Membrane localization and demonstration of isoforms of nucleoside triphosphate hydrolase from *Toxoplasma gondii*

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SUMMARY

Toxoplasma gondii has a unique enzyme, a NTPase, which has a wide specificity toward NTP. In the present study, we produced a monoclonal antibody (IgG_1, 6C6) against the enzyme which could recognize NTPase isozymes among several strains of *T. gondii*. Three avirulent strains of *T. gondii*, ME49, Beverley and Nakayama, were found to have 1 NTPase (63 kDa, pI 6·0), while a virulent strain RH and an avirulent strain Fukaya had 2 isozymes (63 kDa) with different pIs (pIs 6·0 and 6·5 for the former, and pIs 6·2 and 6·4 for the latter, respectively), suggesting that this monoclonal antibody recognizes a common epitope of NTPase among *T. gondii* strains. Furthermore, 6C6 could inhibit NTPase activity in the presence of dithiothreitol in a dose-dependent manner, and immuno-EM study of NTPase revealed that this molecule is located on the surface membrane of *T. gondii* tachyzoites. When Vero cells were co-cultured with tachyzoites pre-treated with 6C6, the number of infected cells significantly decreased, suggesting that 6C6 inhibits invasion of the parasites to host cells. These data suggest that the molecule recognized by 6C6 might be considered a potential candidate antigen for vaccines against *T. gondii* tachyzoites.

Key words: Toxoplasma gondii, monoclonal anti-NTPase, membrane localization, isoforms, inhibited invasion.

INTRODUCTION

Toxoplasma gondii is an ubiquitous pathogen that can cause serious disease in animals and humans, and this protozoan has an extremely wide range of intermediate hosts (Dubey, 1993). Although the infection is generally asymptomatic for healthy individuals, it may lead to serious inflammation such as chorioretinitis or encephalitis, and occasionally death through reactivation of encysted parasites in immunodeficient hosts like patients with acquired immune deficiency syndromes (AIDS)(McCabe & Remington, 1988).

So far several different isolates of *T. gondii* have been reported and they are classified as virulent or avirulent strains based on pathogenicity in mice (Kaufman *et al.*, 1959; Reikvam & Lorentzen-Styr, 1976; Ferguson & Hutchinson, 1981; Sibley & Boothroyd, 1992). Asai, O'Sullivan & Tatibana (1983) have reported the presence of a unique enzyme in *T. gondii* which has a wide specificity towards nucleoside triphosphates. The enzyme consists of 4 subunits with a molecular weight of 63 kDa and has been designated as a nucleoside triphosphate hydrolase (NTPase) (Asai *et al.* 1983). This NTPase exhibits an extremely high enzyme activity which hydrolyses 10 mM of ATP, GTP, ADP *in vitro* in the presence of dithiothreitol compounds but not AMP and PNP (Asai *et al.* 1983). Recently, the existence of isoforms of NTPase was also reported (Asai & Suzuki, 1990; Bermudes *et al.* 1994; Asai *et al.* 1995), and it was suggested that different isoforms of NTPase might be correlated with differences in pathogenicity of the parasite strains (Asai & Suzuki, 1990).

In this study, we produced a monoclonal antibody (6C6) against NTPase which could inhibit the enzyme activity *in vitro* in a dose-dependent manner. The antibody also recognized the isoforms of NTPase in a virulent strain, RH, as well as those in avirulent strains ME49, Beverley, Fukaya and Nakayama. Immuno-EM studies indicated that NTPase was localized on the cell membrane of *T. gondii* tachyzoites, and that pre-treatment of tachyzoites with 6C6 resulted in inhibition of their invasion to Vero cells, suggesting the involvement of the enzyme in infection. To our knowledge, this is the first report of a monoclonal antibody that inhibits the enzyme activity of NTPase *in vitro* and recognizes different isoforms of NTPase among *T. gondii* strains.

MATERIALS AND METHODS

Parasites and antigens

A virulent RH tachyzoite and 4 avirulent strains (ME49, Beverley, Fukaya and Nakayama) of *T. gondii* (generously provided by Drs Y. Suzuki and A. Makioka, Jikei University School of Medicine, Tokyo, Japan) have been maintained routinely by intraperitoneal passage in ddY mice in our laboratory

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according to the method previously described (Asai *et al*. 1987; Suzuki *et al*. 1993). Tachyzoites of *T. gondii* were also grown in *in vitro* cultures with a monolayer of Vero cells as described previously (Hughes, Hudson & Fleck, 1986). Cultured tachyzoites were purified by filtration through 3·0 μm polycarbonate membrane filters (Millipore Co., Bedford, MA), washed 3 times with Tris-buffered saline (TBS, pH 7·5). After the number of the tachyzoites was counted by using a haemocytometer, their pellets were stored at −80 °C until use. For immuno-EM study, 2×10^9 *T. gondii* tachyzoites, freshly collected from 75-cm^2 culture flasks, were used.

Affinity purification of NTPase

Affinity purification of NTPase from *T. gondii* RH tachyzoites was performed with a monoclonal antibody (mAb, IgA subclass, Asai *et al*. 1987) against NTPase of *T. gondii* and Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) according to the method previously described (Sibley *et al*. 1994). Briefly, after the crude *T. gondii* extract suspended in 20 mM Tris buffer (pH 8·0) containing 10 % glycerol was centrifuged, the supernatant was applied to Affi-Gel 10 coupled with anti-*T. gondii* NTPase mAb. After being shaken at 4 °C for 2 h, the gel was washed with Tris buffer, and the elution was performed with 3 M NaSCN solution. The eluted fraction was dialysed against Tris buffer at 4 °C, and stored at −20 °C in Tris buffer containing 50 % glycerol until use.

Production of antibodies against NTPase of T. gondii

Female BALB/c mice aged 6 weeks (Japan SLC. Inc. Hamamatsu, Japan) were injected subcutaneously (s.c.) with 50 μg of affinity-purified NTPase mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI), and boosted 5 times s.c. with 50 μg of NTPase mixed with Freund's incomplete adjuvant (Difco Laboratories) at 2-week intervals. Spleen cells obtained from immunized mice 3-days after the last immunization were fused with NS-1 myeloma cells by using polyethylene glycol and seeded into 96-well multiplates (Köhler & Milstein, 1976). Positive hybridoma clones were screened by dot blotting using purified-NTPase of *T. gondii*, and 20 ng of the NTPase was spotted in strips of nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After being dried and then blocked with 5 % skimmed milk (Difco Laboratories), membranes were washed with PBS (pH 7·5)–0·05 % Tween-20 and incubated with culture supernatants from hybridomas or normal mouse serum, and then an alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG (H + L chain specific, Bio-Rad Laboratories). The presence of anti-*T. gondii* antibody was detected with 1·75 % nitro-blue tetrazolium (NBT, Boehringer Mannheim, Germany) and 2·25 % 5-bromo-4-chromo-3-indolyl phosphate (BCIP, Boehringer Mannheim). Then, a positive hybridoma obtained was subcloned 3 times by limiting dilution and transplanted intraperitoneally into BALB/c mice previously treated with pristane (2,6,10,14-tetramethyl-pentadecane) to collect ascites. The subclass of the mAb designated as 6C6 was identified as IgG_1 by the method previously reported (Watanabe *et al*. 1994). To obtain anti-NTPase immunized serum, a male New Zealand white rabbit (2·6 kg, CLEA Japan, Inc., Tokyo, Japan) was immunized intradermally with 100 μg of affinity-purified NTPase with Freund's complete adjuvant (Difco Laboratories). Booster immunizations were performed twice at 2-weekly intervals and the serum was obtained after 6 weeks by heart puncture under anaesthesia. Anti-*T. gondii* immunized serum was obtained from 2 male SD rats (8 week, Japan SLC, Inc.). The rats were injected s.c. with 200 μg of soluble *T. gondii* (RH) protein mixed with an equal volume of Freund's complete adjuvant, and boosted twice s.c. with 200 μg of soluble *T. gondii* protein mixed with Freund's incomplete adjuvant. The sera were obtained by the method described above.

SDS–PAGE and immunoblotting

SDS–PAGE was performed using 10 % polyacrylamide gel as described by Laemmli (1970). Soluble antigens were prepared from tachyzoites of *T. gondii*, *Plasmodium falciparum*, *P. berghei*, *Babesia rodhaini*, *Theileria* spp., *Leishmania major*, adult worms of *Schistosoma mansoni* and normal mouse lymphocytes by sonication at 5A for 30 sec, twice after freezing and thawing. Ten μg of protein were subjected to electrophoresis and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corp.), using a semi-dry electroblotting apparatus (AE-6670, Atto Corporation, Tokyo, Japan) according to the method previously described (Kyhse-Andersen, 1984). After being blocked with 5 % skimmed milk for 1 h and washed with PBS–0·05 % Tween-20 (PBST) 5 times, PVDF membranes were incubated with 6C6 mAb at room temperature overnight. After washing, the membranes were incubated with an ALP-conjugated goat anti-mouse IgG (H + L chain specific, Bio-Rad Laboratories), and positive bands were detected by NBT and BCIP as described above.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed according to the method previously described

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(O'Farrell, 1975) using a capillary gel electrophoresis apparatus (Daiichi Pure Chemicals Co. Ltd, Tokyo, Japan), with a slight modification. Briefly, 2×10^7 tachyzoites of each strain of *T. gondii* were solubilized in 20 μl of isoelectric focusing sample buffer, and 5 μl of pI marker (Daiichi Pure Chemicals Co., Ltd) were added. Samples were electrophoresed in the first dimension by isoelectric focusing (30 min at 200 V, 3·5 h at 400 V) on gels of $1{\cdot}70 \times 1{\cdot}13 \times 90$ mm cast in capillary tubes. Second dimension SDS–PAGE was performed by using 10–20 % gradient polyacrylamide gels. After the proteins on the gels were electroblotted onto PVDF membranes, positive spots on the membranes were detected by NBT and BCIP as described above.

Assay for nucleoside triphosphate hydrolase

Determination of NTPase activity was performed according to the method previously described with slight modifications (Kobayashi & Anraku, 1972; Asai *et al*. 1983). The reaction mixture and the enzyme in a final volume of 20 μl in a microtitre plate well were incubated for 20 min at 37 °C. The reaction was stopped by adding 0·1 M hydrochloric acid, and the samples were mixed with 0·5 % of ammonium molybdate dissolved in 5 M sulfuric acid, and thereafter, 125 μg/ml of 4-amino-3-hydroxy-1-naphthalenesulfonic acid were added. After incubation for 10 min at 37 °C, hydrolysed inorganic orthophosphate was determined at 630 nm by a microplate reader (Corona Electric Co. Ltd, Ibaraki, Japan) and 20 μl of 1 mM phosphoric acid solution used as a standard. A unit of enzyme activity was defined as the amount of enzyme which hydrolysed 1 μM of ATP in 1 min at 37 °C. To examine the reduction of NTPase activity by using antibodies, affinity-purified NTPase from *T. gondii* RH was mixed with 6C6 mAb, anti-NTPase IgA mAb (Asai *et al*. 1987) or anti-NTPase immunized rabbit serum. Anti-*Pneumocystis carinii* monoclonal antibody (IgG_1, Watanabe *et al*. 1994) was used as a control. The ratios of enzyme to antibody were 1:0·5, 1:1, 1:5, and 1:10 as protein concentrations. The mixture was incubated overnight at 4 °C with gentle shaking and then used for the assay.

Immuno-EM

For immuno-EM, tachyzoites were prepared as described by McLean & Nakane (1974). Briefly, tachyzoites were washed in cold PBS and fixed in 0·075 M phosphate buffer containing 0·01 M periodate, 0·075 M lysine and 2 % paraformaldehyde for 1 h, at 4 °C. After washing with PBS, samples were treated with 0·75 % saponin in PBS for 1·5 h at room temperature and then washed in 0·01 % saponin and 0·2 % glycine in PBS for 10 min, then incubated with 10 % normal goat serum (Dako, Carpinteria, CA) for 30 min. Immunoperoxidase detection was performed according to the method described by Dubremetz, Rodriguez & Ferreira (1985). Immunostaining was carried out by treating tachyzoites with 6C6, anti-*T. gondii* immunized rat serum as a positive or normal mouse IgG_1 (Dako) as a negative control, and incubated overnight at 4 °C. After washing in PBS, samples were reacted with anti-mouse or -rat IgG serum labelled with horseradish peroxidase (Dako) overnight at 4 °C. After being washed with PBS, samples were post-fixed with 1 % glutaraldehyde for 5 min, and then washed 3 times with PBS. After samples were treated with 3,3-diaminobenzidine tetrahydrochloride (DAB) without hydrogen peroxide for 1 h, they were exposed to DAB with hydrogen peroxide for 15 min. After washing with PBS, samples were post-fixed in 1 % OsO_4 in PBS for 30 min, washed in phosphate buffer, dehydrated in ethanol, and then the ethanol was replaced with graded QY1 and Quetol 651 (Nisshin EM Co. Ltd, Tokyo, Japan) and finally embedded in Quetol 651. Ultra-thin sections were placed on copper grids, and then observed with electron microscopy (H-7100, Hitachi, Tokyo, Japan).

Examination of the effects of 6C6 mAb on T. gondii *invasion* in vitro

To examine the effects of 6C6 on parasite invasion, 5×10^4 Vero cells were seeded onto round cover-slips placed in 24-multiwell tissue culture dishes, and monolayers were infected with *T. gondii* tachyzoites (a parasite:cell ratio of 20:1) (Grimwood & Smith, 1992). Tachyzoites were pre-incubated in 50 μg/ml, 100 μg/ml, 200 μg/ml or 350 μg/ml 6C6 mAb for 1 h at 37 °C or an isotype-matched mouse myeloma IgG_1 (Zymed Laboratories, Inc., San Francisco, CA) as control, prior to infection. All antibodies used in the assay were heat inactivated at 56 °C for 30 min before use. After incubation, the monolayer was washed with HBSS 3 times to remove extracellular parasites and re-incubated with 1 ml of RPMI 1640 containing with 10 % heat-inactivated fetal calf serum (Bioserum Ltd, Victoria, Australia) for 3 h. After monolayers were washed and fixed with methanol, they were stained with Giemsa and observed under a light microscope to evaluate intracellular infection by scoring a total of 200 cells per culture. The results were compared for statistical analysis between control and 6C6 mAb groups by Student's *t*-test.

RESULTS

Specificity of a monoclonal antibody

Supernatants from wells with 101 hybridomas were screened by immunoblotting using purified-*T. gondii*

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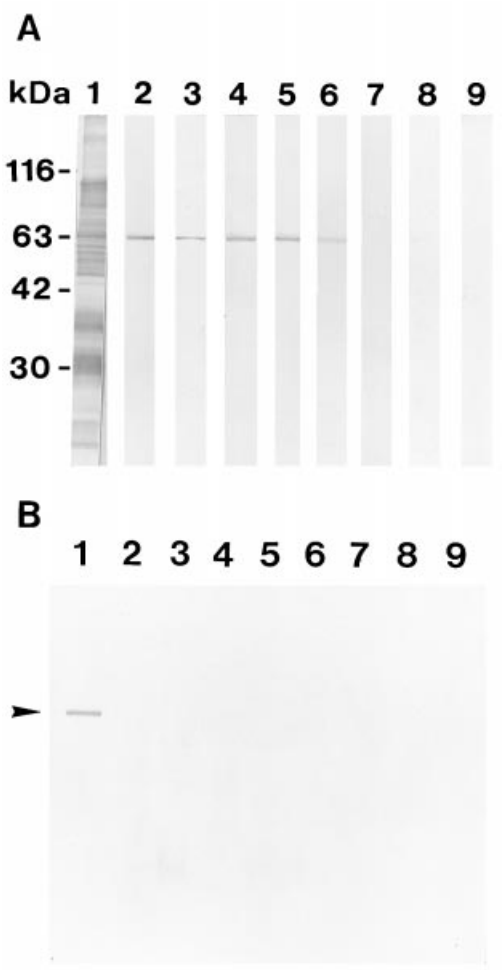

Fig. 1. SDS–PAGE and immunoblot analysis of *Toxoplasma gondii* tachyzoites. (A) *T. gondii* RH tachyzoite antigens reacted with the serum from *T. gondii*-infected mouse (lane 1). 6C6 was reacted with *T. gondii* tachyzoite antigens, RH (lane 2), ME49 (lane 3), Fukaya (lane 4), Beverley (lane 5) and Nakayama (lane 6). *T. gondii* RH tachyzoite antigen did not react with normal mouse serum (lane 7). Vero cell antigen also did not react with 6C6 antibody (lane 8) and normal mouse serum (lane 9). (B) Immunoblot analysis of *T. gondii* and other parasitic antigens with 6C6. Lane 1, *T. gondii*; lane 2, Vero cells; lane 3, murine peritoneal macrophages; lane 4, *Plasmodium falciparum*; lane 5, *P. berghei*; lane 6, *Babesia rodhaini*; lane 7, *Theileria* spp.; lane 8, *Leishmania major*; lane 9, *Schistosoma mansoni*.

NTPase as the antigen. Hybridomas in positive wells were subcloned 3 times by limiting dilution. Finally, 1 monoclonal antibody was identified reactive to 63 kDa of *T. gondii* NTPase protein and named as 6C6. In order to examine the specificity and characteristics of 6C6 mAb, SDS–PAGE and immunoblotting were performed using tachyzoite antigens from RH, ME49, Fukaya, Beverley and Nakayama strains, and were compared with those of serum from *T. gondii*-infected mice or serum from normal mice. The serum from *T. gondii*-infected mice recognized antigens of 70, 63, 60, 56, 52, 44, 36 and 30 kDa in *T. gondii* RH strain (Fig. 1A, lane 1). A 63 kDa band suspected to be an NTPase molecule was found in immunoblotting when 6C6 antibody was reacted with tachyzoite antigens obtained from all *T. gondii* isolates (Fig. 1A, lanes 2–6). On the other hand, no band was found in Vero cell lysates with 6C6 antibody (Fig. 1A, lane 8) or in *T. gondii* tachyzoite antigens with normal mouse serum (Fig. 1A, lane 7). In order to examine cross-reactivities of 6C6 to other parasitic antigens, immunoblotting was performed using soluble proteins obtained from *T. gondii*, *P. falciparum*, *P. berghei*, *B. rodhaini*, *Theileria* spp., *L. major* and *S. mansoni*. A band at 63 kDa was found in the lane of *T. gondii* but not in others (Fig. 1B, lane 1), suggesting that 6C6 antibody had no cross-reactivity with the other parasitic antigens so far examined.

Two-dimensional gel electrophoresis and immunoblotting

Since 6C6 antibody recognized 63 kDa NTPase molecules in virulent and avirulent strains of *T. gondii*, we examined whether these NTPase molecules were identical or not. The results indicated that ME49, Beverley and Nakayama strains showed 1 spot at a molecular mass of 63 kDa, isoelectric point (pI) 6·0, in two-dimensional electrophoresis and immunoblotting (Fig. 2, A–C). However, a virulent strain, RH, and an avirulent strain, Fukaya of *T. gondii* showed 2 spots which had different pIs, pI 6·0 and 6·5 in RH, and pI 6·2 and 6·4 in Fukaya (Fig. 2, D and E), indicating the presence of NTPase isoforms in these strains. Further, to confirm isoform patterns, both specimens were mixed and then two-dimensional gel electrophoresis and immunoblotting patterns were examined. The fact that 4 different isoelectric points were clearly observed (Fig. 2F) indicated that 6C6 mAb could recognize 4 different isoforms of NTPase existing in *T. gondii*.

Inhibition of NTPase activity by antibodies

The inhibition of NTPase activity by antibodies against *T. gondii* was examined *in vitro*. Although ATP diphosphohydrolase activity to ATP *in vitro* was not affected by anti-NTPase IgA class monoclonal or polyclonal antibodies at various ratios of enzyme versus antibody doses, the activity was inhibited by 6C6 antibody in a dose-dependent manner, and residual activity was less than 10% at an antibody ratio of 1:10 (Table 1). When ADP was also used as a substrate, ADP diphosphohydrolase activity was slightly affected by anti-NTPase IgA mAb and polyclonal sera, and the inhibition was limited at ratios of 1:0·5 and 1:1.

However, ADP diphosphohydrolase activity was also completely inhibited by 6C6 in a dose-dependent manner ($P < 0{\cdot}01$). Anti-*P. carinii* mono-

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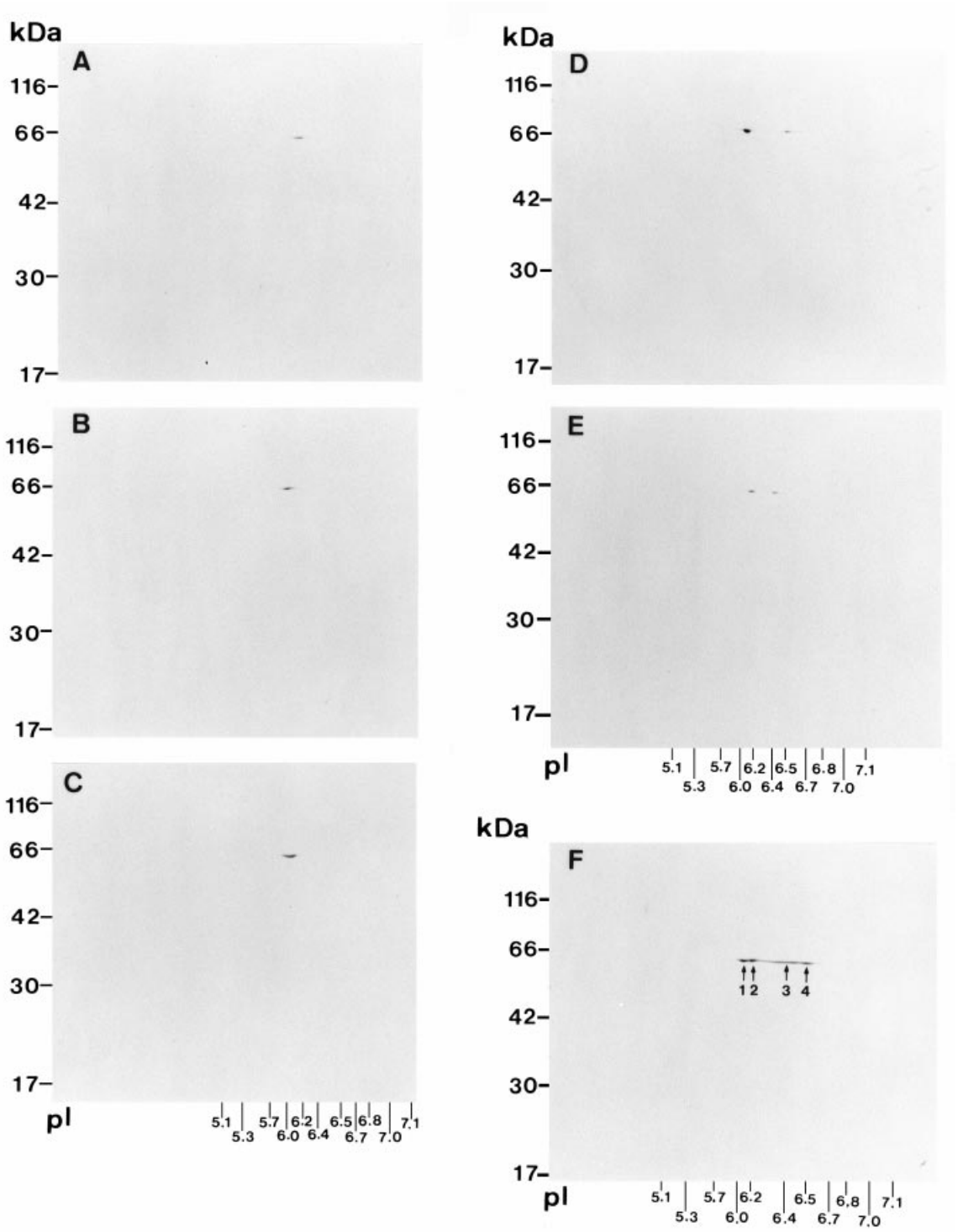

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis and immunoblot analysis of *Toxoplasma gondii* strains using 6C6. (A) ME49; (B) Beverley; (C) Nakayama; (D) RH; (E) Fukaya; (F) mixed RH and Fukaya strains.

clonal antibody used as a control in this experiment showed no inhibition of NTPase (data not shown).

Localization on NTPase of T. gondii *by immunoelectron microscopy*

Immuno-EM was performed to determine the localization of NTPase in tachyzoites. When a serum from a rat immunized with *T. gondii* protein, was used in immuno-EM as positive control, the specific reactions were found in the anterior portions of the rhoptries, dense granules, and the surface membrane of *T. gondii* (Fig. 3C). However, when *T. gondii* tachyzoites were stained with 6C6 in immuno-EM, a specific reaction was observed on the surface membranes of tachyzoites but not in other areas such as dense granules, rhoptries or cytoplasm of *T. gondii* (Fig. 3A). The findings suggested that NTPase

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Table 1. The inhibition of NTPase activity from *Toxoplasma gondii* RH strain by antibodies

	% of residual activity‡ (mean ± S.D.)					
	ATP			ADP		
Ratio of NTPase to antibody†	6C6	mAb IgA	Anti-NTPase serum§	6C6	mAb IgA	Anti-NTPase serum§
1:0·5	78·0 ± 5·1	100	100	36·3 ± 3·2*	46·2 ± 3·1	57·3 ± 7·4
1:1	65·0 ± 2·3*	100	100	17·0 ± 2·2*	55·3 ± 1·1	60·8 ± 4·1
1:5	11·2 ± 1·0*	100	100	0*	91·9 ± 1·6	71·9 ± 0·8
1:10	5·4 ± 2·3*	100	100	0*	100	72·5 ± 2·8

† 1·5 μg of NTPase was used throughout.
‡ All residual activity expressed as percentage of the control having 100 % specific activity without antibodies. The data were obtained from 4 wells.
§ Anti-NTPase immunized rabbit serum.
* $P < 0·01$.

recognized by 6C6 antibody was localized on the surface membrane of tachyzoites of *T. gondii*.

Effect of 6C6 on T. gondii *invasion of Vero cells*

To examine effects of 6C6 on *T. gondii* invasion of host cells *in vitro*, *T. gondii* tachyzoites treated with 50 μg/ml of 6C6 or IgG_1 myeloma protein were added in the monolayer of Vero cells. The percentage of infected cells was 42·0 % in 6C6-treated tachyzoites and 69·6 % in control IgG_1-treated ones ($P < 0·001$). Although a greater inhibitory effect on *T. gondii* invasion was found in tachyzoites treated with 200 μg/ml, a significant difference was not observed between 200 and 350 μg/ml of 6C6 antibody concentration (Table 2). The effect of *T. gondii* invasion was also inhibited by 6C6 in a dose-dependent manner ($P < 0·001$). The percentage of infected cells was 54·8 % in 350 μg/ml of IgG_1 myeloma-treated tachyzoites and 62·1 % in HBSS. However, the decline of the percentage of infected cells treated with 350 μg/ml of IgG_1 myeloma observed, as compared with HBSS-treated cells, might be due to toxicity from the high dose of antibody used.

DISCUSSION

T. gondii is an obligate intracellular protozoan that can invade and replicate in all nucleated mammalian cells and produces an oligomeric protein with dithiol-activated NTPase activity. This enzyme has apyrase activity towards all nucleoside and deoxynucleoside triphosphates, sequentially degrading these substrates to the monophosphate form. Remarkably, it is reported that the NTPase is contained in as much as 3–4 % of total parasite proteins, suggesting a central role of the enzyme in parasite survival and replication (Asai *et al*. 1983).

In the present study, we made a monoclonal antibody against NTPase of *T. gondii*, designated 6C6, which had an inhibitory effect on NTPase dehydrolase activity *in vitro*. To confirm the specificity of this mAb, we examined its cross-reactivity with the other apicomplex or parasite, *S. mansoni*, and mouse lymphoid cells. It has been reported that NTPase in *T. gondii* is closely related to *S. mansoni* ATP diphosphohydrolase (Vasconcelos *et al*. 1993, 1996), potato apyrase (Handa & Guidotti, 1996) and CD 39 antigen of human (Wang & Guidotti, 1996) or mouse lymphoid cells (Vasconcelos *et al*. 1993) at amino acid sequence levels. By using either an antibody against a fusion protein derived from recently cloned nucleoside triphosphate hydrolase of *T. gondii* (Bermudes *et al*. 1994) or an anti-potato apyrase antibody, immunoblot analysis identified *S. mansoni* ATP diphosphohydrolase as a 63 kDa protein (Vasconcelos *et al*. 1993, 1996). Since 6C6 antibody reacted only to *T. gondii* parasites but not to other parasitic antigens and lymphoid cell antigens, it was considered that this mAb was specific for *T. gondii* NTPase.

Regarding the isoform pattern of *T. gondii* NTPase, different patterns were found between virulent and avirulent strains of *T. gondii*. The virulent RH and avirulent Fukaya strains have 2 isoforms but the other avirulent strains have 1 isoform, suggesting that the isoform pattern and the virulence of *T. gondii* might be related to each other. These results corresponded with the finding previously reported (Asai *et al*. 1990).

To our knowledge, this is the first report of a monoclonal antibody capable of inhibiting the hydrolysis activity of *T. gondii* NTPase *in vitro* and distinguishing different isoform patterns of NTPase among virulent and avirulent *T. gondii* strains.

Furthermore, we examined the localization of NTPase in *T. gondii* by immuno-EM with 6C6 antibody. Although a serum from rats immunized with *T. gondii* protein stained rhoptries, dense granules, and the surface membrane, 6C6 showed specific reaction in the surface membrane of *T. gondii*. Since the positive reaction was recognized in

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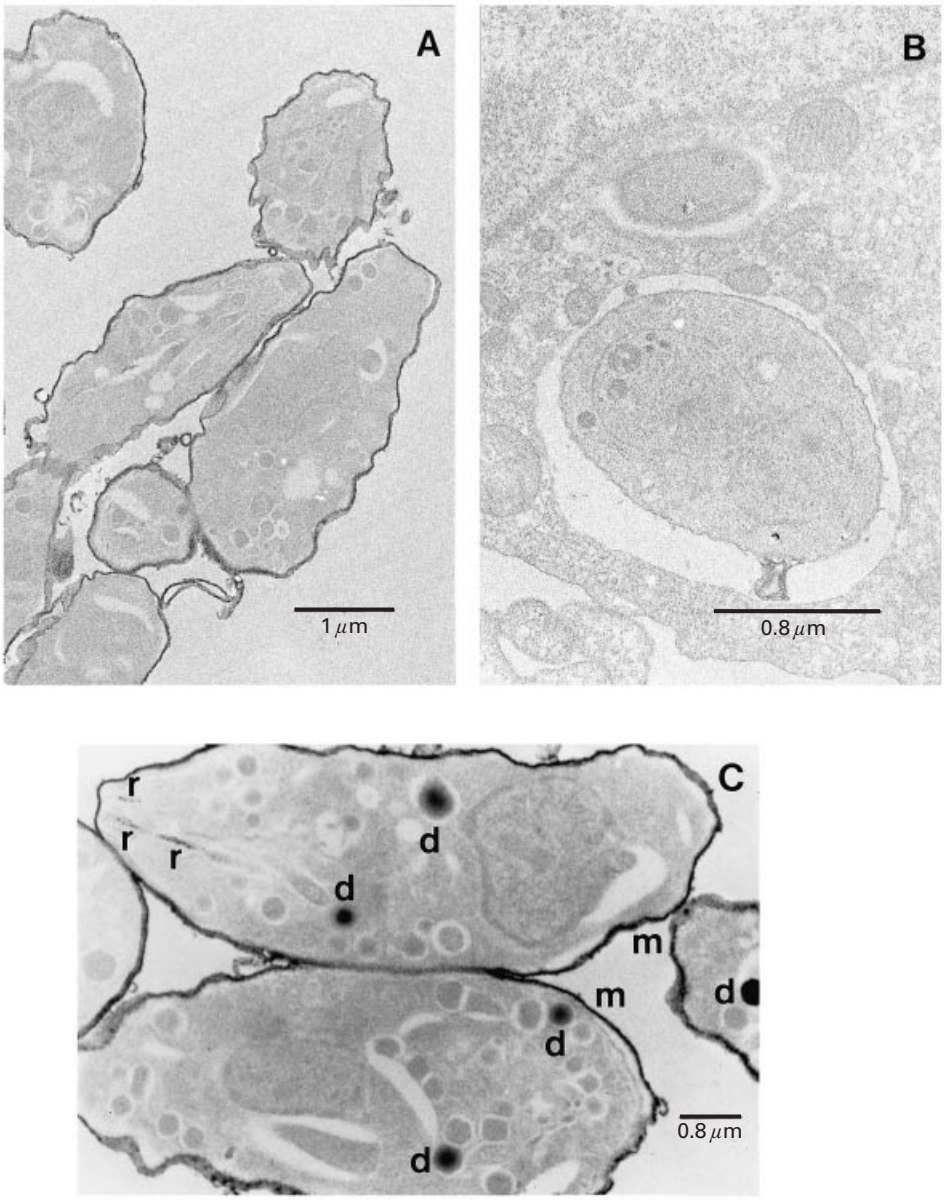

Fig. 3. Localization of NTPase in *Toxoplasma gondii* tachyzoites by immunoelectron microscopy. Positive reactions with 6C6 were found in the surface membrane of *T. gondii* tachyzoites but not in organellae (A). The Vero cell infected with *T. gondii* tachyzoites showed no positive reactions with normal mouse serum (B). Rhoptries (r), dense granules (d), and surface membranes (m) of tachyzoites reacted with rat serum immunized with *T. gondii* protein (C).

inner organellae of *T. gondii* such as rhoptries and dense granules as well as in the surface membrane of *T. gondii*, it was suggested that the antibodies used in these experiments penetrated into the cytoplasm of *T. gondii* tachyzoites. The results strongly suggested that NTPase recognized by 6C6 specifically localized on the surface membrane of tachyzoites but not in the cytoplasm or other organellae. So far, Sibley *et al*. (1994) have examined the localization of NTPase by using immuno-EM with anti-NTPase polyclonal rabbit serum and revealed that the NTPase is located in dense granules, rhoptries, and membranes of parasitophorous vacuoles but not in the cell membrane of tachyzoites (Sibley *et al*. 1994). These findings were inconsistent with ours obtained with immuno-EM. The reason for the discrepancy is not clear, although it might be due to the source and properties of anti-NTPase antibody used in their studies. This point requires further investigation.

In this study, we demonstrated that 6C6 had an inhibitory effect on *T. gondii* invasion into host cells. The greatest inhibition was observed with 200 μg/ml of antibody concentration but not with 350 μg/ml, and the percentage of infected cells in 350 μg/ml of normal IgG was lower than other concentrations of normal IgG and the HBSS control. These findings might suggest that higher concentrations of normal IgG affect the growth of *T. gondii* and Vero cells *in vitro*. Small clumps of tachyzoites were sometimes found in the groups treated with 6C6, normal IgG and HBSS, however, their numbers were not significantly different in each concentration among

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Table 2. Effect of 6C6 on *Toxoplasma gondii* invasion of Vero cells *in vitro*

(5×10^4 Vero cells were cultured with 1×10^6 *T. gondii* tachyzoites which had been treated with 6C6 antibody as described in the Materials and Methods section. Infected cells were counted at 3 h after cultivation and expressed as a percentage.)

Concentration of antibody (μg/ml)	% of infected host cells (mean ± S.D.)	
	6C6	IgG_1
350	17·8 ± 6·0*	54·8 ± 7·3
200	15·9 ± 3·5*	60·8 ± 5·0
100	31·1 ± 2·6*	65·4 ± 2·5
50	42·0 ± 4·0*	69·6 ± 3·3
0 (HBSS)	62·1 ± 5·0	

* $P < 0{\cdot}001$ for 6C6 compared to IgG_1 of each concentration.

them. Although the mechanism of growth inhibition by 6C6 antibody is not clear, the data indicate that this NTPase molecule could be considered a potential candidate antigen for vaccines targeting *T. gondii* tachyzoites. It has been reported that monoclonal antibodies against membrane antigens of parasites such as *Plasmodium* (Hollingdale *et al*. 1984), *Entamoeba histolytica* (Marinets *et al*. 1997), *Giardia lamblia* (Hemphill *et al*. 1996), and *Blastocystis hominis* (Tan *et al*. 1997) can prevent the parasite invasion of host cells. Recent studies on partial purification and immuno-histochemical localization of ATP diphosphohydrolase from *S. mansoni* revealed considerable homology with *T. gondii* NTPase at amino acid sequence level (Vasconcelos *et al*. 1993; Handa & Guidotti, 1996; Vasconcelos *et al*. 1996) and localization of the enzyme on the outer surface of schistosome tegument. In this study, since *T. gondii* NTPase recognized by 6C6 localizes on the surface membrane of tachyzoites as does ATP diphosphohydrolase from *S. mansoni*, the NTPase recognized by this mAb may be a different type of enzyme from that reported by Sibley *et al*. (1994). The function of NTPase localized on the surface membrane of tachyzoites is not clear at present. However, since *S. mansoni* ATP diphosphohydrolase localizes on the outer surface of parasites (Vasconcelos *et al*. 1996), it has been considered that the enzyme may participate in escape mechanisms of the parasites from host defence mechanisms (Capron *et al*. 1982) by splitting ATP or ADP which is cytotoxic and is released on their surface by activated platelets (Bout *et al*. 1986) or cytotoxic T lymphocytes (Filippini *et al*. 1990). In *Toxoplasma* infection, it has been reported that cellular immune responses may play an important role for the host defence mechanism, and that cytotoxic T lymphocytes and platelets may cause lysis of *T. gondii*-infected cells (Yong *et al*. 1991; Khan, Smith & Kasper, 1998). Thus, NTPase in *T. gondii* may participate in escape mechanisms of the parasite from activated cytotoxic lymphocytes like *S. mansoni* ATP diphosphohydrolase. Further studies will be necessary to determine the functional contribution of NTPase to escape host defence mechanisms against *T. gondii*.

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