CHAPTER 1

LITREATURE REVIEW

HISTORY AND ECONOMIC IMPORTANCE OF *NEOSPORA CANINUM*

A *Neospora caninum* like parasite was first discovered in 1984 by Bjerkas et al., in Norway (15). It was then described as an unidentified sporozoan that caused neurological complications in dogs. It was subsequently characterized as *Neospora caninum* in 1988 by Dubey et al., (30) and isolated in cell culture (32). There after, diagnostic histological and serological tests were developed (65) and experimental infections of dogs carried out to demonstrate Koch's postulates (30). Although protozoal abortions had been rampant in Californian dairies since the mid 80's, it was not until 1989 that *N. caninum* was associated with bovine abortions (115). Later, *N. caninum* was also found to affect other species including cats, sheep, goats, mice, gerbils, monkeys, deer, camels and water buffaloes (29). Since then, the parasite has been recognized as an increasingly important cause of bovine abortions and neurological disorders in dogs. This disease had been recorded in Europe, in the Americas, Australia, New Zealand, U.K and some parts of Asia (31, 79). The dog is the definitive host of this parasite (66), although the economic importance of *N. caninum* disease is due to its role in reproductive failure in cattle, an intermediate host.

The economic implications of cattle neosporosis are both direct and indirect (31). The direct losses include cost of loss of the fetus, decrease in milk yield and weight gain, while the indirect costs include time for rebreeding, health costs and costs associated with culling. neosporosis is estimated to cause a loss of $35 million per year to the Californian dairy industry alone (4), $85 million to the Australian dairy industry and $25 million to the Australian beef industry (29).

LIFE CYCLE AND PREVALENCE

Bradyzoites are the nervous tissue cyst forms of the parasite found in affected cattle, dogs and other species. Tachyzoites are the rapidly replicating, asexual stage that can infect several

tissues like the spleen, liver and brain. It is speculated that, upon ingestion of contaminated meat, dogs become infected and excrete the oocysts in their feces, which may contaminate cattle feed (40). Infected cattle pass on the disease to their fetuses about 98% of the time (17, 127). Recrudescence of persistent infection in asymptomatic animals may occur during pregnancy (70, 95). Affected animals continue to transmit the organism to their offspring over several generations leading to cause persistence of infection in the herd (2). Several details concerning how natural transmission occurs, shedding and survival of oocysts in the environment are as yet unknown.

Anderson et al., have found that 42.2 % of aborted bovine fetuses in the U.S were infected with *N. caninum* (4). Sero- prevalence in countries where the disease is endemic varies. It is about 16% in the U.S.A (104), 64% in Brazil, 60% in Egypt and 34.6% in Italy (31). Incidence of *N. caninum* associated abortions ranged from about 12% in Argentina, 39.1% in Brazil, 21% in Switzerland and 77% in Germany (31). In another recent study, it was reported that at least one animal was sero-positive in 90% of the 93 herds examined and the prevalence of cows that were sero-positive for *N. caninum* varied from 2% to 65% among herds in the U.S (104). Due to its widespread incidence and lack of vaccines or control measures, losses caused by this disease have increased greatly, although two decades have passed since the identification of the parasite (3).

TRANSMISSION AND PATHOGENESIS OF *N. CANINUM* IN CATTLE

Two major routes by which transmission of *N. caninum* occurs in cattle are horizontal and vertical. Shedding of oocysts as a consequence of oral infection has not been demonstrated in all hosts. Experimental oral infection has been successful in mice and cattle(75) . Direct cow-to-cow transmission of the disease has not been demonstrated (5).

A comparative study to estimate the relative importance of vertical and horizontal transmission in an affected herd showed that the percentage vertical transmission was 95.2%. However, the percentage of horizontal transmission, as evidenced by sero-conversion of animals that were sero-negative at the beginning of the study, was only 3.8% (24). In a study in Netherlands, it was found that 80% of seropositive heifers produced congenitally infected calves.

Based on such current literature, trans-placental transmission appears to be single most important method of transmission of *N. caninum* (24, 107, 26).

Although vertical transmission occurs about 95% of the time, abortion does not always occur in infected cattle (76). The strain of the parasite, host-parasite relationship and genetic susceptibility of the host may also influence the outcome of infection (17, 88). Very little is known about the pathogenesis of the infection except that lesions are present in the placenta and central nervous system upon experimental infection of cattle and that fetal death can occur within four weeks post infection (17). It is believed that the time of infection may influence the outcome of the pregnancy. Cattle infected in early pregnancy are more likely to abort than cattle infected at mid-gestation, when the fetus is able to mount a more effective immune response (53, 74). Most cattle abortions occur in the second trimester of pregnancy (3, 11). About 5% of affected cows are known to have repeat abortions (126). Sero-positive animals are about 18.9% more at risk for abortion than sero-negative animals (57, 72).

Therefore, an ideal vaccine should be able to prevent vertical transmission and reduce abortions in infected cattle.

HOST IMMUNE RESPONSES

Although neosporosis is primarily a disease of cattle and canines, a large part of research regarding immunity to the disease has been carried out in mice. Since neosporosis is a disease of pregnancy, immune responses in both pregnant and non-pregnant animals have been studied in an attempt to elucidate the role of immune mechanisms in determining the two outcomes i.e vertical transmission or termination of pregnancy.

In non-pregnant cattle infected with *N. caninum,* cell-mediated immunity (CMI) involving IFN-γ and TNF-α seem to play an important role in protection. This was demonstrated by the ability of exogenously administered IFN-γ to inhibit replication of the parasite in bovine brain cells (52). Lymphocytes from infected animals, when stimulated with *N. caninum* antigen (125), exhibited proliferation and secreted IFN-γ. $CD4^+$ T cells have been identified as the key cell type involved in the CMI response (112). The role of antibodies in protection of cattle is undetermined. However, the presence of antibodies in infected animals is of diagnostic value (124).

Protective immune responses in non-pregnant mice are very similar to those in cattle. As in cattle, immunity to *N. caninum is* mediated through the $CD4^+$ T cell and IL-12 in mice (16, 103,112) in protective immunity against *Neospora caninum.* IFN-γ knockout mice are highly susceptible to *N. caninum* infection. IgG2a isotype antibodies are associated with protection because resistant strains of mice are found to develop higher levels of antibody of this isotype(71). The Th1/Th2 balance is important in determining whether the outcome of the disease will be acute or chronic (12, 83, 84). IL-4 seems to have a modulating effect on the toxic effects of IFN-γ, while IFN-γ is indispensable for protection. Increased IL-10 levels correlate with susceptibility. IL-10 is possibly induced by *N. caninum* as a mechanism to evade host IFN-γ dependent responses (84). Mice and cattle that had developed immunity before pregnancy seem to be better protected (69, 82, 94, 125) than those animals exposed to the pathogen during pregnancy. The exact role of antibodies in protection is unknown (36, 116) . However, B cell deficient mice succumb to infection while the wild type do not.

During pregnancy in cattle and mice, it has been found that expression of pro-inflammatory cytokines like IFN-γ and TNF-α levels are down regulated while regulatory cytokines like IL-10, TGF-β and IL-4 are up regulated (35, 92, 98, 110, 114, 123). Fetal trophoblasts and placental tissue secrete cytokines like IL-4, TGF-β and IL-10 (25) and a decrease in IFN-γ levels occurs during mid-gestation (54, 95). Protection against N. *caninum* is mediated by a Th1 response (89). Therefore, it is possible that the depression in the Th1 response is responsible for recrudescence of the disease in pregnant animals and leads to transmission of the parasite. Experimental evidence to prove this hypothesis has not been obtained in mice or cattle (101). However, IL-4 levels do appear to influence vertical transmission rates in mice. Vertical transmission was reduced in mice, previously exposed to *N. caninum* and administered anti-IL-4 antibodies in early pregnancy and then challenged. If there was no previous exposure, administration of IL-4 antibodies did not influence the rate of vertical transmission (70).

Both IgG1 and IgG2a isotype antibodies are present in serum of cows and mice infected with *N. caninum* during pregnancy. However, the IgG2a isotype is predominant in infected, pregnant cattle but in infected mice, the IgG1 levels increased during pregnancy to the same levels as that of IgG2a (6, 101). The relative importance of both antibody isotypes in prevention of vertical transmission or abortions is undetermined.

Based on available literature, rational design of a vaccine for *N. caninum* should be aimed at stimulating protective Th1 type immune responses in the host.

CONTROL OF *N. CANINUM*

Since *N. caninum* is transmitted very efficiently from mother to fetus; effective control of this disease is very complicated. Prevention of exposure of cattle to dogs shedding oocysts and prevention of consumption of infected fetal or placental remains by farm dogs may help to control the disease (27, 28). Culling of infected dams will eventually reduce the disease incidence but the herd may remain highly susceptible to renewed infection. Tolazuril is a chemotherapeutic agent that has been found to be successful in treating acute murine neosporosis, but has not been tested in cattle (60). Therefore, there are no available chemotherapeutic agents that are effective against *N. caninum* in cattle (41). Since neosporosis in adult cattle can be asymptomatic until abortion or stillbirth occurs, it would be difficult to determine when to administer a drug, even if one were available. Prevention of *N. caninum* infection is therefore, unarguably more cost effective and a better alternative.

PROTECTIVE ANTIGENS OF *N. CANINUM*

The knowledge of protective antigens of *N. caninum* in terms of structure and function has been of great importance to vaccine development against nesoporosis since it is the basis of designing effective strategies to interfere with the life cycle of the parasite. The intracellular life-style of Apicomplexan parasites has necessitated the evolution of several specialized, unique proteins in members of this genus.

The important surface antigens that mediate attachment and invasion include the GPI-anchored SAG [surface antigen gene] and SRS [surface antigen related sequences] proteins. There is a 25% homology between NcSAG1 and SRS2 and other SAG and SRS2 genes of apicomplexan parasites, while there is approximately 50% homology between the respective *T. gondii* homologs (49). These proteins are also highly immunogenic and are well recognized by sera from *N. caninum* infected animals (45-47). Monoclonal antibodies to SRS2 inhibit invasion

of trophoblast cells. However *T. gondii* SRS2 (58, 78) knock-outs are still capable of invading host cells indicating a redundancy in the function of these two proteins.

Dense granules are secretory vesicles whose contents are secreted into the parasitivorous vacuole. Localization of these proteins to the vacuole has lead to the deduction that these proteins that are associated with parasitivorous vacuole formation and nutrient acquisition. Some of them are nucleotide triphosphate hydrolases. The NcGRA1 (61) protein was found to have highest homology to *T. gondii* GRA7, while NcGRA2 (63) had highest sequence similarity to *T. gondii* GRA6. These proteins are also found in the supernatants of cultured cells infected with *N. caninum* (10).

Micronemes are secreted proteins that are localized at the apical end of the parasite and released when contact has been established with host cells. These proteins are recognized by serum from infected animals and are found in both the tachyzoite and bradyzoite forms. In *T. gondii*, binding of the tachyzoite to host cells leads to an alteration of the Ca^{++} ionic environment, which causes release of microneme proteins. Some of them contain adhesive domains, such as thrombospondin motifs that help cement the binding of the pathogen to the host cell. Several *N. caninum* microneme proteins such as MIC1, MIC3, M2AP and more recently, MIC4 have been identified and characterized. (59, 80,111). Several of these proteins have been administered as vaccines and have elicited varying degrees of protection against *N. caninum*. Although *N. caninum* has over eleven rhoptries, there is not much information available about the immunogenicity of rhoptrie proteins.

N. CANINUM PROPHYLAXIS BY VACCINATION

A large percentage of the cattle population in the U.S is sero- positive for *N. canium*. For a control program to be effective against cattle neosporosis, measures adopted should be able to interrupt the life cycle of the parasite. The possible ways by which this break can be achieved is to cull infected animals or immunize them with a vaccine that can prevent vertical transmission. In addition, a truly effective *N. caninum* vaccine would also have to be prophylactic in preventing infection in unexposed cattle and in reducing or preventing clinical signs that may include neurological involvement or abortions. Therefore, a large proportion of published literature regarding vaccine development studies for *N. caninum* have addressed one or more of

these aspects and used mice as the laboratory model. Fewer studies have been conducted in cattle, probably because of the prohibitive cost and difficulty of handling cattle.

Since *N. caninum* is an intracellular parasite cell mediated immune responses are considered important for protection, live vaccines have the obvious advantage of being able to stimulate CMI and are likely to persist long enough in the host to elicit long lasting protection. The various types of vaccines that have been researched so far include killed *N. caninum* tachyzoite vaccines; live attenuated vaccines, DNA vaccines, recombinant protein subunit vaccines and virus-vectored vaccines.

It has been shown that vaccination of mice, cattle or ewes with *N. caninum* tachyzoite lysates confers protection against vertical transmission in mice but not in cattle or ewes (56). The extent of this protection depends on the murine strains used for vaccinations and challenge (7, 8, 51, 62).

The one commercially available vaccine in the U.S is an inactivated tachyzoite formulation. Studies to determine the efficacy of this vaccine have been confined to cattle. This inactivated vaccine has been successful in eliciting strong antibody responses and is reported to be safe for use in cattle. It is also reported to reduce the rate of abortion by half (105). There is no available data on its efficacy in preventing primary infection or vertical transmission in cattle. Additions of polygen as an adjuvant for inactivated antigens did not enhance protection or induce complete protection against acute experimental challenge or vertical transmission in cattle and induced a Th2 type response. It has even been suggested that inactivated *N. caninum* vaccines exacerbate disease in mice (7, 8, 13, 56, 105).

A live, temperature sensitive mutant strain of NC-1 generated through chemical mutagenesis was successful as a vaccine by protecting against acute challenge in mice (67). Gamma irradiation of *N. caninum* tachyzoites is known to attenuate the parasite enough to prevent replication but still retain the invasive ability. Such irradiated tachyzoites, when used as a vaccine in mice, conferred complete protection against lethal challenge (99). Immunization with tachyzoites prior to pregnancy appears to confer protection against vertical transmission in cattle (54).

Vectored vaccines that have been developed against *N. caninum* include a vaccinia virus based vaccine expressing SRS2 or SAG1 and a canine herpes virus based vaccine-expressing SRS2 (48).The recombinant vaccinia virus carrying the SRS2 gene was successful in preventing

the disease as well as vertical transmission in mice. The vaccinia SAG1 vaccine did not induce significant levels of protection (85). However, the use of a vaccinia virus recombinant vaccine under field conditions may not be practical. The canine herpes virus based SRS2 vaccine was successful in eliciting antigen specific IgG responses in dogs but protection data is not available for this vaccine (81).

Cannas et al., found that vaccination of mice with DNA vaccines encoding SRS2 and SAG1 did not induce significant protection against acute disease. Protection improved when the DNA vaccines were combined with recombinant antigens. (18,64). Vaccination with a DNA vaccine encoding GRA7 or HSP33 provided partial protection against vertical transmission in mice (64). Addition of a CpG adjuvant improved the GRA7 vaccine's performance from 43% to 86% (55). Further, administration of the vaccine before gestation and boosting during gestation seemed to be more effective than vaccination during pregnancy (8, 54).

Vaccination with recombinant antigens, with or without adjuvant induced Th2 type immune responses and varying degrees of protection. A combination of SRS2 and GRA1 was found to be more efficacious than either antigen alone (21). The antibodies in the serum of mice vaccinated with recombinant proteins alone were only capable of recognizing the recombinant protein and not the native proteins. Vaccination with recombinant MIC1 or MIC3 in combination with RIBI adjuvant was found to confer significant protection against acute challenge in mice (1, 19). It was also reported that immunization with native SRS2 protein induced Th2 type responses and protected mice against vertical transmission (42, 43), while immunization with SRS2 as immune stimulating complexes (ISCOMS) reduced the intensity of infection in mice (93).

While considerable progress has been achieved in vaccine development against *N. caninum* a vaccine that fulfills all the required criteria to be successful in controlling bovine neosporosis i.e. prevention of disease on primary exposure, reduction of abortions, clinical signs and vertical transmission, is yet to be developed. The rapid spread of this disease, the persistance of *N. caninum* in herds and the heavy economic losses caused by it reiterate the urgent need for development of such an effective vaccine.

BRUCELLA ABORTUS STRAIN RB51 AS A VECTOR FOR HETEROLOGUS PROTEIN EXPRESSION

Brucella abortus is a gram-negative intracellular bacterium that causes abortions in cattle. Brucellosis was rampant in the United States in the early 1950's and caused losses of millions of dollars to the cattle industry. The brucellosis eradication program, which was launched in 1954, was instrumental in containing the spread of the disease. The main strategies followed included vaccination with a live attenuated strain 19 vaccine, culling of infected animals and regular serological testing (97). The major disadvantage of the S19 vaccine was that it elicited antibody responses that interfered with serological testing to identify infected cattle for culling. It was also found to occasionally induce abortions in pregnant animals (109).

B. abortus strain RB51 (sRB51) is a stable, rough mutant derived from the virulent strain 2308 (108). It was derived by serial passage of the wild-type strain on rifampicin containing media. It has the major advantage that it does not induce antibodies that interfere with testing. Extensive studies have been carried out to establish the efficacy and safety of this strain as a vaccine (20, 73, 87, 106). Strong Th1 type of immune responses that involve cell mediated immunity and IFN-γ secretion are known as the driving mechanism behind the vaccine efficacy (9, 91, 119). Moreover, heat killed *Brucella* conjugated to a HIV peptide is reported to have stimulated a strong Th1 $CD8^+$ T cell response in MHC-II knock out mice (14, 37, 38, 50,39). Oral administration of sRB51 is known to induce mucosal immune responses (113).

Brucella abortus strain RB51, which is already a successful commercial vaccine, is also efficient as a host for the expression of heterologus proteins (119). The reporter protein, *E. coli* β galactosidase and the HSP65 protein from *Mycobacterium sp* (119) have been expressed in sRB51. Mice vaccinated with sRB51 expressing β-galactosidase (β-gal) developed a β-gal specific IgG2a response and stimulation of splenocytes with β-gal in-vitro induced IFN-γ secretion but not IL-4. Similarly, mice vaccinated with sRB51 expressing HSP65, developed a Th1 profile in the immune response to HSP65, rather than a Th2 profile. Subsequently, several other heterologus proteins, including a few from eukaryotic organisms, have been expressed in sRB51 and vaccination with such recombinant strains has induced a Th1 response to the expressed proteins (44, 90, 117, 118, 120-122).

Immunity to neosporosis involves induction of a potent Th1 type of immune response (12). Therefore testing the hypothesis that *B. abortus* strain RB51 may be an excellent host to express *N. caninum* protective antigens and that vaccination with such recombinant strains is likely to induce a strong Th1 type of response in mice, has been the primary focus of this dissertation. The ability of the recombinant sRB51-Neospora vaccine in preventing acute neosporosis and vertical transmission in mice has also been evaluated.

ANIMAL MODELS FOR *N. CANINUM* RESEARCH

The strain of the parasite and the animal model used for experimental work with *N. caninum* can have a profound influence on the outcome of the research. Mice have been the laboratory animals of choice for the study of *N. caninum* biology, immune responses and vaccine development. Mice are less expensive than sheep, goats or dogs, their tissues are susceptible to tachyzoite invasion, and they develop immune responses that are similar to cattle infected with *N. caninum*. Moreover, they effectively transmit the parasite to their offspring. Mice are the only lab animals for which several gene knockout strains are available, rendering them an indispensable resource immunologic research.

However, the different strains of mice vary greatly in their susceptibility to *N. caninum.* Several mouse strains are resistant and do not exhibit clinical signs of neurological disorders, apathy and mortality. Some strains of mice develop tissue cysts while others do not (102). Out bred Swiss-Webster mice are resistant, while CBA mice develop tissue cyst ten weeks after infection. Qc mice are relatively resistant to experimentally induced neosporosis but are efficient in transmitting the parasite to their offspring (96).

In a study comparing Balb/c, C57BL/6 and B10.D2 mice, Long et al., (71) found that Balb/c and C57BL/6 mice were susceptible to *N. caninum* induced encephalitis while B10.D2 was not. The susceptibility was associated with high IL-4, low IFN-γ, and low IgG2a isotype antibody levels in these two strains of mice (23, 68, 71, 96, 100, 102).

The Balb/c strain of mice has been most commonly used by researchers for investigating the biology and immune responses to *N. caninum* (85). Although Balb/c mice develop encephalitis when infected with *N. caninum* they do not show clinical signs of the disease. C57BL/6 mice on the other hand show clinical signs of the disease, and succumb to lethal

challenge (18, 19). In the above-mentioned experiments infection was carried out by either the intraperitoneal or sub-cutaneous routes

Information regarding vertical transmission in C57BL/6 mice is not available. Swiss Webster mice are reported to have a vertical transmission rate of 85% (68). Sixty-two percent of the litters were infected and on an average 85% of pups in each litter were infected. The percentage transmission of *N. caninum* in Qs mice was determined to be 75% (77). Balb/c mice infected during pregnancy have a vertical transmission rate of 76%, while mice infected before pregnancy had a rate of 50%. Infection of mice before pregnancy appeared to increase the rate of fetal resorptions when compared to infection on day 5 of pregnancy. Reproductive losses could be considered to be a definitve measure of vertical transmission in Balb/c mice (86).

Gerbils are more susceptible to *N. caninum* infection than mice (33). Therefore, it is possible to have a clear-cut lethal challenge model using gerbils for the study of vaccines or chemotherapeutic agents (100). However, no data is available regarding vertical transmission in gerbils. The major disadvantage of using gerbils as a lab animal model for *N. caninum* is that immunological reagents such as antibody conjugates are not readily available for testing of gerbil sera.

Therefore, it is important that an animal model having consistent parameters like an exact challenge dose with known sequelae to challenge, cytokine profiles, antibody responses and percentage vertical transmission be available for carrying out vaccine research with *N. caninum.* As a part of this dissertation the use of gerbils and C57BL/6 mice as animal models to study various aspects of vaccine development against *N. caninum* have been optimized

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CHAPTER 2

DEVELOPMENT OF ANIMAL MODELS FOR *N. CANINUM* VACCINE TESTING

CHAPTER 2-1

GERBIL MODEL OF ACUTE NEOSPOROSIS

Sheela Ramamoorthy, N. Sriranganathan and David S. Lindsay. Vet. Parasitol. 2005 Jan 20; 127(2): 111-4.

ABSTRACT

Experimental infections with the NC-1 strain of *Neospora caninum* were conducted in gerbils (*Meriones unguiculatus*) to determine their acute responses to experimental intraperitoneal infection. Five groups of five female gerbils were used and they were intraperitoneally infected with 1 $x10^6$, $2x10^6$, $3x\ 10^6$, 4 $x10^6$ or 5 $x\ 10^6$ tachyzoites. Gerbils in all groups developed clinical signs of neosporosis, which consisted of inactivity 4–5 days post-inoculation. Morbidity and mortality were observed in all groups. Grossly there was clear fibrinous exudate in the abdominal cavity and adhesions of the spleen and pancreas to the stomach in gerbils suffering from acute neosporosis. The LD_{50} was calculated as 9.3 $x10^5$ tachyzoites per gerbil. The results indicate that gerbils can be used as a suitable model of acute neosporosis. This model can be used to screen candidate treatments and to test the efficacy of vaccines for neosporosis without the need to use histology or PCR to demonstrate treatment efficacy.

INTRODUCTION

Bovine neosporosis is a common cattle production problem worldwide and caused by the protozoan parasite, *Neospora caninum* (9). We are interested in developing effective drug treatments and vaccines to prevent neosporosis. A model that provides clear-cut results in a rapid

manner based on acute infection like that which occurs after inoculation of RH strain or other genotype I strains *Toxoplasma gondii* tachyzoites (17) would be extremely helpful in developing treatments and vaccines for neosporosis. Out-bred mice are resistant to neosporosis (9) and inbred strains are only moderately susceptible to acute neosporosis. Vaccine and drug development studies using mouse models, therefore, vary in the strain of mice used and in the challenge dose (1, 3, 4, 12, 13, 16, 19, 21, 22). This variation in test design makes it difficult to compare the relative efficacy of anti- *Neospora* vaccines and drugs. Models that rely on long observation times post-inoculation and histology or PCR are time consuming and often cost prohibitive.

Gerbils are a logical candidate for a model of acute neosporosis because they are more susceptible to neosporosis than mice. Dubey and Lindsay (8) have demonstrated that gerbils are highly susceptible to severe neosporosis following oral inoculation of *N. caninum* oocysts and others have used this model to demonstrate infectivity of *N. caninum* oocysts (2, 6). Unfortunately, dogs do not excrete many *N. caninum* oocysts in their feces (15, 18, 20) making vaccine and challenge studies difficult to conduct using oocysts. Several studies have demonstrated that gerbils are also susceptible to tachyzoite induced *N. caninum* infections (5, 11) but little quantitative data have been presented on the responses of gerbils to *N. caninum* infection. The present study was done to determine the pathogenicity of the NC-1 strain (7) of *N. caninum* for gerbils given various doses of *N. caninum* tachyzoites by the intraperitoneal (23) route. The NC-1 strain is used worldwide in neosporosis research and is available from the American Type Culture Collection, Manassas, VA (ATCC # 50843). The results indicate that gerbils can be used as a suitable model of acute neosporosis.

MATERIALS AND METHODS

Tachyzoites of the NC-1 strain of *N. caninum* were grown in African green monkey (Cercopithecus aethiops) kidney cells (CV-1 cells, ATCC CCL-70, American Type Culture Collection, Manassas, VA) and processed for inoculation as previously described (14). Female, 6 week-old gerbils were housed as groups of five per cage and fed rodent chow and water *ad*

libitum. They were IP infected with 1x 10^6, 2x 10^6, 3 x 10^6, 4 x10^6 or 5 x 10^6 NC-1 strain tachyzoites in 0.5 ml of Hanks' balanced salt solution without calcium and magnesium (HBSS). Gerbils were examined daily for clinical signs. The study was terminated 20 days post-inoculation (PI).

RESULTS AND DISCUSSION

Gerbils that survived more than 12 days PI lived until the end of the study. Data on mortality days PI are presented for all gerbils in Table 2.1.1. At necropsy of animals that died or were killed because of acute infection, 0.5–1.5 ml of clear fibrinous exudate was present in the abdominal cavity. The carcasses appeared to be dehydrated. Microscopic examination of the liquid collected from the abdominal cavity indicated that numerous inflammatory cells and tachyzoites were present. The pancreas and spleen were usually firmly adhered to each other and to the stomach. The capsule of the liver and spleen appeared thickened and opaque. The intestines were intact. Lungs, heart, and thymus appeared normal. The brains were not examined grossly because of the acute nature of the infections in most gerbils. Gross examination of gerbils killed 20 days PI was not remarkable and no exudate was present in the body cavity. The LD_{50} was calculated using the method of Spearman and Ka¨rber (10). It was determined to be 9.3 x10^5 tachyzoites per gerbil.

The LD_{50} is a widely accepted tool in vaccine and toxicity studies. It is of particular use to manufacturers as it is prescribed in most international pharmacopeias as a test to measure the efficacy of vaccines and drugs. The use of a single challenge strain at the optimal challenge dose in gerbils will help to eliminate variations in data interpretation while evaluating vaccines for neosporosis. The disadvantage of the gerbil model is that it only measures acute infection. Most immunocompetent laboratory rodents are resistant to clinical *N. caninum* infection. Rats, *Psammomys obesus* and Tristrami's jirds, *Meriones tristrami* are susceptible to fatal neosporosis, following IP inoculation of tachyzoites. Fibrinous perotinitious similar to that observed in the present study was reported in both sand rats and jirds (9, 23).

Gerbils are readily available in most developed countries and inexpensive to purchase. This availability and affordability make them good subjects for experimental studies on

neosporosis. The gerbil model presented in this study should be useful in examining and screening chemotherapeutic agents against acute neosporosis. The results are clear cut and routine studies on dose titrations and dose confirmations of chemotherapeutic agents can readily be conducted using IP inoculation of tachyzoites. The intact immune system of gerbils is also advantageous because they can mount an immune response and vaccines against acute neosporosis can be tested using this system. The clear-cut results obtained in a short period of time are an advantage over present rodent models that rely on histological or PCR examination of tissues (12, 21). The lack of reagents for examining TH1 and TH2 immune responses maybe a limiting factor in the use of this gerbil model for the examination of immune mechanisms in response to vaccination but the model can still be used to determine efficacy of vaccination against acute neosporosis.

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Table 2.1.1: Results of intraperitoneal inoculation of gerbils with tachyzoites of *N. caninum*

Gerbil No	Dose of tachyzoites				
	$1x10^6$	**$2x10^6$**	**$3x10^6$**	**$4x10^6$**	**$5x10^6$**
1	D8	D7	D6	D7	D7
2	D10	D9	D7	D8	D8
3	D11	D9	D11	D12	D9
4	D12	K20	K20	K20	D10
5	K20	K20	K20	K20	D10

D- Died, K - Killed

CHAPTER 2-2

OPTIMIZATION OF THE USE OF C57BL/6 MICE AS A LABORATORY ANIMAL MODEL FOR *NEOSPORA CANINUM* VACCINE STUDIES.

S. Ramamoorthy, R.Duncan, D. S.Lindsay and N. Sriranganathan. Under review by Veterinary Parasitology

ABSTRACT

Testing the protective capacity of vaccines for *Neospora caninum* in mice require challenge studies to demonstrate a reduction in severity of clinical signs or prevention of vertical transmission of the parasite, due to vaccination. Laboratory mouse strains differ in their susceptibility to *N. caninum.* The choice of the strain used for research profoundly influences experimental outcome. In this study, C57BL/6 mice were evaluated as a model for *Neospora* vaccine studies.

A lethal challenge model for vaccine testing was developed and the LD_{50} was determined to be 1.5 x 10^7 *N. caninum* tachyzoites/mouse, delivered intraperitoneally. A histological scoring system, involving brain tissue, was developed to determine the extent of protection due to vaccination in sublethally challenged mice.

A vertical transmission model for *N. caninum* vaccine studies was developed by using mice that were infected either two weeks prior to mating or between days 12 –14 of pregnancy. It was found that infection prior to mating reduced the average number of pups per litter. The rate of vertical transmission was 0%, 100% and 90.5 % for the uninfected controls, mice infected during pregnancy and mice infected before mating, respectively, as determined by a *N. caninum* specific PCR.

This study demonstrated that the C57BL/6 strain of mice is a good model for *N. caninum* vaccine studies because it is possible to establish a clear-cut lethal challenge model; moreover, C57BL/6 mice transmit the disease to their offspring at rates that are comparable to other strains of mice.

INTRODUCTION

The apicomplexan parasite *N. caninum* has emerged as a leading cause of bovine abortions during the last decade in the U.S.A. The dairy and beef industries in several parts of the world are severely affected by this disease (9, 29). Mice have been commonly used as a model in experiments conducted to study the biology of this parasite and develop vaccines against this disease (3, 13, 15, 16).

Testing of vaccines and chemotherapeutic agents for their efficacy in preventing neosporosis in mice invariably involves the administration of the treatment followed by challenge with the virulent organism. Most international pharmacopeia standards for vaccine testing require standardization of the challenge dose as a multiple of the lethal $dose_{50}$ (2) of the infectious agent for the laboratory animal used in the study. Such standardization will reduce test-to-test variability and ensure a fair comparison of various vaccines that are developed (23). Researchers working on vaccine development for *N. caninum* have not documented the use of such a lethal-challenge model so far, possibly because most strains of mice do not exhibit clinical signs of neosporosis. Several strains of mice, such as outbred Quakenbush (Qs), inbred Balb/c and C57BL/6 mice have been administered varying challenge doses in vaccine development studies for *N. caninum*, rendering a reasonable comparison of the vaccines impossible (4, 14, 18)

Vaccine efficacy in mice has commonly been determined by sublethal challenge and comparison of protection between the unvaccinated controls and vaccinated mice. The criteria used for such comparison have been the presence or load of the organism in brain tissue as detected by PCR, real-time PCR or immuno-histochemistry (1, 19). The advantage of PCR lies in its sensitivity. However, only a small part of the tissue that is examined would be represented in the result. Moreover, a correlation between the number of parasites in a given tissue and disease severity has not been established (1, 3). In this study, we have attempted to address these issues by developing a histopathological scoring system to determine severity of the disease. This system could be combined with PCR analysis to obtain a more comprehensive protection index.

Since the primary mode of transmission of *N. caninum* is transplacental, the true test of any effective vaccine developed for this disease would lie in its ability to prevent vertical transmission (8). Several strains of mice such as Qs and Balb/c mice have been used in

published research describing vaccine development studies aimed at preventing vertical transmission of *N. caninum*. They were administered varying challenge doses at different times during pregnancy (18, 20). The rates of vertical transmission in these strains of mice varied between 50–91%. Fetal mortality has been correlated with the occurrence of vertical transmission in Balb/c mice (20). However, no such data is available regarding vertical transmission in C57BL/6 mice.

When all the strains of mice used for *N. caninum* vaccine studies thus far are compared (1, 17, 18), it is evident that C57BL/6 mice are probably the most suitable as they not only show clinical signs of the disease but also transmit the parasite to their pups. In this study, we have refined the use of this strain of mice for *N. caninum* vaccine studies in terms of developing a lethal challenge model and a histopathology scoring system to quantify the effects of vaccination and challenge. In addition, we have developed a C57BL/6 pregnant mouse model for vertical transmission studies.

MATERIALS AND METHODS

Parasite culture

NC-1 strain of *N. caninum* was cultured in CV1 cells (ATCC CCL-70, American type culture collection, Manassas, VA) and tachyzoites were purified and counted using previously described protocols (10)

Infection of mice for determination of the LD_{50}

For the first set of titrations, four groups of five 4-6 week old C57BL/6 mice each were inoculated with $1x10^5$, $1x10^6$, $1x10^7$, and $1x10^8$ tachyzoites intraperitoneally. For the second titration, doses of $5x10^6$, $1x10^7$, $2x10^7$, $3x10^7$ and $4x10^7$ tachyzoites were used. All mice were observed for 21 days for clinical signs and mortality.

All animal experimentation was carried out in compliance with the regulations of the animal welfare board of Virginia Tech and the AVMA guidelines, including euthanasia of all animals exhibiting severe signs of debility, whenever warranted.

Histopathology and scoring system

Brain, spleen, lung, heart, muscle, liver and uterus tissues from the mice infected with a lethal challenge dose of 2.5 x 10^7 tachyzoites and a sublethal dose of 5 x 10^6 tachyzoites were examined histologically. Tissues were fixed in 10% buffered neutral formalin (Fischer Scientific, NJ), embedded in paraffin, sectioned at 5μm, and stained with hematoxylin and eosin. Tissue sections were examined by a board certified pathologist, in a double-blind format. Brain lesion scores for sub lethally challenged mice were assigned on the basis of severity of lesions based on a 4-point scale. The lesions were scored by the following scheme: 0 = No lesion. 1 = minimal lesion limited to lymphoplasmacellular meningitis and perivasculitis. 2 = mild lesion including meningitis, perivasculitis, and focal glial cell activation. 3 = moderate lesion including meningitis, perivasculitis, glial cell activation, and rarefaction of the neuropil with macrophage infiltration. 4 = severe lesion including meningitis, perivasculitis, glial cell activation, rarefaction of the neuropil, and focally extensive necrosis. The total number of lesions per sample was divided by the number of sections counted for that sample. The average of the product of the severity score and the number of lesions was used for final data analysis.

Vertical transmission C57BL/6 mouse model

Three groups of seven mice each were used in the study. Mice were randomly assigned to the following groups a) uninfected control mice, b) mice infected with 5x10^6 *N. caninum* i.p. two weeks prior to breeding (designated as the therapeutic model) and c) mice infected with 5x10^6 *N. caninum* i.p between the 12-14^{th} days of pregnancy (designated as the prophylactic model). Fetuses were collected in-utero between days 18-20 of pregnancy and stored at -80^{o}C for PCR detection of *N. caninum.*

PCR for detection of *N. caninum* in fetal tissue:

The entire fetus was macerated in 10% W/V of sterile PBS using a tissue grinder. 200μl of the suspension was used for DNA extraction with the DNA mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. DNA content of each sample was measured using the

Pico Green Kit (Molecular Probes, Eugene, Oregon). 600 ng of DNA was used for each PCR reaction. *N. caninum* specific primers were designed to amplify a 375bp amplicon or a 75bp ampliConAs described in published literature (7, 30). DNA extracted from the fetus of an un-inoculated mouse housed in another room was used as a negative control and DNA extracted from *N. caninum* tachyzoites was used as a positive control. PCR was carried out using the following conditions; 95^{o}C for 5 min, 95^{o}C for 1 min, 50.4^{o}C for 30 sec, 70^{o}C for 30 sec, for 35 cycles followed by extension at 70^{o}C for 5 min. Samples were analyzed on a 1% agarose gel for the presence of a 375 bp or 75 bp *N. caninum* gene 5 amplicon.

Statistical analysis

The LD_{50} was calculated by the Reed and Muench method (22). Differences between the treatment groups for histopathological scoring, the number of pups born in the acute and chronic infection models and the rate of vertical transmission were analyzed by a student T test using the Microsoft Excel package. The statistical level of significance for all tests was set at $p = 0.05$.

RESULTS AND DISCUSSION

In the first set of titrations for the calculation of the LD_{50} for *N. caninum* tachyzoites in C57BL/6 mice, $1x10^5$, $1x10^6$, $1x10^7$, and $1x10^8$ tachyzoites were administered intra-peritoneally in 1ml volume to five mice each. Since all the mice in the $1x10^7$ group survived and none of the mice in the $1x10^8$ group survived, we presumed that the LD_{50} did lie between 10^7 and 10^8 tachyzoites. Therefore, doses of $5x10^6$, $1x10^7$, $2x10^7$, $3x10^7$ and $5x10^7$ were used for the second titration. All of the mice in the $1x10^7$ group survived for 21 days and did not exhibit signs of the disease. All of the mice administered $2x10^7$ tachyzoites died within seven days. Combined data from both titrations are represented in Table 2.2.1. The LD_{50} as determined by the Reed and Muench method was 1.5×10^7 tachyzoites per mouse. Counting of tachyzoites using the hemocytometer is a procedure that is subject to individual experimental error. We, therefore, recommend the use of 2.5×10^7 (1.1 LD_{50}) tachyzoites for lethal challenge experiments. At this

dose 100% mortality was observed in unvaccinated controls within 10 days. It is also evident that extreme care should be taken in preparing the challenge inoculums, as small change in the dose can result in no mortality being observed.

Gottstein et al., (12) have documented the use of a challenge dose of 1 x10^6 tachyzoites of the NC-1 strain of *N. caninum* in C57BL/6 mice as resulting in mild clinical signs of apathy and neuro-muscular disorders in 50% of mice between days 6 to 18 of administration. The authors appear to have chosen this dose as the levels of infection induced, as assessed by PCR, were satisfactory and severe signs that would require mandatory euthanasia could be avoided. The highest dose used in the titration curve was 1x10^7 and at this dose, mice were severely affected until day 20 (12). However, in our study, all the mice administered 2x10^7 tachyzoites died within seven days, while mice administered 1 x10^7 tachyzoites did not. Further, we have been unable to observe clinical signs of the disease in infected mice at a dose of 1x10^7 tachyzoites. A possible explanation for this that our NC-1 strain could be attenuated to some degree due to continuous passage in cell culture. Therefore, it would be prudent for every laboratory to titrate their stock culture before attempting a lethal challenge experiment. The major disadvantage of this method is that purification of a sufficient number of parasites to meet the high challenge dose requirement is laborious. However, the lethal challenge model eliminates the need for a long waiting period for the analysis of tissue samples by PCR or histological methods.

Although there is universal agreement on the need for reduction of the number of animals used for in-vivo testing and abolition of procedures with a lethal end-point, development of alternatives to these tests have not been completely successful. Lethal end-point tests continue to be prescribed for potency testing of vaccines by regulatory bodies such as the OIE (Office de Epizootics) and FDA (Food and drug administration) (24,2, 23).

We had previously described a lethal challenge model for *N. caninum* in gerbils (25). The major disadvantage of using gerbils in *N. caninum* vaccine studies is that immunological reagents such as gerbil specific antibody conjugates are not readily available for this species. An advantage of the gerbil model when compared to the C57BL/6 mouse model is the lower challenge dose (5x10^6 tachyzoites) required to induce mortality, thus reducing the labor involved in preparation of the challenge culture

Mice inoculated with a sub lethal dose (5x10^6) of *N. caninum* tachyzoites did not show clinical signs and survived throughout the observation period. The most significant lesions found

on histopathological examination of their tissues revealed mild/moderate to severe lymphoplasmacellular and granulomatous meningoencephalitis with rarefaction in the brain (Figure 2.2.1 c&d) and mild multi-focal chronic lymphoplasmacellular peritonitis. No significant lesions were found in other organs like liver, spleen or lungs. Therefore, the brain was the organ of choice assigning scores to determine the protection due to vaccination in mice infected with a sub lethal dose of *N. caninum* tachyzoites.

We have demonstrated the use of this scoring system in a study where irradiated *N. caninum* tachyzoites were tested as an attenuated vaccine (27). When the sub lethal challenge model was employed for vaccine testing, scoring of the brain lesions, by the protocol described in the methods section, resulted in an average score of 11.3 for the unvaccinated mice and an average score of 0.5 for mice vaccinated with irradiated tachyzoites.

Mice vaccinated with irradiated *N. caninum* tachyzoites and challenged with a lethal dose of virulent *N. caninum* survived without mortality for 3 weeks post-challenge, while unvaccinated mice died within seven days (27). Histopathological examination of tissues from mice that died from lethal challenge did not reveal any pathological changes in the brain, possibly because the time from inoculation to death was too short and death occurred before brain lesions could develop. The only significant lesions were severe peritonitis and mild, multi-focal, acute hepatocellular necrosis (Figure 2.2.1 1a&1b). It appears that the cause of death in acute murine neosporosis is not related to brain pathology but rather to peritonitis and possibly acute failure of other organs. Similar findings have been reported by others by using PCR and immuno-histochemistry to quantify infection intensities in various tissues (11, 12).

A histopathological scoring system has not been used as a tool to determine the effects of vaccination in *N. caninum* vaccine studies so far. While the clear-cut results that are obtained from the lethal challenge model preclude further histopathological examination, such analyses could be more useful in sub lethal challenge studies. It is less laborious than PCR based methods that require tissue DNA extraction and can be representative of the entire organ used for examination.

Although *N. caninum* vaccines are initially tested in mice, their final intended use is in cattle. In a recent study, Rodriguez et al., (26) reported that at least one animal was sero-positive in 90% of the ninety-three herds examined and that prevalence of cows that were sero-positive for *N. caninum* varied from 2% to 65% among herds (26). For a control program to be effective

these infected animals or their female offspring would either have to be culled or the vaccine used in the program should be able to prevent vertical transmission. Therefore, a truly effective *N. caninum* vaccine would have to be therapeutic in preventing vertical transmission in exposed cattle and be prophylactic in preventing infection in unexposed cattle. Since cattle are the target species of all *N. caninum* vaccines, two types of challenge models were evaluated for the C57BL/6 vertical transmission study. In the therapeutic model, mice were infected with a $5x10^6$ *N. caninum* tachyzoites two weeks prior to mating. In the prophylactic model, mice were infected with the same sublethal dose of *N. caninum* between days 12-14 of pregnancy. This time point was chosen due to the fact that fetal resorptions have been found to be high in mice infected between day 5-10 of pregnancy, causing a reduction in sample size (17).

Five out of seven (71.42%) mice in the uninfected control group and 4 out of 7 (57.14%) mice in the prophylactic model group were found to have sperm plugs after breeding (Table 2.2.2). The average rate of conception for C57BL/6 mice was calculated as 64.28%. Therefore, to obtain the desired sample size of pregnant mice, it would be advisable to initiate experimentation with twice the required number of mice. Four out of 7 mice (57.14%) that were infected before mating conceived. Prior infection with *N. caninum* did not appear to have a detrimental effect on conception.

However, the average number of fetuses collected per pregnant dam in the therapeutic model was 5.25, while that in the uninfected control group and prophylactic model were 8.2 and 8.0 respectively (Table 2.2.2). This reduction in the number of fetuses, which possibly occurred because of increased fetal resorption due to *N. caninum* infection, was statistically significant. Researchers intending to study the therapeutic effect of their vaccine would have to take this effect on sample size into account.

When the percentage of vertical transmission was measured by a *N. caninum* specific PCR of DNA extracted from fetal tissue homogenates, the rates of transmission were 0%, 100% and 90.48% in the uninfected control group, prophylactic group and therapeutic group respectively. Swiss Webster mice were reported to have a transmission rate of 85% (6) , Qs mice 91%(18) and Balb/c mice infected during pregnancy had a transmission rate of 76%, while mice infected before pregnancy had a rate of 50% (21). These rates are comparable to the transmission rates that we have observed in C57BL/6 mice. Therefore, C57BL/6 mice can be considered suitable as lab animal models for such vertical transmission studies.

While we recognize that other variables like age or immune status of mice during pregnancy, time of infection prior to mating, the type of vaccine and vaccination regimen followed i.e., administration before or after mating, can have a great influence on experimental results, the optimization of these parameters have not been attempted in this study. In Balb/c mice challenge with *N. caninum* during pregnancy resulted in fetal mortality. Dead fetuses were PCR positive for *N. caninum*, while live fetuses were not (20). In this study, fetuses that were alive during in-utero collection from challenged mothers were found to be PCR positive for *N. caninum.* It is believed that the different strains of mice have genetically controlled, inherent Th1/Th2 biases (5, 28). C57BL/6 mice are believed to have a Th1 bias, which can influence the outcome of vaccine studies. Moreover, although mice are the best available laboratory animal models for *N. caninum,* there are several differences between the bovine and murine immune systems and placentation.

In conclusion, the use of C57BL/6 mice model for *N. caninum* vaccine studies has several advantages, namely; the availability of an unambiguous lethal challenge model; a sub lethal challenge model that can be assessed by histology and PCR. Moreover, the efficacy of the vaccine in preventing vertical transmission can also be studied in the same strain of mice. The high level of susceptibility of C57BL/6 mice to *N. caninum* infection makes them an invaluable tool for *N. caninum* vaccine studies.

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Table 2.2.1: LD_{50} titration of *N. caninum* tachyzoites in C57BL/6 mice*

Mouse Number	Dose of *N. caninum* tachyzoites							
	$1x10^5$	$1x10^6$	$5x10^6$	$1x10^7$	$2x10^7$	$3x10^7$	$4x10^7$	$1x10^8$
1	K21	K21	K21	K21	D7	D5	D4	D4
2	K21	K21	K21	K21	D5	D5	D3	D4
3	K21	K21	K21	K21	D6	D4	D4	D4
4	K21	K21	K21	K21	D7	D4	D5	D4
5	K21	K21	K21	K21	D5	D4	D5	D3

***** D – Died, K – Killed, Number – Number of days to death or euthanasia

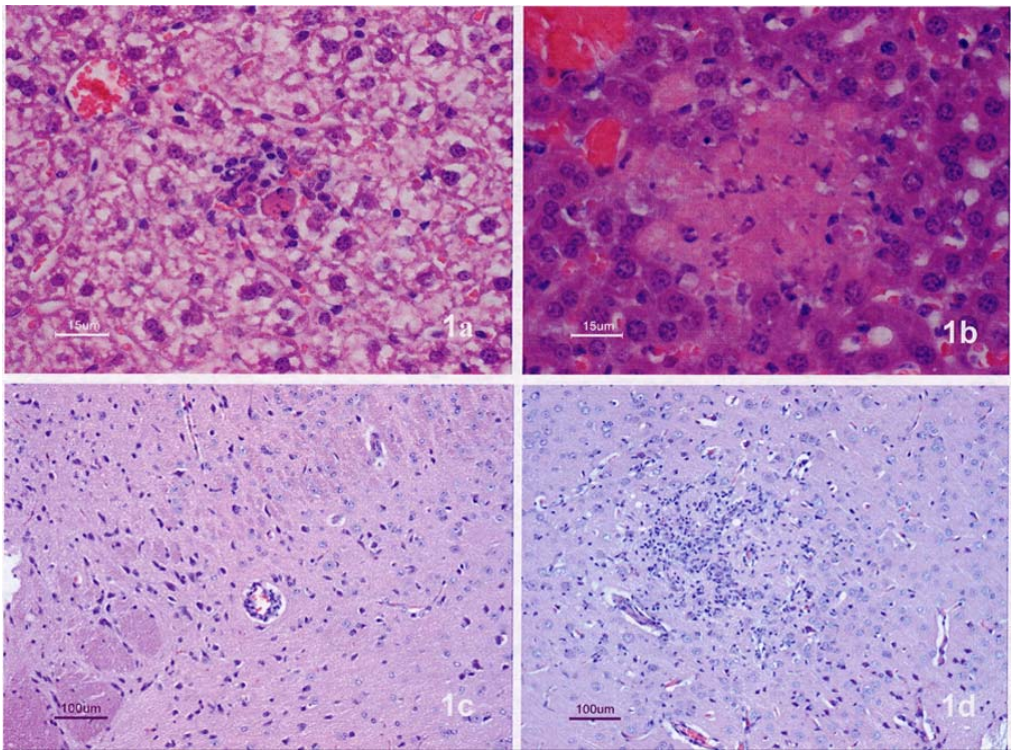

Figure 2.2.1: Histopathological lesions in mice inoculated with a lethal and sublethal dose of *N. caninum* tachyzoites. 1a & 1b – Liver tissue from mice challenged with a lethal dose of *N. caninum*: 1a - Minimal, multifocal sinusoidal lymphocyte aggregation centered on occasional necrotic hepatocytes in a surviving vaccinated mouse, day 21 post challenge .1b - Acute, mild, hepatocellular necrosis in a sick and euthanized unvaccinated mouse, day 7 post challenge, 1c & d – Brain tissue from mice challenged with a sublethal dose of *N. caninum*, 21 days post challenge: 1c – mild lymphoplasmacellular, perivascular cuffing in a vaccinated mouse, 1d - moderate lymphoplasmacellular and granulomatous encephalitis in an unvaccinated mouse.

Table 2.2.2: Vertical transmission model for *N. caninum* in C57BL/6 mice

Treatment	Mouse ID	Preg /Not Preg (+/-)	Rate of concept-tion	No. Of fetuses	Av. No. Of fetuses	No. PCR +	% Vertical Transmi-ssion
Uninfected controls	M1	+	71.42	9	8.2	*	0
	M2	-					
	M3	+		10		*	
	M4	+		9		0	
	M5	+		6		0	
	M6	-					
	M7	+		7		0	
$5x10^6$ *N. caninum* tacyzoites, Day 12-14 of pregnancy, i.p (Prophyl-actic model)	M1	-	57.14		8.0 (p=0.42)		100 (p=0.0002)
	M2	+		9		9	
	M3	+		8		8	
	M4	-					
	M5	-					
	M6	+		8		8	
	M7	+		7		7	
$5x10^6$ *N. caninum* tacyzoites, 14 days before mating, i.p. (Therapeu-tic model)	M1	+	57.14	6	5.25 (p=0.05)	4	90.48 (p=0.002)
	M2	+		2		2	
	M3	-					
	M4	-					
	M5	+		9		9	
	M6	-					
	M7	+		4		4	

* Samples lost. Not included in analysis.

CHAPTER 3

APPROACHES TOWARDS VACCINE DEVELOPMENT AND TESTING FOR PROTECTION AGAINST ACUTE MURINE NEOSPOROSIS

CHAPTER 3-1

EFFICACY OF RECOMBINANT *BRUCELLA ABORTUS* STRAIN RB51 EXPRESSING PROTECTIVE ANTIGENS OF *NEOSPORA CANINUM* AS A VACCINE FOR NEOSPOROSIS

S. Ramamoorthy, N. Sanakkayala, R. Vemulapalli, R. B. Duncan, D. S.Lindsay, G. S. Schurig, S. M. Boyle, N. Sriranganathan. Under review by Infection and Immunity.

ABSTRACT

Bovine abortions caused by the intracellular protozoal parasite *Neospora caninum* are a major concern to cattle industries worldwide. A strong Th1 type of immune response is required for protection against *N. caninum. Brucella abortus* strain RB51 is currently used as the live vaccine against bovine brucellosis. Strain RB51 can also be used as an expression vector that induces Th1 immunity. In this study, putative protective antigens of *N. caninum;* MIC1, MIC3, GRA2, GRA6 and SRS2 were expressed individually in *B. abortus* strain RB51. The ability of each of the recombinant RB51 strains to induce *N. caninum*-specific immunity was assessed in C57BL/6 mice. Mice were immunized by two intra-peritoneal inoculations, 4 weeks apart. Five weeks after the second immunization, spleen cells from the vaccinated mice secreted high levels of interferon-gamma and IL-10 upon *in vitro* stimulation with *N. caninum* whole cell lysate antigens. *N. caninum*-specific antibodies of both IgG1 and IgG2a subtypes were detected in the serum of the vaccinated mice. Mice in the vaccinated and control groups were challenged with $2x10^7$ *N. caninum* tachyzoites i.p and observed for 25 days after vaccination. All unvaccinated control mice died within seven days. Mice in the MIC1 and GRA6 vaccine groups were

completely protected while the mice in the SRS2, GRA2 and MIC3 vaccinated groups were partially protected and experienced 10-20% mortality. These results suggest that expression of protective antigens of *N. caninum* in *B. abortus* strain RB51 is a very promising approach towards the development of a multivalent vaccine against brucellosis and neosporosis.

INTRODUCTION

Neospora caninum an intracellular, apicomplexan parasite, closely related to T*oxoplasma gondii* (14) is the causative agent of neosporosis in cattle. Following it's first thorough characterization, (15), the widespread prevalence of this disease has been documented in Europe, in the Americas, Australia, New Zealand, U.K and some parts of Asia (52). Although the dog is the definitive host of the parasite (28, 32), the economic impact of the disease is attributed to the disease it causes in cattle, an intermediate host. Cattle with neosporosis can abort from three months of pregnancy onwards and the parasite can be transmitted vertically from mother to offspring. The rate of vertical transmission ranges from 80 – 95% (13, 44). Congenitally infected calves may or may not show symptoms but serve as carriers to maintain the disease in the herd (2).

The laboratory mouse has been shown to be a useful model to study the host immune mechanism operative in *N. caninum* infection. Immune responses to *N. caninum* in mice are primarily of Th1 type. CD4+ Tcells appear to play a greater role in protection than CD8+ T cells (47). B cell knockout mice show increased susceptibility to the disease (17) and IgG2a isotype antibodies are associated with protection (4, 48). IFN-γ knock out mice are highly susceptible to *N. caninum* challenge (38). IL-12 has a more transient but protective effect (4). High levels of IL-4 and IL-10 are associated with increased susceptibility to the disease, particularly during pregnancy when Th2 cytokines are up regulated, causing a down-regulation of the protective Th1cytokines (29, 40, 41).

Currently, there are no chemotherapeutic agents available for *N. caninum* infection. Several vaccination studies in mice have been carried out with inactivated and crude lysate preparations of *N. caninum* tachyzoites, with or without adjuvants. These vaccines generate a

Th2 type of response with high antibody titers (3, 5, 43). Administration of purified recombinant proteins like MIC3, SAG1 and SRS2 produce similar results with varying levels of protection (7, 8). DNA vaccines consisting of plasmid DNA encoding *N. caninum* protective antigens and vaccinia virus recombinants expressing SAG1 and SRS2 induce more significant levels of protection (7, 8, 36, 39).

Based on the biology of the parasite and the host protective immune responses, rational vaccine design for *N. caninum* and other apicomplexan parasites would have to take into consideration the targeting of correct antigen presentation pathway and stimulation of the right cytokine milieu (6). A live vaccine that delivers antigen intracellularly and stimulates a Th1 type of response has the obvious advantage of being able to elicit better protection. Selection of the right recombinant antigens or a combination of them is also crucial for the induction of protective immunity by a vaccine (37).

Brucella abortus is a gram negative, intracellular bacterium that primarily affects cattle, but can infect other animals including humans. It causes abortions in cattle and undulant fever in human beings. Immunity to *Brucella* involves both cell-mediated and humoral immune responses (18). Antibodies to the O polysaccharide of the smooth lipopolysaccharide and IFN-γ secreting antigen specific CD4+ and CD8+ T cells are crucial for acquired resistance against brucellosis. *B. abortus* strain RB51 is an attenuated, stable, rough mutant that is being used as a live vaccine against bovine brucellosis in several countries. Protection afforded by this strain is primarily through the induction of robust cell mediated immunity (11, 23, 45).

Previous research in our laboratories demonstrated that strain RB51 can be engineered to express homologous and heterologus proteins. Vaccination of mice with such recombinant RB51 strains leads to induction of Th1 type of immune responses specific to the expressed protein (49, 50). Therefore, strain RB51 can serve as a good vector to express and deliver potential protective antigens of intracellular pathogens where the induction of Th1 type immune responses are essential for acquired resistance.

In this study, we individually expressed five selected putative protective proteins of *N. caninum* in strain RB51 and tested the ability of the recombinant RB51 strains to induce protective immunity against *N. caninum* challenge in a C57BL/6 mouse model.

MATERIALS AND METHODS

Mice:

4-6 week old female C57BL/6 mice were used (Charles River, MA) for all experiments. The mice were housed in a CDC certified BSL3 facility and all experiments were carried out in compliance with the requirements of the animal welfare committee of Virginia Tech and AVMA guidelines.

N. caninum tachyzoite culture:

CV-1 cells (ATCC IZSBS BS CL23) were maintained using RPMI media (Cellgro, VA) containing 10% FBS (Sigma Aldrich, MO) at 37^{o}C in a cell culture incubator at 5% CO_2 tension. Monolayers of CV-1 cells were infected with *N. caninum* NC-1 strain tachyzoites. When the replicating tachyzoites were released from the cells into the media, the culture was harvested by scraping infected monolayers with a rubber policeman and passing it through a 24g needle to lyse cell debris. Purified tachyzoites were obtained by filtering the culture through 0.3 um filters (Osmonics, SD).

Cloning and expression of *N. caninum* MIC1, MIC3, GRA2, GRA6 and SRS2 in *B. abortus* strain RB51:

Total RNA from CV-1 cells infected with *N. caninum* tachyzoites was extracted using a commercial kit (Qiagen Rneasy kit, Qiagen CA). The extracted RNA was used as a template in RT-PCR reactions to amplify the cDNAs encoding MIC1, GRA2, GRA6, SRS2 and MIC3 proteins. A custom designed primer-pair for each open reading frame was used for the cDNA amplification (Table 3.1.1).

The RT-PCR reactions were carried out using the reagents of a commercial kit (SuperScript One-Step RT-PCR system w/Platinum Taq, Invitrogen, CA) and a thermocycler (Bio-Rad, CA). The amplified cDNAs were first cloned into pCR2.1-TOPO vector (Invitrogen, CA) and the inserts were sequenced to confirm the integrity of the nucleotide sequences of the

coding sequences (21, 24, 26, 35, 51). The inserts were subsequently excised from the pCR2.1-TOPO vector using the restriction sites engineered into the primers (Table 3.1.1) and subcloned in a broad-host range plasmid downstream to the *Brucella groE* promoter (in pBBgroE) or under the synthetic hybrid *trc* promoter (in pBBR4Trc) (Figure 3.1.1). Strain RB51 was transformed using standard protocols (33). Transformants were selected on Tryptic soy agar plates containing 100 ug/ml of Ampicillin (pBBTtrc) or 22.5ug/ml of Chloramphenicol (pBBGroE). The expression of the respective *N. caninum* proteins in each of the recombinant RB51 strain was confirmed by Western blot analysis using mouse anti-*N. caninum* serum.

Preparation of the recombinant sRB51-Nesopora vaccines:

Pre-titrated glycerol stocks of the recombinant vaccine strains and strain RB51 carrying plasmid pBBGroE alone, were resuspended in the required volume of PBS to obtain $1x10^9$ CFU/ml for the primary vaccination and $1x10^8$ CFU/ml for the booster vaccination. The actual inoculation dose was determined retrospectively by plating on TSA plates. $1x10^6$/ml *N. caninum* tachyzoites subjected to gamma irradiation with 528Gy was used as a positive control.

Immunization of mice for lethal challenge:

Four to six-week-old female C57BL/6 mice were used for immunization. Groups of randomly allotted mice were vaccinated i.p. with $1x10^9$ CFU *B. abortus s*RB51 expressing MIC1, GRA2, GRA6, SRS2 and MIC3. One group served as the unvaccinated control. Another group was inoculated with the same doses of *B. abortus s*RB51 harboring the plasmid pBBGroE alone (designated as RB51 in all Figures and tables). Irradiated *N. caninum* tachyzoites ($1x10^6$) were used to vaccinate another group of mice as a positive control. The mice were boosted i.p with a log lower dose of the recombinant strains, four weeks later. Serum was collected a week after the booster, to assess antibody responses. Two mice from each group were sacrificed in the tenth week and their splenocytes were used to perform lymphocyte proliferation assays to measure Th1 and Th2 cytokine levels. The remaining mice were subjected to a lethal challenge with $1x10^7$ *N. caninum* tachyzoites. The vaccination and challenge was repeated by following the same vaccination regimen, using a variable number of mice in each group. Pooled results from both experiments are depicted in Figure 3.1.

Immunization of mice for sub lethal challenge:

A group of five mice were vaccinated with $1x10^9$ CFUs of pooled recombinant RB51 strains ($2x10^8$ CFU's each), following the above protocol. In the tenth week they, along with an unvaccinated group of mice and the positive control group, were challenged sub-lethally with $1x10^6$ *N. caninum* tachyzoites. The mice were observed for 30 days after challenge. They were then euthanized by CO_2 asphyxiation and their spleen, liver and brain tissue were used for histopathological analysis.

Serum antibody responses:

Maxisorp ELISA plates (Nalge Nunc NY) were coated with 2µg/ml *N. caninum* lysate solution in coating buffer pH 9.6 overnight at room temperature. The plates were washed thrice with Dulbeccos PBS containing 0.05% Tween20 (Amersham Biosciences NJ). A 1:10 dilution of sera samples in blocking buffer (PBST with 2% BSA (Fisher-Biotech, NJ) and 1.5% skimmed milk powder) was added to the plate and incubated at room temperature for one hour. Anti-mouse IgG, IgG1 and IgG2a conjugated to horse radish peroxidase (Sigma Aldrich, Mo) were used at a 1:2,000 dilution in blocking buffer for one hour as secondary antibodies. Detection was carried out with TMB (KPL, MD). The reaction was stopped using 0.1N HCl. Plates were read in an ELISA plate reader at 460 nm. The values represented are the mean of triplicate samples obtained from three mice in each group. Values from pre-vaccination sera from two mice from each group were used as a blank.

Measurement of cytokines in splenocyte culture supernatants from vaccinated mice:

Lymphocytes from the spleens collected from two vaccinated mice of each group were processed as per previously described procedures (49) .The cultured splenocytes were stimulated with 1 µg/ml of ConA (Sigma Aldrich, Mo), 1 µg/ml of *N. caninum* lysate, $1x10^8$ heat killed sRB51 and RPMI containing 10% FBS and 1% penicillin and streptomycin. The cultures were incubated at 37^oC, for 96 hours, in a CO_2 incubator and plates were centrifuged at 1200 RPM for eight minutes. Cytokine quantification was carried out on the supernatants using a Th1/Th2

cytokine ELISA kit as per the manufacturers' instructions (Cytokine ELISA kit, Ebiosciences, CA). The average of triplicate values from each of the above-mentioned samples is presented (Table 3.1.2). Supernatants from spleen cell cultures of mice vaccinated with the recombinant strains and stimulated with killed sRB51 were pooled in equal volumes and used as a single sample in triplicate (Data not presented). Values of the media stimulated samples from each mouse were used as blank values and subtracted from the other values obtained from the ELISA for analysis and presentation.

Histopathology:

Spleen, liver, and brain samples from animals surviving the lethal and sub lethal challenge were fixed in 10% buffered neutral formaldehyde (Fischer Scientific, NJ). The tissues were routinely processed, embedded in paraffin, sectioned and stained with hematoxylyn and eosin. Stained sections were examined and brain lesions were characterized and scored by a board certified veterinary pathologist, in a double blind study as described in Chapter 2-2. Scores were assigned on the basis of the number of lesions per field as well as the severity of lesions on a scale of 1-10. The average of the product of the severity scores and scores based on the number of lesions is presented.

Statistical analysis:

A two-tailed student T test was carried out using the Microsoft excel package to compare the humoral and cell mediated immune responses of vaccinated mice with those of the unvaccinated ones. A pair-wise comparison of protection based on mortality between vaccinated and unvaccinated mice was performed by Chi-square test using Fischer's exact statistics. Minitab statistical software program (Minitab 12.1, State College, PA, USA) was used to perform this analysis. Data was stratified by treatment groups and Kaplan–Meier survival functions were estimated using the LIFETEST procedure in SAS (SAS Version 9.1 for Windows, SAS Institute, Cary, NC, USA). Kaplan–Meier survival estimates were used to calculate the crude and conditional survival risk for 30 days observation period. The same procedure was used for pair-

wise comparison between sRB51 and other groups. The statistical significance for all tests were set at alpha = 0.05.

RESULTS

Expression of *N. caninum* protective antigens in *B. abortus* strain RB51:

Anti-*N. caninum* mouse polyclonal serum was used to detect *Neospora* protein expression in each recombinant *B. abortus* RB51 strain. Sonicated *N. caninum* lysate was used as a positive control. *B. abortus* strain RB51pBBGroE was used as the vector control. MIC1 was expressed as an approximately 49 kDA protein, GRA2 as a 28 kDA protein, GRA6 as a 36 kDA protein, SRS2 as a 42 kDA protein and MIC3 as a 38 kDA protein (Figure 3.1.2). The molecular weights were in agreement with the expected sizes for these proteins.

Humoral responses to vaccination with recombinant *B. abortus s*RB51 expressing *N. caninum antigens*:

Mice vaccinated with the recombinant strains produced *N. caninum* specific IgG as assessed by the indirect ELISA. Significant levels of IgG2a isotype of antibodies were produced by the GRA2 and SRS2 immunized mice while both IgG2a and IgG1 were produced by the GRA6 and MIC1 immunized mice (Figure 3.1.3). Even though, moderate levels of IgG were also detected in mice immunized with the vector alone (sRB51 group), all the vaccinated groups, except for the MIC3, contained significantly higher levels of antibodies. Similarly, except for the MIC3 groups, mice in all other vaccine groups had significantly higher levels of IgG2a in comparison with the vector group.

Th1 and Th2 cytokine measurement from splenocyte culture supernatants:

IFN-γ, IL-4, IL-10 and IL-12p70 levels were measured from supernatants of splenocyte cultures from all groups of mice using a commercial ELISA kit. Significant levels of IFN-γ were produced in mice vaccinated with the recombinant vaccine, when stimulated with heat-killed

strain RB51 (data not presented) and *N. caninum* lysate. Except for the GRA6 immunized mice, all the other vaccinated mice produced significant levels of IL-10 (Table 3.1.2). IL-12 p70 and IL-4 were not detected. All ConA controls induced significant levels of respective cytokines, while the media controls did not.

Protection of vaccinated mice against lethal challenge with *N. caninum:*

All unvaccinated control mice died within seven days post-challenge. There was no mortality in the MIC1, GRA6 and the positive control groups, while one mouse died in each of the SRS2 and GRA2 groups. Five out of ten mice in the MIC3 group died before the 25th day of observation (Figure 3.1.4). Protection induced by vaccination with all of the recombinant sRB51 strains was statistically significant when compared to the unvaccinated controls. Ten out of fourteen mice in the vector control group survived, indicating that strain *B. abortus* strain RB51 induced a significant degree of non-specific protection against *N. caninum.* Statistical comparison of the protection in the vaccinated groups and the sRB51 vector group, as determined by a Fischer's exact test and Kaplan Meier survival analysis, did not show significant differences. Pooled data from two independent challenge studies is depicted in Figure 3.1.4.

Histopathology -Lethal challenge:

Histopathological examination of brain tissue from surviving mice in the vaccinated groups revealed the presence of mild to moderate lesions and occasional tachyzoites; although no gross lesions or clinical signs were observed during necropsy. The unvaccinated mice that succumbed to acute infection showed gross changes of severe dehydration, sero-fibrinous peritoneal adhesions, acute hepatocellular necrosis and accumulation of fluid in the peritoneal cavity. No lesions were observed in the brain tissue due to the peracute nature of the disease. Lesion scores could not be assigned for comparison of vaccinated groups with the unvaccinated control. Therefore another sub-lethal challenge study was carried out. However, comparison of lesions between mice in the sRB51 group and the other recombinant sRB51-*Neospora* vaccine groups revealed more severe lesions in the brain tissue of mice from the sRB51 group when compared to the sRB51-*Neospora* recombinant vaccine treated mice. Mice from the sRB51

group had an average score of 12, while mice from the GRA2, GRA6, SRS2, MIC1 and MIC3 groups had scores of 8.8, 9.5, 1.4, 5.6 and 10.25 respectively. The average scores of mice from the SRS2 and MIC1 groups were significantly different from the average scores of the mice from the sRB51 group.

Protection of vaccinated mice against sub-lethal challenge with *N. caninum*

The mice in the unvaccinated control group had an average lesion score of 11.33 while mice vaccinated with pooled recombinant sRB51- *Neospora* strains had a score of 5.5; this difference was highly statistically significant. Mice that were vaccinated with RB51 alone and not challenged did not exhibit any lesions, indicating that the vector did not cause tissue pathology

DISCUSSION

The recent emergence of *N. caninum* as a leading cause of bovine abortions and the heavy economic losses caused by this disease has been instrumental in stimulating vaccine research targeting neosporosis. Inactivated tachyzoite lysates, the primary component of the commercially available vaccine, is known to stimulate antibody responses, while it has been demonstrated that Th1 responses are crucial for protection against this disease (5, 38). In this study we have demonstrated that recombinant *Brucella abortus* strain RB51 expressing selected antigens of *N. caninum* induce strong Th1 type responses and provide complete protection against *N. caninum* challenge, in mice.

Identification of protective antigens of *N. caninum* was previously achieved by means of their reactivity to serum from infected animals, ability to induce IFN-γ secretion and T cell proliferation (25, 31, 46), and by comparison to homologous *T. gondii* proteins (16). The antigens that were selected for expression in strain RB51 had been previously characterized and some of them proved to have protective potential.

MIC1 and MIC3 are secreted proteins that mediate *N. caninum* adhesion to host cells (24, 34, 35). Vaccination of mice with recombinant MIC1 antigen is reported to induce superior protection when compared to a MIC1 DNA vaccine, while a DNA vaccine and recombinant vaccine combined immunization regimen appeared to have a negative effect (1). Vaccination of mice with recombinant MIC3 was partially protective and protection appeared to correlate with the presence of MIC3 specific IgG1 antibodies (8). Delivery of the same antigen using sRB51 as a platform induced complete protection against lethal challenge in mice immunized with the MIC1 protein and 50% protection in mice immunized with the MIC3 protein. However mice vaccinated with the MIC1 expressing strain RB51 vaccine developed higher levels of specific antibodies.

Protection provided by strain RB51 expressing MIC3 was significantly less compared to protection in the strain RB51 vector control group. This directly correlates with the presence of low levels of antibodies in mice vaccinated strain RB51 expressing MIC3. Although previous research has established that the Th1 type of immune response is key for protection (38), a possible explanation for the level of protection induced by the recombinant MIC3 vaccine could be the absence of specific IgG1 antibodies in the serum of vaccinated mice. Moreover, it is noteworthy that the challenge dose used in this study was a log higher than that used by Cannas et al.,, even though C57BL/6 mice were used in both studies. Therefore, it appears that although a generalized Th1 response seems to be important for protection against *N. caninum,* targeting a Th2 response with certain individual protective antigens while developing recombinant vaccines may result in induction of better protection.

Dense granule proteins are known to function in nutrient acquisition, and trafficking of wastes between the host cell and *N. caninum* (9, 20). Studies carried out by Dubey et al., indicate that protection against vertical transmission of *N. caninum* in mice vaccinated with a GRA7 DNA vaccine improved substantially upon addition of CpG adjuvant (22, 27) . In gerbils immunized with recombinant GRA1 and GRA2 proteins, 62.5% and 50 % protection against lethal challenge with *N. caninum* was reported (12). In this study, strain RB51 expressing GRA6 induced 100% protection while strain RB51 expressing GRA2 induced 87% protection. It appears that dense granule proteins induce better protection when associated with adjuvants, such as CpG or sRB51, which induce a Th1 response.

The surface antigen gene (SAG) and surface antigen gene related sequence (SRS) antigens have been of particular interest in recombinant vaccine development for *N. caninum.* These proteins are known to bind to host cell sulfated proteoglycans and confer tachyzoites with the ability to invade a variety of host cell types (19, 21). Use of recombinant SAG1 and SRS2 antigens as subunit vaccines did not induce significant protection while combination of the DNA vaccination with the recombinant proteins improved vaccine efficacy (7). Delivery of these two antigens in a vaccinia virus system was able to prevent vertical transmission in mice (36, 39). The protective efficacy of SAG1 was not significant in both studies; hence this antigen was not tested in our study. Recombinant strain RB51 expressing SRS2 conferred 87% protection against *N. caninum* challenge in C57BL/6 mice. It appears that both humoral and cell mediated immunity should be induced for successful vaccination with this antigen.

Overall, our findings support the observation that in addition to the antigen itself or type of immune response (Th1 vs. Th2), a complex interaction of other variables determines protection. These include the strain of mice used, composition of the antigen in terms of signal peptides, B and T cell epitopes, the delivery platform, antigen presentation pathway, adjuvant and the immune status of the host. The determination of the exact mechanism of protection of the recombinant strain RB51- *Neospora* vaccine is not within the scope of this study.

IFN-γ can be considered to be the most important cytokine involved in protection against *N. caninum* (38, 42). The ability of the recombinant RB51- *Neospora* vaccine to protect is likely related to the fact that expression of *N. caninum* protective antigens with strain RB51 or strain RB51 itself, leads to production of very high levels of this cytokine in vaccinated mice. IL-12 p40 was not detected, probably because it occurs early during the immune response. The IL-4 levels were not significantly different from that of the controls.

The non-specific stimulation of immunity against *N. caninum,* as evidenced by absence of mortality in 75% of mice vaccinated with the strain RB51 vector alone, could also be attributed to *IFN-γ* production. However, the rate of parasitic clearance or inhibition of replication appeared to be superior in the mice vaccinated with the sRB51-*Neospora* vaccine, when compared to mice vaccinated with sRB51 alone, based on histopathological analysis of the brain tissue from vaccinated mice that survived lethal challenge. A similar absence of clinical signs in C57BL/6 mice vaccinated with RIBI adjuvant and challenged with $1x10^6$ *N. caninum* tachyzoites has been observed by Cannas et al., (8).

Antibodies are reported to have a protective role against *N. caninum* infections, as evidenced by increased pathology in B cell knockout mice when compared to wild type mice (17). It is speculated that they might help in initial defense against the extra cellular tachyzoites. The protective immune response to *N. caninum* in mice, which is associated with IFN-γ, is known to bias the isotype produced to IgG2a. Studies by Long et al., (30) describe the association of IgG2a isotype antibodies with protection in resistant strains of mice and IgG1 in susceptible strains of mice. As hypothesized, strain RB51-*Neospora* vaccines induced Th1 responses which included high IFN-γ levels and IgG2a type antibodies. Contrary to expectations, our results and results from vaccine studies conducted by other authors indicate that IgG1 antibodies may also be important in inducing complete protection. The induction of the IgG1 isotype antibody appears to vary with the protective antigen used for vaccination and the platform used for vaccine delivery. In this study, mice receiving strain RB51 expressing GRA6 or MIC1 had both isotypes and were better protected.

In confirmation of observations by others, (1, 8, 27), we did could not establish a correlation between the presence of inflammatory changes or tachyzoites in the brain and the presence of clinical symptoms or mortality in mice. Although occasional tachyzoites and lymphoplasmacellular infiltration was recorded on histopathological examination of vaccinated mice, three weeks after challenge, these mice did not exhibit any clinical signs of the disease. However, we were able to establish a clear reduction in inflammatory changes in brain tissue of vaccinated mice when compared to unvaccinated controls, based on histopathological scoring. We also found that the brain was unsuitable for post challenge *N. caninum* histopathology in a lethal challenge model as the time to death was insufficient to cause dissemination of the organism to the brain. However, with a sublethal challenge model, the brain was the only organ that showed appreciable changes, probably because *N. caninum* was cleared from other organs, three weeks after challenge.

The choice of the strain of mice used for *N. caninum* vaccine studies can have a profound effect on the vaccine studies. The C57BL/6 mouse has an inherent Th1 bias in its immune response, while the Balb/c mouse has a Th 2 bias(10). *N. caninum* vaccine studies carried out by various researchers have employed different mouse models and parameters for protection (8, 27, 39, 41), making it difficult to comparatively analyze the efficacies of the various vaccines. The lethal challenge model used in this study was a clear-cut end-point assay, eliminating the need

for PCR analysis to establish protection. The challenge dose used in this study was higher than that used in published studies, thus the requirement for protection was more stringent.

The recombinant strain RB51-*Neospora* vaccine has several advantages when compared to the inactivated vaccine. It is less expensive to produce and preserve and induces cell-mediated immunity. Above all, two important diseases that cause bovine abortions, brucellosis and neosporosis; can be targeted with this one vaccine. In conclusion, the sRB51-*Neospora* vaccine is a novel and commercially viable approach for immunization against *N. caninum.* Future studies with this vaccine will test the efficacy of the vaccine in preventing vertical transmission of *N. caninum* in mice and cattle.

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Table 3.1.1: Cloning of putative *N. caninum* protective antigens in *B. abortus* strain RB51

Gene (F-Forward R-Reverse)	Primer sequence	Length bp	Protein size kd	Tm	Selection Marker	Promo-ter
MIC1-F	TTCCATGGGCCAGTCGGTG GTTTTCG	2278	49	69.32	AMP	TRC
MIC1-R	TTAAGCTTACAATTCAGAT TCACCCGGAG			64.62	AMP	TRC
GRA2-F	FTTCCATGGTCACGGGGAA ACGTTGGATAC	654	28	68.87	AMP	TRC
GRA2-R	TTGAATTCTTAATTGACTTC AGCTTCTGGCGTT			65.87	AMP	TRC
GRA6-F	TTCCATGGCGGAACAATAG AACCCTCGC	582	36	67.64	AMP	TRC
GRA6-R	TTCCATGGCGAACAATAGA ACCCTCGC			67.64	AMP	TRC
MIC3-F	GGATCCATGCGTGGCGGGG CGT	1089	38	58.4	CAT	GROE
MIC3-R	TCTAGAGGATTCCCCTGTC CCAAAATTCG			57.2	CAT	GROE
SRS2-F	AGATCTATGGCGACGCATG CTTGTGTGGTG	1203	42	59.1	CAT	GROE
SRS2-R	TCTAGAACTGCCGTCATGC CCTGTCACAAA			58.6	CAT	GROE

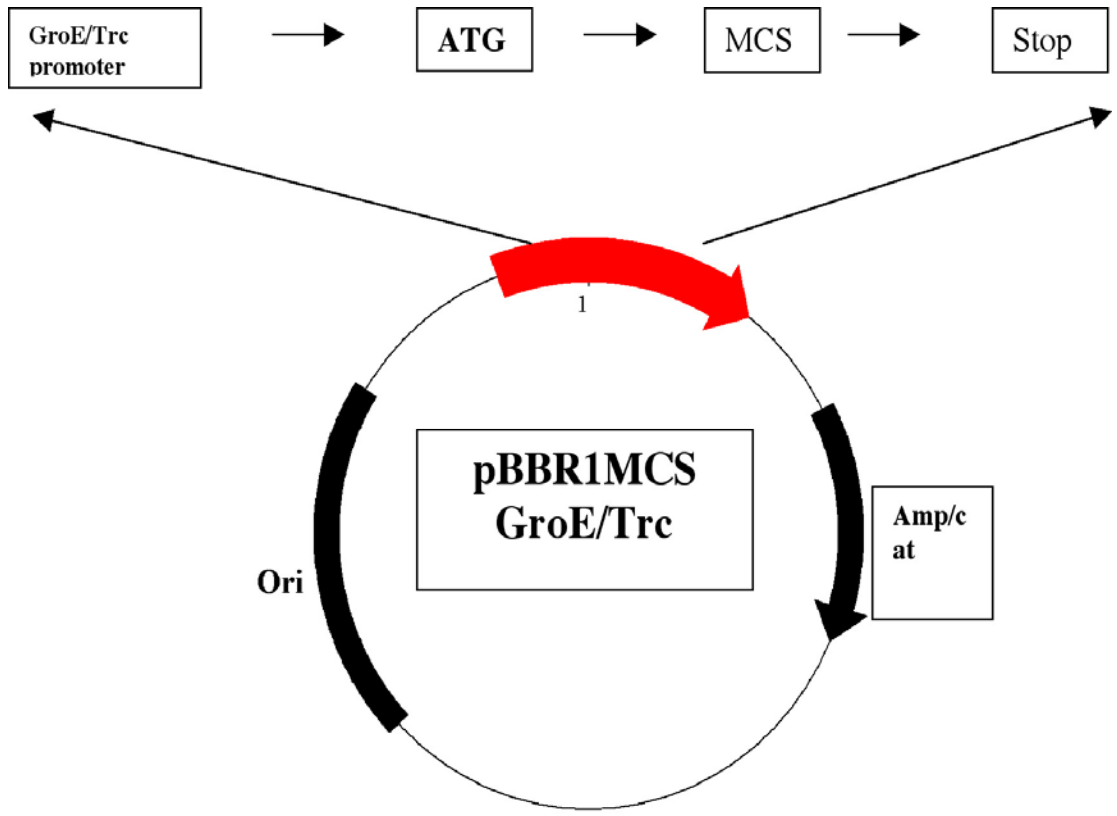

Figure 3.1.1: Plasmid map of pBBr1MCS depicting the GroE/ Trc promoter, multiple cloning site and antibiotic selection genes.

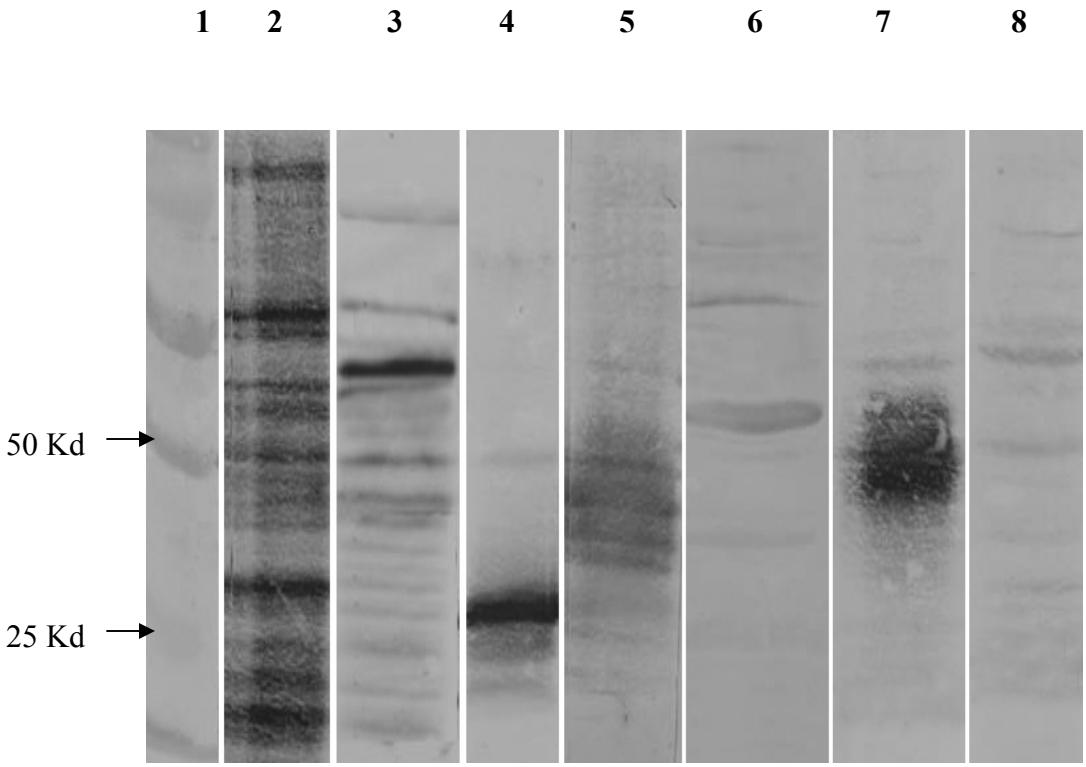

Figure 3.1.2: **Western blot of recombinant *B. abortus* sRB51 expressing *N. caninum* antigens** with polyclonal mouse anti-*Neospora* serum. Lane 1: Molecular Weight Markers, Lane 2: *N. caninum* total lysate (positive control), Lane 3-8 sRB51 clones, Lane 3: MIC1 (49kDa), Lane GRA2 (28kDa), Lane 5:GRA6 (36kDa), Lane 6: SRS2 (42kDa), Lane7: MIC3 (38kDa) Lane 8: *B. abortus* RB51pBBGroE lysate (negative control).

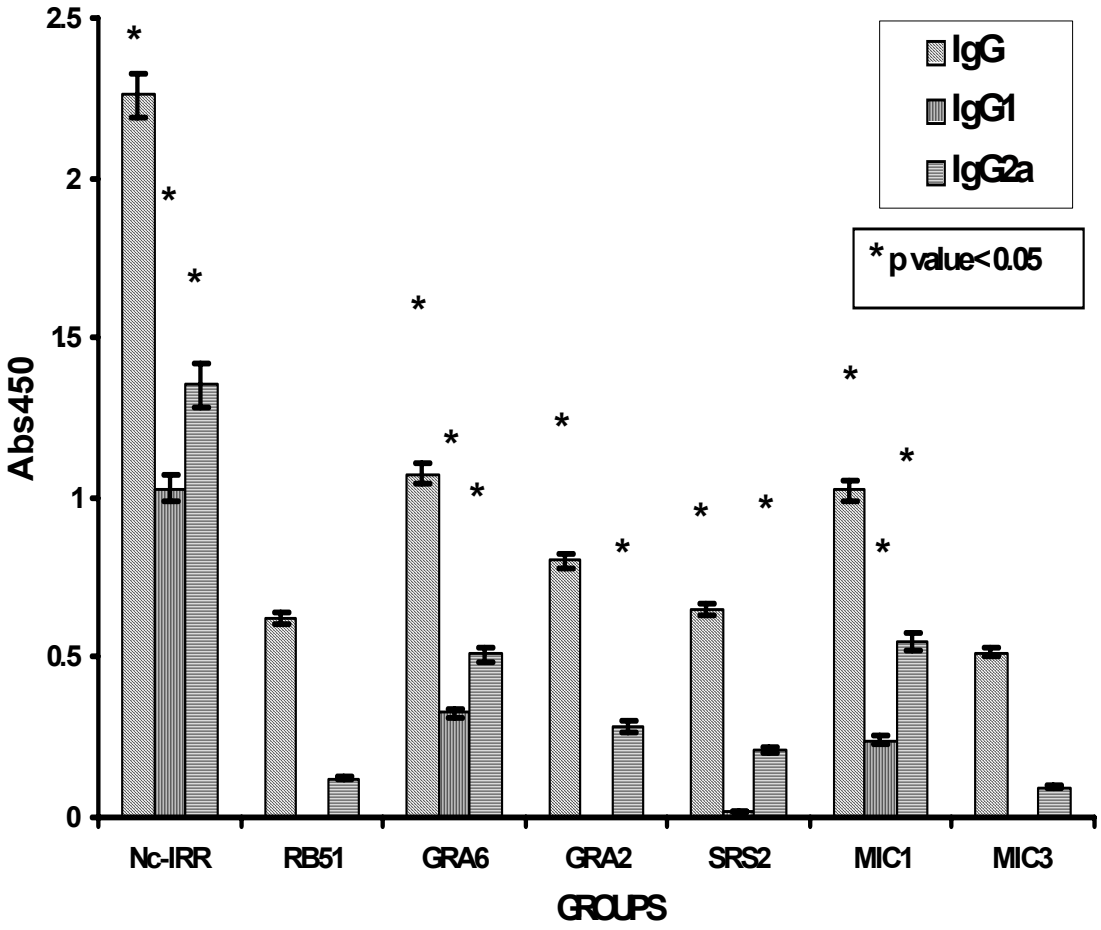

Figure 3.1.3: *N. caninum specific* serum antibody responses: The absorbance values of *N. caninum* specific IgG, IgG1 and IgG2a are depicted as the average of triplicate values after reduction of the values of the PBS group as a blank. Levels of significance that are depicted are based on a comparison with the RB51 group samples.

Table 3.1.2: IFN-γ and IL-10 levels in splenocyte cultures of mice vaccinated with sRB51 and recombinant RB51 strains expressing *N. caninum GRA6*, GRA2, MIC1, MIC3 and SRS2 antigens, stimulated with *N. caninum* tachyzoite lysate

Treatment / Stimulant*	IFN-γ / ng ± S.D	IL-10 / pg ± S.D
RB51/NC	$0.38 \pm 0.01_b$	$174.9 \pm 21.64_b$
GRA6/NC	$7.43 \pm 0.05_a$	$265.8 \pm 17.35_b$
GRA2/NC	$6.69 \pm 0.06_a$	$36.8 \pm 48.71_a$
SRS2/NC	$11.32 \pm 0.03_a$	$934.8 \pm 277.28_a$
MIC1/NC	$8.88 \pm 0.14_a$	$767.4 \pm 171.86_a$
MIC3/NC	$9.20 \pm 0.06_a$	$417 \pm 5.09_b$

* NC – 1 ug/ml *N.caninum* lysate, a – significantly different from unvaccinated mice, b – not significantly different from unvaccinated mice

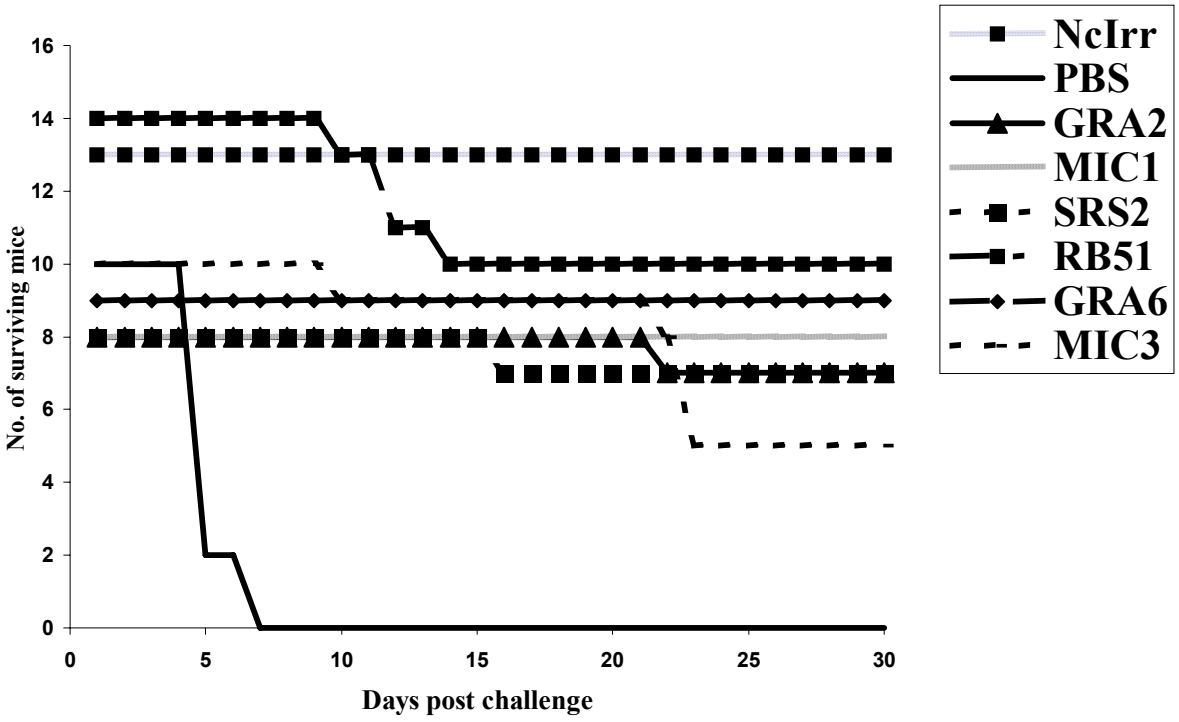

Figure 3.1.4: Post challenge survival of vaccinated mice and unvaccinated controls: All mice were challenged with $2x10^7$ *N. caninum* tachyzoites, i.p. Mortality in the unvaccinated control group was 100%. No mortality was observed in the MIC1, GRA6 and positive control NC-1 group. 1 mouse died in the SRS2 and GRA2 groups each. 4 mice died in the vector control RB51 group. Five mice died in the MIC3 group. All treatment groups were significantly different from the PBS control at $p \leq 0.05$ based on a chi-square test. None of the treatment groups, except PBS ($p=0.0001$), differed significantly from the sRB51 group.

CHAPTER 3-2

VACCINATION WITH GAMMA-IRRADIATED *NEOSPORA CANINUM* TACHYZOITES PROTECTS MICE AGAINST ACUTE CHALLENGE WITH *N. CANINUM*

S. Ramamoorthy, D.S. Lindsay, G. G. Schurig S. M. Boyle, R.B. Duncan, R. Vemulapalli, N. Sriranganathan. J. Eukryot. Microb. 53(2), 2006, pp. 151-156.

ABSTRACT

Neospora caninum, an apicomplexan parasite, is a leading cause of bovine abortions worldwide. The efficacy of gamma-irradiated *N. caninum* strain NC-1 tachyzoites as a vaccine for neosporosis was assessed in C57BL/6 mice. A dose of 528 Gy of gamma irradiation was sufficient to arrest replication but not host cell penetration by tachyzoites. Female C57BL/6 mice were vaccinated with two intraperitoneal inoculations of 1 x 10^6 irradiated tachyzoites at four-week intervals. When stimulated with *N. caninum* tachyzoite lysates, splenocytes of vaccinated mice, cultured 5 and 10 wk after vaccination, secreted significant ($p<0.05$) levels of interferon-gamma, interleukin (IL)-10, and small amounts of IL-4. Antibody isotype specific ELISA of sera from vaccinated mice exhibited both IgG1 and IgG2a isotypes of antibodies. Vaccinated mice were challenged intraperitoneally with 2 x 10^7 *N. caninum* tachyzoites. All vaccinated mice remained healthy and showed no obvious signs of neosporosis up to the 25th day post-challenge when the study was terminated. All unvaccinated control mice died within one week of infection. Gamma-irradiated *N. caninum* tachyzoites can serve as an effective, attenuated vaccine for *N. caninum.*

INTRODUCTION

Neospora caninum is an apicomplexan parasite that affects dogs and cattle (Dubey and Lindsay 1996); dogs are the definitive hosts (McAllister et al., 1998). The parasite causes

neuromuscular paralysis in dogs (Lindsay and Dubey 2000). Cattle may acquire the infection horizontally by consuming feed contaminated with oocysts shed in canine feces (Lindsay et al., 1999a, McAllister et al., 1998). However, vertical transmission is considered the most important mode of transmission. About 90% of infected cattle transmit the disease to their offspring (Dubey 2003). *Neospora caninum* causes mid-term abortions, stillbirths, and neurological complications in calves. This pathogen has a major economic impact on the dairy and beef industries (Dubey 1999). Therefore, development of an effective vaccine to prevent abortions due to neosporosis in cattle is a top research priority.

Immunology studies indicated that CD4+ T cells and interferon-gamma (IFN-γ) are key mediators in a protective immune response against *N. caninum (*Baszler et al., 1999, Nishikawa et al., 2001b, Tanaka et al., 2000). While antibodies that recognize several antigens of the parasite are found in the sera of infected animals (Barta and Dubey 1992), the contribution of antibodies in protection against the disease is not clear (Eperon et al., 1999).

Studies carried out with inactivated vaccines or vaccinations with whole cell lysates of *N. caninum* tachyzoites have shown that they are either ineffective or sometimes partially protective depending on the adjuvant used. The types of responses induced by the above vaccines are primarily humoral. However, cell mediated immunity is important for protection against *N. caninum (*Andrianarivo et al., 1999, 2000, Baszler et al., 2000). Vaccinia virus expressing a *N. caninum* surface protein, SRS2 (surface reactive sequence 2), was able to confer protection against vertical transmission in mice while DNA vaccines encoding *N. caninum* GRA7 (dense granule 7) have been shown to be partially protective (Cannas et al., 2003, Liddell et al., 2003). The practical use of recombinant DNA vaccines is limited due to the complications involved in licensing and the high cost involved in generating lipopolysaccaride-free DNA vaccines.

Attenuated, live vaccines of intracellular parasites are more efficacious than inactivated or recombinant vaccines for intracellular parasites, particularly as antigen presentation and the type of immune response elicited mimic that of a natural infection (Lindsay et al., 1999b, Pefferkorn & Pefferkorn 1976). Gamma-irradiation of parasites, such as *Toxoplasma gondii* (Dubey et al., 1996, Hiramoto et al., 2002, Kook et al., 1995), *Schistosoma japonicum* (Bickle et al., 2001), *Babesia bovis* (Wright et al., 1982), and *Plasmodium gallinaecium* (Egan 1993), indicates that this treatment is a promising method to irreversibly attenuate parasites. The invasive ability of the gamma-irradiated parasite remains intact, while its ability to replicate is

lost due to DNA damage. Thus, the only disadvantage of using a live attenuated vaccine (i.e. that the agent can revert to its virulent state) is eliminated by irradiation. The advantage of the irradiated pathogen is its ability to stimulate specific adaptive immune responses.

In the present study we have determined the optimal radiation dose required for inactivation of the ability of *N. caninum* tachyzoites to replicate while still maintaining their ability to protect mice against lethal challenge. Additionally, we measured selected immune responses in vaccinated mice.

MATERIALS AND METHODS

Parasite culture:

Tachyzoites of *Neospora caninum NC*-1 strain (Dubey et al., 1988) were cultured in African green monkey (*Cercopithecus aethiops*, American Type Culture Collection, CCL-70) kidney (CV-1) cells, grown in RPMI 1640 medium containing 10% fetal calf serum and antibiotics (growth medium) at 37^{o}C in 5% CO_2-95% air (Mitchell et al.,. 2005). Tachyzoites were harvested by scraping infected monolayers with and passing the culture through a 27 g needle to rupture CV-1 cells. This was followed by filtration through 3-µm filters to purify the tachyzoites.

Irradiation:

Purified tachyzoites were resuspended to a concentration of 1 x 10^6 tachyzoites/ml in Hanks balanced salt solution (HBSS). One-ml aliquots were irradiated using a Cobalt source gamma irradiator for 1, 4 or 8 min at 6,600 Rads per min. The effective dose at 8 min was 52,800 Rads or 528 Gy. The irradiated samples were layered onto monolayers of CV-1 cells in 75 cm^2 tissue culture flasks. After 24 h, the flasks were washed five times with RPMI 1640 medium containing 2% fetal calf serum and antibiotics. The monolayers were observed microscopically for the growth of tachyzoites for a period of 4 wk. Media were changed every week for flasks

that did not have growth of tachyzoites. Cell culture supernatants and cell monolayer pellets were collected separately for PCR. Cell culture supernatants were collected by pipetting off the cell culture media and then concentrating the tachyzoites/host cell debris by centrifugation in a 15 ml conical centrifuge tube. The resulting pellet was mixed in 1 ml of HBSS and then placed in a 1.5 ml microfuge tube and re-pelleted by centrifugation. The cell monolayer pellets were collected by scrapping the growth surface of the cell culture flask with a cell scrapper in to 15 ml of HBSS. The cell monolayer/HBSS mixture was pelleted by centrifugation. The entire cell monolayer pellet was used for DNA extraction, as was the pellet from the cell culture supernatants (potentially containing tachyzoites).

PCR analysis for the presence of *N. caninum* DNA:

Neospora caninum specific primers were designed based on published work (Collantes-Fernandez et al., 2002). The primer sequences for the *N. caninum* specific, NC-5 repeats genomic DNA sequence were 5′-CGCTGAACACCGTATGTCGTA-3′ for the forward primer and 5′-TCCTATACCTCCACCACACCTC-3′ for the reverse primer. Tachyzoite DNA was extracted from the cell culture pellets and pelleted cell culture supernatants from cultures inoculated with irradiated *N. caninum* tachyzoites with a QIAamp DNA mini kit (Qiagen, Valencia, CA). PCR was carried out using the following conditions; 95 ^{o}C for 5 min, 95 ^{o}C for 1 min, 57 ^{o}C for 30 sec, 70 ^{o} C for 30 sec, for 35 cycles followed by extension at 70 ^{o}C for 5 min. Samples were analyzed on a 1% agarose gel for the presence of a 75-bp NC-5 repeat amplicon (Kaufmann et al., 1996).

Electron microscopic examination:

A CV-1 cell monolayer grown in a 75-cm^2 flask was infected with 1 x 10^6 *N. caninum* tachyzoites that were irradiated with 528 Gy gamma irradiation. The flask was washed five times with HBSS 24 h after inoculation. After adding fresh growth medium, the flask was incubated for another 48 h at 37^{o}C. The cell monolayer was scraped and pelleted by centrifugation as described above. The pellet was broken in to numerous smaller pellets and fixed in 3% (v/v) glutaraldehyde in PBS (pH 7.4). Pellets were post-fixed in 1% (w/v) osmium tetroxide in 0.1 M

sodium phosphate buffer and rinsed twice with this buffer. The cell pellets were dehydrated in an ethanol series and were cleared by passage through two changes of propylene oxide before embedding in Poly/Bed 812 resin (Polysciences Inc., Warrington, PA). Thin sections were stained with uranyl acetate and lead citrate. Samples were examined with a Zeiss 10CA TEM operating at 60 kV and digital images were taken using an ATM camera system (Advanced Microscopy Techniques Corp., Danvers, MA).

Alamar-blue dye reduction assay for metabolic activity:

Alamar-blue measures metabolic activity by change in fluorescence (Ahmed et al.,. 1994). Tachyzoites were collected from CV-1 cells and resuspended in HBSS at a concentration of 1 x 10^6 /ml. One 2-ml aliquot was irradiated for 528 Gy. Twelve 90-µl replicates were dispensed in a 96-well tissue culture plate. Twelve 90-µl replicates each of un-irradiated tachyzoites and HBSS were also dispensed in 24 other wells. The plate was incubated at 37 ^oC in a CO_2 incubator and 10 µl of Alamar blue (Biosource, Camarillo, CA) were added to all wells after 15 min. The plate was returned to the CO_2 incubator for 8 h. The plate was read in a Cytofluor II multi-well plate reader (Perseptive Biosystems, Framington, MA) at 530 nm excitation and 590 nm emission wavelengths (Zhi-Jun et al., 1997). The difference in fluorescence between the irradiated and un-irradiated tachyzoites was assessed by a two-sample T test.

Vaccination:

Four to 6 wk-old female C57BL/6 (Charles River Laboratories, Wilmington, MA) mice were used for vaccination. Nine mice served as unvaccinated controls. Twelve mice were vaccinated intraperitoneally (i.p.) with 1 x 10^6 *N. caninum* tachyzoites irradiated with 528 Gy. The mice were boosted i.p. with the same dose of irradiated *N. caninum tachyzoites* 4 wk later. At 5 and 10 wk post-vaccination, two mice from each group were first bled to collect serum and then euthanized to aseptically remove their spleens for establishing in vitro splenocyte cultures. The serum was collected to assess *N. caninum* specific antibody responses. At 10 wk post-vaccination, five mice were lethally challenged i.p. with 2 x 10^7 *N. caninum* tachyzoites and three

were sub-lethally challenged i.p. with 1 x 10^6 *N. caninum* tachyzoites. The mice were observed for symptoms and mortality for a period of 25 days after challenge. Thereafter, they were euthanized by CO_2 asphixiation and their spleen, pancreas, liver, brain and heart tissues collected for histopathology.

***Neospora caninum* specific serum antibody ELISA:**

Maxisorb ELISA plates (Nalge Nunc, Rochester, NY) were coated with 2 µg/ml of *N. caninum* lysate solubilized in coating buffer, pH 9.6, overnight at room temperature. The plates were washed thrice with Dulbecco's phosphate buffered saline containing 0.05% (v/v) Tween-20 (Amersham Biosciences, Piscataway, NJ) (PBST). A 1:50 dilution of each serum sample in PBST with 2% (w/v) bovine serum albumen (Fisher-Biotech, city, NJ) and 1.5% (w/v) skimmed milk powder was added to the plate and incubated at room temperature for 1 h. Anti-mouse IgG, IgG1a, and IgG2a conjugated to alkaline phosphatase (Sigma Aldrich, St. Louis, MO) were used as secondary antibodies at a 1:2000 dilution in blocking buffer for 1 h. Detection was carried out using a substrate solution of Tetra Methyl Benzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The reaction was stopped by adding 50 µl of 0.1N HCl. The intensity of the developed color was measured in an ELISA reader at 460 nm.

Interleukin (IL)-12, IL-10, IL-4, and IFN-γ production by cultured splenocytes:

The spleens collected from the control and vaccinated mice were processed to obtain splenocytes and cultures were setup as previously described (Vemulapalli et al., 1998). The splenocytes were stimulated with either 1 µg/ml of ConA (Sigma Aldrich, Saint Louis, MO) or 1 µg/ml of *N. caninum* lysate or cell culture growth medium. The cultures were incubated at 37 ^oC, for 96 h. The plates were centrifuged at 1200g for 8 min. The supernatants were collected and assayed for selected cytokines. Quantification of cytokines in the supernatants was carried out using a Th1/Th2 cytokine ELISA kit as per the manufacturer's instructions (Ebiosciences, San Diego, CA).

Histopathology:

Spleen, pancreas, liver, lung, and brain samples were fixed in 10% buffered formaldehyde (Fischer Scientific, Fairlawn, NJ). The tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. All tissues were evaluated by a board-certified veterinary pathologist (RBD) who was blinded to treatments.

Statistical analysis:

A 2-tailed Student's t test was carried out using the Microsoft Excel package to compare the immune responses of vaccinated mice with those of the unvaccinated ones; $p \leq 0.05$ was considered significant.

RESULTS

Irradiation dose, viability, and morphology:

The effect of different doses of gamma-radiation on the ability of *N. caninum* tachyzoites to invade cells and cause culture infection was determined using a *N. caninum* -specific PCR assay. Irradiation with 528 Gy for 8 min resulted in complete attenuation of the parasites. Higher doses caused disintegration of the tachyzoites. PCR analysis of CV-1 monolayers infected with irradiated tachyzoites and culture supernatants revealed that the cells and supernatants from the cultures infected with tachyzoites irradiated for 1 and 4 min supported amplification of *N. caninum* DNA after incubation for 4 and 7 days respectively. *Neospora* DNA was detected only in the cells and not in the cell culture supernatants in the 8-min irradiated samples even after incubation for 4 wk. Therefore, irradiation for 8 min caused the tachyzoites to lose reproductive ability but still retain invasive capabilities (Figure 3.2.1)

Electron micrographs of control (N=9) and irradiated *N. caninum* (N = 20) tachyzoites indicated differences were present (Figure. 3.2.2). Both were located inside intracellular

parasitophorous vacuoles. However, irradiated tachyzoites appeared swollen and more rounded. Some appeared to be degenerating or to have degenerated. Numerous lipid/carbohydrate-like bodies were present in the cytoplasm of some tachyzoites. Some irradiated tachyzoites appeared to have undergone division because two tachyzoites were present in two of the 20 micrographs examined. The integrity of the nucleus and most of the organelles was lost in these degenerated, irradiated tachyzoites. Control tachyzoites appeared normal and had features typical of intracellular *N. caninum* tachyzoites.

Presence of metabolic activity after irradiation:

The mean metabolic activity readings of irradiated and non-irradiated tachyzoites were recorded after 8 h. The mean fluorescence units for control tachyzoites were 708 ± 32 while those for irradiated tachyzoites were 604 ± 51 (Figure 3.2.3). The irradiated tachyzoites did not show a significantly decreased metabolic activity when compared to the un-irradiated tachyzoites ($p=0.7$)

Immune responses to vaccination:

Neospora caninum-specific antibody responses in mice sera were detected by ELISA 5 wk after vaccination. These antibodies consisted of both IgG1 and IgG2a isotypes, suggesting that a mixture of Th1 and Th2 types of immune responses were elicited by the vaccination (Figure 3.2.4).

Splenocytes from the vaccinated mice secreted significant levels of IFN-γ and IL-10 upon in vitro stimulation with *N. caninum* lysates (Table 3.2.1); significant levels of IL-12 p40 and IL-4 were not detected (data not shown). As expected, splenocytes stimulated with ConA secreted all of the above-mentioned cytokines, while those stimulated with culture media did not.

Protection in mice:

After challenge with 2 x 10^7 *N. caninum tachyzoites*, all the vaccinated mice (N = 5) survived for a period 25 days. All mice in the unvaccinated control group (N = 5) died within 7

days after challenge (1 died 5 days and 4 died 7 days post-challenge). Lesions were observed in the pancreas, liver, and spleen of these mice and were consist with acute neosporosis. Mild to moderate lymphoplasmacellular meningoencephalitis was found in the brains of the vaccinated and lethally challenged mice (N = 5).

All sub-lethally challenged mice survived challenge. The brain tissue of the mice sub-lethally challenged with 1 x 10^6 *N. caninum* tachyzoites (N=2) was normal in one mouse and the other had mild meningoencephalitic changes, while the unvaccinated controls sub-lethally challenged mice exhibited moderate to severe lymphoplasmacellular and granulomatous meningoencephalitis.

DISCUSSION

Cattle and other hosts for *Neospora caninum* require a predominantly CD4+ T cell-mediated, Th1 type of immune response involving IFN-γ (Nishikawa et al., 2001b, Tanaka et al., 2000) for protection. Antibodies may be important as the initial line of defense against the tachyzoites before they penetrate host cells (Nishikawa et al., 2000). To elicit an effective immune response, an attenuated vaccine would have to mimic a natural infection, particularly in regard to intracellular antigen expression, processing, and presentation. The failure of recombinant proteins or lysates to induce complete protection may be attributed to the inability to induce CMI (Baszler et al., 2000). Moreover, vaccination against a *Neospora* challenge with a single protein is less effective than vaccination with a combination of proteins (Cannas et al., 2003). In the absence of a well-defined and completely protective parasitic antigen, an attenuated vaccine that contains a complete complement of protective antigens is a logical choice as an effective *N. caninum* vaccine (Lindsay et al., 1999b).

Our results demonstrated that irradiation of *N. caninum* tachyzoites causes attenuation of the parasite by preventing multiplication while not altering their invasive ability. Both PCR analysis and electron micrographs of CV-1 cell monolayers infected with the irradiated tachyzoites revealed the presence of intracellular parasites. There was no amplification of tachyzoites in cell culture after irradiation with 528 Gy. Lesser irradiation doses lead to

incomplete attenuation as some of the parasites were still able to multiply and were detected in the cell culture supernatant by PCR analysis after incubation of the culture for one week. Similar attenuation by irradiation has been reported with other parasites, namely *T. gondii* (Hiramoto et al., 2002), *P. berghei* (Chatterjee et al., 2001) and *C. parvum* (Yu et al., 2003): the dose used varied from 200 Gy to 50,000 Gy. Such irradiated parasites were also reported to be metabolically active (Hiramoto et al., 2002) and protective against challenge with the virulent strains. Irradiated *Toxoplasma* sporozoites penetrated enterocytes and lamina propria cells and formed parasitophorous vacuoles in these cells (Kook et al., 1995). The Alamar blue dye reduction assay indicated that irradiated *N. caninum* tachyzoites were metabolically active for at least 8 h after irradiation.

When mice have been immunized with killed *N. caninum* parasitic lysates or whole tachyzoites, others have established that IgG2a-type antibodies and IFN-γ are involved in the immune response to *N. caninum (*Baszler et al., 2000, Long et al., 1998). Vaccination with killed parasites, with or without adjuvants, conferred varying degrees of protection and induced Th2 responses (Lunden et al., 2002, Baszler et al., 2000). Since extensive work has been carried out in characterizing immune responses and protection induced by killed parasites, we have studied these parameters for irradiated tachyzoites.

Previous studies using Balb/c, C57BL/6, and B10.D2 mice established that an IgG2a-type of antibody associated with a Th1 immune response is indicative of resistance to *N. caninum (*Long et al., 1998). Isotype-specific ELISA analysis of sera from mice vaccinated with irradiated *N. caninum* tachyzoites demonstrated the presence of both IgG2a and IgG1, indicating that a protective Th1 type of response was being stimulated. In contrast, it has been shown that antigen lysates mixed with Freund's complete adjuvant induced Th2-type responses and exacerbated the disease (Baszler et al., 2000). The success of the irradiated vaccine in inducing Th1 type of responses is probably due to the fact that the in vivo expressed protective antigens are presented intracellularly, as is the case with natural infections.

Stimulation of cultured splenocytes from vaccinated mice using *N. caninum* lysate induced secretion of highly significant amounts of IFN-γ and IL-10. However, IL-12 p40 and IL-2 were not detected. The reason for this can be attributed to the time of sampling, since these cytokines are normally up regulated in the early stages of the response. IFN-γ, CD4, and CD8 +T cells are important in providing protection against *N. caninum (*Khan et al., 1997). IFN-γ is

responsible for activation of macrophages, nitric oxide production, killing of infected cells, and activation of antigen-specific T cells. IFN-γ knock out mice succumb to acute infection while exogenous administration of IFN-γ helps in recovery. MHC-II expression, which has been determined to be critical in protection against *N. caninum,* was also affected in the IFN-γ knockout mice (Nishikawa et al., 2001). However, abrogation of CD4+ and CD8+ T cell activity did not affect IFN-γ levels, indicating that the activation of IFN-γ may involve other pathways (Tanaka e al. 2000). While the exact mechanism by which IFN-γ was induced by vaccination with irradiated tachyzoites was not determined, this is possibly the single most important factor that contributes to the vaccine's protective ability.

Interleukin-10, considered to be a Th2 cytokine by some researchers, was also found to be highly up-regulated following vaccination with irradiated *N. caninum.* Other studies have shown that it is secreted from day 7 post-infection (Khan et al., 1997). Interleukin-10 levels have also been found to be higher in mouse strains that are more susceptible to *N. caninum* and when IL-12 secretion is blocked *in-vitro* or *in-vivo*. Interleukin-10 knockout mice were not susceptible to virulent *N. caninum* infection, indicating that it is not a critical cytokine in host responses to *N. caninum (*Ritter et al., 2002). However, the potential benefits of high IL-10 levels include mitigation of the toxic effects of IFN-γ, helping to keep tissue damage in check and maintain a healthy Th1/Th2 cytokine balance. Interleukin-4 knock out mice were found to have a moderately increased susceptibility to *N. caninum infection* in the same study (Ritter et al., 2002). However, only very low levels of this cytokines were detected.

All the mice in the vaccinated group were protected against the lethal challenge of 2×10^7 *N. caninum* tachyzoites. While the unvaccinated controls died within 7 days of challenge, the vaccinated mice did not exhibit any signs of morbidity for 25 days post-challenge. Complete protection, such as recorded in this study, has not been shown with any of the *Neospora* vaccines that have been developed so far (Cannas et al., 2003, Liddell et al., 2003, Nishikawa et al., 2001a, 2001c).

Histopathological lesions from vaccinated mice, euthanized after the observation period, included mild to moderate lymphoplasmacellular meningoencephalitis and tachyzoites in the brain. Similar findings have been observed in mice vaccinated with irradiated *T. gondii* (Dubey et al., 1996, Hiramoto et al., 2002). The presence of lesions did not have any correlation with presence of overt clinical signs such as inappetance, cachexia or neuromuscular disorders.

Further work needs to be carried out to determine the efficacy of this irradiated vaccine in preventing vertical transmission in cattle. A possible disadvantage of the vaccine, when compared to a live, attenuated vaccine, may be that the irradiated tachyzoites may not persist in the host for as long as a live vaccine (Lindsay et al., 1999b). This problem can be offset by booster immunizations at the required time intervals. In conclusion, irradiated *N. caninum* tachyzoites are useful as a practical vaccine to prevent neosporosis.

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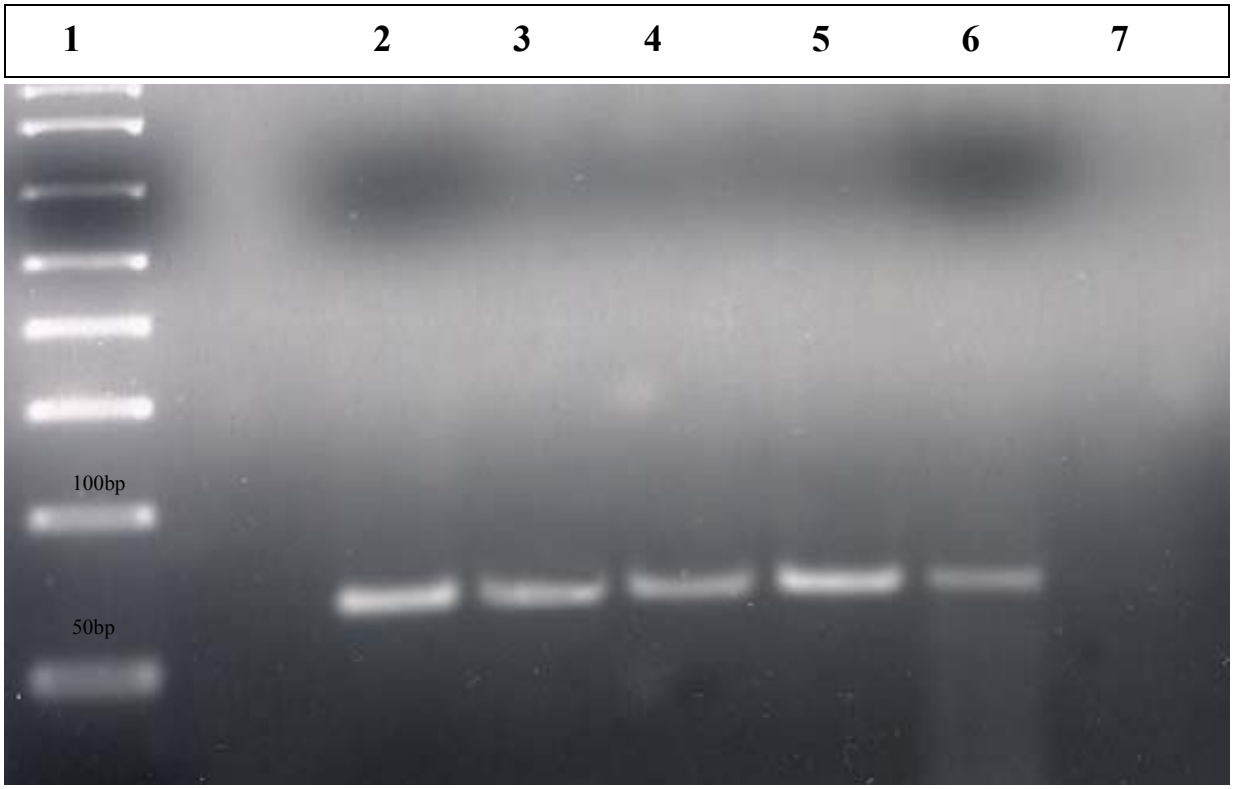

Figure 3.2.1: Results of *Neospora caninum* PCR on CV-1 cells and cell culture supernatants inoculated with gamma irradiated tachyzoites of *Neospora caninum*. 1. DNA ladder, 2. Infected CV-1 cell pellet 1 min irradiated tachyzoites (4 days PI). 3. Supernatant from 1 min irradiated tachyzoites cell culture (4 days PI). 4. Infected CV-1 cell pellet 4 min irradiated tachyzoites (7 days PI). 5. Supernatant from 4 min irradiated tachyzoites cell culture (7 days PI). 6. Infected CV-1 cell pellet 8 min irradiated tachyzoites (4 wk PI). 7. Supernatant from 8 min irradiated tachyzoite cell culture (4 wk PI).

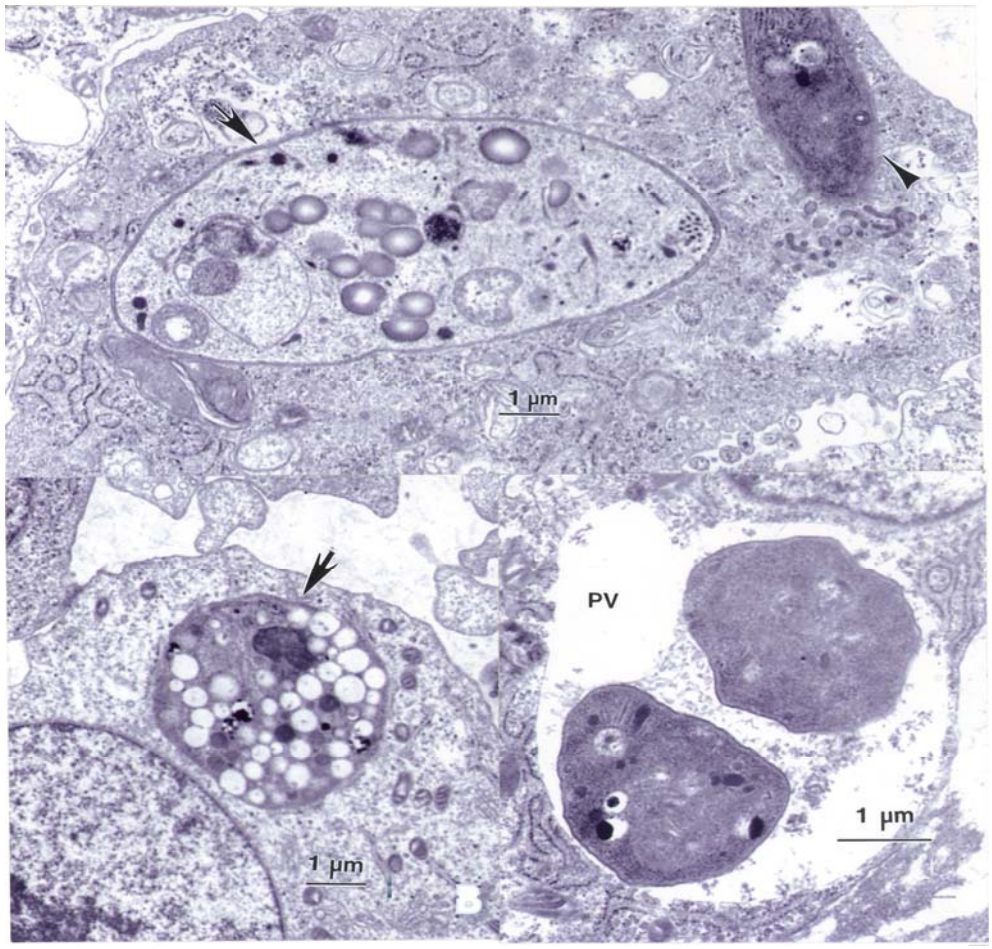

Figure 3.2.2: Transmission electron micrographs of irradiated *Neospora caninum* tachyzoites. A. Longitudinal section of a tachyzoite (arrow) that is undergoing degeneration. There are numerous lipid/carbohydrate-like bodies in the tachyzoite cytoplasm. Note that a portion of another tachyzoite (arrowhead) is present in this micrograph **B.** Cross section of a tachyzoite. Note the numerous lipid/carbohydrate-like bodies in the cytoplasm. **C**. Cross section of two tachyzoites indicating that division has occurred. The parasitophorous vacuole (PV) is labeled.

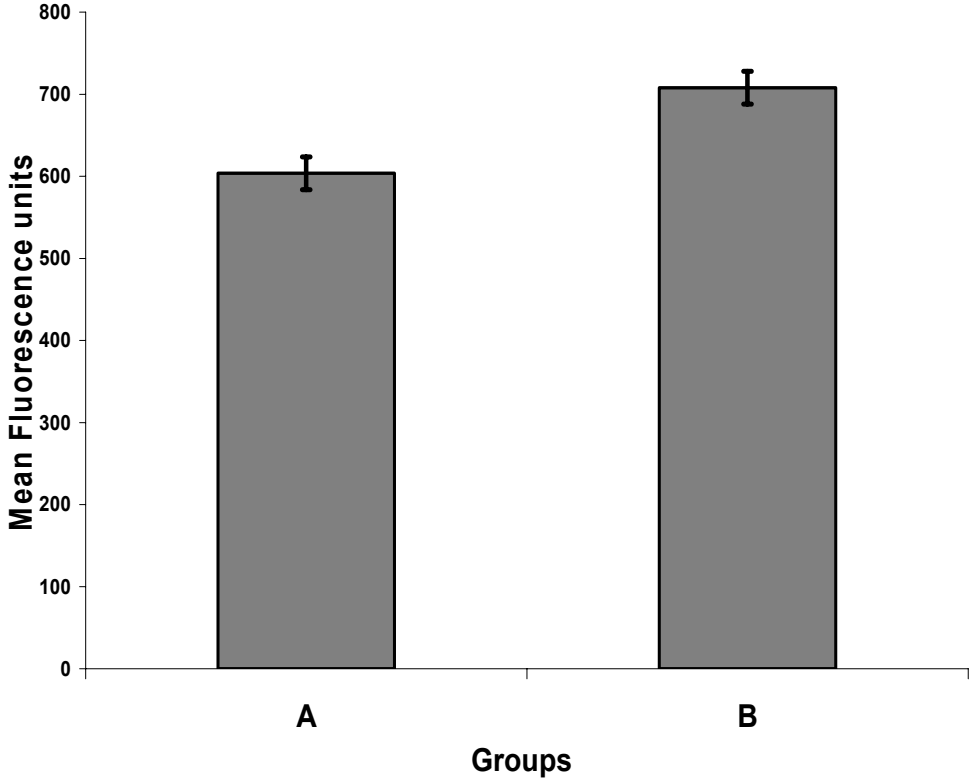

Figure 3.2.3.Measurement of metabolic activity by the Alamar blue dye reduction method: The mean fluorescence unit measurement of 12 replicates of irradiated samples (A), taken 8 hr after irradiation and control tachyzoites (B) after subtraction of blank values are depicted. The difference between the irradiated and unirradiated tachyzoites was not significant (p>0.05). Error bars represent the SEM.

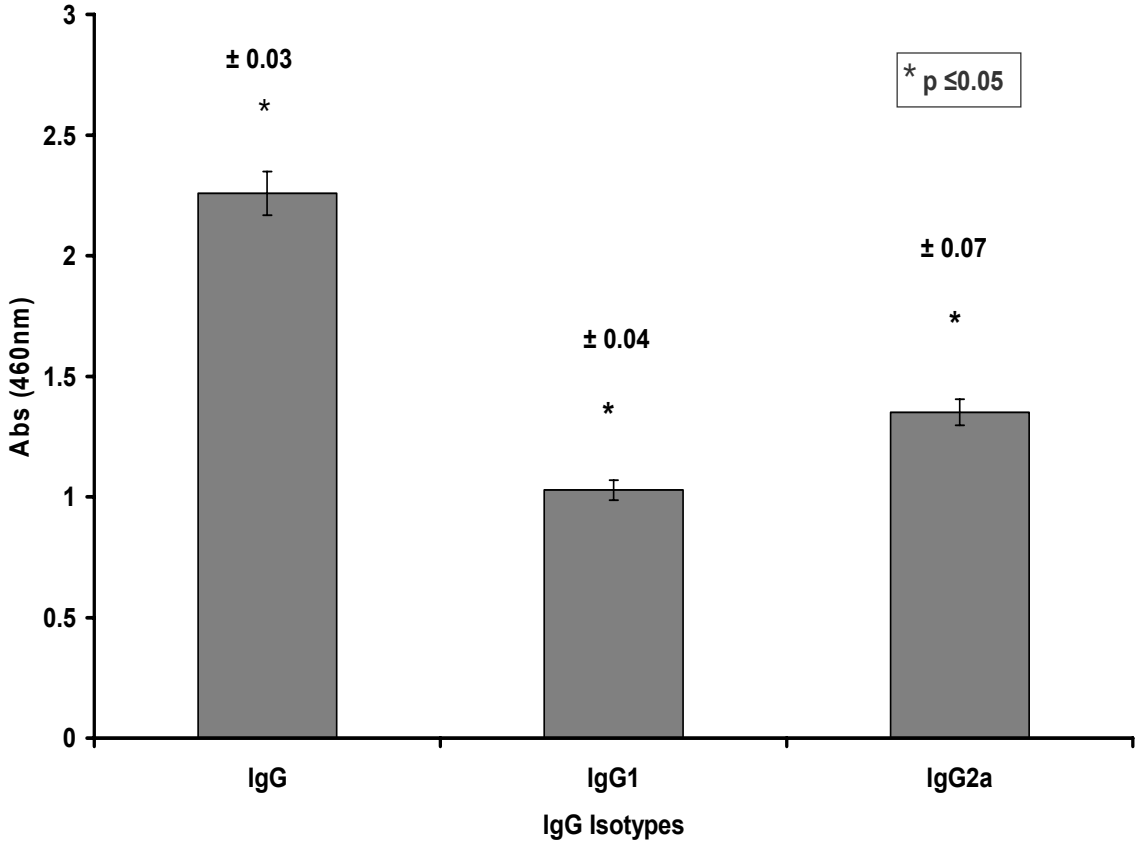

Figure 3.2.4: *Neospora caninum* specific antibody responses in mice vaccinated with irradiated tachyzoites depicting N. *caninum* specific total IgG, *N. caninum* specific IgG1 and *N. caninum* specific IgG2a. Error bars represent the SEM. The values are an average of triplicate readings from each mouse after subtracting the normal mouse serum value, obtained as pre-bleed sera, as a blank.

Table 3.2.1. Cytokine profiles of mice vaccinated with irradiated *Neospora caninum* tachyzoites measured at 5 and 10 weeks after infection *.

Cytokine	Time point (Weeks post vaccination)	Vaccination – Nc Irr Stimulant –Nc Lysate	Vaccination- PBS Stimulant – Nc Lysate
IFN-γ (ng) ± SD	5	7.76 ± 0.04 p=0.0003	0.473 ± 0.020
IFN-γ (ng) ± SD	10	16.33 ± 0.07 p=0.00005	0.428 ± 0.51
IL-10 (pg) ± SD	5	1060.2 ± 82.11 p=0.002	331. 8 ± 12.9
IL-10 (pg) ± SD	10	2004 ± 241.95 p= .004	341.4 ± 74.19

*The values are a representation of the mean of triplicate readings from two mice, obtained after subtraction of the average values from the group vaccinated with PBS and stimulated with media, as a blank.

CHAPTER 3-3

PREVENTION OF VERTICAL TRANSMISSION OF *NEOSPORA CANINUM* IN MICE VACCINATED WITH *BRUCELLA ABORTUS* STRAIN RB51 EXPRESSING *N. CANINUM* PROTECTIVE ANTIGENS

S. Ramamoorthy, N. Sanakkayala, R. Vemulapalli, D. S. Lindsay, G. S. Schurig, S. M. Boyle, N. Sriranganathan. Formatted for Infection and Immunity.

ABSTRACT

Bovine abortions caused by the apicomplexan parasite *Neospora caninum* have been responsible for severe economic losses to the cattle industry. Infected cows either experience abortion or transmit the parasite transplacentally at a rate of about 95% to fetuses that are carried to term. Therefore, *N. caninum* (NC) vaccines that can prevent vertical transmission to ensure disruption in the life cycle of the parasite transmission in a herd will greatly aid in the management of neosporosis in the cattle industry.

Brucella abortus strain RB51, the official vaccine for bovine brucellosis, can also be used as a vector to express plasmid encoded *N. caninum* proteins. *N. caninum* protective antigens MIC1, MIC3, GRA2, GRA6 and SRS2 were expressed in strain RB51. Female C57BL/6 mice were vaccinated with each recombinant sRB51 vaccine or irradiated tachyzoites, boosted four weeks later and then bred. Antigen specific IgG1 and IgG2a antibodies were detected in the serum of vaccinated pregnant mice. Spleen cells from these mice secreted significant levels of IFN-γ and IL-10 upon invitro stimulation with *N. caninum* antigen lysate. Vaccinated mice were challenged with 5 x 10^6 *N. caninum* tachyzoites between days 11-13 of pregnancy. Brain tissue was collected from pups three weeks after birth and examined for the presence of *N. caninum* by a semi-nested PCR. Protection against vertical transmission elicited by the RB51-GRA6, RB51-MIC3, irradiated tachyzoite vaccine, RB51-GRA2, RB51-MIC1, pooled sRB51-*Neospora* vaccine and RB51-SRS2 groups was 45%, 38%, 37%, 34%, 18%, 18% and 7% respectively.

Thus, *B. abortus* strain RB51 expressing the specific *N. caninum* antigens induced partial protection against vertical transmission of *N. caninum* in mice.

INTRODUCTION

The widespread incidence of bovine neosporosis in different parts of the world has resulted in major economic losses to the dairy and beef industries. One of the clinical forms of the disease is abortion in pregnant cattle. A survey of 93 beef and diary herds from 20 states in the U.S.A in 2002 revealed that at least one animal was sero-positive in 90% of the herds tested and that the rate of sero-prevalence within herds ranged from 2-65% (28). The dog has been identified as the definitive host of *N. caninum* (17). Contamination of cattle feed with oocysts excreted by dogs is believed to result in horizontal transmission of Neosporosis from dogs to cattle. About 77-98% of infected cattle are known to transmit the parasite to their offspring throughout their reproductive life. Thus, vertical transmission is the most important factor in maintaining the disease in herds (6). An effective vaccine against N. *caninum* should prevent vertical transmission to be highly effective.

During pregnancy in cattle and mice, it has been found that expression of pro-inflammatory cytokines like IFN-γ and TNF-α level are down regulated while regulatory cytokines like IL-10, TGF-β and IL-4 are up regulated. Fetal trophoblasts and placental tissue secrete cytokines like IL-4, TGF-β and IL-10 and a decrease in IFN-γ levels occurs during mid-gestation (4,8,11). Protection against N. *caninum* is mediated by a Th1 response (2). Therefore, it is possible that the depression in the Th1 response is responsible for recrudescence of the disease in pregnant animals and leads to transmission of the parasite. Experimental evidence to prove this hypothesis has not been obtained in mice or cattle. However, IL-4 levels do appear to to cause a depression in cell mediated immunity and influence vertical transmission rates in mice (23). However, the exact mechanisms by which vertical transmission occurs and the immune responses that facilitate protection against such transmission have not been characterized.

Brucella abortus is a gram-negative bacterium that is also a significant cause of abortions in cattle. *B. abortus* strain RB51 is a stable, rough strain that is an established vaccine

for this disease (29). Previous studies have shown that strain RB51 can be engineered to express heterologous proteins and that specific immune responses to the expressed protein are strongly Th1 biased (30). We have previously described the expression of *N. caninum* MIC1, MIC3, GRA2, GRA6 and SRS2 protein in strain RB51. Antigen specific IgG1 and IgG2a antibodies and antigen specific T cells secreting significant levels of IFN-γ and IL-10 were elicited in mice vaccinated with the recombinant RB51 strains. Challenge with a lethal dose of *N. caninum* tachyzoites resulted in complete protection in mice vaccinated with the RB51-MIC1 and RB51-GRA6 strains and partial protection in mice vaccinated with the GRA2, MIC3 and SRS2 (26).

Currently, there are no known chemotherapeutic agents that are effective against cattle neosporosis. Detailed efficacy data regarding prevention of vertical transmission has not been published for the one commercial, inactivated vaccine available in the USA. Vaccination of mice with tachyzoite lysates has been shown to completely prevent vertical transmission in mice (15). However, vaccination with tachyzoite lysates did not induce protection against vertical transmission in cattle or ewes (12,22). Experimental infection of cattle with live parasites six weeks before pregnancy prevented vertical transmission, when the cattle were challenged mid-gestation. Both cell mediated and humoral responses were detected in the cattle, after challenge (12). Vaccination of Balb/c mice with a GRA7 DNA vaccine combined with CpG as adjuvant is reported to have reduced vertical transmission by 87% (13) while a vaccinia virus expressing SRS2 vaccine provided 83% protection (19). When compared to these recombinant vaccines, the added advantage of the *B. abortus s*RB51 based vaccine is that two important abortifacient diseases of cattle can be prevented simultaneously. Since cattle exposed to live tachyzoites experienced protection against vertical transmission, we incorporated gamma irradiated tachyzoites as a control in this study to evaluate the efficacy of the recombinant sRB51 vaccine in preventing vertical transmission of *N. caninum.* The other advantages of using a live attenuated tachyzoite vaccine, or a live, recombinant vaccine over the inactivated tachyzoite lysate vaccine are the ease of production and targeting of a combined cellular and antibody response as against the antibody biased response in the case of the lysate vaccine (3).

We had previously described the use of gamma-irradiated tachyzoites as a vaccine for *N. caninum.* When *N. caninum* tachyzoites were irradiated with gamma irradiation equivalent to 528Gy, they were found to lose their ability to replicate. However, they were still able to penetrate and invade host cells. Strong antibody and Th1 type cell mediated responses were

elicited in mice vaccinated with this attenuated vaccine. The vaccinated mice were completely protected against lethal challenge with virulent *N. caninum* (24).

With the decline in the incidence of bovine brucellosis following the implementation of nationwide control program, *N. caninum* has emerged as one of the leading causes of bovine abortions in the U.S and in several parts of the world. Approximately 42.2 % of aborted bovine fetuses in the U.S were infected with *N. caninum* (6). The rate of sero-prevalence in the U.S.A in 2003 was about 16% (6). Due to its widespread incidence and lack of vaccines or control measures, losses caused by this disease have increased greatly, during the two decades that have passed since the identification of the parasite.

In this study, we have explored the hypothesis that vaccination of mice with the recombinant sRB51-*Neospora* strains expressing *N. caninum* antigens will induce strong Th1 type responses and prevent vertical transmission of *N. caninum.*

MATERIALS AND METHODS

Parasite culture and preparation of irradiated *N. caninum* tachyzoites

NC-1 strain of *N. caninum* was cultured in CV1 cells (ATCC CCL-70, American type culture collection, Manassas, VA). The tachyzoites were purified and counted and irradiated using previously described protocols (7).

Cloning and expression of *N. caninum* MIC1, MIC3, GRA2, GRA6 and SRS2 antigens in *B. abortus* strain RB51 were previously described in Chapter 3-2.

Pre-titrated glycerol stocks of the recombinant vaccine strains and strain RB51 carrying plasmid pBBGroE alone, were resuspended to obtain $1x10^9$ CFU/ml in PBS for the primary vaccination and $1x10^8$ CFU/ml for the booster vaccination. Titers of the resuspended vaccines were verified by plating serial dilutions on TSA plates.

Immunization, mating and challenge of mice

4-6 week old female C57BL/6 mice were housed (Jackson Laboratory, Charles River, MA) in a CDC certified BSL3 facility and all experiments were carried out in compliance with the requirements of the animal welfare committee of Virginia Tech as per AVMA guidelines.

Eight groups of ten mice each were treated as follows: a) unvaccinated controls; b) irradiated *Neospora* ($1x10^6$) tachyzoites, i.p; c) sRB51 vector control group; d) RB51-MIC1; e) RB51-MIC3; f) RB51- GRA6; g) RB51-GRA2; h) RB51-SRS2. Combination of recombinant RB51 MIC1, MIC3, GRA6, GRA2, SRS2 strains ($2x10^8$ cfu each – designated as pooled recombinant vaccine). All recombinant RB51 strains were administered at a dose of $1x10^9$ CFU in 1ml volumes i.p. A ten fold lower dose was administered as a booster; four weeks post primary immunization for all groups.

One week after the booster vaccination, bedding from cages housing male mice was transferred to the cages housing females for 48 hours to induce estrus. Subsequently, one male and two females were kept in the same cage for 72 hours and the females were examined for the presence of a sperm plug. Two non-pregnant females and two pregnant females each from the unvaccinated controls, sRB51pBBGroE, irradiated *N. caninum* tachyzoites and pooled recombinant vaccine groups were euthanized between day 9-11 of pregnancy for collection of serum, splenocyte culture and cytokine assays. The remaining sperm plug positive females were challenged with 5×10^6 *N. caninum* tachyzoites between days 11-13 of pregnancy. Pregnant dams were placed in individual cages 72 hours before the expected time of parturition. The number of pups born alive or stillborn and any deaths during the 21-day observation period were recorded. All surviving pups were sacrificed on day 21 and brain samples were collected for PCR analysis. Samples were stored at -80^oC until processed. Stringent procedures were followed to avoid sample contamination.

Serum antibody responses

Maxisorp ELISA plates (Nalge Nunc NY) were coated with 50µl of 2µg/µl *N. caninum* lysate solution in coating buffer (carbonate buffer pH 9.6) overnight at room temperature. The plates were washed thrice with Dulbeccos PBS containing 0.05% Tween20 (Amersham

Biosciences NJ). A 1: 10 dilution of sera samples in blocking buffer (PBST with 2% BSA (Fisher-Biotech, NJ) and 1.5% skimmed milk powder) was added to the wells and incubated at room temperature for one hour. Anti-mouse IgG, IgG1a and IgG2a antibodieds conjugated to horse radish peroxidase (Sigma Aldrich, MO) were used at a 1:2000 dilution in blocking buffer for one hour as secondary antibodies. Detection was carried out with TMB (KPL, MD). The reaction was stopped using 0.1N HCl. Plates were read in an ELISA reader (Perkin Elmer, MA) at 460 nm. The values represented are the mean of triplicate samples obtained from two mice in each group. Values obtained for the mice vaccinated with PBS were used as the blank. Pre-vaccination sera from two mice from each group were also tested to establish that the mice did not contain cross-reactive antibodies.

Measurement of cytokines in splenocyte culture supernatants from vaccinated mice

Splenic lymphocytes from two pregnant vaccinated mice from the irradiated tachyzoite vaccine group, pooled sRB51-*Neospora* vaccine group, RB51 vector group and unvaccinated controls were processed as previously described (30). The cultured splenocytes were stimulated in duplicate with 1 µg/ml of ConA (Sigma Aldrich, Mo) or 1 µg/ml of *N. caninum* lysate or RPMI containing 10% FBS and 100 IU each of penicillin and streptomycin. The cultures were incubated at 37^{o}C, for 96 hours, in a CO_2 incubator. The plates were centrifuged at 1,200 RPM for eight minutes and the supernatants were collected. Cytokine quantification was carried out using a Th1/Th2 cytokine ELISA kit as per the manufacturers' instructions (Ebiosciences, CA). The average of triplicate values from each of the above-mentioned samples is presented. Values of the media stimulated samples from each mouse were used as blank values and subtracted from the other values obtained from the ELISA for analysis.

Semi-nested PCR for detection of *N. caninum* in pup brain tissue

Brain tissue samples were macerated in 10% w/v of sterile PBS, using asceptic technique. 200µl of the lysates were used for DNA extraction using the Qiagen DNA mini kit (Qiagen, Valencia, CA) following the manufacturers instructions. The DNA in each sample was quantified using Pico-Green (Molecular Probes, Eugene, OR). For the first step of amplification,

200 ng of template DNA was amplified using Ready-mix Taq PCR mix (Sigma, Valencia, CA). The primer sequences used in this reaction were GTGCGTCCAATCCTGTAAC for the forward primer and CAGTCAACCTACGTCTTCT for the reverse primer for the amplification of a 500bp *N. caninum* gene fragment from the NC-5 gene. 5µl of the reaction mixture from the first amplification was used for the second step of the PCR. The primer sequences in the second reaction were ACTGATGACGGGGGAGATTA for the forward primer; while the reverse primer remained the same; an internal 375bp band was amplified by the second reaction. Both reactions were amplified using a Tm of 49^{o}C. DNA extracted from tachyzoites was used as a positive control. One sample that did not contain any DNA was used as a no template control. Two hundred ng of naive mouse brain DNA spiked with DNA equivalent to 2.5 tachyzoites was used as a control to rule out false negative reactions.

Statistical analysis

A two-tailed student T test (Microsoft Excel package) was carried out to compare immune responses of vaccinated mice with those of the unvaccinated ones. Chi square analysis was used to compare vertical transmission rates and pup survival rates (Excel software). The cut off for statistical significance was set at a p value of 0.05 for all tests.

RESULTS

Antibody responses in pregnant mice

ELISA carried out using serum collected from vaccinated mice between day 9-11 of pregnancy, revealed the presence of *N. caninum* antigen specific IgG1 and IgG2a antibody isotypes. Except for the IgG1 levels in mice vaccinated with the strain RB51-MIC3 and the vector control sRB51, the IgG, IgG1 and IgG2a levels in all treatment groups were significantly different from those of the unvaccinated control group (Figure 3.3.1). When compared with the

sRB51pBBGroE control group, all values in treated groups were significantly different, except for IgG2a levels in the mice immunized with RB51-MIC3.

Cytokine levels in vaccinated pregnant mice:

Measurement of cytokine levels in supernatants of splenocyte cultures derived from pregnant, unvaccinated mice, stimulated with *N. caninum* lysate revealed significant ($p \leq 0.05$) levels of IL4, IL-10 and IFN-γ, when compared to non-pregnant, unvaccinated mice. This comparison was carried out to establish differences in cytokine profiles that could be attributed to pregnancy in unvaccinated mice. Comparison of cytokines levels between a) pregnant-unvaccinated and pregnant-vaccinated mice was carried out to determine immune responses to vaccination in pregnant mice (p value designated as p_a in Table 3.3.1) b) non-pregnant-vaccinated and pregnant-vaccinated mice was carried out to determine the effect of pregnancy on immune responses to vaccination (p value designated as p_b in Table 3.3.1). When cytokine levels in pregnant unvaccinated mice were compared to pregnant vaccinated mice, IL-4 levels in mice vaccinated with irradiated tachyzoites and the pooled recombinant sRB51- *Neospora* vaccine were not significant (Ref p_a values), while IFN-γ and IL-10 levels were significantly different. Only IL-10 concentration in pregnant mice vaccinated with irradiated tachyzoites were significantly higher (Ref p_b values) than non-pregnant mice administered the same treatment (Table 3.3.1). Significant levels of IFN-γ were produced in mice vaccinated with the recombinant vaccine, when stimulated with heat-killed strain RB51 (data not presented). All ConA controls induced significant levels of the respective cytokines while the media controls did not.

Litter size and pup survival in vaccinated and challenged mice

The average litter size in treatment groups varied from 5.75 to 8.6 (Table 3.3.2). However, the differences in litter size of the treatment groups when compared to the unvaccinated control group were not statistically significant. Pup survival rates in vaccinated groups ranged from 50-95.5%. Although the number of pups that survived until 21 days post-partum in some vaccinated groups such as RB51-MIC3 (95.5%), RB51-GRA6 (90.7%) and NC-

Irr (86.7%) were considerably higher than those that survived in the unvaccinated control group (73.91%) but still the differences were not statistically significant (Table 3.3.2). The percentage pup survival in the MIC1 group was 80%, in the GRA2 group 73.8% and in mice administered the pooled recombinant vaccine the pup survival rate was 82.6%. Mice administered the RB51-SRS2 vaccine had a survival rate that was significantly lower than that of the unvaccinated controls. Mice immunized with sRB51 alone were less protected than unvaccinated mice and had a pup survival rate of 61.5.

Protection against vertical transmission in vaccinated mice

Based on the *N. caninum* specific semi-nested PCR of DNA extracted from brain tissue homogenates collected from pups 21 days after birth, protection against vertical transmission elicited by the irradiated tachyzoite, RB51- MIC1, RB51-MIC3, RB51-GRA6, RB51-GRA2 , RB51-SRS2 immunized and the pooled recombinant groups was 34%, 18%, 38%, 43%, 34% ,7% and 18% respectively. Significant protection against vertical transmission was observed in all immunized mice except for those immunized with the RB51-MIC1 and RB51-SRS2 vaccines, (Table 3.3.3) based on Chi-square analysis. There was no significant protection in any of the treatment groups in comparison to the sRB51 vector control group

DISCUSSION

The mechanism of and factors influencing vertical transmission of N. *caninum* have yet been completely understood. However, it is well recognized that prevention of this route of transmission is crucial to controlling neosporosis (6). It is believed that a physiological Th2 bias occurs in the pregnant dam (4, 8, 14). Such immune-modulation may cause an exacerbation of diseases, such as neosporosis, that require a Th1 response for resistance

Although a mixed IgG1 and IgG2a response is elicited upon infection of non-pregnant mice (2), it is believed that protection against murine neosporosis is associated with Th1 type immunity and therefore, presence of IgG2a isotype antibodies is considered to be beneficial (16).

In mice infected during pregnancy, both IgG1 and IgG2a antibody isotypes were detected and neither isotype was predominant (27). However, it is not clear which isotype is important in preventing vertical transmission. Mice that were vaccinated with sRB51-*Neospora* vaccine strains and the irradiated tachyzoite vaccine and sampled between days 9-11 of pregnancy had significant levels of both IgG1 and IgG2a isotype (except RB51-MIC3) antibodies (Figure 3.3.1). Pregnancy appeared to promote IgG1 responses in this treatment group in mice vaccinated with RB51-SRS2, as we have previously shown that significant levels if IgG1 were not induced by vaccination with RB51-SRS2 in non-pregnant mice (26) . Although significant levels of IgG1 and IgG2a were found in the serum of vaccinated, pregnant mice, the RB51-SRS2 and RB51-MIC1 vaccines did not induce significant protection against vertical transmission (Table 3.3.3). Based on this lack of correlation, it is difficult to determine the significance of either isotype in protecting against vertical transmission. However, other researchers have had considerable success in preventing vertical transmission by vaccination with tachyzoite lysates (15). When SRS2 was administered either through a vaccinia vector (20) or as native antigen (9), strong IgG1 responses were elicited (20). It has also been found that antibodies to SRS2 prevent attachment of tachyzoites to trophoblast cells (10). Therefore, while it is difficult to establish a generalized correlation of protection with the antibody isotype, it appears that certain *N. caninum* antigens perform better as vaccines if they elicit an IgG1 response and others when they elicit an IgG2a response.

It is previously known that pregnant mice have elevated levels of IL-4 and IL-10 (4, 8). In this study, we also found higher levels of IFN-γ in pregnant mice (Table 3.3.1). While very high levels of IFN-γ are considered detrimental for pregnancy, Delassus et al., have also found that a systemic elevation of both Th1 and Th2 cytokines does occur in early murine pregnancy (1, 5).

IFN-γ and IL-12 are very critical to the host immune response against *N. caninum* infections (2). Pregnant mice infected with *N. caninum* during pregnancy experience an increase in IL-4 levels and a decrease in IFN-γ levels in comparison to infected, non-pregnant mice (23). However, Rettinger et al., did not find evidence to support the hypothesis that modulation of the immune response during pregnancy might be responsible for vertical transmission (27). Vaccination of non-pregnant mice with irradiated tachyzoites and recombinant RB5-*Neospora* vaccine induced antigen specific immune cells secreting highly significant levels of IFN-γ and

IL-10, but not IL-4 (26). In pregnant, vaccinated mice, although IL-4 was detected, the levels were not significant when compared to unvaccinated pregnant mice (Table 3.3.1). This could be attributed to the Th2 depression that could be a result of high IFN-γ levels induced by vaccination or to the fact that the Th2 bias of pregnancy is more localized than systemic (1).

There was no significant reduction in the IFN-γ or IL-10 levels (Table 3.3.1) due to pregnancy, indicating that, as expected, vaccination with the recombinant sRB51-*Neospora* vaccine and the irradiated vaccine elicited a strong Th1 immune response that did not compromise pregnancy. However, it appears that the induction of a strong Th1 response alone is not sufficient to induce complete protection against vertical transmission, as the protection induced by these vaccines was not complete. However, except for the RB51-MIC1 and RB51-SRS2 vaccines, the efficacy of the other vaccines in reducing vertical transmission was statistically significant when compared to unvaccinated controls. None of the vaccinated groups were significantly different from the sRB51 control group except for mice vaccinated with RB51-SRS2, which were significantly less protected compared to sRB51. Therefore, sRB51 was responsible for induction of non-specific protection, which can be attributed to its capacity to induce high IFN-γ levels

It is possible that adopting measures to improve the Th1 response to these vaccines may improve protection as reported by Dubey et al., wherein the protection elicited by NC-GRA7 DNA was improved from 46% to 85% by the addition of CpG (13). In contrast, vaccination with native SRS2 induced higher levels of IL-4 than IFN-γ and was effective in reducing vertical transmission in Balb/c mice (9). In the sRB51 context, the SRS2 vaccine performed poorly, eliciting just 7% protection against vertical transmission in C57BL/6 mice. Therefore, it is evident that not all protective antigens will be effective as vaccines when administered in a context that induces a strong Th1 response or when expressed in sRB51. It is very likely that the GRA proteins work well in a Th1 context while SRS2 requires a Th2 context to be effective, a judicious and balanced induction of both responses may lead to complete protection. It is also possible that the strain of mice used can strongly influence the outcome of the experiment.

It is also evident that the mechanisms of protection and protective antigens involved in preventing acute neosporosis are not the same as those involved in preventing vertical transmission. The strain RB51-MIC1 vaccine was able to provide complete protection against a highly lethal challenge with *N. caninum* tachyzoites in vaccinated mice, but was unable to

protect against vertical transmission. Similarly, crude tachyzoite lysates were able to induce complete protection against vertical transmission but exacerbated the disease when used as a vaccine against acute disease (3, 15). It is known that strong Th2 responses are induced by vaccination with tachyzoite lysates, while the irradiated and sRB51-based vaccines induce a strong Th1 type immunity. Therefore, it appears that a Th1 response is required for protection against disease; while a Th2 response may be required for protection against vertical transmission. It is possible that the parasite uses this strategy to evade the host immune response. Like other apicomplexan parasites, it is also possible some *N. caninum* immuno-dominant antigens or specific epitopes of the same antigen can stimulate strong but ineffective CMI or antibody responses and act as decoys in subverting the host immune response (21).

Protection against vertical transmission induced by administration of the pooled recombinant vaccine was lower than that induced by sRB51 alone. It is possible that the poorly performing strains such as RB51-SRS2 or RB51-MIC1 induced immune responses that interfered with the protective responses that could be induced by the other strains. It is also likely the reduction in effective vaccination dose of individual recombinant strains to $2x10^8$ CFU each (as opposed to the dose of $1x10^9$ CFU's administered to mice vaccinated with the individual strains), which was necessary to achieve a composite dose of $1x10^9$ CFUs may be responsible for this reduction in protection against vertical transmission.

Susceptibility to *N. caninum* has a genetic predisposition in mice (16). Balb/c mice do not show clinical signs of the disease, are not susceptible to lethal challenge and are relatively resistant to the disease. However, most of the *N. caninum* vaccine studies have been conducted in Balb/c mice. Therefore, a comparison of the efficacy of the irradiated and sRB51-*Neospora* vaccines and the other vaccines cannot always be made. C57BL/6 mice, on the other hand, are highly susceptible to *N. caninum* infection. They succumb to acute infection and the rate of transmission in mice infected between 9-11 days of pregnancy is 95-100% (25). It is possible that if the irradiated and sRB51-*Neospora* vaccines were tested in Balb/c mice, better rates of protection could have been achieved, as the mice are inherently more resistant to the disease.

Another possible reason for differences in protective potential of the selected recombinant strains could be due to the differences in the amounts of recombinant proteins expressed by them. However, we have followed the standard protocol of dose determination for

vectored vaccines by optimizing the dose of the vector, in this case CFU's of strain RB51, rather than the dose of the heterologous *N. caninum* protein.

Using the C57BL/6 pregnant mouse model for vaccine testing, we did not find a significant difference in the average number of pups per litter in vaccinated or unvaccinated groups (Table 4.2). These results are in keeping with our previous findings that challenge of pregnant C57BL/6 mice during mid-gestation did not cause a reduction of litter size due to resorptions (25).

Nishikawa et al., (20) were able to establish a correlation between high fetal mortality and vertical transmission in Balb/c mice. In this study, although survival rates were somewhat higher in vaccinated mice when compared to the controls, the differences were not significant except for RB51-SRS2 vaccine, where the mortality was higher than that of the control group (Table 3.3.2). It appears C57BL/6 and Balb/c mice differ in that mid-gestation infection of the dam does not induce fetal death in C57BL/6 mice. Therefore, pup survival is probably not a good indication of protection against vertical transmission in the C57BL/6 model.

In BALB/C pregnant mouse model, brain tissue from pups that survived the vaccine trial was always PCR negative (20). Unlike Balb/c mice, in C57BL/6 mice, brain tissue of some surviving pups were PCR positive, indicating that transmission has taken place, while it was negative in other surviving pups, indicating protection by the vaccine (Table 3.3.3). However, brain tissue from all dead pups was PCR positive. Therefore, the C57BL/6 pregnant mouse model probably bears a closer resemblance to the transmission model in the cow, in that infection in mid-gestation usually results birth of clinically normal calves that are still infected. While it is likely that the results from this study will translate well in cattle studies, they cannot be compared to vaccines that have been tested in other strains of mice.

Therefore, it appears that the success of a vaccine in preventing vertical transmission is determined by a very complex interplay of several factors such as stimulation of the right type, duration and robustness of the maternal immune response and the ability of the fetus to mount an effective immune response while not compromising pregnancy (11). The fact that significant levels of protection were elicited in selected vaccinated groups against both acute challenge and vertical transmission indicate that these approaches hold great promise for the prophylaxis against neosporosis.

The irradiated vaccine is likely to be more successful in cattle since it is known that experimental exposure of cattle to *N. caninum* tachyzoites protects against vertical transmission. However, this vaccine will be more expensive to produce as it involves scaling up cell cultures to obtain the required amounts of tachyzoites and downstream tachyzoite purification. The recombinant sRB51 based vaccine will be easy to produce, store, cost-effective and more importantly, target two important diseases that cause bovine abortions.

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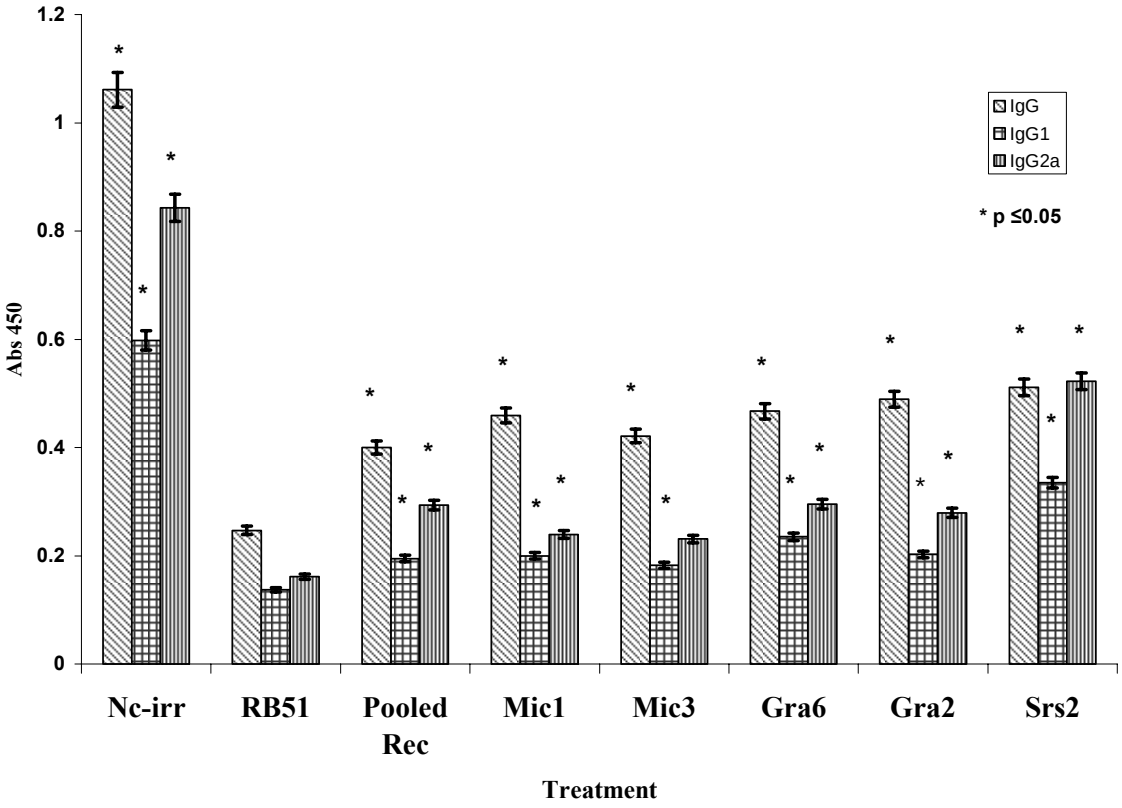

Figure 3.3.1: Post vaccination antibody responses in pregnant C57BL/6 mice. Values are averages of triplicate samples derived from two mice in each treatment group. Levels of significance were determined by a T test with $\alpha = 0.05$ for a comparison of treatment groups with sRB51 immunized mice

Table 3.3.1: Cytokine levels in vaccinated, pregnant mice as measured in splenocyte culture supernatants stimulated with *N. caninum* lysate.

Treatment / Stimulant NC-1	IL-4 (pg)	IL-10 (pg)	IFN-γ (pg)
Non-Pregnant, Unvaccinated	9.94 ± 11.57	254.38 ± 34.01	412.50 ± 9.54
Pregnant, Unvaccinated	139.10 ± 18.92 p = 0.03	1151.60 ± 2.50 p = 0.02	1108.50 ± 18.03 p = 0.006
RB51 Non-Pregnant	ND	174.9 ± 21.63	724.36 ± 36.25
RB51 Pregnant	110.51 ± 36.20 p_a = 0.26 p_b = 0.16	959.20 ± 57.35 p_a = 0.12 p_b = 0.02	1792.50 ± 256.68 p_a = 0.29 p_b = 0.22
Pooled RB51-NC Non-pregnant	ND	491.4 ± 8.82	6012 ± 99.01
Pooled RB51-NC Pregnant	89.23 ± 15.66 p_a = 0.12 p_b = 0.144	893.09 ± 270.42 p_a = 0.015 p_b = 0.19	4563.75± 165.79 p_a = 0.0451 p_b = 0.28
Nc-Irr Non-pregnant	3.35 ± 0.28	1060.20 ± 82.11	7759.30 ± 716.98
Nc-Irr Pregnant	70.41 ± 5.47 p_a = 0.12 p_b = 0.145	2515.08 ± 188.93 p_a = 0.0003 p_b = 0.0002	7243.50 ± 394.24 p_a = 0.0005 p_b = 0.510

P_a – p value for the comparison between vaccinated pregnant mice and unvaccinated pregnant mice in the same treatment group.
P_b - p value for the comparison between non-pregnant mice and pregnant mice in the same group
Values depicted are mean values of triplicate determinations ± the standard deviation from two mice in each group

Table 3.3.2: Litter size and survival data in treated and control mice challenged between days 11-13 of pregnancy.

Group	No of pregnant mice	Total No of pups	Av. No of pups /litter A	No. of pups dead within 20 days of birth	% Survival
Unvaccinated control	4	27	6.8	5	73.9
NC-Irr	4	30	7.5	4	86.7
RB51	5	39	7.8	15	61.5
RB51-MIC1	5	41	8.2	8	80
RB51-MIC3	3	22	7.3	1	95.5
RB51-GRA6	5	43	8.6	4	90.7
RB51-GRA2	5	42	8.4	11	73.8
RB51-SRS2	4	28	7	14	50_A
Pooled RB51/NC	4	23	5.8	4	82.6

A – Significantly different from the unvaccinated control group at $\alpha = 0.05$

Table 3.3.3: Protection against vertical transmission in vaccinated mice.

Group	Total samples for analysis [A]	No of PCR + samples [B]	% Transmission	% Protection
Unvaccinated control	23	22	95	–
NC-Irr	30	19	63	37 [C]
RB51	32	22	69	31 [C]
RB51-MIC1	38	31	82	18
RB51-MIC3	21	13	62	38 [C]
RB51-GRA6	40	22	57	45 [C]
RB51-GRA2	39	26	66	34 [C]
RB51-SRS2	16	15	93	7 [D]
Pooled RB51/NC	23	19	82	18

A - Does not include samples lost due to cannibalization or autolysis.

B – Including brain samples from dead pups. All brain DNA samples from dead pups were PCR positive.

C – Significantly different from unvaccinated controls as determined by Chi-square analysis

D - Significantly different from RB51 controls as determined by Chi-square analysis

GENERAL CONCLUSIONS

N. caninum is an important pathogen that affects cattle, sheep and goats. The reproductive losses caused by *N. caninum* in cattle are responsible for severe economic losses in many parts of the world (3). There are no known chemotherapeutic agents or effective vaccines against cattle Neosprosis. The existing inactivated vaccine stimulates strong antibody responses and its efficacy in preventing vertical transmission has not been determined. Therefore there is a very urgent need to develop an effective vaccine against *N. caninum.*

Based on the biology of the parasite and its life cycle, an effective *N. caninum* vaccine should be able to: a) stimulate strong Th1 type immune responses b) protect against infection in naïve animals; c) reduce or abolish clinical signs such as abortions in infected animals and d) prevent vertical transmission of the parasite. Most of the vaccines described so far have been able to satisfy some of these criteria but none meet all of the criteria. In this study, we have developed two vaccines against *N. caninum*, an expensive, gamma irradiated, live tachyzoite vaccine (7) and a less expensive and more feasible recombinant *Brucella abortus* strain *RB51* based vaccine, that have largely satisfied all the above-mentioned criteria.

Several strains of mice have been used as laboratory animal models for the testing of *N. caninum* vaccines. There is a large variation in the genetic susceptibility of mice to *N. caninum* and therefore, the efficacies of the vaccines tested in studies, where different strains of mice have been used cannot be compared (2, 5, 6). We have addressed the need to have a uniform testing procedure in a susceptible strain of mice by optimizing the use of C57BL/6 mice for *N. caninum* vaccine testing. We determined the LD_{50} for lethal challenge in this strain of mice and determined vertical transmission parameters such as rate of transmission corresponding to time of infection and dose. We also determined variation in localization and tissue pathology in mice based on dose and time. This information was used to develop a scoring system to determine protection conferred by vaccines as a complement for determination of parasite load by real time PCR. These findings have important application in the vaccine and chemotherapeutic agent development research and to standardize the testing of *N. caninum* vaccines. We have also standardized the gerbil lethal challenge dose as gerbils are more susceptible to *N. caninum* than mice (8) and can be useful for drug-development studies.

We are the first to show that eukaryotic proteins can be successfully expressed in the strain RB51 system and that the Trc promoter is as effective as the GroE promoter in driving strong expression of heterologus proteins in strain RB51. This knowledge paves the way for the future development of several multivalent vaccines for diseases that affect cattle using strain RB51 as a vector.

Since *N. caninum* requires strong cell mediated immunity involving IFN-γ for protection, we hypothesized that *B. abortus* strain RB51 expressing *N. caninum* protective antigens should stimulate strong Th1 type of immune responses and protect mice against lethal challenge with virulent *N. caninum.* We have clearly demonstrated that strong Th1 responses were indeed stimulated by vaccination and that vaccinated mice were protected against lethal challenge. The recombinant strain RB51-*Neospora* vaccines have several advantages when compared to inactivated vaccines. They are less expensive to produce because they do not require cell culture. Moreover, they induce the desirable type of cell-mediated immunity. Above all, two important diseases that cause bovine abortions, brucellosis and neosporosis, can be targeted with this one vaccine. Therefore, the sRB51-*Neospora* vaccine is a novel and commercially viable approach for immunization against *N. caninum.*

We have also shown that gamma irradiation of *N. caninum* tachyzoites abolishes replicative properties, while retaining the ability of tachyzoites to invade cells. Attenuated vaccines are considered superior to inactivated vaccines for immunization against intracellular parasites (4) as they stimulate cell mediated, rather than antibody-mediated immunity (1). When used as a vaccine, irradiated tachyzoites stimulated strong Th1 type immune responses and completely protected mice against lethal challenge. The irradiated vaccine can serve as an excellent alternative for the commercially available inactivated vaccine (7). It has the added advantage that commercial licensing by the USDA is easier for conventional vaccines when compared to recombinant vaccines. However, the yield of tachyzoites from cell culture is low and production of this vaccine will be expensive. Down stream purification of tachyzoites will further add to the cost. The stability and preservation of such irradiated tachyzoite preparations will also have to be further studied before commercialization.

We have demonstrated that the irradiated tachyzoite vaccine and the recombinant sRB51 based vaccines were able to partially prevent vertical transmission of *N. caninum.* Additionally, the vertical transmission vaccine study has yielded valuable insights into the host immunology

involved in vertical transmission. As the vaccine strains that protected very well against acute challenge did not necessarily protect against vertical transmission, it is likely that the immune responses required for preventing vertical transmission are different from those required for preventing the acute form of the disease. Further studies to improve protection against vertical transmission and testing of the vaccines in cattle can lead to the development of one of the most complete *N. caninum* vaccines available to date. Some strategies that can be adopted to improve protection against vertical transmission include incorporation of adjuvants that can improve Th1 or Th2 responses or using the sRB51 based vaccine for priming followed by recombinant antigen boost.

In summary, we have developed two excellent vaccines against neosporosis that are not only effective but commercially viable alternatives to the existing inactivated vaccine.

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ADDENDUM -1

STABILITY OF RECOMBINANT PLASMIDS pBBGroE AND pBBTrc ENCODING *NEOSPORA CANINUM* PROTECTIVE ANTIGENS IN *BRUCELLA ABORTUS* STRAIN RB51.

S. Ramamoorthy, S. M. Boyle, G. S. Schurig, N. Sriranganathan. Short communication formatted for Veterinary Microbiology

ABSTRACT

The instability of cloned plasmid DNA in recombinant strains of bacteria is of major concern in the commercialization of vaccines. In this study, the stability of plasmids, pBBGroE and pBBTrc, which are used for protein over expression in *Brucella* species, was determined. Mice were inoculated with a pooled culture of *Brucella abortus* strain RB51 transformed with plasmids pBBGroE or pBBTrc encoding *Neospora caninum* MIC1, MIC3, SRS2, GRA2 and GRA6 antigens. Some infected mice were sacrificed two weeks after inoculation and *Brucella* organisms isolated from spleen. Others were administered a booster dose and sacrificed a week later. Spleen homogenates were plated on the antibiotic free and antibiotic containing trypticase soy agar plates and the CFU difference was considered equivalent to plasmid loss. PCR of plasmid DNA extracted from the isolated cultures revealed the presence of all the five constructs. There was a loss of approximately 35% of plasmids in the first two weeks and a loss of approximately 15 % of plasmids a week after booster. This study determined that pBBGroE and pBBTrc are fairly stable in the host and are suitable for vaccine development in *B. abortus* strain RB51.

INTRODUCTION

Recombinant DNA technology and recombinant DNA vaccine development have advanced rapidly in the last few decades. New generation vaccines like naked DNA vaccines (1), vectored vaccines (2), recombinant subunit vaccines or purified recombinant proteins used as vaccines, all rely upon manipulation of host protein expression machinery by the introduction of extra-chromosomal plasmid DNA into the host cell.

A major problem in the commercialization and production of recombinant vaccines is plasmid instability. Expression of heterologus proteins can be a stress factor for the host cell and cause a disruption of its normal homeostasis. The stability of plasmid within host cells depends on the type of plasmid, the host and the environment (5). Inherent characteristics of the plasmid such as the sequence including the promoter type, selective marker and copy number can influence stability. Higher levels of transcription are associated with lower stability. Similarly, certain host cells are less amenable to transformation and have poor retention of foreign DNA (4). Environmental conditions such as the culture medium, temperature and pH can also indirectly influence the stability of the plasmid in recombinant vaccines (11), as they are associated with host cell metabolism and division.

pBBR1MCS is a broad-host range plasmid that can be used to efficiently transform *Brucella* species (3, 6). It is a low copy number plasmid that is stable in six *Brucella* species. *B. abortus* strain RB51 is a stable, rough mutant of *B. abortus* wild type strain 2308, which is used as the official vaccine against cattle brucellosis in the United States. Strain RB51 can be used as a vector to express heterologus and homologous proteins to improve vaccine efficacy or create multivalent vaccines (10). It was found that the pBBR1MCS based plasmid harboring either a GroE or Trc promoter was able to successfully drive the expression of *N. caninum* protective antigens MIC1, MIC3, SRS2, GRA2 and GRA6 in strain RB51 (7).

In this study, the stability of the recombinant sRB51-*Neospora* vaccine strains were evaluated to ensure that expression of the encoded antigens persisted long enough to sufficiently stimulate host immunity.

MATERIALS AND METHODS

Preparation of the recombinant sRB51-*Neospora* strains for inoculation

A pre-titrated glycerol stock of each individual recombinant strain was re-suspended to $2x10^8$ CFU/ml in PBS for the primary vaccination and $2x10^7$CFU/ml in PBS for the booster vaccination as described in Chapter 3-1. Initial titers were determined using TSA plates free of antibiotics. 200ul of each solution was pooled to arrive at 1ml of inoculum containing a total of $1x10^9$ CFU/ml for the primary vaccination and $1x10^8$CFU/ml for the booster vaccination. The final re-suspended solution was titrated on TSA plates containing 22.5 μg/ml of chloramphenicol for the GroE promoter containing constructs (RB51-SRS2 and RB51-MIC3) and in TSA plates containing 100μg/ml of Ampicillin for the Trc promoter containing constructs (RB51-MIC1, RB51-GRA2 and RB51-GRA6) to verify the administered dose.

Immunization of mice

Four female C57BL/6 mice of the 4-6 week age group were administered $1x10^9$ CFU of the pooled recombinant vaccine strains in 1ml volumes intrapertitoneally. The mice were housed in a certified BSL3 facility and all animal experimentation was carried out in compliance with the animal welfare act and AVMA regulations. Two mice were sacrificed after two weeks and their spleens were homogenized in 2ml of Tryptic soy broth. The two remaining mice were boosted with $1x10^8$ CFU's of pooled sRB51 expressing *Neospora* antigens [$2x10^7$] of each recombinant strain. The mice were sacrificed after a week and their spleens processed for the re-isolation of RB51 strains and identification of plasmids by PCR.

Re-isolation of the recombinant RB51 strains

RB51 strains were re-isolated from spleen homogenates by plating 100ul of the homogenates on TSA plates in triplicate, with and without the respective antibiotics used for

plasmid selection. The average difference between the two counts was considered to be a measure of plasmid loss.

PCR for detection of plasmids in re-isolated recombinant sRB51- *Neospora* cultures

Enriched culture plates containing the re-isolated colonies were washed with 10 ml PBS. Bacterial cells were pelleted by centrifugation at 3000 RPM for 10 mins. 200ul of the pellet was used for plasmid DNA extraction with the QIAamp kit; (Qiagen CA). PCR was carried out using the following conditions; 95^{o}C for 5 min, 95^{o}C for 1 min, Tm for 30 sec, 70^{o}C for 30sec, for 35 cycles followed by extension at 70^{o}C for 5 min. The primer sequences and Tm for each pair are as previously described (7) .

Statistical analysis

A 2-tailed student T test was carried out using the Microsoft Excel package to determine if plasmid losses were significant or not; the level of significance was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Examination of the re-isolated colonies, obtained from spleen homogenates of mice inoculated with the pooled recombinant vaccine strains by PCR with a specific primer for each of the encoded genes, revealed that all the five constructs were present in the mice (Figure A.1.1). Attempts to verify protein expression using a western blot were not successful as some of the bands that were not very different in molecular weights, merged in to each other. However, the fact that strong, antigen specific immune responses are elicited in mice up to ten weeks post vaccination (7) is indicative of the fact that sufficient quantities of antigen was being produced.

N. caninum MIC1, GRA2 and GRA6 genes were cloned in the pBBTrc plasmid with the ampicillin resistance gene for selection; while the MIC3 and SRS2 genes were cloned in the pBBGroE plasmid with the chloramphenicol resistance gene for selection. Therefore, spleen

homogenates were plated on TSA plates without antibiotics and TSA plates with chlorampenicol and ampicillin.

There appears to be a loss of plasmid in about 33% of the vaccine strain two weeks after vaccination, and a loss of about 15% in cells isolated a week after administration of the booster. The average loss was about 25% and this difference was statistically significant (Table A.1.1). Although there were a higher percentage of ampicillin resistance genes encoding bacteria in the initial inoculum, the recovery of both ampicillin and chloramphenicol resistant colonies was approximately the same, indicating that the ampicillin resistant cells were cleared more quickly. However, there was no major difference between the two plasmids in their stability.

Elzer et al., (3) have shown that there is no loss of pBBR1MCS in *B. abortus* 2308, one week after inoculation. Although the plasmids and *B. abortus* strain used in this study are different, the comparatively higher loss of plasmid recorded in this study can be attributed to the expression of the heterologus protein. It is also possible that the plasmid might still be present in the cells but may not express sufficient levels of the antibiotic resistance gene. Therefore, further work needs to be carried out to verify the proportion of cells that have actually lost the plasmid. This can be achieved by isolating DNA from individual colonies and carrying out a PCR to determine the presence or absence of plasmid. This work should be considered a preliminary study to determine the stability of pBBR1MCS based plasmids in strain RB51.

The immunological significance of this plasmid loss is unknown as the vaccinated mice mounted satisfactory immune responses to the expressed proteins. The stability of the pBBRMCS derived plasmids in sRB51 appears to be more stable compared to commonly used plasmids such a pBR322 based plasmids in other systems such as the *Salmonella* and yeast expression systems (2, 12). However, several approaches, such as increasing the copy number (8), modulating promoter strength or addition of telomere sequences for mammalian expression systems have been found to improve plasmid stability. In conclusion, the broad-host range plasmids pBBGroE and pBBTrc are found to be fairly stable for heterologus protein expression in *B. abortus* strain RB51.

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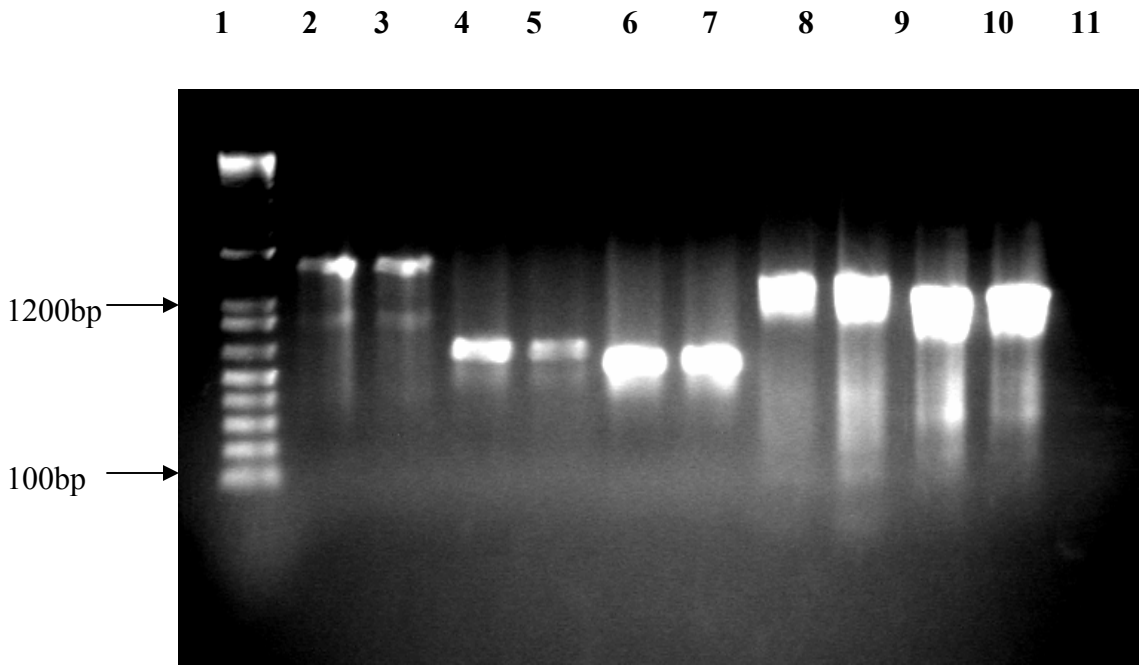

Figure A.1.1: PCR for detection of plasmids in re-isolated recombinant sRB51- *Neospora* cultures: Lane1-Ladder, Lane2 –Primary dose MIC1 (2278bp), Lane3- Boosted MIC1 (2278bp), Lane4 – Primary dose GRA2 (654bp), Lane5- Boosted GRA2 (654bp), Lane6 – Primary dose GRA6 (582bp), Lane7- Boosted GRA6 (582bp), Lane 8 – Primary dose SRS2 (1203bp), Lane9- Boosted SRS2 (1203bp), Lane10–Primary dose MIC3 (1089bp), Lane11-Boosted MIC3 (1089bp)

Table A.1.1: In Vivo Stability of pBBGroE and pBBTrc in mice vaccinated with recombinant sRB51 transformed with pBBGroE and pBBTrc encoding *N. caninum* antigens.

Time point	Dose CFU's/ml	TSA CFU's /spleen	TSA Cm CFU's/ spleen	TSA amp CFU's/ spleen	Sum of TSA Cm+ TSA amp	% Loss*
2 weeks after primary vaccination	$1x10^{9}$	3.8×10^{3}	1.2×10^{3}	1.31×10^{3}	2.51×10^{3}	33 % p=0.001
1 week after boost	$1x10^{8}$	1.78×10^{4}	8.38×10^{3}	6.66×10^{3}	1.51×10^{4}	15 % p=0.03

* - % loss = $\frac{[\text{Sum TSA Cm CFU + TSA Amp CFU}]}{\text{TSA CFU}} \times 100$

ADDENDUM-2

MECHANISMS OF THE PROTECTIVE IMMUNE RESPONSE STIMULATED BY THE RECOMBINANT *BRUCELLA ABORTUS* STRAIN RB51 BASED *NEOSPORA* VACCINE AGAINST MURINE NEOSPOROSIS

S. Ramamoorthy, R.B. Duncan, R. Vemulapalli, D. S.Lindsay, G. S. Schurig, S. M. Boyle, N. Sriranganathan. Formatted for Journal of Parasitology.

ABSTRACT

Neospora caninum is a protozoan parasite that causes bovine abortions and thereby, devastating economic losses to the cattle industry. Protective host immune responses to this parasite are believed to be primarily mediated by the cellular component and involve IFN-γ and IL-12. While B cell knockout mice eventually succumb to infection, the exact contribution of antibodies towards protection is unkown.

We previously developed a recombinant *Brucella abortus* strain RB51 based vaccine for *N. caninum*. This vaccine was highly efficacious in protecting mice against acute neosporosis. In this preliminary study, we have examined the mechanism of protection of this vaccine.

Either T cells or serum from vaccinated mice were transferred to two sets of naïve mice, which were then challenged either lethally or sublethally. All lethally challenged mice succumbed to infection, suggesting that both T cell and antibody responses may be required for protection. Brain tissues from sublethally challenged mice were examined three weeks after challenge. The brain lesion scores in mice transferred with T cell or serum from mice vaccinated with the sRB51-*Neospora* vaccine were not different from scores in unvaccinated mice, while they were higher than scores in vaccinated and challenged mice. Therefore, it is possible that both components of the immune system may be required for complete protection by the sRB51-*Neospora* vaccine.

INTRODUCTION

Neospora caninum is an intracellular parasite that affects dogs and cloven-footed animals including cattle. In dogs, it causes neuromuscular paralysis while in cattle it results in abortions and stillbirths. These reproductive losses have a severe economic impact on the cattle industry (3). There are currently no chemotherapeutic agents that are effective against cattle neosporosis. While an inactivated *N. caninum* vaccine is commercially available, it is known to stimulate strong antibody responses, while it is believed that cell mediated immunity is more important for protection against neosporosis (1, 6).

Brucella abortus is a gram-negative bacterium that is also an important cause of abortions in cattle. *B. abortus* strain RB51 is a stable, rough; mutant that is an effective and established vaccine against bovine brucellosis. Previous studies have shown that sRB51 can be engineered to express heterologus proteins and that immune responses to the expressed protein is strongly Th1 biased (11). We have previously described the expression of *N. caninum* MIC1, MIC3, GRA2, GRA6 and SRS2 protein in strain RB51 (8). Antigen specific IgG1, IgG2a and significant levels of IFN-γ and IL-10 were elicited in mice vaccinated with the recombinant strains. Challenge with a lethal dose of *N. caninum* tachyzoites resulted in complete protection in mice vaccinated with the RB51-MIC1 and RB51-GRA6 strains and partial protection in mice vaccinated with the RB51-GRA2, RB51-MIC3 and RB51-SRS2, based on pooled results from two separate experiments (8).

We have also investigated the use of irradiated *N. caninum* tachyzoites as an attenuated vaccine for neosporosis. Vaccination of mice with irradiated tachyzoites, elicited Th1 type immune responses and completely protected mice against challenge (7).

Therefore, while the protection elicited by the irradiated tachyzoite and sRB51-*Neospora* vaccines is based on stimulation of predominantly Th1 type responses, it is known that inactivated vaccines stimulate Th2 type responses. Baszler et al., have reported that such a Th2 type immune response exacerbated clinical signs of the disease in mice (2). Although it is established that IL-12 and IFN-γ are indispensable in protection of the mice against neosporosis, it was also found that C57BL/6 B cell knock out mice eventually succumbed to infection (4). Since the role of B cell and antibodies in protection against murine neosporosis is controversial,

we have investigated the relative importance of cell mediated and antibody based immunity stimulated by a recombinant *B. abortus* strain RB51 based *Neospora* vaccine in inducing protection of mice against challenge with *N. caninum* in this study. The irradiated tachyzoite vaccine was used as a positive control, since it is known that CD4+T cell responses are elicited upon infection of mice with *N. caninum* (10). Similarly, it was expected that the T cell response stimulated by the recombinant RB51-*Neospora* vaccine would be a major player in the protection elicited by the vaccine.

MATERIALS AND METHODS

Preparation of the recombinant sRB51-*Neospora* strains for inoculation:

Individual recombinant vaccine strains (i.e RB51-MIC1, RB51-MIC3, RB51-GRA2, RB51-GRA6 and RB51-SRS2) were grown in TSA containing 22.5 μg/ml of chloramphenicol or 100μg/ml of Ampicillin and titred using TSA plates free of antibiotics. The pre-titrated glycerol stock of each individual recombinant strain was re-suspended to $2x10^8$ CFU/ml in PBS for the primary vaccination and $2x10^7$ CFU/ml in PBS for the booster vaccination. Two hundred ul of each of the recombinant RB51 strains was pooled to arrive at 1ml of inoculum containing a total of $2x10^9$ CFU/ml for the primary vaccination and $2x10^8$ CFU/ml for the booster vaccination. The final re-suspended solution was titrated on TSA plates containing 22.5 μg/ml of chloramphenicol for the GroE promoter containing constructs (RB51-SRS2 and RB51-MIC3) and on TSA plates containing 100μg/ml of Ampicillin for the Trc promoter containing constructs (RB51-MIC1, RB51-GRA2 and RB51-GRA6) to confirm the actual administered dose.

Vaccination of mice and adoptive transfer of T cells and serum:

Four to six week old, female C57BL/6 mice were used for immunization and adoptive transfer. The mice were housed in a certified BSL3 facility and all procedures were carried out in compliance with the animal welfare act and AVMA guidelines. Eight mice were vaccinated with

$1x10^9$ pooled recombinant vaccine and four mice were vaccinated with $1x10^6$ irradiated *N. caninum* tachyzoites, in 1ml volumes, intra-peritoneally. The eight mice vaccinated with the recombinant vaccine were administered a log lower, booster dose of the pooled recombinant vaccine, while the other four mice vaccinated with irradiated tachyzoites were administered the same dose of irradiated *N. caninum* tachyzoites, four weeks after the primary dose.

A week after the booster vaccination, the mice were sacrificed. Serum was collected and pooled. T cells were purified from splenic extracts using the mouse T cell enrichment columns [R&D systems, Minneapolis, MN]. Purity of the preparation was checked by flow cytometry using monoclonal antibodies to the CD3 [T cell specific] and CD19 [B cell specific] surface markers. $1x10^7$ T cells were transferred to four mice by intraperitoneal inoculation. Based on published literature, the accepted dose of T cells for such transfers can range from $5x10^6$ to 7.5 x 10^7 cells (9, 10). Four hundred ul each of pooled serum was transferred to four other mice. Two mice from each group were challenged lethally with $2x10^7$ *N. caninum* tachyzoites and the other two mice from each group was challenged sub lethally with $1x10^6$ *N. caninum* tachyzoites, within three hours of the transfer procedure. For the irradiated vaccine, the mice were subject to sub lethal challenge only. The lethally challenged mice were observed until death, while the sub lethally challenged mice were sacrificed after 4 weeks. Organs such as spleen, liver, muscle, heart, lung, brain and uterus were collected for histopathology.

Histopathological analysis and scoring:

Organs collected from the mice were preserved in formalin and processed. A board certified pathologist, according to previously described procedures, carried out examination and scoring of tissues (7).

Statistical analysis:

A 2-tailed student T test was carried out using the Microsoft Excel package to determine if the differences in the scores were significant; $P \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

We have previously shown that mice vaccinated with the RB51-MIC1 and RB51-GRA6 strains were completely protected while mice vaccinated with RB51-MIC3, RB51-GRA2 and RB51-SRS2 strains were partially protected when challenged with a lethal dose of $2x10^7$ *N. caninum* tachyzoites. Mice vaccinated with pooled recombinants were also completely protected against lethal challenge (unpublished results). Therefore, only pooled recombinant vaccines were used in this experiment. However, both the unvaccinated controls and mice that were adoptively transferred with either T cells or serum obtained from mice vaccinated with the pooled sRB51-*Neospora* strains succumbed to lethal challenge within six days, suggesting that both T and B cell responses may be required for the protection (Table A.2.1).

As verified by flow-cytometry T cells purified from vaccinated mice using an enrichment column were 98% pure. Tissues from mice that were adoptively transferred with T cells or with serum from vaccinated mice and subjected to a sub lethal challenge were analyzed and scored histopathologically. It was found that the unvaccinated controls had a score of 11.55. Mice given T cells or serum from the mice vaccinated with irradiated tachyzoites had a score of 2 and 8 respectively. Mice given T cells or serum from mice vaccinated with sRB51 expressing *N. caninum* antigens had a score of 13 and 10 respectively (Table A.2.2). However, the lesion scores of mice which were vaccinated with the recombinant vaccine and subjected to sub-lethal challenge was only 5.5, when compared to a score of 11.55 for the unvaccinated controls. All the lethally challenged mice did not have any brain lesions, as there was insufficient time before mortality for development of lesions.

Therefore, the mechanism of protection for the irradiated vaccine appears to involve a predominantly T cell response, while for the sRB51-*Neospora* recombinant vaccine neither T cells nor serum alone conferred protection. Since the vaccinated control mice were protected, one possibility is that the synergistic action of both T cells and B cells is required for protection. The other is that innate immunity stimulated by sRB51, which may involve toll like receptors and stimulation of IFN-γ production may be a major player in protection. Such an innate immune response has been demonstrated to be important in protection against *Cryptosporidium parvum*, another apicomplexan parasite (5). SCID mice infected with *Cryptosporidium parvum* survived

for long periods of time, while IFN-γ–SCID double knock out succumbed to infection very quickly.

Although the dose of T cells used for transfer falls within the accepted range, the proportion of *N. caninum* specific T cells in the transfered population were probably higher in mice vaccinated with irradiated tachyzoites as these mice were exposed to the full spectrum of parasitic antigens, while mice vaccinated with the pooled recombinant vaccine were effectively exposed only to five *N. caninum* antigens. Morevover, as the number of mice used in this preliminary study is small; it would be difficult to draw definite conclusions. The positive controls in this experiment were normal vaccinated mice, while mice in which equivalent amounts of both T cells and serum were transferred would have been more appropriate as controls to determine if sufficient cells were transferred. A sRB51 vector control group was not employed due to time and resource constraints and therefore, it was not possible to determine how much of protection can be attributed to the adjuvant effect of sRB51. The inherent limitation of the adoptive transfer technique is that it is difficult to determine the exact number of cells or serum that might be sufficient for protection.

Another recent publication by Spencer et al., (9) describes the exacerbation of tissue pathology in naïve mice adoptively transferred with $CD8^+$ T cells from a mouse immunized with *N. caninum.* A similar cause could be attributed to the high lesion scores in the mice transferred with T cells ($CD4^+$T cells and $CD8^+$T cells) from the sRB51-*Neospora* immunized mice in this study. However, transfer of cells from mice vaccinated with irradiated *N. caninum* did not cause a similar increase in lesion scores. In fact, the scores of this group were significantly lower than that of the unvaccinated control mice.

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Table A.2.1: Lethal challenge results of mice adoptively transferred with T cells or serum from vaccinated mice.

Group	No. Of mice	Type of transfer	Outcomes Survival –S Death –D	Histopath Score
Unvaccinated Control	1	None	D	No Lesions
RB51-NC Vaccine	2	T cell transfer	D	No Lesions
RB51-NC Vaccine	2	Serum transfer	D	No Lesions

Table A.2.2: Brain histopathology scores in mice adoptively transferred with T cells or serum from vaccinated mice and challenged sublethally with 1x10^6 *N. caninum* tachyzoites

Treatment	No. of mice	Type of Transfer	Score
Unvaccinated control	3	None	11.56±9.07
Pooled recombinants	4	None	5.5±5.50
Pooled recombinants	2	T cells	13±2.82
Pooled recombinants	2	Serum	10±0
Nc-Irr	2	None	0.5±0.70
Nc-Irr	2	T cells	2±2.82
Nc-Irr	2	Serum	8±0