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DEVELOPMENT OF MONOCLONAL ANTIBODIES SPECIFIC FOR GLYCATED PRION PROTEIN

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Transmissive spongiform encephalopathies (TSE) are neurodegenerative diseases characterized by depositions of abnormally folded prion protein (PrP^{TSE}) in brain. PrP^{TSE} is at present the only specific biochemical marker of human and animal TSE. As deposits of PrP^{TSE} remain in the body for long periods, there is substantial chance of them being nonenzymatically modified by glycation. The detection of glycated PrP^{TSE} may have potential to serve as a diagnostic marker. Monoclonal antibodies specific for carboxymethyl lysine/arginine-modified prion protein were prepared. Recombinant human prion protein (rhPrP) was bacterially expressed and purified by affinity chromatography. rhPrP was modified by glyoxylic acid that introduces carboxymethyl groups on lysine and arginine residues present within the molecule of the protein. Modified rhPrP (rhPrP-CML) was used for immunization of 6 mice, and 960 hybridoma cells were prepared. Screening of cell supernatants resulted in the selection of four promising clones. One of them (EM-31) strongly reacts with human and mouse recombinant PrP-CML, and three other clones react also with CML in vitro modified human and mouse brain PrP. Besides possible implication in TSE diagnostics, the antibodies may serve as tolls to advance our knowledge regarding the role of glycation in the prion pathophysiology.

Transmissive spongiform encephalopathies (TSE) are neurodegenerative diseases characterized by depositions of abnormally folded prion protein (PrP^{TSE}) in brain. The most frequent human TSE is Creutzfeldt–Jakob disease (CJD), and the diagnosis can only be definitively confirmed postmortem on brain tissues. Risk of CJD transmission during neurosurgical interventions and other invasive procedures constitutes a serious problem. There have been reported cases of CJD transmission due to transplantations of dura mater or cornea and due to use of pituitary hormones or insufficient sterilization of surgical tools (Brown et al. 2006; Armitage et al. 2009). PrP^{TSE} is at present the only specific molecular marker of TSE. Detection of PrP^{TSE} usually depends on treatment of sample with proteinase K or denaturing agents, which allows distinguishing PrP^{TSE} from normal cellular prion protein (PrP^{C}) (Grassi et al. 2008). These procedures are time-consuming and difficult to standardize. According to our hypothesis, glycated prion protein may represent a new type of TSE marker that could be visualized directly by a specific monoclonal antibody without need for proteinase K cleavage step.

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Glycation is a nonenzymatic binding of glucose or other reducing sugars to free amino groups of proteins. Initially created reversible Schiff bases slowly form more stable and covalently bound Amadori products that remain in the organism for a long time without being cleaved. Once formed, Amadori products undergo further chemical rearrangements and oxidations, which results in formation of advanced glycation end products (AGE) (Monnier and Cerami 1981). Glycation occurs mostly within side-chain amino groups of lysines and arginines. One well-characterized AGE product is *N*-(carboxymethyl)lysine (CML). AGE play a key role in the pathogenesis of chronic diabetes mellitus complications (Brownlee et al. 1984) and glycated hemoglobin HbA1c is the most important diagnostic marker in diabetes compensation. Glycation occurs in low amounts even in healthy individuals on membrane proteins of senescent erythrocytes (Ando et al. 1999), most likely including also PrP^{C} (Panigaj et al. 2011). Erythrocytes live long (120 d), and due to the lack of a protein synthesis they do not replenish their proteins, leaving them susceptible to glycation. Glycation was reported on protein deposits in brains of patients with neurodegenerative diseases such as Alzheimer and Parkinson disease, systemic amyloidosis, and prion diseases (Miranda and Outeiro 2009). Protein deposits, remaining in the body for a long time, are continuously exposed to glucose and thus undergo glycation process. Studies using pan-specific anti-AGE antibodies have already demonstrated the presence of glycation in prion brain deposits (Choi et al. 2004; Sasaki et al. 2002). The role of glycation in prion pathogenesis is poorly understood. Modification of PrP^{C}/PrP^{TSE} with AGEs may change their properties and affect their role in the disease process. Availability of antibodies specific for glycated prion protein, not reacting with other glycated targets, is important step in the effort directed on the elucidation of these pending questions. This study was aimed on development of monoclonal antibodies specific for glycated human prion protein.

METHODS AND MATERIALS

Expression and Purification of Recombinant Prion Proteins

pRSET A plasmids with engineered thrombin cleavage site at the N-terminal histidine tail and containing human sequences for full-length prion protein 23–231 or prion fragments 81–231, 90–231, and 121–231 were kindly provided by Kurt Wüthrich (Zürich, Switzerland). After transformation, *Escherichia coli* BL21(DE3) (Stratagene) were cultivated in Luria broth medium containing ampicilin (100 μg/ml). Recombinant protein expression was induced by 1 m*M* isopropyl *β*-D-galactopyranoside. Cells were harvested, resuspended in phosphate-buffered saline (PBS), sonicated and inclusion bodies were isolated by centrifugation as described previously (Pavlicek et al. 2007). The recombinant prion proteins (rhPrPs) were purified by affinity chromatography on Cu^{2+} resin (TALON, Clontech) in denaturing conditions (8 *M* urea). The purity of the proteins was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and ultraviolet (UV) spectrophotometry. Full-length mouse recombinant prion protein (rmPrP) was produced as described previously (Pavlicek et al. 2007).

Glycation of Recombinant Prion Protein

In order to obtain carboxymethyl lysine-modified rhPrP (rhPrP-CML) for immunization, full-length rhPrP was incubated 16 h at 4°C with 45 m*M* glyoxylic acid in 40 m*M* phosphate buffer, pH 8, containing 1 m*M* sodium cyanoborohydride (Ando et al. 1999). Glycation of rmPrP was also performed by its incubation with 1 *M* D-ribose (rmPrP-AGE) under nitrogen (7 d, 37°C).

Immunization of Mice

Six *Prnp -/- (Zürich I)* mice (EMMA, Monterondo, Italy) were immunized sc on d 0, 14, and 28 with 30 μg rhPrP-CML in PBS with Freund's adjuvant. A final booster of 5 μg

antigen in PBS was administered iv on d 64. Mice were sacrificed on d 68 and splenic cells from the 2 best responding mice were used for fusion with myeloma cells. Nine hundred and sixty hybridoma cells were prepared and used for primary screening.

ELISA

For screening, rhPrP and rhPrP-CML were diluted to 1 μg/ml in 50 m*M* sodium bicarbonate buffer, pH 9.6, and coated to NUNC U96 MaxiSorp plates. For testing of specificity of monoclonal antibodies (MAbs), rPrP, rPrP-CML, and bovine serum albumin (BSA)-CML were diluted to 1 μg/ml and rmPrP-AGE to 100 μg/ml. Wash steps were carried out with 0.05% Tween 20 in PBS (PBS/T) and the blocking step with 1% BSA in PBS/T. Mice sera were diluted 1:5000 to 1:40,000 and MAbs 1:500 to 1:128,000 in 0.1% BSA/PBS; hybridoma supernatants were tested undiluted. The conjugate (Goat anti mouse IgG [GAM] -horseradish peroxidase [HRP], Jackson ImmunoResearch or Caltag) was diluted to a final concentration 0.125 μg/ml in PBS/T and plates were developed with TMB substrate (Amresco). Reaction was stopped with 1 *M* H_2SO_4 and absorbance was measured at 405 nm.

Western Blot (WB)

Human brain homogenate (hBh) was prepared and the Western blot (WB) was carried out as described previously (Panigaj et al. 2011). Eventually, proteins were glycated directly on the nitrocellulose membrane (16 h at 4°C) by 50 m*M* glyoxylic acid and 50 m*M* sodium cyanoborohydride. Membrane was blocked and incubated for 1 h at room temperature with clone supernatants diluted 1:10. Monoclonal antibodies (MAbs) 3F4 (Chemicon, Inc., Temecula, CA) and AG4 (TSE Resource Center, Compton, UK) were used as positive controls.

Screening Procedure

Fifty-three clones selected in primary screening by enzyme-linked immunosorbent assay (ELISA) were tested in secondary screening by WB on rhPrP and rhPrP-CML. Tertiary screening was conducted by WB and dot-blot on biological material, such as 1% normal hBh, 1% mouse brain homogenates (mBh, WT, and Prnp -/-), human platelets (10^9/ml), red blood cell ghosts (10^{10}/ml), and BSA (0.5 mg/ml). Selected samples were glycated directly on the membrane as described earlier.

RESULTS AND DISCUSSION

Development of antibodies against prion protein was often hampered by its poor immunogenicity due to the highly conserved primary structure of the protein among different mammal species. To overcome this potential problem, Prnp -/- mice were used and immunized with rhPrP-CML. CML represents the end-stage product of protein glycation and is the most abundant AGE antigen in vivo (Reddy et al. 1995). Out of six immunized mice, two had high, three medium, and one low titer antibody response to the immunization. The reactivity of antibodies to hrPrP-CML in sera of both high-responding mice was numerically higher than the reactivity to unmodified hrPrP (e.g., $A_{405} = 1.5$ versus $A_{405} = 1.1$ at 5×10^{-5} dilution), suggesting the presence of antibodies directed against CML (data not shown). Primary ELISA screening of 960 hybridomas resulted in the selection of 53 clones demonstrating production of MAbs with different affinities to unmodified and CML modified rhPrP. Secondary WB screening selected 12 clones, and among them 4 clones (7A8, 1C3, 1C12, and 1F3) displayed high specificity to rhPrP-CML. Experiments initially concentrated on characterization of MAb EM-31 produced by clone 7A8, which demonstrated the highest reactivity to rhPrP-CML. MAb EM-31 displayed strong binding to hrPrP-CML on WB, while its binding to unmodified hrPrP was negligible (Figure 1a). EM-31 did not bind to BSA-CML, either on WB (Figure 1a) or ELISA (Figure 1f), suggesting that the binding is specific for PrP-CML. The antibody cross-reacted with mrPrP-CML(Figure 1a) and also

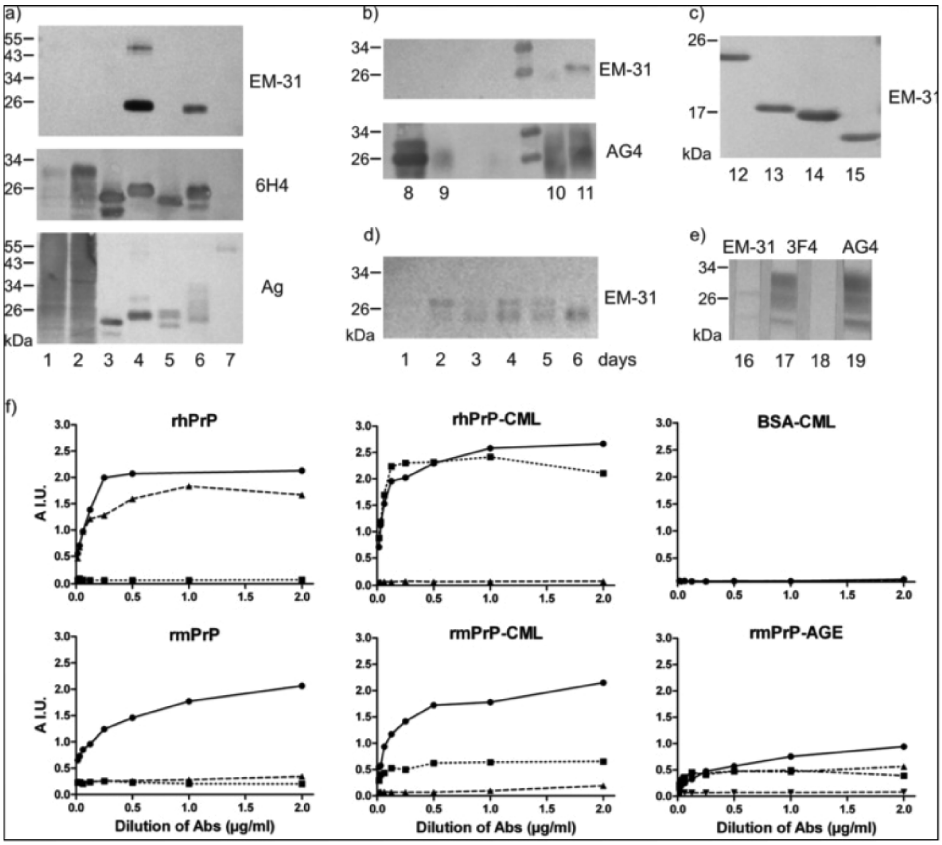

FIGURE 1. Characterization of MAb EM-31 binding to glycated prion proteins by Western blot and ELISA. (a) Three identical blots developed with EM-31, control prion antibody 6H4, or stained for proteins with colloidal silver (Ag). Lines: 1, normal mouse brain homogenate (mBh); 2, normal human brain homogenate (hBh); 3, recombinant mouse PrP (rmPrP); 4, glycated rmPrP (rmPrP-CML); 5, recombinant human PrP (rhPrP); 6, glycated rhPrP (rhPrP-CML); 7, glycated BSA (BSA-CML). EM-31 reacts only with glycated rPrPs. (b) Two identical blots developed with MAb EM-31 and control prion MAb AG4. Lines: 8, reduced rmPrP-CML; 9, reduced rmPrP-AGE (glycated by incubation with ribose); 10, nonreduced rmPrP after incubation with ribose in Tris buffer (glycation inhibited); 11, glycated nonreduced rmPrP after incubation with ribose in phosphate buffer. Reactivity of EM-31 with rmPrP-CML and rmPrP-AGE is lost after reduction of samples with dithiothreitol. (c) EM-31 reacts with C-terminal fragments of rhPrP-CML molecule: 23–231 (lane 12), 81–231 (lane 13), 90–231 (lane 14), and 121–231 (lane 15). (d) Time course of rmPrP modification by incubation with 1 *M* D-ribose detected by EM-31. (e) EM-31 has only a weak affinity to PrP in glycated human brain homogenate (hBh-CML) (lane 16). MAb 3F4 is used as a control of glycation. It binds to PrP in nonglycated hBh (lane 17), but not in hBh-CML (lane 18). MAb AG4 is a glycation insensitive control detecting PrP in hBh-CML (lane 19). (f) Demonstration of the specificity of EM-31 for glycated prion protein by ELISA. Wells of the plate were coated by rhPrP, rmPrP, rhPrP-CML, rmPrP-CML, rmPrP-AGE, or BSA-CML and developed with increasing concentrations of MAbs: EM-31 (rectangles, dotted line), glycation-sensitive 3F4 (triangles, dashed line), and glycation-insensitive AG4 (circles, full line). Labeling in rmPrP-AGE figure is AG4 (circles, full line), 3F4 (inverted triangles, dashed line), and two batches of EM-31 (rectangles, dashed and dotted line, and triangles, dashed and double dotted line). EM-31 does not bind to unmodified proteins and the reactivity of 3F4 to rhPrP is lost after its modification. Neither MAb binds to glycated BSA.

with mrPrP-AGE prepared by glycation of the protein with D-ribose (Figures 1b and 1d). This confirmed that the antibody recognizes also rPrP glycated by milder, more natural procedure. EM-31 does not bind to reduced rhPrP-CML nor to reduced rmPrP-CML or rmPrP-AGE (Figure 1b), which implicates that the epitope of EM-31 is not linear and may include the disulfide bond between Cys179 and Cys214. EM-31 reacted with all rhPrP-CML fragments tested, including $rhPrP_{121–231}$-CML, confirming the location of its epitope on C-terminal part of PrP molecule (Figure 1c). Importantly, EM-31 did not cross-react with any protein on blots of normal hBh or mBh (Figure 1a). Recently, Panigaj et al. (2011) obtained evidence that the epitope of MAb 3F4 ($PrP_{106–112}$) on PrP^C on the membrane of human red

blood cells is covalently modified, likely by glycation. However, EM-31 did not bind to human red blood cell or platelet PrP^C on WB (data not shown). This raises a question regarding the location of glycation sites in the PrP molecule. Human mature PrP contains 10 lysine and 10 arginine residues, which are fairly scattered along its sequence and can in theory be glycated. Previous study by Choi et al. (2004) demonstrated the presence of glycation in one or more lysine residues of the PrP^{TSE} N-terminus. Whether also other putative glycation sites are modified during the disease process remains to be elucidated. Interestingly, EM-31 bound poorly to in vitro glycated PrP^C in hBh (Figure 1a). This was not produced by improper modification of PrP, as MAb 3F4 lost its reactivity (Panigaj et al. 2011), while the protein was still recognized by glycation-insensitive MAb 6H4 (Figure 1a). A plausible explanation of this phenomenon may lie in the shielding effect of glycosylation of asparagines N_{181} and N_{197} in PrP^C, which is not present in recombinant protein. While this finding may limit the usefulness of EM-31 for the detection of in vivo glycated glycosylated isoforms of PrP^C/PrP^{TSE}, it also suggests that EM-31 may represent a useful tool for studies of glycation of unglycosylated isoform of the protein. Recently, properties of MAbs produced by the other selected hybridoma clones 1C3, 1C12, and 1F3 were analyzed. Generally, MAbs exhibited properties similar to those of EM-31 with two important distinctions. MAbs were able to recognize not only hrPrP-CML (Figure 2a), but also glycosylated PrP^C-CML on blots of in vitro modified hBh (Figure 2b) or mBh (not shown). In addition, their binding was not sensitive to protein reduction by dithiothreitol (not shown). The specificity of their binding to glycated rhPrP was confirmed by ELISA (Figure 2d). All clones reacted more strongly with rhPrP-CML than with $rhPrP_{90–231}$-CML or $rhPrP_{81–231}$-CML

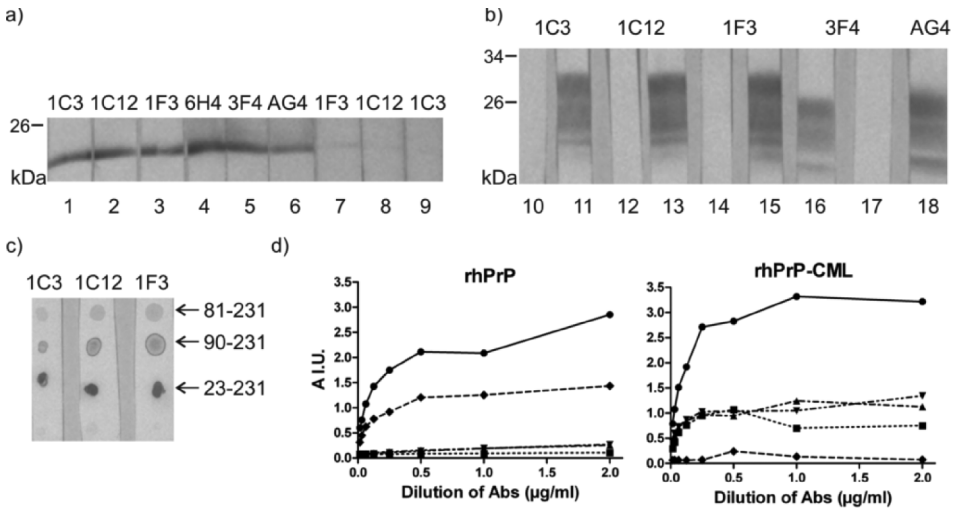

FIGURE 2. Three hybridoma supernatants demonstrate affinity to PrP in the in vitro glycated human brain homogenate. (a) Western blot of rhPrP was cut in strips and strips 1–3 were glycated by incubation with glyoxylic acid while strips 4–9 were left untreated. Supernatants 1C3, 1C12, and 1F3 demonstrate strong reactivity with the PrP on treated strips (1–3), but only faint reactivity with untreated rhPrP (7–9). The presence of the rhPrP is confirmed by MAbs 6H4, 3F4, and AG4 (4–6). (b) Clones 1C3, 1C12, and 1F3 recognize PrP in the in vitro glycated human brain homogenate (hBh-CML, lanes 11, 13, 15), but not in untreated hBh (lanes 10, 12, 14). MAb 3F4 is used as a control for glycation. It binds to PrP in nonglycated hBh (lane 16), but not in hBh-CML (lane 17). MAb AG4 is a positive control for PrP in hBh-CML (lane 18). (c) The reactivity of all three clones with glycated C-terminal fragments $rhPrP_{81–231}$ and $rhPrP_{90–231}$ on dot-blots is weaker than with $rhPrP_{23–231}$-CML. (d) Demonstration of 1C3, 1C12, and 1F3 reactivity with rhPrP-CML by ELISA. Wells of the plate were coated with rhPrP or rhPrP-CML and developed with increasing dilutions of MAb: 6H4, glycation insensitive (circle, full line); 3F4, glycation sensitive (diamond, dashed line); 1C3 (inverted triangle, dashed and double dotted line); 1C12 (triangle, dashed and dotted line); and 1F3 (rectangle, dotted line). Reactivity of 3F4 after glycation is lost, while the reactivity of the MAbs produced by the tested clones increases.

(Figure 2c), suggesting that their epitopes could be located on N-terminal half of the PrP molecule; however, this point must be independently verified.

In conclusion, it was shown that the development of monoclonal antibodies specific for glycated prion protein is feasible. At the present time, studies are continuing in the basic characterization of MAbs produced by clones 1C3, 1C12, and 1F3, while the MAb EM-31 was purified and made commercially available (EXBIO Praha a.s., Vestec, Czech Republic). Our project is primarily aimed at the development of antibodies useful for detection of in vivo glycated PrP^{TSE}, so an important next step will be to test our MAbs on samples of CJD patients and TSE-infected laboratory animals. Eventual success of these studies may lead to the development of a new diagnostic test for prion diseases. Beside this, our antibodies may serve as basic tools in elucidation of the role of glycation of prion protein in the TSE pathogenesis.

Glycation is thought to stabilize protein deposits in different neurodegenerative disorders including TSE (Miranda and Outeiro 2009). On the other hand, glycation of PrP^{C} is likely ongoing in red blood cells (Ando et al. 1999; Panigaj et al. 2011) and may modify its properties, e.g., affinity for PrP^{TSE}. As red blood cells possess the majority of cell-associated PrP^{C} in blood, not only in humans (Panigaj et al. 2011) but also in laboratory mice and nonhuman primates (Holada et al. 2007), such a change in its properties could play an important role in the transmission of TSE by blood transfusion. Detailed studies of the effect of glycation on the structure and stability of recombinant PrP should provide useful starting data, which will further our understanding of this interesting area of prion pathophysiology.

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