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Short communication

Delayed type hypersensitivity induced by intradermal inoculation of a *Neospora caninum* tachyzoite antigen in previously exposed cattle

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ABSTRACT

The aim of this study was to evaluate delayed type hypersensitivity (DTH) induced by the intradermal inoculation of a *Neospora caninum* tachyzoite soluble lysate in cattle previously exposed with the protozoa. Four experimental groups were selected according to the prior exposure to *N. caninum* antigen. All cows were intradermally injected with a *N. caninum* tachyzoite soluble lysate and skinfold thickness growth at the inoculation sites was measured at 0, 24, 48, 72 and 96 h post inoculation (hpi). Additionally, specific antibodies and IFN-γ production were assessed. Cows experimentally infected with live *N. caninum* tachyzoites and cows naturally exposed to *N. caninum* developed skin reactions compatible with DTH between 24 and 96 hpi ($p < 0.05$). Moreover, cows inoculated with an experimental *N. caninum* vaccine and cows without evidence of exposure to *N. caninum* did not show a significant increase in skin thickness ($p > 0.05$). Furthermore, serological status of the animals was not modified due to the intradermal inoculation. The highest IFN-γ production was observed at 15 days after intradermal inoculation ($p < 0.05$). Therefore, these results suggest that cattle previously exposed to *N. caninum* develop a reaction compatible with DTH which could be useful as *in vivo* cell mediated immunity parameter for assessed bovine neosporosis.

1. Introduction

Neospora caninum, an intracellular protozoan parasite from the phylum Apicomplexa, is considered one of the most frequently diagnosed bovine abortifacient pathogens worldwide. Great advances have been achieved since its discovery in the 80´s (Dubey et al., 2007). Indeed, the knowledge related to the parasite, epidemiology, immunopathogenesis and diagnosis allowed the design of some control measures able to reduce its impact on reproductive losses in cattle (Dubey and Schares, 2011; McAllister, 2016; Almeria et al., 2016). Nonetheless, several aspects of the disease are still partially understood. Moreover, an effective chemotherapeutic agent or vaccine is not available to treat or prevent bovine neosporosis (Dubey and Schares, 2011; McAllister, 2016; Almeria et al., 2016).

Several authors have suggested that cell-mediated immune mechanisms (CMI), predominantly Th1-type responses, have a key role in the control of the *N. caninum*. The production of cytokines such as interferon gamma (IFN-γ), is an indicator of such immune response, established as an important cytokine in host defenses against *N. caninum* (Khan et al., 1997; Lunden et al., 1998; Long and Baszler, 2000). However, *N. caninum* also triggers a Th2 immune response (Dubey et al., 2007). Thus, the balance between these types of immune responses is important to the effective control of *N. caninum* infection (Nishiwaka, 2017).

Delayed type hypersensitivity (DTH) reaction plays an important role against intracellular pathogens; however, there is only one study describing DTH after the inoculation of an antigenic extract of *N. caninum* tachyzoites in the footpad of BALB/c mice (Omata et al., 2006). Until now, no information is available regarding a DTH in *N. caninum*-infected cattle. The aim of this work was to evaluate the development of the DTH after intradermal injection of a *N. caninum* tachyzoite soluble lysate in cattle previously sensitized.

2. Materials and methods

2.1. Animals and study design

Twelve British cattle breeds (*Bos taurus*) were included in the

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Table 1
Experimental groups and characteristics of sensitization prior to intradermal inoculation of *N. caninum* tachyzoite soluble lysate.

Group	Serological status*	Sensitization	Antigen	Sensitization-Intradermal test interval
A	Positive	Experimental	Live *N. caninum* tachyzoite	12 months
B	Positive	Experimental	*N. caninum* tachyzoite soluble lysate	12 months
C	Positive	Natural	Live parasites	Unknown
D	Negative	Non sensitized	Non exposed	–

* Including the serological status of their progeny.

present study. All animals belonged to a herd with 23% of *N. caninum* seropositive animals and with the occurrence of endemic abortions due to this infection. However, none of the cows included in this study had a history of abortion. The herd was brucellosis and tuberculosis free and a vaccination program against foot and mouth disease and reproductive diseases were routinely performed. In addition, cows were *T. gondii* seronegative using a micro-agglutination test (Desmonts and Remington, 1980). All animals were handled in strict accordance with the guidelines of good animal practice and animal welfare defined by the Animal Ethics Committee, at National Institute of Agricultural Technology (INTA), Argentina.

Cows were assigned to 4 different experimental groups (3 cows in each group) as stated in Table 1. Group A cows had been experimentally infected with live *N. caninum* tachyzoites in a previous experiment (Hecker et al., 2013). Group B cows had been experimentally vaccinated with 100 µg of *N. caninum* tachyzoite soluble lysate plus adjuvant as reported by Mansilla et al. (2016). Group C cows were naturally exposed to *N. caninum* and Group D cows were *N. caninum* seronegative. Animals from groups C and D were defined not only testing for specific antibodies in themselves (commercial ELISA kit (CIVTEST™, Hipra, Spain), but also for the serological status of their progeny. Indeed, the progeny of animals from group C and D were seropositive and seronegative respectively.

Each cow received four intradermal inoculations with three different antigenic concentrations of a *N. caninum* tachyzoite soluble lysate and as control, inoculation of Vero cell extract (described in Section 2.3). Animals were daily clinically monitored after the intradermal inoculation.

2.2. N. Caninum tachyzoite soluble lysate for the intradermal inoculation

The NC-1 strain of *N. caninum* (Dubey et al., 1988) was used to produce the tachyzoite soluble lysate for the intradermal inoculation. NC-1 was maintained under continuous passage in Vero cells and collected when 80% of the cells were infected. Later, 1×10^9 NC-1 tachyzoites were purified using Sephadex columns (GE Healthcare) and pelleted by centrifugation at 1500 × *g* for 10 min. Parasite pellets were re-suspended in 1 ml of 10 mM Tris hydrochloride pH 7.0 containing 2 mM of phenylmethylsulfonyl fluoride (Sigma Chemical Co. St. Louis, MO, USA), disrupted by three freeze–thaw cycles, and sonicated with 6 × 30 s bursts on ice at maximum setting using a sonic cell disruptor (Sonifier 450, Branson Ultrasonic Co. USA). Protein content was determined using the Micro BCA protein assay method (Pierce, Rockford, USA) and the supernatant aliquoted and cryopreserved at −80 °C. The *N. caninum* tachyzoite soluble lysate was adjusted to different concentrations (0.5, 1.0 and 2.0 mg/ml) to be used in the intradermal test.

2.3. Intradermal test

Twelve months after the experimental sensitization (groups A and B), four areas of 9 cm^2 at the border of the anterior and middle third of the neck region of each animal were shaved and intradermally inoculated with 0.1 ml of the following formulations: 0.5, 1.0, 2.0 mg/ml of *N. caninum* tachyzoite soluble lysate. As inoculation control, 1.0 mg/ml of Vero cell lysate was injected. The distance between inoculation sites was 8–10 cm approximately.

Each injection site was visually inspected and skinfold thickness was measure in each inoculation site at 0, 24, 48, 72 and 96 hpi. Skinfold thickness measurements were obtained using a caliper, according to the methodology used in tuberculin test in cattle (Office Internationale Epizooties – OIE, 2004). All measurements were obtained by the same operator. Skin thickness growth was calculated as the difference between pre-inoculation and post-inoculation skin thickness measurement.

2.4. Samples

Blood samples (20 ml) with heparin were collected from the jugular vein at 0, 15 and 21 days post intradermal inoculation for IFN-γ assay. Similarly blood samples (10 ml) without heparin were collected from the jugular vein at 0, 15 and 45 days post intradermal inoculation. Serum samples were obtained by centrifugation at 1600 × *g* for 10 min and kept at −20 °C until assessed.

2.5. IFN-γ assay

Immune stimulation of heparinized blood samples was performed as previously described (Serrano-Martínez et al., 2007). Briefly, duplicate aliquots of 0.9 ml of heparinized blood, collected from each cow at 0, 15 and 21 days after intradermal test were cultured in duplicate wells in 24-well tissue culture plates (Cellstar Greiner, Monroe, USA). Heparinized blood samples were also cultured with 0.1 ml of PBS (unstimulated control) or concanavalin A (Con-A, Sigma) at 10 mg/ml to assess their ability to respond to stimulation and secrete IFN-γ, or with native antigen extract from the NC-1 strain (10 mg/ml). Plates were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Supernatants were collected and assessed for IFN-γ content using a commercial sandwich iELISA (BOVIGAM, CSL, Victoria, Australia) following manufacturer's instructions. IFN-γ concentrations in supernatants were analyzed using a spectrophotometer (Labsystems Multiskan Plus). A standard curve, derived from a series of dilutions of a recombinant bovine standard (SerotecTM, Oxford, UK), was used to estimate IFN-γ levels in the test samples.

2.6. N. caninum-specific antibody response

Serum samples were assessed for specific IgG antibodies using a commercial kit following the recommendations suggested by the manufacturer (CIVTEST™, Hipra, Spain). Serum samples were analyzed in duplicate and the mean value of the optical density (OD) was converted into a relative index percent (RIPC) using the following formula: RIPC = (OD405 sample − OD405 negative control) / (OD405 positive control − OD405 negative control) × 100. RIPC values higher than 10 indicate seropositivity.

Subclasses of IgG_1 and IgG_2 antibodies were determined by an in house ELISA as described by Moore et al. (2011). Anti-bovine IgG1 or IgG_2 mAbs (1:100; Serotec™, Oxford, UK) were used. For IgG_1 and IgG_2, a kinetic reading was determined at an OD 405 when the *N. caninum* high-positive control with anti-IgG1 reached 1.0 ± 25%. Data were expressed as the OD IgG_1/IgG_2 ratio.

2.7. Statistical analysis

Skin thickness growth was analyzed which a repeated measures design with a split-plot arrangement of treatments, using a linear mixed model (PROC GLIMMIX procedure, SAS Studio v3.6, SAS Institute Inc. Cary, NC, USA). Prior exposure to *N. caninum* antigens (main plot effect), intradermal inoculation treatment (subplot effect), measurement time and their interactions were included as fixed effects. A first order autoregressive covariance structure was used, to account for the correlation between the measurements made within the same animal (main plot) and inoculation point (subplot). A significant thickness increase was considered when the model-estimated least square mean (LS mean) was significantly higher than zero after performing a one sample, one tailed Student's t-test. When significant thickness increase was determined, multiple comparisons were made.

ELISA titers were analyzed by using a general linear model (proc. GLIMMIX). Prior exposure to *N. caninum* antigens, blood-sampling time and their interaction were included as fixed effects. A continuous first order autoregressive covariance structure was assumed between samples collected from the same animal. IFN-γ levels at day 15 were also analyzed by using a general linear model, only including the prior exposure to *N. caninum* antigens as fixed effect.

Covariance structures were selected according to the Akaike' s selection criterion. The symmetry and homoscedasticity of the residuals were assessed by graphical methods, modelling the heteroscedasticity when necessary. Multiple comparisons were adjusted using the Bonferroni method.

3. Results and discussion

3.1. Intradermal test

Neither clinical signs nor changes in rectal temperature were detected in any cows throughout the study. Groups A and C cows developed an increased skin thickness over time (Fig. 1). Although a previous study reported the development of a DTH reaction in BALB/c mice infected with *N. caninum* (Omata et al., 2006), DTH in cows that had been naturally or experimentally sensitized with *N. caninum* antigens is described here for the first time. DTH assay is accepted as an *in vivo* cell-mediated immunological test for investigating the specific immunity against many pathogens including intracellular parasites.

Group A cows increased skin thickness at 48 and 72 hpi when 0.5, 1.0 and 2.0 mg/ml of *N. caninum* tachyzoites soluble lysate were injected ($p < 0.05$). Group C cows increased skin thickness at 24, 48, 72 and 96 hpi when using 0.5, 1.0 and 2.0 mg/ml of soluble lysate ($p < 0.05$). These results suggest that a significantly increased skin reaction could be induced by using antigen concentrations ranging from 0.5 to 2.0 mg/ml. In agreement with these findings, a concentration of 0.3 mg/ml of *T. gondii* antigen (Daryani et al., 2003) and 5 mg/ml of *N. caninum* (Omata et al., 2006) were required to induce DTH in mice. Whether less than 0.5 mg/ml can be inoculated in order to induce DTH in naturally *N. caninum* exposed cattle will be further investigated.

In the present study, a significant increase in the skin thickness was recorded ranging from 48 to 72 hpi and 24 to 96 hpi in experimental and naturally *N. caninum* infected cows, respectively. In agreement with these findings, *N. caninum* infected mice showed a significant footpad reaction between 6 and 72 h after intradermal test (Omata et al., 2006). Similarly, when DTH test is used for the diagnosis of bovine tuberculosis or *T. gondii*-infected human beings (Francis, 1958; Jirovec and Jira, 1961), DTH reaction usually developed between 24 to 72 hpi.

Group D cows did not show a significant skin thickness increased growth at any concentrations of the *N. caninum* tachyzoite soluble lysate. Moreover, no reaction was evidenced when the Vero cell lysate was inoculated in animals from all groups, except from Group A cows at 24 hpi (Fig. 1). On the other hand, cross reactions with other common protozoan parasites should be further investigated; however, all animals included in the trial were *T. gondii* seronegative. In addition, taking into account the high bovine *Sarcocystis sp*. prevalence reported by previous studies from several countries, including Argentina (Moré et al., 2011), these cows were most probably *Sarcocystis sp*. infected. Therefore, the fact that no increase in skinfold thickness was observed in the control group would suggest that the reaction is only due to previous *N. caninum* live parasites exposure.

Protective immune response was associated with DTH-positive mice after challenge with *T. gondii* (Araujo, 1991; Daryani et al., 2003). Omata et al. (2006) reported that experimentally *N. caninum* emulsion immunized mice did not show any DTH reaction. In agreement, no mild DTH reaction was observed in experimentally *N. caninum* vaccinated

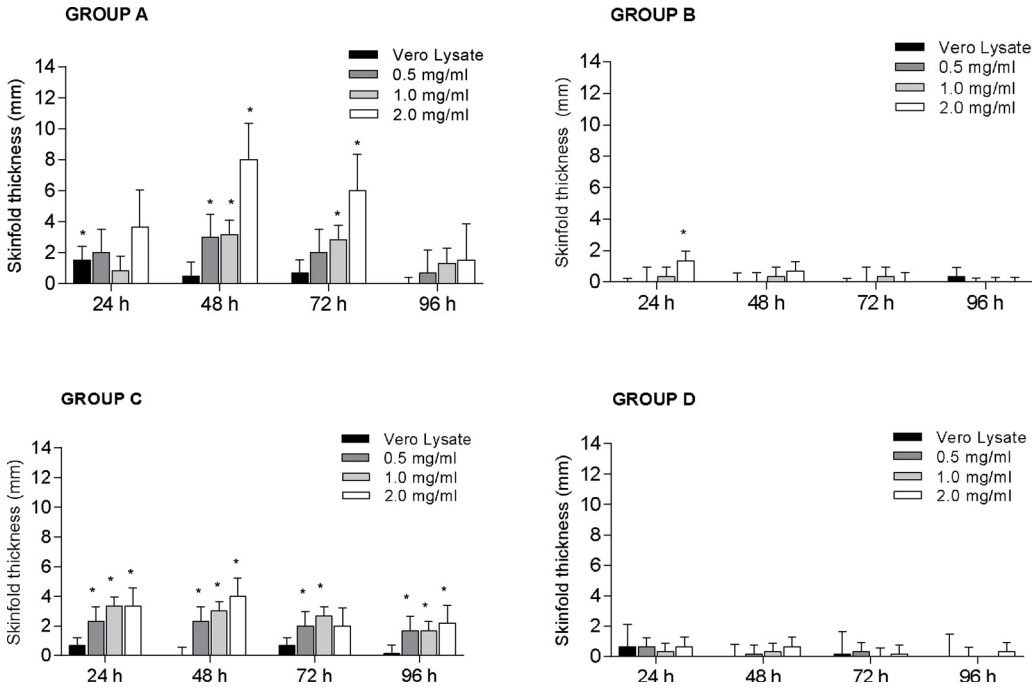

Fig. 1. Skinfold thickness growth (LS means ± SE) of the different experimental groups at 24, 48, 72 and 96 h post intradermal inoculation (hpi). Group A: cows experimentally infected with live *N. caninum* tachyzoites; Group B: cows vaccinated with an experimental *N. caninum* vaccine; Group C: cows naturally infected with *N. caninum*; Group D: cows *N. caninum* seronegative. (*) Significant skinfold thickness growth respect to 0 hpi ($p < 0.05$).

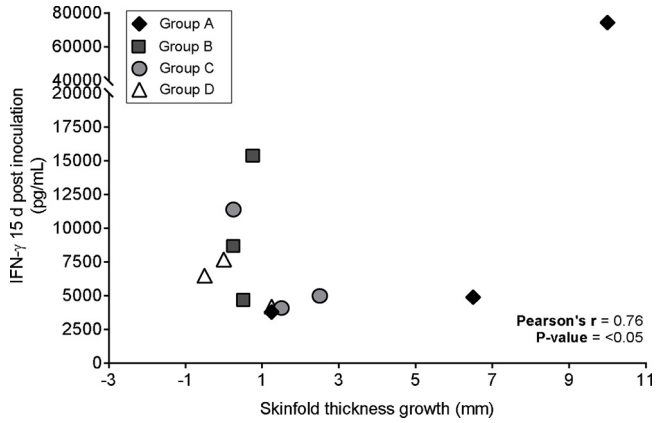

Fig. 2. Scatter plot showing the positive correlation between IFN-γ at 15 days post intradermal inoculation and skinfold thickness growth at 72 hpi when 2 mg/ml of *N. caninum* lysate was inoculated. Group A: cows experimentally infected with live *N. caninum* tachyzoites; Group B: cows vaccinated with an experimental *N. caninum* vaccine; Group C: cows naturally infected with *N. caninum*; Group D: cows *N. caninum* seronegative.

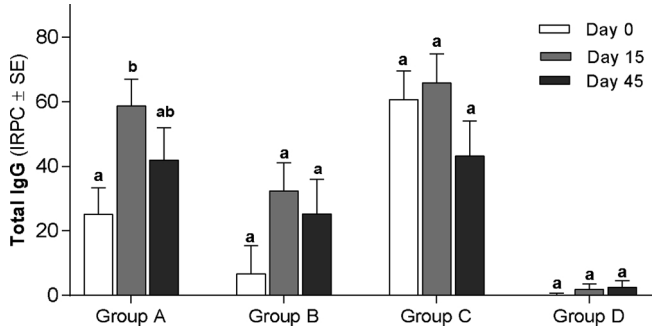

Fig. 3. *N. caninum*-specific IgG response (LS means ± SE) at 0, 15 and 45 days after intradermal inoculation with *N. caninum* soluble lysate. Group A: cows experimentally infected with live *N. caninum* tachyzoites; Group B: cows vaccinated with an experimental *N. caninum* vaccine; Group C: cows naturally infected with *N. caninum*; Group D: cows *N. caninum* seronegative. The mean value of the optical density (OD) was converted into a relative index percent (RIPC). Different letters within the same group indicate statistically significant differences ($p < 0.05$).

with a similar lysate (Mansilla et al., 2013). Cattle experimentally or naturally exposed to live parasites generally develop immunity against *Neospora* abortion (Innes et al., 2001; Almería et al., 2017), interestingly only groups A and C animals developed the skinfold growth compatible with a DTH reaction.

3.2. IFN-γ assay

Solid protective immunity is associated with IFN-γ production in *N. caninum*-experimentally infected cattle previous mating (Innes et al., 2001; Hecker et al., 2013). Moreover, IFN-γ plays a key role in the immunity against *N. caninum* and is usually an indicator of infection in mice and cattle (Khan et al., 1997; Long et al., 1998; Hemphill et al., 2006). In this study, IFN-γ specific production was higher on 15 days after intradermal test in all experimental groups ($p < 0.05$). Positive correlation between IFN-γ specific production at 15 days after intradermal test and skinfold thickness elicited at 48 and 72 hpi was observed, independently of the inoculated antigen concentration ($p < 0.05$) (Fig. 2). On the other hand, no correlation between skinfold thickness due to inoculation of a Vero lysate and production of IFN-γ at day 15 was observed ($p > 0.05$). Therefore, DTH test may be useful for determining the cellular immune function or evaluating protective immune responses to *N. caninum*.

3.3. N. Caninum-specific antibody responses

No differences were found in IRPC values in any group at any time post intradermal inoculation ($p > 0.05$), except for group A animals, where the IRPC (± SE) increased from 25.1 ± 0.5 at 0 hpi to 58.7 ± 11.5 at 15 days after intradermal inoculation (Fig. 3). In the same way, analysis of IgG sub-isotypes based on the IgG_1/IgG_2 ratio showed no statistical differences at 15 and 45 days post intradermal inoculation. Noteworthy, intradermal inoculation of *N. caninum* soluble lysate did not induce systemic specific antibody response. Indeed, this experimental inoculation route seems to be safe in order to maintain the status of seronegative cattle without interfering with serological diagnosis (Dubey et al., 2007). These findings confirm that a previous *N. caninum* exposure before intradermal test is essential for the development of a specific DTH reaction.

Further characterization of the DTH reaction in bovine neosporosis, including determination of many other immune mediators, genotyping and phenotyping of the cellular immune responses, will be performed in future experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

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