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Inhibitory effects of a monoclonal antibody (MAb-001) on *in vitro* oxygen consumption and multiplication of the pathogenic haemoflagellate, *Cryptobia salmositica* Katz

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Abstract

A monoclonal antibody (MAb-001), against a surface glycoprotein on *Cryptobia salmositica* inhibited the multiplication and oxygen consumption of both virulent and avirulent strains of the parasite. The classical cysteine proteinase inhibitor (E-64) and a cysteine proteinase activator (EDTA) affected the *in vitro* multiplication of *C. salmositica*. Concentrations of E-64 higher than 10 μM reduced the multiplication of *C. salmositica* while 5 mM of EDTA enhanced its multiplication. We propose that the cysteine proteinase is an important metabolic enzyme in *C. salmositica* and that binding of MAb-001 to the enzyme inhibited parasite multiplication and reduced oxygen consumption.

Keywords: *Cryptobia salmositica*, MAb, multiplication, oxygen, consumption.

Introduction

The antigenic composition of numerous parasitic membranes has been studied in order to develop rational antiparasitic strategies (James, Levy & Ristic 1981; Reduker, Jasmer, Goff, Perryman, Davis & McGuire 1989; Torian & Lukehart 1987). The surface membrane of *Cryptobia salmositica* contains sialic acid residues, phosphate groups and protein glycoconjugates as demonstrated by enzymatic treatment of parasites with neuraminidase, phospholipase C or trypsin (Vommaro, Attias, Silva-Filho, Woo & De Souza 1997; Feng & Woo 1998a). However, the antigenic composition of *C. salmositica* is not well known. Twenty-one polypeptide bands (21–200 kDa) were detected by Woo & Thomas (1991) and a monoclonal antibody (MAb-001) was developed to identify a protective 200 kDa glycoprotein (Cs-gp200) on the membrane of *C. salmositica* (Feng & Woo 1996). The epitope consists of a polypeptide, a carbohydrate, a phospholipid and a phosphatidylinositol residue which anchors the polypeptide to the surface membrane. The antibody (MAb-001) also recognizes the carbohydrate part of Cs-gp200 and cross-reacts therefore with other glycoproteins on/in *C. salmositica* (Feng & Woo 1998b).

Cysteine proteinases have been shown to be important in parasite development and in pathogenesis (Berasain, Goni, McGonigle, Dowd, Dalton, Frangione & Carmona 1997; Coombs & Baxter 1984; Coombs, Hart & Capaldo 1982; Yamakami, Hamajima, Akao & Tadakuma 1995). The cysteine proteinase in *Entamoeba histolytica* has cytopathic effects on mammalian cells (Keene, Hidalgo, Orozco & McKerrow 1990) while that of *Plasmodium falciparum* degrades haemoglobin (Rosenthal 1995).

Cysteine proteinase inhibitors such as epoxysuccinyl-leucyl-agmatine (E-64) have been used to demonstrate the importance of cysteine proteinases in parasite metabolism. E-64 rapidly inactivates cysteine proteinases such as papain, cathepsin B (Inaba, Hirayama & Fujinaga 1979) and calpain (Sugita, Ishiura, Suzuki & Imaghari 1980; Suzuki, Tsuji & Ishiura 1981; Suzuki 1983). It is thought to act by irreversibly modifying the sulphydryl

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group on the Cys-25 active site of the enzyme by alkylation of the amino acid. Zuo & Woo (1997) showed that the activity of the cysteine proteinase from *C. salmositica* is inhibited by E-64.

As MAb-001 inhibits cysteine proteinase activity (Zuo, Feng & Woo 1997) and *C. salmositica* respires aerobically (Thomas, Ballantyne & Woo 1992), the goals of the present study were to examine more closely the role of cysteine proteinase in oxygen consumption and multiplication of *C. salmositica.* This was accomplished by exposing the parasite to MAb-001 and E-64 and monitoring their effects on the *in vitro* oxygen consumption and multiplication.

Materials and methods

Parasites

The strain of *C. salmositica* (T4, cloned substrain) was initially isolated from *Piscicola salmositica*, a leech vector found in spawning coho salmon, *Oncorhynchus kisutch* (Walbaum), in streams on Vancouver Island (Woo 1979). The parasite has been maintained by serial passages in rainbow trout and has retained its pathogenicity. Passage is done by peritoneal blood inoculations of uninfected rainbow trout with the parasite. The avirulent strain of *C. salmositica* was developed from the virulent strain by continuous serial *in vitro* culture (Woo & Li 1990). It was cultured in minimum essential medium (MEM) supplemented with 15% foetal bovine serum and 0.025 M Hepes buffer (pH 7.2–7.4) at 10–12 °C (Woo & Thomas 1991).

In the present study, the pathogenic strain was freshly isolated from the blood of an infected rainbow trout, *Onchorhynchus mykiss* (Walbaum), and was cultured and subcultured in MEM for no more than 2 months. Short-term culture of the parasite does not reduce its pathogenicity to trout and is a way to obtain large numbers of parasites free of host cells (Woo & Thomas 1992).

Cell lysate preparation of *C. salmositica*

All procedures were carried out at 4 °C. Briefly, parasites (avirulent strain) from cultures (35 mL flasks, grown until parasites reached maximum biomass) were washed and suspended in phosphate buffered saline (pH 7.2) containing 0.2% (v/v) Triton X-100. The suspension was sonicated in an ice bath for 7 min, followed by three freeze and thaw cycles of the parasite suspension at –20 °C allowing for thorough disruption of the cell membrane. The mixture was then centrifuged at 7500 ***g*** for 15 min and the supernatant pooled and used as antigen for the enzyme-linked immunosorbent assay (ELISA) (Sitja-Bobadilla & Woo 1994).

Monoclonal antibody

The MAb-001 that reacted against a 200 kDa surface antigen on *C. salmositica* was produced as described previously (Feng & Woo 1996). It was purified by affinity-chromatography using the protein G resin (Pharmacia Biotechnology, Uppsala, Sweden). A sodium phosphate binding buffer (pH 7.2) was used and the purified monoclonal was eluted with 20 mM sodium phosphate + 0.5 M sodium chloride (pH 7.2) solution (Munoz, O'Shea-Alvarez, Perez-Garcia, Weinbach, Moreno, De La Torre, Wagos & Tovar 1992). The degree of purification was determined using an ELISA (Sitja-Bobadilla & Woo 1994). The protein concentration of *C. salmositica* antigen and MAb-001 preparations were determined using the method of Bradford (1976).

ELISA values of unpurified and purified MAb-001

The protein concentration of sonicated *C. salmositica* crude lysate was diluted to 5 μg mL^{-1} with carbonate buffer (pH 9.8) and used as antigen (Sitja-Bobadilla & Woo 1994). Wells of a 96 well ELISA plate were coated with 50 μL of parasite antigen and the plate was incubated overnight at 4 °C. The wells were washed five times with Tween20-PBS (TPBS) and then blocked with 200 μL of 5% skimmed milk in PBS for 1 h at 37 °C. The primary antibody (MAb-001, unpurified or purified, 50 μL per well) was added to appropriate wells and incubated at 37 °C for 4 h followed by the secondary antibody [goat-anti-mouse-IgG conjugated with alkaline phosphatase (Bio-Rad Laboratories, CA, USA)] diluted 1:3000 in 3% skimmed milk in TPBS, incubated at 37 °C for 1 h before the enzyme substrate (50 μL per well of p-nitrophenyl phosphate in diethylamine buffer, pH 9.8) was added. Colour development was observed after 1 h; a yellow colour indicated a positive reaction. The optical density (OD) was read at 450 nm using a V-max kinetic microplate

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reader. Negative controls were (a) wells without antigen, (b) primary antibody, (c) second antibody, (d) wells with enzyme substrate only or (e) wells with mouse preimmune serum as primary antibody.

The effect of MAb-001 on the *in vitro* multiplication of *C. salmositica*

All procedures were conducted under sterile conditions and at 4 °C unless otherwise indicated. Pathogenic and avirulent strains of *C. salmositica* were harvested by centrifugation (4 °C) at 7500 ***g*** for 12 min and washed three times in cold phosphate buffered saline (PBS, pH 7.2). Parasites were resuspended in 10 mL of MEM in 35 mL culture tissue flasks such that the final suspension contained 5×10^5 parasites mL^{-1}.

Monoclonal antibody was diluted with PBS (1.006 mg/10 mL) to the point where it did not agglutinate living *C. salmositica* (pathogenic and avirulent strains), as observed under a light microscope, even after 24 h. Monoclonal antibody (200 μL) was added to 10 mL MEM (1.006 mg/10 mL) in each flask which contained 5×10^5 mL^{-1} parasites ($n = 10$ per group). Two control groups were used. In the first control group, MAb-001 was substituted with an equivalent amount of hybridoma medium (Feng & Woo 1996). The second control group was an IgM (1.006 mg/10 mL) antibody specific for *Pseudomonas aeruginosa* lipid A membrane protein (Rocchetta & Lam 1997). As it was not possible to obtain an IgG1 class antibody (like MAb-001), an IgM was used. The parasite was cultured for 12 days and parasite samples (20 μL per sample, three samples per flask) were taken every 4 days. The samples were diluted and loaded into both chambers (10 μL) of a haemocytometer and parasite numbers were determined by routine haemocytometry (Archer 1965).

Effect of the cysteine proteinase inhibitor (E-64) on *in vitro* multiplication of *C. salmositica*

Parasites were added to 10 mL of MEM in 35 mL culture flasks so that the final concentration of parasites was 5×10^5 parasites mL^{-1}. To each of three experimental groups ($n = 10$) 5, 10 and 20 μM of E-64 (Sigma, St Louis, MO, USA) dissolved in dimethyl sulphoxide (DMSO) were added. The first control group ($n = 10$) consisted of parasites with no E-64, the second control group contained parasites with no DMSO and the third control group had different volumes (2.5, 5.0 and 10.0 μL) of DMSO.

To estimate parasite numbers, the parasite was cultured for 24 days and three 20 μL samples were taken every 4 days from each flask and counted by haemocytometer by loading into both of its chambers (10 μL).

Effects of EDTA on *in vitro* multiplication of *C. salmositica*

Parasites were added to 10 mL of MEM in 35 mL culture tissue flasks so that the final concentration of parasites was 5×10^5 parasites mL^{-1}. The two experimental groups ($n = 10$ per group) either contained 1 or 5 mM of ethylenediaminetetraacetic acid disodium salt (EDTA) (Sigma, St. Louis, MO, USA) while the control group had no EDTA.

The parasites were incubated for 18 h and parasite samples (20 μL) were taken after the first hour and every 4 h thereafter. The samples were diluted with PBS and counted as described previously.

In vitro oxygen consumption of *C. salmositica* incubated with MAb-001 (unpurified or purified)

Parasites (pathogenic and avirulent strains) were harvested by centrifugation (4 °C), at 7500 ***g*** for 12 min and washed three times in cold (4 °C) PBS (pH 7.2). They were resuspended in 10 mL of MEM in 35 mL culture tissue flasks (three replicates per treatment, i.e. three flasks per treatment) so that the final suspension contained approximately 5×10^5 parasites mL^{-1}. The three experimental groups were: (a) avirulent strain with unpurified MAb-001 and MEM (1.006 mg/10 mL), (b) avirulent strain with purified MAb-001 in MEM (1.006 mg/10 mL) and (c) pathogenic strain with unpurified MAb-001 in MEM (1.006 mg/10 mL).

The five control groups were: (a) avirulent strain with hybridoma medium (200 μL) added to MEM, (b) pathogenic strain with hybridoma medium added to MEM, (c) hybridoma medium, (d) avirulent strain incubated with IgM (1.006 mg/10 mL) specific for *P. aeruginosa* membrane lipid A protein and (e) avirulent strain in MEM to which neither hybridoma medium nor MAb-001 was added.

Oxygen consumption (nmol oxygen h^{-1}) was measured at 10 °C using a Clark Electrode Oxygraph (Sable Systems, Salt Lake City, UT, USA) calibrated

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with air-saturated MEM. The parasite suspension (1.8 mL containing 4×10^6 mL^{-1} parasites) from each group (taken from each of three replicates) was added to the water-jacketed cell and oxygen consumption was determined for 1 h. Measurements were taken prior to the addition of MAb-001 or hybridoma medium and then 1, 3, 7 and 12 days after the start of the experiment.

The *in vitro* oxygen consumption of *C. salmositica* after exposure to purified MAb-001 was measured. The three control groups were: (a) *C. salmositica* incubated with unpurified MAb-001 for 24 h but not washed, (b) *C. salmositica* incubated with hybridoma medium for 24 h and then washed and (c) *C. salmositica* incubated with hybridoma medium for 24 h and not washed.

Oxygen consumption (nmol oxygen h^{-1}) was measured at 10 °C using a Clark Electrode Oxygraph calibrated with air-saturated modified MEM. The parasite suspension (1.8 mL containing 4×10^6 mL^{-1} parasites) from each group was added to the water jacketed cell and the oxygen consumption was determined for 1 h. Measurements were also taken 12, 24 and 48 h after the parasites were washed with PBS.

Statistical analysis

One way analysis of variance (ANOVA) was used to determine the statistical differences between control and experimental assays. Multiple comparisons among means were made with Duncan's Multiple Range Test. Results were considered significant if $P < 0.05$ (Steel & Torrie 1980).

Results

Purification of MAb-001

Protein concentration of unpurified and purified MAb-001 was standardised prior to ELISA. Before purification, MAb-001 had an ELISA OD value of 0.312 ± 0.041 ($n = 6$) and after purification 1.113 ± 0.125 ($n = 6$). Purified MAb-001 had a fourfold increase in activity.

Effects of MAb-001 on the *in vitro* multiplication of *C. salmositica*

Both virulent and avirulent strains were active after addition of diluted unpurified MAb-001. However, after 4 days the MAb-001 exposed parasites were sluggish (as observed using a light microscope) and did not multiply. Both strains of parasite multiplied readily in the absence of the antibody. Significant ($P < 0.05$) differences were detected between the experimental groups (with MAb-001) and control groups after 4, 8 and 12 days culture (Fig. 1). There were no significant differences between control groups (hybridoma medium and an IgM MAb-001 against *P. aeruginosa* membrane protein) or between experimental groups (virulent and avirulent strains) exposed to MAb-001.

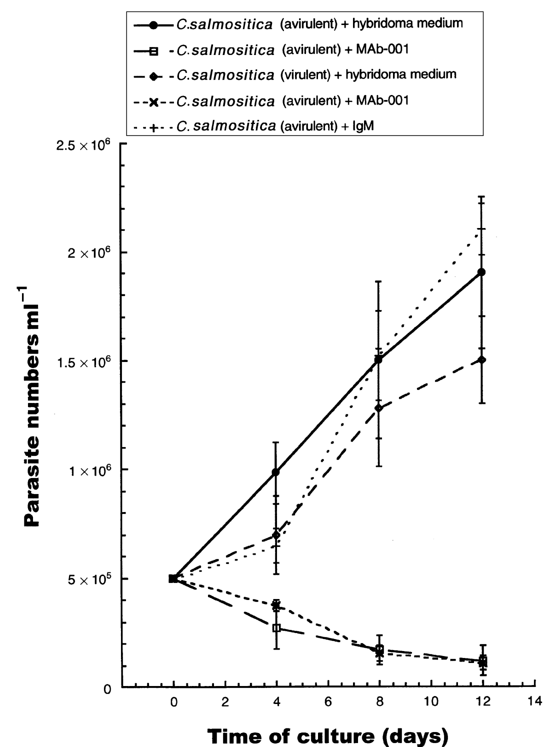

Figure 1 Effects of unpurified MAb-001 on the *in vitro* multiplication of *C. salmositica*. Measurements taken from 10 replicates (flasks). After 4 days the MAb-001 exposed parasites did not multiply. Significant ($P < 0.05$) differences were detected between experimental groups (with MAb-001) and control groups after 4, 8 and 12 days of culture.

Effects of E-64 on the *in vitro* multiplication of *C. salmositica*

The avirulent strain did not multiply when cultured in 10 μM E-64, or in higher concentrations; however, it multiplied readily in the absence of E-64 or in 5 μM of E-64 or with equivalent amounts of DMSO (Fig. 2). There were no significant differences between control groups

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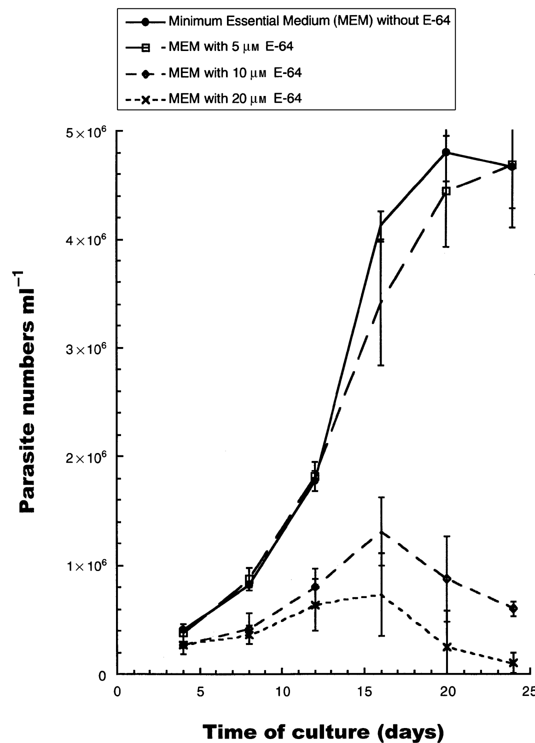

Figure 2 Effects of E-64 on the *in vitro* multiplication of *C. salmositica.* Measurements taken from 10 replicates (flasks). *C. salmositica* did not multiply when cultured in 10 µM E-64, or higher concentrations. Significant differences ($P < 0.05$) were detected between two experimental groups (10 µM or 20 µM E-64) and controls, and also the experimental group with 5 µM of E-64 after 8, 12, 16, 20 and 24 days of culture.

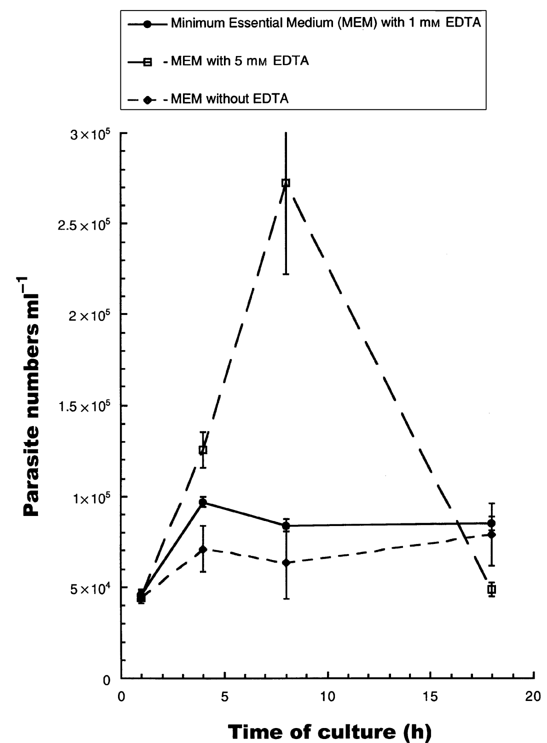

Figure 3 Effects of EDTA on the *in vitro* multiplication of *C. salmositica.* Measurements taken from 10 replicates (flasks). Significant differences ($P < 0.05$) were observed between *C. salmositica* cultured in 5 mM of EDTA and those cultured either in 1 mM EDTA or no EDTA after 4 and 8 h of culture. However, before 18 h the number of parasites in 5 mM of EDTA dropped significantly and was not significantly lower than the control groups or the group cultured in 1 mM of EDTA.

(cultured with DMSO and without E-64 or DMSO) or parasites cultured with 5 µM E-64 or between experimental groups (parasites cultured with 10 or 20 µM E-64).

Effects of EDTA on the *in vitro* multiplication of *C. salmositica*

Parasites in 5 mM of EDTA multiplied significantly better compared with the control groups (without EDTA) or parasites with 1 mM of EDTA (at 4 and 8 h of culture) (Fig. 3). There were no significant differences between the control groups and parasites cultured in 1 mM of EDTA at 1, 4, 8 and 18 h.

In vitro oxygen consumption of *C. salmositica* in MAb-001

Prior to the addition of MAb-001, or hybridoma medium, there were no significant differences in oxygen consumption between experimental and control groups. Also, there were no significant differences between control groups (without MAb-001) at 1, 3, 7 and 12 days. There were no significant differences between parasites treated with purified MAb-001 and unpurified MAb-001 1, 3 and 7 days after culture; but parasites in purified MAb-001 consumed significantly more oxygen after 12 days culture (Fig. 4).

In vitro oxygen consumption of *C. salmositica* after exposure to MAb-001

There were no significant differences between groups treated with hybridoma medium. Also there were no significant differences between groups treated with MAb-001 (washed and not washed) at 10, 24 and 48 h. However, when cells had been exposed to MAb-001 (and washed) they had

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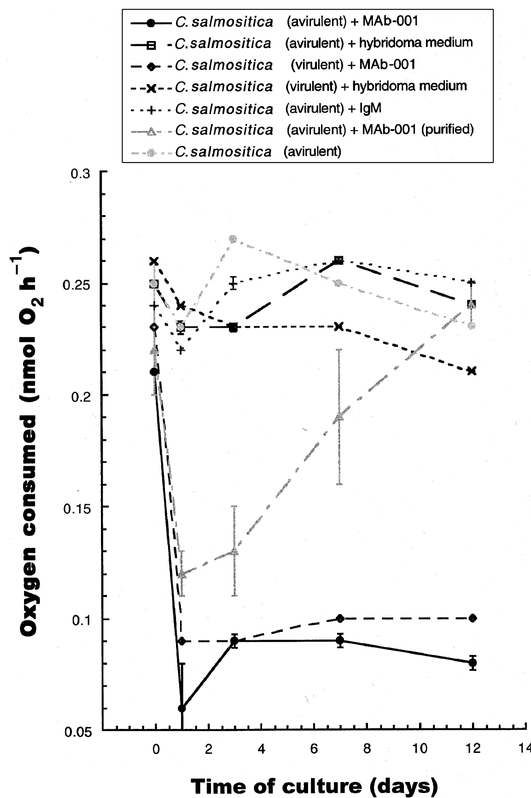

Figure 4 Effects of MAb-001 on the *in vitro* oxygen consumption of *C. salmositica.* Measurements taken from five replicates (flasks). *C. salmositica* (virulent and avirulent strains) cultures in unpurified MAb-001 had significantly ($P < 0.05$) lower oxygen consumption than control groups after 1, 3, 7 and 12 days of culture. Similarly, the avirulent strain, cultured with purified MAb-001, had significantly lower oxygen consumption than control groups.

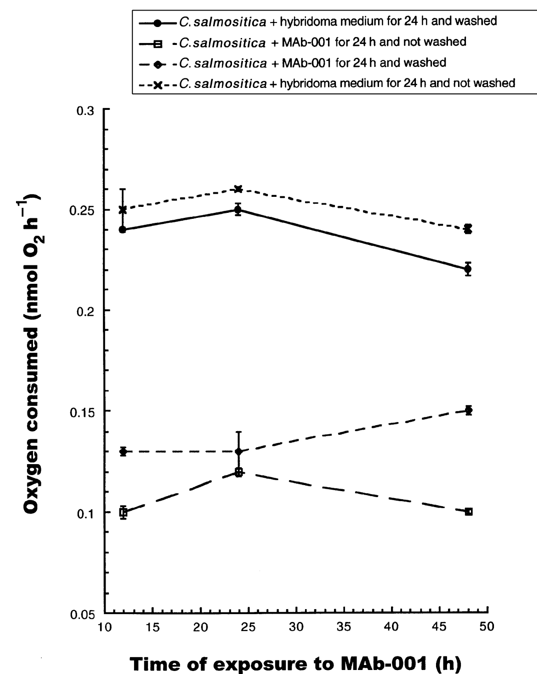

Figure 5 Oxygen consumption of MAb-001 exposed *C. salmositica* with or without washing. Measurements taken from five replicates (flasks). No significant difference between groups treated with MAb-001 (washed and not washed) at 10, 24 and 48 h. Parasites exposed to MAb-001 (and washed) had significantly ($P < 0.05$) lower oxygen consumption than those treated with hybridoma medium after 12, 24 and 48 h of culture.

significantly lower oxygen consumptions than groups treated with hybridoma medium after 12, 24 and 48 h culture (Fig. 5).

Discussion

This study has shown that the MAb-001 (unpurified and purified) inhibited the *in vitro* oxygen consumption of *C. salmositica* (pathogen and avirulent strains). A two-fold decrease in O_2 consumption was observed in the parasite after treatment with the MAb-001. The group treated with purified MAb-001 also exhibited a twofold decrease in oxygen consumption. However, after 7 days its O_2 consumption steadily rose and at day 12 it was similar to control values. This is different from the unpurified MAb-001 which inhibited oxygen consumption of *C. salmositica* well into day 12. This difference could be explained by the fact that a 10-fold larger volume of unpurified antibody was added than purified. Although the activity of the purified MAb-001 is fourfold higher it would not be as readily available in the medium for the parasites as the unpurified antibody. This would allow the unaffected *C. salmositica* to multiply without inhibition in the group treated with purified antibody compared with the group treated with unpurified antibody. In addition, washing parasites after exposure to MAb-001 still resulted in oxygen inhibition. The oxygen consumed by parasites after washing was similar to the oxygen consumed by MAb-001 treated parasites without washing. The inhibition of oxygen consumption observed when *C. salmositica* was treated with MAb-001 was substantial. This would indicate that the antibody was taken into the parasite or it was bound irreversibly on its surface membrane.

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The classical cysteine proteinase inhibitor E-64 inhibited the *in vitro* multiplication of *C. salmositica.* There was a fivefold decrease in parasite numbers observed at day 24 between 10 and 20 μm E-64 treated groups and untreated groups. As E-64 is a cysteine proteinase inhibitor it is likely that the inhibition of multiplication observed is either directly or indirectly a result of the inhibitor acting on the cysteine proteinase. Therefore, it is most likely that the inhibitor was taken into the parasite. Webster & Grab (1988) showed that monoclonal antibodies (IgG and IgM) specific for surface glycoproteins of *Trypanosoma brucei* bound to these glycoproteins were pinocytosed by the parasite. Similar results were observed with antibodies against *Leishmania* spp. (Doyle, Behin, Mauel & Rowe 1984), *T. cruzi* (Abelha, Azevedo & Teixeira 1981), *T. lewisi* (Cherian & Dusanic 1978) and *E. histolytica* (Aust-Kettis & Sundqvist 1980). Russo, Grab, Lonsdale-Eccles, Shaw & Williams (1994) further showed that monoclonal antibodies were also internalized in *T. brucei* even in the absence of antigen–MAb-001 complex. They suggested this was through directional endocytosis and involved the flagellar pocket. Once endocytosed, the antibody became bound to the lysosomal cysteine proteinase in *T. brucei* (Mbawa, Gumm & Fish 1991; Russo *et al.* 1994). A similar mechanism for the internalization of MAb-001 may be postulated for *C. salmositica.* Monoclonal antibody is taken into the parasites possibly through pinocytosis. Once inside it binds to the cysteine proteinase and inhibits its activity similar to the action of E-64. However, the possibility that the inhibition of oxygen consumption is in part indirectly a result of membrane-antigen cross-linking events and signal-transduction cannot be excluded.

Zuo & Woo (1998) showed that the cysteine proteinase in *C. salmositica* is similar to the lysosomal cysteine proteinase in *T. brucei* and suggested that it is mainly located in lysosomes. Binding of MAb-001 to the cysteine proteinase and inhibition of its activity, as shown *in vitro* by Zuo *et al.* (1997), would result in inhibition of oxygen consumption and multiplication as observed in the present study.

The apparent importance of the cysteine proteinase in the *in vitro* multiplication and survival of *C. salmositica* has led us to the sequencing of the cysteine proteinase gene of *C. salmositica* and to the production of a recombinant protein. This protein would be a good candidate vaccine and antibodies produced against it may be used for therapeutic purposes.

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