Title

Identification and characterization of cross-reactive antigens from *Neospora caninum* and *Toxoplasma gondii*

Running title

Cross-reactive antigens between *N. caninum* and *T. gondii*

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SUMMARY

Murine monoclonal antibodies against *Neospora caninum* tachyzoite were produced to identify the cross-reactive antigens between *N. caninum* and *Toxoplasma gondii*. Ten mAbs recognizing cross-reactive antigens of both parasites were obtained and tentatively classified into 6 different groups based on their reactivity patterns in an indirect fluorescent antibody test and Western blot analysis. Three mAbs in group 1 recognized antigens located on the surface of parasites with molecular masses ranging from 28 to 76 kDa; one mAb in group 2 recognized antigens located on interior organelles of parasites with a molecular mass of 50 kDa; one mAbs in group 3 recognized antigens located on interior organelles of parasites with molecular masses of 35 kDa and 14 kDa; three mAbs in group 4 recognized antigens located on interior organelles with a molecular mass of 64 kDa; one mAb in group 5 recognized antigens located on surface of parasites with an unknown molecular mass; one mAb in group 6 recognized antigens located on apical end of parasites with an unknown molecular mass. The mAbs in groups 1, 2, 3, and 5 showed inhibitory effects on the growth of the two parasites *in vitro* in a concentration-dependent manner. A cDNA expression library prepared from *N. caninum* tachyzoite mRNA was immunoscreened with the mAb panel. Three kinds of proteins, protein disulfide isomerase (PDI), heat-shock protein 70 (HSP70), and ribosomal protein 1 (RP1), were identified as cross-reactive antigens recognized by mAbs in groups 2, 3, and 4, respectively. Some of the proteins could be useful in developing vaccines or drugs for controlling the diseases caused by the two parasites.

Key words: *N. caninum*; *T. gondii*; cross-reactive antigen; protein disulfide isomerase; heat-shock protein; ribosomal protein 1.

INTRODUCTION

Neospora caninum and *Toxoplasma gondii,* two closely related apicomplexan parasites, can infect many warm-blood vertebrates (Dubey, 1998; Dubey et al., 1999). *T. gondii* has long been recognized as an important pathogen in humans and animals. However, compared with *T. gondii*, *N. caninum* was recently regarded as a major cause of bovine neonatal mortality and abortion (Dubey & Lindsay, 1996). Because these two organisms have nearly identical morphology and can cause similar pathology and disease, *N. caninum* has often been incorrectly identified as *T. gondii* (Dubey, 1999). Both neosporosis and toxoplasmosis caused by *N. caninum* and *T. gondii*, respectively, have been recognized as economically important diseases with considerable impact on the livestock industry.

Since *N. caninum* was identified as an etiologic agent of neosprosis and was found to be close to *T. gondii*, a great deal of research has been conducted to determine the differences and the nature of the relationship between *N. caninum* and *T. gondii* (Lindsay et al., 1993; Baszler et al., 1996; Howe et al., 1998, 1999). For a better understanding of the relationship between the two parasites and for the development of future control measures, detailed information about cross-reactive antigens for the infection process in *N. caninum* and *T. gondii* is necessary. In addition, the characterizations of the common antigens of *N. caninum* and *T. gondii* are important prerequisites for detailed analyses of the common mechanisms of parasite adhesion to and invasion of host cells.

The objectives of the present study were to identify and characterize cross-reactive antigens, which can be responsible for important functions during parasite-host interactions, by using monoclonal antibodies. Several mAbs recognizing both *N. caninum* and *T. gondii* tachyzoites were produced, and 3 kinds of proteins, disulfide isomerase (PDI), heat-shock protein 70 (HSP70), and ribosomal protein 1 (RP1), were identified as cross-reactive antigens by using the mAb panel.

MATERIALS AND METHODS

Culture and purification of parasites

The NC-1 strain of *N. caninum* and the RH strain of *T. gondii* were cultured in Vero cell monolayers in a minimum essential medium (MEM) supplemented with 8% fetal bovine serum (FBS) and 50 µg/ml kanamycin at 37°C in a 5% CO_2 air environment. For the purification of tachyzoites, the parasites were scraped from the flask and then passed through a 27G needle and a 5.0 µm filter (Millipore, USA). They were then washed in phosphate-buffered saline (PBS). The pellet was stored at –30°C until use.

Production and purification of monoclonal antibodies

N. caninum tachyzoite lysate was used as an antigen. The primary immunization of BALB/c mice (8 weeks old) consisted of an intraperitoneal injection of the antigen (1 x 10^8 tachyzoites/mouse) with Freund's complete adjuvant. Successive immunizations were done intraperitoneally twice with the same antigen with Freund's incomplete adjuvant. A mouse with the highest antibody titer against *N. caninum* tachyzoite, as indicated by an indirect fluorescent antibody test (IFAT), was selected for hybridoma production. Mouse spleen cells were fused with SP2 myeloma cells, and hybridomas were cultured in a GIP medium (Wako, Tokyo, Japan) with hypoxantine, aminopterin, and thymidine (HAT) (Sigma, MO, USA) in 96-well plates. Two to three weeks after fusion, screening for hybridoma-producing antibodies was performed by IFAT and Western blot analysis. Hybridoma-producing mAbs against *N. caninum* and *T. gondii* tachyzoites were identified and cloned three times by limiting dilution. Ascitic fluid was produced in incomplete Freund's adjuvant-primed BALB/c mice. Purification of the mAbs was performed by 50% ammonium sulphate precipitation. The isotype of the mAbs was determined using a mouse mAb isotyping kit (Amersham Biosciences, NJ, USA).

Indirect fluorescent antibody test

Both purified tachyzoites and parasites grown in Vero cells were analyzed by an IFAT, respectively. For purified tachyzoites, *N. caninum* and *T. gondii* tachyzoites (5 x 10^4) were mounted on slides, dried and fixed with acetone before use. For parasites in Vero cells, monolayers of Vero cells grown on 14-well glass slides were infected with freshly purified *N. caninum* and *T. gondii* tachyzoites (800 cells/well), respectively. After 2-3 days incubation at 37°C with 5% CO_2, infected cells were fixed with 3% paraformaldehyde in PBS for 30 min and were then permeated with 0.3% Triton X-100 in PBS for 30 min. The slides were subsequently incubated in blocking solution (PBS containing 1% BSA and 50 mM glycin) for 30 min. Both slides with purified parasites and parasites grown in Vero cells were incubated with hybridoma-cultured medium, followed by incubation with a goat anti-mouse immunoglobulins (IgG+IgA+IgM) Alexa-488 antibody (Molecular Probes, OR, USA) diluted 1:500 in blocking solution. The location of the antigens on parasites reacted with mAbs was observed with fluorescent microscopy and confocal laser microscopy.

SDS-PAGE and Western blot analysis

Purified *N. caninum* and *T. gondii* tachyzoites were suspended in PBS and mixed with a sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% ß-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) under reducing conditions. The samples were run on 15% polyacrylamide gel. After SDS-PAGE, the proteins in the gel were electrically transferred onto a membrane. The membrane was blocked with PBS containing 3% skim milk and then incubated with a hybridoma-cultured medium at 37°C for 60 min. Then, the membrane was washed three times with PBS and then incubated with peroxidase-conjugated goat anti-mouse IgG, IgA, and IgM (Southern Biotechnology, AL, USA) diluted 1:500 in PBS containing 3% skim milk. After washing with PBS three times, the bands on the membrane were visualized by incubation with diaminobenzine.

In vitro assay for the inhibitory effects of mAbs on the growth of the parasites

Tachyzoites (1 X 10^4) of *N. caninum* and *T. gondii* expressing green fluorescent protein (GFP) (Nishikawa et al., 2003; Kaneko et al., 2004; Xuan et al., Manuscript in preparation) were incubated with purified mAbs at different concentrations of 0.5, 0.1, 0.05, and 0.01 mg/ml at 37°C for 1 h. Isotype-matched mouse mAbs (IgG1, IgG2a, IgG2b, or IgM) against *Babesia caballi* (Ikadai et al., 1999) were used as a control. After incubation, the parasites were added to confluent monolayers of Vero cells grown in 24-well plates. After incubation at 37°C for another 1 h, infected Vero cells were covered with 1% low-melting agarose gel diluted in MEM supplemented with 1% FBS. After 48 h, the number of plaques of each well was counted with fluorescent microscopy. The percentage of inhibition was calculated by the following formula: [the number of parasites at mouse mAb control – the number of parasites at tested mAb treatment]/ [the number of parasites at mouse mAb control] %. All assay experiments were repeated two times.

Construction and immunoscreening of the N. caninum cDNA library and cDNA sequencing

Total RNA was prepared from *N. caninum* tachyzoites (1 x 10^9) by acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987), and then poly(A)$^+$ RNA was purified with Oligotex-dT 30 (Takara, Tokyo, Japan). The cDNA was synthesized by using a Zap-cDNA synthesis kit, ligated to an Uni-ZAP XR vector, and packaged by using a Gigapack III packaging system according to the manufacturer's instructions (Stratagene, CA, USA). The *N. caninum* cDNA library (10^7 PFU) was screened with hybridoma-cultured medium, and the *in vivo* excision of the cDNA inserts from lambda phage to plasmid pBluescriptSK(+) were according to the manufacturer's instructions (Stratagene). Both strands of the cDNA were sequenced by using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit, and then analyzed with an ABI3700 DNA sequencer (Applied Biosystems, CA, USA).

Sequence data were analyzed with a computer program (MacVector, Oxford Molecular, CA, USA).

Nucleotide sequence accession numbers

Nucleotide sequence data reported in this article are available in the GenBank, EMBL and DDBJ databases under the accession numbers AB178220, AB178286, and AB178287.

RESULTS

Production of mAbs recognizing both N. caninum and T. gondii tachyzoites

A total of 384 wells from 5 microplates containing hybridomas were subjected to screening of mAbs recognizing cross-reactive antigens by IFAT with purified tachyzoites. Culture media from all 384 wells were IFAT-positive for *N. caninum* tachyzoites. Of 384 wells, 10 wells were IFAT-positive for *T. gondii* tachyzoite. After cloning of hybridoma cells by limiting dilution, 10 mAbs recognizing cross-reactive antigens between the two parasites were obtained and subjected to further analyses. These 10 mAbs were tentatively classified into 6 different groups based on the results of IFAT and Western blot analyses (see below), and their properties are summarized in Table 1.

Localization of antigens on parasites recognized by mAbs

The localization of antigens on *N. caninum* and *T. gondii* tachyzoites recognized by mAbs was analyzed by IFAT with both purified tachyzoites and parasites grown in Vero cells (Table 1 and Fig. 1). mAbs in groups 1 and 5 reacted with the surface of both parasites (Fig. 1A and 1E). Group 2 and 3 mAb-coated tachyzoites of both parasites exhibited clearly scattered fluorescent dots inside the parasites, and most of the fluorescent dots were around the surface of the tachyzoites (Fig. 1B and 1C). The fluorescence exhibited by the tachyzoites that had been reacted with mAbs in group 4 was

different from the reactive patterns observed in group 2 and group 3 mAbs: it seemed to be suffused inside the tachyzoites (Fig. 1D). One mAb in group 6 reacted with the apical end of the two parasites (Fig. 1F).

Western blot analysis of antigens recognized by mAbs

Western blot analysis was performed to identify the antigens recognized by mAbs. As shown in Fig. 2 and Table 1, mAbs in group 1 reacted with several major proteins with molecular masses ranging from 28 to 76 kDa in the two parasites; mAb in group 2 recognized a 50 kDa protein; mAb in group 3 recognized 35 kDa and 14 kDa proteins; mAbs in group 4 recognized a 64 kDa protein. The proteins that had been reacted with mAbs in IFAT in groups 5 and 6 were not detectable by Western blot analysis in both reducing (Fig. 2) and non-reducing (data not shown) conditions.

Inhibitory effects on the growth of N. caninum and T. gondii tachyzoites

As shown in Table 2, 10 mAbs were tested to examine their inhibitory effect on *N. caninum* and *T. gondii* tachyzoite growth *in vitro*. mAbs in groups 1, 2, 3, and 5 had more than a 90% inhibitory effect on the growth of the two parasites at a 0.5 mg/ml concentration compared with the control mAbs. The different lower concentration of mAbs from 0.01 to 0.1 mg/ml was used in the inhibition assay to examine if the inhibition effects were dependent on the concentration of the mAbs. The results showed that the inhibition rate decreased corresponding to the reduction in the concentration of mAbs.

Identification of genes encoding cross-reactive antigens

A cDNA expression library prepared from the mRNA of *N. caninum* tachyzoites was immunoscreened with mAbs recognizing cross-reactive antigens between *N. caninum* and *T. gondii*. A total of 32 positive clones were obtained by immunoscreening of the cDNA library with mAbs in groups 2 (15 clones), 3 (7 clones), and 4 (10 clones). The clones with the longest cDNA in each group were chosen for sequencing. The amino acid

sequences deduced from the nucleotide sequences show high homology with the PDI of *T. gondii* (Genbank accession number AB178220*)*, the RP1 of *Plasmodium falciparum* (Genbank accession number AB178286), and the HSP70 of *T. gondii* (Genbank accession number AB178287) (Table 3).

DISCUSSION

Several reports have shown that there are serological cross-reactions and protective cross-reactions between *N. caninum* and *T. gondii* (Kasper & Khan, 1998; Lindsay et al., 1998; Nishikawa et al., 2002). These works suggested that the two parasites might possess cross-reactive antigens important for host immunity against the two parasites. However, only a few cross-reactive antigens have been reported to date: a 42 kDa protease of *T. gondii*, which is a phoptry protein, has been confirmed to show immunological cross-reactivity with *N. caninum* (Ahn et al., 2001); the nucleoside triphosphate hydrolase (NTPase) of *N. caninum*, which is a dense granule protein, has been described as showing antigenic cross-reactivity with the NTPases of *T. gondii* (Asai et al., 1998); another antigen, the BAG5 of *T. gondii*, which is a bradyzoite-specific antigen, has shown cross-reactivity with the bradyzoites of *N. caninum* (Mcallister et al., 1996). On the other hand, antigens sharing common epitopes involving a protective immunity have not been identified.

In the present study, we focused on the search for cross-reactive antigens between *N. caninum* and *T. gondii* with mAbs against *N. caninum* tachizoites. Ten mAbs recognizing the cross-reactive antigens of both parasites were obtained and classified into 6 different groups based on their reactivity patterns in IFAT and Western blot analysis. After immunoscreening of a cDNA library of *N. caninum* with the mAbs, three kinds of proteins, PDI, HSP70, and RP1, were identified as cross-reactive antigens.

PDI is a multifunctional protein of the endoplasmic reticulum (RE) known to be involved in the formation, breakage, and rearrangements of disulfide bonds during the folding of secreted proteins. PDI not only catalyzes the

folding and isomerization of proteins, but it also acts either as a chaperone or an anti-chaperone depending on its concentration. It is also essential for assisting unfolded or incorrectly folded proteins to attain their native state (Gilbert, 1998). The functions of PDI in protozoan parasites, such as *T. gondii*, *Leishmania major*, and *Plasmodium falciparum*, have been described (Florenta et al., 2000; Achour et al., 2002; Meek et al., 2002a, 2002b). Conserved regions of PDI are targeted by the natural IgA antibody in humans infected with *T. gondii*, which would be involved in defense against pathogens (Meek et al., 2002b). In the present study, mAbs recognizing the PDI of *N. caninum* and *T. gondii* showed inhibitory effects on the growth of both parasites. In addition, the specific inhibitors of PDI, such as zinc bacitracin and tocinoic acid, have shown inhibitory effects on the growth of *L. major in vitro* (Achour et al., 2002). Therefore, the targeting of PDI would offer new opportunities for the prevention and chemotherapeutic strategies for the control of *N. caninum* and *T. gondii* infections.

Ribosomal protein is a structural protein that plays an essential role in the ribosomes. The sera against the ribosomal phosphoprotein P0 of the human malarial parasite *P. faliparum* (PfP0) inhibited the invasion of erythrocytes by merozoites in a concentration-dependent manner (Sing et al., 2002). The same antibodies protected mice against a challenge with a virulent strain of *Plasmodium* (Singh et al., 2002). RP1, one of the ribosomal proteins, might have potential protective immunogenicity for *N. caninum* and *T. gondii* infections, since the mAb recognizing RP1 had significant growth inhibitory effects on the growth of the two parasites.

The HSP70 of protozoan parasites, such as *Leishmania*, *Trypanosoma*, and *Plamodium*, is believed to play a fundamental role during infection and has been demonstrated to possess antigenic properties (Rico et al., 1998). It has been proposed that HSP70 of *T. gondii* may have potential as an antigen for vaccines and may protect a host against *T. gondii* infection (Yang et al., 1997; Chen et al., 2000; Mun et al., 2000). Further work should be performed in

order to determine the protective effects against both *N. caninum* and *T. gondii* infections.

Generally, protein on the surface of intracellular pathogens is believed to play a critical role in infection, since it represents the initial interaction between the pathogen and the host cell surface molecules and the host immune response. All of the mAbs produced in this study, which recognize the surface antigens of the two parasites, had significant inhibitory effects on the growth of the parasites *in vitro* in a concentration-dependent manner. This result suggested that the conserved immunodominant antigens of the two parasites would be present on the surface. In this study, mAbs 9E8 in group 2 and 10F7 in group 3, which respectively reacted with PDI and RP1 proteins located inside the parasites, also had significant inhibitory effects on the growth of the parasites. It is important to examine how an antibody can react with an antigen located inside a live parasite. The mechanism is still unknown. However, it is possible that *T. gondii* PDI exits the endoplasmic reticulum bound to its targets, such as SGA1, and relates to surface proteins functioning as a chaperone for these membrane proteins (Hehl et al., 2000), since the binding of PDI-specific IgA antibodies to the exterior of parasites clearly demonstrated the presence of PDI on the surface (Meek et al., 2000). PDI or RP1 may also participate in adhesion to host cells during the initial phase of infection.

In this study, we could not identify the genes encoding any surface proteins or apical protein recognized with mAbs in groups 1, 5, and 6, from the cDNA expression library in *E. coli*. The mAbs in group 1 recognized several proteins with molecular weights ranging from 28 to 76 kDa. These proteins might be a glycoprotein containing a different size of sugar chain. On the other hand, the mAbs in groups 5 and 6 did not recognize any proteins by Western blot analysis, although they showed clear fluorescent staining on the parasites by IFAT. The antigens recognized with mAbs in groups 5 and 6 were expected to share conformational epitopes. Because neither glycoprotein nor conformational protein can be expressed in *E. coli*, further

immunoscreening using a cDNA expression library in eucaryotic cells needs to be carried out.

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FIGURE LEGENDS

Fig. 1. Localization of the cross-reactive antigens on *N. ccaninum* and *T. gondii* tachyzoites recognized with mAbs by IFAT with both purified tachyzoites (left) and parasites grown in Vero cells (right). Tachyzoites reacted with 9D12 in group 1 (A), 9E8 in group 2 (B), 10F7 in group 3 (C), 10A10 in group 4 (D), 10G6 in group 5 (E), 10F10 in group 6 (F), respectively.

Fig. 2. Western blot analysis of the cross-reactive antigens of *N. caninum* (A) and *T. gondii* (B) recognized with mAbs. Purified tachyzoites lysates were run on SDS-PAGE gel, transferred into nitrocellulose membranes and probed with 9D12 in group 1 (lane 1), 9E8 in group 2 (lane 2), 10F7 in group 3 (lane 3), 11A10 in group 4 (lane 4), 10G6 in group 5 (lane 5), and 10F10 in group 6 (lane 6), respectively.