated at 37 °C for 25 min. The plates were then washed four times with PBS-0.05% Tween, and developed with OPD buffer (Sigma). The reaction was stopped with 3 M of sulphuric acid and the plates were read at 490 nm.

2.3.2. Peptide ELISA

50 μl/well of a 10 μg/ml Aβ or tau peptide (Tocris Biosciences) solution in coating buffer (35 mM NaHCO3, 15 mM Na2CO3, pH 9.6) was added to high binding, 96 well plates (Greiner) and incubated for 1 h at 37 °C then washed 3 times with PBS-0.05% Tween 20, and then blocked with SuperBlock (SB; Pierce) for 1 h at room temperature. After decanting the SB, 50 μl of PrioAD12, 13 or 120 antibody diluted in PBS-0.05% Tween 20 was added and incubated for 1 h at 37 °C. The plates were then washed 3 times with PBS-0.05% Tween and a 1/1000 dilution of horseradish-peroxidase (HRP) conjugated goat anti-llama IgG (Bethyl Laboratories) was added for 25 min at 37 °C and the plates were again washed 4 times with PBS-0.05% Tween. Finally, the plates were developed with OPD buffer (Sigma) until optimum development occurred, when the reaction was stopped with 3 M sulphuric acid prior to spectrophotometric reading at 490 nm.

2.3.3. Sandwich ELISA

Medium binding, 96 well ELISA plates (Greiner) were coated with 50 μl of a 1 μg/ml ICSM18 (Beringue et al., 2003) antibody solution in coating buffer. The plates were incubated for 1 h at 37 °C then washed 3 times with PBS-0.05% Tween, and then blocked with SuperBlock (SB; Pierce) for 1 h at room temperature. After decanting the SB, antibody-treated ScGT1 cell lysates diluted in PBS-0.05% Tween with protease inhibitors (Roche) were added and incubated for 1 h at 37 °C. The plates were then washed 3 times with PBS-0.05% Tween and a 1 μg/ml of biotinylated ICSM35 (Beringue et al., 2003) was added for 1 h at 37 °C and the plates were again washed 3 times with PBS-0.05% Tween before a 1/1000 dilution of horseradish-peroxidase (HRP) conjugated anti-mouse IgG (Sigma) was added for 25 min at 37 °C and the plates were again washed 4 times with PBS-0.05% Tween. Finally the plates were developed with OPD buffer until optimum development occurred and the reaction was stopped with 3 M sulphuric acid prior to spectrophotometric reading at 490 nm.

2.4. Cellular fractionation

In order to isolate proteins from PrioV3-treated-human brain endothelial microvascular endothelial cell (MVEC) lines, we followed the published protocol by Abulrob et al. (2005). Briefly, 6 wells containing confluent MVEC cell lines exposed to 25 μg PrioV3 for 30 min were used to prepare plasma membrane fractions. MVEC cells were washed with solution A (Abulrob et al., 2005) (0.25 M sucrose, 1 mM EDTA, and 20 mM Tricine, pH 7.8) and a pellet was collected by centrifugation at 1400 g for 5 min (Beckman GS-6, GH-3.8 rotor). The pellet was re-suspended then homogenized in solution A using pre-chilled Dounce homogenizer before centrifugation at 1000 g for 10 min (Eppendorf 5415R). Supernatants were collected and layered on 23 ml of 30% Percoll solution then ultra-centrifuged at 83,000 g for 30 min (Beckman SW 28 Ti). The pellet was then sonicated and mixed with 50% Optiprep (final concentration: 23%) in solution B (0.25 M sucrose, 6 mM EDTA, and 120 mM Tricine, pH 7.8). The re-suspended pellet was added to the bottom of the Beckman tube and layered with a linear 20–10% Optiprep gradient and centrifuged at 52,000 g for 90 min (Beckman SW 28 Ti). 50% Optiprep in solution B was then mixed with the top 5 ml of the gradient and then over layered with 2 ml of 5% Optiprep in solution A and centrifuged at 52,000 g for 90 min (Beckman SW 28 Ti). Finally, the gradient was fractionated into 1.25 ml fractions and each fraction of the final gradient was resolved on SDS-PAGE under reducing conditions.

2.5. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE pre-cast gels (Invitrogen) were used. Samples to be electrophoresed were diluted 1:1 in 40 μl sample buffer and boiled for 5 min in screw-cap Eppendorf tubes. The samples were spun for 5 s at 14.000 rpm in a microfuge before being loaded on the gel. The gels were electrophoresed at a constant voltage of 200 V for 1 h. Following electrophoresis, gels were blotted onto Invitrolon PVDF (Invitrogen) at 18 V for 45 min. Following blotting, the membranes were rinsed in PBS-tween (0.05%) before being transferred to blocking solution for 60 min at room temperature. The membranes were again rinsed in PBS-tween (0.05%) to remove all traces of blocking solution. 1 μg/ml of mouse anti-clathrin, anti-caveolin, anti-PrP 3F4 primary antibody or a 1/1000 anti-llama-HRP conjugated antibody were added and incubated for 1 h at room temperature. Blots incubated with mouse anti-clathrin, anti-caveolin or anti-PrP 3F4 were washed then incubated in anti-mouse HRP-conjugated antibody diluted at 1 in 20,000 dilution. The membranes were washed and developed using the Hybond-chemiluminescence (ECL) system (GE Healthcare), according to the manufacturer's instructions. Signal development times ranged from 1 s to 30 min.

2.6. In vitro effect of PIPLC on transmigration of antibodies

MVEC cells were used to assess the transmigration of PrioV3 (or control antibodies) through the blood–brain barrier (BBB). MVEC cells were seeded on collagen-coated Falcon tissue culture inserts in the top chamber (1 μm pore size). The bottom chamber of the insert assembly contained appropriate medium (with no cells). 0.5 U/ml of Phosphoinositide phospholipase C (PIPLC) was added for 2 h followed by addition of 25 μg PrioV3 to the upper chamber and 200 μl aliquots were collected from the bottom chamber at hourly intervals from 1 h to 18 h. PrioV3 in the aliquots was detected by ELISA.