Detection of typical and atypical bovine spongiform encephalopathy and scrapie prion strains by prion protein motif-grafted antibodies

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To evaluate further the reactivity of prion-specific monoclonal antibodies containing the 89–112 or 136–158 prion protein (PrP) polypeptides, immunoprecipitations were performed on brain extracts from Italian bovines, sheep and goats with transmissible spongiform encephalopathies. No binding of IgG 89–112 or IgG 136–158 to PrP in normal brain extracts was detected. Conversely, both reagents immunoprecipitated PrP from bovine and bovine amyloidotic spongiform encephalopathies, and from typical and atypical scrapie brain extracts. The immunoprecipitated PrP bands mirrored the Western blot (WB) profile of the different prion strains, indicating universal affinity of two independent PrP regions for disease-associated PrP conformers regardless of species source and strain properties. Immunoprecipitation with motif-grafted antibodies increased the sensitivity of conventional detection methods based on centrifugation followed by WB, which was confirmed by assay of diluted samples using both methods. These reagents or derivative molecules may thus find broad applications in prion detection and research.

Prion diseases or transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative disorders affecting both humans and animals. The most extensively studied prion disorders – Creutzfeldt–Jakob disease (CJD) and new variant Creutzfeldt–Jakob disease (nvCJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats and chronic wasting disease in elk and deer – are characterized by the accumulation of abnormal isoforms (PrPSc) of the host encoded cellular prion protein (PrPc) in the central nervous system (CNS) (Prusiner, 1998). PrPc is fully sensitive to proteolytic degradation, whereas PrPSc is only partially degraded by proteases; this property is exploited by most TSE diagnostic methods in combination with the use of monoclonal antibodies (mAbs) that bind prion protein (PrP) at different epitopes. The patterns of resistance to proteinase K (PK) digestion and reactivity with PrP mAbs have also been used to characterize different prion strains in previous studies (Jacobs et al., 2007; Klingeborn et al., 2006; Kuczius et al., 2007).

The generation of high-affinity antibodies specifically distinguishing between PrPc and PrPSc conformations was described some years ago (Moroncini et al., 2004). Such reagents, generated by independently grafting polypeptides corresponding to mouse PrP regions 89–112 or 136–158 into the heavy chain complementarity-determining region 3 (HCDR3) of the recipient human IgG b12 (Burton et al., 1994), specifically immunoprecipitate infectious fractions of PrPSc and PrPc27–30 but not PrPc from the brain tissue of humans and rodents affected by prion diseases.

A subsequent immunohistochemical study confirmed the ability of 89–112 PrP-grafted IgG to specifically detect PrPSc in untreated tissue sections from genetic, acquired and sporadic CJD (sCJD) (Moroncini et al., 2006). The reactivity of these antibodies has not yet been tested on tissue taken from cattle, sheep and goats infected by prions.

In cattle naturally affected by BSE, traditionally considered a single pathological entity characterized by one electrophoretic PrPSc pattern (Wilesmith et al., 1991; Stack et al., 2002), at least two new distinct molecular PrPSc forms have been detected (Biacabe et al., 2004; Casalone et al., 2004); these are defined as H-type and L-type according to whether the molecular size of the PrP un-glycosylated band

Received 8 September 2008
Accepted 1 December 2008
is higher or lower, respectively, than that of classical BSE. The Italian L-type was designated bovine amyloidotic spongiform encephalopathy (BASE) owing to its immunohistochemical pattern of PrPSc deposits (Casalone et al., 2004). Recently, the two atypical BSE forms have been described worldwide (Polak et al., 2004; Buschmann et al., 2006; Richt et al., 2007; Terry et al., 2007). In Europe, a number of unusual isolates identified in small ruminants were classified as atypical scrapie because of their unusual epidemiological, diagnostic and molecular features. In particular, scrapie-Nor98 cases, first detected in Norway in 1998, were later found and characterized in other European countries (Benestad et al., 2003; Buschmann et al., 2004; Everest et al., 2006). The classical PrP electrophoretic profile of scrapie displays three glycoforms (di-, mono- and un-glycosylated) ranging in molecular mass between 30 and 20 kDa, whereas Nor98 cases are typified by faster migration of PrP bands after PK digestion and by one smaller fragment of approximately 12 kDa.

This study was designed to evaluate the reactivity of the hybrid PrP-IgG mAbs, herein termed IgG 89–112 and IgG 136–158, with scrapie, scrapie-Nor98, BSE and BASE prions in biological samples from naturally infected animals identified by the Italian TSEs surveillance system. Brain samples from sheep, goats and cattle that were positive by TSE rapid tests (Prionics Check Western or Prionics Check LIA; Bio-Rad TeSeE Sheep and Goat) and confirmed as positive by histological (haematoxylin and eosin), immunohistochemical (IHC) and Western blot (WB) analysis, as described by Caramelli et al. (2001) and Bozzetta et al. (2004), were selected on the basis of epidemiological criteria and tissue availability. Twelve classical scrapie cases (nine sheep and three goats) and 12 atypical scrapie cases characterized as Nor98 (ten sheep and two goats) isolated from animals of different age, breed, geographical origin and susceptible genotype (Table 1) were selected for this study, along with four BSE-positive and three BASE-positive cattle. Brain samples from two healthy sheep, one goat and two bovines were collected and stored frozen for use as negative controls.

In order to obtain an even distribution of PrP in the samples of each selected case, different specimens of CNS tissue taken from the same brain were initially pooled, homogenized at 10 % w/v in Tris-buffered saline (TBS)/1 % Triton X-100 or 10 % Sarcosyl, split into aliquots and stored at -280 °C prior to the experiments.

Two distinct methods for immunoprecipitation (i.p.) of the CNS samples were employed. For samples taken from cases characterized by strong or medium intensity signals at confirmatory tests, i.p. was carried out as previously described (Moroncini et al., 2007).

### Table 1. Genotypes of TSE-positive sheep and goat samples

<table>
<thead>
<tr>
<th>Case number</th>
<th>TSE type</th>
<th>Species</th>
<th>Sheep genotype at codons 136, 154 and 171</th>
<th>Other mutations</th>
<th>Goat genotype</th>
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<tbody>
<tr>
<td>1</td>
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<td>Sheep</td>
<td>ARQ/AHQ</td>
<td>127G/V-141L/F</td>
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<td>ARQ/ARQ</td>
<td>112M/T</td>
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<td>ARQ/ARQ</td>
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<tr>
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<td>ARQ/VRQ</td>
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<td>ARQ/ARQ</td>
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<tr>
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<td>Goat</td>
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<td>141L/F</td>
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<td>Goat</td>
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<td>154R/H; 240P/S</td>
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<td>ARR/ARQ</td>
<td></td>
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*wt, Genotype homologous to ovine wild-type.
2004), using 50 μl 5% brain homogenate mixed with either IgG 89–112 or IgG 136–158 or the parental IgG b12 reagents at 5 μg ml⁻¹ in 500 μl reaction volume. Tosyl-activated paramagnetic beads (Dynal) coupled to polyclonal goat anti-human IgG F(ab’2) were added to the antibody–homogenate mixture and incubated overnight at 4 °C. Beads were then washed four times in washing buffer (TBS containing 1% Triton X-100) and once with TBS before separation by magnet. Collected beads were resuspended in 20 μl Laemmli buffer and boiled for 10 min at 99 °C prior to separation and WB.

For samples taken from cases characterized by weak or very weak signals at confirmatory tests, i.p. was preceded by a preliminary ultracentrifugation step to enrich the sample for PrPSc. Brain homogenate (10%) in 10% Sarcosyl was split into two aliquots, one undergoing i.p. followed by WB (the WB i.p. aliquot), the other undergoing WB without prior i.p. (the WB aliquot). Both aliquots were clarified by centrifugation at 22 000 g for 20 min at 10 °C and the supernatants were incubated with 40 μg PK ml⁻¹ for 1 h at 37 °C with continuous shaking. Digestion was stopped by adding phenylmethylsulphonyl fluoride (PMSF) to the samples. After ultracentrifugation at 215 000 g for 1 h at 10 °C, the supernatants were discarded and the pellet of the WB i.p. aliquot was resuspended in 50 μl TBS/1% Triton X-100 buffer, then subjected to i.p. followed by WB; the pellet of the WB aliquot was resuspended in 50 μl ultrapure water and directly subjected to WB.

Brain homogenates prepared from one typical and one atypical scrapie case characterized by very weak signals at confirmatory tests were diluted logarithmically and serially, respectively, to evaluate the sensitivity of the two methods described above. Due to the very weak signal of the Nor98 case, logarithmic dilutions were made only for the classical scrapie case. Serial dilutions of the atypical scrapie sample were 1:4, 1:8, 1:16, 1:32; logarithmic dilutions of the typical scrapie sample were 10⁻¹, 10⁻², 10⁻³, 10⁻⁴. Both series of dilutions were prepared in duplicate for the WB i.p. and WB methods. Both aliquots were mixed with Laemmli buffer, boiled for 10 min at 99 °C, then separated by SDS-PAGE on a 12% minigel (acylamide/bisacrylamide ratio of 37.5:1) and transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). Blots were blocked with 5% (w/v) BSA in TBS for 1 h at room temperature. Blotted ovine or bovine PrP was detected by P4 (0.1 μg ml⁻¹; R-Biopharm) (Harmeyer et al., 1998) or 6H4 (0.2 μg ml⁻¹; Prionics) (Schaller et al., 1999) mAbs, respectively, followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Invitrogen). Membranes were developed with chemiluminescence reagent (Immunostar, Bio-Rad) onto Hyperfilm ECL (GE-Healthcare) or visualized by a UVI Prochemi (Uvitec) analysis system. PrP bands were quantified using UVI Bandmap software (Uvitec).

No binding of IgG 89–112 or IgG 136–158 to PrPSc was detected in the normal brain extracts of small ruminants and cattle taken as negative controls. Conversely, both motif-grafted reagents immunoprecipitated PrP from undigested and PK-digested BSE, BASE, scrapie and scrapie-Nor98 brain extracts of all cases examined in this study. Under identical experimental conditions, the parental IgG b12 did not show reactivity with any of the four prion strains (data not shown). Moreover, IgGs 89–112 and 136–158 were completely unreactive with PrPSc after its denaturation by heating in the presence of SDS (data not shown).

The immunoprecipitated PrP bands detected by WB replicated the characteristic molecular patterns of the four different prion strains assayed, indicating affinity of these antibodies for disease-associated PrP conformers regardless of species source and strain properties (Fig. 1). This was further confirmed by densitometric analysis of PrP bands obtained from i.p. of the BSE and BASE samples (Fig. 1b), indicating that the different glycoform ratios between the strains was maintained (data not shown).

There were no significant differences in the i.p. sensitivity due to genotype variations (Table 1) between scrapie and Nor98 cases.

The WB signals of weak or very weak typical and atypical scrapie cases (1/12 and 9/12, respectively) were enhanced following addition of the i.p. step (Fig. 2a). These findings were confirmed by serial and logarithmic dilutions performed to evaluate the sensitivity of the two methods (Fig. 2b). When the brain homogenate of the atypical case was serially diluted, the WB i.p. method was able to detect PrP up to the 1:16 sample dilution, with a faint signal at the 1:32 dilution, whereas the WB method detected some PrP traces only up to the 1:8 sample dilution (Fig. 2b, i–ii). When the classical scrapie sample was logarithmically diluted, the WB i.p. method revealed PrP up to the 1×10⁻⁴ dilution, whereas the WB method retained sensitivity only up to the 1×10⁻² dilution (Fig. 2b, iii–iv). These observations were supported by densitometric measurement of the immunoblotted bands (data not shown), which confirmed detectable levels of PrP at these sample dilutions.

The data from this study, together with previous data (Moroncini et al., 2004, 2006; Lau et al. 2007; Solforosi et al. 2007), confirm the identification of two independent regions of mouse PrP sequence that possess intrinsic specificity and affinity for epitopes found on PrPSc and PrP27–30 molecules encountered in prion diseases of animals and humans. Grafting such sequences into a recipient IgG scaffold led to the generation of hybrid PrP antibody reagents that can bind to different prion strains (79A, RML, 263K, Gerstmann–Straussler–Scheinker disease, vCJD, sCJD, BSE, BASE, scrapie and scrapie Nor98) under non-denaturing conditions, indicating the presence of common conformational motifs across this wide spectrum of prions, and universal affinity of PrP 89–112 and PrP 136–158 for PrPSc regardless of species source, strain properties and specific glycoforms. In support of
this, the use of conventional mAbs to immunoblot PrP immunoprecipitated by such motif-grafted antibodies revealed bands mirroring the canonical molecular profile of the different prion strains assayed. In fact, the glycoform ratio between the di- and mono-glycosylated bands and the molecular mass of the un-glycosylated band of BSE and L-type BSE, the major identification criteria of atypical forms of BSE (Jacobs et al., 2007), were both preserved upon immunoprecipitation, as was the characteristic 12 kDa band of atypical scrapie.

Ultrastructural studies of the complex between PrP\textsuperscript{C} and PrP\textsuperscript{Sc} will permit identification of the epitopes shared by different prion strains that are required for interaction with the 89–112 and 136–158 regions of PrP.

The discovery of such PrP regions provides new areas for basic prion research and opens prospects for generating reagents that can discriminate between disease-associated and normal conformations of PrP. This is particularly relevant for animal prion disease surveillance systems.
which require novel diagnostic tools not strictly dependent upon PK pre-treatment of samples. In fact, recent studies have reported cases where PK resistance may not represent the optimal criteria to identify a sample as prion-infected. Groschup et al. (2007) identified two natural scrapie cases in ARR/ARR sheep characterized by biochemical and transmission properties that were similar to cases of classic scrapie but with lower PK resistance than pathological PrP. Jacobs et al. (2007) observed lower PK resistance in L-type and H-type atypical BSE isolates. In such cases, the use of motif-grafted antibodies may help to establish diagnosis, since they can discriminate between normal and pathological PrP, regardless of PK pre-treatment of samples. This particular property may also prove useful for defining cases whose diagnosis remains uncertain even after confirmatory WB because of insoluble aggregates of PK-resistant, normal PrP in the sample; this was recently demonstrated in the brain specimens of healthy humans, bovines and hamsters (Yuan et al., 2006).

Besides improving test specificity, the motif-grafted reagents may enhance the sensitivity of standard detection methods such as WB or ultracentrifugation followed by WB. This would be extremely useful when suspected prion-positive cases are not confirmed by routine diagnostic assays. In these cases, the simple addition of an immunoprecipitation step based on motif-grafted antibodies may enrich the sample for PrPSc prior to WB, potentially enabling a selected test to reach the necessary detection threshold without loss of specificity. The analysis of a larger set of atypical cases will be required to determine the statistical significance of this described gain in sensitivity. For these reasons, the use of PrP motif-grafted antibodies or derivative molecules, e.g. 89–112 or 136–158 polypeptides (or shorter fragments within those sequences) coupled to tags enabling solid phase adsorption, may find practical applications for solving difficult, yet not uncommon, diagnostic problems that many prion disease surveillance centres encounter in their daily activities.

Acknowledgements

The motif-grafted antibodies IgG 89–112, IgG 136–158 and the parental antibody IgG b12 were supplied, respectively, by R. Anthony Williamson and Gil Abalos, and by Dennis Burton and Ann Hessell (The Scripps Research Institute, La Jolla, CA, USA). This work was supported by the Italian Ministry of Health (annual agreement for Italian TSEs NRL; grant: Regione Marche 14, Ricerca Finalizzata 2003) and by the United States Department of Agriculture (USDA) (ARS Cooperative Agreement No. 58-3625-4-F217).

References


