

## High Titers of Mucosal and Systemic anti-PrP Antibodies Abrogates 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<sup>C</sup>, to a toxic and infectious form, PrP<sup>Sc</sup>. None of the prionoses currently have an effective treatment. Some forms of prion disease are thought to be spread by oral ingestion of PrP<sup>Sc</sup>, 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<sup>Sc</sup> scrapie strain 139A. These methodological refinements lead to a significantly improved therapeutic response. 100% of mice with a high mucosal anti-PrP titer IgA and a high systemic IgG titer, prior to challenge, remained without symptoms of PrP infection at 400 days (long-rank test  $p < 0.0001$  versus sham controls). The brains from these surviving clinically asymptomatic mice were free of PrP<sup>Sc</sup> 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

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## Keywords

Scrapie; Immunization; Salmonella vaccine strain; Creutzfeldt-Jakob disease; Bovine spongiform encephalopathy; Chronic wasting disease

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## Introduction

Prion disease 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<sup>C</sup> (C for cellular) to a pathological and infectious conformer known as PrP<sup>Sc</sup> (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 CJD (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, 4 cases in Ireland, 3 in USA, 2 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 1 thousand to about 136 thousand 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 (Liberki et al., 2001). However like BSE, CWD is transmissible to non-human primates and transgenic mice expressing human PrP<sup>C</sup> (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<sup>Sc</sup> 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 classical 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 yrs 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<sup>Sc</sup> long before PrP<sup>Sc</sup> 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<sup>Sc</sup> 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 towards PrP<sup>C</sup> (Polymenidou et al., 2004), although in our studies the increase in the incubation period correlated well with the antibody titers against PrP<sup>C</sup>. 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<sup>Sc</sup> 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). 30% 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<sup>Sc</sup>. We report that 100% of mice with a good mucosal IgA 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 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 1,200g for 20 minutes at 15°C, washed once with sterile PBS, centrifuged again and diluted to  $1 \times 10^{11}$  CFU/ml.

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 (PrP $\times$ 2). A control group of 10 mice were orally immunized with *S.typhimurium* LVR01 carrying pTECH without the PrP insert. The mice were subject to a 3 hr food fast and each exposed via gavage to  $2 \times 10^{10}$  viable cells of the vaccine strain in 0.36M NaHCO<sub>3</sub>, pH 8.3 in a 0.5ml 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 3 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 6 inoculations, 4 with live Salmonella and 2 with dead Salmonella). 45 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 $\mu$ l of a 1:10 dilution of a brain homogenate of the mouse-adapted scrapie strain 139A. This time point for challenge was chosen as a maximal humoral immune response is expected 6 to 8 weeks after the original vaccination. An additional control group of 10, age-matched CD-1 mice received the oral challenge of 139A scrapie strain, without first receiving either the vaccine or the Salmonella vaccine strain. Mice were bled prior to the first vaccination (T0), the week prior to oral challenge with scrapie strain 139A (T1), and at the time of sacrifice (T Final). In addition to being bled, at the same time points feces were collected from the mice in PBS pH 7.2, 0.25% SDS/1mM PMSF in order to quantitate gut IgA titers, as we described previously (Goni et al., 2005). Mice were sacrificed when they scored positive for clinical signs of prion for three consecutive weeks using a test of motor co-ordination by observers blinded to the experimental status of the animals, using an established protocol (Goni et al., 2005; Pankiewicz et al., 2006). In addition, mice clinically healthy 400 days following challenge, were sacrificed to assess for the presence of sub-clinical prion infection. Mice were clinically assessed once a week starting 100 days following the 139A scrapie strain challenge. The analysis of clinical symptoms consists of observing the activity level and competency of the mice on an apparatus containing a series of parallel bars (3 mm in diameter) placed 7 mm apart. The initial clinical findings are a reduction in activity and/or the ability of the mice to traverse the parallel bars. This clinical endpoint correlates with the pathological development of CNS scrapie infection (Kimberlin and Walker, 1989; Aucouturier et al., 2001; Carp et al., 1984; Goni et al., 2005; Pankiewicz et al., 2006). The plasma samples were tested for specific antibodies against recPrP by enzyme-linked immunosorbent assay (ELISA). The brains from ketamine/xylazine anaesthetized mice were removed, and the right hemisphere was immersion-fixed overnight in periodate-lysine-paraformaldehyde, whereas the left hemisphere was snap frozen for Western blots. The fixed brain hemispheres were subsequently transferred to a solution containing 20% glycerol and 2% dimethylsulfoxide in 0.1 M sodium phosphate buffer, and stored at 4°C until sectioned. Serial coronal sections (40  $\mu$ m) were cut and series of sections at 0.2 mm intervals were obtained for histology. The diagnosis of prion disease was confirmed by staining brain sections with cresyl violet, immunostaining with a monoclonal anti-PrP antibody 6D11 (Spinner et al., 2007) and by the detection of proteinase K resistant PrP on Western blots as previously described (Sigurdsson et al., 2003a; Pankiewicz et al., 2006).

### Antibody Levels

IgG and IgA serum antibody levels to recPrP were determined by a 1:125 dilution of plasma in duplicate, in which 50  $\mu$ l/well of 1 $\mu$ g/ml mouse recPrP in 50 mM ammonium bicarbonate pH 9.6 was coated overnight onto microtiter wells. Antibodies were detected by a goat anti-mouse IgG or IgA linked to horseradish peroxidase (Bethyl Lab. Inc., Montgomery, TX ) and 3,3',5,5'-tetramethylbenzidine (Pierce Biotech. Inc., Rockford, IL) was the substrate.

In the feces supernatant extract, IgA levels to recPrP were determined in a 1:30 dilution of the extract in PBST, using the above protocol. The titers of specific anti-PrP IgA were normalized, correlated to the total IgA in each sample to account for the differing levels of collected feces and extracted IgA from these feces homogenates. The total IgA levels in each feces extract

( $\mu\text{g/ml}$ ) were determined using a mouse IgA quantitation kit and following the manufacturer's instructions (Bethyl Lab. Inc., Montgomery, TX).

## Data Analysis

The Kaplan and Meier survival curve of the vaccinated and control animals was analyzed by the logrank test (GraphPad Prism, version 4; GraphPad Inc., San Diego CA). The anti-PrP IgA and IgG level results were analyzed by two-way repeated measures ANOVA followed by Bonferroni post-hoc test (GraphPad Prism).

## Results

### IgG and IgA anti-PrP Response to Vaccination

At T1 the vaccinated mice were divided into groups depending on the serum IgG and feces IgA titer. A high serum IgG titer was defined as an ELISA OD reading at 450nm of  $>0.19$  from plasma diluted 1:125. A high IgA feces titer was defined as an ELISA OD reading of  $>0.2$  from a 1:30 diluted sample. These cut off values for IgG and IgA were chosen based on a retrospective analysis of data from our original mucosal vaccination study (data not shown), which indicated that these values tended to separate out animals with a prolonged survival versus those that did not (Goni et al., 2005). These cut off values were then applied prospectively for the mouse population reported in this manuscript. Of the 50 mice vaccinated with the LVR01 PrP $\times 2$  expression vector 14 were in the high IgG, high IgA group (28%); 10 in the low IgG, high IgA group (20%), 12 in the high IgG, low IgA group (24%) and 14 in the low IgG, low IgA group (28%). The plasma IgG titers in the 5 groups (the 4 vaccinated animals group plus controls) at T0, T1 and TF are shown in figure 1. Two-way ANOVA analysis showed that the difference between the groups was significant ( $p < 0.0001$ ). Bonferroni post-tests showed that the T1 values for the 4 vaccinated groups differed from their respective T0 values significantly ( $p < 0.001$ ). The plasma IgG values dropped at TF; however, they remained significantly above the T0 values ( $p < 0.001$ ). The gut IgA titers in the 5 groups (the 4 vaccinated animals group plus controls) at T0, T1 and TF are shown in figure 2. . Two-way ANOVA analysis showed that the difference between the groups was significant ( $p < 0.0001$ ). Bonferroni post-tests showed that the T1 values for the 2 vaccinated groups with a high T1 IgA titer differed from their respective T0 values significantly ( $p < 0.001$ ). The group with a high IgG and low IgA at T1 also differed significantly from the T0 IgA titer ( $p < 0.05$ ). At TF the two vaccinated groups with a high IgA at T1 differed significantly from their prior T0 value ( $p < 0.001$  for the high IgA and high IgG group;  $p < 0.05$  for the high IgA and low IgG group). The drop off of specific anti-PrP IgA and IgG titers at TF are consistent with the phenomena of immunosenescence and is expected. With aging specific humoral immunity responses are reduced while there is an increase in autoantibodies (LeMaout et al., 1997; Gruver et al., 2007); hence we observed a slight increase of anti-PrP titers in the old control non-vaccinated animals at TF representing an autoimmune response, while the specific anti-PrP IgG and IgA titers in the vaccine responder groups with initial high titers dropped off.

### Survival of Vaccinated Mice

Figure 3 shows the Kaplan and Meier survival curve of the different groups. At 400 days post-inoculation 100% of the animals in the high IgG, high IgA group ( $n=14$ ) were free from clinical symptoms ( $p < 0.0001$  using the logrank test [GraphPad Prism, version 4; GraphPad Inc., San Diego CA]). The two control groups that either received the *S. typhimurium* LVR01 without PrP expression or were not exposed to *Salmonella* were combined in a single control group, as there was no difference in survival between these control groups. By 205 days post-oral challenge with scrapie strain 139A all the animals in the control groups ( $n=20$ ) and in the low IgG, low IgA group ( $n=14$ ) had shown clinical signs of prion infection which were confirmed by Western blotting. The mice in the low IgG, high IgA group ( $n=10$ ) had a slightly longer

survival (median survival was 198 days versus 194 for the control group), but this difference was not statistically significant. In the high IgG, low IgA group, 4 out of the 12 mice (33%) were without clinical symptoms of infection at 400 days ( $p=0.02$  versus control group).

### Histological and Western Blot Evaluations

Histological and Western blot evaluations of all the brains of treated and control animals which were clinically ill, did not reveal any apparent differences in the degree of spongiform change or PrP<sup>Sc</sup> levels at the time of sacrifice. Figure 4A shows a representative section through the dentate gyrus of the hippocampus showing the characteristic spongiform change of prion infection in a control animal, while in a clinically asymptomatic animal (figure 4B) there is an absence of pathology. In addition, there were no signs of organ inflammation or other toxicity that could be associated with vaccination or autoimmunity evident by histological analysis (data not shown). The lack of PrP<sup>Sc</sup> in the brains of clinically asymptomatic animals was confirmed by Western blotting as can be seen in Figure 5. There was no apparent difference in the levels of PrP<sup>Sc</sup> in the brains of vaccinated animals that had a longer survival versus control animals. Hence, mucosal immunization with LVR01 expressing PrP in animals which ultimately showed clinical signs of infection reduced the dosage of PrP<sup>Sc</sup> entry and/or PrP<sup>Sc</sup> propagation, but ultimately similar pathology and PrP<sup>Sc</sup> levels were obtained.

### Discussion

The creation of vaccines is one of the greatest successes of medical and veterinary science. Currently, there is a great need for the development of a vaccine that is effective against exogenous prion infection. A mucosal vaccination approach is particularly suitable for prion infection, since the gut is the main route of entry of prion infection in many forms of prion disease (Beekes and McBride, 2007; Wisniewski et al., 2007). In our prior studies of mucosal vaccination for prion infection we used an attenuated Salmonella vector expressing one or two copies of murine PrP. CD-1 mice were thrice immunized intragastrically followed by oral challenge with 139A scrapie (Goni et al., 2005). We showed animals were able to mount an appreciable antibody response to endogenous PrP and significantly 30% of the mice had protection against infection. These results suggested that the type of antibody against PrP might be important when neutralizing the infectious prion particle.

In the present study we have optimized the vaccination protocol to obtain a more consistent immune response. This was done by increasing the number of immunizations (to six) prior to challenge with the prion agent, which resulted in higher anti-PrP antibodies compared to our previous study. Importantly, we also determined the specific anti-PrP systemic IgG and gut IgA response immediately prior to scrapie challenge. This information was used to separate the animals into low and high responders. We show that in the animal group with the comparatively high gut IgA and high serum IgG (figure 3) full protection against prion infection via an oral route was possible. The fact that animals in the low gut IgA and high serum IgG group had the same survival rate (33%) as in our previous study (Goni et al., 2005) suggests that factors besides the absolute quantity of the antibody response may be important for efficacy of the vaccination. Our results highlight the promise of an immunomodulatory approach to prevent prion infection.

It has long been known that the immune system plays an important role in prion infection (Aguzzi and Sigurdson, 2004; Aucouturier et al., 2000; Wisniewski and Sigurdsson, 2007; Beekes and McBride, 2007). Since the prion protein is a self-antigen present on the membranes of neurons and cells of immunological origin, the immune system typically is unresponsive to the infectious agent, acting paradoxically to aid the spread of infection. Many forms of immunosuppression have been shown to prolong the incubation period after peripheral infection (Aguzzi and Sigurdson, 2004; Aucouturier et al., 2000; Wisniewski and Sigurdsson,

2007; Beekes and McBride, 2007). However, in experimental settings components of the immune system are capable of quenching infection. The effectiveness of anti-PrP antibodies was first shown in vitro, where antibodies were able to clear neuroblastoma cells from prion infectivity (Enari et al., 2001; Peretz et al., 2001; Pankiewicz et al., 2006). In an in vitro experimental model system it was shown that transgenic mice expressing single-chain variable fragments of a monoclonal anti-PrP antibody could protect animals against an intraperitoneal infection with PrP<sup>Sc</sup> (Heppner et al., 2001). The first study to show that active immunization in a non-genetically altered mouse model could reduce the level of PrP<sup>Sc</sup> was published by Souan et al. (Souan et al., 2001), where PrP fragments with complete Freund's adjuvant (CFA) was used as an immunogen. It was shown in immunized mice, in which PrP<sup>Sc</sup> infected neuroblastoma cells were injected into the upper back area, that the level of PrP<sup>Sc</sup> in the neuroblastoma cells was reduced. In the first study more directly applicable to livestock or humans, it was shown that use of the whole mouse PrP recombinant protein with CFA as an immunogen was able to prolong the incubation period of disease; an effect that was dependent on the anti-PrP IgG titer (Sigurdsson et al., 2002). A number of subsequent studies have shown that tolerance to PrP can be broken in non-genetically modified mice resulting in modest anti-PrP titers (Gilch et al., 2003; Nikles et al., 2005). These titers could be greatly increased by using stronger immunogenic formulations, such as multiple antigen peptides using PrP peptide fragments (Arbel et al., 2003), CpG oligodeoxynucleotides as an immune stimulant (Spinner et al., 2007) and viral delivery systems (Handisurya et al., 2007). None of these immunization approaches have been tested to show effectiveness against prion infection challenge. However, two groups have shown that PrP fragments applied as immunogens in multiple booster protocols in wild-type mice can lead to slight prolongations of the incubation period of prion infection (Ishibashi et al., 2007; Arbel et al., 2003), consistent with the prior Sigurdsson et al. study (Sigurdsson et al., 2002). Although these various studies clearly demonstrate that it is possible to overcome self tolerance in mice to PrP and induce specific anti-PrP IgG, none of these approaches is capable of producing more than a modest prolongation of the incubation period of prion infection.

An ideal means of using immunomodulation to prevent prion infection is by mucosal immunization (Goni et al., 2005; Sakaguchi and Arakawa, 2007; Wisniewski et al., 2007). One important reason for this is that the gut is the major route of entry for prion diseases such as CWD, BSE and vCJD (Beekes and McBride, 2007). Furthermore mucosal immunization can be designed to induce primarily a humoral immune response, avoiding the cell mediated toxicity that was seen in the human AD vaccine trial (Wisniewski and Sigurdsson, 2007). In addition, mucosal vaccination has the advantage that it is unlikely to induce significant immune response within the brain. Although it has been shown that reduced levels or absence of CNS PrP<sup>C</sup> by, for example, conditional ablation by genetic manipulation of neuronal PrP<sup>C</sup> (Mallucci et al., 2007) can prevent clinical prion infection, it is likely that the immunological targeting of neuronal PrP would be associated with inflammatory toxicity. We first showed the successful use of mucosal vaccination in prion infection using a *Salmonella* delivery system (Goni et al., 2005). Live attenuated strains of *Salmonella enterica* have been used for many years as mucosal vaccines against salmonellosis and as delivery systems for the construction of multivalent vaccines with broad applications in human and veterinary medicine (Mastroeni et al., 2001). A main advantage for this system is that the safety of human administration of live attenuated *Salmonella* has been extensively confirmed in humans and animals (Tacket et al., 2000; Kirkpatrick et al., 2006). These bacterial vectors are genetically altered by multiple deletions and therefore are unable to revert to a pathological state. Ruminants and other veterinary species can be effectively immunized by the oral route using attenuated *Salmonella*, to induce humoral mucosal responses (Villarreal-Ramos et al., 1998; Chabalgoity et al., 2000). The potential effectiveness of using a mucosal vaccination approach has been subsequently confirmed by another group who used intranasal immunization in Balb/c mice with a recombinant PrP 90–231 fragment and cholera toxin as an adjuvant; although this approach only prolonged the

incubation period and did not protect animals following oral challenge of 139A scrapie (Bade et al., 2006). In the present study we show that after using a multiple immunization protocol and selecting mice with comparatively higher immune responses it is possible to attain complete protection against prion infection. We found considerable variation in the levels of the specific anti-PrP IgA and IgG among individual mice. This is likely to be in part related to the greater technical difficulties of administering a mucosal vaccine (Neutra and Kozlowski, 2006). There is variation in the intra-gut survival of the Salmonella, their expression of PrP and their ultimate penetration of the gut. Individual differences in the degree of immune responsiveness, also contributes to the final variation of anti-PrP levels. This variability in the response to our mucosal vaccine will need to be overcome prior to any widespread veterinary use. In addition, our results show that it is important to generate not only a gut IgA anti-PrP response but also a systemic IgG anti-PrP response. The comparatively high anti-PrP IgA might prevent or significantly reduce prion entry through the gut; whereas the systemic anti-PrP IgG could subsequently inhibit the replication of a reduced inoculum of prion agent. The fact that the presence of one of these components on its own was not sufficient to provide complete protection against oral challenge with prions supports this assertion.

Development of vaccination approach for conformational disorders that target self-antigens is a delicate balancing act between effective inhibition of the conversion process and lack of auto-immune side effects. Our reported results show that complete abrogation of prion infection is possible, at least in our model system, suggesting the promise of an active mucosal vaccination approach to prevent prion infection from an oral source. Further refinements to increase the degree of humoral immunity induced and to target the response more specifically against the PrP<sup>Sc</sup> conformation are underway.

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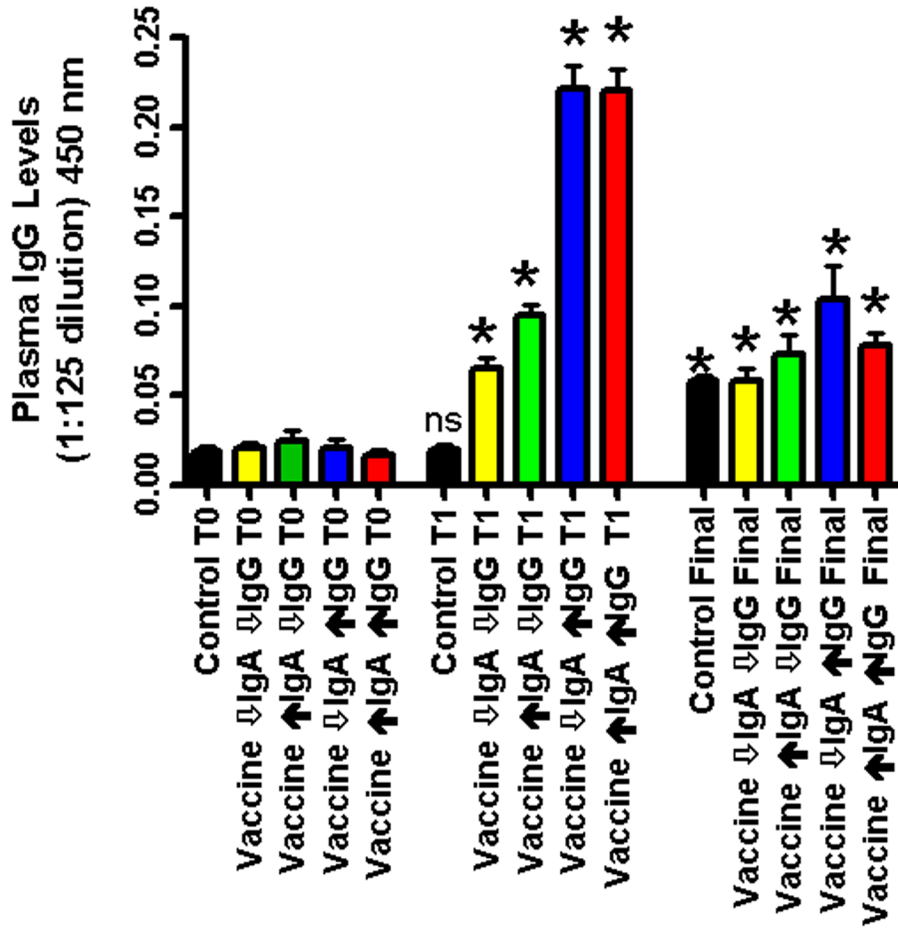
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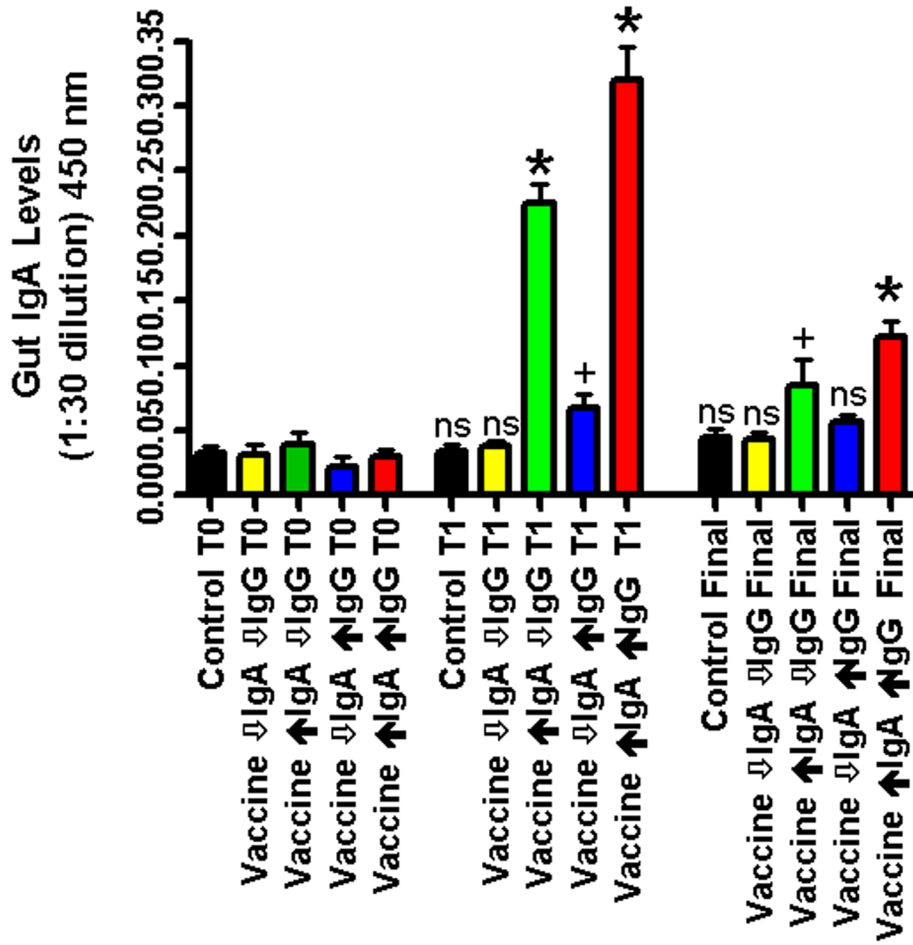
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## List of Abbreviations

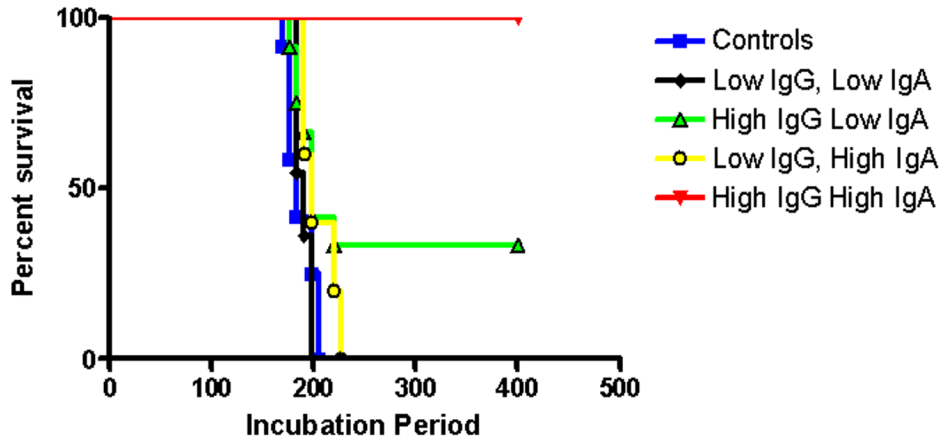
- PrP<sup>C</sup>, prion protein cellular  
PrP<sup>Sc</sup>, prion protein scrapie  
Ig, immunoglobulin  
BSE, bovine spongiform encephalopathy  
vCJD, variant Creutzfeldt-Jakob disease  
CWD, chronic wasting disease  
recPrP, recombinant prion protein  
CFU, colony forming units  
GI, gastro-intestinal  
PBST, phosphate buffered saline, 0.1% Tween-20  
GI, gastrointestinal



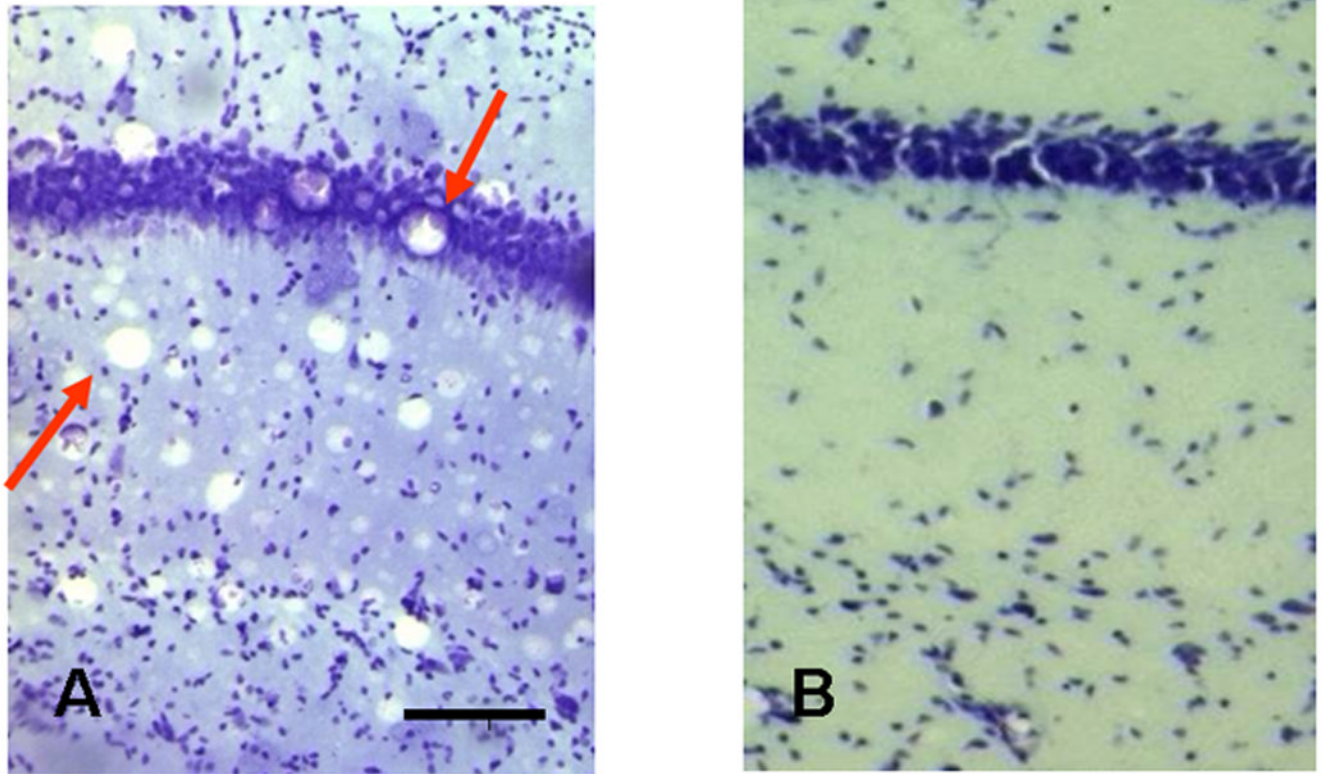
**Figure 1.** Shows the plasma IgG titers in the 5 groups (the 4 vaccinated animals group plus controls) at T0, T1 and TF. Two-way ANOVA analysis showed that the difference between the groups was significant ( $p < 0.0001$ ). Bonferroni post-tests showed that the T1 values for the 4 vaccinated groups differed from their respective T0 values significantly ( $p < 0.001$ ). The plasma IgG values dropped at TF; however, they remained significantly above the T0 values ( $p < 0.001$ ). (\*  $p < 0.001$ ; ns: not significant)



**Figure 2.** Shows the gut IgA titers in the 5 groups (the 4 vaccinated animals group plus controls) at T0, T1 and TF. Two-way ANOVA analysis showed that the difference between the groups was significant ( $p < 0.0001$ ). Bonferroni post-tests showed that the T1 values for the 2 vaccinated groups with a high T1 IgA titer differed from their respective T0 values significantly ( $p < 0.001$ ). The group with a high IgG and low IgA at T1 also differed significantly from the T0 IgA titer ( $p < 0.05$ ). At TF the two vaccinated groups with a high IgA at T1 differed significantly from their prior T0 value. (\*  $p < 0.001$ ; +  $p < 0.05$ ; ns: not significant)

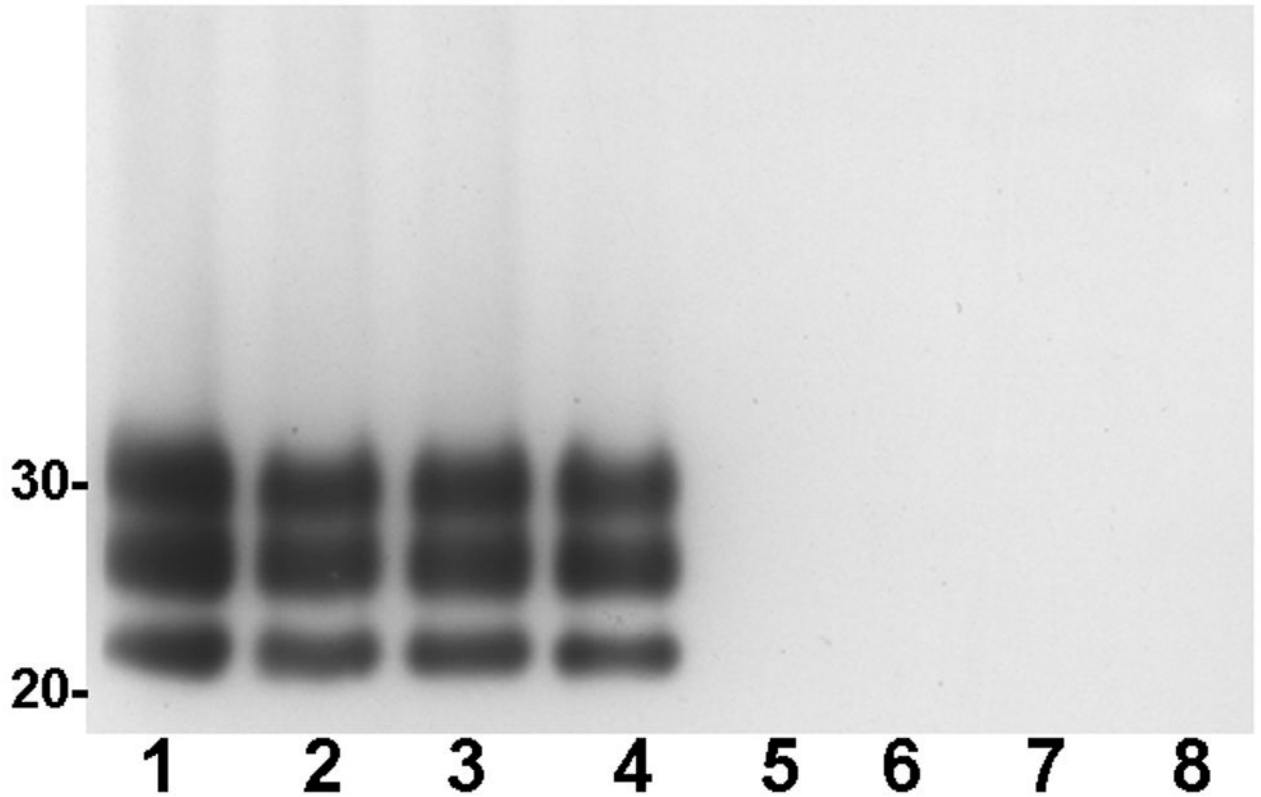


**Figure 3.** Shows the Kaplan and Meier survival curve of the different groups. At 400 days post-inoculation 100% of the animals in the high IgG, high IgA group (n=14) were free from clinical symptoms (solid red line in graph,  $p < 0.0001$  using the logrank test [GraphPad Prism, version 4; GraphPad Inc., San Diego CA]). By 205 days post-oral challenge with scrapie strain 139A all the animals in the control groups (n=20, solid blue line in graph) and in the low IgG, low IgA group (n=14, solid black line in graph) had shown clinical signs of prion infection which were confirmed by Western blotting. The mice in the low IgG, high IgA group (n=10, yellow short, long dashed line in graph) had a slightly longer survival (median survival was 198 days versus 194 for the control group), but this difference was not statistically significant. In the high IgG, low IgA group 4 out of the 12 mice (33%) were without clinical symptoms of infection at 400 days (green, short dashed line in graph,  $p = 0.02$  versus control group).



**Figure 4.**

A shows a representative section through the dentate gyrus of the hippocampus showing the characteristic spongiform change of prion infection in a control animal (see arrows). In a clinically asymptomatic animal (figure 4B) there is an absence of pathology. Scale bar = 100 microns.



**Figure 5.**

Shows a Western blot of proteinase K treated brain homogenates developed using anti-PrP 6D11 (Spinner et al., 2007). Each sample consisted of exactly 20  $\mu$ g of brain homogenate which was incubated with 1  $\mu$ g of proteinase K for 1 hr at 37°C, prior to loading onto gels. In lanes 1 and 2 brains from representative control mice were used. In lanes 3 and 4 brains from representative animals which were clinically sick and showed spongiform change on histological examination from the high IgA, low IgG group were used. There is no apparent difference in the level of the PrP<sup>Sc</sup> bands in lanes 1 and 2 versus 3 and 4. In lanes 5 through 8 representative brains from mice sacrificed 400 days post 139A challenge from the high IgG, high IgA group were used. These animals had no clinical signs of prion infection and no PrP<sup>Sc</sup> was detectable in their brains. The position of molecular weight markers is shown on the left side of the figure.