Modulation of proteinase-K resistant prion protein by prion peptide immunization

Lina Souan[1], Yuval Tal[2], Yacov Felling[3], Irun R. Cohen[1], Albert Taraboulos[2] and Felix Mor[1]

[1] Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel
[2] Department of Molecular Biology, Hebrew University, Hadassah Medical School, Jerusalem, Israel
[3] Department of Pathology, Hadassah University Hospital, Jerusalem, Israel

Prion diseases are caused by conformational alterations in the prion protein (PrP). The immune system has been assumed to be non-responsive to the self-prion protein, therefore, PrP autoimmunity has not been investigated. Here, we immunized various strains of mice with PrP peptides, some selected to fit the MHC class II-peptide binding motif. We found that specific PrP peptides elicited strong immune responses in NOD, C57BL/6 and A/J mice. To test the functional effect of this immunization, we examined the expression of proteinase-K-resistant PrP by a scrapie-infected tumor transplanted to immunized syngeneic A/J mice. PrP peptide vaccination did not affect the growth of the infected tumor transplant, but significantly reduced the level of protease-resistant PrP. Our results demonstrate that self-PrP peptides are immunogenic in mice and suggest that this immune response might affect PrP-scrapie levels in certain conditions.

Received	4/2/01
Revised	4/4/01
Accepted	15/5/01

Key words: T cell / Neuroblastoma / Antibody

1 Introduction

The transmissible spongiform encephalopathies (TSE) such as scrapie of sheep and Creutzfeldt-Jakob disease of humans are caused by prions [1, 2]. The prion protein can assume two distinct isoforms: the normal prion protein (PrP^{C}) and its pathological isoform (PrP^{Sc}) [3–5]. PrP^{Sc}, which is the only known component of the infectious prion [6, 7], is formed post-translationally in the host cell [4, 8], probably by the refolding of normal PrP^{C} into a β-sheet-rich abnormal conformation [9–14]. Although the two PrP isoforms appear to be identical in amino acid sequence [15], their biophysical properties are very different: PrP^{C} is readily soluble in most detergents and is completely degraded by proteinase K, whereas PrP^{Sc} is insoluble in detergents and possesses a protease resistant core called PrP 27–30 [16, 17].

[I 21723]

L.S. and Y.T. equally contributed to this study.

Abbreviations: BCA: Bicinchoninic acid **PrP:** Prion protein **PrP^{C}:** The normal isoform of the prion protein **PrP^{Sc}:** The scrapie PrP isoform **hsp-60:** Heat shock protein 60 **TSE:** Transmissible spongiform encephalopathies

An important question is whether autoimmunity might play some role in prion disease. However, it is generally believed that immune reactions are not likely to be involved in prion disease; inflammation has not been reported in scrapie [18]. Indeed, PrP knockout mice were used to produce monoclonal antibodies to mouse PrP [19]. However, the apparent lack of specific immune reactions to prions does not mean that the immune system is not involved in the natural history of these diseases. It has recently been reported that B cells and dendritic cells are involved in the transport of prions from peripheral sites to the central nervous system [20, 21]. In addition, refined techniques have demonstrated the activation of microglia and the infiltration of T cells in the central nervous system (CNS) before the onset of clinical signs in mouse scrapie [22]. A recent study showed that macrophages can delay scrapie by sequestering and preventing PrP^{Sc} from reaching the spleen, which is a peripheral site for scrapie replication [23]. The question remains, however, whether an immune response can be raised against self-PrP protein, or its peptides.

In this report, we describe the induction of immunity to PrP peptides in three strains of mice: NOD, C57BL/6 and A/J. The choice of peptide used for immunization of NOD mice was guided by our characterization of the peptide-binding motif of the NOD MHC class II binding groove [24, 25]. We also studied C57BL/6 mice, because these

0014-2980/01/0808-2338$17.50+.50/0

mice have been used in the best-described models for scrapie, and A/J mice, which provide a novel model for studying PrP in a scrapie-infected neuroblastoma (N2a; see below). In these mice, we used a library of overlapping 20-mer peptides that span the mouse PrP sequence.

The N2a neuroblastoma is useful for studying some aspects of prions *in vivo* (Y. Tal, unpublished data). The neuroblastoma line N2a, which is a sub-clone of the C1300 neuroblastoma that arose spontaneously in the spinal cord of an A/J mouse in 1940, has been passaged *in vitro* since 1967 [26]. N2a cells, as well as their scrapie-infected counterparts ScN2a [3], grow rapidly when inoculated into A/J mice and give rise to large subcutaneous tumors (up to several grams) within 3 weeks of inoculation. The ScN2a tumors produce high amounts of protease-resistant PrP that express the biochemical characteristics of PrP^{Sc}. Here we studied the effect of immunizing A/J mice with self-PrP peptides on the growth and PrP content of these tumors.

2 Results

2.1 PrP peptides are immunogenic for NOD mice

By visual inspection, we searched the mouse prion sequence for peptides that contain the NOD ($I\text{-}A^{g7}$) MHC class II binding motif [24, 25], and found two peptides: p131–150, and p211–230 (Table 1). We immunized NOD mice with these two peptides, in addition to three other PrP peptides that do not contain a NOD motif: p31–50, p182–202, and p118–137.

To assay the T cell response, popliteal lymph nodes (LN) were removed 10 days after immunization, and the cells were tested in a standard proliferation assay against the immunizing peptide. We found that p31–50 and p118–137 did not induce any detectable T cell response (data not shown), while p182–202 elicited a stimulation index (SI) of only 2. In contrast, both p131–150 and p211–230 induced strong T cell responses (SI $>$ 5). T cell lines specific for p131–150 and p211–230 were generated by repeated cycles of stimulation with the immunizing peptide. Fig. 1 shows the proliferation profiles of these lines at the fourth *in vitro* stimulation in response to various concentrations of the immunizing peptide. The lines did not respond to other peptides (data not shown).

2.2 PrP peptides are immunogenic for C57BL/6 mice

Having found that self-PrP peptides are immunogenic in autoimmune prone NOD mice, we turned to C57BL/6 mice, which are not unusually prone to autoimmune disease. Because the MHC class II motif of the C57BL/6 strain is not well characterized, we tested overlapping 20-mer peptides spanning the mouse PrP sequence, and some non-overlapping PrP sequences that were available (see Table 2). Fifty micrograms of each peptide were emulsified in CFA and injected in the hind footpads of C57BL/6 mice.

Table 1. The sequences of mouse prion peptides containing the NOD (IA^{g7}) MHC class II binding motif [24, 25][a)]

Prion peptide	Amino acid sequence		
		MHC anchor positions	
		1 2 3 **4** 5 **6** 7 8 **9**	
p131-150	SAMSRP	**M** I H F G **N** D W **E**	DRYYR
p211-230	QMCVTQY	**Q** K E S Q **A** Y Y **D**	GRRS

a) Peptides are aligned according to the proposed anchor positions (bold).

Ten days after injection, LN cells were examined for their proliferative responses to the immunizing peptides. The most immunogenic peptides in C57BL/6 mice were p131–150 and p211–230, followed by p31–50 and p151–170. Proliferation assays of the various lines at the second stimulation cycle showed the following stimulation indexes: p31–50 = 6; p151–170 = 4; p131–150 = 11;

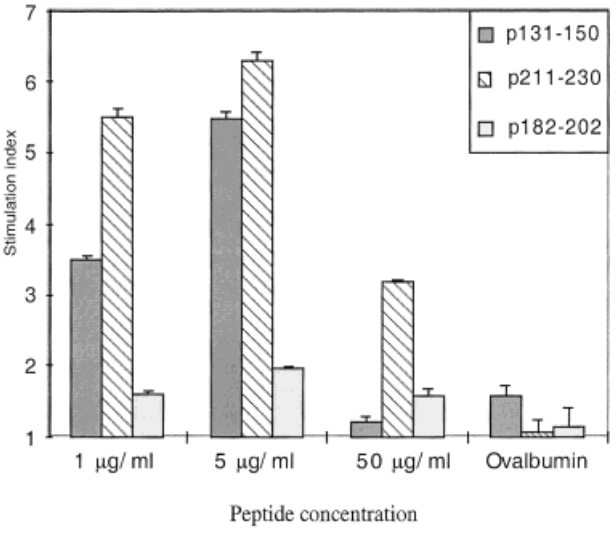

Fig. 1. NOD T cells proliferate to PrP peptides. NOD mice were immunized, and the proliferative responses of their T cell lines to p131-150, p211-230 and p182-202 were examined after the fourth *in vitro* stimulation. Proliferation is shown as the stimulation index+SD.

Table 2. The sequences of the prion peptides used in this study

PrP peptide	Sequence
p11-30	L F V T M W T D V G L C K K R P K P G G
p31-50	W N T G G S R Y P G Q G S P G G N R Y P
p51-70	P Q G G T W G Q P H G G G W G Q P H G G
p71-90	S W G Q P H G G S W G Q P H G G G W G Q
p91-110	G G G T H N Q W N K P S K P K T N L K H
p118-137	A G A V V G G L G G Y M L G S A M S R P
p121-140	V G G L G G Y M L G S A M S R P M I H F
p131-150	S A M S R P M I H F G N D W E D R Y Y R
p151-170	E N M Y R Y P N Q V Y Y R P V D Q Y S N
p182-202	I T I K Q H T F T T T T K G E N F T E T D
p191-210	T T K G E N F T E T D V K M M E R V V E
p211-230	Q M C V T Q Y Q K E S Q A Y Y D G R R S

p211–230 = 25. Other peptides, p11–30, p51–70, p71–90, p91–110, p121–140, p191–210, p182–202 and p118–137 did not induce any detectable T cell proliferation (data not shown). T cell lines to p131–150 and p211–230 were generated, and Fig. 2 shows their proliferative responses during the sixth *in vitro* stimulation cycle to the respective peptide and to the control antigen ovalbumin. Thus, the same PrP peptides were strongly immunogenic in both NOD and in C57BL/6 mice.

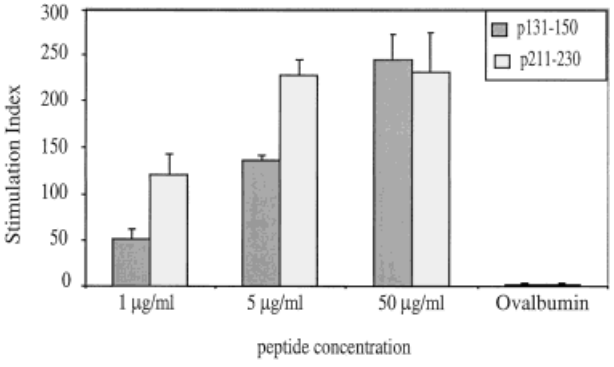

Fig. 2. C57BL/6 T cells proliferate to PrP peptides. Proliferative response of C57BL/6 T cell lines to p131-150, and p211-230 after the sixth stimulation cycle *in vitro*, as described in the legend to Fig. 1.

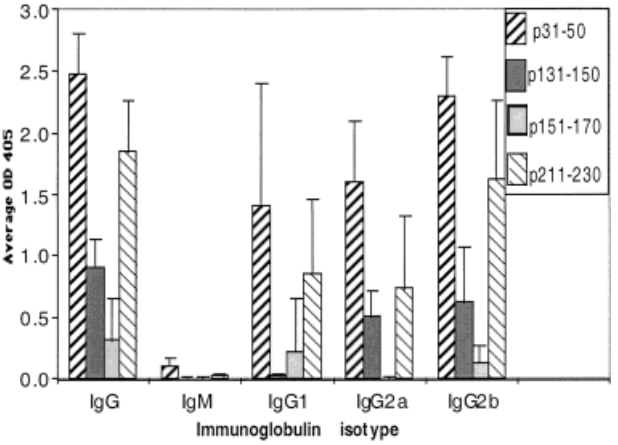

Fig. 3. The antibody response to PrP peptides in C57BL/6 mice. Mice were immunized and serum antibodies (at a 1:100 dilution) were analyzed for binding to PrP peptides in a standard ELISA assay. Immunoglobulin isotypes were measured using isotype specific secondary antibodies. The bars indicate the mean OD 405+SD.

2.3 Antibodies to prion peptides

We measured peptide-specific antibodies developing 2 months after the immunization of C57BL/6 mice. Mice injected with p31–50 and p211–230 produced the highest antibody levels (both IgG2a and IgG2b), followed by p131–150 and p151–170 (Fig. 3). The antibodies were specific for the immunizing peptide measured using a panel of PrP peptides (data not shown).

2.4 PrP peptides are immunogenic for A/J mice

Both the N2a neuroblastoma line, which expresses cell surface PrP^{C} [27], and their scrapie-infected analog, ScN2a, which in addition to PrP^{C} produces large amounts of PrP^{Sc} [3], can grow in syngeneic A/J mice as subcutaneous tumors (Tal et al. unpublished results). Our finding that self-PrP peptides induced both B and T cell immune responses raised the question of whether such autoimmunization could affect either the growth of the tumors or their expression of PrP. In these experiments, we used the stably transfected populations N2a-C10 and ScN2a-C10, which overexpress a chimeric PrP that is recognized by the mAb 3F4.

To investigate these questions, we first studied the immune response of A/J mice to PrP peptides. Groups of A/J mice were immunized with p31–50, p131–150, p151–170, p211–230, p182–202, or p118–137 peptides (Table 2) in CFA. Of these peptides, p211–230 was again the strongest T cell immunogen, whereas p131–150 elic-

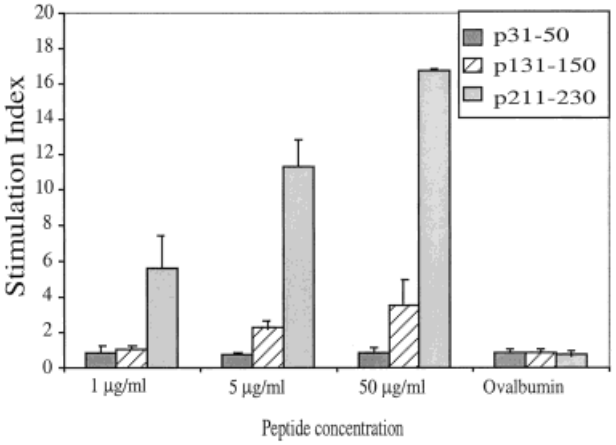

Fig. 4. A/J T cells proliferate to PrP peptides. Proliferative responses of A/J T cell lines to p131-150, or p211-230 after the second stimulation cycle *in vitro*, as described in the legend to Fig. 1.

ited a weak T cell response. The other peptides elicited no detectable T cell response (data not shown). We generated T cell lines, and Fig. 4 shows the proliferation profiles following the second *in vitro* stimulation with the respective peptides. Fig. 5 shows that the immunized mice produced specific antibodies against both of these peptides and not to other PrP peptides (data not shown). However, despite the T cell and antibody responses, there was no detectable effect noted on the growth of the N2a-C10 or ScN2a-C10 tumors (data not shown). Moreover, histological examination showed no morphological differences between the tumors in the immunized or the control mice (data not shown).

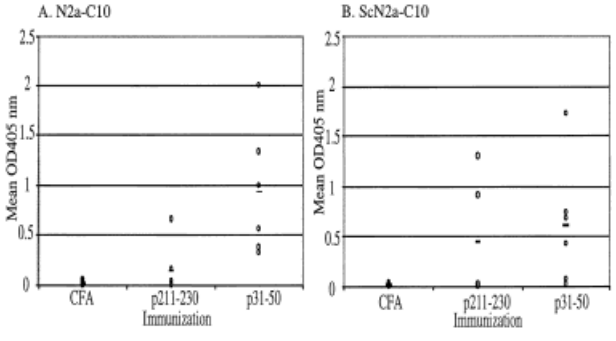

Fig. 5. Quantification of the total IgG antibody produced in A/J mice immunized with p31-50 or p211-230 or CFA. A/J mice were immunized with the PrP peptide on day 0 and boosted on day 10, and then injected with the tumor N2a-C10 tumor (panel A) or with the ScN2a-C10 tumor (panel B) on day11.

2.5 Immunization to PrP peptides reduces the expression of protease-resistant PrP in tumors

We assessed the influence of the immunization with self-PrP peptides on the amounts of the PrP isoforms in the tumors. The amount of PrP^C in the non-infected N2a-C10 tumors was not affected by immunization with the PrP peptides (Fig. 6, A). However, the proteinase K-resistant PrP content in the ScN2a-C10 (clone 4) tumors was markedly reduced in the mice immunized with p31–50 (about 60% reduction) and in those immunized with p211–230 (70–80% reduction), compared to control animals that were immunized with CFA alone (Fig. 6, B). No apparent change was observed on the total content of normal PrP of the ScN2a-C10 tumors (data not shown). Similar results were obtained in repeated experiments using several different sections of each tumor (data not shown).

Experiments were also done using a different clone of ScN2a-C10, clone 21,which expresses about fivefold more PrP^{Sc} than does clone 4. We found that this high-expressor clone showed a more modest reduction (~20%) in protease-resistant PrP in the mice immunized with the PrP peptides compared to controls (data not shown). This suggests that a high load of PrP^{Sc} in clone

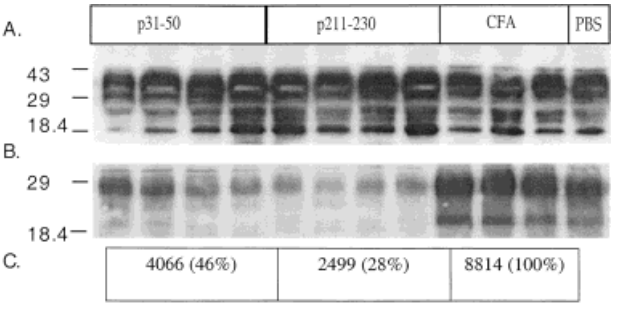

Fig. 6. Reduction in protease-resistant PrP in peptide-immunized mice. From each frozen tumor, a sample of about 100 mg was lysed and prepared as decribed in Sect. 4, and 15 μl from the lysate solution was loaded on 12% polyacrylamide-SDS gels. Samples from ScN2a-C10 tumors were treated with proteinase K prior to loading on the gels. The gels were then blotted on PVDF membranes, and both PrP^C and PrP^{Sc} were detected using rabbit PrP antiserum RO73. Band intensities were further measured using a Umax Astra 1220S scanner, and densitometry was analyzed using the Tina 2.1 Raytest program. (A) The PrP^C content in the N2a-C10 tumor. The PrP^C was completely degraded by proteinase K (data not shown). (B) The protease resistant PrP content in the ScN2a-C10 tumor. (C) The mean band intensities in (B) expressed in percents of the intensities in the CFA control group.

21 of ScN2a-C10 may affect the immune-mediated reduction in protease-resistant PrP in these experiments.

3 Discussion

Much work on the prion protein has focused on the molecular pathogenesis of TSE [28–35]. Studies on the role of the immune system in prion disease have been directed to the contribution of various cellular components to the peripheral replication of the abnormal prion protein and its transport to the CNS [21, 36]. These studies concluded that T cells do not participate in the transport of prions to the CNS, while both B cells and follicular dendritic cells are critical for this process [21, 36, 37]. To our knowledge, a specific immune response to the self-prion protein or to its peptides has not been reported previously.

We show here that self-PrP peptides can elicit both T- and B cell responses in various mouse strains. We found that p131–150 and p211–230 elicited a considerable T cell response in the three mouse strains examined, NOD (Fig. 1), C57BL/6 (Fig. 2), and A/J (Fig. 4). Although these peptides were chosen because they were predicted to fit the MHC-II (IA^{g7}) NOD binding motif, it is interesting that they were immunogenic in the two other strains as well.

While the experiments involving NOD and A/J mice were terminated relatively quickly, the immunized C57BL/6 mice were followed for up to 9 months. During this period, we were unable to detect any clinical abnormalities in the immunized animals.

To gain some insight into the possible effects of prion auto-immunization on the expression of PrP^{Sc}, we used a novel model of a scrapie infected A/J neuroblastoma tumor line. Although this model is far removed from the natural history of scrapie, it does provide a relatively convenient and expeditious survey procedure. Moreover, the tumor model might be useful for analyzing peripheral effects that could be masked in the CNS. The classical *in vivo* models of scrapie infectivity require several hundred days to obtain results.

Using this model, we found that the autoimmune response to PrP was associated with a reduced level of protease-resistant PrP in scrapie-infected tumors; the level of proteinase K-resistant PrP was decreased by ~60% in the p31–50 immunized group, while the reduction in the p211–230 immunized group was even higher (70–80%), compared to the control immunized mice (Fig. 6). Interestingly, this reduction was not accompanied by any detectable change in PrP^{C}, or in the growth of the tumors. A similar analysis of proteinase K resistant PrP protein was done on lysates from the spleens of the tumor bearing mice, but the splenic material was negative in all groups of mice (data not shown). It remains to be seen whether the protease K-resistant PrP might spread from the tumor to the lymphoid organs at a later stage. In any case, it would now be justified to undertake the more lengthy work with experimental scrapie.

The mechanism of protease-resistant PrP down-regulation in the tumors remains to be elucidated. That p211–230, which is a strong T cell immunogen, reduced protease-resistant PrP better than did p31–50 suggests that specific T cells or antibodies may be involved. However, while histological examination clearly demonstrated some scattered lymphocytes in sections of the tumors (staining with anti-CD3; data not shown), neither the number of the infiltrating lymphocytes nor their distribution seemed to be altered in the samples of tumors taken from the immunized animals. Further work will be needed to determine if specific anti-PrP lymphocytes are among the cells that infiltrate the tumors in PrP-peptide immunized animals, and whether the affect on PrP expression can be adoptively transferred by lymphocytes. We attempted to detect antibodies binding *in situ* in the tumors of the unimmunized and control animals by immunocytochemistry. However, this could not be done technically because the tumors appeared to bind large amounts of immunoglobulins nonspecifically in both the immunized and control animals. Further work needs to be done to elucidate the mechanisms of the immune effect.

Recent work on a mouse model of Alzheimer's disease has shown that immunization with a beta-amyloid peptide can prevent the accumulation of amyloid in the brain [38, 39]. Amyloid formation is also part of the pathogenic mechanism in prion diseases [40], and our work suggests that the immune responses to prion peptides should be investigated for their possible influence on amyloid formation in prion diseases.

In summary, we demonstrate here that immunizing mice with self-prion peptides induces both T- and B cell immune responses, and that two of the PrP peptides (p131–150 and p211–230) are immunogenic in several strains of mice regardless of their MHC genotype. This immune response to prion peptides was associated with a significant decrease in the load of protease-resistant PrP produced in an infected tumor. These results suggest that it would be important to study the possible effects of PrP peptide immunization on the evolution of prion disease.

4 Materials and methods

4.1 Animals

Inbred female NOD ($H2^{g7}$) and C57BL/6 ($H2^{b}$), were supplied by Harlan laboratories, Israel. Male A/J ($H2^{a}$) mice were supplied from Jackson Laboratories, Bar Harbor, ME.

4.2 Cell culture

Cell culture reagents were purchased from Biological Industries (Bet Haemek, Israel) or from Bio-Lab (Jerusalem, Israel). Tissue culture plates were obtained from Miniplast (Ein Shemer, Israel) or Nunc (Denmark). Mouse neuroblastoma ScN2a cells were obtained as described [3]. N2a-C10 and ScN2a-C10 cells [41] stably express MHM2-PrP[42], which is a mouse-Syrian hamster chimeric PrP. ScN2a-C10 cells were further cloned to improve their PrP^{Sc} production. Two clones of ScN2a-C10 (clones 4 and clone 21) were used in this research. Cells were grown at 37°C in DMEM-16 (low glucose) supplemented with 10% fetal calf serum with 1% penicillin /streptomycin and 2% L-glutamine.

4.3 Peptides

We synthesized a library of 20-mer peptides that span the mouse prion protein sequence and overlap in 10 amino acids p1–10 to p231–254, as well as two additional peptides p182–202 and p118–137. Table 2 shows the sequence of these peptides. Peptides were synthesized using Fmoc (N-a-flourenylmethoxycarbonyl) chemistry, using the AMS422 automated synthesizer (ABIMED, Langenfeld, Germany) as described [43]. Peptides were examined for purity by HPLC and their composition was confirmed by amino acid analysis. For NOD immunization, we searched the mouse prion sequence for MHC class II ($I\text{-}A^{g7}$) motifs [24, 25] and found two peptides that contained an $I\text{-}A^{g7}$ anchoring motif, p131–150 and p211–230, Table 1. For the C57BL/6 and A/J mice, we used several peptides from our peptide library.

4.4 Antibodies

Rabbit anti-mouse R073 binds to both mouse PrP and MHM2-PrP [5, 44]. Antibodies were used at a dilution of 1:5,000 of the serum or the ascites fluid. Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

4.5 Immunizations

To assay immune responses, peptides were dissolved in PBS (1 mg/ml) and mixed with CFA in a 1:1 (v/v) ratio. For studies that did not involve subsequent tumor inoculation, 8-week-old female mice were immunized once by injecting 50 μl of the peptide emulsion in each hind footpad (each animal was thus injected with 50 μg of peptide). For the tumor studies in A/J male mice, 8-week-old animals were boosted as described in Sect 4.9. Complete Freund's adjuvant (CFA) was prepared by mixing incomplete adjuvant (IFA) with 4 mg/ml of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI).

4.6 T cell lines

Draining popliteal lymph node (LN) cells were removed on day 10 after immunization, and single-cell suspension was prepared by pressing the organs through a fine wire mesh. To establish antigen-specific T cell lines, LN cells were stimulated with the immunizing peptide (5 μg/ml) for 3 days in stimulation medium: DMEM supplemented with β-mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 u/ml), streptomycin (100 μg/ml), nonessential amino acids (1 ml/100 ml), and autologous serum, 1% (v/v) [45]. Following stimulation, the T cell blasts were seeded in propagation medium (identical to stimulation medium without autologous serum, but supplemented with 10% fetal calf serum), and 10% (v/v) supernatant of Con A-stimulated spleen cells (containing T cell growth factors [45]). Five days after seeding, the cells (5×10^{5}/ml) were re-stimulated with the peptide (5 μg/ml) and irradiated splenocytes as antigen presenting cells (5×10^{6}/ml) for 3 days in stimulation medium. Lines were expanded by repeated stimulation with peptides and irradiated splenocytes as antigen-presenting cells every 10–12 days [46], for the number of cycles indicated for each result. The cells were analyzed for their specificity to immunizing peptides in a proliferation assay at each stimulation cycle.

4.7 T cell proliferation

T cell proliferation assays were performed by mixing 5×10^{4} cells (from each line) with irradiated (2,500 rad) splenocytes as antigen-presenting cells (2.5×10^{6}/ml) in stimulation medium. The cells were incubated for 3 days at 37°C in humidified air containing 7% CO_2 in the presence of 1 μg/ml, 5 μg/ml, or 50 μg/ml peptide, or either 10 μg/ml ovalbumin or 1.25 μg/ml Con A as negative and positive controls, respectively. The assay was performed in 96-microtiter wells ($Nunclon^{TM\text{-}Delta}$-surface, Denmark). Each well was pulsed with 1 μCi of [^{3}H]thymidine (5.0 Ci/mmol, Amersham, UK) for the final 18 h. The cultures were then harvested using a MicroMate 96 Cell Harvester and the cpm were determined using a Matrix 96 Direct Beta Counter using avalanche gas (98.7% helium; 1.3% C_4H_{10}) ionization detectors (Packard Instrument Company, Meriden, CT).

4.8 Determination of PrP-peptide antibodies

C57BL/6 and A/J mice, which were immunized with either PrP peptides in CFA or control immunized with CFA alone, were bled and sera were collected. An ELISA assay for anti-PrP peptide antibodies was performed using a standard

protocol [47]. Briefly, 10 μg/ml of the peptide was used to coat the wells of the ELISA plate in a coating buffer consisting of 0.05 M $NaHCO_3$, pH 9.6. The plates were incubated overnight at 4°C. Next day, the wells were blocked with 7% milk for 2 h at 37°C, then washed four times with PBS/0.05% Tween 20. Dilutions of the sera were made at 1:10, 1:100, 1:1,000 in 1% milk/PBS/0.05% Tween 20 (diluting solution) and the sera were then incubated overnight at 4°C. Next day, the plates were washed four times and then incubated for 2 h at 37°C with 1:1,000 anti-mouse IgG-alkaline phosphatase-conjugated antibody. Thereafter, the plates were washed five times, and developed with phosphatase substrate tablets (p-nitrophenyl phosphate, disodium, 5 mg per tablet plus filler, Sigma, MI, USA). The plates were read using an *anthos htII* ELISA-plate reader at 405 nm.

4.9 Immunization and tumor growth in A/J mice

Naive A/J mice were immunized in both hind footpads with 50 μl of p31–50 or p211–230 prion peptides (Table 2) emulsified in CFA, as described in Sect. 4.5. Ten days later, the mice were boosted subcutaneously with the same peptide in CFA. On the next day, the animals were inoculated with the neuroblastoma cells, as follows: The cells utilized for inoculation were grown to confluency in 10 cm petri dishes. One or two days prior to their inoculation, samples of these cells were analyzed by Western blotting, and the presence of protease-resistant PrP^{Sc} was confirmed. Immediately before their inoculation, the cells were rinsed three times with ice-cold sterile saline, and then scraped in 1 ml saline. Cell suspensions from all the plates were pooled prior to their inoculation. Mice were briefly sedated with halothane, and 400 μl of the suspension (containing 5×10^6 cells) were then injected subcutaneously in the upper back area. The animals were monitored daily for the appearance of tumors. On days 12, 17, and 20 after the inoculation of the cells, the dimensions of the tumors were measured with a caliper and recorded. The cross sections of the tumor were calculated by multiplying their length by their width. Tumors usually appeared between days 9 and 12-post inoculation. In most cases, they grew steadily to a size of up to 2–3 grams on day 21. In some mice (about 10–20% of the animals), the tumors involuted spontaneously. These involutions occurred whether or not the animals had been immunized.

The tumor experiments were terminated on day 33 (21–22 days after tumor inoculation). The mice were anesthetized with ethyl ether, bled, and then sacrificed. Tumors were then removed and weighed. About one third of each tumor was transferred to tubes containing buffered formalin (4% formaldehyde in PBS) for histological examination (data not shown). The remaining portion was frozen in liquid nitrogen, and then stored at –20°C. The frozen samples were used for biochemical analyses.

4.10 PrP analysis

Lysates from the tumors were prepared as follows: tissue pieces weighing approximately 100 mg were cut from frozen tumors and homogenized at a concentration of 10% w/v in "standard" lysis buffer (0.5% Triton X-100, 0.5% Na-deoxycholate, 150 mM NaCl, 10 mM Tris-Cl, pH 7.5, 10 mM EDTA): For this purpose, the tissue was first incubated in lysis buffer on ice for 30 min. It was then homogenized in a 1 ml dounce homogenizer equipped with a tight fitting glass pestle (Wheaton, Millville, NJ), followed by sonication in a bath sonicator (Ultra Turrax P8, IKA, Labortechnik, Germany) three times for 20-s periods, and then incubated again for 30 min on ice. Insoluble debris were removed by a 30-s spin in an Eppendorf microfuge at 14,000 rpm, at room temperature. The supernatant was then transferred to a fresh tube and the protein concentration was determined using the BCA method (Pierce, Rockford, IL). Tumor lysates were normalized for their protein content prior to Western analysis.

4.11 Immunoblot analysis

The PrP isoforms were characterized and separated as described [17]**.** To digest away the PrP^C while enriching the lysates for PrP^{Sc}, the lysates were incubated for 2 h with 20 μg/ml proteinase K (F. Hoffmann-La Roche Ltd Basel, Switzerland) at 37°C. The reaction was stopped by cooling the samples on ice and adding PMSF to 2 mM for 30 min. To study the total levels of PrP in a sample, proteolysis was omitted. SDS-PAGE and Western blot analysis of the PrP isoforms were carried out as described [41, 48]. Western blots were developed with the rabbit PrP antiserum R073 [44] using the ECL reaction, and films were scanned using a Umax Astra 1220S scanner, and densitometry was analyzed using the Tina 2.1 Raytest program software (Isotopenmeagerilte GmbH, Germany).

References

1 **Prusiner, S. B.,** Novel proteinaceous infectious particles cause scrapie. *Science* 1982. **216:** 136û144.

2 **Prusiner, S. B., Scott, M. R., DeArmond, S. J. and Cohen, F. E.,** Prion protein biology. *Cell* 1998. **93:** 337û348.

3 **Butler, D. A., Scott, M. R., Bockman, J. M., Borchelt, D. R., Taraboulos, A., Hsiao, K. K., Kingsbury, D. T. and Prusiner, S. B.,** Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins. *J. Virol.* 1988. **62:** 1558–1564.

4 **Borchelt, D. R., Scott, M., Taraboulos, A., Stahl, N. and Prusiner, S. B.,** Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells. *J. Cell Biol.* 1990. **110:** 743–752.

5 **Taraboulos, A., Serban, D. and Prusiner, S. B.,** Scrapie prion proteins accumulate in the cytoplasm of persistently infected cultured cells. *J. Cell Biol.* 1990. **110:** 2117–2132.

6 **Kellings, K., Meyer, N., Mirenda, C., Prusiner, S. B. and Riesner, D.,** Further analysis of nucleic acids in purified scrapie prion preparations by improved return refocusing gel electrophoresis. *J. Gen. Virol.* 1992. **73:** 1025–1029.

7 **Prusiner, S. B.,** Molecular biology of prion diseases. *Science* 1991. **252:** 1515–1522.

8 **Taraboulos, A., Rogers, M., Borchelt, D. R., McKinley, M. P., Scott, M., Serban, D. and Prusiner, S. B.,** Acquisition of protease resistance by prion proteins in scrapie-infected cells does not require asparagine-linked glycosylation. *Proc. Natl. Acad. Sci. USA* 1990. **87:** 8262–8266.

9 **Caughey, B. W., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F. and Caughey, W. S.,** Secondary structure analysis of the scrapie-associated protein PrP 27–30 in water by infrared spectroscopy. *Biochemistry* 1991. **30:** 7672–7680.

10 **Gasset, M., Baldwin, M. A., Lloyd, D., Gabriel, J.-M., Holtzman, D. M., Cohen, F., Fletterick, R. and Prusiner, S. B.,** Predicted α-helical regions of the prion protein when synthesized as peptides form amyloid. *Proc. Natl. Acad. Sci. USA* 1992. **89:** 10940–10944.

11 **Pan, K. M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J., Cohen, F. E. and Prusiner, S. B.,** Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc. Natl. Acad. Sci. USA* 1993. **90:** 10962–10966.

12 **Cohen, F. E., Pan, K. M., Huang, Z., Baldwin, M., Fletterick, R. J. and Prusiner, S. B.,** Structural clues to prion replication. *Science* 1994. **264:** 530–531.

13 **Gasset, M., Baldwin, M. A., Fletterick, R. J. and Prusiner, S. B.,** Perturbation of the secondary structure of the scrapie prion protein under conditions that alter infectivity. *Proc. Natl. Acad. Sci. USA* 1993. **90:** 1–5.

14 **Huang, Z., Prusiner, S. B. and Cohen, F. E.,** Scrapie prions: a three-dimensional model of an infectious fragment. *Fold Des.* 1996. **1:** 13–19.

15 **Stahl, N., Baldwin, M. A., Teplow, D. B., Hood, L., Gibson, B. W., Burlingame, A. L. and Prusiner, S. B.,** Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. *Biochemistry* 1993. **32:** 1991–2002.

16 **Prusiner, S. B., McKinley, M. P., Bowman, K. A., Bolton, D. C., Bendheim, P. E., Groth, D. F. and Glenner, G. G.,** Scrapie prions aggregate to form amyloid-like birefringent rods. *Cell* 1983. **35:** 349–358.

17 **Meyer, R. K., McKinley, M. P., Bowman, K. A., Braunfeld, M. B., Barry, R. A. and Prusiner, S. B.,** Separation and properties of cellular and scrapie prion proteins. *Proc. Natl. Acad. Sci. USA* 1986. **83:** 2310–2314.

18 **Prusiner, S. B.,** The prion diseases. *Sci. Am.* 1995. **272:** 48–51, 54–57.

19 **Williamson, R. A., Peretz, D., Smorodinsky, N., Bastidas, R., Serban, H., Mehlhorn, I., DeArmond, S. J., Prusiner, S. B. and Burton, D. R.,** Circumventing tolerance to generate autologous monoclonal antibodies to the prion protein. *Proc. Natl. Acad. Sci. USA* 1996. **93:** 7279–7282.

20 **Brown, K. L., Stewart, K., Ritchie, D. L., Mabbott, N. A., Williams, A., Fraser, H., Morrison, W. I. and Bruce, M. E.,** Scrapie replication in lymphoid tissues depends on prion protein-expressing follicular dendritic cells. *Nature Med.* 1999. **5:** 1308–1312.

21 **Klein, M. A., Frigg, R., Raeber, A. J., Flechsig, E., Hegyi, I., Zinkernagel, R. M., Weissmann, C. and Aguzzi, A.,** PrP expression in B lymphocytes is not required for prion neuroinvasion. *Nature Med.* 1998. **4:** 1429–1433.

22 **Betmouni, S., Perry, V. H. and Gordon, J. L.,** Evidence for an early inflammatory response in the central nervous system of mice with scrapie. *Neuroscience* 1996. **74:** 1–5.

23 **Beringue, V., Demoy, M., Lasmezas, C. I., Gouritin, B., Weingarten, C., Deslys, J. P., Andreux, J. P., Couvreur, P. and Dormont, D.,** Role of spleen macrophages in the clearance of scrapie agent early in pathogenesis. *J. Pathol.* 2000. **190:** 495–502.

24 **Reizis, B., Eisenstein, M., Bockova, J., Konen Waisman, S., Mor, F., Elias, D. and Cohen, I. R.,** Molecular characterization of the diabetes-associated mouse MHC class II protein, I-Ag7. *Int. Immunol.* 1997. **9:** 43–51.

25 **Reizis, B., Eisenstein, M., Mor, F. and Cohen, I. R.,** The peptide-binding strategy of the MHC class II I-A molecules. *Immunol. Today* 1998. **19:** 212–216.

26 **Augusti-Tocco, G. and Sato, G.,** Establishment of functional clonal lines of neurons from mouse neuroblastoma. *Proc. Natl. Acad. Sci. USA* 1969. **64:** 311–315.

27 **Stahl, N., Borchelt, D. R., Hsiao, K. and Prusiner, S. B.,** Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell* 1987. **51:** 229–240.

28 **Moore, R. C., Lee, I. Y., Silverman, G. L., Harrison, P. M., Strome, R., Heinrich, C., Karunaratne, A., Pasternak, S. H., Chishti, M. A., Liang, Y., Mastrangelo, P., Wang, K., Smit, A. F., Katamine, S., Carlson, G. A., Cohen, F. E., Prusiner, S. B., Melton, D. W., Tremblay, P., Hood, L. E. and Westaway, D.,** Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. *J. Mol. Biol.* 1999. **292:** 797–817.

29 **Cohen, F. E. and Prusiner, S. B.,** Pathologic conformations of prion proteins. *Annu. Rev. Biochem.* 1998. **67:** 793–819.

30 **DebBurman, S. K., Raymond, G. J., Caughey, B. and Lindquist, S.,** Chaperone-supervised conversion of prion protein to its protease- resistant form. *Proc. Natl. Acad. Sci. USA* 1997. **94:** 13938–13943.

31 **Laurent, M. and Johannin, G.,** Molecular clues to pathogenesis in prion diseases. *Histol. Histopathol.* 1997. **12:** 583–594.

32 **Edenhofer, F., Rieger, R., Famulok, M., Wendler, W., Weiss, S. and Winnacker, E. L.,** Prion protein PrPc interacts with molecular chaperones of the Hsp60 family. *J. Virol.* 1996. **70:** 4724–4728.

33 **Prusiner, S. B.,** Biology and genetics of prion diseases. *Annu. Rev. Microbiol.* 1994. **48:** 655–686.

34 **Westaway, D., Cooper, C., Turner, S., Da Costa, M., Carlson, G. A. and Prusiner, S. B.,** Structure and polymorphism of the mouse prion protein gene. *Proc. Natl. Acad. Sci. USA* 1994. **91:** 6418–6422.

35 **Tatzelt, J., Zuo, J., Voellmy, R., Scott, M., Hartl, U., Prusiner, S. B. and Welch, W. J.,** Scrapie prions selectively modify the stress response in neuroblastoma cells. *Proc. Natl. Acad. Sci. USA* 1995. **92:** 2944–2948.

36 **Montrasio, F., Frigg, R., Glatzel, M., Klein, M. A., Mackay, F., Aguzzi, A. and Weissmann, C.,** Impaired prion replication in spleens of mice lacking functional follicular dendritic cells. *Science* 2000. **288:** 1257–1259.

37 **Brandner, S., Klein, M. A. and Aguzzi, A.,** A crucial role for B cells in neuroinvasive scrapie. *Transfus. Clin. Biol.* 1999. **6:** 17–23.

38 **Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandevert, C., Walker, S., Wogulis, M., Yednock, T., Games, D. and Seubert,**

P., Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* 1999. **400:** 173–177.

39 **Schenk, D. B., Seubert, P., Lieberburg, I. and Wallace, J.,** Beta-peptide immunization: a possible new treatment for Alzheimer disease. *Arch Neurol.* 2000. **57:** 934–936.

40 **Lorenzo, A. and Yankner, B. A.,** Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. *Proc. Natl. Acad. Sci. USA* 1994. **91:** 12243–12247.

41 **Naslavsky, N., Stein, R., Yanai, A., Friedlander, G. and Taraboulos, A.,** Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform. *J. Biol. Chem.* 1997. **272:** 6324–6331.

42 **Scott, M. R., Kohler, R., Foster, D. and Prusiner, S. B.,** Chimeric prion protein expression in cultured cells and transgenic mice. *Protein Sci.* 1992. **1:** 986–997.

43 **Elias, D.,** The NOD mouse: a model for autoimmune insulin-dependent diabetes. **In Cohen, I. R. and Miller, A. (Eds.),** *Autoimmune Disease Models: A Guidebook*, *Academic Press, San Diego* 1994, pp. 147–161.

44 **Serban, D., Taraboulos, A., DeArmond, S. J. and Prusiner, S. B.,** Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins. *Neurology* 1990. **40:** 110–117.

45 **Mor, F., Lohse, A. W., Karin, N. and Cohen, I. R.,** Clinical modeling of T cell vaccination against autoimmune diseases in rats. Selection of antigen-specific T cells using a mitogen. *J. Clin. Invest.* 1990. **85:** 1594–1598.

46 **Mor, F. and Cohen, I. R.,** Shifts in the epitopes of myelin basic protein recognized by Lewis rat T cells before, during, and after the induction of experimental autoimmune encephalomyelitis. *J. Clin. Inves.* 1993. **92:** 2199–2206.

47 **Figueiredo, A. C., Cohen, I. R. and Mor, F.,** Diversity of the B cell repertoire to myelin basic protein in rat strains susceptible and resistant to EAE. *J. Autoimmun.* 1999. **12:** 13–25.

48 **Taraboulos, A., Scott, M., Semenov, A., Avrahami, D., Laszlo, L., Prusiner, S. B. and Avraham, D.,** Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. *J. Cell Biol.* 1995. **129:** 121–132.

Correspondence: Irun R. Cohen, Department of Immunology, The Weizmann Institute of Science, P.O. Box 26, Rehovot 76100, Israel
Fax: +972-8-934–4103
e-mail: irun.cohen@weizmann.ac.il