

Characterization of a New Gene *wx2* in *Toxoplasma gondii*

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Abstract Using hybridization techniques, we prepared the monoclonal antibody (Mab) 7C3-C3 against *Toxoplasma gondii*. The protection tests showed that the protein (Mab7C3-C3) inhibited the invasion and proliferation of *T. gondii* RH strain in HeLa cells. The passive transfer test indicated that the antibody significantly prolonged the survival time of the challenged mice. It was also shown that the antibody could be used for the detection of the circulating antigen of *T. gondii*. After immunoscreening the *T. gondii* tachyzoite cDNA library with Mab7C3-C3, a new gene *wx2* of *T. gondii* was obtained. Immunofluorescence analysis showed that the WX2 protein was located on the membrane of the parasite. Nucleotide sequence comparison showed 28% identity to the calcium channel α -1E unit and shared with the surface antigen related sequence in some conservative residues. However, no match was found in protein databases. Therefore, it was an unknown gene in *T. gondii* encoding a functional protein on the membrane of *T. gondii*. Because it has been shown to have a partial protective effect against *T. gondii* infection and is released as a circulating antigen, it could be a candidate molecule for vaccine or a novel target for new drugs.

Keywords *Toxoplasma gondii*; *wx2* gene; monoclonal antibody; protection; immunofluorescence analysis

Toxoplasma gondii is an opportunistic pathogen that infects nearly all mammals and avian species [1]. It can cause congenital infection with a high incidence of complications in pregnancies [2,3]. There is a high infection rate in human, approximately 22% of the population of the USA is infected, but severe disease in adults exists mainly in immunosuppressed patients, such as those with AIDS. *Toxoplasma* encephalitis is the most severe manifestation and the main cause of death [4–6]. Recent reports linking *Toxoplasma* to first-episode schizophrenia and cryptogenic epilepsy are drawing more attention to the study of this parasite's pathology, fueling speculation that long-term effects of infection are currently underestimated [7,8].

Nevertheless, there are difficulties in prevention, diagnosis and treatment of the disease. Drug therapy is not satisfactory because of the toxicity of drugs, need for long-term treatment and rapid re-infection by the parasite; moreover, there is no drug that can kill the encysted bradyzoites [9,10]. The development of vaccines and new therapeutic drugs has therefore become a priority strategy to control toxoplasmosis.

Here we report a new gene, *wx2*, whose product reacts with the monoclonal antibody (Mab) 7C3-C3 against *T. gondii* prepared by our laboratory. Our research showed that Mab7C3-C3 had a partial protective effect against *T. gondii* by inhibiting the invasion and proliferation of the parasite. According to the results of immunofluorescence analysis, the WX2 protein was located on the membrane of the parasite, and this is consistent with the prediction of bioinformatics analysis. The sequence homology analysis showed an identity of 28% to the calcium channel α -1E unit and some conservative residues shared common sequence with the surface antigen related sequence. However, we did not find any hit on protein databases.

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Therefore, we propose that the protein might have an unknown function on the membrane of the parasite which can affect the invasion and proliferation of the parasite in host cells.

Materials and Methods

Cell culture and parasites

The *T. gondii* RH strain was purchased from the Medical School of Shanghai Jiaotong University (Shanghai, China) and kept in our laboratory. The parasite was propagated in Kunming mouse and human foreskin fibroblast cell grown in Dulbecco's modified Eagle's medium (DMEM) with *L*-glutamine, supplemented with 10% dialyzed fetal bovine serum.

Antibody and hybridization technique

Kunming mice were infected with *T. gondii* I (TAI) by intraperitoneal injection. Three days later, peritoneal fluid was collected and the parasites were purified by filtering CF-11 cellulose, centrifuged at 500 *g* for 10 min and stored at -20°C for immunizing animals.

Kunming mice were infected with *T. gondii* II (TAII) by intraperitoneal injection. Three days later, peritoneal fluid was collected and the parasites were purified by filtering CF-11 cellulose, and centrifuged 500 *g* for 10 min. After freezing and thawing three times, it was done by ultrasonication, centrifuged at 10,000 *g* for 30 min, and stored at -20°C .

A female BALB/c mouse (12 weeks old) was immunized once with 500 μg of TAI by intravenous injection and its spleen cells were fused with SP2/0 cells 4 d later. The fusion of myeloma cells and isolated spleen cells was carried out by a routine procedure [11]. To obtain pure positive hybridoma clones, the hybridoma cells were cloned and subcloned three times by limiting dilution in 96-well cell culture plates and cultured in selective medium. Mabs were produced from both monolayer cell culture and mouse ascites.

For the production of ascites in BALB/c mouse and purification of Mabs, the positive cells were expanded and propagated in petrolatum liquidum primed mouse. Five hundred microliters of petrolatum liquidum was injected intraperitoneally into each mouse. After two weeks, 5.0×10^6 hybridoma cells were injected intraperitoneally into each primed mouse. The ascites fluid was harvested at the time of maximum ascites production (10–15 d after injection). The cells were removed by centrifugation at 3000 *g* for

20 min.

The Mabs from ascites fluid were purified by ammonium sulfate precipitation followed by anion exchange chromatography. The ammonium sulfate was slowly added to 45% saturation concentration to ascites fluid with gentle stirring on ice bath. After stirring the mixture for at least 6 h at 4°C , the pellet was collected by centrifugation at 10,000 *g* for 15 min, resuspended in 10 mM sodium phosphate (pH 7.5) and dialyzed against the same buffer to remove the ammonium sulfate. The solution was applied to a DEAE-52 column equilibrated with 10 mM sodium phosphate (pH 7.5). The antibodies could be eluted by 50 mM sodium phosphate buffer (pH 7.5) containing 40 mM NaCl. The culture supernatant was condensed by polyethylene glycol. The purity and the specificity of antibodies were further evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Subset of the antibody was detected by enzyme linked immunosorbent assay (ELISA).

Goat anti-rabbit immunoglobulin (Ig) G (fluorescein-isothiocyanate-coupled) was obtained from Boster (Wuhan, China). Goat anti-mouse IgG (IgM) (cy3-coupled) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse were Zymed products (San Diego, USA). Mab P24 (a known plasmosin of *T. gondii*) and polyclonal antibody P30 (rabbit origin) against *T. gondii* were both gifts from Dr. Shuhong LUO (University of Illinois, Urbana-Champaign, USA).

SDS-electrophoresis and Western blot analysis

The electrophoresis system was essentially the same as that described by Laemmli [12]. TAI (14 μg) was mixed with 10 μl of 125 mM Tris-HCl (pH 7.0), 10% (*W/V*) β -mercaptoethanol, 20% (*V/V*) glycerol, 6.0% (*W/V*) SDS and 0.4% (*W/V*) bromophenol blue as tracking dye, and boiled for 5 min prior to application to 10% SDS-polyacrylamide gels. Electrophoresed proteins were transferred to a nitrocellulose membrane by the method of Towbin *et al.* [13] with a Bio-Rad transblot apparatus (Boston, USA). The membrane was blocked using 5% non-fat milk powder in TPBS (0.1% Tween-20, 80 mM Na_2HPO_4 , 20 mM NaH_2PO_4 , 100 mM NaCl, pH 7.5) overnight at 4°C . A 1:10 dilution of Mab7C3-C3 in TPBS was applied at 37°C for 2 h. The nitrocellulose membrane was washed three times for 15 min each with TPBS, incubated with HRP-rabbit anti-mouse IgG (M+A) antibody (1:5000) and detected by an enhanced chemiluminescence (ECL) detection kit (Boster) and visualized on an X-ray film. Controls were incubated with normal serum of mice (1:100) and Mab P24 against *T. gondii* (1:100).

Protection test

Two thousand *T. gondii* RH strain tachyzoites were incubated with diluted (1:10) Mab7C3-C3 at 37 °C for 4 h, then they were added to HeLa cells and incubated at 37 °C for 12 h. Samples were fixed and stained with Giemsa dye. The number of intracellular tachyzoites and the cell infection rate in 50 HeLa cells parasitophorous vacuoles total were calculated at 100× magnification [14].

Two groups of Kunming mice, A1 and A2, were injected intraperitoneally with diluted Mab7C3-C3 (1:2) at a dosage of 0.5 ml (500 µg) per mouse. Group A1 was challenged with 2000 *T. gondii* tachyzoites on the same day of Mab injection. Group A2 was challenged with the same number of *T. gondii* tachyzoites 72 h later. Mice in the control groups B1 and B2 were injected with cell culture medium. The negative control groups C1 and C2 were injected with immune serum against *Schistosoma japonicum* (1:10). The survival time was recorded and death rate was calculated.

cDNA library and immunoscreening

The *T. gondii* tachyzoite cDNA library was provided by Dr. Shuhong LUO (the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: *T. gondii* cDNA Library. Catalog Number: 1896, Lot Number: 399001) [15].

Immunoscreening was carried out using a picoBlue immunoscreening kit (Stratagene, La Jolla, USA) according to the manufacturer's instructions. Approximately 2×10^5 p.f.u. per plate of the amplified library was used for plaque lifting. The library was grown on NZY agar for 3–5 h at 37 °C. Then IPTG induction was carried out at 37 °C for 3–5 h by placing nitrocellulose membranes soaked with 10 mM IPTG onto the surface of the agar. For secondary screening, 600 p.f.u. of the cDNA library was screened. After blocking the non-specific binding site with 1% bovine serum albumin (BSA) for 1 h, the membranes were allowed to react with diluted (1:20) Mab7C3-C3 (which was absorbed by the schizolysis solution of *Escherichia coli* in advance) for 3 h and washed with 0.05% TBST. The membranes were further incubated with anti-mouse HRP-IgM at a dilution of 1:2000. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate substrate were used for the visualization of the positive signal. The positive plaques were subjected to further secondary screening until the isolated clones were obtained. The cDNA inserts of the positive clone were then excised from the lambda phages to obtain the plasmid DNA. After two rounds of screening, the clones that remained positive were chosen

to be excised *in vitro*, followed by plasmid DNA extraction, polymerase chain reaction (PCR) and sequencing. Universal primers T3 and T7 were designed. PCR was carried out in a total reaction volume of 100 µl with the following conditions: 94 °C for 4 min, 35 cycles of 94 °C for 1 min 20 s, 51 °C for 1 min 20 s, 72 °C for 2 min, and 72 °C for 8 min.

Construction of recombinant vector of pcDNA and expression in mammalian cells

Plasmid pcDNA3 was a gift from Dr. Shuhong LUO and kept in our laboratory. Primers were synthesized by Shanghai Sangon Biological Engineering Technology Service Co. (Shanghai, China). *EcoRI* and *XhoI* are products of NBI (Plymouth, USA).

For construction of *wx*-pcDNA3, the open reading frame of the *wx2* gene obtained by PCR was used as a template, and a pair of primers [GCGAATTCCTTGCG-GAGACACATG (forward; *EcoRI* shown as underlined) and CCGCTCGAGATTCGTTTAGAGAGG (reverse, *XhoI* shown as underlined)] were designed. PCR was carried out in a total reaction volume of 100 µl for 94 °C for 4 min, 35 cycles of 94 °C for 1 min 20 s, 53 °C for 1 min 20 s, 72 °C for 2 min, and 72 °C for 8 min. PCR product was digested with *EcoRI* and *XhoI*, and ligated into *EcoRI*/*XhoI* digested pcDNA3. The recombined plasmid was characterized by double digestion and sequencing (Sangon).

For transient expression in mammalian cells, COS-7 cells were maintained under the conditions recommended by American Tissue Culture Collection (Manassas, USA). One day before transfection, 2×10^6 cells were subcultured onto 100 mm×20 mm plates resulting in approximately 90% confluence. Cells were transfected with 10 µg of DNA premixed with Lipofectamine 2000 (Invitrogen, Shanghai, China). It was carried out using a Lipofectamine 2000 transfection kit according to the manufacturer's instructions (Invitrogen). At 48 h after transfection, cells were harvested, schizolysed and stored at –20 °C. The expression products were evaluated by SDS-PAGE and Western blot analysis. Methods were the same as described above.

Immunofluorescence analysis

Parasites fixed with 4% formaldehyde were allowed to adhere to polylysine-coated coverslips, permeabilized with 0.3% Triton X-100 for 20 min, blocked with 2.5% BSA in phosphate-buffered saline (PBS), and then incubated with the antibody (Mab7C3-C3) and polyclonal antibody P30 (1:100) at 37 °C for 2 h. They were washed three times with PBS followed by incubation in fluorescent isothiocyanate-coupled goat anti-rabbit IgG and cy3-coupled goat

anti-mouse IgM (IgG) as secondary antibodies together. Positive controls were incubated with Mab P24 (1:100) and polyclonal antibody P30 (1:100) against *T. gondii* [16]. Immunofluorescence images were obtained with an Olympus fluorescence microscope (Tokyo, Japan). The confocal images were collected with a Zeiss LSM 510 confocal microscope (Carl Zeiss SMT, Thornwood, USA) and analyzed by LSM 510 software. In these experiments, the aim was to co-localize the P30 (P30 is a known membrane protein of *T. gondii*) and the protein that Mab7C3-C3 reacted with. The negative control was cell culture medium.

Sandwich-ELISA used to detect *T. gondii* circulating antigen (cAg) in sera of rabbits

Four New Zealand rabbits were immunized with 500 µg of TAI emulsified in an equal volume of Freund's complete adjuvant for primary immunization through hypodermic injection. The same volume of solution was injected hypodermically at intervals of 2 weeks. After three or four immunizations, the sera were collected and stored at -20 °C.

Ten New Zealand rabbits were infected with *T. gondii* RH strain, 2000 tachyzoites each. After 4 d, the rabbits received a daily treatment of sulfadiazine (300 mg/kg body weight) and pyrimethamine (15 mg/kg body weight), lasting for 30 d. The sera were collected from the third day of infection, mixed, and stored at -20 °C.

Rabbit anti-sera (1:100) against *T. gondii* were prepared in carbonate buffer (pH 9.6) and 100 µl of this solution was used per well to coat the ELISA plate (Nunc, Naperville, USA). After incubation at 4 °C overnight, wells were rinsed with PBST (pH 7.4) and blocked with 3% BSA in PBS at 4 °C overnight. After rinsing with PBST, 100 µl of *T. gondii*-infected rabbit serum prepared with 3% BSA in PBS was added and incubated for 2 h at 37 °C. After the plate was rinsed with PBST, 100 µl of purified Mab7C3-C3 was added and incubated for 2 h at 37 °C. After washing with PBST, HRP-anti-mouse IgM antibody (1:3000) was added and incubated for 2 h at 37 °C. After washing with PBST, the enzymatic reaction was developed using 4-nitrophenyl phosphate as the substrate. The absorbance values were recorded at 405 nm using an ELISA plate reader (Biotech, Texas, USA). The negative control was the normal rabbit serum and the blank control was PBST [17].

Statistical analysis

SPSS statistical analysis software (SPSS, Chicago, USA) and Student's *t*-test were used for statistical analysis. All

experimental data are presented as mean±SD. *P* values less than 0.05 were considered as significant and are marked with an asterisk (*).

Results

Western blot analysis of Mab7C3-C3

In our research, Mabs against *T. gondii* RH strain were produced by the hybridoma technique. Seven strains of hybridoma secreting Mab against *T. gondii* were established including 7C3-C3. The subclass of Mab7C3-C3 was IgM. Western blot analysis showed the protein molecular mass of Mab7C3-C3 and the specific binding ability to *T. gondii* RH strain antigen (Fig. 1).

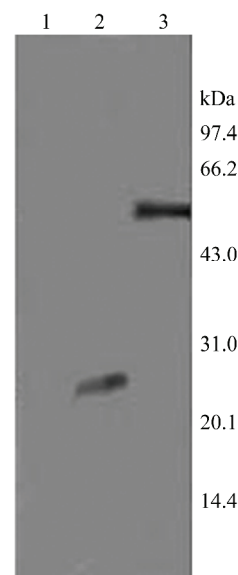


Fig. 1 Molecular weight of the monoclonal antibody (Mab) 7C3-C3 against *Toxoplasma gondii* RH strain using Western blot analysis

1, negative control (reacted with serum of normal mice), for which no band appeared; 2, the positive control of Mab P24 against *T. gondii* has a band at 24 kDa; 3, the band for Mab7C3-C3 appears at 49 kDa.

Protective effect of Mab7C3-C3

The protective test *in vitro* and passive transfer tests were used to observe the protective effect of the protein. The infection rate and the mean parasite number per parasitophorous vacuole were calculated in a total of 50 fields. The survival times were recorded in the Kunming mice passively immunized with the protein previously. It showed that the protein could inhibit the invasion and

Characterization of recombinant plasmid wx2-pcDNA3 and recombined protein

The characterization of the recombinant plasmid wx2-pcDNA3 and the recombined protein expressed in mammalian cells (Figs. 3–5). According the results of

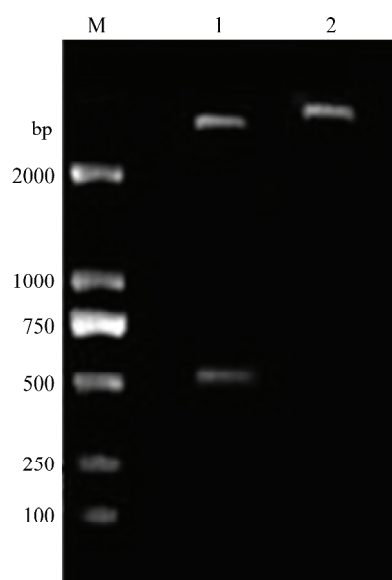


Fig. 3 Confirmation of the construction of the recombinant vector of wx2-pcDNA3

M, DL 2000 DNA marker; 1, recombinant plasmid wx2-pcDNA3 double digested by *Eco*RI and *Xho*I. Two bands appeared at 561 bp and 5 kb; 2, recombinant plasmid wx2-pcDNA3, not double digested by *Eco*RI and *Xho*I. Only one band appeared at approximately 5.4 kb.

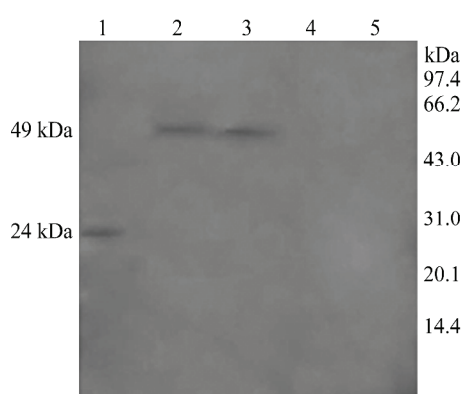


Fig. 4 Western blot analysis of protein WX2 expressed in COS-7 cells

1, monoclonal antibody (Mab) P24 against *Toxoplasma gondii* reacted with antigen of *T. gondii* (positive control); 2, Mab7C3-C3 reacted with the antigen of *T. gondii*; 3, cell schizolysis solution of expressed products of gene wx2 in COS-7 cells; 4, the negative control (supernatant of COS-7 cell culture); 5, cell culture medium. The experiment group had bands at 49 kDa. The control had no band appeared. The positive control had a band at 24 kDa.

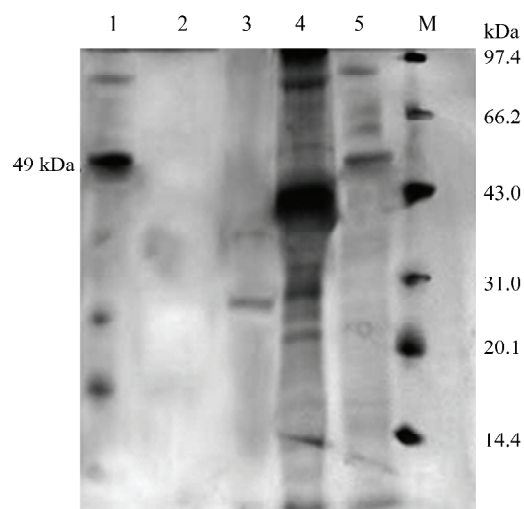


Fig. 5 SDS-PAGE analysis of the supernatant of cell culture of Mab7C3-C3 and the COS-7 cell culture

1, supernatant of cell culture of monoclonal antibody (Mab)7C3-C3; 2, cell culture medium; 3, Mab P24 against *Toxoplasma gondii*; 4, soluble antigen of *T. gondii*; 5, supernatant of the COS-7 cell culture in which protein WX2 was expressed (48 h).

double digested, and the sequencing result of recombinant plasmid wx2-pcDNA3 showed that the construction was successful. The western blot result showed that the recombinant protein can react with Mab7C3-C3.

Immunofluorescence microscopy

Confocal immunofluorescence images showed that the protein that reacted with Mab7C3-C3 was on the membrane of the parasite. The positive controls are P30 and P24 which both are known proteins of *T. gondii* (Fig. 6).

Analysis of *T. gondii* cAg

Sandwich-ELISA was used to detect the *T. gondii* cAg in the sera of rabbits infected with *T. gondii*. The results showed that the cAg of *T. gondii* in infected rabbits' sera was detected on days 3–27. The cAg reached its peak on day 11 and dropped quickly afterwards, but it was still detectable on day 27 (Fig. 7). It also showed that the protein WX2 was released as a cAg in sera.

Discussion

Bioinformatics is one of the main technological strategies used to investigate the function of new genes and the products of gene expression [18–20]. Some bioinformatics

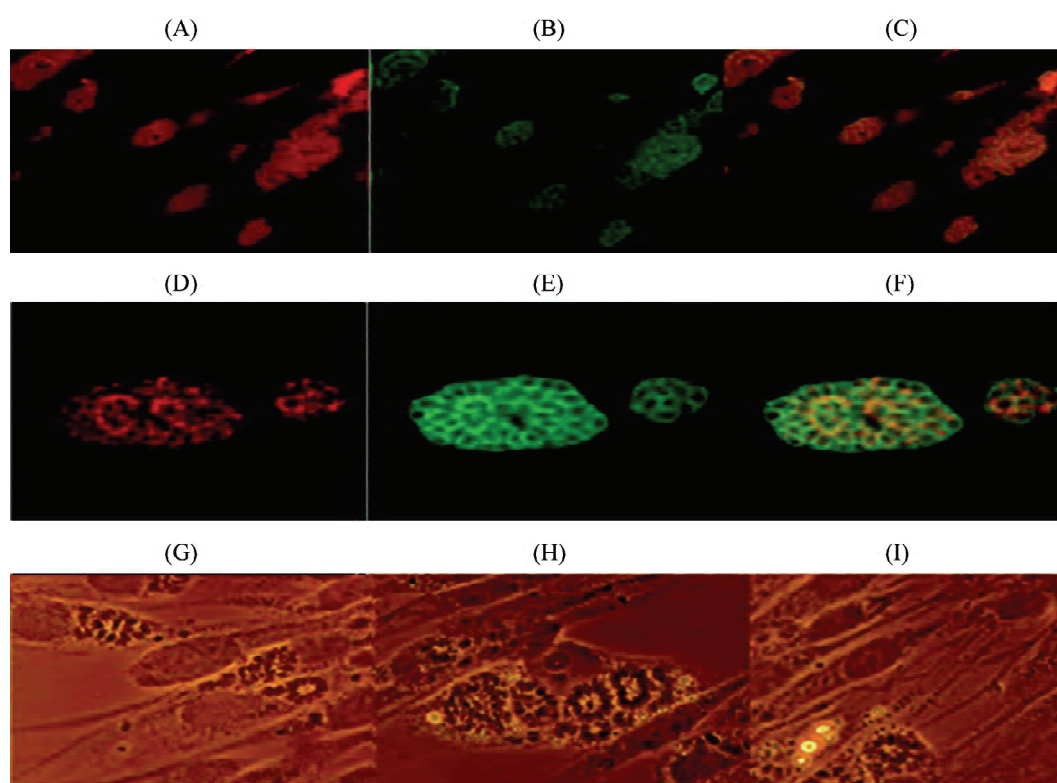


Fig. 6 Confocal immunofluorescence images of the protein that reacted with Mab7C3-C3

(A–C) Monoclonal antibody (Mab)7C3-C3 reacted with the protein on the membrane of the parasite *Toxoplasma gondii* and the location of P30 (a membrane protein of *T. gondii*). Red fluorescence is the protein location of Mab7C3-C3 reacting on the parasites (A), green fluorescence is the location of P30 on the parasites (B), and (C) is the co-location of the protein where Mab7C3-C3 reacted and P30 on the parasites. (D–F) Mab P24 (a known plasmosin of *T. gondii*) and P30 located on *T. gondii* intracellular tachyzoites. Red fluorescence is the location of P24 on the cytoplasm of the parasites (D), green fluorescence is the location of P30 on the parasites (E), and (F) is the co-location of P24 and P30 on the parasites. (G–I) The negative controls are cell culture medium without fluorescence. Magnification, 1000 \times .

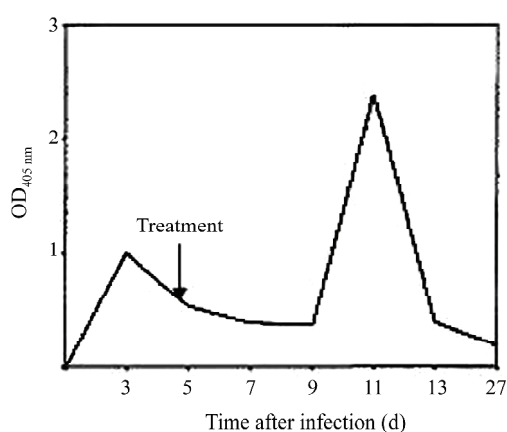


Fig. 7 Measurement of circulating antigen (cAg) of *Toxoplasma gondii* in infected rabbit sera

The cAg of *T. gondii* in infected rabbit sera was detectable from day 3 till day 27, and reached its peak at day 11 but dropped quickly afterwards. From day 5 on, the rabbits were treated with sulfadiazine 300mg/kg·d and pyrimethamine 15mg/kg·d and the treatment lasted 30 days.

approaches are very useful for complex, model genomes including human, rat, and mouse [21–23]. They are also useful for searching for the candidate molecules of vaccines and diagnostic antigens by screening cDNA libraries with Mabs or polyclonal antibodies [24,25]. Many studies have been done. For instance, the *ROP1* gene was obtained through immunoscreening of the *T. gondii* tachyzoite cDNA library using Mabs against *T. gondii* ROP1 [26]. Bohne *et al.* obtained the 30 kDa-specific bradyzoite antigen in cells through immunoscreening of the *T. gondii* cDNA library using Mabs against *T. gondii* bradyzoite [27].

In this study, through hybridization techniques, we prepared Mab7C3-C3. The protection test showed that the Mab inhibited the invasion and proliferation of *T. gondii* in HeLa cells. Passive transfer tests also showed that it significantly prolonged the survival time of the passively immunized mice. Although the protein could not prevent the death of the immunized animals, it might enhance the

immune function when combined with other membrane proteins that possess a protective function. It is worth studying further. Raizman and Neva detected circulating antigens in the sera of infected mice and rabbits [28]. Circulating antigens are considered to be markers to the infection of *T. gondii*. Villavedra *et al.* also verified the molecular mass of the cAg among 109–94, 67–47, 35–31, and 28–21 kDa in BALB/c mice infected with *T. gondii* RH strain [29]. Our report has also shown that Mab7C3-C3 can be used for the detection of the cAg in the acute stage of toxoplasmosis.

We obtained the gene sequence of *wx2* by immunoscreening the *T. gondii* tachyzoite cDNA library with the Mab7C3-C3 against *T. gondii*. By searching GenBank and the *Toxoplasma* database, it was defined as a new gene with a signal peptide of *T. gondii*. According to the alignment (<http://www.embi.net>), the gene *wx2* has a sequence identity of 28% with the voltage-dependent R-type calcium channel and some conservative residuals shared with the surface antigen related sequence. The WX2 protein has also been identified to be located on the membrane of the parasites through immunofluorescence analysis and been tested to be released as a cAg. This is consistent with the bioinformation prediction of the gene sequence. However, no distinguished hit has been found in protein databases that can verify which family the protein belongs to. In conclusion, we surmise that the gene is one of the unknown genes of *T. gondii*, of which more than 70% are unknown [30]. The corresponding protein, which can affect invasion and proliferation in host cells, might be a functional molecule on the membrane of *T. gondii* that could be considered as a candidate molecule for vaccine development or as the target for new drugs. The exact function and mechanism of the gene needs to be studied further.

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