Studies on a species-specific epitope in murine, ovine and bovine prion protein

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Transmissible spongiform encephalopathies are fatal neurodegenerative disorders which are linked to abnormal isoforms of the prion protein (PrP), which is expressed in different cells of various mammalian species. Susceptibility to disease and reduced transmission rates upon the first passage to another species are thought to be a result of functional and biochemical differences of the PrP as a consequence of amino acid sequence among species. In 1985 an epidemic of bovine spongiform encephalopathy (BSE) started after accidental transmission of scrapie by feeding infected sheep and goat meat and bone meal products to cattle. In this report we present data demonstrating species-specific epitopes in bovine, ovine and murine PrP that are based on amino acid substitutions at positions 108 and 110. Rabbit antisera to synthetic peptides representing amino acid sequence 108 to 123 of PrP of cattle, sheep and mice reacted strongly with modified PrP of the homologous host but not, or only poorly, with PrP of heterogeneous origin. Cross-reactivity was observed, however, with antisera to bovine and ovine peptide sequences 102 to 117, thus stressing the importance of the location of the amino acid substitution in synthetic peptides used for immunization. Based on these data, BSE PrP and ovine and murine scrapie PrP can be distinguished from each other, and these differences might help elucidate the species barrier effect.

Bovine spongiform encephalopathy (BSE) and scrapie in sheep and goats belong to the group of transmissible spongiform encephalopathies (TSEs) and resemble Kuru, Creutzfeld-Jacob disease and Gerstmann-Sträussler-Scheincker syndrome in man. After incubation for several years there is progressive degeneration of central nervous functions and animals die within 1 to 6 months of the onset of clinical symptoms. The causative agents copurify from brains of infected individuals with fibrils composed of prion proteins (PrP). These display many virus-like features, such as strain variation and mutation, but differ from conventional viruses in being exceptionally resistant to heat, u.v. and ionizing radiation and many chemical disinfectants (Asher et al., 1986).

Sequencing PrPs led to the discovery of a gene for an analogous cellular protein of unknown function. Many efforts have therefore been made to immunologically and biochemically characterize PrPs. Rodents, to which TSEs are easily transmitted, served as model animals in many studies and their PrPs have been investigated extensively. Hamster cellular PrP of Mr 33K to 35K (PrP<sub>33</sub> to 35K) is post-translationally modified in the Golgi apparatus by cleavage of a signal peptide (22 amino acids) at the amino terminus and by the exchange of a part of the carboxy terminus with a glycolipid phosphatidylinositol anchor. Cysteines at positions 177 and 212 are linked by disulphide bonds and asparagines at 181 and 197 are glycosylated. Cellular PrP is then transported to the cell surface and bound to the membrane (Caughey et al., 1991).

Amino acid sequences of PrP<sub>e</sub> are quite conserved within the species and in man amino acid mutations are frequently associated with disease (Kretzschmar et al., 1992; Goldfarb et al., 1992). Approximately 5% amino acid differences exist among the known sequences of PrP from hamster, mouse, rat, sheep and cattle (Oesch et al., 1991; Goldmann et al., 1990, 1991). Studies using transgenic mice expressing both murine and hamster PrP genes, which encode 16 amino acid differences among 254 PrP residues, have linked PrP with incubation times in different species (Prusiner et al., 1990). Transgenic mice expressing hamster PrP exhibit incubation times...
Fig. 1. Reactivity of antisera generated by amino acid sequence 108 to 123 of bovine (×), ovine (Δ) and murine (○) PrP and by 219 to 238 (■) of murine PrP, against synthetic peptide antigens representing amino acid sequence 108 to 123 of bovine (a), ovine (b) and murine (c) PrP, and sequence 219 to 238 (d) of murine PrP in an ELISA.

characteristic of hamsters after inoculation with hamster prions (Scott et al., 1989). Moreover, variations in the primary, secondary and tertiary structure of PrP could explain the species barrier (Kimberlin, 1991). This major obstacle to inter-species transmission of these infectious agents often results in complete failure to transmit or in greatly extended incubation periods of strains at the first, compared to the second or third, passage (Kimberlin, 1991). A similar phenomenon was observed when scrapie strains were passaged in different mouse and hamster breeds (Lowenstein et al., 1990; Fraser et al., 1991). In hamster, three breed-specific amino acid replacements in the PrP gene in the vicinity of codons 115 to 125 are thought to be responsible for this effect (Lowenstein et al., 1990).

To determine the conformational influence of species-specific amino acid substitutions at regions close to the proteinase K cleavage site, we generated rabbit antisera to synthetic peptides corresponding to positions 108 to 123 of bovine (THGQWNKPSKPKTNMK) (Ra1, Ra2), ovine (SHSQWNKPSKPKTNMK) (Ra3, Ra4) and murine (THNQWNKPSKPKTNLK) (Ra5, Ra6) PrP. An antiserum directed to amino acids 219 to 238 of murine PrPc (MERVVEQMCVTQYKESQAY) (Ra7) served as a control serum in all experiments. In addition, two antisera to synthetic peptides corresponding to positions 102 to 117 of ovine (WGQGSSHQWNKPSK) (Ra1755) and bovine (WGQGSGTHGQWNKPSK) (RaIV/2) PrP were used (generously contributed by H. Diringer). Amino acid positions were calculated after aligning known PrP sequences of man, rat, hamster, mouse, sheep and cattle.

Peptides were synthesized using an automated peptide synthesizer (Milligen 9050) and Fmoc chemistry. After coupling to keyhole limpet haemocyanin (KLH) (Sigma), peptide conjugates were emulsified in complete Freund’s adjuvant (Sigma) and injected sub- and intracutaneously at days 0, 14, 28, 42, 56 and 70 into outbred chinchilla rabbits. Each vaccine dose represented 200 μg of peptide per animal. Rabbits were bled when antibody titres were deemed adequate.

Antibody response to peptides was determined by ELISA. In brief, 96-well ELISA plates were coated with 200 ng peptide per well, blocked with 5% non-fat dry milk and incubated with the primary rabbit antibody for 2 h. Binding was detected by a second incubation with
horseradish peroxidase-conjugated goat antibody to rabbit IgG (Dianova) for 1 h and was visualized by the conversion of o-phenylenediamine substrate (Sigma) to product in the presence of excess hydrogen peroxide.

All antibodies elicited by amino acids 108 to 123 displayed excellent antibody binding to homologous peptides with detection cut-off values of > 1:100000 and plateau levelling-off margins of approximately 1:3000 (Fig. 1). Half-maximal absorbance levels were achieved with serum dilutions of > 1:25800 for Ra1, Ra2, Ra5 and Ra6 and > 1:12800 for Ra3 and Ra7. Antisera RaIV/2 and Ra1755, raised against the ovine and bovine sequences of amino acids 102 to 117, exhibited little reactivity in ELISA to the 108 to 123 sequence (data not shown). The close relationship of epitopes of amino acids 108 to 123 of different species was illustrated by the cross-reactivity of antisera to this ovine and bovine peptide (Fig. 1). Mouse peptide 108 to 123 was, however, only poorly recognized by sera to corresponding bovine and ovine sequences. Control antiserum to the carboxy-terminal region of mouse PrP exhibited significant reactivity with the peptide to which it was raised, but not to peptides of the interior PrP sequences.

Cross-reactivity in ELISA was confirmed by immunoblotting with PrP antigens. Murine brains were collected from terminally ill NMRI mice infected intraperitoneally with 0.1 ml of 10% homogenate of a Merino sheep suffering from scrapie (S142). Sheep brain material originated from cases of scrapie in two Suffolk sheep (S703, S16). Isolate S16 and brain stem material from a natural BSE case in a Holstein-Friesian cow (isolate BSE Friend) were generously contributed by Drs M. Dawson

Fig. 2. Immunoblot of PrP on nitrocellulose strips with antisera to peptides representing the sequence of residues 102 to 117 (a) and 108 to 123 (b) of bovine PrP. Antisera were preadsorbed with synthetic peptide (concentration indicated in µg/ml) representing amino acids 108 to 123 of bovine (i), ovine (ii) or murine (iii) PrP sequences, or with the peptide of residues 219 to 238 of murine PrP (iv). Protein standards are shown on the left.
Fig. 3. Immunoblot of PrP$^{\text{BSF}}$ (a) and ovine PrP$^{\text{SSE}}$ (b) with antisera directed against synthetic peptides representing amino acid sequences 102 to 117 of bovine (lanes 1; RaA/IV2) and ovine (lanes 3; Ra1755) PrP, 108 to 123 of bovine (lanes 2; Ra2), ovine (lanes 4; Ra3) and murine (lanes 5; Ra6) PrP, and 219 to 238 of murine PrP (lanes 6; Ra7). Mr standards are shown on the left.

and A. Scott (Central Veterinary Laboratory, Weybridge, U.K.). All cases were confirmed by histopathology, isolates $703$, S16 and BSE Friend also by electron microscopy and/or by mouse inoculation ($703$ and S142).

Ovine and murine brains were homogenized (10%) in Tris-buffered saline (TBS). Crude cell detritus was pelleted by centrifugation at 500 g for 10 min and fibrils were sedimented at 20000 g for 30 min. Pellets were resolubilized in TBS containing 1% Zwittergent 3-14 (Calbiochem), pelleted at 100000 g for 1 h and resuspended in 10 mM-Tris-buffered solution pH 8.5, containing 5 mM-CaCl, 0.1% Zwittergent 3-14 and 10% NaCl. Subsequently, extracts were treated with 8 μg/ml staphylococcus nuclease (Boehringer Mannheim) followed by 4 μg/ml proteinase K (Serva) for 5 min at 37 °C. Finally, fibrils were pelleted through a 20% sucrose cushion for 20 min at 540000 g. PrP$^{\text{BSF}}$ was purified according to a method introduced by Hilmert & Diringer (1984) and modified by Kitamoto et al. (1989).

Extracts were boiled (5 min/95 °C) in 125 mM-Tris-buffered sample solution, pH 6.8, containing 1% SDS, 100 mM-DTE (Bio-Rad) and 20% glycerol. Proteins were separated by discontinuous preparative SDS-PAGE (13% monomer) (Laemmli, 1970), transferred to nitrocellulose membranes (Burnette, 1981) and denatured by incubation in 3 M-guanidinium thiocyanate (Sigma) (10 min). Excess binding capacities of the membranes were blocked by 5% non-fat dry milk in PBS containing 0.1% Tween 20 (30 min) and sheets were cut into narrow strips. Strips were probed (for 2 h at 22 °C) with the untreated antisera to synthetic peptides diluted 1:1000 (except RA7 1:200) with PBS-Tween, or with antibody previously incubated for 1 h at 37 °C with 0, 20, 100 or 500 μg peptide/ml. Following extensive washings, strips were incubated with alkaline phosphatase-conjugated goat antibody to rabbit IgG (Medac), diluted 1:2000 in PBS-Tween, for 1 h at 22 °C and were washed again. Antibody binding was visualized by nitro-tetrazolium blue chloride/5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt (Fluka) substrate conversion.

Antisera to synthetic peptides of amino acid sequences 102 to 117 and 108 to 123 of mouse, sheep and bovine PrP reacted well in the immunoblot with partially denatured PrP$^{\text{SSE}-27-30K}$ of the corresponding species. Reactions were completely inhibited by pre-absorbing the sera with corresponding species' peptides of amino acids 108 to 123 (Fig. 2). Inhibition, however, was also possible between antiserum and peptides of heterogeneous species. Thus cross-reactivity seen in ELISA was confirmed by the immunoblots (Table 1). Similarly, the reaction of antiserum with the carboxy-terminal region of murine PrP was inhibited by the peptide of murine PrP sequence 219 to 238, but not by ovine and bovine peptides of residues 108 to 123. Reactivity was diminished, however, by the murine peptide of residues 108 to 123, indicating an as yet unexplained antigenic relationship between the interior and carboxy-terminal regions of murine PrP.

Antisera raised against synthetic peptides were finally tested for their reactivity with PrP originating from heterologous species. Interestingly, antiser to the BSE peptide of residues 108 to 123 displayed no (Ra2) or only very poor (Ra1) reactivity with PrP originating from other species, while antiserum RaAIV/2 to BSE peptide representing residues 102 to 117 cross-reacted strongly with ovine PrP and to a lesser extent with murine PrP.

### Table 1. Inhibition* of anti-peptide antibody binding to bovine, ovine and murine PrP by preincubation with synthetic peptides in immunoblot

<table>
<thead>
<tr>
<th>Antiserum to PrP antigen (sequence)</th>
<th>Peptides representing amino acid sequences</th>
<th>BSE 108 to 123</th>
<th>Scrapie 108 to 123</th>
<th>Mus 219 to 238</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSE (102 to 117)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BSE (108 to 123)</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
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<tr>
<td>Scrapie (102 to 117)</td>
<td>+/-</td>
<td>-</td>
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<tr>
<td>Scrapie (108 to 123)</td>
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<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Murine (108 to 123)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Murine (219 to 238)</td>
<td>-</td>
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* +, Complete inhibition by < 100 μg/ml synthetic peptide; +/-, inhibition by 100 to 500 μg/ml synthetic peptide; -, no inhibition by 500 μg/ml synthetic peptide.
Although cross-reactivity to murine PrP was less evident, species-specific antisera to ovine PrP were generated by shifting the target sequence by six amino acids, from 102 to 117 to 108 to 123. Antisera to the mouse peptides of sequence 108 to 123 strongly bound to murine PrP and exhibited no (Ra5) or only a very faint banding reaction (Ra6) with the ovine and bovine PrP (Fig. 3, Table 2). No species-specific reactivity was seen with antiserum to the carboxy-terminal region of murine PrP. Although the immunoblot detection level of antiserum to this region was generally lower than the reactivity of antisera to the internal peptides, PrP of either bovine, ovine or murine origin was equally well recognized.

These data clearly demonstrate a species-specific epitope on PrP<sub>BSE</sub> and ovine and murine PrP<sub>Sc</sub>, located close to the proteinase K cleavage site. Cross-reactivity in ELISA and in competitive immunoblots of antisera to the corresponding peptides representing amino acid sequences of different species, presumably resulted from the peptides’ high conformational flexibility in aqueous solution and from their limited flexibility preserved even after coupling onto the surface of ELISA plate wells. Hence, conformational flexibility partially restored the peptides’ lack of affinity to antibodies, whereas no antibody reaction is observed with rather rigid PrP molecules which contain amino acid sequences fixed in unique conformations. Coupling peptides to KLH, however, must produce epitopes similar in shape to those present, on amino-terminal truncated isoforms of PrP. In contrast, only a minority of peptide molecules of the carboxy-terminal sequences are bound to KLH in a conformation similar to PrP, because reaction levels of antisera were much lower in immunoblots as compared to ELISA.

Species-specific epitopes have been reported in PrP<sub>BSE</sub> and ovine and murine PrP<sub>Sc</sub> with valine at position 125 in the target epitope of these MAbS might account for this effect. Lowenstein et al. (1990) showed species-restricted activity of a MAb to hamster but not to human or mouse PrP. This MAb is directed against an epitope spanning amino acids 150 to 156 and species specificity is presumably generated by replacing methionine at position 152 (hamster) by isoleucine (mouse, man) (Lowenstein et al., 1990).

Results of this present study now suggest a species-specific epitope in ovine and bovine PrP in the vicinity of amino acid 110. Presumably, the secondary or tertiary structure is modified by substitution of glycine in PrP<sub>BSE</sub> with serine, which has an additional hydroxyl group. The human PrP gene also codes for serine at this position, whereas hamster, mouse and rat, as well as the PrP from cow as well as in Syrian, Chinese and Armenian hamster PrP.

The amino acid replacement of methionine by leucine at position 122, as well as a missing glycine, obviously resulted in the lower cross-reactivity levels in ELISA of the antisera to synthetic mouse peptides of residues 108 to 123 with the peptides constituting ovine and bovine amino acid sequences. However, a faint banding reaction with immunoblotted ovine and bovine PrP was detected by antisera to this mouse peptide, which is presumably due to a subset of antibodies stimulated by constant regions present in the peptide sequences used for vaccination.

Other studies revealed no variations in the amino acid sequence at positions 108 and 110 of PrP<sub>BSE</sub> in different cow breeds. Similarly, no diversity in sheep genes at this site has been reported (Goldmann et al., 1990, 1991). Hence, peptides of amino acid sequence around position 110 are prime candidates for vaccines to generate species-specific antibodies. Our study revealed, however, that sequence variations alone in synthetic peptides are not sufficient to elicit specific antibodies. Equally important in this

| Table 2. Binding of anti-peptide antibodies to bovine, ovine and murine PrP* |
|-----------------|-----------------|-----------------|-----------------|
| Antiserum to PrP antigen (sequence) | PrP<sub>BSE</sub> | PrP<sub>Sc-ovine</sub> | PrP<sub>Sc-mouse</sub> |
| BSE peptide (102 to 117) | + + + | + + + | + |
| BSE peptide (108 to 123) | + + + | - | - |
| Scrapie peptide (102 to 117) | - | + + + | + |
| Scrapie peptide (108 to 123) | - | + + + | - |
| Murine peptide 2 (108 to 123) | +/− | +/− | + + + |
| Murine peptide 1 (219 to 238) | + | + | + |

* + + +, Very strong band in immunoblot; + +, strong band in immunoblot; +, weak band in immunoblot; +/−, faint band in immunoblot; −, no antigen detection in immunoblot.
epitope is the location of the substitution in the peptide used for vaccination. Antibodies directed to residues 102 to 117 displayed little or no species specificity, whereas vaccines of peptides of residues 108 to 123, harbouring the variable region at the amino terminus, worked well.

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References


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