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Footpad reaction induced by *Neospora caninum* tachyzoite extract in infected BALB/c mice

Y. Omata [a,*], H. Kamiya [a], R. Kano [a], Y. Kobayashi [b], R. Maeda [a], A. Saito [a]

[a] *Department of Basic Veterinary Science, School of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan*
[b] *Department of Pathobiological Veterinary Science, School of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan*

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Abstract

Little information is available regarding a delayed type hypersensitivity (DTH) reaction in neosporosis. In this study, we examined the elicitation of a DTH reaction in mice infected with *Neospora caninum* by inoculation of the footpad with tachyzoite antigens. The footpads of BALB/c mice infected with *N. caninum* and those of non-infected were injected with either the tachyzoite extract, or paraformaldehyde-fixed tachyzoites. In mice inoculated with *N. caninum* antigens on day 7 p.i. swelling peaked at 6 h after injection of the tachyzoite extract. In mice inoculated on days 14, 28 and 56, swelling was observed between 6 and 72 h afterwards. Mice immunized with the tachyzoite extract plus adjuvant showed peak footpad swelling at 6 h post injection, and the swelling had decreased at 24 h or later. In contrast, mice injected before infection showed no specific swelling. In sections of footpads injected with the tachyzoite extract, exudate had accumulated at 6 h post injection and clusters of infiltrated lymphocytes were observed at 48 h post injection. In mice administered anti-$CD4^+$ cell monoclonal antibodies swelling had decreased at 24 h post injection of the extract. These results indicate that mice infected with *N. caninum* produce a DTH reaction, which is a good indicator of the development of type 1 immune responses.
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Keywords: Delayed type hypersensitivity reaction; Infection; *Neospora caninum*

1. Introduction

Neospora caninum (*N. caninum*), a *Toxoplasma gondii*-like apicomplexan intracellular parasitic protozoa, causes severe neuromuscular disease and repeated abortion through transplacental transmission in livestock and companion animals (Dubey and Lindsay, 1996). This parasite can penetrate many kinds of mammalian host cells, and it has been shown to be capable of infecting a wide range of species. It has been reported that immunity to *N. caninum* involves a predominantly type1 immune response (Khan et al., 1997; Long et al., 1998; Lunden et al., 1998; Marks et al., 1998; Baszler et al., 1999; Long and Baszler,

* Corresponding author. Tel.: +81 155 49 5356; fax: +81 155 49 5359.
E-mail address: yomata@obihiro.ac.jp (Y. Omata).

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2000; Tanaka et al., 2000). The production of cytokines, such as interferon-γ (IFN-γ) and interleukin-4 (IL-4) is an indicator of the reactivity of type 1 or type 2 cells, respectively. The increases in serum levels of IFN-γ and IL-4 in mice after infection with *N. caninum*, however, is transient and relatively lower than those observed in mice infected with *T. gondii* (Shibahara et al., 1999; Kano et al., 2005). Therefore, assay methods for the detection of $CD4^+$ cells specifically reacting to *N. caninum* are needed to better understand the development of the cell-mediated immune responses in the infected animals without any sacrificing them. Delayed type hypersensitivity (DTH) involves T cells, especially sensitized $CD4^+$ cells, and macrophages dependent reaction, while inflammation is granulocyte dependent. In toxoplasmosis, infected mice produce a DTH reaction, which is related to protective immune responses, and $CD4^+$ cell dependent (Araujo, 1991, 1992; Daryani et al., 2003). In this study, we investigated mice infected with *N. caninum* to see if they would show the footpad reaction when injected with tachyzoite antigens.

2. Materials and methods

Eight-week-old female BALB/c mice were purchased from Japan CLEA (Tokyo, Japan) and bovine angio-endothelial cells (BAE cells) were grown in Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal bovine serum (D-MEM10FBS) and non-essential amino acids (ICN Biomedicals Inc., Aurora, Ohio, USA).

Tachyzoites of *N. caninum* which had been isolated from sheep (Kobayashi et al., 2001; Koyama et al., 2001) were maintained by continuous passage in BAE cell cultures, and collected from the culture supernatant. They were washed three times by centrifugation at 1200 *g* for 10 min in phosphate buffered saline (PBS). The parasite suspension was passed through a 3 μm polycarbonate filter (Nuclepore; Corning Coster Corporation, Tokyo, Japan) to remove the host cells and cell debris.

To prepare paraformaldehyde-fixed tachyzoites, tachyzoites were fixed with chilled PBS containing 4% paraformaldehyde for 15 min on ice, then washed three times by centrifugation at 1200 *g* for 10 min in PBS, and adjusted to 2×10^8/ml in PBS.

To prepare *N. caninum* tachyzoite extract, a suspension of approximately 5×10^8 tachyzoites in 1 ml of PBS containing 2 mM PMSF and aprotinin at 1000 units/ml was frozen and thawed five times repeatedly. This was then ultrasonicated, and centrifuged at 12,000 *g* for 10 min. The supernatant was stored at −80 °C until use. The amount of protein in the supernatant was measured using Coomassie Plus Protein Assay Reagent kit® (Pierce, Rockford, IL).

An immunoblotting assay was conducted to determine the antigenic components in the paraformaldehyde-fixed tachyzoites and *N. caninum* extract. Briefly, 10^8 fixed tachyzoites were suspended in 100 μl SDS-PAGE sample buffer, and amount of the extract at concentrations of 250, 50 and 25 μg/50 μl were mixed with an equal volume of SDS-PAGE sample buffer. The 10 μl samples of these preparations were treated with SDS-PAGE using a 10% gel and transblotted onto a polyvinylidene difluoride microporous membrane (Immobilon-P; Millipore Inc., Tokyo, Japan). After blocking with PBS containing 3% bovine serum albumin, the membranes were then immersed in 200-fold diluted sera of mice inoculated with *N. caninum* on day 28 p.i., or mice which had not been inoculated. The membranes were then reacted with 1000-fold diluted horseradish peroxidase conjugated anti-mouse IgG (Bio-Rad Ltd., Hercules, California) at 4 °C for 18 h. Antibodies were visualized by means of the peroxidase reaction using diaminobenzidine-4 HCl in 0.1 M Tris–HCl pH 7.4 containing 0.03% H_2O_2. The molecular masses (M.m.) of electrophoresed and transblotted proteins were estimated based on the running distance compared with molecular weight markers (Kaleidoscope prestained standards: Bio-Rad Ltd.). Assay controls consisted of samples in which the primary antibody was replaced with non-immune mice sera.

Twenty mice were inoculated intraperitoneally (i.p.) with 2×10^6 *N. caninum* tachyzoites and 10 mice were inoculated with 100 μl of an emulsion of the *N. caninum* tachyzoite extract (at a concentration of 250 μg/50 μl) and an equal volume of adjuvant (Titer Max gold; Titer Max USA Inc., Norcross, GA). At specified periods after inoculation, five mice which had been infected with *N. caninum* and five mice which had been immunized were injected in the footpad with 50 μl of PBS containing specified amounts of the *N. caninum* protein extract, or paraformaldehyde-fixed tachyzoites (10^7/50 μl) or the

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