FULL PAPER *Parasitology*

Monoclonal Antibodies Preventing Invasion of *Neospora caninum* Tachyzoites into Host Cells

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ABSTRACT. Twelve monoclonal antibodies (MAbs) against *Neospora caninum* tachyzoites were produced to specify the antigens related to the invasion of tachyzoites into host cells. In the assay to evaluate the inhibition activity, all these MAbs prevented the cultured Vero cells from the invading by the tachyzoites. These MAbs recognized approximately a 73 kDa antigen in Western blot analysis. Immunofluorescence assay and immune electron microscopy revealed that this 73 kDa antigen is a part of the surface antigens of *N. caninum* tachyzoite, and that the tachyzoite antigen identified plays an important role for invasion of host cells.
KEY WORDS: invasion process, monoclonal antibody, *Neospora caninum*, surface antigen.

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Neospora caninum is a coccidian protozoon first isolated from the brain of a puppy by Dubey in 1988, which developed paralysis in the hind limbs [5]. Dogs infected with this parasite mainly show neurological symptoms and infected cows are attached with abortion and stillborn, so serious economical damages are produced [4]. In 1998, McAllister *et al.* reported that definitive host of the protozoon is dogs [14]. However, the life cycle of *N. caninum* is not completely clarified yet, and the procedures for prevention and treatment of neosporosis also are not established. The pathogenesis of *N. caninum* is known to be caused by multiplication of parasites resulting destruction of many kinds of host cells by invasion [2, 3, 20]. To develop the prevention and treatment of neosporosis, analyses of antigens related with the invasion process into host cells are required. Antigens of some protozoan parasites belonging to phylum Apicomplexa, such as *Toxoplasma gondii* participate in invading the host cells and were thought to be surface antigens, secretory proteins and those in the structures of apical complex such as microneme, rhoptry and dense granule [6, 11, 15, 18]. Also in *N. caninum*, some antigens, possibly affecting the invasion, were studied, and the antigens on the body surface [9, 10], dense granule [7], microneme [12, 17, 19] and rhoptry [1], glycoconjugates, glycoproteins [8] and subtilisin-like serineprotease [13] were reported until now. But the roles of these antigens in invasive process remain unclear in detail.

In this study, we prepared monoclonal antibodies (MAbs) against *N. caninum* tachyzoites which obstruct the invasion of tachyzoites into host cells, and further, we tried to specify the antigen of *N. caninum* concerning with attachment and invasion into the host cells.

MATERIALS AND METHODS

Parasites: *Neospora caninum* JPA-1 strain used was isolated and passaged by Yamane *et al.* in the National Institute of Animal Health, Japan [23]. The isolate was inoculated into monolayer Vero cells maintained in Eagle's minimum essential medium (MEM; NISSUI PHARMACEUTICAL Co., Japan) supplemented with 10% fetal calf serum (FCS; HARLAN SERA-LAB, England), 2 mM L-glutamine (Wako Pure Chemical Industries, Japan), 1% MEM Vitamin solution (GIBCO BRL, U.S.A.), 13 mM sodium hydrogen carbonate ($NaHCO_3$) (Wako Pure Chemical Industries), and 4 μl/m*l* Fungizone (GIBCO BRL). The tachyzoites cultured were used for examination.

Purification of tachyzoites: The tachyzoites cultured were purified according to an improved method of Yamane *et al.* [25]. Briefly, Vero cells infected with the tachyzoites were disrupted and mixed with Dulbecco's phosphate buffered saline (PBS^-; NISSUI PHARMACEUTICAL Co.) containing 30% isotonic percoll (Amersham Pharmacia Biotech, England), and then PBS^- containing 50% and 80% isotonic percoll respectively, were injected under the layer and centrifuged at 1,250 × g for 30 min at 4°C. Tachyzoites were collected from a layer between 50% and 80% isotonic percoll and washed in PBS. The purified tachyzoites were sonicated three times for 2 min at 40 w and provided for production of monoclonal antibodies (MAbs), sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) and determination of isotype of MAbs.

Preparation of monoclonal antibodies: Seven-week-old female BALB/c mice were immunized three times with *N. caninum* tachyzoite antigen to produce MAbs. In the first immunization, mice were intramuscularly inoculated with 0.5 m*l* of 2.5×10^6 tachyzoites antigen adsorbed with aluminum hydroxide gel and in the second immunization, intraperitoneally with 0.5 m*l* of the same immunogen. In the last immunization, 0.2 m*l* of 1.0×10^6 tachyzoites antigen was intravenously inoculated. After the immunization, the spleen was removed from the mice, and splenocytes were collected and fused with myeloma (P3-X63-Ag8-U1 : P3U1) cells in PEG/DMSO SOLUTION (Sigma, U.S.A.).

The hybridomas were cultured in Hybridoma-SFM (SFM; GIBCO BRL) containing 10% FCS, 1% MEM vitamin solution (GIBCO BRL), 29 mM $NaHCO_3$ (Wako Pure Chemical Industries), 50 μg/m*l* gentamicin, 4 μ*l*/m*l* fungizone and 1% hypoxanthine aminopterin thymidine suppliment (HAT; GIBCO BRL) at 37°C under 5% CO_2 atmosphere. Fourteen days after the cell fusion, screening was performed using supernatant of hybridomas by the inhibition assay mentioned later. Hybridomas were cloned by the limiting dilution and picking up method under a stereomicroscope. By inoculation of cloned hybridomas (2.0×10^6) intraperitoneally into BALB/c mice treated with Prestene (2,6,10,14-tetramethylpentadecane; Sigma, U.S.A.), MAbs with high titer were collected from the peritoneal fluid of mice. The isotypes of MAb were analyzed by means of the Mouse MonoAB ID KIT (ZYMED LABORATRIES, U.S.A.).

Inhibition assay: The inhibition assay was carried out for the evaluation of inhibition activity of MAbs which obstruct *N. caninum* tachyzoites from invading into host cells. Briefly, 100 μ*l* of *N. caninum* tachyzoite suspension adjusted with MEM to 2.0×10^6/m*l* was treated with our equal volume of supernatant of each hybridoma at 37°C for 1 hr and inoculated into monolayered Vero cells, then they were incubated in 96 well microculture plates at 37°C under 5% CO_2 atmosphere for 4 weeks. The inhibition ability of MAb was judged whether the tachyzoites could invade and multiplicate in Vero cells or not.

SDS-PAGE and Western blotting: SDS-PAGE and Western blotting were performed in accordance with the methods of Weber and Osborn [22] and Towbin *et al.* [21]. Briefly, the antigens of *N. caninum* tachyzoites were separated by SDS-PAGE under reducing condition and transferred on nitrocellulose membrane (Amersham Pharmacia Biotech). After treated with skim milk (Wako Pure Chemical Industries) to block non-specific binding of antibody, the membrane was reacted with MAb as the first antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Cappel, U.S.A.) as the second antibody. The antigen-antibody reaction was detected using ECL Western Blotting Detection System (Amersham Pharmacia Biotech).

Indirect immunofluorescence assay (IFA): IFA was performed according to the method mentioned by Yamane *et al.* [24]. *N. caninum* tachyzoites fixed with 2% (w/v) paraformaldehyde in PBS on the slides were incubated with MAb or anti-*N. caninum* tachyzoites or normal mouse serum as the positive or negative control against *N. caninum* tachyzoite at 37°C for 1 hr. The slide glasses were washed and dried, and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:100, Leinco Technologies, U.S.A.), at 37°C for 1 hr. The bound region of *N. caninum* tachyzoites with MAb was surveyed with a fluorescence microscope.

Immune electron microscopy: Tachyzoites were pre-fixed in mixed solution of 0.05% (w/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2) for 4 hr at 4°C. The fixed tachyzoites were immersed in incubation buffer (0.1 M PB supplemented with 0.8% bovine serum albumin and 0.25% gelatin) supplemented with 5% BSA and 5% normal goat serum for 30 min at 4°C to block non-specific binding of MAbs to tachyzoites. Then the tachyzoites were kept with 100 times volume of MAbs overnight at 4°C, and after washed in incubation buffer, they were immersed in goat anti-mouse IgG mixed with colloidal gold (1:30; 1 nm in diameter; Auriongp-ultrasmall, Wageningen) for 2 hr at room temperature (R.T.). The tachyzoites were washed, and then post-fixed with 2% glutaraldehyde in 0.1 M PB for 20 min at 4°C and subsequently in 1% (w/v) osmium tetroxide in 0.05 M PB (pH 7.2) for 30 min at R.T. After washing, the colloidal gold labelling of the tachyzoites was intensified using AURION R-Gent SE-LM (Aurion, Wageningen, The Netherlands) mixed with 50% (w/v) arabic gum solution. Finally, the tachyzoites were washed and dehydrated with an ethanol series, and then immersed in propylene oxide and embedded in resin (Polysciences, Inc, Warrington, PA). Ultrathin sections were stained with uranyl acetate and examined with a JEM-1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

RESULTS AND DISCUSSION

In the present study, 12 MAbs against *N. caninum* tachyzoites were produced (3/NCT/1–12). When the inhibition assay was performed as a screening to select MAbs with an activity to inhibit cell invasion of tachyzoites, tachyzoites incubated with supernatant of hybridoma without inhibition activity invaded Vero cells and destroyed the cells. On the other hand, in the assay using 3/NCT/1–12 MAbs no tachyzoites invaded nor destroyed Vero cells (Fig. 1). Of 3/NCT/1–12 MAbs obtained in this examination, MAb 3/NCT/10 was an isotype of IgM with κ Light chain, and all the other MAbs were isotypes of IgG_1 with κ Light chain (Table 1). When IFA was performed to confirm the region of *N. caninum* tachyzoite combined with respective 3/NCT/

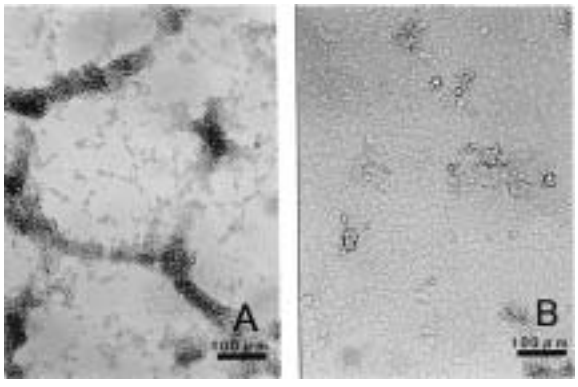


Fig. 1. Vero cells 26 days after inhibition assay to produce MAbs having an ability to obstruct *N. caninum* tachyzoite from invading Vero cells. (A) Vero cells assayed by antibody having no inhibition activity. (B) Vero cells assayed by MAb 3/NCT/2. Destruction of Vero cells by *N. caninum* tachyzoites is less than in A.

Table 1. The characterization of MAbs that prevent *N. caninum* tachyzoites from invading into Vero cells

Monoclonal antibodies (MAbs)	Region of parasite identified by MAbs	Molecular weight determined by MAbs	Isotypes of MAbs
3/NCT/1	surface	73.0	IgG_1, κ L chain
3/NCT/2	surface	73.0	IgG_1, κ L chain
3/NCT/3	surface	73.0	IgG_1, κ L chain
3/NCT/4	surface	73.0	IgG_1, κ L chain
3/NCT/5	surface	73.0	IgG_1, κ L chain
3/NCT/6	surface	73.0	IgG_1, κ L chain
3/NCT/7	surface	73.0	IgG_1, κ L chain
3/NCT/8	surface	73.0	IgG_1, κ L chain
3/NCT/9	surface	73.0	IgG_1, κ L chain
3/NCT/10	surface	73.0	IgM, κ L chain
3/NCT/11	surface	73.0	IgG_1, κ L chain
3/NCT/12	surface	73.0	IgG_1, κ L chain

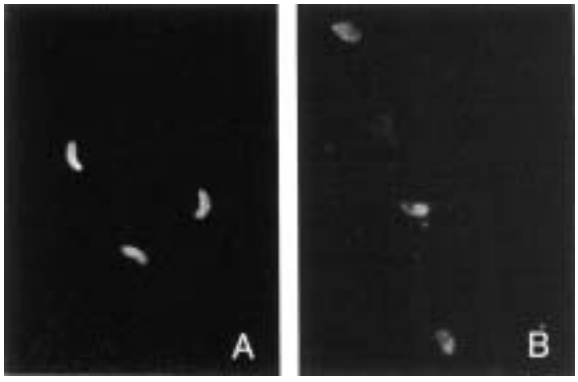


Fig. 2. Indirect immunofluorescence assay (IFA) of *N. caninum* tachyzoites incubated with antibody which is bound to *N. caninum* tachyzoite or MAbs. (A) IFA incubated with anti-*N. caninum* tachyzoites mouse serum. Fluorescent dye is observed all over the surface of tachyzoites. (B) IFA incubated with MAb 3/NCT/5. Fluorescent dye is specifically recognized in one part of surface of tachyzoite.

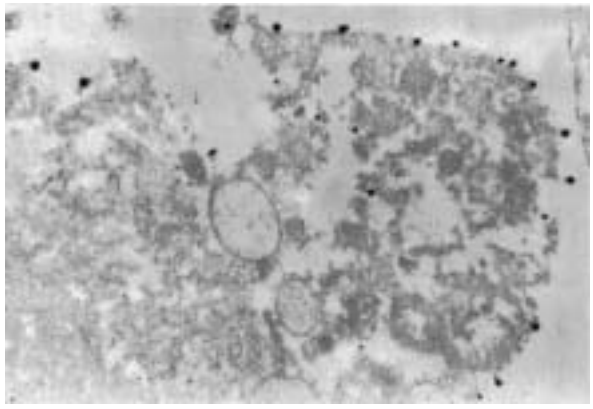


Fig. 3. Immune electron micrograph of *N. caninum* tachyzoite incubated with MAb 3/NCT/2. Colloidal gold particles are found on the surface of *N. caninum* tachyzoite.

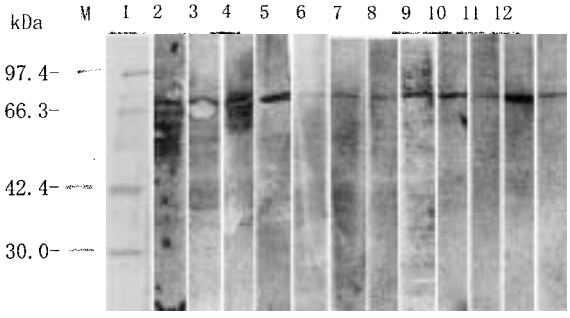


Fig. 4. Western blot analysis of recognition site of *N. caninum* tachyzoite antigen with MAbs 3/NCT/1–12 (lane 1–12) that inhibits the cell invasion by *N. caninum*. All the MAbs recognized approximately 73 kDa antigens of *N. caninum* tachyzoites (arrow).

1–12 MAbs, fluorescein isothiocyanate bound with MAb was localized on a part of surface area of tachyzoite (Fig. 2). In immune electron microscopy using respective 3/NCT/1–12 MAbs, the colloidal gold was detected on the surface of the tachyzoites (Fig. 3). To identify the molecular mass of tachyzoite antigen bound with the MAbs, Western blotting was carried out and revealed that all the MAbs recognized approximately 73 kDa antigen of tachyzoites (Fig. 4). From the above result, 3/NCT/1–12 MAbs prepared in the present study had the activity to inhibit cell invasion and recognized *N. caninum* tachyzoite surface antigen of 73 kDa. This result leads a view that 3/NCT/1–12 MAbs would bind with the 73 kDa surface antigen of tachyzoites and prevent the invasion of tachyzoites into Vero cells physically or chemically. In the tachyzoites of *Toxoplasma gondii*, surface proteins as well as those from microneme, rhoptory, dense glanule, and enzymes which activate microneme in the apical complex are known as the proteins related to the invasion process [6, 11, 16, 18]. Since *N. caninum* is very closed to *T. gondii* in morphology, it is expected that the same or

very similar proteins of *N. caninum* tachyzoite participate in the invasion into host cells like in *T. gondii*. As the proteins related to cell invasion in *N. caninum*, Nc-p36 [10] and Nc-p43 [9] existing on the surface of tachyzoite, NcMIC3 [12, 17, 19] in microneme, GRA2 [7] in dense glanules, and NC-SUB1 [13] which is one of the proteases were confirmed. In the inhibition assay using the MAbs obtained in this study, invasion by the tachyzoites into the host cells was highly inhibited, so that it is considered that the surface antigen identified might have the function of attachment, recognition or secretion. As all of these 3/NCT/1–12 MAbs screened by the inhibition assay recognized the only one antigen, it is thought that the antigen identified in the present study would appear when tachyzoites invade host cells. For that reason, it is suggested that this antigen is necessary in the invasion process of *N. caninum* tachyzoites and plays an important role in the process.

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