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Low efficacy of NcGRA7, NcSAG4, NcBSR4 and NcSRS9 formulated in poly-ε-caprolactone against *Neospora caninum* infection in mice

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ABSTRACT

The protective efficacy of vaccination with *Neospora caninum* recombinant antigens was evaluated in Balb/c pregnant and non-pregnant mouse models of neosporosis. A major immunodominant dense granule protein (NcGRA7) and three bradyzoite-specific surface antigens (NcSAG4, NcBSR4 and NcSRS9) were expressed in *Escherichia coli* and encapsulated within poly-ε-caprolactone (PCL) nanoparticles for the first time. Good efficiencies of entrapment (greater than 50%) were obtained for all encapsulated proteins. Moreover, antigenicity was unaffected after formulation. Afterwards, separate groups of mice were immunised with the nanoparticles and were then challenged with *N. caninum* tachyzoites. High IgG1 and IgG2a antibody levels of anti-*N. caninum* and specific antibodies directed against recombinant proteins were developed by all of the immunised groups. Mice previously inoculated with encapsulated rNcGRA7 produced significant levels of IFN-γ. However, in general, a low production of IFN-γ was detected. This may indicate a failure in the complete liberation of antigens after immunisation or an incorrect balance of the Th1/Th2 response to combat acute neosporosis during pregnancy. In fact, high morbidity and mortality rates were observed in dams. Moreover, vertical transmission was not prevented, and high neonatal mortality rates occurred similarly among the groups. Despite the global absence of efficacy, the study reveals some results of positive efficacy regarding dams and pups' survival and parasite presence for NcSRS9 recombinant protein. Furthermore, vaccination with rNcGRA7 encapsulated alone or combined with rNcSAG4 resulted in a slight decrease of parasite presence in non-pregnant mice. These promising results are further discussed to suggest new approaches that may be more suitable to test vaccine formulations based on bradyzoite stage-specific proteins.

1. Introduction

Neospora caninum is an obligate intracellular protozoan parasite closely related to *Toxoplasma gondii*, which has been recognised worldwide as a major cause of infectious bovine abortion [1,2]. Currently, there are no effective methods for the control of neosporosis, and thus, vaccine development for neosporosis prevention in cattle remains the main objective in several research efforts. Although bovine models are more suitable for the study of neosporosis and immunoprophylactic control measures, the availability of inbred strains of mice led to the extensive use of different mouse models for an initial screening of vaccines owed to the reduced cost, easy handling and reduced time of pregnancy. In previous studies, live-parasite vaccines have shown good results in experimental trials [3–5]. However, live vaccines have several disadvantages due to their high cost of production, stability along the distribution process and safety. Inactivated vaccines are a good option because they can show promising levels of protection against vertical transmission [6,7]. However, their production is also expensive. For these reasons, recombinant vaccines may represent a good alternative in terms of a lower cost of manufacturing and safety, as well as a focussed election of the relevant antigens to selectively target effective immune responses [8]. In this sense, a repertoire of surface and secretory *N. caninum* proteins have been previously selected and tested in mouse models with variable results such as NcGRA7, NcROP2, NcMIC1 and NcPDI, among others [9–14]. When choosing the antigens as vaccine targets for this study, we focused on proteins involved in two essential events in the pathogenesis of neosporosis: the lytic cycle and the establishment of the parasite into the host's tissues. Thus, we employed the recombinant NcGRA7 protein [15], which is a highly immunogenic dense granule protein involved in invasion and associated with the active replication of the parasite as well as the establishment of the

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parasitophorous vacuole in the host cell [16,17]. NcGRA7 has been previously evaluated in vaccine formulations with variable protection results [13,18–20]. We followed the same approach with the protein NcSAG4, which is expressed early in bradyzoite conversion [21] and has been tested in vaccine formulations with the water-in-oil Titermax Gold Adjuvant without any protection results [20]. Additionally, this is the first time that two bradyzoite late-expressed-specific proteins, NcBSR4 [22] and NcSRS9 [23], were employed as vaccine candidates. It is known that these proteins play a role in maintaining parasite persistence in host tissues and are involved in the reactivation within the intestinal cycle in *T. gondii* [24,25], indicating that they may be good vaccine targets. Neither TgSRS9 nor TgBSR4 have been previously tested in vaccines against toxoplasmosis and neosporosis. However, other bradyzoite-specific proteins, such as TgBAG1 and TgMAG1, showed protection in a mouse model of toxoplasmosis [26].

Another critical variable in designing formulations against intracellular protozoan is the use of potent adjuvants that can boost antigen immunogenicity and induce an appropriate cell-mediated immune response [27]. Several adjuvants have been tested in vaccine assays against intracellular parasites like *N. caninum*, with promising results [11,18,19,28]. It is known that polymer particles prepared from both synthetic and natural macromolecules have shown great potential as new generation adjuvants [29]. In the present study, we employed poly-ε-caprolactone (PCL), a biodegradable and biocompatible polymer that has been previously tested as an adjuvant with good results against *Streptococcus equi* and *Brucella ovis* [30,31].

The objectives of our work were first, to entrap four different recombinant proteins of *N. caninum* within PCL nanoparticles prepared by the water/oil/water (w/o/w)-method together and to characterise these formulations in terms of protein loading, morphology, size and physical state of all nanoparticles. Second, the vaccine efficacy of these new formulations was evaluated in pregnant and cerebral mouse models to establish their efficacy against vertical transmission and chronic infection of *N. caninum*. In the present work, we attempted to improve the efficacy of rNcGRA7 and rNcSAG4 with a new adjuvant [20], and to obtain a possible synergetic effect with the mixture NcGRA7 and rNcSAG4 or the mixture of the poorly immunogenic rNcBSR4 and rNcSRS9 proteins.

2. Materials and methods

2.1. Cloning and purification of recombinants proteins

Both NcGRA7 and NcSAG4 proteins were produced in a prokaryotic expression vector system following previously described procedures with few modifications [15,21]. Both proteins were cloned into a new plasmid called pET-45b (+) (Novagen, Germany). NcSAG4 was cloned without the N-terminal signal peptide and GPI-anchor to modify its possible posterior folding and to improve its immunogenicity [20]. In contrast, rNcBSR4 and rNcSRS9 were cloned into a prokaryotic expression vector, pRSET-C (Invitrogen, USA), and expressed as polyhistidine-tagged fusion proteins in *Escherichia coli*, as previously described [22,23].

After the bacteria were lysed, rNcGRA7 was purified from the soluble fraction by immobilised metal ion affinity chromatography (IMAC) using HisTrapHP columns (GE Healthcare, USA) [15]. rNcSAG4, rNcSRS9 and rNcBSR4 were extracted from inclusion bodies and denatured with a binding buffer containing 20 mM phosphate salts, 8 M urea and 40 mM imidazole [32]. These recombinant proteins were obtained using an on-column refolding and purification procedure based on a method previously reported [33] that consisted of a descent urea gradient followed by an elution with a high imidazole concentration buffer. The concentration and purity of both recombinant proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a standard BSA scale (Roche, Switzerland) and by western blot with a monoclonal anti-T7 tag antibody (Novagen, Germany) and polyclonal anti-rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 mouse sera [21–23,32]. Protein concentrations were measured using Quantity One software (v.7.2, BioRad, USA). All the proteins were stored at −80 °C until use.

2.2. Encapsulation of recombinant proteins

2.2.1. Preparation of nanoparticles

Nanoparticles were prepared by a multiple ($w_1/o/w_2$) emulsion solvent evaporation method, as described by Sinha et al. [34] with some modifications. Briefly, 1 mg of rNcSAG4, rNcSRS9 or rNcBSR4 (hydrophobic proteins) in 1 ml of an elution buffer (NaCl [50 mM], NaH_2PO_4 [1 mM], Na_2HPO_4 [1 mM], imidazole [500 mM], glycerol [20%], glucose [5%] at pH 7.4) were mixed with 60 mg of a non-ionic surfactant Pluronic F-68® (Sigma, USA). In parallel, 150 mg of poly-ε-caprolactone (PCL; Aldrich) was dissolved in 2.5 ml trichloromethane (Scharlau, USA). Then, the aqueous phase containing the antigen was dispersed in the organic phase of the polymer by sonication for 20 s (Microson™ Ultrasonic Cell Disruptor, USA) to obtain the inner emulsion (w_1/o). This emulsion was then mixed again at high speed with 8 ml of a continuous aqueous phase containing 0.5% (w/v) polyvinyl alcohol (PVA; Mw: 115,000, BDH-Supplies). The resulting multiple emulsions ($w_1/o/w_2$) were stirred with a bladder agitator in a fume hood for 2 h at room temperature to facilitate the evaporation of the organic solvent. Finally, the resulting nanoparticles were centrifuged, washed and freeze-dried using 5% mannitol as a cryoprotector. The resulting nanoparticles were named NP1-rNcSAG4, NP1-rNcBSR4 and NP1-rNcSRS9. Empty PCL nanoparticles were prepared in the same way in the absence of antigen (NP1).

For the encapsulation of the soluble rNcGRA7 protein in PCL nanoparticles (NP2-rNcGRA7), the previous protocol was modified. In this case, the inner aqueous emulsion was obtained after mixing 1 mg/ml of antigen in 0.5 ml with 300 μl of calcium chloride (10 mg/ml in water; Sigma, USA), 100 ml Protasan UP G113 (10 μg/ml water; NovaMatrix/FMC, Norway) and 60 mg Pluronic F-68® (Sigma, USA). The organic phase of PCL and the multiple emulsions were prepared following the protocol mentioned above. Similarly, the nanoparticles were purified and freeze-dried, as described above. Empty PCL nanoparticles (NP2) were also prepared in the same way and used as a control. Before freeze-drying, 20 μl samples from all formulations were collected for the characterisation of the particles.

2.2.2. Characterisation of nanoparticles and quantification of antigen loading

The size, zeta potential (particles superficial charge) and polydispersity of the nanoparticles were determined immediately after the preparation, using photon correlation spectroscopy by a Malvern Nano ZS instrument (Malvern Instruments Ltd., UK). The diameter of nanoparticles was determined after dispersion in ultrapure water (1:10 dilution) and measured at 25 °C with a dynamic light scattering angle of 90°. The average particle size was expressed as the volume mean diameter (V_{md}) in nanometres (nm), and the average surface charge in milliVolts (mV).

The particles were purified by centrifugation (17,000 × *g*, 20 min at RT), then the supernatant and the precipitated nanoparticles were stored separately until the quantification by SDS-PAGE gel with their previous-quantified protein scale. For this purpose, the nanoparticles within the pellet were dissolved with trichloromethane and acetone (3:1, v/v) by shaking overnight on a magnetic stirrer. Then, the organic solvent was evaporated. The