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Tracking *Neospora caninum* parasites using chimera monoclonal antibodies against its surface antigen-related sequences (rNcSRS2)

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Neosporosis, an infectious disease of cattle and dogs, causes an abortion in cattle, which has a major damage on the dairy industry worldwide. Tracking of *Neospora caninum* parasite that is responsible for neosporosis is required for the prevention of this infectious disease. We developed three chimera monoclonal antibodies consist of variable regions of murine antibody and constant regions of human antibody against *N. caninum*. Recombinant surface antigen-related sequence 2 (rNcSRS2) of *N. caninum* was expressed in silkworm larvae, and immunized in mice to obtain phage displaying antibody library. Through three rounds of selection, three antibodies, A6, E1 and H3, were isolated and bound to rNcSRS2 with nanomolar to micromolar affinity. In immunofluorescent staining assays, A6 and E1 bound to *N. caninum* strain Nc-Liv, demonstrating a successful tracking of the parasite. H3 clone bound to rNcSRS2 but not to a truncated protein without glycosylphosphatidylinositol (GPI) anchor domain in the carboxyl terminal. Amino acid sequences of A6 and E1 were similar, but that of H3 differed in the CDR-H1 region, which might be the reason of their difference of affinity. These antibodies are thought to be useful for prevention of cattle from neosporosis.

[**Key words:** Neosporosis; Parasite; Antibody; Phage display; Immunofluorescent staining]

Neosporosis is a coccidian disease caused by *Neospora caninum* in a variety of animals such as cattle, horses, deer, dogs, and so on (1), induces an abortion in cattle, which is the reason for the huge economic damage on the dairy industry in many countries (2,3). The major mode of transfection of *N. caninum* is transplacental parasite transmission that infects in the herd over successive generations (4,5), but horizontal transmission between cattle was not observed. There are three infectious stages in the life cycle of *N. caninum*: tachyzoite, tissue cysts, and oocysts. Tachyzoites and tissue cysts are the stages found in the intermediate hosts and they occur intracellularly (6); the unsporulated oocysts are found in domestic dogs, which are the only known definitive host for *N. caninum* (7).

Like other coccidian parasites, tachyzoites of *N. caninum* also contain the characteristic organelles such as dense granules, rhoptries, and micronemes. Those organelles produce important proteins that play important roles in infection of host cells (8). On the other hand, proteins located on the surfaces are considered to play very important roles in the infection process. They may induce the interaction with the host cell and subsequently help the parasites adhere to and invade the host cell. Surface antigen 1(NcSAG1) of *N. caninum* is the immunodominant surface antigen of tachyzoites, which is involved in the attachment of the parasite to host cells (9,10). There are also many surface proteins structurally related to NcSAG1, which are designated NcSAG1-related sequences (NcSRSes). NcSRS2, with a molecular weight of 37 kDa, elicit strong antibody response in infected animals and is an attractive candidate for diagnosis and vaccine antigen (10). In vitro studies have shown that blocking these proteins can limit the parasite's ability to attach to and invade host cells (11–13). In addition, in vivo studies also showed that the recombinant NcSRS2 (rNcSRS2) has protective effects against encephalitis and transplacental transmission (14).

So far, we have successfully expressed rNcSRS2 in silkworms (15). Because NcSRS2 was reported to have high antigenicity and plays critical roles in *N. caninum* transmission, we immunized mice in this study with purified proteins and developed three chimera monoclonal antibodies consist of variable regions of murine antibody and constant regions of human antibody against the parasite by employing phage display technology. The binding capacity of antibodies to parasites and the potential in practical applications were subsequently investigated.

MATERIALS AND METHODS

Materials The *Escherichia coli* strains XL10-Gold for cloning and amplification of phagemid, and TG-1 for displaying antibody on M13 phage were purchased from Agilent Technologies (La Jolla, CA, USA). Phagemid pDong1/Fab (16), helper phage KM13 and non-suppressor *E. coli* strain HB2151 were provided kindly by Dr. Hiroshi Ueda of Chemical Resources Laboratory of Tokyo Institute of Technology.

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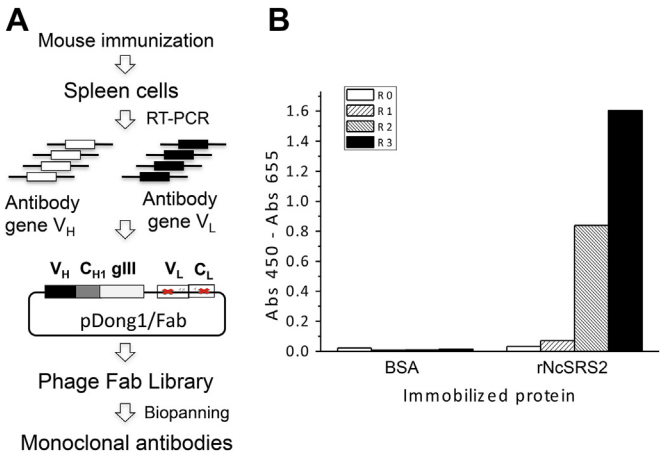

FIG. 1. Scheme for the development of murine anti-*N. caninum* monoclonal antibodies (A) and the enrichment of rNcSRS2-specific clones (B). ELISA was performed to confirm the enrichment of rNcSRS2-specific clones. In ELISA, rNcSRS2 (0.5 μg/ml) and BSA (10 μg/ml) were immobilized on a 96-well microplate, respectively. HRP/anti-M13 monoclonal antibody conjugate was used as the secondary antibody. R0 stands for the original phage library, whereas R1, R2, and R3 stand for the amplified Fab-phage in Rounds 1, 2, and 3 of biopanning, respectively.

The *N. caninum* Nc-Liv strain (no. 50845) and Vero cell (no. CCL-81) were purchased from ATCC (Rockville, MD, USA). Restriction and modification enzymes were purchased from Takara-Bio (Shiga, Japan), Toyobo (Osaka, Japan), Roche Diagnostics (Tokyo, Japan), or New England Biolabs (Tokyo, Japan). Oligonucleotides were synthesized either by Operon (Tokyo, Japan) or Invitrogen (Tokyo, Japan). Other chemicals, reagents, and antibodies, unless otherwise indicated, were obtained from Sigma–Aldrich (St. Louis, MO, USA) or Wako Pure Chemical Industries (Osaka, Japan).

Immunization of mice with rNcSRS2 The strategy for developing monoclonal antibodies is shown in Fig. 1A. Firstly, mice were immunized with rNcSRS2. After the quantitation of peptide-specific antibodies in sera, the variable region genes of the antibody heavy (V_H) and light (V_L) chains were prepared and cloned to a phagemid vector to perform phage display selection. The rNcSRS2 was expressed in silkworm according to previous report (15) and purified. Two inbred BALB/c mice (Japan SLC, Inc., Hamamatsu, Shizuoka, Japan) were immunized with purified rNcSRS2 four times at 2-week intervals with a dose of 100 μg through the subcutaneous route. The protein solutions were emulsified with a Freund's complete adjuvant (Rockland Immunochem, Gilbertsville, PA, USA) to increase the efficiency of immunization. After the last immunization, blood samples were taken by tale bleeding and the rNcSRS2-specific antibodies in sera were confirmed by an enzyme-linked immunosorbent assay (ELISA) with immobilized rNcSRS2 on a microplate as described by Dong et al. (17). The experiments with animals were carried out in the Animal House of Shizuoka University in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Shizuoka University and were approved by the Committee on the Ethics of Animal Experiments of Shizuoka University (permit number: 24-11).

Construction of phage display antibody library The total RNA was extracted from spleen cells of immunized mice with TRIzol (Invitrogen, Tokyo, Japan). The genes of V_H and V_L of antibodies were amplified using PrimeScript One step RT-PCR kit ver. 2.0 (Takara) according to the manufacturer's protocol. The mouse IgG-specific primers are synthesized based on the common antibody primer sequences (17). The PCR products were then purified using Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare). The purified V_L fragments were digested with restriction enzymes *Sal*I and *Not*I and were purified and ligated into a phagemid pDong1/Fab digested with the same enzymes using T4 DNA ligase at 16°C for 1 h. After confirmation of the inserted V_L sequence of several clones out of the obtained ones, the V_H genes were inserted into the V_L-inserted phagemid library using restriction enzymes *Sfi*I and *Xho*I. Electroporation-competent *E. coli* TG-1 cells were transformed with the ligation product and plated on 2× YTAG agar (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, pH 7.2, supplemented with 100 μg/ml ampicillin, 1% glucose, and 1.5% agar) plates overnight at 37°C. The size of library was estimated from the number of colonies on the plate. *E. coli* TG-1 cells, transformed with the phagemid, were cultivated in 4 ml of 2× YTAG overnight at 37°C. Ten milliliters of 2× YTAG were inoculated with 100 μl of the overnight culture at 37°C at 200 rpm until OD_{600} reached ~0.5, when helper phage KM13 (18) was added with a multiplicity of infection (MOI) of 20. After incubation at 37°C for 30 min without shaking, the culture was centrifuged at 3700×*g* for 15 min. The *E. coli* pellet was resuspended in 50 ml of 2× YTAK (2× YT medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin) and incubated overnight with shaking at 30°C. The overnight culture was centrifuged at 10,800×*g* for 30 min. Ten milliliters of PEG/NaCl solution (20% polyethylene glycol 6000, 2.5 M NaCl) was added to 40 ml of supernatant, and the mixture was incubated on ice for 1 h. After incubation, the mixture was centrifuged at 6000×*g* for 30 min. The pellet was resuspended in 2 ml of PBS and centrifuged at 15,000×*g* for 10 min to pellet cell debris, and the supernatant was collected as a Fab-displaying phage solution.

Enrichment and selection of monoclonal antibodies For antibody selection, 100 μl of rNcSRS2 (1 μg/ml in PBS) was immobilized on a microplate at 4°C overnight. It was washed three times with 200 μl of PBST, and then blocked with MPBS (PBS containing 2% skim milk) for 2 h, followed by adding 10^{12} colony forming unit (cfu) phage in 100 μl PBS and incubated for 1 h at room temperature. Phages bound to the microplate were eluted with 100 μl of 1.0 mg/ml TPCK-treated trypsin (Sigma–Aldrich) in PBS after washing with PBST for six times. *E. coli* TG-1 cells (OD_{600} = 0.5 in 700 μl culture) were infected with 100 μl of eluted phage solution and cultured in 10 ml of 2× YTAG medium at 37°C with shaking at 200 rpm. When OD_{600} reached 0.5, the KM13 helper phage was added at an MOI of 20, and incubated for 30 min at 37°C without shaking. After being centrifuged at 3700×*g* for 10 min, the pellet was resuspended in 50 ml of 2× YTAK medium and incubated with vigorous shaking at 30°C overnight. The culture supernatant was prepared by centrifugation at 10,800×*g* for 30 min, and phages were precipitated with 0.2 volume of PEG/NaCl on ice for 1 h. After centrifugation at 6000×*g* for 30 min, the pellet was resuspended in PBS and used as a source of Round 1 (R1) phage. Round 2 (R2) antibody selection from R1 phage was performed as described above and R2 phage was obtained. From the R2 phage library, Round 3 (R3) selection was also carried out to generate R3 phage. The enrichment of rNcSRS2-specific phage-antibody among the original phage library (R0), R1, R2, and R3 phages was confirmed with a polyclonal phage ELISA.

After the increase of binding capability of phage was confirmed, 96 infected *E. coli* clones at the 3rd biopanning were picked up and cultivated for making monoclonal phage. A phage ELISA was performed for 96 individual clones to select rNcSRS2-specific phage-antibodies. Nucleotide sequence of positive clones was read by Greiner Bio One (Tokyo, Japan) with primer M13rv (5′-GGAAACAGCTATGACCATG-3′) for V_H and primer VLseq (5′-CACTGGCTGGTTTCGCTAC-3′) for V_L, and was analyzed using a GENETYX software (Genetyx Corporation, Tokyo, Japan).

Expression of Fab fragments of positive clones pDong1/Fab was designed for the convenient expression of the Fab fragment after selection of positive clone by placing a TAG amber codon (19) between V_H–C_H1 and gene III of phage. With this design, Fab is expressed as a fusion protein with protein gIII of phage in suppressor strain like *E. coli* TG1, resulting in the display of Fab on the surface of phage. However, with a non-suppressor strain, such as *E. coli* HB2151, the Fab fragment will be expressed as a soluble fragment (16). Because the genes for C_H1 and C_L gene in pDong1/Fab system were from human IgG (C_H1 and $C_κ$), Fabs expressed in this study were murine-human chimera fragments. In brief, 200 μl of exponentially growing *E. coli* HB2151 was infected with 10^9 cfu of phage for 30 min at 37°C. Infected *E. coli* cells were pelleted by centrifuge at 5000×*g* for 10 min, resuspended in 4 ml 2× YT medium containing 100 μg/ml of ampicillin (2× YTA), and cultivated for 3 h at 37°C. Four hundred milliliters of 2× YTA medium was inoculated with the 4-ml culture and cultivated at 37°C with shaking. Once the OD_{600} reached 0.5, isopropyl β-D-thiogalactoside (IPTG), with a final concentration of 1 mM, was added and cultivated further overnight at 30°C. The *E. coli* cells were harvested by centrifugation at 4000×*g* for 20 min at 4°C. The periplasmic fraction was extracted according to a general protocol. His-tagged Fabs were purified from the periplasmic fraction and concentrated supernatant with TALON Co^{2+}-immobilized resin (Takara-Bio) according to the instructions provided by the manufacturer. Because one-step purification was not enough to achieve purity, Fabs were furthermore purified with an anti-FLAG M2 affinity gel (Sigma–Aldrich) according to the instructions provided by the manufacturer. The purified Fabs were analyzed using SDS-polyacrylamide gel electrophoresis as described by Laemmli (20).

ELISA analysis The antigen-binding capacity of phage-displayed Fab fragments was tested with ELISA. The microplates (NUNC, Langenselbold, Germany) were coated overnight with 100 μl of rNcSRS2 (0.5 μg/ml) per well or 10 μg/ml of BSA in PBS at 4°C. The plate was blocked at 25°C for 2 h with 2% MPBS, washed three times with PBST, and incubated with 100 μl/well of MPBS containing 10^9–10^{10} cfu of Fab-displaying phage at 25°C for 1 h. The plate was washed three times with PBST and incubated with 100 μl/well of 5000-fold diluted HRP/anti-M13 monoclonal conjugate (from Sheep; GE Healthcare UK limited, Little Chalfont, UK) in MPBS at 25°C for 1 h. The plate was then washed three times with PBST and developed with 100 μl/well of 3,3′,5,5′-tetramethylbenzidine (TMBZ) substrate solution. After incubation for an appropriate time, the reaction was stopped by adding 50 μl/well of 10% sulfuric acid, and the absorbance was read using a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm with 655 nm as a control.

For evaluation of IC_{50} of phage Fabs, competitive ELISAs were performed in which free rNcSRS2 in a series of concentration levels competed with immobilized rNcSRS2 to bind Fab-phages.