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HIGH TITERS OF MUCOSAL AND SYSTEMIC ANTI-PrP ANTIBODIES ABROGATE ORAL PRION INFECTION IN MUCOSAL-VACCINATED MICE

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Abstract—Significant outbreaks of prion disease linked to oral exposure of the prion agent have occurred in animal and human populations. These disorders are associated with a conformational change of a normal protein, PrP^{C} (C for cellular), to a toxic and infectious form, PrP^{Sc} (Sc for scrapie). None of the prionoses currently have an effective treatment. Some forms of prion disease are thought to be spread by oral ingestion of PrP^{Sc}, such as chronic wasting disease and variant Creutzfeldt-Jakob disease. Attempts to obtain an active immunization in wild-type animals have been hampered by auto-tolerance to PrP and potential toxicity. Previously, we demonstrated that it is possible to overcome tolerance and obtain a specific anti-PrP antibody response by oral inoculation of the PrP protein expressed in an attenuated Salmonella vector. This past study showed that 30% of vaccinated animals were free of disease more than 350 days post-challenge. In the current study we have both optimized the vaccination protocol and divided the vaccinated mice into low and high immune responder groups prior to oral challenge with PrP^{Sc} scrapie strain 139A. These methodological refinements led to a significantly improved therapeutic response. 100% of mice with a high mucosal anti-PrP titer immunoglobulin (Ig) A and a high systemic IgG titer, prior to challenge, remained without symptoms of PrP infection at 400 days (log-rank test $P<0.0001$ versus sham controls). The brains from these surviving clinically asymptomatic mice were free of PrP^{Sc} infection by Western blot and histological examination. These promising findings suggest that effective mucosal vaccination is a feasible and useful method for overcoming tolerance to PrP and preventing prion infection via an oral route

Key words: scrapie, immunization, salmonella vaccine strain, Creutzfeldt-Jakob disease, bovine spongiform encephalopathy, chronic wasting disease.

Prion diseases are a unique category of illness, affecting both animals and humans, where the underlying pathogenesis is related to a conformational change of a normal, self-protein called PrP^{C} (C for cellular) to a pathological and infectious conformer known as PrP^{Sc} (Sc for scrapie). Currently all prion diseases are without effective treatment and are universally fatal. Interest in prion disease has greatly increased since the emergence of bovine spongiform encephalopathy (BSE) in England and the resulting appearance of variant Creutzfeldt-Jakob disease (vCJD) in human populations. BSE arose from the feeding of cattle with prion-contaminated meat and bone meal products, while vCJD developed following entry of BSE into the human food chain (Manson et al., 2006; Butler, 2006). Since the original report in 1995 a total of 201 probable or confirmed cases of vCJD have been diagnosed, 165 in the Great Britain, 21 in France, four cases in Ireland, three in USA, two in Netherlands and one each in Italy, Canada, Japan, Saudi Arabia, Portugal and Spain (Sadowski et al., 2008). It has been difficult to predict the expected future numbers of vCJD. Mathematical analysis has given a range from 1000 to about 136,000 individuals who will eventually develop the disease (Sadowski et al., 2008; Smith et al., 2004).

In North America a significant emerging prion infection is chronic wasting disease (CWD). This disease is now endemic in Colorado, Wyoming and Nebraska and continues to spread to other parts in the US, first in the Midwest but has now been detected as far east as New York State (Williams, 2005; Aguzzi and Sigurdson, 2004). Transmission of CWD is thought to be mainly via an oral route (Beekes and McBride, 2007). The occurrence of CJD among three young deer hunters from this same region raised the speculation of transmission of the CWD to humans (Belay et al., 2004). Autopsy of these three subjects did not show the extensive amyloidosis characteristic of the vCJD and CWD (Liberski et al., 2001). However like

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Abbreviations: BSE, bovine spongiform encephalopathy; CFA, complete Freund's adjuvant; CWD, chronic wasting disease; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; PrP^{C}, prion protein cellular; PrP^{Sc}, prion protein scrapie; recPrP, recombinant prion protein; vCJD, variant Creutzfeldt-Jakob disease.

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BSE, CWD is transmissible to non-human primates and transgenic mice expressing human PrP^C (Marsh et al., 2005; Tamguney et al., 2006). Therefore the possibility of such transmission needs to be closely monitored. CWD is similar to BSE in that the peripheral titers of the prion agent are high. PrP^{Sc} has been detected in blood, muscle and saliva of CWD infected deer (Angers et al., 2006; Mathiason et al., 2006).

The prion protein is a self-antigen; hence, prion infection is not known to elicit a classic immune response. In fact the immune system is involved in the peripheral replication of the prion agent and its ultimate access to the CNS (Aucouturier et al., 2000; Sigurdsson and Wisniewski, 2005). This interval during which time the prion agent replicates peripherally, without producing any symptoms, is quite long, lasting many months in experimental animals and up to 56 years in documented human cases associated with cannibalistic exposure to the prion agent (Collinge et al., 2006). Lymphatic organs such as the tonsils, lymph nodes or gut-associated lymphoid tissue (GALT) contain high concentrations of PrP^{Sc} long before PrP^{Sc} replication starts in the brain (Brown et al., 2000; Mabbott and MacPherson, 2006; Beekes and McBride, 2007). An emerging therapeutic approach for prion infection is immunomodulation (Sakaguchi and Arakawa, 2007; Sasson et al., 2005; Wisniewski et al., 2007).

Currently, there is no treatment that would arrest and/or reverse progression of prion disease in non-experimental settings, although many approaches have been tried (Trevitt and Collinge, 2006). Earlier *in vivo* studies had shown that infection with a slow strain of PrP^{Sc} blocked expression of a more virulent fast strain of PrP, mimicking vaccination with a live attenuated organism (Manuelidis, 1998). In tissue culture studies, anti-PrP antibodies and antigen binding fragments directed against PrP have been shown to inhibit prion replication (Enari et al., 2001; Peretz et al., 2001; Pankiewicz et al., 2006). While we first demonstrated that active immunization with recombinant PrP delayed the onset of prion disease in wild-type mice, the therapeutic effect was relatively modest and eventually all the mice succumbed to the disease (Sigurdsson et al., 2002). This limited therapeutic effect may be explained by the observation that antibodies generated against prokaryotic PrP often do not have a high affinity toward PrP^C (Polymenidou et al., 2004), although in our studies the increase in the incubation period correlated well with the antibody titers against PrP^C. Our follow-up passive anti-PrP immunization study confirmed the importance of the humoral response, showing that anti-PrP antibodies are able to prolong the incubation period (Sigurdsson et al., 2003b). Subsequently, other investigators, using a much higher antibody dosage, were able to completely prevent disease onset in mice exposed to PrP^{Sc} provided passive immunization was initiated within a month of exposure (White et al., 2003). This type of approach could eventually be used immediately following accidental exposure to prevent future infection. However, passive immunization has not been found to be effective closer to the clinically symptomatic stages of prion infection. Also passive immunization would be an approach that is too costly for animal prion diseases.

We were the first to demonstrate the effective use of active mucosal vaccination to prevent prion infection (Goni et al., 2005). Thirty percent of animals immunized with an attenuated salmonella strain expressing the whole PrP protein were free of disease after challenge with the prion agent. Mucosal immunization has subsequently been confirmed to be partially protective against prion infection by another group who used a recombinant PrP fragment and a cholera toxin adjuvant as their vaccine (Bade et al., 2006). In the present study we have extended our vaccination approach using the live attenuated strain of *Salmonella typhimurium*, LVR01, expressing the mouse PrP gene. Following a more extensive oral vaccination protocol, we divided the mice into high and low responders, prior to challenge with PrP^{Sc}. We report that 100% of mice with a good mucosal immunoglobulin (Ig) A and systemic IgG response were protected from oral prion infection.

EXPERIMENTAL PROCEDURES

Construction of a recombinant salmonella vaccine strain expressing tandem copies of PrP

The construction of the *S. typhimurium* aroC LVR01 and PrP expression by this vector were as previously described (Chabalgoity et al., 2000; Goni et al., 2005). The plasmid constructs encoding two copies of PrP insert were introduced into salmonella LVR01 by electroporation. The expression of recombinant prion protein (recPrP) by the salmonella LVR01 strain was assessed by SDS-PAGE and Western blotting using monoclonal anti-PrP 6D11 (Spinner et al., 2007), as previously reported (Goni et al., 2005).

Animal and vaccination protocols

Prior to inoculation into mice, the bacteria were cultured overnight on Luria broth at 37 °C with continuous shaking. The bacterial suspensions were centrifuged at $1200\times g$ for 20 min at 15 °C, washed once with sterile PBS, centrifuged again and diluted to 1×10^{11} colony forming units/ml.

All animal experiments were approved by the NYU School of Medicine Institutional Animal Care and Use Committee. The experiments were designed to minimize the number of animals used and their suffering. A group of 50 female CD-1 mice, 6 weeks of age, were orally immunized with *S. typhimurium* LVR01 expressing pTECH and a double copy of the mouse PrP (PrPx2). A control group of 10 mice was orally immunized with *S. typhimurium* LVR01 carrying pTECH without the PrP insert. The mice were subject to a 3 h food fast and each exposed via gavage to 2×10^{10} viable cells of the vaccine strain in 0.36 M $NaHCO_3$, pH 8.3 in a 0.5 ml volume mixed in a 6:1 ratio with alum ($[Al(OH)_3]$, Alhydrogel, Superfos Biosector, Denmark), as previously described (Goni et al., 2005). The oral vaccinations were repeated weekly for a further three inoculations, followed by two further boosts with dead LVRO1 salmonella expressing the PrP protein on days 35 and 42 after the original vaccination (for a total of six inoculations, four with live salmonella and two with dead salmonella). Forty-five days after the original mucosal vaccination the mice were separated into groups based on their respective feces and plasma anti-PrP IgA and IgG titers. The four groups of vaccinated mice were animals with: high IgG and high IgA; high IgG and low IgA; high IgA and low IgG; or low IgA and low IgG. Seven weeks after the original vaccination the mice were orally challenged via gavage with 200 μl of a 1:10 dilution of a brain homogenate of the mouse-adapted scrapie strain 139A. This time point for challenge