

Conjugation of isometamidium chloride to antibodies and the use of the conjugate against the haemoflagellate, *Cryptobia salmositica* Katz, 1951: an immuno-chemotherapeutic strategy

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Abstract

The trypanocidal drug isometamidium chloride (Samorin) was conjugated to polyclonal and monoclonal antibodies produced against the pathogenic haemoflagellate *Cryptobia salmositica*. Under *in vitro* conditions the unconjugated drug normally accumulates rapidly in the kinetoplast in the parasite; however, once it was conjugated to antibodies (either polyclonal or monoclonal) it was found throughout the parasite. Isometamidium conjugated to polyclonal antibodies lysed *C. salmositica* under *in vitro* conditions, but parasites were not agglutinated. In contrast, isometamidium conjugated to monoclonal antibodies (against a 200 kDa surface membrane glycoprotein) did not lyse *C. salmositica*, but parasites were agglutinated. Because of the low efficacy of the monoclonal conjugate against the parasite *in vitro*, its cryptobiocidal effect was not evaluated further. The infectivity of *C. salmositica* (incubated either in culture medium or whole blood) was reduced in fish after *in vitro* exposure to isometamidium conjugated to polyclonal antibodies. Parasitaemias were reduced in infected chinook salmon, *Oncorhynchus tshawytscha*, after treatment with isometamidium conjugated to polyclonal antibodies.

Keywords: *Cryptobia*, isometamidium, treatment, antibodies, conjugates

Introduction

Cryptobia salmositica Katz, 1951 (Kinetoplastida, Bodoniidae) is a pathogenic haemoflagellate that causes disease and mortality in all Pacific salmon species, *Oncorhynchus* spp., on the West coast of North America (Woo 1992, 1994). Transmission of the parasite occurs through the bite of a blood-sucking aquatic leech, *Piscicola salmositica* (Becker & Katz 1965) or directly between infected fish (Bower & Margolis 1983; Woo & Wehnert 1983). Within Canada, the parasite occurs in the Fraser River system and in rivers on both the East and West coasts of Vancouver Island (Bower & Margolis 1984).

High mortality has been associated with *Cryptobia* infections of wild post-spawning rainbow trout, *O. mykiss* (Walbaum), and pre-spawning chinook salmon, *O. tshawytscha* (Walbaum), and in at least one hatchery in Washington State, USA the annual loss of broodstock is 50–60% (Woo & Poynton 1995). In Canada, an outbreak occurred in 1997 and large numbers of chinook salmon smolts and broodstock died in a hatchery and in sea cages on Vancouver Island. Another outbreak of cryptobiosis occurred in Caspian salmon, *Salmo trutta caspius* Kessler, in 1996 at the Ardon Hatchery, Republic of North Ossetia-Alania, Russia (see Woo 1998). The most recent outbreak of cryptobiosis occurred in chinook salmon in early 2001; these were pre-harvest fish (about 3 kg) held in sea cages off Vancouver Island, Canada (Bruno & Woo 2002).

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A major goal of current research on parasitic diseases in fish is vaccine development, as mass vaccination represents an ideal means of controlling parasitic infection. However, despite the widespread consensus concerning the need for immunization against parasitic diseases, there are no commercial vaccines against parasitic diseases in fish. In general, vaccines against parasites may not always be practical and it usually takes several years to develop and test a vaccine. Chemotherapy may be a more rapid and alternative approach to control parasitic diseases. However, chemical control (prophylactic and therapeutic) may not be a good long-term strategy if fish are for human consumption. Thus, future areas of research should focus on a better understanding of the disease process and to protect fish from disease outbreaks with minimal use of chemicals.

Our control strategies against salmonid cryptobiosis include breeding of *Cryptobia*-resistant fish (Forward, Ferguson & Woo 1995), elevation of water temperatures to control parasitaemia in fish (Woo, Wehnert & Rogers 1983; Bower & Margolis 1985) and vaccination (Li & Woo 1995). The vaccine is a live attenuated strain (Woo & Li 1990) that protects adult and juvenile rainbow trout from cryptobiosis for at least 2 years (Sitja-Bobadilla & Woo 1994; Li & Woo 1995) however, the vaccine is not therapeutic (Li & Woo 1995). Ardelli & Woo (1999, 2001a) recently demonstrated that isometamidium chloride, a drug used routinely to treat mammalian trypanosomiasis (Kinabo, Bogan, McKellar & Murray 1989), is therapeutic against *C. salmositica* in rainbow trout and chinook salmon. Ardelli & Woo (2001b) demonstrated that under *in vitro* conditions, isometamidium inhibited multiplication of *C. salmositica*, reduced its oxygen consumption, caused a rapid decantation of kinetoplast DNA, and lysed the parasite. If isometamidium chloride is to be used to treat infected broodfish its effects on the quality and quantity of eggs and sperms should be evaluated. Thus, a method to reduce the amount of drug delivered to fish would be desirable.

Serotherapy (passive transfer of immune serum) has been used to treat infectious diseases (Kuby 1992). There are many difficulties associated with serotherapy which include obtaining large quantities of antisera of sufficient specificity, side-effects associated with the use of crude antisera (i.e. development of an anti-idiotypic response, anaphylactic reactions) (Schroff, Foon, Beatty, Oldham & Morgan 1985), and the slow action of serotherapy

against a number of parasitic diseases (i.e. malaria, trypanosomiasis). Experimental serotherapy with monoclonal antibodies has been conducted against many protozoan parasites including *Trypanosoma cruzi* (Heath, Martins & Hudson 1990; Franchin, Periera-Chioccola, Schenkman & Rodrigues 1997), *Ichthyophthirius multifiliis* (Lin, Clark & Dickerson 1996) and *C. salmositica* (Feng & Woo 1997). The benefits of serotherapy and chemotherapy have been combined in cancer treatment in the form of immunotoxins (Schroff *et al.* 1985). The conjugation of a drug to a specific antibody has the benefits of specificity, reducing the amount of toxin required and reducing damage to host tissue.

The objectives of the present study were to (1) conjugate isometamidium chloride to monoclonal and polyclonal *C. salmositica* antibodies, (2) examine the *in vitro* effects of the conjugates on *C. salmositica* and the effects of such treatment on the infectivity of the parasite, and (3) examine the efficacy of the conjugates against *C. salmositica* in chinook salmon.

Materials and methods

Culture of *C. salmositica*

A cloned strain of pathogenic *C. salmositica*, isolated from *P. salmositica*, was used to infect rainbow trout held at 10 °C (Woo 1979). To culture pathogenic *C. salmositica*, blood from an infected trout was aseptically inoculated into sterile tissue culture bottles containing minimum essential medium (MEM). The MEM was supplemented with Hank's salts, L-glutamine, 25 mM HEPES buffer and 25% heat-inactivated foetal bovine serum. After 3 days (to allow red cells to settle) the MEM containing *C. salmositica* was withdrawn from the flask and inoculated into sterile flasks containing MEM to establish a cell-free culture. Flasks were incubated at 10 °C for no more than 8 weeks as the parasite maintains its pathogenicity to trout only in short-term culture (Woo & Thomas 1991). The non-pathogenic *C. salmositica* was cloned and has been maintained in MEM since 1989. This attenuated strain is infective to fish but does not cause disease (Woo & Li 1990).

Enumeration of *C. salmositica*

Cryptobia salmositica (from blood or culture) were counted using a haemocytometer (Archer 1965).

Blood or MEM containing parasites was diluted in cold-blooded vertebrate ringers (CBVR), dispensed into both chambers of the haemocytometer and counted under a microscope (ocular 10× and objective 16×). Low parasitaemias were detected using the haematocrit centrifuge technique (Woo & Wehnert 1983).

Production of anti-*C. salmositica* antibodies

Ten rainbow trout were immunized with 100 000 non-pathogenic *C. salmositica* (vaccine strain of the parasite) and then challenged with 100 000 pathogenic *C. salmositica* at 4 weeks after vaccination. Blood samples were obtained and antibody titre was determined using an ELISA (Sitja-Bobadilla & Woo 1994). When ELISA OD values were high, fish were then exsanguinated and the γ -globulin fraction (containing antibodies) was separated from plasma using affinity chromatography or dialysis (see below). Antibodies were purified and then analysed for purity, molecular weight and protein concentration.

Monoclonal antibodies were produced against a membrane glycoprotein (Feng & Woo 1996). Essentially, spleen cells from one BALB/c mouse immunized against *C. salmositica* were fused with NS-1 myeloma cells to produce hybridomas. The hybridomas were cloned and screened for production of anti-*C. salmositica* antibodies. One hybridoma was selected and the antibody produced (IgG1 with κ light chain) was against a 200 kDa surface membrane glycoprotein. It was designated MAb-001. Large volumes of MAb-001 were produced using a Cell-Pharm™ mini bioreactor system (Unisyn Technologies, Canberra Packard Canada Ltd, Mississauga, ON, Canada) (Feng & Woo 1997).

Purification of anti-*C. salmositica* antibodies

Polyclonal antibodies were purified according to Johnson (1990) using diethylamino ethyl (DEAE) Affi-Blue gel serum IgG purification columns and desalting columns (Bio-Rad Laboratories, Mississauga, ON, Canada). Plasma was desalted and the γ -globulin fraction was separated using DEAE purification columns. Fractions were collected and the absorbance at 280 nm (200 μ L aliquot from each fraction) was determined using a spectrophotometer. Effluent tubes containing the unbound protein were combined. The process was repeated

until all desalted plasma had been purified. Purified antibodies were concentrated on a bed of polyethylene glycol (mol wt 7000–9000) at 4 °C until the volume in the fraction was reduced to 1.0 mL. After concentration, the solution was removed from the dialysis tubing and stored at 4 °C until use.

In an alternative method, 0.4 mL of plasma was added to a centrifuge tube containing 9.6 mL of a precipitating buffer (193 g of ammonium sulphate and 40 g NaCl L⁻¹ in distilled water). The sample was mixed (2 min at 24 °C) and then centrifuged (1 h; 300 g at 4 °C). After centrifugation, the supernatant was removed, the precipitate re-suspended and centrifuged as before. The final precipitate was dissolved in 100 μ L of 0.15 M NaCl, and dialysed (24 h at 4 °C) against 0.15 M NaCl. After dialysis, the insoluble protein was removed by centrifugation (300 g at 4 °C). The purified antibodies were concentrated using Centricon-10 centrifugal concentrators (Amicon Fisher Scientific, Unionville, ON, Canada). Monoclonal antibodies (MAb-001) were concentrated from hybridoma supernatant using Centricon-10 centrifugal concentrators with a 100 000 molecular weight cut-off.

SDS-PAGE

Antibodies were assessed for purity using one-dimensional SDS-PAGE with a 10% running gel and a 4% stacking gel. Purified antibody samples were mixed 1:2 with Laemmli sample buffer (Bio-Rad), heated for 2 min in 100 °C water, and allowed to cool. Samples were dispensed to the reservoirs of the stacking gel and electrophoresed (in Tris-glycine-SDS buffer) with a pre-stained standard at 100 V for 1 h. Gels were stained overnight with Coomassie blue and then de-stained (Feng & Woo 1997).

Zone electrophoresis

Zone electrophoresis was used to determine the separation of serum proteins. A 1% (w/v) agarose (dissolved in 0.02 M barbital buffer) gel was poured onto the hydrophilic side of a gel bond film (Sigma, St Louis, MO, USA). Samples (20 μ g mL⁻¹ of unpurified trout immune plasma, or purified antibodies, or bovine serum albumin, or bovine serum γ -globulin) were dispensed to the wells and electrophoresed at 100 V (10 V cm⁻¹ field strength) until the bromophenol blue (1% w/v) was

4 cm from the well. The gel was soaked in fixative (20% TCA in 20% acetic acid solution) for 30 min, soaked in distilled water, pressed, dried, stained with Coomassie blue and then de-stained (Oudin 1980).

Western immunoblot

Protein samples ($2.5 \mu\text{g mL}^{-1}$ of sonicated *C. salmositica*) were electrophoresed using SDS-PAGE (see above) and then transferred to nitrocellulose (2 h at 100 V) for Western immunoblot analysis (Feng & Woo 1997).

Conjugation of polyclonal and monoclonal antibodies to isometamidium chloride

Antibodies were conjugated to isometamidium chloride using the methods of Johnson (1990) as follows. The reaction mixture contained 10 mg mL^{-1} of antibody in 0.15 M NaCl and a 10% volume of $0.5 \text{ M carbonate-bicarbonate}$, pH 9.5. Thirty micrograms of isometamidium mg^{-1} of protein was added to the reaction mixture and rotated gently for 1.5 h with cooling in ice. Unconjugated isometamidium was removed by washing with Sephadex G25 (Sigma, St Louis, MO, USA).

To initially assess conjugation of isometamidium to antibodies, a suspension of Sephadex G25 was prepared in PBS. The Sephadex was allowed to settle and the gel slurry was poured onto a microscope slide and surplus fluid removed. Twenty microlitres of conjugate were applied about 1 cm from one end. After 20 min the slide was examined under a fluorescent microscope equipped for incident illumination but without the objective lens installed.

To assess the specificity of the conjugate, pathogenic *C. salmositica* were incubated in the following treatments for 1 min: (1) isometamidium, (2) polyclonal antibody isometamidium conjugate (PAIC), (3) monoclonal antibody isometamidium conjugate (MAIC), or (4) unconjugated antibodies and drug (polyclonal – PAI or monoclonal – MAI) and then used in the following experiments.

In vitro effects of PAIC and MAIC on *C. salmositica*

To each well ($n = 10$) of a 96-well tissue culture plate was added 1500 pathogenic *C. salmositica*

from culture (washed three times in CBVR) in $25 \mu\text{L}$ of CBVR followed by $25 \mu\text{L}$ of twofold serial dilutions of each of the following treatments: (1) PAIC or MAIC, (2) isometamidium in PBS, (3) PAI or MAI, and (4) polyclonal or monoclonal antibodies. Control wells had parasites with no treatment in either MEM or saline. Plates were incubated for 3 h at 10°C after which wells were examined for living parasites using an inverted microscope (ocular $10\times$ and objective $10\times$).

Infectivity of *C. salmositica* after *in vitro* exposure to PAIC

Experiment 1

To each well ($n = 10/\text{treatment}$) of a 96-well tissue culture plate was added 5000 pathogenic *C. salmositica* from culture (washed three times in CBVR) followed by $25 \mu\text{L}$ of each of the following treatments: (1) PAIC ($30 \mu\text{g}$ isometamidium conjugated to 10 mg mL^{-1} protein in 1 mL of buffer), (2) isometamidium ($30 \mu\text{g}$ in 1.0 mL PBS), (3) PAI (10 mg mL^{-1} protein in 1.0 mL PBS + $30 \mu\text{g}$ isometamidium), (4) polyclonal antibodies (10 mg mL^{-1} in 1.0 mL PBS), and (5) controls with saline. Plates were incubated for 3 h, examined using an inverted microscope for living parasites, and then the contents of each well were inoculated into 50 disease free chinook salmon. The 50 chinook salmon were divided into five groups ($n = 10/\text{group}$): Group A ($249.14 \pm 81.11 \text{ g}$; inoculated with PAIC-treated parasites), Group B ($219.15 \pm 85.13 \text{ g}$; inoculated with isometamidium-treated parasites), Group C ($248.34 \pm 54.71 \text{ g}$; inoculated with PAI-treated parasites), Group D ($221.93 \pm 97.59 \text{ g}$; inoculated with polyclonal antibody-treated parasites), and Group E ($232.41 \pm 76.23 \text{ g}$; untreated controls). The weights of the fish did not differ significantly ($P = 0.8683$) among the groups. Parasitaemias were determined weekly and fish were monitored for mortality.

Experiment 2

Experiment 1 was repeated using pathogenic *C. salmositica* from culture (see above). *In vitro* conditions were as described for experiment 1. Fifty chinook salmon were divided into five groups ($n = 10/\text{group}$): Group 1 ($187.12 \pm 38.15 \text{ g}$; inoculated with PAIC-treated parasites), Group 2

(192.21 ± 52.44 g; inoculated with isometamidium-treated parasites), Group 3 (198.97 ± 93.84 g; inoculated with PAI-treated parasites), Group 4 (182.42 ± 52.09 g; inoculated with polyclonal antibody-treated parasites), and Group 5 (197.09 ± 41.68 g; untreated controls). Weights did not differ significantly between the two groups ($P = 0.7145$). Parasitaemias were determined weekly and fish were monitored for mortality.

Experiment 3

Experiment 1 was repeated using pathogenic *C. salmositica* from the blood of a fish and diluted such that 25 µL of blood contained 5000 pathogenic *C. salmositica*. *In vitro* exposure conditions were as described for experiment 1. Fifty chinook salmon were divided into five groups ($n = 10$ /group): Group F (251.53 ± 58.09 g; inoculated with PAIC-treated parasites), Group G (257.08 ± 52.08 g; inoculated with isometamidium-treated parasites), Group H (249.09 ± 70.94 g; inoculated with PAI-treated parasites), Group I (259.80 ± 71.23 g; inoculated with polyclonal antibody-treated parasites), and Group J (262.40 ± 63.44 g; untreated controls). Weights did not differ significantly between the groups ($P = 0.5712$). Parasitaemias were determined weekly and fish were monitored for mortality.

Efficacy of PAIC against *C. salmositica* in chinook salmon

A 1.0 mL stock solution was made of each of the following treatments: (1) PAIC (containing 10 mg mL⁻¹ of protein and 300 µg mL⁻¹ of isometamidium), (2) PAI (containing 10 mg mL⁻¹ protein and 300 µg mL⁻¹ of isometamidium), and (3) isometamidium (300 µg mL⁻¹ in 1.0 mL of sterile, double-distilled water). A 0.2-mL aliquot of each of the treatments was inoculated intraperitoneally into chinook salmon as follows.

Forty disease-free chinook salmon were divided into four groups ($n = 10$ /group; Group K – 252.25 ± 51.02 g, Group L – 250.89 ± 35.62 g, Group M – 258.29 ± 54.09 g, Group N – 254.48 ± 49.25 g). Each fish was inoculated with 5000 pathogenic *C. salmositica* from the blood of a fish. Fish were bled weekly (0.2 mL fish⁻¹) for 3 weeks and parasitaemias, packed cell volume (PCV) and antibody titres were determined. At 3 weeks post-inoculation (see Ardelli & Woo 2001a), fish in

Group K were inoculated with PAIC, fish in Group L were inoculated with PAI, fish in Group M were inoculated with isometamidium, and fish in Group N were inoculated with saline (untreated controls). Blood samples were obtained at 48 h post-treatment and at 1, 2, and 3 weeks after treatment. Parasitaemias and PCV (used as an indicator of disease) were determined and fish were monitored for mortality.

Statistics

To analyse the effects of treatment on parasite infectivity, as well as the efficacy of the conjugate, a two-way or two-factor repeated measures analysis of variance (RM-ANOVA) was used. The factors time and treatment were considered for each experimental group. When the treatment effects were not normally distributed, the Friedman RM-ANOVA on ranks was used. The two-way RM-ANOVA was used to test the hypothesis of no differences between the groups, but was not able to determine which groups were different, or the sizes of these differences. A Tukey multiple comparison test was used to isolate the differences by performing comparisons between the experimental groups. The Tukey test was used for multiple comparisons because it controls the errors of all comparisons simultaneously therefore, it is less likely to determine that a difference was statistically significant. Significance was evaluated at $P = 0.05$.

Results

Purification of polyclonal anti-*C. salmositica* antibodies

Anti-*C. salmositica* antibodies were purified using affinity chromatography and protein precipitation and dialysis. There were numerous bands before antibodies were purified, however, as the purification process continued, the number of bands decreased (Fig. 1). Zone electrophoresis of the purified antibodies indicated that the sample contained mainly γ -globulin. Proteins in the purified sample migrated a short distance towards the cathode and this migration pattern was similar to that in the control which contained bovine γ -globulin (Fig. 2). The purified polyclonal antibodies recognized three discrete bands of *C. salmositica* (Western immunoblot) and the antigen ranged from 45 to 200 kDa (Fig. 3). The purity, specificity and molecular weight of MAb-001 were assessed in an

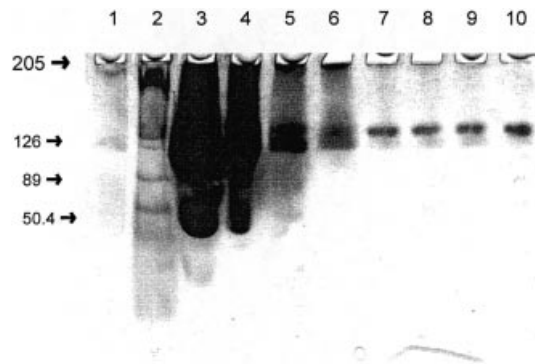


Figure 1 SDS-PAGE of purified trout immunoglobulin. Lane 2 – SDS-PAGE broad range molecular weight standard; Lanes 3 and 4 – unpurified trout plasma; Lanes 5, 7 and 9 – trout plasma purified using affinity chromatography; Lanes 6, 8 and 10 – trout plasma purified using protein precipitation and dialysis. Plasma in Lanes 5–10 were threefold purified.

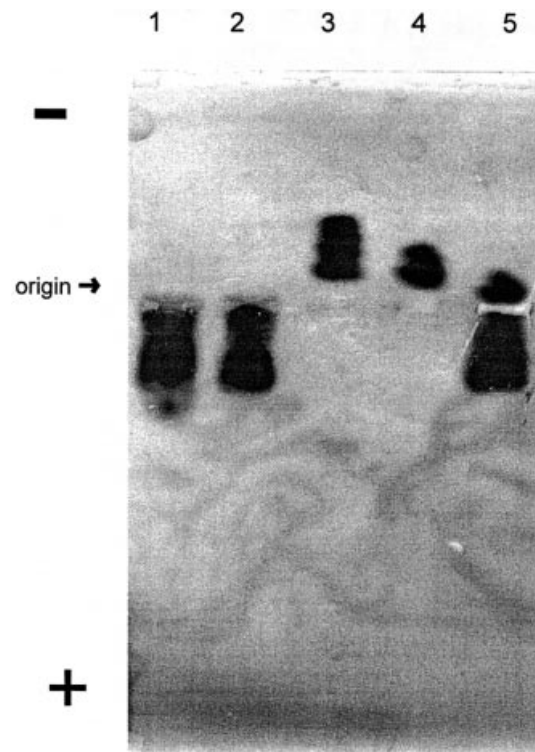


Figure 2 Zone electrophoresis of trout plasma showing separation of serum proteins. The γ -globulin fraction moves towards the cathode and albumin moves furthest towards the anode. Lanes 1 and 2 – bovine serum albumin; Lane 3 – affinity purified goat antitrou trout immunoglobulin; Lane 4 – purified trout plasma; Lane 5 – unpurified trout plasma.

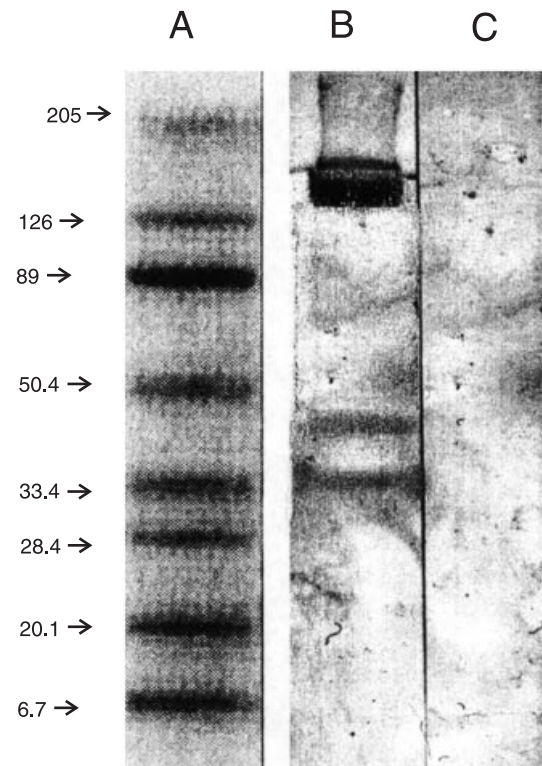


Figure 3 Western immunoblot of purified trout immunoglobulin. Lane A – broad range molecular weight standards (kDa); Lane B – purified anti-*C. salmositica* antibodies from rainbow trout plasma; Lane C – plasma from naive trout.

earlier study (Feng & Woo 1996). The MAb-001 recognizes a glycoprotein (gp200) on the surface of *C. salmositica*.

Conjugation of polyclonal antibodies to isometamidium and its localization in *C. salmositica*

Antibody conjugated to isometamidium was seen as a discrete spot several centimetres from the origin while unbound drug remained in the Sephadex at the point of application. To confirm conjugation of isometamidium to polyclonal antibodies, *C. salmositica* was incubated with isometamidium and with PAIC or MAIC (Fig. 4), and then viewed using phase contrast and fluorescent microscopy. Unconjugated isometamidium accumulated in the kinetoplast of the parasite (Fig. 4a). In contrast, isometamidium conjugated to antibodies was distributed over the body surface including the flagellum (Fig. 4b).

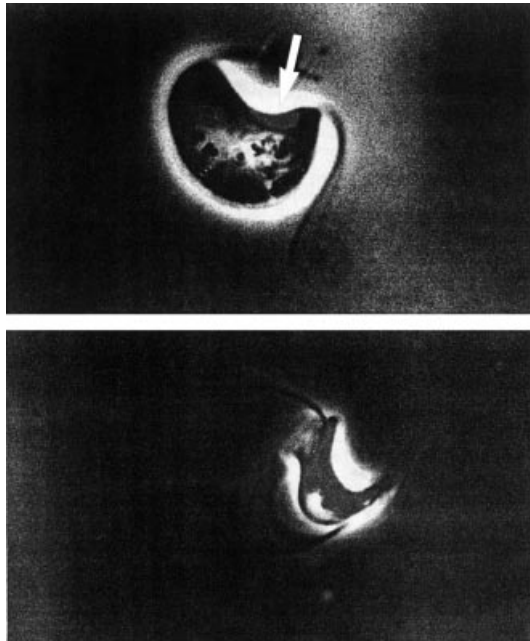


Figure 4 Phase contrast and fluorescent microscopy of *Cryptobia salmositica* after *in vitro* exposure to isometamidium. (a) *Cryptobia salmositica* exposed to isometamidium. Note the accumulation of isometamidium in the kinetoplast (→); (b) *C. salmositica* exposed to isometamidium conjugated to anti-*C. salmositica* polyclonal antibodies.

***In vitro* effects of polyclonal antibody and monoclonal antibody conjugates**

Polyclonal antibody-isometamidium conjugates

The PAIC (purified using affinity chromatography or protein precipitation) lysed *C. salmositica* under *in vitro* conditions, however, the conjugate did not agglutinate the parasite. In contrast, parasites incubated with PAI lysed and also agglutinated *C. salmositica*. Antibody alone did not lyse *C. salmositica*, however, parasites were agglutinated. Isometamidium lysed the parasite under *in vitro* conditions. The PAIC had a higher lytic titre (average 1:16) in comparison to PAI (average 1:8) or to isometamidium (average 1:8). The agglutinating titre of anti-*C. salmositica* antibodies was 1:8. Control wells containing parasites incubated with buffers used in affinity chromatography did not have living parasites. In contrast, wells containing buffers used in protein precipitation still had living parasites.

Monoclonal antibody-isometamidium conjugate

The MAIC did not lyse *C. salmositica*, however, parasites were agglutinated. This was similar to MAI and antibody alone. The agglutinating titre of MAIC (average 1:4), MAI (average 1:4), and antibody (average 1:4) were similar. Because of the low efficacy of MAB-001 under *in vitro* conditions, it was not used in *in vivo* experiments.

Infectivity of *C. salmositica* after *in vitro* exposure to the antibody-drug conjugate

Experiment 1

Conjugation of isometamidium to polyclonal antibodies reduced the infectivity of *C. salmositica*. The PAIC was more effective than drug alone, antibody alone, or PAI. At 7 weeks post-inoculation (end of study) of treated parasites, four of ten fish inoculated with PAIC had detectable infections (Group A). All fish in groups with parasites exposed to isometamidium alone, or PAI, or with no treatment (infected controls) had detectable infections. Parasitaemias remained low in Group A (PAIC) until 6 weeks after infection, when they began to increase. In contrast, parasitaemias in fish in Groups B, C, D, and E continued to increase until 6 weeks after inoculation, and then declined. Also, there was mortality in all groups, except in Group A (PAIC) (Table 1).

A two-way RM-ANOVA was used to determine significant differences between the different treatments. Significant differences were not detected between the groups at 1–2 and 5–7 weeks post-inoculation of treated parasites. At 3 weeks post-inoculation, significant differences ($P = 0.0327$) were detected between Groups A (PAIC) and C (PAI) and Groups B (drug), D (antibody) and E (infected controls). Similarly, at 4 weeks post-inoculation, parasitaemias were significantly lower in Group A (PAIC) in comparison with Groups B, C, D and E.

Experiment 2

This study was conducted to confirm experiment 1 and used a different batch of the antibody-drug conjugate. Parasitaemias in Group 1 (PAIC) remained low throughout the course of the study. At 7 weeks post-inoculation, only one of ten fish had a detectable infection. In contrast, parasitaemias continued to increase in Group 2 (drug),

Table 1 Infectivity of cultured *C. salmositica* to chinook salmon after *in vitro* exposure to isometamidium chloride conjugated to anti-*C. salmositica* polyclonal antibodies (experiment 1)

| Weeks after infection | Group A PAIC | Group B Isometamidium | Group C PAI | Group D Antibody | Group E Untreated controls |
|-----------------------|---|--|-------------------------------------|--|--|
| 1 | 2/10 ^a 0.8 ± 1.75 ^b 0% ^c | 9/10 1.7 ± 1.34 0% | 8/10 3.1 ± 3.18 0% | 8/10 2.7 ± 2.45 0% | 8/10 2.6 ± 3.06 0% |
| 2 | 6/10 0.7 ± 0.67 0% | 5/10 3.7 ± 5.96 0% | 2/8 0.7 ± 1.49 20% | 10/10 11.3 ± 5.72 0% | 10/10 23.8 ± 13.56 0% |
| 3 | 6/10 1.8 ± 2.44 0% | 10/10 18 755 ± 34 482 0% | 6/8 5.0 ± 7.3 0% | 10/10 220 000 ± 122 076 0% | 10/10 221 250 ± 212 708 0% |
| 4 | 3/10 1.7 ± 3.37 0% | 10/10 361 250 ± 461 257 0% | 6/8 36 252 ± 58 465 0% | 10/10 1 963 750 ± 1 285 893 0% | 10/10 3 331 250 ± 3 107 735 0% |
| 5 | 2/10 2750 ± 86 613 0% | 10/10 3 901 250 ± 8 454 280 0% | 7/8 859 378 ± 2 056 605 0% | 10/10 162 500 000 ± 154 373 735 0% | 10/10 82 913 750 ± 167 854 295 0% |
| 6 | 4/10 1 192 500 ± 1 062 583 0% | 10/10 44 457 222 ± 124 969 327 10% | 7/8 78 126 ± 172 268 0% | 10/10 80 535 714 ± 94 281 430 30% | 10/10 77 835 937 ± 140 609 987 20% |
| 7 | 4/10 20 362 500 ± 61 841 0% | 10/10 11 365 627 ± 26 435 638 10% | 7/8 5 171 878 ± 14 577 828 0% | 10/10 56 250 000 ± 60 454 011 0% | 10/10 3 218 750 ± 4 660 966 20% |

^a Number of infected fish/number of fish inoculated.^b Mean parasitaemia ± SD; parasitaemia was determined using the haematocrit centrifuge technique (for low parasitaemias) or a haemocytometer (for high parasitaemias).^c Percentage mortality.**Table 2** Infectivity of cultured *C. salmositica* to chinook salmon after *in vitro* exposure to isometamidium conjugated to anti-*C. salmositica* antibodies (experiment 2)

| Weeks after infection | Group 1 PAIC | Group 2 Isometamidium | Group 3 PAI | Group 4 Antibody | Group 5 Untreated controls |
|-----------------------|---|----------------------------------|-----------------------------|----------------------------------|----------------------------------|
| 1 | 1/10 ^a 0.30 ± 0.95 ^b | 7/10 1.80 ± 2.78 | 0/10 0 | 0/10 0 | 8/10 6.60 ± 4.72 |
| 2 | 1/10 0.30 ± 0.95 | 6/10 8.10 ± 5.28 | 2/10 1.80 ± 3.91 | 0/10 0 | 10/10 48 750 ± 45 814 |
| 3 | 2/10 0.50 ± 1.27 | 7/10 33 250 ± 38 207 | 2/10 1950 ± 4310 | 0/10 0 | 10/10 35 625 ± 29 978 |
| 4 | 2/10 0.30 ± 0.675 | 8/10 302 000 ± 254 142 | 3/10 2500 ± 5270 | 3/10 5558 ± 16 665 | 10/10 5 487 500 ± 5 439 838 |
| 5 | 2/10 0.60 ± 1.58 | 10/10 9 395 000 ± 16 925 911 | 4/10 54 740 ± 112 499 | 4/9 556 944 ± 1 500 001 | 10/10 3 175 000 ± 3 639 196 |
| 6 | 1/10 3750 ± 11 858 | 10/10 13 475 000 ± 15 298 624 | 4/10 503 750 ± 1 030 810 | 4/10 2 600 000 ± 5 577 465 | 10/10 3 243 750 ± 5 416 196 |
| 7 | 1/10 11 250 ± 35 575 | 10/10 18 305 000 ± 52 500 000 | 4/10 928 860 ± 1 326 575 | 4/10 44 383 334 ± 129 150 260 | 10/10 27 051 250 ± 56 209 714 |

^a Number of infected fish/number of fish inoculated.^b Mean parasitaemia ± SD; determined by HCT or haemocytometer.

Group 3 (PAI), Group 4 (antibody), and Group 5 (untreated controls). All fish in Groups 2 and 5 had a detectable infection, while only four of ten fish had detectable infections in Groups 3 and 4.

However, parasitaemias were high in Groups 3 and 4 (Table 2).

Comparison of the different groups using an RM-ANOVA revealed a significant difference. To isolate

Table 3 Infectivity of *C. salmositica* to chinook salmon after *in vitro* exposure to isometamidium in whole fish blood

| Weeks after infection | Group F PAIC | Group G Isometamidium | Group H PAI | Group I Antibody | Group J Untreated controls |
|-----------------------|---|----------------------------------|-------------------------------|-------------------------------|---------------------------------|
| 1 | 1/10 ^a 0.20 ± 0.63 ^b | 9/10 3.50 ± 2.68 | 2/10 0.30 ± 2.68 | 0/10 0 | 10/10 6.40 ± 5.34 |
| 2 | 1/10 0.50 ± 1.58 | 9/10 18.80 ± 12.92 | 2/10 0.70 ± 5.00 | 0/10 0 | 10/10 13 764 ± 18 101 |
| 3 | 1/10 0.70 ± 2.21 | 10/10 146 250 ± 112 120 | 2/10 3.8 ± 11.33 | 1/10 1.2 ± 3.79 | 10/10 820 000 ± 428 304 |
| 4 | 3/10 1.80 ± 3.55 | 10/10 3 700 000 ± 3 300 394 | 2/10 22 502 ± 71 150 | 2/10 1.70 ± 4.72 | 10/10 12 765 000 ± 7 718 517 |
| 5 | 3/10 35 000 ± 110 679 | 10/10 16 825 000 ± 13 098 312 | 2/10 235 000 ± 708 695 | 2/10 28 750 ± 71 698 | 5/5 11 800 000 ± 16 933 832 |
| 6 | 4/10 150 000 ± 422 788 | 6/6 17 218 750 ± 32 821 504 | 1/9 236 111 ± 708 333 | 2/10 287 500 ± 612 514 | 3/3 11 000 000 ± 10 373 305 |
| 7 | 4/10 637 501 ± 1 280 800 | 3/3 2 854 166 ± 4 673 869 | 2/9 3 611 111 ± 10 833 333 | 2/10 3 875 000 ± 8 300 978 | 1/1 18 750 000 |

^a Number of infected fish/number of fish inoculated.

^b Mean parasitaemia ± SD; determined by HCT or haemocytometer.

the group or groups that differed from each other, a Tukey test was used. Significant differences were not detected between the groups between 1 and 6–7 weeks after inoculation with drug treated parasites. However, differences were detected between the groups between 3 and 5 weeks after inoculation. At 3 and 4 weeks post-inoculation, parasitaemias were significantly lower ($P = 0.001$) in Groups 1, 3 and 4 than in Groups 2 and 5. At 5 weeks post-inoculation of treated parasites, the parasitaemia was significantly lower ($P = 0.0434$) in Group 1 (PAIC) as compared with the other groups.

Experiment 3

In vitro exposure of *C. salmositica* (in whole fish blood) to PAIC also altered the infectivity of the parasite. At 7 weeks post-inoculation of treated parasites, only four of ten fish in Group F (PAIC) had a detectable infection. Parasitaemias continued to increase in fish in Group F, but they were lower than parasitaemias in the remaining groups (Table 3). There was 10% mortality in Group F (PAIC), 70% in Group G (drug), 30% in Group H (PAI), 20% in Group I (antibody), and 100% mortality in Group J (untreated controls).

Repeated measures-ANOVA was used to determine significant differences between treatments. There was no significant difference between treatments at week 6 ($P = 0.184$); treatments were not tested at week 7 as there was 100% mortality in the uninfected controls. Significant differences were found between the treatments at weeks 1 through

5. At 1 week post-treatment, parasitaemias were significantly higher in Group J (untreated controls) as compared with Group F (PAIC), Group H (PAI) or Group I (antibody). Similarly, parasitaemias were significantly higher in Group G (drug) in comparison with Group F (PAIC) or Group I (antibody). Similar results were seen at 2 and 3 weeks post-inoculation, as parasitaemias were significantly higher ($P = 0.001$) in Group J (untreated controls) compared with Groups F, H and I and Group G (drug) had significantly higher parasitaemias than either Groups F (PAIC) or I (antibody). At 4 weeks after treatment, significant differences ($P = 0.001$) were detected between Group J (untreated) and Groups F, H and I and between Group G and Groups F, H and I. At 5 weeks post-inoculation, there were significant differences in parasitaemia between Group G and between Groups F, H and I.

Efficacy of polyclonal antibody-isometamidium conjugate against cryptobiosis

Parasitaemias in Groups K (PAIC), L (PAI), M (drug), and N (untreated controls) were low between 1 and 3 weeks after infection and at 48 h post-treatment. Parasitaemias continued to increase in all groups and were highest in the untreated controls (Group N). At 2 weeks after treatment, parasitaemias declined in Groups K and L, but increased in Group L 1 week later. Parasitaemias were always lower in fish treated with the PAIC (Group L) in comparison with the other groups

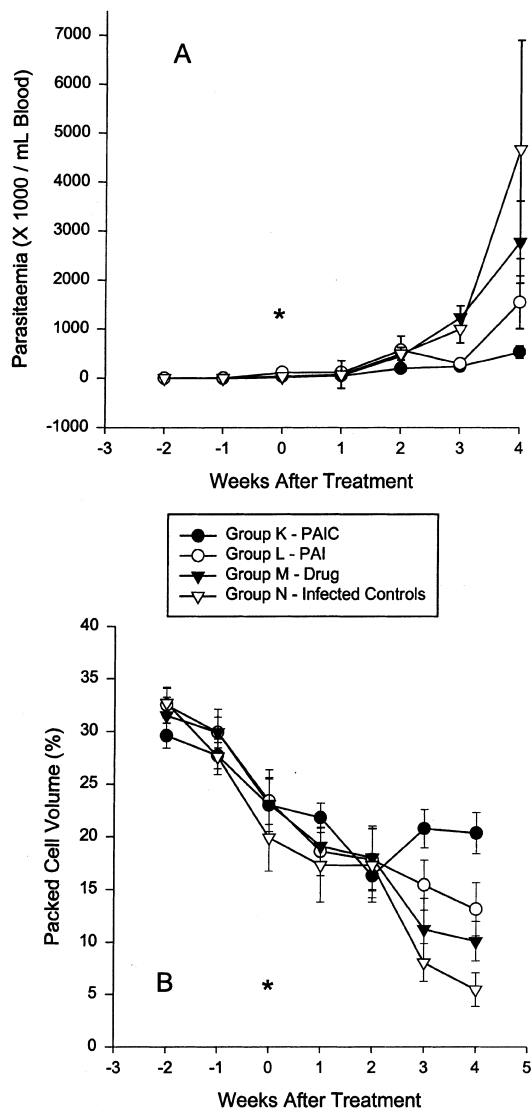


Figure 5 Parasitaemias and packed cell volume in chinook salmon treated with isometamidium chloride conjugated to anti-*C. salmositica* antibodies. (a) Parasitaemia; (b) packed cell volume. * Treatment of fish.

(Fig. 5a). Mortality was 20% in Group K, 40% in Group L and 50% in Groups M and N.

Similar to the parasitaemia, PCV was higher in Group K as compared with Groups L, M or N between 3 and 4 weeks after treatment. However, there was not a significant difference (Fig. 5b).

Discussion

In chemotherapy the ideal delivery system should increase the therapeutic effects of the drug by

improving its delivery to the site(s) of infection, by protecting it from host metabolism, and by decreasing its toxicity to the host. In the present study, isometamidium conjugated to polyclonal antibodies reduced the infectivity of *C. salmositica* (either from culture or from the blood of fish) as parasitaemias were lower in fish inoculated with PAIC-treated parasites.

The lower parasitaemias in fish inoculated with parasites exposed to the drug-antibody conjugate were partly because of the reduced size of the inoculum because the conjugate lysed many of the parasites during the *in vitro* incubation. Parasite lysis was caused by the drug (Ardelli & Woo 2001b) as the antibody would not lyse the parasite because there was no complement, but would coat the parasite and cause agglutination. Antibody-coated parasites inoculated into a fish would probably activate complement resulting in lysis of the parasite and would also be engulfed by phagocytic cells. Also, the number of infective parasites would be reduced because antibodies, especially MAb-001, inhibit metabolic enzymes (e.g. cysteine protease) and subsequently cause cell death (Feng & Woo 1996; Zuo, Feng & Woo 1997).

An intramuscular dose of 1.0 mg kg^{-1} of isometamidium was therapeutic in rainbow trout during pre-clinical and chronic disease (Ardelli & Woo 1999). Also, all juvenile chinook salmon treated at 2 and 3 weeks post-infection survived while 100% of untreated control fish died from cryptobiosis. A higher dose (2.5 mg kg^{-1}) eliminated the infection in 30% of adult fish and parasitaemias were significantly reduced in remaining fish (Ardelli & Woo 2001a). In the present study the concentration of isometamidium delivered to chinook salmon (either in the conjugate, in a mixture of antibodies and drug, or isometamidium alone) was $6.0 \times 10^{-5} \text{ } \mu\text{g kg}^{-1}$. This dose of isometamidium alone (delivered intraperitoneally) did not lower the parasitaemia, while parasitaemia in fish treated with a mixture of isometamidium and antibodies declined 1 week post-treatment and remained lower than in the other groups. This was probably the result of the antibody or the synergistic effects of drug and antibody, as isometamidium alone did not reduce the parasitaemia. A reduction in parasitaemia after injection of immune plasma is not unusual. Feng & Woo (1997) showed significant reduction in parasitaemias in *C. salmositica* infection 48 h after intraperitoneal inoculation of MAb-001. Fish are

thought to have one immunoglobulin isotype, which has the properties of IgM and is thus able to activate complement (van Muiswinkel 1995). The effects of the antibody might not last long as the polyclonal antibodies were produced in rainbow trout and an anti-isotype response might occur in chinook salmon.

Immunotoxins have been constructed as cancer chemotherapeutic agents by chemically or genetically linking a monoclonal antibody to a protein toxin (Siegall, Wolff, Gawlak, Leland, Chace & Mixan 1995). These immunotoxins have been used to treat B-cell neoplasms such as non-Hodgkin's lymphomas, melanoma and colorectal cancer. In all cases, the best clinical responses were in patients with a minimal tumour burden and resulted in 30% tumour reduction (Siegall *et al.* 1995). Attempts have been made to use this approach against haemoparasites. Gelonin has been linked to human transferrin as a form of directed targeting against *Plasmodium falciparum* (Surolia & Misquith 1996). Also, immunotoxins have been constructed with IgG antibodies against *T. cruzi* surface antigens by hybridization with abrin and ricin A chains. The conjugates worked well under *in vitro* conditions, however, they did not reduce the parasitaemia or increase survival of mice infected with *T. cruzi* (Santana & Teixeira 1989; Teixeira & Santana 1990).

In the present study affinity chromatography and protein precipitation were used to purify polyclonal antibodies. Both methods worked well as antibodies were relatively free of albumin and were specific for *C. salmositica*. Conjugation of isometamidium to the purified antibodies was successful, however, the buffers used in affinity chromatography were toxic to the parasite. As a result, only antibodies purified using a combination of protein precipitation and dialysis were used for further studies as these buffers were not toxic to *C. salmositica*.

Polyclonal antibodies conjugated to isometamidium lysed *C. salmositica in vitro*, but the antibodies no longer agglutinated the parasite. A mixture of antibodies and drug not only lysed the parasite but also agglutinated it. The basic structure of an antibody molecule consists of two identical light chains and two identical heavy chains, which are linked by disulphide bonds. The heavy and light chains contain both variable and constant regions. Within the variable regions are three hypervariable subregions, or complementarity-determining regions. Antibody specificity depends largely on

the structure of the complementarity determining regions, which are the antigen binding sites. It is unclear why the drug-conjugated antibodies did not agglutinate parasites. Perhaps the conjugation procedure altered the hypervariable regions, or blocked sites within these regions such that agglutination could not occur. Also, the lack of agglutinating activity may be because of restricted flexibility in the hinge-region of the antibody caused by the bound isometamidium, thus making it difficult for the antibody to assume the required angle for optimal cross-linking of epitopes on the parasite. Finally, the number and distribution of epitopes or the locations of some epitopes in deep pockets of the parasite may have made it difficult for antibodies (with bound drug) to attach. Fluorescent image analysis of the conjugated drug showed it was located throughout the parasite. It is likely that the drug-conjugated antibodies bind to epitopes on the surface of *C. salmositica* and are then internalized via endocytosis by the parasite. The conjugate was both in the cytoplasm and kinetoplast after the parasite was exposed to the conjugate while isometamidium was localized in the kinetoplast. This is similar to what is seen in pathogenic trypanosomes of African mammals as isometamidium binds specifically to kinetoplast DNA (Shapiro 1993). Antibodies have been shown to interact with *Leishmania* (Doyle, Behin, Mauel & Rowe 1984) and *T. brucei* (Russo, Grab, Lonsdale-Eccles, Shaw & Williams 1993) and they bind specifically to epitopes on the cell surface (variable surface glycoproteins) and the flagellum. Clearance of the bound antibody complexes followed a directional movement, with clearance of immune complexes from the cell surface onto the flagellum and finally into the flagellar pocket where they were endocytosed.

The present study demonstrated that conjugation of polyclonal antibody-isometamidium conjugate was an effective method for delivering the drug to the parasite under *in vitro* conditions. Although the drug conjugate did not significantly lower the parasitaemias in infected fish, parasitaemias were still lower than in those fish inoculated with just the drug or the drug antibody mixture. Further studies will be required to determine the optimal dose of the antibody-drug conjugate for effective treatment. This approach would likely result in reducing the side-effects of chemotherapy and the immunochemotherapeutic strategy might be useful against other parasitic diseases, especially those in which the pathogens are in the blood.

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