

Characterization of a 200 kDa glycoprotein (Cs-gp200) on the pathogenic piscine haemoflagellate *Cryptobia salmositica*

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ABSTRACT: The 200 kDa antigenic doublet of the pathogenic haemoflagellate *Cryptobia salmositica* Katz, 1951, was detected using a monoclonal antibody (MAB-001) in 1-dimensional SDS-PAGE. This antigenic doublet is a glycoprotein since it was susceptible to both protease K and to sodium *m*-periodate oxidation and is designated Cs-gp200. It has a PI (isoelectric point) value of about 5.5 (using 2-dimensional gel electrophoresis). It migrated faster under reducing conditions than under non-reducing conditions and was partitioned into the aqueous phase in Triton X-114 phase separation. It is a secretory-excretory product since it was detected in a non-protein culture medium with *C. salmositica*. These results suggest that the Cs-gp200 is a glycoprotein consisting of carbohydrate determinants and conformational polypeptide with internal disulphide bonds. It is a hydrophilic antigen, is a secretory product of the parasite, and plays an important role in antibody elicitation in immunized fish.

KEY WORDS: *Cryptobia salmositica* · Haemoflagellate · Monoclonal antibody · Antigen · Glycoprotein

INTRODUCTION

Monoclonal antibodies have been used to identify antigens of parasitic protozoa from mammals (Parish et al. 1985, Scharfstein et al. 1986, Kink & Chang 1988, Wood et al. 1989, Tachibana et al. 1990, Grimwood & Smith 1992, Precigout et al. 1993, Campbell & Fubert 1994). The location and distribution of some specific antigens in parasites have been determined using monoclonal antibodies (Aikawa et al. 1986, Favalord et al. 1993). Some of these antigens have been used to develop vaccines (Boyle et al. 1982, Saul et al. 1984, Oikawa et al. 1992).

Cryptobia salmositica Katz, 1951 causes salmonid cryptobiosis and it has been found in all species of Pacific salmon on the west coast of North America (Woo 1987, 1994). The clinical signs of the disease include exophthalmia, general oedema, splenomegaly, abdominal distention with ascites, and anorexia (Woo 1979, Li & Woo 1991). *C. salmositica* causes mortality

in experimentally and naturally infected salmonids (Woo 1987) and it has also been identified as a lethal pathogen in semi-natural and intensive salmon culture facilities (Bower & Thompson 1987). The pathogenic *C. salmositica* was attenuated by continuous *in vitro* culture (Woo & Li 1990) and the avirulent parasite does not cause disease but protects adult and juvenile *Oncorhynchus mykiss* from disease (Woo & Li 1990, Sitja-Bobadilla & Woo 1994, Li & Woo 1995).

Little is known about the antigenic composition of *Cryptobia salmositica*. Woo & Thomas (1991) showed that *C. salmositica* has 21 polypeptide bands (21 to 200 kDa in 1-D SDS-PAGE) and these were detected using rabbit anti-*C. salmositica* polyclonal antibodies. Verity & Woo (1996) identified a common cytoplasmic antigen (49 kDa) in haematozoic *Cryptobia* spp. using a monoclonal antibody (MAB-007) against *C. salmositica*.

The main objective of the present study was to characterize an antigen recognized by a protective monoclonal antibody (MAB-001) which was produced against *Cryptobia salmositica* (see Feng & Woo 1996).

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MATERIALS AND METHODS

Preparation of antigen. *Cryptobia salmositica* from the blood of an infected rainbow trout was cultured for no more than 10 wk at 10°C in Minimum Essential Medium (MEM; Gibco Life Technologies Inc., Grand Island, NY, USA) supplemented with 25% foetal bovine serum (FBS; Gibco) (Woo & Li 1990). The parasite cultured for 10 wk is still able to cause disease in rainbow trout (Woo & Thomas 1991) and this strain is referred to as the pathogenic strain in the present study. Parasites were harvested by washing 3 times at 4°C (centrifugation at $10\,000 \times g$ for 15 min each time) in cold blooded vertebrate Ringer's solution (CBVR) and resuspended in cold CBVR. Parasite numbers were determined using a haemocytometer (Archer 1965). After the last wash, 0.5% Triton X-100 in ice cold 10 mM Tris-HCl, pH 7.4, 150 mM NaCl with 2 mM EDTA was added, vortexed for 10 s, incubated for 1 h at 4°C, then centrifuged at $7\,000 \times g$ for 10 min at 4°C. The supernatant was saved and stored at -100°C. The total protein concentration was determined according to Bradford (1976).

For Triton X-114 solubilization of the pathogenic *Cryptobia salmositica*, detergent and aqueous phase fractions were obtained from temperature-dependent phase separation of the parasite extract (incubated 1 h at 37°C) (Bordier 1981). This was followed by centrifugation at $120 \times g$, 37°C in a Brinkmann eppendorf microfuge on a 6% sucrose cushion, in 0.06% Triton X-114 (in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl). Fractions were analyzed using SDS-PAGE and immunoblotting.

To collect secreted antigen, 1×10^8 *Cryptobia salmositica* in log-phase of culture (no round forms present) were washed 3 times with Ringer's saline, resuspended in 200 ml high glucose DMEM and cultured at 10°C for 24 h. Cells were then pelleted by centrifugation at $9\,000 \times g$ for 25 min at 4°C and the supernatant was carefully removed. The supernatant was then filtered through a 0.2 µm filter and concentrated with a 100 000 MW cut-off microconcentrator (Amicon, Beverly, MA, USA). The concentrated supernatant was stored at -20°C until needed.

Monoclonal antibody. The monoclonal antibody was produced in the earlier study (Feng & Woo 1996). Briefly, spleen cells from one pathogenic *Cryptobia salmositica* immunized BALB/c mouse were fused with NS-1 myeloma cells to produce hybridomas. The hybridomas were cloned and screened for production of antibodies. One positive hybridoma was selected and the antibody produced (IgG1 with kappa light chain) was designated MAb-001 and used in the present study.

Electrophoresis and Western immunoblotting. Parasite proteins were separated using a discontinuous gel

system (Laemmli 1970) in a mini gel apparatus (Bio-Rad Laboratories). Unless otherwise stated, 10% acrylamide gels were used. For reducing SDS-PAGE, samples were dissolved in buffer containing 150 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol and 20% glycerol. For non-reducing SDS-PAGE, samples were dissolved in the same buffer without 2-mercaptoethanol. Gels were stained with either Coomassie brilliant blue or silver stain. Molecular mass was estimated by comparison with commercial molecular weight standards (Bio-Rad).

Two-dimensional gel electrophoresis was performed using the method as described by O'Farrell (1975). Isoelectric focusing gels were cast and placed in the running chamber and prefocused for 1 h at 200 V. Membrane extracts of pathogenic *Cryptobia salmositica* were applied to the top of the column. Isoelectric focusing was allowed to proceed for 20 h at 750 V. The focused gels were applied to the top of a 13% slab gel with a 5% stacking gel. The gels were run for 1.5 h at 100 V, and were either stained with silver stain or blotted and probed with the monoclonal antibody.

After electrophoresis, the separated proteins were transferred to nitrocellulose membrane (Towbin et al. 1979). Electrophoretic transfer was for 2 h at 100 V. The membrane was oxidized with 5 mM sodium *m*-periodate (unless otherwise stated) after transfer, blocked with 5% skimmed milk in Tris-buffered saline (TBS), washed in TBS, and incubated in MAb-001 overnight at room temperature. A nitrocellulose membrane incubated in hybridoma culture medium was used as a negative control. The membranes were washed in Tween-20 Tris-buffered saline (TTBS) followed by TBS (2 washes each, 5 min per wash), and then incubated for 1 h in goat anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad) diluted 1:3000 in 3% skimmed milk/TTBS. Membranes were washed as before, and incubated in enzyme substrate, 5-bromo-4-chloro-3-indolyl phosphate/Nitroblue tetrazolium (Bio-Rad) for 20 min.

Periodate oxidation. Periodate oxidation was carried out as described by Woodward et al. (1985). This was to demonstrate carbohydrates in the antigen recognized by MAb-001. Following blotting, the nitrocellulose sheet was cut into a number of strips and adjacent pairs of strips were used as experimental and control strips. All strips were rinsed briefly with 50 mM sodium acetate buffer (pH 4.5) after which control strips were incubated in the same buffer for 1 h. Experimental strips were exposed to varying concentrations of periodate (0.1 to 20 mM) in buffer (pH 4.5) for 1 h in the dark at room temperature (about 20°C). Both the control and experimental strips were rinsed with sodium acetate buffer and exposed to 50 mM sodium borohydride in TBS for 30 min at room temperature. After 3 washes

with TTBS, the strips were blocked with 5% skimmed milk in TBS for 1 h at room temperature, and then exposed to MAb-001, followed by goat anti-mouse IgG and to its substrate.

Protease K digestion. Protease digestion was performed to confirm the polypeptide nature of the antigen (Parish et al. 1985). Protease K in 50 mM Tris-HCl, pH 8.0, in different concentrations were incubated with blotted strips at 37°C for 1 h after antigen transfer. The strips were washed in TTBS once and 12% trichloroacetic acid (TCA) to inactivate the protease, blocked, incubated with MAb-001 followed by goat anti-mouse IgG and its substrate.

RESULTS

Biochemical characterization of the antigen recognized by MAb-001

Carbohydrate moiety. MAb-001 recognized about 15 bands (1-D gel electrophoresis; molecular mass ranging from 40 to 200 kDa) of *Cryptobia salmositica* before periodate oxidation. Following treatment with

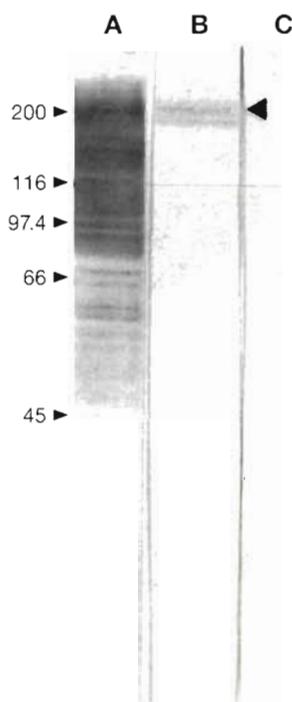


Fig. 1. Immunoblot analysis of the reactivity of *Cryptobia salmositica* lysate with MAb-001 after 5 mM sodium periodate oxidation (lane B); lane A: Ag-MAb reaction before oxidation; lane C: antigen incubated with hybridoma culture medium. Arrow to right indicates the 200 kDa antigen recognized by MAb-001 (lane B). Numbers on the left indicate the molecular weights (kDa) of protein standards (Bio-Rad, high range)

sodium periodate (0.5, 1, 5, 10, or 20 mM ml⁻¹), there were reductions in the number of bands recognized by MAb-001 (0.5 mM, 10 bands; 1 mM, 6 bands; 5 mM, 2 bands). At a concentration of 5 mM ml⁻¹ periodate, MAb-001 only recognized a doublet antigen of molecular weight 200 kDa (Fig. 1). At concentrations greater than 5 mM ml⁻¹ periodate, MAb-001 binding was completely eliminated and no bands were observed. No reactions were obtained with hybridoma culture medium. The sensitivity of the antigen to periodate oxidation indicate that there is a carbohydrate moiety in the MAb-001 recognized antigen.

Polypeptide component. There were no reactions between the antigen and MAb-001 after incubation with both 5 mM sodium periodate and protease K (Fig. 2); however, separate incubation of the antigen with either 5 mM sodium periodate or protease K, MAb-001 will still probe out either the carbohydrate or the polypeptide components of the antigen. There were no reactions between the antigen and hybridoma culture medium. These results show that the antigen

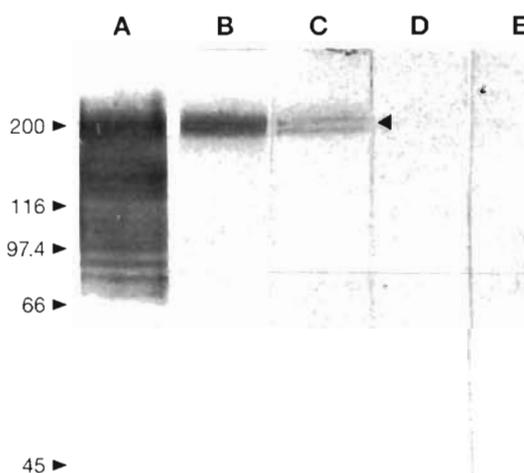


Fig. 2. Immunoblot analysis of the reactivity of antigen of *Cryptobia salmositica* with MAb-001 after protease K digestion. Lane A: Ag-MAb reaction before sodium *m*-periodate oxidation; lane B: reaction of MAb-001 after protease K digestion; lane C: after sodium *m*-periodate oxidation; lane D: after both sodium *m*-periodate oxidation and protease K digestion; lane E: *C. salmositica* antigen incubated with hybridoma culture medium as a negative control. Arrow to right indicates the 200 kDa antigen recognized by MAb-001. Numbers on the left indicate the molecular weights (kDa) of protein standards (Bio-Rad, high range)

(200 kDa) recognized by MAb-001 is a glycoprotein with a polypeptide backbone and carbohydrate moieties. This antigen is designated as Cs-gp200 in all subsequent studies.

Native and reduced antigen. Under non-reducing condition, the migration of the native protein (heated or not heated) was significantly slower than that of the reduced form of the antigen. Under reducing conditions and without heating, Cs-gp200 migrated at the same rate as the native antigen. However, the reduced antigen moved faster than the native antigen after the sample was boiled at 90°C for 10 min. This indicates that the antigen is not a linear polypeptide and there are disulphide bonds in the protein. The change in antigen migration after reducing and heating is because of unfolding of the protein due to the treatments (Fig. 3).

Isoelectric point and subunits. The total membrane protein profile is quite complex (Fig. 4A). After 2-D gel electrophoresis (silver staining), the 200 kDa antigen again showed up as a doublet and was not associated with the other proteins. However, MAb-001 recognized 6 spots (Fig. 4B) before and 2 spots (Fig. 4C) after

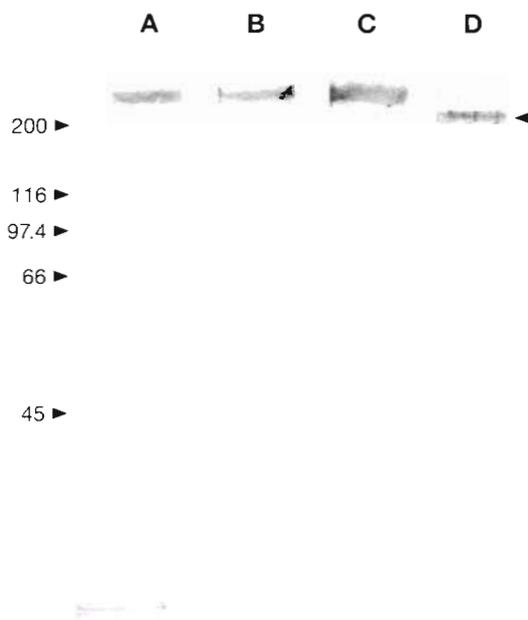


Fig. 3. Immunoblot analysis of the effects of reducing and non-reducing conditions on protein migration. Lane A: not heated and not reduced; lane B: heated but not reduced; lane C: reduced but not heated; lane D: heated and reduced. After SDS-PAGE, antigen was transferred onto nitrocellulose and all strips were oxidized with 5 mM sodium periodate in order to show the MAb specific antigen. Numbers on the left indicate the molecular weights (kDa) of protein standards (Bio-Rad, high range)

6 mM periodate oxidation. The 2 spots (1 major and 1 minor indicating the 2 subunits) are located at PI (isoelectric point) 5.4 to 5.5.

Solubility of the Cs-gp200 in Triton X-114. Since the antigen is a glycoprotein and is located on the membrane of the parasite, we investigated the solubility of the antigen in Triton X-114 (Fig. 5). The antigen was partitioned into the aqueous phase suggesting that it is a hydrophilic protein.

Excretory-secretory products from parasites to culture medium. After incubation of live parasites in a non-protein medium, the parasites were removed from the medium, and the medium was analyzed using SDS-PAGE and Western blot. The culture medium contained a number of polypeptides (30 to 200 kDa) including the 200 kDa antigen which was recognized by MAb-001 (Fig. 6). This shows that the 200 kDa antigen is also released by living parasites.

Antibodies against the 200 kDa antigen in polyclonal antisera

This antigen was also recognized by antisera from trout (which had recovered from a *Cryptobia salmositica* infection) indicating that this antigen also elicits the production of antibody in fish during infection (Fig. 7).

DISCUSSION

Periodate oxidation has been used to characterize surface-exposed epitopes on *Chlamydomonas* (see Woodward et al. 1985) and to denature a wide range of carbohydrate moieties in parasites (Omer-Ali et al. 1986, Lustigman et al. 1990, Ravindran et al. 1990, Zihao et al. 1991). In the present study, MAb-001 recognized about 15 polypeptide bands before periodate oxidation. However, the antibody binding decreased with increasing concentrations of sodium periodate (0.5 to 20 mM) and binding was completely lost at concentrations higher than 5 mM. Following 5 mM periodate treatment, MAb-001 only detected a doublet (200 kDa) and the reduction of bands indicates the presence of carbohydrate in the antigen. Further studies on the epitope of Cs-gp200 by coupling the antigen with biotin-hydrazide and deglycosylating the protein with glycosidases showed that the N-glycan was attached to the antigen (Feng & Woo 1997b). Since carbohydrates alone can be antigenic and some carbohydrate moieties are not sensitive to periodate oxidation, we digested blot strips with protease K after oxidation. The band disappeared after protease K digestion suggesting the antigen recognized by MAb-001 is a glycoprotein and contains a carbohydrate and

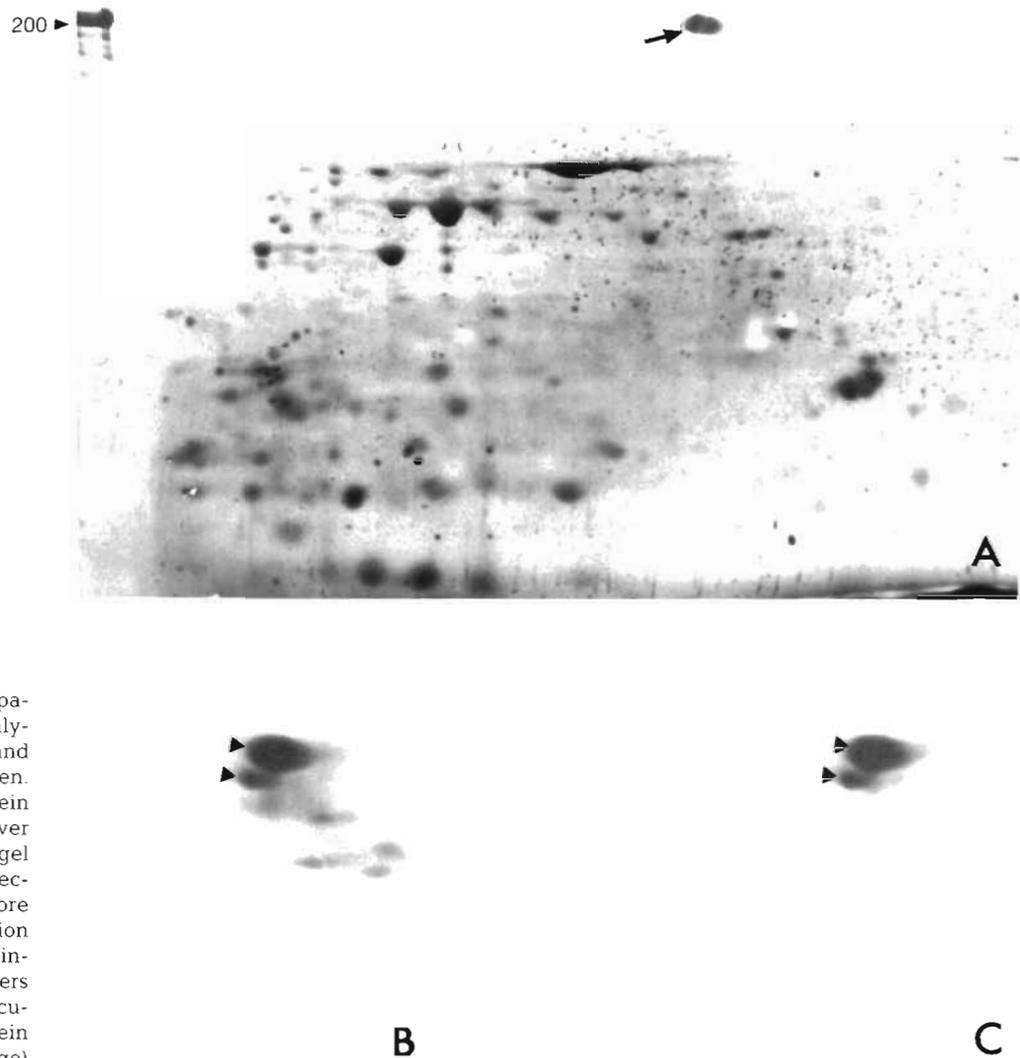


Fig. 4. Two-dimensional separations and immunoblot analysis of isoelectric point and isoforms of 200 kDa antigen. (A) Total membrane protein profiles in the 2-D gel (silver stain); the acidic end of the gel is to the right. (B) Antigen recognized by MAb-001 before oxidation. (C) After oxidation (regional). The arrow heads indicate the antigen and numbers on the left indicate the molecular weights (kDa) of protein standards (Bio-Rad, high range)

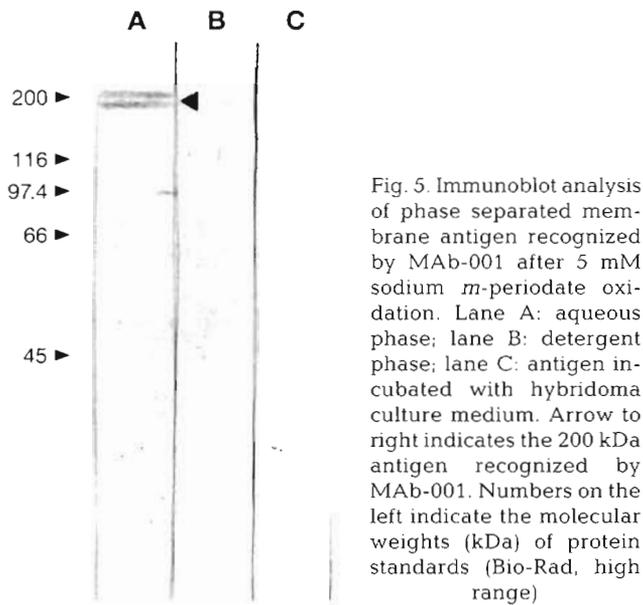
a polypeptide antigenic determinant. Since the antigen migrated faster under reducing conditions than under non-reducing conditions, it is likely that the antigen is a conformational polypeptide with disulphide bonds. Another study showed that reduction of the antigen using DTT, and carboxymethylation using iodoacetic acid significantly decrease the reaction between the antigen and MAb-001 (Feng & Woo 1997b). This further provides confirmation of existence of internal disulphide bonds.

The antigen has an isoelectric point of 5.3 to 5.5 indicating that it is an acidic protein. Chaudhuri & Chang (1988) reported that the gp63 glycoprotein in *Leishmania mexicana* is an acid protease. Further studies on Cs-gp200 have indicated that it is likely to be a metallo-protease (Zuo et al. 1997).

In the phase separation using Triton X-114, the antigen was partitioned into the aqueous phase suggesting that the antigen is hydrophilic and is not an integral

membrane protein. The precise way the 200 kDa antigen is bound to the membrane is not clear. However, Bordier et al. (1986), who characterised the major surface proteins of *Leishmania major* and *L. donovani*, found that the phospholipid anchors of the protein were susceptible to cleavage by phosphatidylinositol-specific phospholipase C from *Bacillus cereus*. The cleavage resulted in the generation of the hydrophilic form from the amphiphilic form of the protein. We also found that the epitope of our antigen was susceptible to phospholipase C, which is specific for phosphatidylinositol (Feng & Woo 1997b). Therefore it is possible that our antigen also contains a lipid anchor and that this anchor is released before the antigen becomes soluble in the lytic buffer during the preparation of whole cell lysate.

Our present study showed that Cs-gp200 is a secretory-excretory antigen because it was found to be released by live parasites (centrifuged and filtered with



0.22 μ m pore size membrane to avoid parasite contamination). Study of the location of antigen using immunogold labelling showed that the antigen was located on the cell membrane and in cytoplasmic vac-

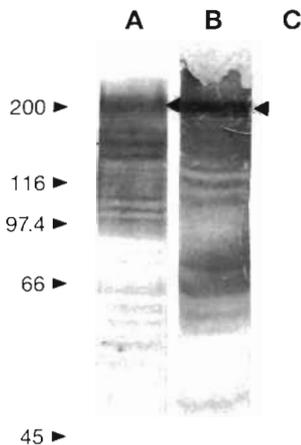


Fig. 6. Immunoblot detection of excretory/secretory (E-S) products of *Cryptobia salmositica*. Lane A: cell lysate of *C. salmositica*; lane B: E-S products after 24 h culture; lane C: non-protein culture medium. Arrow to right indicates the 200 kDa antigen recognized by MAb-001. Numbers on the left indicate the molecular weights (kDa) of biotinated protein standards (Bio-Rad, high range)

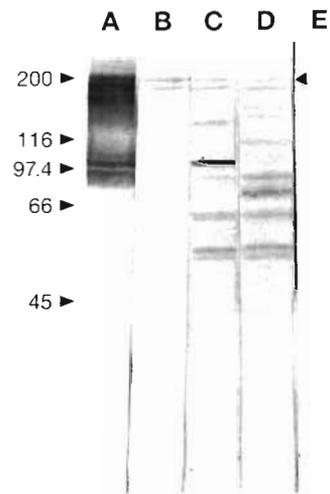


Fig. 7. Immunoblot analysis of the immunogenicity of 200 kDa antigen. Lane A: antigen recognized by MAb-001 before oxidation. Lane B: after oxidation. Lane C: antigen recognized by trout anti-*C. salmositica* serum from infected fish. Lane D: trout anti-*C. salmositica* serum from vaccinated fish. Lane E: representative of preimmune serum from mouse and fish. Arrow to right indicates the 200 kDa antigen recognized by MAb-001. Numbers on the left indicate the molecular weights (kDa) of protein standards (Bio-Rad, high range)

uoles (unpubl. data). This suggests that Cs-gp200 can be secreted from cytoplasm of parasite or from cell membrane. MAb-001 also recognized a number of polypeptides from medium in which we had cultured the parasite. This would indicate cross-reactions between MAb-001 and other glycoproteins that are secreted/excreted by the parasite. Parasites such as *Schistosoma mansoni*, *Giardia lamblia*, *Ichthyophthirius multifiliis* and *Trypanosoma cruzi* release excretory-secretory materials into the host (Nash et al. 1981, 1983, Ouaiissi et al. 1990, Xu et al. 1995). We believe that these excretory antigens may be important for the development of protective immunity by the host. The antigen recognized by MAb-001 was also recognized by antisera from trout and the antibody could be involved in protection against *Cryptobia salmositica*. We showed that MAb-001 inhibits parasite multiplication and kills them under *in vitro* conditions (Feng & Woo 1996). Also, MAb-001 is therapeutic when injected into trout at the acute phase of the disease (Feng & Woo 1997a).

Acknowledgements. This study was supported by grants from the Department of Fisheries & Oceans and the Natural Science and Engineering Research Council of Canada to P.T.K.W. We thank Drs J. S. Lam and L. Mutharia for advice on hybridoma technology, and Dr J. Bogart and Miss L. Rye for advice in taking photomicrographs.

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Editorial responsibility: Wolfgang Körting,
Hannover, Germany

Submitted: June 26, 1997; Accepted: November 11, 1997
Proofs received from author(s): January 29, 1998