INTERLEUKIN-12 RESPONSE TO NCSRS2 IMMUNIZATION OF BALB/C MICE AGAINST *Neospora caninum*[*]

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ABSTRACT:- GONÇALVES, K.N.; ANDREOTTI, R.; PAIVA, F.; PONTES, E.R.J.C.; LIMA-JUNIOR, M.S.C.; OSHIRO, L.M.; MATOS, M. DE F.C. **Interleukin-12 response to NcSRS2 immunization of BALB/c mice against *Neospora caninum*.** [Resposta da interleucina-12 na imunização de camundongos BALB/c com NcSRS2 contra *Neospora caninum*]. *Revista Brasileira de Parasitologia Veterinária*, v. 17, supl. 1, p. 215-219, 2008. Embrapa Gado de Corte, BR 262, km 4, Caixa Postal 154, Campo Grande, MS 79002-970, Brazil. E-mail: andreott@cnpgc.embrapa.br

The apicomplexan parasite *Neospora caninum* can cause abortions and is recognized as an important causative agent responsible for economic and reproductive losses in the cattle industry. Humoral immune response was investigated in BALB/c mice by using recombinant NcSRS2 expressed in *Escherichia coli* as polyhistidine-tagged fusion proteins. NcSRS2 is the major immunodominant tachyzoite surface antigen of *N. caninum*. Separate groups of female BALB/c mice were infected subcutaneously with (P) PBS, (N) recNcSRS2, (NIL) recNcSRS2 plus interleukin-12 or (NF) recNcSRS2, in Freund's adjuvant. Serological analysis showed that the antibodies produced by immunization recognized native protein from *N. caninum* tachyzoites and that, 14 days after the initial immunization, NcSRS2-specific antibodies were present in all sera tested from the groups N, NIL and NF. NcSRS2 with Freud's adjuvant led to a stronger immune response, as measured by IgG1 and IgG2a levels, than did other formulations (NF > NIL > N > P; $p < 0.001$), with a Th2 bias. The results corroborate the potential use of recombinant protein NcSRS2 as a vaccine aimed at reducing congenital transmission. Further studies are required to identify new adjuvants capable of improving the induction of Th1 immune response.

KEY WORDS: Nc1-p43, immune response, IgG1, IgG2a, vaccine.

RESUMO

O parasita apicomplexo *Neospora caninum* pode causar abortos e é reconhecido como agente importante responsável por perdas econômicas e reprodutivas na produção de bovinos. A resposta imune humoral foi investigada em camundongos BALB/c usando-se a proteína recombinante NcSRS2 expressa em *Escherichia coli* contendo uma cauda de histidina. NcSRS2 é o principal antígeno de superfície com efeito imunodominante em taquizoítas de *N. caninum*. Grupos de camundongo BALB/c fêmeas foram inoculados com (P) PBS, (N) recNcSRS2, (NIL) recNcSRS2 mais interleucina-12 e (NF) recNcSRS2 com adjuvante de Freund. A análise sorológica mostrou que os anticorpos produzidos pela imunização reconheceram a proteína nativa de taquizoíta de *N. caninum* e que, 14 dias após a primeira imunização, anticorpos específicos para NcSRS2 estavam presentes em todos os soros testados dos grupos N, NIL e NF. Com base na resposta imune de IgG1 e IgG2a, a proteína com adjuvante de Freud provocou resposta mais forte do que as demais formulações (NF > NIL > N > P; $p < 0,001$), com desvio para Th2. Os resultados corroboram o uso potencial da proteína recombinante NcSRS2 como vacina, visando a redução de transmissão congênita. Estudos adicionais são necessários para identificar adjuvantes que favoreçam a indução de resposta imune Th1.

PALAVRAS-CHAVE: Nc1-p43, resposta imune, IgG1, IgG2a, vacina.

INTRODUCTION

Neospora caninum, an apicomplexan protozoan, was first identified by Dubey et al. (1988) and has been described worldwide. It can infect a wide range of intermediate and definitive host animals and has been recognized as an important causative agent responsible for economic and reproductive

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losses in the livestock industry, especially in dairy cattle (DUBEY, 2003).

Mice are frequently used as a laboratory animal model in *N. caninum* studies. Inbred BALB/c mice are susceptible to both *N. caninum*-induced encephalitis and congenital transmission, in a dose-dependent manner (LINDSAY et al., 1995; LONG et al., 1996).

Immune responses to intracellular parasites include humoral as well as cellular mechanisms. Protective immunity is achieved mainly through cell-mediated responses characterized by type 1 T-helper (Th1) cells and interleukin-2 (IL-2), IL-12, and IFN-ã production (INNES et al., 2000), whereas susceptibility to encephalitis and congenital transmission is associated with type 2 T-helper (Th2) cytokines, such as IL-4 and IL-10, and production of IgG1 antibodies (LONG et al., 1998, 2000).

To date, research into the development of effective vaccines for preventing neosporosis has yielded mixed results (HALDORSON et al., 2005). In a mouse model for encephalitis, immunization with tachyzoite sonicate resulted in a Th2 immune response and neurological disease (BASZLER et al., 2000). Immunization of mice with various recombinant surfaces and dense granule proteins led to protection against systemic disease (CHO et al., 2005).

Two surface proteins (NcSAG1 and NcSRS2) and two dense granule proteins (NcDG1 and NcDG2) from the parasite have been regarded as candidates for effective vaccines capable of conferring immunity against *N. caninum* (INNES et al., 2002). Protein NcSRS2, also named Nc1-p43, has proved to be more functionally involved in the adhesion process and in host cell invasion (HEMPHILL, 1996). Anti-NcSRS2 monoclonal antibodies (mAbs) may inhibit invasion of *N. caninum* into host cells, *in vitro* (NISHIKAWA et al., 2000).

In mice, NcSRS2 expressed in recombinant vaccinia virus has reduced the parasite load in tissues following challenge (NISHIKAWA et al., 2001a). It has also decreased congenital transmission in females vaccinated before pregnancy and challenged during pregnancy (NISHIKAWA et al., 2001b). HALDORSON et al. (2005) demonstrated that, in its native form, NcSRS2 induced protection against *N. caninum* congenital transmission in mice.

Despite the availability of vaccines against neosporosis, they have not been capable of controlling the disease. The purpose of the present study was determine whether recombinant NcSRS2 associated with IL-12 might induce a specific immune response to *N. caninum*.

MATERIAL AND METHODS

Parasites

Neospora caninum isolate NC-1 (DUBEY et al., 1988) was used to challenge mice and to prepare an antigen formulation for immunization. The parasite was propagated in Vero cells using Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), at 37 ºC, in a humidified atmosphere of 5% CO_2 in air. When 80% of Vero cells had been infected with *N. caninum* tachyzoites, the cell monolayers were removed and twice washed with phosphate-buffered saline (PBS) solution at 1000 rpm centrifugation for 10 min.

Recombinant NcSRS2 (Nc1-p43)

The antigen domain of NcSRS2, which is located in the distal C-terminal two thirds of the molecule, was amplified using the polymerase chain reaction, in accordance with AHN et al. (2003).

The amplified DNA was cloned into pet100/D-TOPO vectors (Invitrogen Tech, Carlsbad, CA), which were then used to transform *E. coli* of the TOP10 strain (Invitrogen Tech, Carlsbad, CA). Once the correct orientation of the insert was confirmed, plasmids were inserted for transformation into *E. coli* BL21 Star (DE3) (Invitrogen Tech, Carlsbad, CA). The *E. coli* cells in the log phase were treated with 0.75-mM isopropyl á-D-thiogalactoside (IPTG) for 4.5 h at 30 °C, to induce expression of fused fragment of NcSRS2 (LIMA-JUNIOR et al., 2007).

The protein was purified using the Ni-NTA Purification System (Invitrogen Tech, Carlsbad, CA) and the recombinant protein was confirmed based on molecular weight, by means of SDS-PAGE and Western blotting.

Western blotting

The first Western blot assay was performed to confirm whether the recombinant protein had been purified in a nickel column. The protein was separated on 12% SDS-PAGE gel and transferred onto nitrocellulose (NC) sheets (Schleicher and Schuell, Keene, NH). The NC sheets were blocked with 5% skimmed milk in TBS/0.05% Tween-20 overnight at 4 ºC and then incubated for 1-2 h with 1:5000 diluted anti-HisG antibodies (Invitrogen Tech, Carlsbad, CA) produced in mice. After incubation and washing, NC was incubated with 1:20,000 diluted alkaline phosphatase conjugate goat anti-mouse IgG antibody (Sigma, St Louis, MO) and then soaked in BCIP/NBT substrate solution (Sigma, Saint Louis, MO) for 15 min and washed with water.

The second Western blot step was performed as above to find out whether mice antibodies produced in response to immunization recognized antigens in *N. caninum* tachyzoites. The extract of sonicated *N. caninum* tachyzoites following separation on 12% SDS-PAGE was used as the antigen.

Experimental design of immunization and challenge

All the procedures involving use of animals were approved by the Ethics Committee of Universidade Federal de Mato Grosso do Sul.

Seven-week-old female BALB/c mice were obtained from a colony maintained at Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. The mice were housed in four groups of 12, fed rodent chow daily and given water *ad libitum*. Prior to any immunization, they were checked for absence of *N. caninum* immunoglobulins by means of the indirect enzyme-

linked immunosorbent assay (ELISA). The experimental groups were treated as described below.

Each mouse was inoculated subcutaneously with a 200-ml volume as follows: Group 1 (P) was given PBS alone; Group 2 (N), 20 mg of recombinant NcSRS2; Group 3 (NIL), 20 mg recombinant NcSRS2 plus 0.5 g of IL-12 (Biosource, Invitrogen); and Group 4 (NF), 20 mg of recombinant NcSRS2 emulsified in Freund's complete adjuvant. After 15 days, inoculation was repeated in all groups with the same doses, except in Group 4, which received 20 mg of recombinant NcSRS2 in Freund's incomplete adjuvant.

Serology

All mice were bled from the supraorbital vein before the first inoculation and again on days 7, 14, 21 and 30 after immunization. Sera from each sample were stored at –20 ºC until use.

To evaluate humoral immune responses, individual sera were analyzed three times for NcSRS2-specific IgG1 and IgG2a, by means of ELISA. Briefly, 3 mg/ml of purified recombinant protein were placed in 96-well ELISA plates (Corning Costar 3590) with 100 ml of recombinant protein per well. The plates were incubated overnight at 4 ºC. After washing five times with PBS/0.05% Tween-20, the plates were blocked with 3% skimmed milk and incubated for 1 h at 30 °C. The sera (1:100) were incubated for 1 h at 30 ºC. After washing, 1:1000 diluted horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG1 or IgG2a (Zimed) were added and incubated for 30 min. After the last washing, 50 ml of o-phenylene diamine (OPD) substrate solution were added. The reaction was stopped by adding 50 ml of H_2SO_4. Absorbencies were read at 490 nm with an ELISA reader.

Statistical analysis

The significance of differences between the controls (Group 1) and each immunized group (Groups 2-4) was determined using ANOVA and the *t* test (LSD). The results were expressed as means ± standard deviation (SD). The significance level was taken as $p < 0.01$. The computer statistical programs Epi Info and BioStat 5.0 were used for data analysis.

RESULTS AND DISCUSSION

The recombinant protein NcSRS2 (Nc1-p43) obtained from *E. coli* culturing was affinity-purified in nickel-charged agarose resin. SDS-PAGE revealed the profile of a 29-kDa protein. Western blotting using HisG tag antibodies confirmed the recombinant nature of the protein (Figure 1), as described by Lima-Junior et al. (2007).

The antibody produced by mouse immunization with the recombinant protein NcSRS2 was used as the first antibody in a Western blot test, in which a pool of sonicated native protein from *N. caninum* tachyzoites was run. In this assay, the first antibody recognized antigens from *N. caninum* tachyzoites (Figure 2). In a cerebral infection model, immunization with recombinant NcSRS2 resulted in

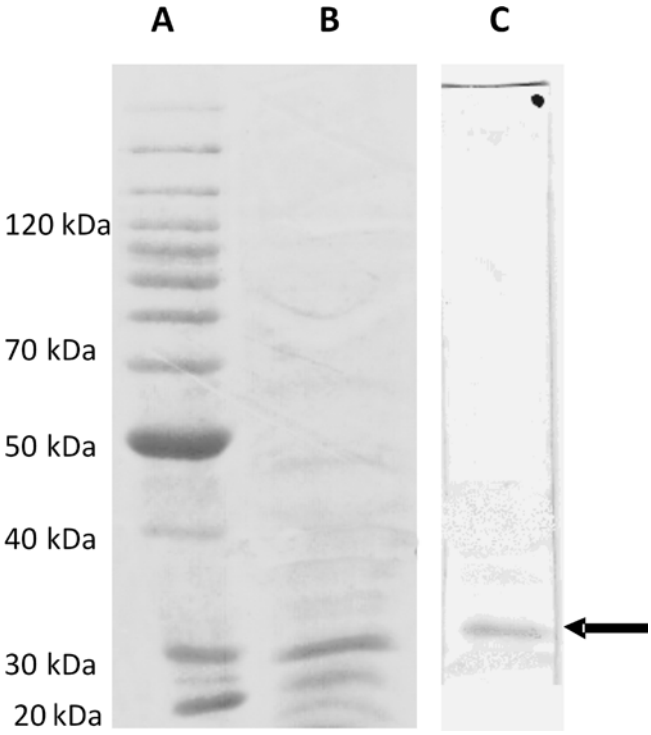

Figure 1. (A) Molecular weights of markers. (B) Coomassie blue-stained SDS-PAGE (12% gel) of the expressed NcSRS2 fragment, purified by means of affinity chromatography in a nickel column, showing a 29-kDa band. (C) HisG tag detected by means of Western blotting.

production of antibodies to recombinant but not native protein (HALDORSON et al., 2005).

Mice were immunized with recombinant NcSRS2 in different formulations, using the protein alone (N), in conjunction with IL-12 (NIL), or with Freund's adjuvant (NF). The antibody profile is shown in Figures 3A and 3B, considering the median of each group from ELISA.

Fourteen days after the initial immunization, NcSRS2-specific antibodies were detected in all sera tested from groups N, NIL, and NF. Controls (P) did not react to the antigen at any time during the experiment.

On day 14, IgG1 immune responses were statistically higher in the NF animals than in other groups ($p < 0.001$). On day 21 and day 30, NF > NIL > N > P ($p < 0.001$) (Table 1).

Immune response evaluation based on IgG2a levels showed that on day 14 NF > NIL = N > P ($p < 0.001$); on day 21 and day 30, NF > NIL > N > P ($p < 0.001$) (Table 1).

Antibodies play a role in cytotoxicity mechanisms, opsonophagocytosis and complement-dependent lysis, which are important for controlling widespread parasites (TAYLOR et al., 2001). This action might explain the reduction of *N. caninum* congenital transmission in experimentally challenged BALB/c mice, associated with a Th2 immune response (HALDORSON et al., 2005). Since NcSRS2 was shown to be more functionally involved in the adhesion and invasion process, anti-NcSRS2 antibodies might inhibit *N. caninum* invasion into host cells (HEMPHILL, 1996) and thus might be used as a vaccine.

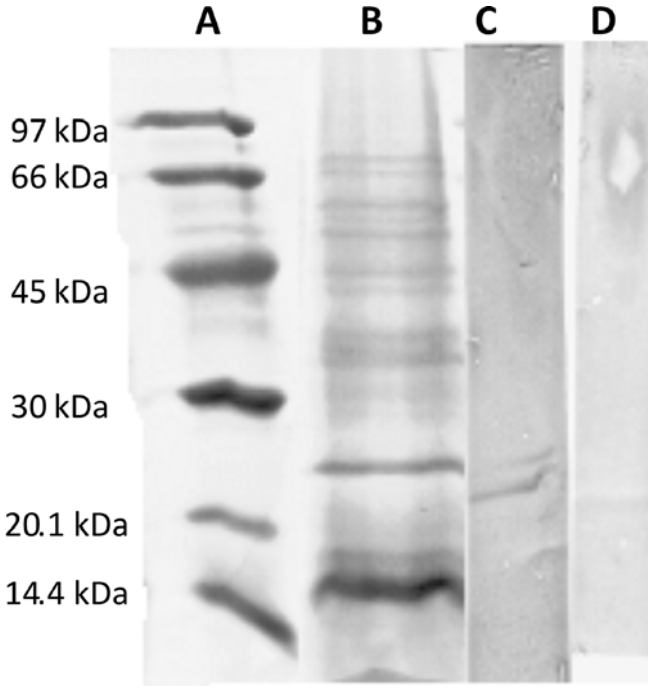

Figure 2. (A) Molecular weights of markers. (B) Coomassie blue-stained SDS-PAGE (12% gel) of sonicated *N. caninum* tachyzoites. (C) *N. caninum* protein detected in NcSRS2-positive mouse serum detected by means of Western blotting. (D) *N. caninum* protein detected in NcSRS2-negative mouse serum detected by means of Western blotting.

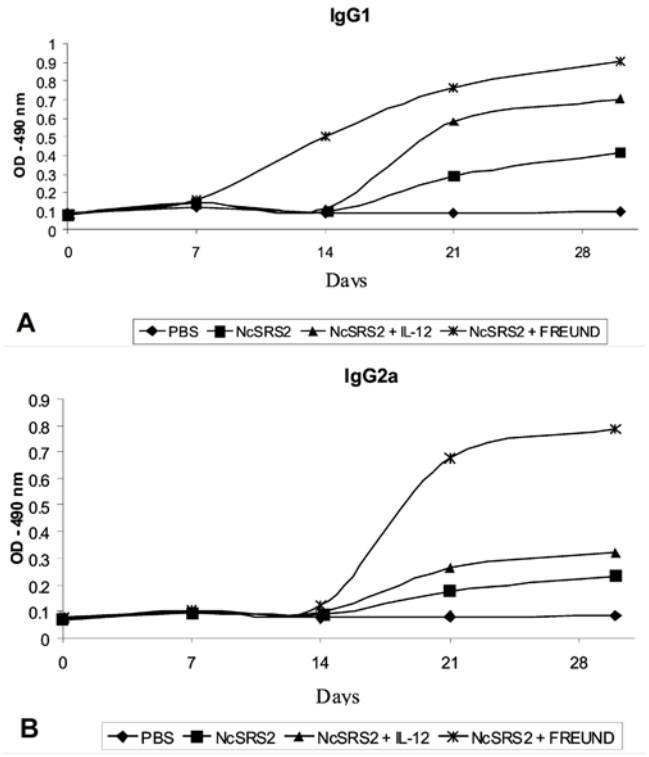

Figure 3. NcSRS2-specific antibody isotype responses in mice vaccinated with different formulations of recombinant NcSRS2. (P) PBS alone (controls); (N) NcSRS2; (NIL) NcSRS2 plus IL-12; (NF) NcSRS2 emulsified in Freund's complete adjuvant.

In neosporosis, congenital transmission resulting in clinically normal but infected calves is the most common means of perpetuating infection within a herd (DUBEY, 2003). In the present investigation, IL-12 was not expressed in considerable amounts, nor was a Th1 immune response elicited. However, the results revealed that antibody expression was high when the mice were stimulated with Freund's adjuvant, although a Th2 bias was observed (Figure 4). According to Haldorson et al. (2005), native NcSRS2 protein can induce protective immunity, also with a Th2 bias.

Our results corroborate the potential of the recombinant protein NcSRS2 for use as a vaccine aimed at reducing congenital transmission of *N. caninum* and encephalitis caused by this parasite. Further studies are needed, however, to reveal new adjuvants capable of improving induction of Th1 immune responses.

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Table 4. Mean ± standard deviation of IgG1 and IgG2a values, with regard to time and group: (P) PBS alone (controls); (N) NcSRS2; (NIL) NcSRS2 plus IL-12; (NF) NcSRS2 emulsified in Freund's complete adjuvant.

Time(days)		Group					Statistically significant difference
		P	N	NIL	NF	*P*	
IgG1	0	0.09±0.01	0.08±0.01	0.08±0.01	0.08±0.02	0.1202	None
	7	0.12±0.02	0.14±0.03	0.15±0.02	0.16±0.03	<0.0001	P < N = NIL = NF
	14	0.09±0.01	0.10±0.02	0.11±0.02	0.50±0.10	<0.0001	P = N = NIL < NF
	21	0.10±0.02	0.29±0.06	0.58±0.11	0.77±0.13	<0.0001	P < N < NIL < NF
	30	0.10±0.01	0.42±0.08	0.71±0.08	0.91±0.07	<0.0001	P < N < NIL < NF
IgG2a	0	0.07±0.01	0.07±0.01	0.07±0.01	0.07±0.01	0.0917	None
	7	0.09±0.02	0.10±0.02	0.10±0.02	0.10±0.02	0.0794	None
	14	0.08±0.01	0.09±0.01	0.10±0.02	0.12±0.02	<0.0001	P < N < NIL < NF
	21	0.08±0.01	0.18±0.03	0.26±0.04	0.68±0.13	<0.0001	P < N < NIL < NF
	28	0.08±0.01	0.23±0.05	0.32±0.06	0.79±0.11	<0.0001	P < N < NIL < NF

Note: *p* d" 0.01 indicates a statistically significant difference. ANOVA and *t* tests (LSD).

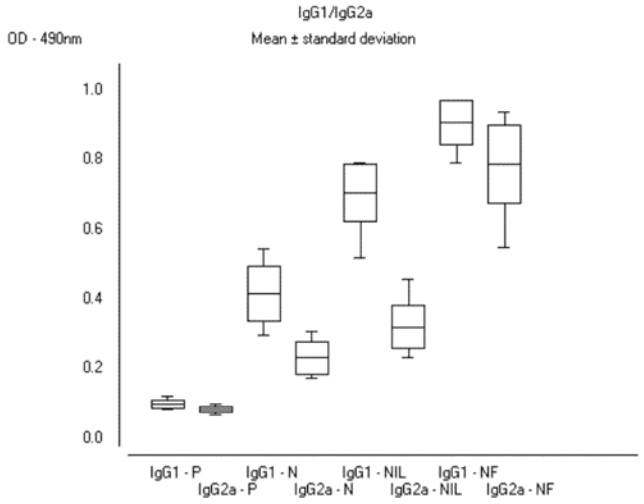

Figure 4. Effect of NcSRS2 immunization with different formulations on antibody subclass responses. (P) PBS alone (controls); (N) NcSRS2; (NIL) NcSRS2 plus IL-12; (NF) NcSRS2 emulsified in Freund's complete adjuvant. IgG1 and IgG2a levels of individual sera were determined by means of ELISA 30 days after the last immunization.

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