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Complete Freund's Adjuvant Immunization Prolongs Survival in Experimental Prion Disease in Mice

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We recently reported that immunization of mice with certain self-prion protein peptides induced specific T-cell and B-cell immune responses; importantly, this immunization was associated with a decrease in the number of protease-resistant PrP^{Sc} particles recoverable in a transplanted, scrapie-infected syngeneic tumor. The present study was carried out to determine whether immunization with the immunogenic PrP peptides might influence the natural history of experimental scrapie in mice. We immunized C57BL/6 mice with self-prion peptides in complete Freund's adjuvant (CFA) or with CFA alone as a control and then infected the mice with mouse-adapted scrapie by injection either intraperitoneally or intracerebrally. We report here that immunization with CFA, irrespective of whether prion peptides were present in the inoculum, resulted in marked prolongation of survival of the mice, whether the challenge was intracerebral or intraperitoneal. Mice in the immunized and control groups that died contained equivalent amounts of PrP^{Sc}. Thus, CFA immunization has a therapeutic effect in experimental scrapie in mice, possibly by reducing the rate of PrP^{Sc} accumulation in the brain.
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Key words: transmissible spongiform encephalopathies; scrapie; immunotherapy; Freund's adjuvant; PrP^{Sc}

Prion diseases are a group of neurodegenerative diseases caused by the accumulation of abnormal prion protein (Prusiner, 1995). Previous work on the immune system's role in prion disease has centered on the mechanisms of prion transport from the periphery to the central nervous system (CNS; Klein et al., 1997; Mabbott et al., 1998; Brown et al., 1999; Sy and Gambetti, 1999; Montrasio et al., 2000). These studies pointed to B cells (Klein et al., 1997) and dendritic cells (Brown et al., 1999; Sy and Gambetti, 1999; Montrasio et al., 2000) as the main players in the traffic of the pathological isoform of PrP (PrP^{Sc}) from the peripheral lymphoid organs to the CNS. The paradigm, until recently, was that the immune system is tolerant to the prion protein; one cannot induce an effective immune response to the protein (Porter et al., 1973). Indeed, PrP null mice were used for immunization to raise monoclonal antibodies to the mouse prion protein (Krasemann et al., 1996; Williamson et al., 1996).

We characterized, on the basis of our previous experience in documenting immune reactivities to self-proteins in experimental autoimmune diseases (Mor and Cohen, 1993, 1995; Mor et al., 1996), MHC class II peptide-binding motifs in Lewis rats (Reizis et al., 1996) and NOD mice (Reizis et al., 1997). Synthetic prion peptides, selected on the basis of their positive motifs, were found to be immunogenic in both rats (Souan et al., 2001a) and mice (Souan et al., 2001b). We found, in a neuroblastoma model expressing PrP^{Sc}, that prion peptide immunization resulted in a decrease in the tumor content of PrP^{Sc} in A/J mice (Souan et al., 2001b).

We therefore undertook to test the effect of prion peptide immunization on the natural history of scrapie in inoculated mice. We found that both prion peptide and control complete Freund's adjuvant (CFA) immunization led to a significant prolongation of survival of the mice, with similar amounts of PrP^{Sc} in the brain. The protective effect was seen in both intraperitoneally and intracerebrally inoculated mice. The possible mechanisms of these findings are discussed.

MATERIALS AND METHODS

Animals

Inbred female C57BL/6 ($H2^b$) were supplied by Harlan Laboratories, Israel.

The first two authors contributed equally to this study.

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Peptides

Mouse prion peptides p31–50 (WNTGGSRYPGQG-SPGGNRYP) and p211–230 (QMCVTQYQKESQAYY-DGRR) were synthesized by Fmoc chemistry, using the AMS422 automated synthesizer (ABIMED, Langenfeld, Germany). Peptides were examined for purity by high-performance liquid chromatography (HPLC), and their composition was confirmed by amino acid analysis.

Antibodies

Rabbit antiserum R073 to PrP (Serban et al., 1990) was used in Western blots at a dilution of 1:5,000. Secondary antibody was from Jackson Immunoresearch (West Grove, PA).

Immunizations

Peptides for immunizations were dissolved in phosphate-buffered saline (PBS; 1 mg/ml) and mixed with CFA in a 1:1 (v/v) ratio. Naive 8-week-old female C57BL/6 mice were immunized in both hind footpads with 50 μl of the peptide emulsion in CFA (each animal was thus injected with 50 μg of peptide). Ten days later, the mice were boosted subcutaneously with the same peptide in CFA. On the next day, the animals were inoculated with either the RML scrapie brain homogenate or PBS as negative control as described under Scrapie Inoculation below. CFA was prepared by mixing incomplete adjuvant (IFA) with 4 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI).

Scrapie Inoculation

A 10% brain homogenate was prepared by extracting the brain from an RML-diseased mouse. The brain was then homogenized in sterile PBS. The homogenate was next diluted 1 to 10 into vials with a 1 ml tuberculin syringe. On the next day, subsequently to boosting the mice, each animal was briefly sedated with halothane, and 30 μl of either the scrapie brain homogenate or PBS was injected intracerebrally with a tuberculin syringe. Animals were monitored three times per week for the first 3 months and then daily until signs of experimental scrapie appeared.

Brain Preparation

Mice were sacrificed by ethyl ether anesthesia to avoid damaging the brain tissue. The brain was then taken out. One half (the cut was made along the longitudinal cerebral fissure) was immediately frozen in liquid nitrogen for biochemical analysis, and the other half was placed in 4% formaldehyde buffer (in PBS) for histological analysis.

Scrapie Disease Scoring

Animals were kept under S.P.F. conditions and checked three times per week for the first 3 months and then daily until signs of experimental scrapie appeared. Scrapie diagnosis was carried out by the conventional parameters, and the neurological symptoms of the mice were double checked by another technician blinded to experimental groups. Scrapie symptoms can be summarized as follows: bradykinesia (slow movement and reflexes), dysmetria (strut, abnormal gait, high-step, Volkswagen strut), tremor (trembling or "short shivering"), ataxia (wobbling, poor balance, toppling), proprioceptive deficits (clasp foot), tilted head, masked facies (blank stare), tail rigidity, kyphosis (hunched back posture), loss of deep pain sensation (no reaction to tail pinching), and stupor. Two of the previous symptoms were considered sufficient to diagnose the mouse as "scrapie sick." When a mouse was diagnosed with the disease, it was examined twice per day. Wet food was added to the cage when the mice became too weak to reach the food and water. Whenever possible, mice were sacrificed shortly before death. However, on a few occasions, animals were found dead in the cage.

PrP Analysis

Brain homogenates were prepared as follows: half of the brain (frozen in liquid nitrogen) was 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) by first incubating the tissue in lysis buffer on ice for 30 min. Then, the tissue lysate was homogenized in an electrical homogenizer. Insoluble debris was removed by a 30 sec 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 a BCA kit (Pierce, Rockford, IL). Brain lysates were then normalized for their protein content prior to Western analysis.

Western Analysis of the Prion Protein

The PrP isoforms were characterized and separated as described elsewhere (Meyer et al., 1986). To digest away PrP^{C} (the normal isoform of PrP) while enriching the lysates for PrP^{Sc}, lysates were incubated for 2 hr with 20 μg/ml proteinase K at 37°C. The reaction was stopped by cooling the samples on ice and adding phenylmethylsulfonyl fluoride (PMSF) to 2 mM for 30 min. To study the total levels of PrP in a sample, proteolysis was omitted. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis of the PrP isoforms were carried out as described elsewhere (Taraboulos et al., 1995; Naslavsky et al., 1997).

RESULTS

CFA Immunization Prolongs Survival in Intraperitoneal Scrapie-Challenged Mice

Groups of C57BL/6 mice were immunized with p31–50 (known to induce antibody production) or p211–230 (known to induce antibody- and antigen-specific T cells in this strain of mice) emulsified in CFA or with CFA alone. On day 10 after immunization, the mice were boosted with the same antigen. On the following day, the mice were inoculated intraperitoneally with RML-scrapie brain homogenate (Carp and Callahan, 1986) and were then examined for signs of scrapie developing over time. We found that control, nonimmunized mice survived for only an average of 232 days, and mice immunized with either of the PrP peptides survived for 250 days, whereas mice immunized with CFA alone lived to 251 days (Fig. 1). The prolonged survival was statistically highly significant (CFA vs. PBS, $P = 0.0027$), indicating that CFA immunization affected the disease.

We measured the content of PrP^{Sc} in the brains of the mice at death and found no difference in amount

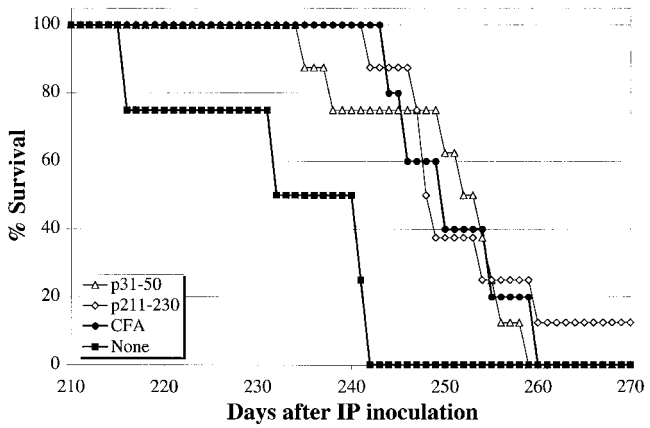

Fig. 1. Effects of immunization with CFA or PrP peptides on the survival rate of C57BL/6 mice inoculated intraperitoneally with RML scrapie. Statistical analysis (log-rank test, GraphPad Prizm): p31–50 vs. PBS, $P = 0.0124$; p211–230 vs. PBS, $P = 0.0006$; CFA vs. PBS, $P = 0.0027$.

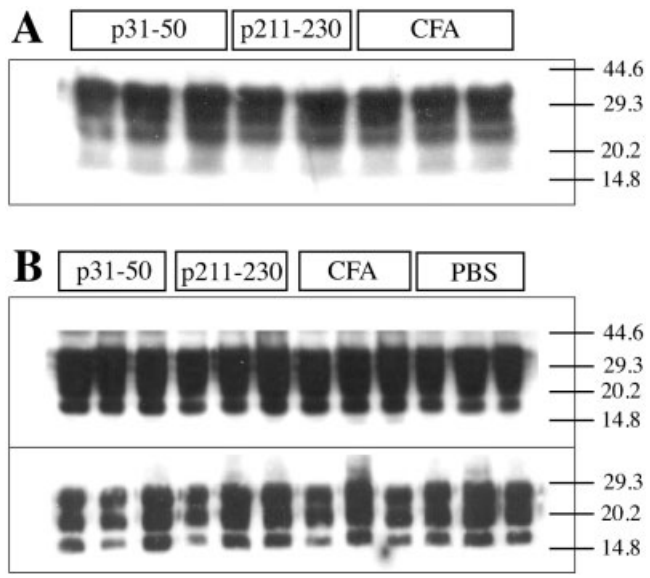

Fig. 2. Immunization does not appreciably alter the brain level of protease-resistant PrP^{Sc} at the time of death. The brains from terminally ill i.p.-inoculated animals were lysed, and their PrP content was analyzed by Western blotting. The samples were normalized for protein concentration as measured by the BCA method. **A:** Control mice were immunized but not inoculated with brain homogenate. It can be easily seen that PrP^{C} level did not vary between groups. **B:** Mice were immunized and inoculated as described. In the lower panel, the brain lysates were treated with proteinase K (20 μg/ml, 2 hr, 37°C) prior to electrophoresis to digest away PrP^{C}. The blot was developed with the PrP antiserum R073. The various forms of immunization did not affect the levels of PrP^{Sc} appreciably.

between the various groups (Fig. 2). This suggests that the immunized mice died with the same load of PrP^{Sc} that was associated with death in the control mice. Hence, it is conceivable that the immunization did not increase the

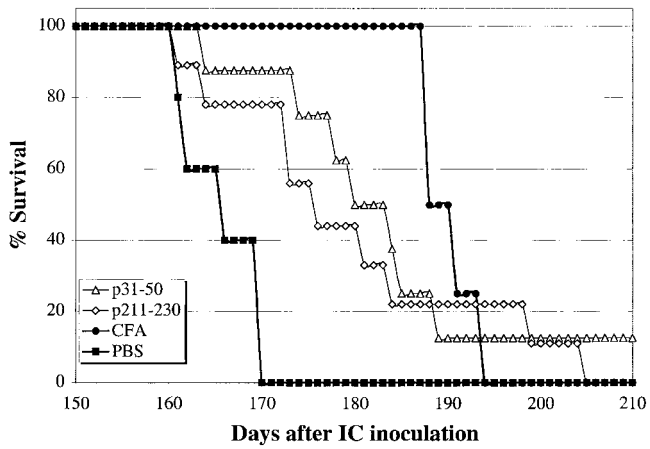

Fig. 3. Effects of CFA or PrP peptide immunization on the survival rate of C57BL/6 mice inoculated i.c. with RML-scrapie. Statistical analysis of survival (log-rank test, GraphPad Prizm): p31–50 vs. PBS, $P = 0.0025$; p211–230 vs. PBS, $P = 0.011$; CFA vs. PBS, $P = 0.0065$.

ability of the mice to tolerate more PrP^{Sc} but reduced the rate of PrP^{Sc} accumulation. This conclusion is compatible with our previous finding of a reduction of PrP^{Sc} in the transplanted neuroblastoma model (Souan et al., 2001b).

CFA Immunization Prolongs Survival in Mice Challenged Intracerebrally With Scrapie

It is conceivable that immune activation could have impeded transport of PrP^{Sc} by immune cells from the periphery to the brain. We therefore tested the effect of immunization on intracerebrally inoculated scrapie, bypassing transport from the periphery. The control, non-immunized mice survived to day 166 after challenge. Mice that had been immunized with either peptide in CFA survived for 179 days, whereas the mice immunized with CFA alone survived for 190 days (Fig. 3). The prolongation of survival was statistically significant (CFA vs. PBS, $P = 0.0065$). Thus, immunization with CFA, with or without immunogenic prion peptides, had a beneficial effect on mice injected intracerebrally with PrP^{Sc}. The PrP^{Sc} content of brains from experimental and control groups at the time of death contained similar amounts of the aberrant protein, again arguing for a reduced rate of accumulation of the PrP^{Sc} in the CFA- and peptide/CFA-immunized mice.

DISCUSSION

This study was designed to test for the immunological effects of prion peptide immunization on the natural history of experimental prion disease in mice. On the basis of our experience with an implanted neuroblastoma model (Souan et al., 2001b), we expected that the peptide immunization would have a therapeutic effect. However, we found that both peptide and CFA immunization exerted a similar protective effect on survival of mice. It remains to be determined how CFA immunization prolongs survival by apparently reducing the rate of PrP^{Sc} accumulation.

Since the protective effect was seen in mice inoculated both i.p. and i.c., it would seem that the underlying mechanism is local rather than being related to the inhibition of propagation from the spleen to the CNS. Microglial cells are a crucial component of plaques in all prion diseases (Barcikowska et al., 1993). It might be possible that CFA immunization activates microglial cells, leading to increased phagocytosis of scrapie protein, averting the scrapie from killing neurons. CFA is composed of killed mycobacteria and mineral oil, and its inoculation in experimental animals has myriad effects (Billiau and Matthys, 2001). The mycobacteria in CFA induce a polarized Th1 response, and CFA is also known to activate the innate immune system. Previously, protective effects of CFA immunization have been reported in diabetes in NOD mice (Qin et al., 1993; Calcinaro et al., 1997), in experimental autoimmune encephalomyelitis (Weber and Hempel, 1987), and in nematode infection (Robinson et al., 1996). One of the major immunogenic antigens in mycobacteria is the 65 kDa bacterial variant of the 60 kDa heat shock protein HSP60 (Kaufmann et al., 1987). Remarkably, HSP60 was suggested to function as a chaperone in the formation of PrP^{Sc} (DebBurman et al., 1997; Stockel and Hartl, 2001). Bacterial and mammalian HSP60 variants are highly homologous and immunologically cross-reactive (Zugel and Kaufmann, 1999), so it is possible that a specific immune response to HSP60, induced by CFA, leads to some decrease in the availability of the PrP^{Sc} protein.

At present, we do not know whether HSP65, or any other molecule present in CFA, or the immune stimulation induced by such immunization is responsible for the beneficial effect in C57BL/6 mice challenged with scrapie. Future experiments using other adjuvants (such as IFA, alum, CpG DNA, Ribi Adjuvant System) prior to scrapie inoculation, might help to clarify the mechanism of the apparently reduced rate of PrP^{Sc} accumulation induced by CFA. Recent work using monoclonal antibodies demonstrated the ability of antiprion protein antibodies to clear PrP^{Sc} from tissue culture neuroblastoma cells (Enari et al., 2001; Peretz et al., 2001). Transgenic mice expressing antiprion antibodies were protected from scrapie (Heppner et al., 2001). The results presented here indicate that activating the immune system can affect the course of experimentally induced scrapie. Further analysis of this effect may lead to the development of novel means to affect the course of prion diseases in animals and in humans.

Note added in proof:

In agreement with our results, recent work published since our paper was submitted, documented a beneficial effect of CpG administration on survival of scrapie inoculated mice [Sethi S, Lipford G, Wagner H, Kretzschmar H. 2002. Postexposure prophylaxis against prion disease with a stimulator of innate immunity. Lancet 360:229–230.]

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