Prion Protein Peptides as Vaccines

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Abstract: Investigations into the immunogenicity of the prion protein are ongoing. To combat its pathological isoform without affecting the cellular protein is a challenge in prion research. We here summarize the studies in which prion protein peptides have been used for immunization, to thus determine the most immunogenic parts of the prion protein.

Key Words: Prion protein, peptides, epitope prediction, immune response, vaccine.

1. INTRODUCTION

The misfolded isoform of the cellular prion protein (PrP^C), designated as PrP^{Sc} (scrapie), is the sole cause of the inevitably lethal, transmissible spongiform encephalopathies (TSEs). This prion protein was first introduced to the scientific community in 1982 by Stanley B. Prusiner, who was later awarded the Nobel Prize in Medicine for his 'protein-only' hypothesis. According to this widely accepted hypothesis, the infectious isoform, PrP^{Sc}, has the same primary structure, but an increased β-sheet content, in comparison with PrP^C, and it can catalyze the conversion of the normal (self-) PrP^C into the misfolded PrP^{Sc}. This misfolded protein can then form insoluble aggregates in the brain, which are resistant to detergents and proteases. To date, the structure of PrP^{Sc} has not yet been resolved, despite many and persistent efforts [1].

PrP^C has a molecular weight of 35-36 kDa, and it is a sialoglycoprotein that is expressed in a variety of cell types. In human, the primary translation product consists of 253 amino acids. Residues 1-22 constitute a very hydrophobic signal sequence that is normally cleaved before PrP^C reaches the cell surface. Residues 231-253 are cleaved prior to the addition of the glycosylphosphatidylinositol (GPI) anchor that localizes PrP^C to functionally specialized membrane domains [2]. These so-called lipid rafts have been suggested to be critical for PrP^C conversion into the misfolded PrP^{Sc} and for PrP^{Sc} infection [2, 3]. Of the remaining residues of PrP^C, amino acids 23-125 form an unfolded, highly dynamic domain, while residues 126-230 form the C-terminal part. This latter is folded into a series of three α-helices and a small two-stranded β sheet, which are stabilized by a disulphide bond linking the Cys 179 and Cys 214 residues [4]. Apart from the GPI-anchor attachment, posttranslational modifications can include the addition of oligosaccharide chains at amino acids Asn 180 and/or Asn 197 [5]. The structure of PrP^C is schematically presented in Fig. (**1**).

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PrP^C is expressed not only on cells of neuronal tissues, but also on those of the immune system, including primary and secondary lymphoid organs. Repression of this glycoprotein during lymphocyte differentiation has been reported to have an influence on positive and negative lymphocyte selection, and therefore on the establishment of immune tolerance, which leads to the deletion of prion-specific lymphocytes in the periphery [6, 7]. As a result of self-tolerance to PrP^C, prion-specific T-cells are deleted in the thymus or are rendered anergic, which leads to peripheral tolerance. An impact of PrP^C on the immune system processes is indicated by an activation-induced up-regulation of PrP^C on mouse and human lymphocytes [8, 9] and its accumulation on dendritic cells (DCs) at peptide-dependent T-cell–DC interaction sites [10]. The role of PrP^C release into the extracellular milieu and its possible conversion into PrP^{Sc} remains to be established [11]. In prion diseases described in human and in animals, the immune system is not normally activated, due to the similarity between the pathological and the normal cellular prion proteins [12]. Furthermore, it has been suggested that T-cells of the natural and the acquired immune system, including macrophages, DCs and lymphocytes, are involved in prion uptake, and its accumulation and transport from the periphery to the central nervous system. However, how crucial these roles are still remains a matter of discussion [12]. Nevertheless, it has been shown that defense mechanisms against the infectious agent can be activated experimentally [13-16]. It is a challenge to explore the conditions under which the immune system might act against PrP^{Sc} instead of supporting its spread.

In general, B-lymphocytes produce immunoglobulins that specifically recognize conformational or linear epitopes, or peptide fragments of proteins. The range of amino acids in the epitopes that contribute to antibody–antigen binding can vary from four to more than 20 [17]. T-cell activation, however, requires peptide presentation on the highly polymorphic major histocompatibility complex (MHC) molecules, which in human correspond to the human leukocyte antigens (HLA), by antigen-presenting cells (i.e. macrophages, DCs and B-cells). For binding to MHC I or MHC II, peptides of 8-10 amino acids and 12-28 amino acids are optimal, respectively. Activation of MHC/ peptide-specific CD8+ (MHC I)

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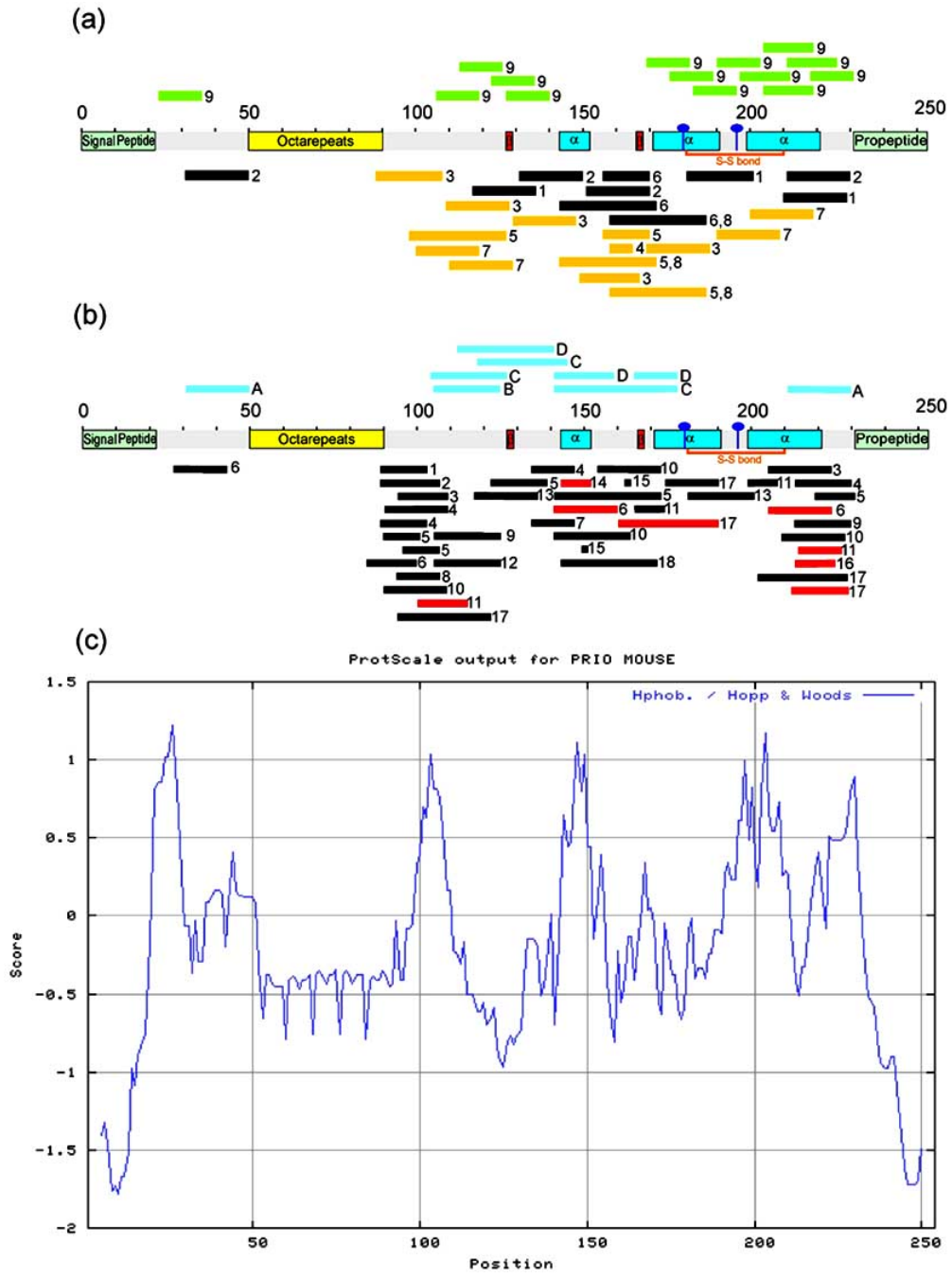

Fig. (1). Immunogenic prion peptides from the different species plotted according to their positions in PrP^{C}, which is shown schematically. (**a**) Only the most immunogenic peptides listed in Table **1** are depicted. T-cell stimulatory prion peptides determined in wild-type mouse (black), the *Prnp*$^{0/0}$ mouse (yellow), and human *in-vitro* (green) models; (**b**) B-cell stimulatory prion peptides raising polyclonal sera titers of 10^3-10^5 (black), $>10^5$ (red), and PrP peptides used for active immunizations (blue). Peptides numbering corresponds to studies described in Table 2 and Table 3 (peptides denoted with letters). (**c**) Hopp-Woods hydrophilicity plot of the mouse PrP sequence (UniProtKB/Swiss-Prot P04925), obtained using the ProtScale tool (http://expasy.org/tools/protscale.html). The hydrophilic parts of the protein are plotted above zero. The amino acid positions in PrP correspond to those on Fig. **(1a, b)**.

and CD4+ (MHC II) T-cells induces cytotoxic and helper functions, respectively. For T-cell stimulation, it is believed that only the primary, and not the secondary, structure is crucial for immunogenicity. In addition, when administered alone, B-cell responses to peptides are weak and require T-cell help for optimal antibody production. Helper T-cells secrete cytokines and provide co-stimulatory signals, which induce B-cell proliferation, immunoglobulin switching, and antibody secretion. During this process, antibody-secreting plasma cells and memory B-cells are produced. Correspondingly, memory T-cells develop from a pool of naïve T-lymphocytes that have weak effector functions, but that maintain some of their naïve properties. These memory B-cells and T-cells induce a more rapid immune response upon secondary infection or vaccine boost.

To overcome self-tolerance and induce an immune response against PrP, one of the strategies has been the immunization of the *Prnp* knock-out mouse (*Prnp*$^{0/0}$). These mice do not express PrP^{C} on their cell surfaces, and they have therefore been used as an experimental model for the lack of immune tolerance. They have been successfully used for the production of PrP-specific monoclonal antibodies and for the study of T-cell responses, despite controversial data regarding the effectiveness of their immune system (see [18] for review).

To date, no efficient treatments for TSEs are available. Passive or active vaccination (e.g. of livestock) would represent a desirable solution. With the goal of active vaccine development in mind, the immune response has to be studied in wild-type animals. It has been difficult, although not impossible [19, 20], to obtain immune responses upon immunization with the whole PrP. However, in contrast to vaccination with the whole recombinant PrP (recPrP) or with isolated PrP^{Sc} fibrils, PrP peptides (linked to a carrier molecule) are better immunogens and are safer to handle [21].

Here, we summarize a considerable number of studies in which prion peptides have been used as antigens to stimulate humoral and/or cellular immune responses in laboratory animals and/or in *in-vitro* models. Our aim is to determine whether specific parts of PrP are better targets than others for the design of peptide-based vaccines against TSE. The amino acid numbering of all of the peptides cited below corresponds to mouse PrP, to avoid any confusion (see Tables **1**-**3**).

2. T-CELL RESPONSES TO PRION PROTEIN PEPTIDES

Cellular immunogenicity to peptides from rat, sheep, mouse and human PrP has been examined in nine different studies [22-30]. In general, lymphocytes from previously immunized mice were isolated and restimulated *in vitro*, with their peptide-specific T-cell responses determined. Proteins or peptides used for immunization are designated as such in Table **1**. From more than 90 different peptides tested, only 48 were successful in stimulating T-cells (Table **1**). The most immunogenic ones among these are depicted in Fig. (**1a**), according to their position in PrP.

2.1. Prediction of T-Cell Epitopes

The application of the whole PrP elicits an immune response against all of the available HLA/MHC molecules, whereas each peptide corresponds to mostly one epitope. The core sequence of the peptide that interacts with the HLA II binding cleft is nine amino acids long and allows a flexible number of flanking peptide residues. The traditional way to scan the whole antigen sequence is to synthesize overlapping peptide fragments and to assess their immunogenicity. To save time and costs, modern vaccine development strategies use various computer algorithms as HLA–peptide binding-prediction tools, which are based on existing binding data [31].

In the studies listed in Table **1**, four out of nine groups selected their prion peptides for immunization and/or restimulation using relevant binding-prediction models to preferably work with potent immunogens [22-25]. In particular, one study used a human 7-mer peptide that was predicted to bind to MHC I in mice [22]. In two other studies, 20-mer peptides were selected for investigations in rat and mice [23, 24]. One group used human 15-mer prion peptides that were predicted to bind up to 25 HLA-DR molecules, offering a wide cross-selection of human HLA class II specificities [25]. These peptides provided higher T-cell responses than randomly chosen peptides in the animal models [24]. In the human *in-vitro* system, however, eight out of 10 HLA II binding peptides induced cellular immune responses, although proliferation was not significantly higher compared to the unselected control peptides [25]. We can conclude from these studies that a particular peptide that is selected according to an appropriate binding-prediction tool does not necessarily increase the possibility of obtaining positive results.

2.2. Major T-Cell Epitopes of the Prion Protein

Three main experimental animal and human models have been described: I) autologous, i.e. peptide and wild-type animal of the same origin; II) PrP^{C} knock-out, i.e. the *Prnp*$^{0/0}$ mouse that lacks PrP^{C} expression; and III) the human *in-vitro* model.

I) The Autologous Model

Autologous conditions have been set up in various wild-type mouse strains and in a rat system (Table **1**). Due to self-tolerance, no immunogenicity of PrP-derived fragments was expected in this animal system. Interestingly, in all of these studies, peptides from the C-terminal PrP^{C} region (amino acids 131-230) induced T-cell responses; in general, the N-terminal part of PrP^{C} was not immunogenic. The same was explored when the peptides were used in the presence of the adjuvant CpG-oligodeoxynucleotides (CpG), as a toll-like receptor 9 (TLR9) agonist [26, 27]. Surprisingly, none of these mouse models showed autoimmune reactions [24, 26, 27]. The reason for this could be that the C-terminal region of PrP^{C} is under-represented in the proteosome digest, according to findings in the human system [32]. Therefore, self-tolerance to peptides derived from this part of PrP^{C} might be insufficient, and T-cell anergy can be overcome upon activation [25]. To date, the rat model provides the only report where immunization with prion peptides has led to severe skin inflammation, in 15%-25% of elderly rats. The delay in these symptoms is not characteristic of autoimmune diseases [23].

II) The PrP^{C} Knock-Out Model

Sheep-, human- and mouse-derived peptides have been investigated under PrP^{C}-free conditions. The immunogenic peptides covered the region of amino acids 109-209 of PrP^{C}, and the non-responding peptides were derived from the dynamic N-terminal part (Table **1**).

The exchange of only one amino acid in the peptide can lead to a different presentation on MHC and consequently induce immune responses [33]. Therefore, PrP peptides derived from other species are processed and presented in a distinct way to the mouse MHC molecules. In particular, six out of 22 sheep-derived PrP peptides were immunogenic in

Table 1. Cellular Immunity to Prion Peptides from Different Species

Peptide Position on PrP[1]	Nu[2]	Peptide Origin	Animal(s) for Immunization/ Human *In-Vitro* System	No. of Amino Acid Differences[3]	Binding Prediction	T Cell Proliferation[4]	Protein/Peptide Used for Immunization [5]	[Ref]
117-136	1	Rat	Lewis rats	0	MHC II	SI: 2	ra118-137	[23]
181-201				0	MHC II	SI: 78	ra182-202	
210-229				0	MHC II	SI: 22	ra211-230	
131-150	2	Mouse	NOD mice	0	MHC II	SI > 5	mo131-150	[24]
182-202				0	--	SI: 2	mo182-202	
211-230				0	MHC II	SI > 5	mo211-230	
31-50			C57BL/6 mice	0	--	SI: 6	mo31-50	
131-150				0	--	SI: 11	mo131-150	
151-170				0	--	SI: 4	mo151-170	
211-230				0	--	SI: 25	mo211-230	
131-150			A/J mice	0	--	SI weak	mo131-150	
211-230				0	--	SI strong	mo211-230	
88-108	3	Sheep	*Prnp*$^{0/0}$ mice	4	--	SI low	shPrP	[29]
109-128				0	--	SI high	mPrP	
129-148				2	--	SI low	shPrP	
149-168				0	--	SI low	shPrP	
149-168				0	--	SI high	mPrP	
169-188				1	--	SI middle	shPrP	
129-148			C57BL/6 mice	2	--	SI middle	shPrP	
158-165	4	Human	*Prnp*$^{0/0}$ mice	1	MHC I	SI > 10	hu159-166KLH	[22]
158-165				1	MHC I	SI > 10	plasmid (huPrP)	
98-127	5	Mouse	*Prnp*$^{0/0}$ mice	0	--	SI: 1.88	PrP pDNA	[28]
98-127				0	--	SI low	mo98-127	
143-172				0	--	SI: 3.86	PrP pDNA	
143-172				0	--	SI middle	mo143-172	
156-170				0	--	SI: 4.27	mo143-172	
156-170				0	--	SI: 9.47	mo158-187	
158-187				0	--	SI: 4.03	PrP pDNA	
158-187				0	--	SI middle	mo158-187	
143-172	6	Mouse	C57BL/6 mice	0	--	F: 67	mo143-172/CpG	[26]
156-170				0	--	F: 60	mo143-172/CpG	
156-170				0	--	F: 55	mo158-187/CpG	
158-187				0	--	F: 223	mo158-187/CpG	
90-109	7	Human	*Prnp*$^{0/0}$ mice	2	--	SI < 2	I: α- or β-PrP	[30]
100-119				2	--	SI ≤ 2	I: α- or β-PrP	
110-129				1	--	SI ≥ 2	I: α- or β-PrP	
150-169				3	--	SI < 2	I: α- or β-PrP	
160-179				2	--	SI < 2	I: α- or β-PrP	
170-189				0	--	SI < 2	I: α- or β-PrP	
190-209				0	--	SI ≥ 2	I: α- or β-PrP	
200-219				0	--	SI ≤ 2	I: α-PrP	
210-230				6	--	SI < 2	I: α- or β-PrP	

(Table 1. Contd….)

Peptide Position on PrP[1]	Nu[2]	Peptide Origin	Animal(s) for Immunization/ Human *In-Vitro* System	No. of Amino Acid Differences[3]	Binding Prediction	T Cell Proliferation[4]	Protein/Peptide Used for Immunization [5]	[Ref]
143-172	8	Mouse	*Prnp*$^{0/0}$ mice	0	--	F: 97	mo143-172/ CpG	[27]
158-187				0	--	F: 306	mo158-187/CpG	
158-187			C57/BL6 mice	0	--	F: 201	mo158-187/CpG	
23-36	9	Human	healthy donors (*in-vitro*)	0	--	SI > 2	P: hu23-36	[25]
106-119				2	HLA II	SI > 2	P: hu107-120	
113-126				0	--	SI > 2	P: hu114-127	
120-133				0	HLA II	SI > 2	P: hu121-134	
127-140				1	HLA II	SI > 2	P: hu128-141	
169-182				0	HLA II	SI > 2	P: hu170-183	
176-189				0	--	SI > 2	P: hu177-190	
183-196				0	--	SI > 2	P: hu184-197	
190-203				0	--	SI > 2	P: hu191-204	
197-212				0	HLA II	SI > 2	P: hu198-213	
204-219				3	--	SI > 2	P: hu205-220	
211-226				4	--	SI > 2	P: hu212-227	
218-231				4	--	SI > 2	P: hu219-231	

[1]Numbering of peptides corresponds to mouse PrP; peptides used for restimulation *in vitro*; [2]Numbering depicted in Fig. **(1a)**; [3]Substitutions or deletions compared to the corresponding mouse peptide; [4]SI: Stimulation Index, F: Frequency of IFN-γ^+ T cells/10^6 splenocytes; SI were taken as such from their respective articles and values ≥2 were considered as positive. When values were not specified, the extent of T-cell proliferation was indicated as low, middle or high according to their description. [5]P: in the human *in vitro* system peptides were used for priming only.

the *Prnp*$^{0/0}$ mouse, whereas only one of them induced a T-cell response in wild-type mice under the same conditions. Interestingly, sheep peptides with no amino acid differences to their mouse analog provided the best T-cell stimulation [29].

The stimulatory capacity of mouse-derived prion peptides was investigated in the presence or absence of CpG. In both studies, the most immunogenic peptide consisted of amino acids 158-187 [27, 28]. Two different vaccination approaches were used to study human-derived prion peptides. Immunizations performed with a prion peptide linked to keyhole limpet hemocyanin (KLH; 159-166KLH) induced high antigen-specific T-cell responses [22]. T-cells from mice immunized with rα-PrP and rβ-PrP recognized peptide residues 110-129 and 190-209 [30].

KLH is a large protein, which is known to contain various antigenic structures; therefore, it elicits strong, non-specific, immune responses. Both KLH and CpG enhance the preservation of antigen-specific T-cells. As described in wild-type mice, it was mainly peptides from the C-terminal region that activated the cellular immune system, which indicates that the N-terminus has only a few immunogenic epitopes. As expected, no side effects have been detected in the *Prnp*$^{0/0}$ mouse [22, 27-30].

III) The Human In-Vitro *Model*

For human PrP studies, an *in-vitro* system provides the only possible approach. In non-immunized individuals, the prion peptides that can stimulate specific T-cells out of a large pool of lymphocytes can be determined. To date, there has been only one study that has investigated the human T-cell tolerance in an autologous *in-vitro* system. For this, peripheral blood mononuclear cells were isolated from whole blood and cultured in the presence of prion peptides, and their antigen-specific proliferative responses were determined. The immunogenic peptides of human PrP^C lie mainly in the central and C-terminal parts of the molecule, i.e. between amino acid residues 106-140 and 169-231. Further studies are required to support and extend these findings [25].

3. B-CELL RESPONSES TO PRION PROTEIN PEPTIDES

Humoral immune responses to more than 80 different PrP peptides have been tested across 24 studies [23, 24, 26, 27, 34-55]. The most successful immunizations are summarized in Table **2**. The peptides were derived from mouse, hamster, rat, cow, pig and human PrPs. The immunized animals were predominantly rabbits and mice, but in some studies chickens, goats and sheep were also used for immunization.

3.1. Prediction of B-Cell Epitopes

Although a few B-cell epitope prediction algorithms are available, the use of such algorithms for the choice of peptides was not a part of even the most recent studies reviewed here.

The majority of B-cell epitope-prediction algorithms are based on theoretical characteristics that have been deduced from the amino acid sequence (i.e. hydrophilicity, flexibility,

Table 2. Humoral Immunity to Prion Peptides from Different Species

Peptide Position on PrP[1]	Nu[2]	Peptide Origin	Animal(s) for Immunization	No. of Amino Acid Differences[3]	Carrier System	Titer Against Peptide[4]	Reactivity with				[Ref]
							Denatured[5]		Native[6]		
							PrP^C	PrP^{Sc}	PrP^C	PrP^{Sc}	
89-103	1	Mouse	Rabbit	3	OA	10^5	/	+	/		[34]
89-107	2	Mouse	Rabbit	3	/	10^5	/	+	/		[35]
94-109	3	Cattle	Rabbit	3	KLH	10^5	/	+	/		[36]
		Sheep		4			/	+			
		Mouse		2			/	+			
205-224		Mouse		3			/	+/-			
89-103	4	Mouse	Sheep	3	OA	/	+	/	/		[37]
90-109		Cattle	Rabbit	3			/	/			
			Mouse	3			/	/			
134-147		Cattle	Rabbit	1			+	/			
			Mouse	3			+	/			
213-230		Cattle	Rabbit	3			+	/			
			Mouse	6			+	/			
90-101	5	Sheep	Rabbit	3	KLH	/	/	+	/		[38]
96-107				3			/	+			
122-139				0			/	+			
141-173				0			/	+			
219-231				4			/	+			
27-43	6	Sheep	Mouse	1	KLH	$\geq 10^4$	+	+	/	/	[39]
85-100				4		$\geq 10^4$	+	+	+	/	
141-160				1		$\geq 10^5$	+	+	+	/	
205-224				2		$>10^5$	+	+	+/-	/	
134-147	7	Cattle	Mouse	2	KLH	/	+ *	+ *	/		[40]
94-107	8	Human	Rabbit	3	MAP	10^5	/	+	/		[41]
105-125	9	Human	Mouse	2	KLH	/	/	+	/		[42]
213-230				6			/	+			
90-109	10	Cattle	Rabbit	3	BSA	10^5	+	+	/		[43]
141-164				2		10^4-10^5	+	+			
154-173				2		10^4-10^5	+	+			
209-228				2		10^4-10^5	+	+			
100-115	11	Mouse	Rabbit	2	MAP	10^5	/	+	/		[44]
100-115		Hamster		2		10^6	/	+			
165-174		Mouse		1		10^4	/	+			
199-208		Mouse		2		10^5	/	+			
213-226		Mouse		4		10^6	/	+			
105-125	12	Human	Mouse	2	KLH	/	+	/	+	+	[45]
117-136	13	Rat	Rat	0	/	Low	/				[23]
181-201						High	Possible autoimmune side effects observed, but not proven.				

(Table 2. Contd….)

Peptide Position on PrP[1]	Nu[2]	Peptide Origin	Animal(s) for Immunization	No. of Amino Acid Differences[3]	Carrier System	Titer Against Peptide[4]	Reactivity with				[Ref]
							Denatured[5]		Native[6]		
							PrP^C	PrP^{Sc}	PrP^C	PrP^{Sc}	
143-152	14	Human	Mouse	1	MAP	10^5 –10^6	+	/	+	/	[46]
149-151	15	Any	Rabbit	0	KLH	/	/		+	+	[47]
162-164		Mammal	Goat						+	+	
213-225	16	Human	Mouse	3	KLH	10^6	-	+	-	+	[48]
94-122	17	Cattle	Rabbit	3	/	10^5	+ *	+ *	/		[49]
160-190				2		10^6	+ *	- *			
174-190				1		10^4	+ *	/			
202-229				5		10^4-10^5	+ *	- *			
212-229				3		10^5-10^6	+ *	- *			
143-151	18	Mouse/	Rabbit	1	Papilloma virus	10^2-10^3	/		+	/	[50]
		Rat	Rat	0		10^2-10^3			-	/	

[1]Numbering of peptides corresponds to mouse PrP; [2]Numbering depicted in Fig. **(1b)**; [3]Substitutions or deletions compared to the corresponding mouse peptide; [4]Titers were determined by ELISA in studies 2-18 and by dot blotting in study 1; [5]Denaturating techniques used for PrP detection: immunohistochemistry and/or Western blotting; [6]Native techniques used for PrP detection: immunoprecipitation, ELISA, native Western blotting and/or dot blotting; OA: ovalbumin; KLH: keyhole limpet hemocyanin; MAP: multiple antigenic peptide systems; BSA: bovine serum albumin; *Reactivity with recPrP.

accessibility) and from the PrP secondary structure (i.e. turns) [56-59]. The usefulness of these tools was severely criticized by Blythe and Flower [60], who performed a comparative study that showed that the ability to predict immunogenic peptides by methods based on sequence analysis is no better than random. They further stated that their findings were not surprising, due to the complexity of antibody-antigen interactions.

Recently, B-cell epitope prediction algorithms that also take into account the three-dimensional structure of a protein have been described [61-65]. Once the structure of the pathological isoform, PrP^{Sc}, is resolved, these algorithms can potentially be used for the prediction of epitopes specifically exposed in PrP^{Sc}.

3.2. Carrier Systems

To prolong their exposure to the immune system and to enhance helper T-cell activation, peptides are usually administered linked to a carrier molecule. The majority of studies have used KLH as the carrier protein as it is a potent immunogen. Our own observations have led us to believe that the molar ratio between peptide and carrier has to be high enough to ensure a good anti-peptide response. However, its undeterminable molecular weight complicates the assessment of this ratio [66]. In certain cases it may be difficult to tell whether the reason for the low anti-peptide response is a sub-optimal conjugation of the peptide to KLH, or indeed the lower immunogenicity of a certain peptide.

Multiple antigen peptide (MAP) systems that comprise an oligomeric branching lysine core to which the peptides are bound have been shown to efficiently stimulate anti-peptide immune responses [41, 44, 46]. In comparison with big proteinaceous carriers, the MAP system offers the advantage of a low anti-carrier response, which is required when the goal is active vaccine development.

3.3. Major B-Cell Epitopes of the Prion Protein

In theory, longer peptides are more likely to assume a conformation that is similar to that of the original protein, while short peptides (less than 20 amino acids) tend to be more mobile and hence able to adopt a variety of conformations [67]. Therefore, when immunization is performed with protein peptides that are exogenous to the immunized animal, it is reasonable to choose longer peptides. However, if the peptide belongs to a self or self-similar protein, the strategy of choosing longer peptides would be more likely to result in unresponsiveness of the immune system due to B-cell tolerance. Indeed, in the studies reviewed, the majority of the peptides that induced efficient anti-PrP responses were composed of 20 amino acids or less. When immunizing with longer PrP peptides, or even with the whole PrP, the immune response will depend more on structural differences between the 'donor' and the 'host' PrPs [19].

In Fig. (**1b**), the peptides are plotted according to their positions in PrP^C. Interestingly, the majority of the immunogenic peptides that have been shown to cross-react with the whole molecule of PrP in at least one immunological method fall into one of three regions of PrP: amino acids 90-120, 140-170 or 200-230.

Although titres of polyclonal sera against peptides are arbitrary data, it is outstanding that four out of the eleven peptides that form the C-terminal cluster raised titres of about 10^6. This high immunogenicity of the C-terminus did not depend on the origin of the peptide used (human, cattle, sheep or mouse), the immunized animal (mouse, rabbit), or the carrier system. In specific studies, the peptides were administered alone [49], linked to KLH [39, 48], or incorporated into a MAP system [44].

With two exceptions [39, 24], there have been no reports of successful immunization with peptides from the N-

Table 3. Active Immunizations of Mice and Hamsters with PrP Peptides

Peptide Position on PrP[1]	Nu[2]	Peptide Origin	Animal(s) for Immunization	No. of Amino Acid Differences[3]	Carrier System	Titer Against Peptide	Protective Effect	[Ref]
31-50 131-150 151-170 211-230	A	Mouse	Mouse	0	/	High Low Low High	Reduction of PrP^{Sc}, but not PrP^{C} in neuroblastoma tumors. No autoimmune side effects.	[24]
105-125	B	Mouse	Mouse	0	KLH	10^3	Prolongation of survival of immunized mice. No autoimmune side effects.	[51]
104-127 118-145 141-178	C	Hamster	Hamster	0	KLH	+ + +	Some prolongation of survival.	[52]
112-141 141-159 165-178	D	Hamster	Mouse	1 0 1	BCP	10^4 10^4 10^4	Some prolongation of survival.	[53]

[1]Numbering of peptides corresponds to mouse PrP; [2]Numbering depicted in Fig. **(1b)**; [3]Substitutions or deletions compared to the corresponding mouse peptide; KLH: keyhole limpet hemocyanin; BCP: blue carrier protein; "/", not done.

terminal part of PrP^{C}. Whether the reason for this is indeed the lower immunogenicity of the N-terminus or merely the lack of immunizations performed with peptides from this part of PrP is difficult to determine, due to the tendency against the reporting of negative results. However, in the study by Gregoire *et al*. [27], upon immunization with 13 overlapping peptides (covering the whole PrP sequence), the immune responses of BALB/c mice were compared to the immune responses of $Prnp^{0/0}$ mice, which are not tolerant to PrP. While in this *Prnp* knock-out mouse the N-terminal part of PrP was immunogenic, there was no B-cell response to the peptides chosen from the PrP sequences N-terminal of amino acid 98 in the wild-type mice. More data supporting the non-immunogenicity of the N-terminus were reported by the same group after their immunization of $Prnp^{0/0}$ and wild-type mice with the PrP-pDNA, corresponding to the same PrP peptides: while in the $Prnp^{0/0}$ mouse the highest responses were seen for the peptides that cover the sequence from 23-112 of mouse PrP, in wild-type mice there were no responses to any of these 13 peptides.

3.4. Anti-Peptide Antibodies Cross-Reactivities with the Whole PrP^{C} Molecule

The peptides that elicited antibodies that were cross-reactive with the whole PrP molecule corresponded in general to the hydrophilic parts of PrP (Fig. (**1b, c**)). In all of the PrP isoforms, the highly hydrophobic parts tend to 'hide' from the hydrophilic environment, which makes the access of antibodies to these epitopes less likely.

The majority of the immunizations that have been reported resulted in antibodies that were not only reactive to the administered peptide, but also to the whole PrP molecule. However, the reactivity of these antibodies against the whole PrP molecule was normally restricted to non-self conformations[1] of PrP, i.e. to native or denatured PrP^{Sc} [24, 34-45, 47, 48], to denatured PrP^{C} [21, 37, 39, 43, 45, 46], to recombinant PrP [40, 46, 49] and to native PrP^{C} of other species [39, 45, 46], and depended on the plasticity of the corresponding protein region (Table **2**). These antibodies did not react (or were not tested) with native self PrP^{C}, except in one study, where a faint reaction with native self PrP^{C} was detected [50]. In accordance with these observations no autoimmune reactions were proven. B-cell tolerance is oriented against self-conformations, rather than against self-sequences; therefore, peptides that differ from the 'host' sequence in a single amino acid can also elicit strong anti-peptide responses [39, 46]. It is therefore not surprising that a higher number of amino-acid differences between the 'donor' and the 'host' peptide sequences did not necessarily correlate with a higher anti-peptide titre, nor with a higher degree of cross-reactivity with the whole PrP (Table **2**). Also, the majority of the polyclonal and monoclonal antibodies were not species specific; however, some were more promiscuous in their specificity than others, depending on the plasticity of the epitope.

3.5. Active Immunization with PrP Peptides

Recently, four studies have reported on the active immunization of mice and hamsters with PrP peptides prior to infection with PrP^{Sc} (Table **3**). Although the polyclonal serum titres were not very high (10^3-10^4) or not explicitly determined, all of the immunizations resulted in a short prolongation of survival [51-53] or in a reduction of PrP^{Sc} in neuro-

[1]We use the term 'non-self conformations' for all of the conformations of the PrP that arise during the disease or that are artificially produced during manipulation of the PrP outside the human/animal body. These conformations were not present in the organism when the immune tolerance was being formed.

blastoma tumors induced in mice by subcutaneous injection of PrP^{Sc}-infected neuroblastoma cells [24]. With better antibody titers, a more efficient prevention of the disease would be expected. It has been recently demonstrated that the efficiency of disease prevention depends on the anti-PrP antibody titer [20]. Autoimmune side effects in immunized animals were not seen in any of the active immunization studies, which strengthens our hypothesis that normally only antibodies against non-self conformations of PrP peptides are recruited. The way in which these antibodies work has not been analyzed. Some of them were probably directed against conformations present in PrP^{Sc}, thus slowing the spread of the infectious agent. Therefore, there is hope that immunizations with peptides that provoke high and preferably native PrP^{Sc}-directed immune responses will also provide better protection from the disease.

CONCLUSIONS

The aim of peptide vaccine development is the identification of immunogenic peptides that can induce both T-cell and B-cell immune responses. To avoid the complex problem of self-tolerance, B-cell and T-cell epitopes of the PrP have been studied in the *Prnp* knock-out mouse models. The findings that PrP^{C} is involved in immune functions [10] limit the assessment of prion peptide immunogenicity. Therefore, the results obtained in the *Prnp* knock-out mouse, which are often contradictory, should be interpreted carefully. Studies from wild-type mice have contributed more reliable data.

When the first prion peptide immunizations were performed, exogenous peptides were preferably used, probably due to the assumption that self-peptides would not stimulate an efficient response, or worse that the response would be of an autoimmune nature (Table **2**). Later, it became clear that self-peptides are equally immunogenic and that immune tolerance mechanisms effectively prevent autoimmune reactions by restricting activated B-cell clones only to those reactive with non-self conformations of PrP [21, 27]. While the conformation can be crucial for B-cell tolerance, T-cell tolerance is thought to be based on primary structure alone. Nevertheless, it has been suggested that peptides that can form α-helical structures in solution are often good T-cell stimulators, meaning that peptide secondary structure can also have a role in T-cell responses [68].

Interestingly, even self-peptides that efficiently stimulated T-cells did not provoke any autoimmune reactions. A plausible explanation for this is that upon administration of peptide antigens in the classical way, CD4+ T-cells are stimulated, while the activation of CD8+ T-cells that could result in cytotoxic reactions is normally not triggered. However, for the administration of peptides by means that promote CD8+ T-cell activation (e.g. peptide expressed on viral particles [50]), more caution needs to be taken. To address the issue of T-cell autoimmunity to PrP peptides properly, more investigations remain to be performed, and in particular, more attention needs to be given to the type of T-cells activated.

The application of peptide-prediction tools was assumed to facilitate the search for the optimal T-cell and B-cell epitopes. However, whether such prediction algorithms indeed offer any advantages for vaccine design is not yet clear, since their use has been considered only in a few of the PrP peptide immunization studies.

Since the N-terminus appears to contribute to the conversion from PrP^{C} to PrP^{Sc} [2, 3, 69], peptides from this part of PrP represent an interesting target for vaccine development. However, from our comparative study, we conclude that immunogenic PrP peptides that elicit antibodies that cross-react with the whole PrP arise from the central and C-terminal parts of PrP. More precisely, they tend to fall into one of the three amino acid clusters: 90-120, 140-170 or 200-230. These regions correspond in general to the hydrophilic parts of PrP, while a very hydrophilic part located in the N-terminus (amino acids 20-50) does not appear to be immunogenic. Interestingly, T-cell epitopes that should not be influenced by hydrophilicity are also located mostly in the central and C-terminal parts of PrP. A possible explanation for the low immunogenicity of the N-terminus is a better digestion and presentation of this highly mobile part during lymphocyte development, which results in more complete immune tolerance.

Successful passive immunizations against TSE with anti-PrP antibodies have already been reported [70]. Based on the studies reviewed here, we can conclude that active vaccination with PrP could offer a solution for the currently incurable TSEs. It is important, however, that the peptides are carefully chosen on the basis of their immunogenicity as well as their ability to mimic PrP^{Sc} conformational epitopes. The application of various epitopes represented by a mixture of PrP peptides or by a multivalent vaccine should also be considered in future studies.

ABBREVIATIONS

BCP	=	Blue carrier protein
BSA	=	Bovine serum albumin
CpG	=	CpG-oligodeoxynucleotides
DCs	=	Dendritic cells
HLA	=	Human leukocyte antigen
KLH	=	Keyhole limpet hemocyanin
MAP	=	Multiple antigenic peptides system
MHC	=	Major histocompatibility complex
OA	=	Ovalbumin
Prnp	=	Prion protein gene
PrP^{C}	=	Cellular prion protein
PrP^{Sc}	=	Pathological isoform of prion protein (scrapie prion protein)
recPrP	=	Recombinant prion protein
TLR	=	Toll-like receptor
TSE	=	Transmissible spongiform encephalopathy

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