Journal of NeuroVirology (1999) 5, 401–413

http://www.jneurovirol.com

Immune surveillance and antigen conformation determines humoral immune response to the prion protein immunogen

Richard Rubenstein*,[1], Richard J Kascsak[1], Michael Papini[1], Regina Kascsak[1], Richard I Carp[1], Giuseppe LaFauci[1], Rob Meloen[2] and Jan Langeveld[2]

[1]*Department of Virology, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, New York, NY 10314, USA;* [2]*Institute for Animal Science and Health, Edelhertweg 15, P.O. Box 65 NL-8200 AB, Lelystad, The Netherlands*

Transmissible spongiform encephalopathies (TSE) are progressive degenerative disorders of the central nervous system. PrP^{Sc} is a TSE-specific marker derived from the host-encoded glycoprotein, PrP^{c}. The generation of antibodies to PrP plays an important role in the diagnosis of these diseases. In this study the role of the PrP immunogen and the species being immunized was examined in relation to specific epitopes. Various mammals (mice, hamsters, rabbits and PrP null mice) were immunized with formic acid-treated PrP^{Sc} isolated from mice, hamsters and sheep. Both the species being immunized and the source of immunogen played an important role in the antibody response. Response to a limited number of linear epitopes was seen among the various immunized animals. One region in the C-terminal portion of PrP appeared highly immunogenic in all species. Comparison of immunoreactivity and the pepscan-defined linear epitope sites suggests both linear and conformational directed responses in many of the animals. Information on the forces directing immune responses to PrP will lead to a better understanding of host-PrP interactions. It will also assist in the development of new strategies for generating additional tools for immunodiagnosis.

Keywords: prion protein; PrP antibodies; rabbit PrP; pepscan; continuous and discontinuous epitopes

Introduction

The immunodiagnosis of the transmissible spongiform encephalopathies or prion diseases is of critical importance due to the transmissibility of these conditions and their fatal prognosis. Scrapie in sheep and goats is the prototype of these diseases and has been recognized for hundreds of years. The human forms of prion disease include Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, Kuru and fatal familial insomnia (reviewed in Brown and Gajdusek, 1991; Collinge and Palmer, 1997; Pocchiari, 1994). Human prion diseases occur in sporadic, iatrogenic and inherited forms with an overall incidence of 1–2 per million in the general population throughout the world (Brown *et al*, 1987). Several years ago, this group of diseases spread from sheep to cattle probably as a result of changes in the rendering of supplements fed to cattle (Wilesmith *et al*, 1988). This outbreak in cattle has subsequently led to prion disease appearing in felines, zoo animals, antelopes and recently in young human adults (reviewed in Bradley, 1997; Collinge and Palmer, 1997). The hallmark of all prion diseases is the conversion of a host membrane glycoprotein, termed PrP^{c} or PrP^{sen}, into a protease resistance insoluble form, termed PrP^{Sc} or PrP^{res}, which is associated with infectivity (Gabizon *et al*, 1987; Hilmert and Diringer, 1984; Prusiner *et al*, 1982). The presence of PrP^{Sc} is now recognized as a universal marker for prion disease.

PrP^{c} is linked by its glycosyl phosphatidylinositol (GPI) anchor to the cell surface of all mammalian cells (Stahl *et al*, 1987; Caughey *et al*, 1989). As a consequence of this cell surface location, this protein is readily seen by immune surveillance. All mammalian species are tolerant to their homologous (self) PrP and to any homologous sites on PrP

*Correspondence: R Rubenstein
Received 28 October 1998; revised 29 December 1998; accepted 27 January 1999

from other species. Animals will respond immunologically to those sites on PrP viewed as nonself by their immune systems.

The immunological detection of PrP employing various immunoassays affords the sensitivity and specificity for the rapid and accurate diagnosis of prion disease. The monoclonal antibody (Mab) 3F4, which recognizes both PrP^{c} and PrP^{Sc} from hamsters, humans and feline, has been widely used for diagnosis (Kascsak *et al*, 1993). It is important to have available to the research and medical communities a large repertoire of antibodies to PrP for its specific identification in a wide range of immunoassays. This manuscript describes the immune response to PrP in several animal species (rabbits, mice and hamsters). Examination of this response by various immunoassays, pepscan analysis and PrP sequence comparisons among different species provides insight into the forces which direct these responses and a means to better understand the relationship between PrP and the host.

Results

In order to view the anti-PrP responses in rabbits within the context of nonself recognition for each species used for immunization, the PrP sequence must be defined. Therefore, sequencing of the NZW rabbit PrP gene was performed. The ORF of the rabbit PrP gene from a NZW rabbit brain cDNA library coded for a 254 amino acid protein (GenBank accession number AF015603) (Figure 1). Characterization of the PrP ORF from a NZW rabbit heart genomic library has previously been reported (Loftus and Rogers, 1997) and shown to encode a protein of 252 amino acids. The rabbit PrP sequence obtained in this study differs from that reported previously by an additional two glycine residues at positions 54 and 93 (Figure 1). At the amino acid level, rabbit PrP shares 88–93% homology with other mammalian species. A comparison of the amino acid sequences of rabbit PrP (254 amino acids) with hamster PrP (254 amino acids) and mouse PrP (254 amino acids) shows differences at 29 and 30 positions, respectively. Furthermore, a collective comparison between the aligned amino acid sequence of rabbit PrP and those of several other mammalian species [bovine, hamster (Armenian, Chinese, Syrian), human, mink, mouse, sheep] indicates that there are only nine amino acid positions (12, 45, 112, 178, 224, 229, 235, 237 and 239) where the rabbit sequence differs from all the other species (Figure 1). Rabbits were immunized with either PK-treated PrP^{Sc} or PrP synthetic peptides from mouse, hamster, chicken and human. All rabbits receiving mammalian formic acid-treated PrP^{Sc} (FA-PrP^{Sc}) responded by producing antibodies which were immunoreactive to the same types of mammalian species. When purified mouse and hamster FA-PrP^{Sc} served as antigen for rabbit immunizations, pepscan analysis revealed only six linear epitopes present in anti-PrP antibodies from five different rabbits (amino acids 99–103, 104–108, 182–191, 190–198, 201–207 and 221–226) (Table 1). The majority of these sites are present in the C-terminal region of the protein. Since there is a 94% homology between mouse and hamster PrP, it is not surprising that hamster FA-PrP^{Sc} generated a response with overlapping epitopes to mouse antigen-generated antibody (Table 1; Figure 2a and b). Western blotting revealed that antibodies generated in rabbits using mouse and hamster FA-PrP^{Sc} reacted against both isoforms of PrP from each of the species to which these antibodies were immunoreactive (bovine, feline, hamster, human, mouse, rat and sheep) and did not react to PrP from ferret, mink, chicken, and, of course, rabbit (Tables 1 and 3). Furthermore, all but one of the linear epitopes against PrP each represent sites with one or two amino acid differences between antigen source (hamster or mouse) and responding species (rabbit) (Table 1). One epitope (rabbit ME7-3, Table 1) is directed against a site that is identical in both the immunogen and the host. This, together with additional studies described below, indicates that parameters other than linear self-nonself differences may also play a role in determining epitope response.

Hamsters were immunized with mouse FA-PrP^{Sc} and generated a strong immune response. Pepscan analysis revealed the epitope specificity of this response (Table 2). In the three immunized hamsters, response to only three of the 13 nonself sites (comparing hamster and mouse PrP 27-30) were generated (Table 2); position 132–139 (strongly reactive) in hamster H-1 (Figure 2c) and sites 103–113 (weakly reactive) and 201–207 (strongly reactive) in hamster C3. In addition to these sites, hamsters C7 and C3 also responded (weakly reactive) to sites conserved in both hamsters and mice which includes amino acids 93–103 (C7) and 92–99 (C3). This was in fact the only linear epitope recognized by hamster C7 antibody. This suggests that these sites are either conformationally different between mice and hamsters or sequestered. The antibodies produced in hamsters to mouse PrP are unique in that they are the only anti-PrP antibodies which recognize rabbit PrP^{c} in immunoassays (Table 3).

Antibodies were also generated in mice to hamster-derived FA-PrP^{Sc}. Mabs produced to hamster PrP in mice expressing PrP have been previously described (Kascsak *et al*, 1993; Rogers *et al*, 1991). The polyclonal sera from these animals were not available for analysis. Two Mabs to hamster PrP have been previously generated in our laboratory (Table 3; Kascsak *et al*, 1993). Mab 3F4 recognizes hamster, feline and human PrP, whereas Mab 7G5 will immunoreact with only hamster PrP. The

precise epitopes seen by these antibodies have been mapped by pepscan analysis (Figure 3). The reactive core sequences are TNMKHM for 3F4 and YRPVDQYNN for 7G5.

In order to overcome the self-nonself limitation in antibody response, PrP null mice were also immunized with three (mouse, sheep and hamster) mammalian forms of PrP (Table 4). PrP null

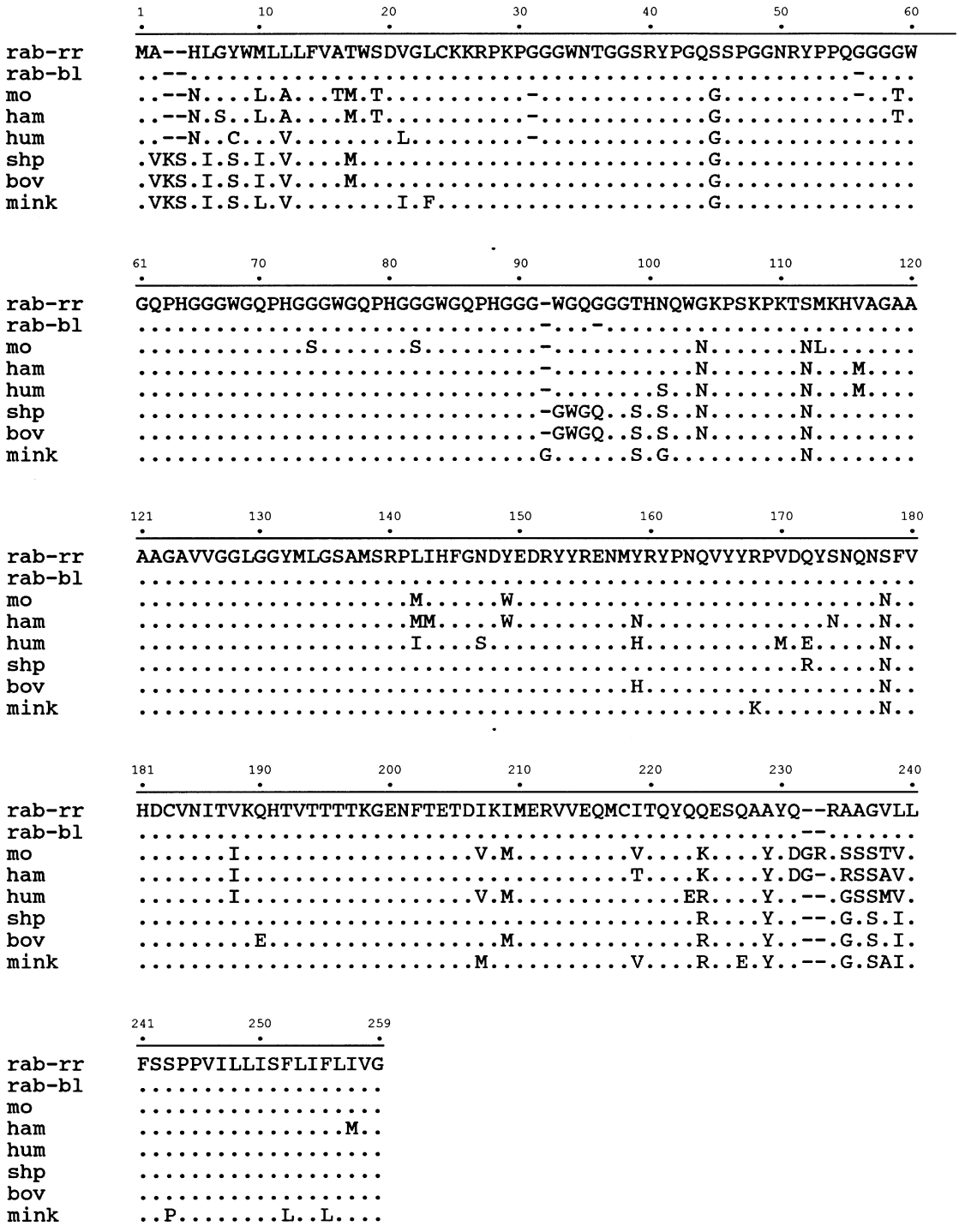

Figure 1 Alignment of the predicted protein sequences of the PrP genes from rabbit [rab-rr; rabbit sequence as determined by Rubenstein *et al* (this manuscript), rab-bl; rabbit sequence as determined by Loftus and Rogers (1997)], mouse (mo), hamster (ham), human (hum), sheep (shp), bovine (bov), and mink.

mice do not express PrP^{c} on their cell surface and, therefore, should view the entire PrP molecule as nonself. The two mice (KO2 and KO45) which were immunized with murine FA-PrP^{Sc} responded to a similar linear site on mouse PrP which included the sequence DVKMME (Table 4; Figure 2d). This appears to be a highly immunogenic site since two rabbits (78295 and ME7-1) and a hamster (C3) immunized with mouse PrP react to this same site. Both the KO2 and KO45 antisera react with PrP^{c} and PrP^{Sc} from a wide range of species including bovine, feline,

Table 1 Pepscan analysis of rabbit antibodies to FA-PrP^{Sc}

Rabbit antibody	*PrP antigen source*	*Amino acid sequence*[a]	*Codon difference in host PrP*	*Reactivity*[b]
78295	Mouse	92–GGTHNQWNKPSKPKTNLKH–110	N99G	All mammals except ft, mink, rab
		178–CVNITIKQHTVTTTTK–193	I183V	
		195–ENFTETDVKMMERVVEQM–212	V202I, M204I	
		213–CVTQYQKESQAYYDGRRSSS–232	Y224A	
ME7-1	Mouse	196–NFTETDVKMMERVVEQM–212	V202I M204I	All mammals except ft, mink, rab
ME7-3	Mouse	180–NITIKQHTVTTTTKG–194	I183V	All mammals except ft, mink, rab
		187–TVTTTTKGENFTETD–201	None	
		213–CVTQYQKESQAYYDGRRSSS–232	Y224A	
79608	Hamster	97–NQWNKPSKPKTNMKHMAGA–115	N108S	All mammals except ft, mink, rab
79607	Hamster	215–TTQYQKESQAYYDGRRSS–232	Y225A	All mammals except ft, mink, rab

[a]Amino acid sequence represents the composite of overlapping 12-mer peptides used to determine the epitope (underlined). [b]Reactivity, as measured by Western blotting, was analyzed against PrP^{Sc} and/or PrP^{c}, ft, ferret; rab, rabbit.

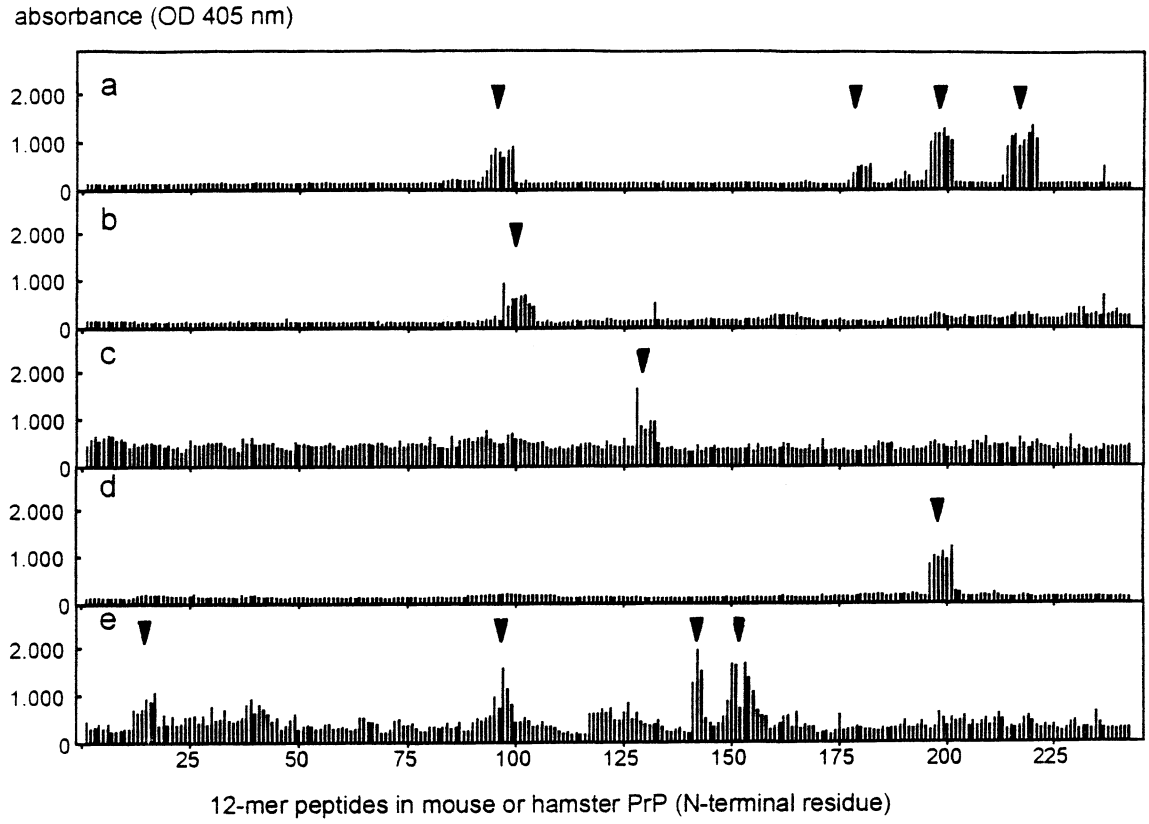

Figure 2 Pepscans of rabbit (**a**, 78295; **b**; 79608), hamster (**c**, H-1) and null mouse (**d**, KO2; **e**, KOctbt) antisera raised against mouse (**a**, **c**, **d**), or hamster (**b**, **e**) FA-PrP^{Sc}. The sequence of PrP used for peptide synthesis was either murine PrP (**a**, **c**, **d**) or hamster PrP (**b**, **c**). Linear epitopes are defined by peaks of absorbance (arrowheads) which indicate regions of immunoreactivity as described in Materials and methods.

hamster, human, mink, mouse, rat and sheep (Tables 3 and 4). PrP null mice receiving both sheep and mouse FA-PrP^{Sc} immunogens (KO5 and 94-6) were highly reactive to both DVKMMER (mouse site) and DIKIMER (sheep site). One of these mice (94-6) also responded to an additional site (MYRYPNQ) which is identical in mouse and sheep PrP. Null mouse (KO20) receiving only sheep PrP responded to a single site, YEDRYYREN (148–156). Null mouse (KOctbt) receiving hamster PrP immunogen responded to three sites, QWNKPSKPKTN (98–108), NDWEDRYYRE (143–152) and NMNRYPNQ (153–160) (Table 4, Figure 2e).

Despite the nearly 50% sequence homology between mammalian and chicken PrP, antibodies produced to mammalian (hamster, human or mouse) PrP did not immunoreact with chicken PrP^{c}. Likewise, antibody to chicken PrP did not react with any of the mammalian forms of PrP^{c} (Table 3) and PrP^{Sc} (data not shown). In addition, PrP null mice produced a species-specific immune response to

Table 2 Pepscan analysis of hamster antibodies to FA-PrP^{Sc}

Hamster antibody	*PrP antigen source*	*Amino acid sequence*[a]	*Codon difference in host PrP*
C7	Mouse	92–GGTHNQWNKPSKP–104	None
H-1	Mouse	128–MLGSAMSRPMIHFGND–143	I138M
C3	Mouse	90–OGGGTHNQWNKPS–102	None
		102–SKPKTNLKHVAGA–114	L108M, V111M
		196–NFTETDVKMMERVVEQM–212	V202I, M204I

[a]Amino acid sequence represents the composite of overlapping 12-mer peptides used to determine the epitope (underlined).

Table 3 Reactivity by Western blotting against PrP^{c} from various species

	Bov	*Fe*	*AHam*	*CHam*	*SHam*	*Hum*	*Mink*	*Mo*	*Rab*	*Rt*	*Shp*	*Chk*
Mab7G5	–	–	+	+	+	–	–	–	–	–	–	–
Mab3F4	–	+	+	–	+	+	–	–	–	–	–	–
PrP Null Mouse	+	+	+	+	+	+	+	+	–	+	+	–
Rabbit PrP peptide 505	+	+	+	+	+	+	–	+	–	+	+	–
Rabbit PrP peptide 524	+	+	+	+	+	+	–	+	–	+	+	–
Rabbit anti-MoPrP	+	+	+	+	+	+	–	+	–	+	+	–
Rabbit anti-SHamPrP	+	+	+	+	+	+	–	+	–	+	+	–
Rabbit anti-ChkPrP	–	–	–	–	–	–	–	–	–	–	–	+
Hamster anti-MoPrP	+	+	–	+	–	–	+	+	+	+	+	–

Bov, bovine; Chk, chicken; Fe, feline; AHam, armenian hamster; CHam, Chinese hamster; SHam, Syrian hamster; Hum, human; Mo, mouse; Rab, rabbit; Rt, rat; Shp, sheep.

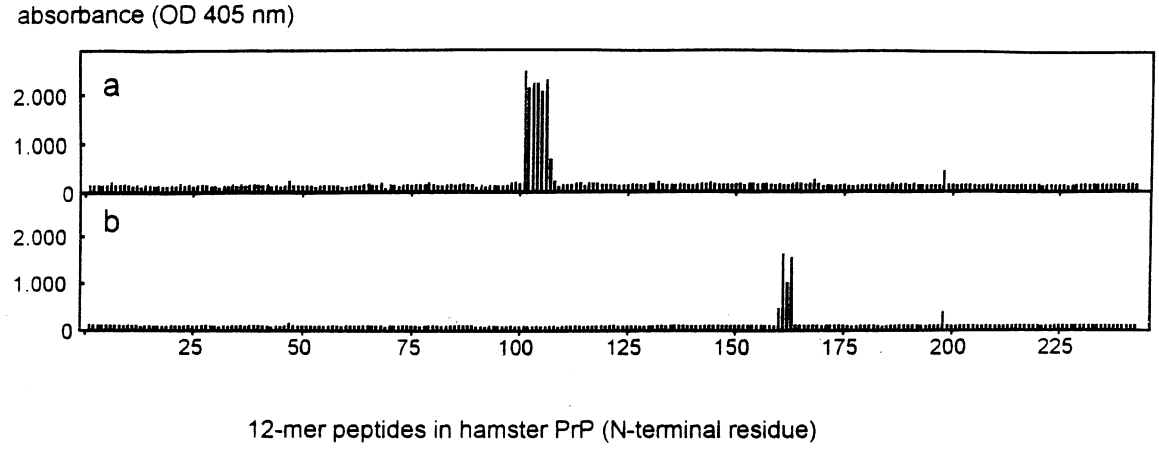

Figure 3 Pepscans of monoclonal antibodies 3F4 (**a**) and 7G5 (**b**). The sequence of PrP used for peptide synthesis was murine PrP. Peaks of absorbance indicating regions of immunoreactivity define the linear epitopes as described in Materials and methods.

Table 4 Pepscan analysis of null mouse antibodies to FA-PrP^{Sc}

Null Mouse antibody	*PrP antigen source*	*Amino acid sequence*[a]	*Reactivity by sequence*	*Reactivity by immunoassay*[b]
KO45	Mouse	195–ENFTETDVKMMERV–208	hum, mo, rt	bov, fe, ham, hum, mink, mo, rt, shp
KO2	Mouse	196–NFTETDVKMMERVVEQM–212	hum, mo, rt	bov, fe, ham, hum, mink, mo, rt, shp
KO5	Mouse and sheep[c]	19–TDVGLCKKRPKPGG–33 (S) 196–TKGENFTETDVKM–208 (I) (I) 202–TETDVKMMERVVEQMCV–218 (I) (I) (I)	bov, ft, ham, hum, mink, mo, rab, shp	ham, mo, shp
94-6	Mouse and sheep[c]	152–YYRENMYRYPNQVYYRP–168 199–ENFTETDVKMMERVVEQM–216 (I) (I)	ft, hum, mink, mo, rab, shp	ND
KO20	Sheep	145–GNDYEDRYYRENMYR–159	bov, fe, ft, hum, mink, rab, shp	ham, mo, shp
KOctbt	Hamster	97–NQWNKPSKPKTNM–109 141–FGNDWEDRYYRENM–154 149–YYRENMNRYPNQVYYRPV–166	bov, fe, ham, hum, mo, rt, shp	ham, mo, shp

bov, bovine; fe, feline; ft, ferret; ham, hamster; hum, human; mo, mouse; rab, rabbit; rt, rat; shp, sheep. [a]Amino acid sequence represents the composite of the overlapping 12-mer peptides used to determine the epitope (underlined). [b]Indicates reactivity, by Western blotting, against PrP^{Sc} and PrP^{c} only for those species tested. [c]Amino acid symbols in parenthesis indicate the sheep PrP sequence differences compared to the mouse sequence. ND, not done.

both PrP isoforms similar to the one seen in rabbits or hamsters (Tables 3 and 4). The limited response (five epitopes using three different immunogens) was quite similar to the range seen in rabbits (six epitopes) and hamsters which express PrP on their immune cells.

The polyclonal antisera raised in mice, hamsters or rabbits to various PrP antigens were also examined by Western blot analysis. Each blot contained individual lanes with FA-PrP^{Sc} obtained from infected mice, hamsters or sheep (Figures 4 and 5). Rabbit antisera generated to mouse or hamster FA-PrP^{Sc} was immunoreactive to PrP^{Sc} (Figure 4A and B) and PrP^{c} (Table 3) from all three species. However, Western blotting indicated that this reactivity appeared to be more intensive to the antigen used as immunogen (i.e. the animal immunized with mouse PrP reacted more intensely with mouse PrP). Antibody response appeared to be directed to all three isoforms of PrP^{Sc} (27–30 kDa diglycosylated form, 22–23 kDa monoglycosylated form, and 18–19 kDa unglycosylated form). Mice immunized with hamster FA-PrP^{Sc} (Figure 4C) reacted very strongly with the immunogen (hamster PrP), only weakly with sheep PrP and not at all with self antigen (mouse PrP). Hamster anti-mouse PrP^{Sc} antisera reacted strongly to the immunogen (mouse PrP), only weakly to sheep PrP and not at all to hamster PrP (Figure 4D).

The Western blot activity of antibody to PrP generated in PrP null mice produced a slightly different profile. Antisera raised in PrP null mice receiving either mouse or hamster FA-PrP^{Sc} reacted strongly to both antigens and not just the immunogen (Figure 5A and B). In contrast to mice which express the PrP gene, these mice responded well to mouse PrP. Unexpectedly, the immune surveillance system of null mice was able to distinguish among the different sources of PrP. Antisera from the null mice immunized with mouse or hamster FA-PrP^{Sc} reacted very poorly with sheep PrP (Figure 5A and B). However, animals immunized with sheep-derived FA-PrP^{Sc} generated an intense response directed to sites on the immunogen, a less intense response to mouse PrP, and only minimal reactivity against hamster PrP (Figure 5C). In addition, although PrP null mouse antisera was immunoreactive against PrP^{c} from most species analyzed as described above (Tables 3 and 4), differences in the intensity of reactivity (data not shown) paralleled that described for PrP^{Sc}. These differences in PrP immunoreactivity appear to be a result of response to both linear and non-linear sites. For example, mouse KO20 immunized with sheep PrP did not produce a response to linear sites on mouse or hamster PrP.

Discussion

This report describes the antibody response to PrP in several mammalian species and the use of these antibodies for the identification and characterization of PrP. The use of pepscan analysis reproducibly reveals antigenic regions in the primary sequence of proteins by testing antisera for their binding to complete sets of overlapping solid phase peptides with a distinct length (Geysen *et al*, 1984,

1985, 1987) (in this case 12-mers) and played a major role in defining host-immunogen interactions. The linear epitope response to PrP isolated from three different species is shown in Figure 6 which graphically depicts the limited responses which are generated. The antigen source and the species being

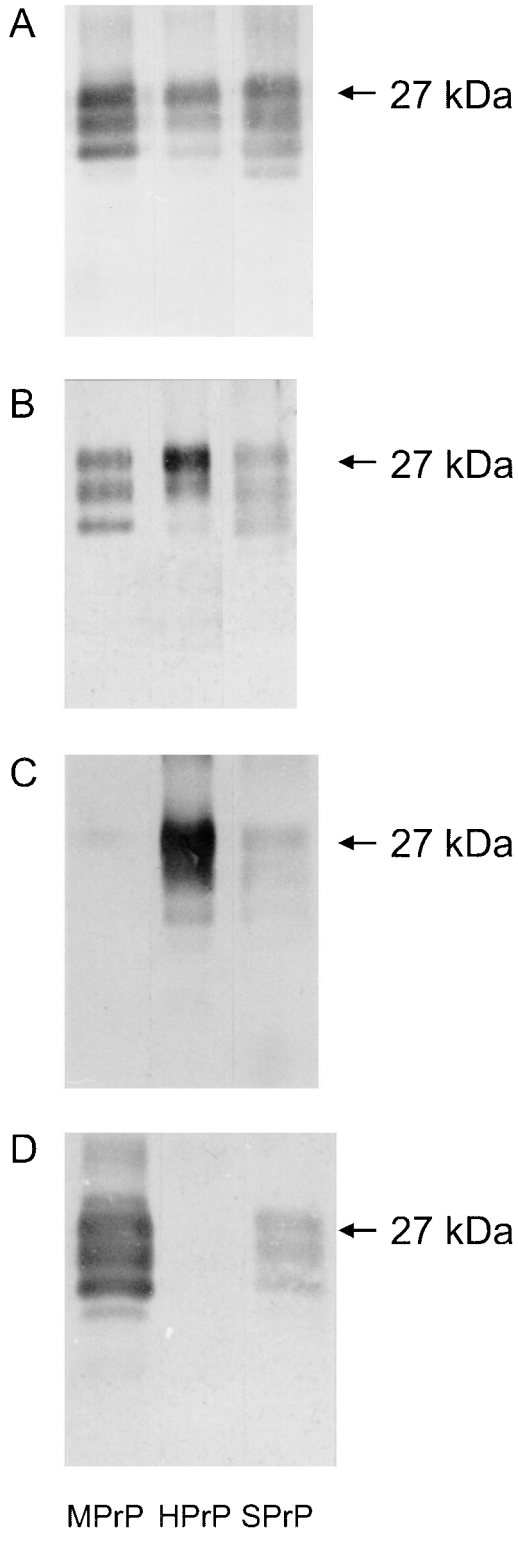

Figure 4 Western blot analysis with various antisera. Following electrophoresis of purified FA-PrP^{Sc} from ME7 scrapie strain-infected mice (MPrP), 263K scrapie strain-infected hamsters (HPrP) and natural cases of sheep scrapie (SPrP), immunoblotting was performed using rabbit anti-mouse FA-PrP^{Sc} antisera (78295) (**A**), rabbit anti-hamster FA-PrP^{Sc} antisera (79607) (**B**), Balb/CJ mouse anti-hamster FA-PrP^{Sc} antisera (**C**), and hamster anti-mouse FA-PrP^{Sc} antisera (C7) (**D**).

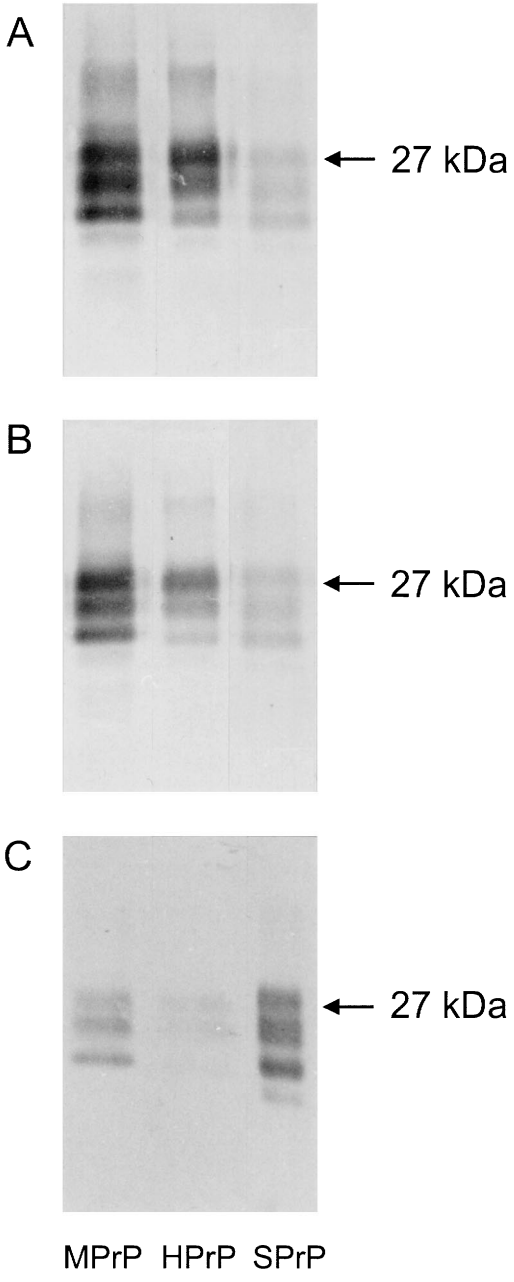

Figure 5 Western blot analysis using antisera from PrP null mice. Following electrophoresis of FA-PrP^{Sc} from ME7 scrapie strain-infected mice (MPrP), 263K scrapie strain-infected hamsters (HPrP) and natural cases of sheep scrapie (SPrP), immunoblotting was performed using antisera from PrP null mice immunized with hamster FA-PrP^{Sc} (KOctbt) (**A**), mouse FA-PrP^{Sc} (KO2) (**B**), and sheep FA-PrP^{Sc} (KO20) (**C**).

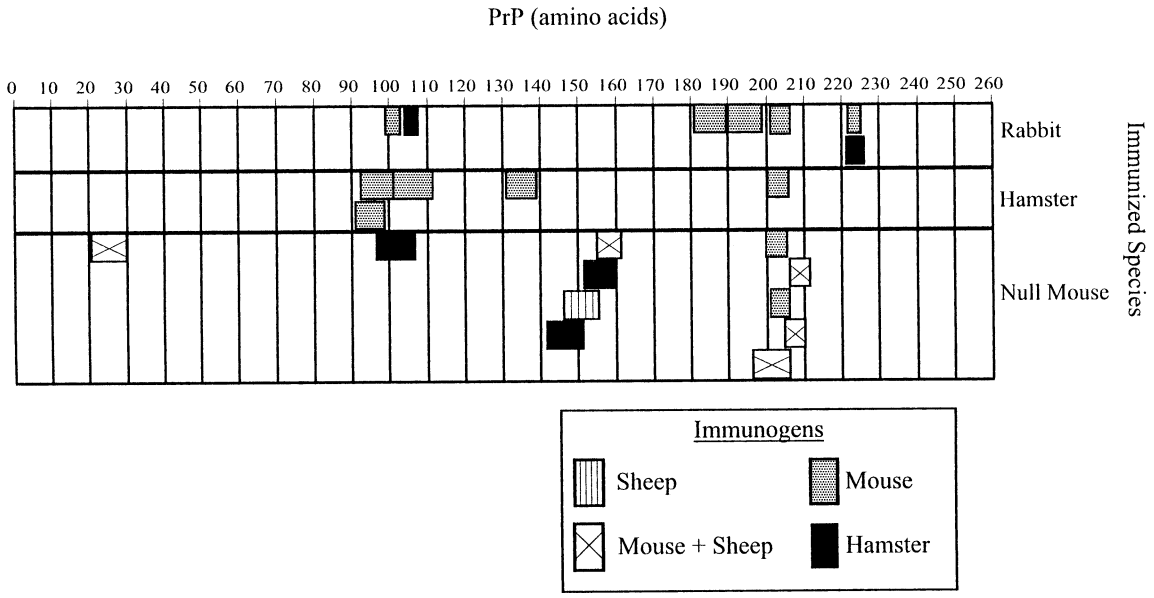

Figure 6 Representation of the prion protein indicating the linear epitope positions, as defined by pepscan analysis, for the antisera from each immunized species-immunogen combination. The width of each box indicates the number of amino acids comprising each epitope.

immunized influenced the specificity of the response both for linear as well as conformational epitopes. Several laboratories have produced rabbit antibodies to PrP (Barry *et al*, 1986; Bendheim *et al*, 1984; Farquhar *et al*, 1989; Kascsak *et al*, 1987; Serban *et al*, 1990). When mouse or hamster PrP were used as immunogen for rabbits, the specificity of the response for linear epitopes was not only remarkably similar but was restricted to a small number of sites (total of six in five different rabbits). This limited response was unexpected since the amino acid sequence differences between rabbit and mouse or hamster PrP are 22 and 21, respectively, in the PrP 27–30 protein. This restricted response suggests that certain amino acid sequences are more immunogenic than others and/or the role of forces other than those related to self-nonself differences are involved. Only linear sites were measured in this manner and our results suggest that responses to non-linear sites are also being generated. While the PrP^{Sc} immunogens have been formic acid-treated and dissolved in zwitterionic detergent-containing buffer, it would appear that conformational sites of the FA-PrP^{Sc} can still play a role in driving the epitope response.

The distinguishing properties between the native cellular form of the prion protein and the prion disease-specific form are presumably the result of their conformational differences. The conversion of PrP^{c} to PrP^{Sc} probably involves the conformational rearrangement of a predominantly α-helical protein to a β-pleated sheet (Pan *et al*, 1993; Prusiner, 1991, 1997; Prusiner *et al*, 1990). FA-PrP^{Sc}, used as immunogen in our studies, is susceptible to protease digestion and therefore similar to PrP^{c}. Presumably, the formic acid treatment causes a structural rearrangement of the PrP^{Sc} to a PrP^{c}-like conformation. NMR characterization of the recombinant murine prion protein (rmPrP^{c}) has recently been reported (Billeter *et al*, 1997; Riek *et al*, 1997). The three-dimensional structure of this protein consists of an N-terminal flexible coil, three α-helical regions, and an antiparallel β-pleated sheet. A comparison of the mouse PrP amino acid sequence with 23 other mammalian species indicated regions of variability which were divided into different classes based on their locations in the three-dimensional structure and their chemical properties. Examination of the defined linear epitopes generated in rabbits, hamsters and mice with respect to the structure of rmPrP^{c} indicates that the regions of self-nonself recognition occurs mainly within the α1 and α3 helices. Furthermore, of the amino acid residues which were classified, those responsible for this recognition were mainly of the B class. The B class residues contain exclusively hydrophobic side chains which are in contact with other hydrophobic residues (Billeter *et al*, 1997). Therefore, because of their limited surface accessibility, and assuming rmPrP^{c} and FA-PrP^{Sc} are folded in the similar manner, these sites would not have been expected to play a major role in intermolecular interactions, and presumably antibody generation.

The amino acid sequence of the rabbit PrP gene reported here is similar to that previously reported

(Luftus and Rogers, 1997). There are nine positions where the rabbit PrP sequence differs from all other species. In view of the reports that rabbits are not susceptible to prion diseases (Gibbs *et al*, 1979) and that the conversion of PrP^{c} to PrP^{Sc} is a necessary event for the infectious process, it would seem that one or more of these amino acid differences is interfering with this event. Since it has previously been shown that neither deletion of the N-terminal 66 amino acids nor truncation of the C-terminus at the GPI signal peptide interferes with the conversion of PrP^{c} to PrP^{Sc} (Rogers *et al*, 1993), only five of the remaining amino acid differences (112, 178, 224, 229 and 235) would seem to modulate rabbit susceptibility to prion disease. Studies using scrapie-infected mouse neuroblastoma cells have shown that a change at a single amino acid residue can inhibit the conversion of recombinant PrP^{c} to PrP^{Sc} (Priola and Chesebro, 1995). Furthermore, additional studies have shown that the efficiency of *in vitro* conversion is dependent on the homology of the PrP combinations (Raymond *et al*, 1997).

Self-nonself differences also influence the response in mice or hamsters. Mab 3F4 and Mab 7G5 (Kascsak *et al*, 1993) do not react with PrP from the species in which they were generated (mice). This is also true for Mab F89/160.1.5 (O'Rourke *et al*, 1998) generated in mice to a synthetic peptide representing residues 146–159 of the bovine prion protein. This Mab is reported to react with PrP from only sheep, cattle, mule deer and elk. In addition to Mab F89/160.1.5, three Mabs were produced in mice to hamster PrP by Rogers *et al* (1991). These three Mabs were epitope mapped using recombinant vaccinia virus expressing chimeric PrP; Mab 27-2 is similar to Mab 3F4, Mab 7D4 is similar to Mab 7G5 but may involve asparagine sites at both 155 and 170 of the hamster PrP sequence (Mab 7G5 recognizes asparagine at 170), and Mab 13A5 recognizes a site at 139 involving methionine. Despite the potential for a response to many more sites, Mabs have only been produced to these three sites using mice which express PrP.

The response to both linear and non-linear sites is evident in PrP null mice and hamsters immunized with mouse FA-PrP^{Sc}. Response was monitored in six PrP null mice receiving either mouse, sheep or hamster FA-PrP^{Sc}. While a total of five linear epitopes could be assigned to all of these antibodies, the immunoreactivity of these antisera clearly indicated reactivity to non-linear sequences. The limited number of linear epitopes was surprising since the entire PrP molecule should be seen as foreign by the PrP null mouse. The non-linear sequences defined by these polyclonal antibodies may represent single or multiple sites. For example, sera defined by the epitope DVKMMER will react with certain species which contain this linear sequence (human, mouse, and rat). However, the reactivity of KO2 and KO45 antibodies with PrP isoforms from additional species indicates the presence of non-linear epitopes either defined by the DVKMMER sequence or by another undefined noncontinuous set of amino acids. This is also suggested by the KO5 antibody which contains an epitope to the N-terminal region of PrP. Pepscan analysis indicates reactivity to a site (amino acids 22–30) which would have been removed by the protease treatment of the immunogen. In addition, hamster C7 responded to a linear site identical in mice and hamsters. This may represent a situation in which this site is linear on the formic acid-treated mouse PrP but, in its natural state in the hamster is either hidden or folded.

The generation of responses to both continuous and noncontinuous epitopes is further indicated by Western blot analysis. The comparatively lower antibody reactivity to heterologous antigens may reflect the conformational differences, which are a consequence of protein sequence heterogeneity, resulting in altered antibody binding. Reactivity to both linear and conformational epitopes is indicated in the response of PrP null mice. Antibodies produced in null mice to sheep FA-PrP^{Sc} react with a wide range of mammalian PrP but display a greater immunoreactivity to the autologous immunogen than heterologous PrP antigens.

PrP null mouse KO20 responded to a site, defined by amino acids 148–156, which is similar to the site recognized by Mab 6H4 (Korth *et al*, 1997). Mab 6H4 was generated in null mice receiving recombinant bovine PrP while KO20 received sheep FA-PrP^{Sc}. Mab 6H4 immunoreacts with both PrP isoforms of bovine, sheep, mouse and human. In our hands, hybridomas derived from the fusion of anti-mouse PrP-producing lymphocytes from PrP null mice and murine myeloma cells do not survive (results not shown). It is likely that the antibody reacts with the prion protein present on the surface of the hybridoma cells and interferes with the ability of these cells to function and/or survive. It is unclear as to why the clones that produce Mab 6H4, which immunoreacts with mouse PrP, are able to survive.

The small number of epitopes recognized by the various antibodies described in this report suggests further constraints on the response to PrP. In addition to self-nonself constraints, mechanisms involving conformational differences among species also appear to participate in this immune response. Conformational differences among species appear to foster the recognition of non-linear epitopes. Of particular interest is the epitope DVKMMER (hum, mo, rt) or DIKIMER (ham, rab, shp) since it is highly immunogenic regardless of the antigen source or the animal being immunized. It would appear that conformational differences among PrPs from different species are able to limit and target the immune response to PrP. The polyclonal antibody response to PrP reflects re-

sponses to both linear and conformational sites on the PrP immunogen and is a consequence of both the immunogen source and the species being immunized.

The humoral immune response is one of the primary defenses against invasion by exogenous molecules or micro-organisms. Prions circumvent this process by being composed solely or mainly of host (self) PrP. Animals are naturally tolerant to molecules seen as self and do not mount a humoral response to PrP (Kascsak *et al*, 1985). The immune responses generated in this study employed PrP immunogens which would be viewed as foreign (non-self), i.e., mouse PrP into hamsters, mouse or hamster PrP into rabbits, and various sources of PrP into PrP null mice which view all PrP as foreign. All antibodies generated in this study react with both PrP isoforms and therefore other criteria are needed to distinguish the two isoforms (Bendheim *et al*, 1988). Attempts to generate a specific PrP^{Sc} antibody have been unsuccessful until a recent report by Korth *et al* (1997). This antibody, designated 15B3, was generated against recombinant bovine PrP and may provide insight into conformational and biophysical differences among the PrP isoforms.

The need for the rapid and sensitive diagnosis of prion disease has become more evident with the recent outbreaks in Great Britain. The ever present threat to all parts of the globe cannot be ignored. An understanding of how and why animals respond immunologically to PrP will not only assist in our goal to develop improved diagnostic tools, but also provide further insight into PrP-host interaction and immune surveillance of exogenous PrP. These studies also contribute to our understanding of how the infectious agent is viewed by the invaded host. Understanding of this interaction can help to formulate strategies to modulate or prevent prion-host cell interaction and disease.

Materials and methods

Animals
Balb/CJ and C57BL/6J mice were obtained at 4–6 weeks of age from Jackson Laboratories, Bar Harbor, Maine, USA. Syrian LVG/LAK hamsters were obtained at 4–6 weeks of age from Charles River Laboratories (Wilmington, MA, USA.). New Zealand White (NZW) rabbits at 8–10 weeks of age were obtained from Hazelton Research Products (Denver, PA, USA). PrP null (knockout) mice were the kind gift of Dr Charles Weissman, Zurich, Switzerland and were immunized at 4–6 weeks of age.

Characterization of the rabbit PrP gene
An NZW rabbit brain cDNA library was prepared in λgt10 (Clontech, Palo Alto, CA, USA). Approximately 5×10^5 phage plaques were screened by hybridization to the 470 bp *Nco*I-Sau3AI fragment of plasmid pHaPrP as previously described (Goldmann *et al*, 1990). Following the isolation and amplification of three positive clones, the DNA was purified and digested with Eco RI (Sambrook *et al*, 1989). The 1.8–2.5 kb inserts were subcloned into pBluescript KS^+ vector (Stratagene, La Jolla, CA, USA). Analysis by restriction digestion and cycle sequencing (GIBCO BRL, Gaithersburg, MD, USA) confirmed these to be overlapping clones which contained the sequence for the PrP open reading frame (ORF).

Scrapie strains
The ME7 mouse-adapted scrapie strain was kindly provided by Dr Alan Dickinson (ARC and MRC Neuropathogenesis Unit, Edinburgh, Scotland) and was propagated in C57BL/6J mice. Hamster-adapted scrapie strain 263K was provided by Dr Richard Kimberlin (SARDAS, Edinburgh, Scotland) and propagated in LVG/LAK hamsters. Brains of sheep naturally infected with scrapie were kindly provided by Dr Allen Jenny (National Veterinary Services Laboratory, Ames, IA, USA). Preparation of inoculum, injection, scoring and sacrifice of animals were as previously described (Carp and Callahan, 1981; Carp *et al*, 1990).

Preparation of PrP antigen
PrP^{Sc} was isolated from the brains of clinically affected hamsters or mice and from the brains of scrapie infected sheep by a modification of the method of Hilmert and Diringer (1984) as previously described (Rubenstein *et al*, 1994). The procedure involved detergent extraction in 10% sarcosyl, differential centrifugation, extraction and re-pelleting in 10% NaCl and treatment with proteinase K (PK). The final pellet from 12 g of brain contained 300–500 μg of PrP^{Sc} (Rubenstein *et al*, 1994) as determined by the micro BCA protein assay (Pierce, Rockford, IL, USA). PrP^{Sc} was solubilized by formic acid treatment (Kascsak *et al*, 1987), dried in a speed-vac (Savant Instruments, Holbrook, NY, USA) and dissolved in tris-buffered saline containing 0.1% of the zwitterionic detergent, sulfobetaine (SB) 3–14. All PrP^{Sc} used as immunogen in this study was treated in this manner rendering it protease sensitive.

PrP^{c} was partially purified from uninfected brain material using a modification of the method described by Bendheim *et al* (1988). This method involved treatment with 10% sarcosyl, differential centrifugation, and subcellular fractionation using a discontinuous sucrose gradient. The synaptic plasma membrane fraction was utilized without immunopurification.

Immunization
The purified formic acid-treated PrP^{Sc} (FA-PrP^{Sc}) was emulsified in Hunter's Titer Max (Vaxcel, Norcross, GA, USA) and each animal received 10–20 μg of PrP subcutaneously in multiple sites.

Each animal received 3–4 immunizations at 2- to 3-week intervals. Antibody response was monitored 7–10 days following the third immunization by ELISA and Western blot analysis.

Pepscan
Three complete sets of overlapping 12-mer peptides with sequences based on that of mouse (Locht *et al*, 1986), hamster (Robakis *et al*, 1986), and sheep (Goldmann *et al*, 1990) PrP were synthesized onto polyethylene according to established procedures and tested for binding by antibody in an ELISA-like test as previously described (Geysen *et al*, 1984). Each vertical line on the pepscans represent a 12-mer of the designated PrP sequence from the N-terminus to the C-terminus. The criteria for assigning a site as antigenic was as follows: absorbance value should be at least twice the background and there should be two or more neighboring peptides that reach this value. The background was taken as twice the average absorbance value of 20 consecutive low reacting peptides for which the coefficient of variation (CV) is below 20% of the average value (CV=standard deviation/average $\times$ 100).

ELISA
Indirect ELISA assays were performed as previously described (Kascsak *et al*, 1987). Briefly, FA-PrP^{Sc} was bound to Falcon 96 well ELISA plates (Becton Dickinson Labware, Lincoln Park, NJ, USA) at 1 μg/ml in phosphate-buffered saline (PBS). Following binding of antigen overnight at 4°C, unbound sites were blocked using 10% normal goat sera in PBS containing 2% Tween 20. Primary antibodies were diluted in PBS with 1% normal goat sera and 0.2% Tween 20 and incubated at 37°C for 2 h. Secondary antibodies conjugated with alkaline phosphatase, obtained from either BioSource International (Camarillo, CA, USA) (goat anti-rabbit and goat anti-mouse) or Accurate Chemical and Scientific Corp (Westbury, NY, USA) (rabbit anti-hamster), were added for 1 h at 37°C. Conversion of nitrophenol phosphate was measured at 405 nm employing a 7520 Cambridge Systems ELISA reader (Cambridge Technology, Inc, Cambridge, MA, USA).

Western blot
Electrophoresis and Western blotting of proteins were performed as previously described (Kascsak *et al*, 1987). Briefly, after adding 2% SDS and 0.5% β-mercaptoethanol to the PrP sample (FA-PrP^{Sc}, PK-treated PrP^{Sc}, PrP^{c}), the sample was electrophoresed on 12% Laemmli SDS polyacrylamide gels. Proteins were electrophoretically transferred to Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) and processed for reactivity with the various antisera.

The following antisera used in Table 3 were generous gifts: rabbit anti-hamster PrP^{Sc} from Dr James Hope (Compton Laboratories, Compton, Berkshire, UK) and rabbit anti-chicken synthetic PrP peptide from Dr David Harris (Harris *et al*, 1993). The rabbit antisera to synthetic peptides refer to amino acids 100–111 (R505) and amino acids 223–234 (R524) of the ovine PrP sequence (van Keulen *et al*, 1995). Other antisera in Table 3 refer to reagents analyzed by pepscan and were generated as part of this current study.

Acknowledgments

The authors wish to thank Ms JoAnne Lopez Stocker for her excellent preparation of the manuscript. This work was supported in part by the United States Department of Agriculture (98-9136-0101-CA), the Bayer Corporation, and the New York State Office of Mental Retardation and Developmental Disabilities.

References

Barry RA, Kent SBH, McKinley MP, Meyer RK, DeArmond SJ, Hood LE, Prusiner SB (1986) Scrapie and cellular prion proteins share polypeptide epitopes. *J Infect Dis* **153:** 848–854.

Bendheim P, Barry RA, DeArmond SJ, Stites DP, Prusiner SB (1984). Antibodies to a scrapie prion protein. *Nature (London)* **310:** 418–421.

Bendheim PE, Potempska A, Kascsak RJ, Bolton DC (1988). Purification and partial characterization of the normal cellular homologue of the scarpie agent protein. *J Infect Dis* **158:** 1198–1208.

Billeter M, Riek R, Wider G, Hornemann S, Glockshuber R, Wuthrich K (1997). Prion protein NMR structure and species barrier for prion diseases. *Proc Natl Acad Sci USA* **94:** 7281–7285.

Bradley R (1997) Animal prion diseases. In: *Prion Diseases*. Collinge J, Palmer MS (eds). Oxford University Press: Great Britain, pp 89–129.

Brown P, Cathala F, Raubertas RF, Gajdusek DC, Castaigne P (1987). The epidemiology of Creutzfeldt-Jakob disease: conclusion of a 15-year investigation in France and review of the world literature. *Neurology* **37:** 895–904.

Brown P, Gajdusek DC (1991) The human spongiform encephalopathies: kuru, Creutzfeldt-Jakob disease, and the Gerstmann-Straussler-Scheinker syndrome. In: *Transmissible Spongiform Encephalopathies: Scrapie, BSE and Related Human Disorders.* Chesebro BW (ed). Springer-Verlag: Berlin, pp 1–20.

Carp RI, Callahan SM (1981). In vitro interaction of scrapie agent and mouse peritoneal macrophages. *Intervirology* **16:** 8–13.

Carp RI, Kim YS, Callahan SM (1990). Pancreatic lesions and hypoglycemia-hyperinsulinemia in scrapie-infected hamsters. *J Infect Dis* **161:** 462–466.

Caughey B, Race RE, Ernst D, Buchmeier MJ, Chesebro B (1989). Prion protein (PrP) biosynthesis in scrapie-infected and uninfected neuroblastoma cells. *J Virol* **63:** 175–181.

Collinge J, Palmer MS (1997) Human prion diseases. In: *Prion Diseases.* Collinge J, Palmer MS (eds). Oxford University Press: Great Britain, pp 18–56.

Farquhar CF, Somerville RA, Ritchie LA (1989). Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *J Virol Met* **24:** 215–222.

Gabizon R, McKinley MP, Prusiner SB (1987) Purified prion proteins and scrapie infectivity copartition into lysosomes. *Proc Natl Acad Sci USA* **84:** 4017–4021.

Geysen HM, Meloen RH, Barteling SJ (1984) Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proc Natl Acad Sci USA* **81:** 3998–4002.

Geysen HM, Barteling SJ, Meloen RH (1985). Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein. *Proc Natl Acad Sci USA* **82:** 178–182.

Geysen HM, Rodda SJ, Mason TJ, Tribbick G, Schoofs PG (1987). Strategies for epitope analysis using peptide synthesis. *J Immunol Methods* **102:** 259–274.

Gibbs Jr CJ, Gajdusek DC, Amyx H (1979). Strain variation in the viruses of Creutzfeldt-Jakob disease and kuru. In: *Slow Transmissible Diseases of the Nervous System,* Vol. 2. Prusiner SB, Hadlow WJ (eds). Academic Press: New York, pp 87–110.

Goldmann W, Hunter N, Foster JD, Salbaum JM, Beyreuther K, Hope J (1990). Two alleles of a neural protein gene linked to scrapie in sheep. *Proc Natl Acad Sci USA* **87:** 2476–2480.

Harris DA, Huber MT, van Dijken P, Shyng S-L, Chait BT, Wang R (1993). Processing of a cellular prion protein: identification of N- and C-terminal cleavage sites. *Biochemistry* **32:** 1009–1016.

Hilmert H, Diringer H (1984). A rapid and efficient method to enrich SAF-protein from scrapie brains of hamsters. *Biosci Rep* **2:** 165–170.

Kascsak RJ, Rubenstein R, Merz PA, Carp RI, Wisniewski HM, Diringer H (1985). Biochemical differences among scrapie-associated fibrils support the biological diversity of scrapie agents. *J Gen Virol* **66:** 1715–1722.

Kascsak RJ, Rubenstein R, Merz PA, Tonna-DeMasi M, Fersko R, Carp RI, Wisniewski HM, Diringer H (1987). Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. *J Virol* **61:** 3688–3693.

Kascsak RJ, Tonna-DeMasi M, Fersko R, Rubenstein R, Carp RI, Powers JM (1993). The role of antibodies to PrP in the diagnosis of transmissible spongiform encephalopathies. In: *Transmissible Spongiform Encephalopathies-Impact on Animal and Human Health.* Brown F (ed). Dev. Biol. Stand., Vol. 80, Basel, Karger, pp 141–151.

Korth C, Stierli B, Streit P, Moser M, Schaller O, Fischer R, Schulz-Schaeffer W, Kretzschmar H, Raeber A, Braun U, Ehrensperger F, Hornemann S, Glockshuber R, Riek R, Billeter M, Wuthrich K, Oesch B (1997) Prion (PrP^{Sc})-specific epitope defined by a monoclonal antibody. *Nature* **390:** 74–77.

Locht C, Chesebro B, Race R, Keith JM (1986) Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent. *Proc Natl Acad Sci USA* **83:** 6372–6376.

Loftus B, Rogers M (1997). Characterization of the prion protein (PrP) gene from rabbit; a species with apparent resistance to infection by prions. *Gene* **184:** 215–219.

O'Rourke KI, Baszler TV, Miller JM, Spraker TR, Sadler-Riggleman I, Knowles DP (1998). Monoclonal antibody F89/160.1.5 defines a conserved epitope on the ruminant prion protein. *J Clin Microbiol* **36:** 1750–1755.

Pan K-M, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Flettrick RJ, Cohen FE, Prusiner SB (1993). Conversion of α-helices into β-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci USA* **90:** 10962–10966.

Pocchiari M (1994). Prions and related neurological diseases. In: *Molecular Aspects of Medicine,* Vol. 15, Baum H, Azzi A, Jordan VC, Ozawa T, Rice-Evans C (eds). Elsevier Science Ltd: Great Britain, pp 195–291.

Priola SA, Chesebro B (1995). A single hamster PrP amino acid blocks conversion to protease-resistant PrP in scrapie-infected mouse neuroblastoma cells. *J Virol* **69:** 7754–7758.

Prusiner SB (1991). Molecular biology of prion diseases. *Science* **252:** 1515–1522.

Prusiner SB (1997). Prion diseases and the BSE crisis. *Science* **278:** 245–251.

Prusiner SB, Bolton D, Grot DF, Bowman KA, Cochran SP, McKinley MP (1982). Further purification and characterization of scrapie prions. *Biochemistry* **21:** 6942–6950.

Prusiner SB, Scott M, Foster D, Pan K-M, Groth D, Mirenda C, Torchia M, Yang S-L, Serban D, Carlson GA, Hoppe PC, Westaway D, DeArmond SJ (1990). Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* **63:** 673–686.

Raymond GJ, Hope J, Kocisko DA, Priola SA, Raymond LD, Bossers A, Ironside J, Will RG, Chen SG, Petersen RB, Gambetti P, Rubenstein R, Smits MA, Lansbury Jr PT, Caughey B (1997). Molecular assessment of the potential transmissibilities of BSE and scrapie to humans. *Nature* **388:** 285–288.

Riek R, Hornemann S, Wider G, Glockshuber R, Wuthrich K (1997). NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231). *FEBS Letts* **413:** 282–288.

Robakis NK, Sawh R, Wolfe GC, Rubenstein R, Carp RI, Innis MA (1986). Isolation of a cDNA clone encoding the leader peptide of prion protein and expression of the homologous gene in various tissues. *Proc Natl Acad Sci USA* **83:** 6377–6381.

Rogers M, Serban D, Gyrus T, Scott M, Torchia T, Prusiner SB (1991). Epitope mapping of the syrian hamster prion protein utilizing chimeric and mutant genes in a vaccinia virus expression system. *J Immunol* **147:** 3568–3574.

Rogers M, Yehiely F, Scott M, Prusiner SB (1993). Conversion of truncated and elongated prion proteins into the scrapie isoform in cultured cells. *Proc Natl Acad Sci USA* **90:** 3182–3186.

Rubenstein R, Carp RI, Ju W, Scalici C, Papini M, Rubenstein A, Kascsak R (1994). Concentration and distribution of infectivity and PrP^{Sc} following partial denaturation of a mouse-adapted and a hamster-adapted scrapie strain. *Arch Virol* **139:** 301–311.

Sambrook J, Fritsch EF, Maniatis T (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Serban D, Taraboulos A, DeArmond SJ, Prusiner SB (1990). Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins. *Neurology* **40:** 110–117.

Stahl N, Borchelt DR, Hsiao K, Prusiner SB (1987). Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell* **51:** 229–240.

van Keulen LJ, Schreuder BE, Meloen R, Poelen van den Berg M, Mooij-Harkes G, Vromans ME, Langeveld JP (1995). Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. *Vet Pathol* **32:** 299–308.

Wilesmith JW, Wells GAH, Cranwell MP, Ryan JBM (1988). Bovine spongiform encephalopathy: epidemiological studies. *Vet Rec* **123:** 638–644.