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Transgenic *Neospora caninum* strains constitutively expressing the bradyzoite NcSAG4 protein proved to be safe and conferred significant levels of protection against vertical transmission when used as live vaccines in mice

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ABSTRACT

At present, there is no effective treatment or vaccine to prevent vertical transmission or abortion associated with *Neospora caninum* infection in cattle. Different vaccine formulations have been assayed, and live vaccines have shown the most promising results in terms of protection. Previously, transgenic *N. caninum* tachyzoites expressing the bradyzoite stage-specific NcSAG4 antigen in a constitutive manner (Nc-1 SAG4^c) were obtained and showed a reduced persistence of parasite in inoculated mice. Thus, the present study evaluates the Nc-1 SAG4^{c}1.1 and Nc-1 SAG4^{c}2.1 transgenic strains and the Nc-1 wild-type (WT) strain to determine their protective efficacy against vertical transmission and cerebral neosporosis in mice. Consequently, dams were immunized twice with 5×10^5 tachyzoites of each strain and challenged with 2×10^6 tachyzoites of a heterologous and virulent isolate at 7–10 days of gestation. The Nc-1 SAG4^{c}1.1 strain offered less protection than the other transgenic strain (Nc-1 SAG4^{c}2.1) or their ancestor (Nc-1 WT). Indeed, 40%, 7% and 5.6% of the postnatal deaths corresponded to pups from dams vaccinated with Nc-1 SAG4^{c}1.1, Nc-1 SAG4^{c}2.1 and Nc-1 (WT) strains, respectively. In comparison, the non-immunized challenge group had a 100% mortality rate. In addition, mice were protected against congenital transmission; vertical transmission rates were 45%, 11.1% and 10.8% in the Nc-1 SAG4^{c}1.1, Nc-1 SAG4^{c}2.1 and Nc-1 WT immunized groups, respectively, vs. 94.9% in the non-vaccinated infected group. However, this protection against the postnatal mortality and the vertical transmission was not associated with a consistent Th1 or Th2-type immune response. Nonetheless, the Nc-1 SAG4^{c}2.1 strain appears to be the best candidate for use as a live vaccine, as evidenced by results demonstrating its high levels of protection against vertical transmission and its lower persistence in mice, making this transgenic strain safer than Nc-1 WT.

1. Introduction

Bovine neosporosis has emerged as a major disease responsible for abortion and congenital infection in cattle worldwide [1]. Economic losses include loss of calves, an increased calving interval, reduced stock value and increased likelihood of culling [2–4]. Vaccination appears to be the most cost-efficient strategy to control neosporosis in cattle [5]. Moreover, experimental mouse models of neosporosis offer an economical and convenient system for testing potential vaccine candidates [6].

Killed vaccines are generally regarded as safe, but they are usually unable to stimulate the required mechanisms of cell-mediated immunity [7–9]. Moreover, vaccines based on recombinant proteins or DNA vectors usually fail to confer enough protective immunity to avoid *Neospora* infection and congenital transmission [10–16], likely due to their inability to induce an appropriate response [17]. However, in pregnant mouse models, some recombinant proteins involved in the invasion and/or adhesion processes showed only partial protection against transplacental transmission to pups. For example, NcSRS2 had a 0% rate of parasite detection in pups [11], whereas NcMIC10 had a 33% rate of protection against transplacental transmission [6]. In addition, the rhoptry protein NcROP2 reduced both neonatal mortality and parasite burden [18].

Conversely, live vaccines are more protective against neosporosis because they can better stimulate both humoral and cell-mediated responses in mice and cattle [9,19,20]. However, these live vaccines may have safety problems. In fact, several procedures have been developed to obtain low virulence strains, including using naturally attenuated isolates from infected animals [21,22], temperature-sensitive mutants [23], γ-irradiated parasites

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[24] or live vaccines attenuated by long-term maintenance in cellular culture [25].

Thus, in the present study, two genetically modified strains that constitutively express the bradyzoite-specific antigen NcSAG4 in the tachyzoite stage, Nc-1 SAG4^{c}1.1 and Nc-1 SAG4^{c}2.1 [26], were used for the first time as live vaccines in mice. The hypothesis of this study was that the lower persistence exhibited by these strains would make them safer than their ancestor Nc-1 WT. The ability to protect against vertical transmission of the parasite and cerebral neosporosis was tested in both pregnant and non-pregnant mouse models, respectively, for both transgenic and Nc-1 WT strains.

2. Materials and methods

2.1. Parasites and tachyzoite soluble extract

Tachyzoites of transgenic parasites Nc-1 SAG4^{c}1.1 and Nc-1 SAG4^{c}2.1 [26], their ancestor Nc-1 WT and the high virulent challenge isolate Nc-Liv [27], were grown by continuous passage in MARC-145 cell culture following standard procedures [28]. For the purpose of immunization, parasites were harvested before vacuole rupture, passed through 21-gauge needles and centrifuged. The number of viable tachyzoites was estimated by Trypan blue exclusion. Tachyzoites used for PCR controls were prepared as previously described [29]. *Neospora caninum* soluble extract was prepared from previously purified tachyzoites [30].

2.2. Vaccination assay, samples and data collection

Tachyzoites from the strains used in the immunizations (Nc-1 SAG4^{c}1.1, Nc-1 SAG4^{c}2.1, and Nc-1 WT; Table 1) were resuspended in phosphate buffered saline (PBS) at a dose of 5×10^5 tachyzoites in a final volume of 200 μl per mouse and were subcutaneously (s.c.) injected into mice. The challenge isolate (Nc-Liv) was prepared following the same protocol for immunization at a dose of 2×10^6 tachyzoites in a final volume of 200 μl per mouse.

To evaluate for protection against vertical transmission and cerebral neosporosis conferred by live vaccines, a pregnant and a non-pregnant mouse models were employed. Eight-week-old female mice (Harlan Interfauna Ibérica) were divided into five groups (G1: $n = 25$; G2: $n = 24$; G3: $n = 29$; G4: $n = 25$; G5: $n = 25$) and immunized twice s.c.; a booster dose was given three weeks after the initial immunization. Besides, five mice were randomly selected 10 days after the booster (prior to challenge and gestation) (5 mice from G1, G2 and G3 immunized groups and 6 mice from G4 and G5 representative of non immunized control groups) and five dams were selected at the end of the experiment (post-challenge) from each group to process their spleens for splenocyte culture to analyze cellular immune response [33].

In the pregnant mouse model, mating and gestation were carried out as previously described [31,32]. Immunizations and the challenge injection were performed as reported by Aguado-Martinez et al. [33]. Dams and pups were sacrificed with CO_2 gas on day 50 post-partum (P.P.) to evaluate the humoral immune response in the offspring without the interference of colostral antibodies [26,34]. Mice that did not become pregnant were left for a non pregnant mouse model (G6, G7, G8, G9 and G10) (Table 1).

In the non-pregnant mouse model, mice were challenged three weeks after the booster dose. They were sacrificed at day 30 post-challenge (p.c.) (chronic phase of infection), and tissue samples (brain and lungs) and blood were collected.

All mice were inspected daily throughout the experiment for clinical signs of neosporosis.

2.3. Vaccine safety, efficacy and immune response evaluation

Vaccine safety was determined in the mice sacrificed 10 days after the booster (prior to challenge and gestation) by visual inspection for skin ulcerations at the inoculation site or systemic reactions. The presence of parasites in the mice brain and lungs was also investigated by PCR prior to challenge. Microsatellite analysis was used to discriminate the presence of the challenge or the immunization strains (Nc-1 vs. Nc-Liv isolates) in infected mice after challenge (dams, pups and non-pregnant mice), in order to verify the safety of immunization strains at the given doses.

To check vaccine efficacy in the pregnant mouse model, data concerning litter size, hebdomadal mortality (the number of full-term dead pups from birth until day 2 P.P.), postnatal mortality (the number of dead pups from day 2 to day 50 P.P.), morbidity (growth rate and neosporosis-associated clinical signs) and vertical transmission rate (PCR detection of *N. caninum* in pups' brain and lungs) were recorded. Mortality, neosporosis-associated clinical signs and the presence of the parasite in the brain and lungs were also assessed in dams. The vaccine's efficacy in non-pregnant mice was studied by evaluating clinical signs of neosporosis and for the presence of *N. caninum* DNA in the lungs and brain in the chronic phase of infection.

The humoral immune response was analyzed before and after challenge in both the pregnant and non-pregnant models by measuring specific serum IgG1 and IgG2a against the entire tachyzoite extract and detecting specific antibodies against recombinant NcSAG4 (rNcSAG4) and NcGRA7 (rNcGRA7) proteins. The cellular immune response was also studied prior to and after challenge by measuring IFN-γ and IL-10 cytokines levels in the supernatants from splenocyte cultures.

2.4. DNA extraction and PCR techniques for parasite detection

Realpure Extracción DNA Genómico (Durviz) was used to extract DNA from 10 to 20 mg of mouse tissue. The presence of parasite DNA was investigated in mouse tissues by nested PCR on the ITS-1 region of *N. caninum* [35]. Amplification products were visualized by 1.8% agarose gel electrophoresis and ethidium bromide staining.

2.5. Microsatellites analysis for isolate identification

Approximately 300 ng of DNA extracted from PCR-positive brains from dams, pups and non-pregnant mice, was used as a template for the amplification by nested PCR of 12 previously described microsatellites [36]. For automated allele sizing, all reverse primers in the secondary PCR were fluorescently end-labeled. Amplified products were prepared with HiDi formamide and Gene Scan-500 (LIZ) Size Standards (Applied Biosystems). The size of the fluorescent PCR product was determined using a 48-capillary 3730 DNA analyzer (Applied Biosystems) and analyzed with GeneMapper® V 3.5 Software [37].

2.6. Humoral immune responses in mice sera

N. caninum-specific serum isotypes IgG1 and IgG2a were determined by ELISA using a soluble *N. caninum* tachyzoite antigen (0.5 μg/well) and anti-mouse IgG2a or IgG1 peroxidase-conjugated as secondary antibody (1:5000; Southern Biotechnology) [38]. Antibody responses developed against native NcSAG4 and NcGRA7 proteins, which are usually employed as markers of chronic or acute phase infection, respectively, were evaluated by ELISA using rNcSAG4 and rNcGRA7 proteins (0.1 μg/well) and anti-mouse IgG peroxidase-conjugated as secondary antibody (1:5000; Sigma