Immunological and Therapeutic Strategies against Salmonid Cryptobiosis

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Salmonid cryptobiosis is caused by the haemoflagellate, Cryptobia salmositica. Clinical signs of the disease in salmon (Oncorhynchus spp.) include exophthalmia, general oedema, abdominal distension with ascites, anaemia, and anorexia. The disease-causing factor is a metalloprotease and the monoclonal antibody (mAb-001) against it is therapeutic. MAb-001 does not fix complement but agglutinates the parasite. Some brook charr, Salvelinus fontinalis cannot be infected (Cryptobia-resistant); this resistance is controlled by a dominant Mendelian locus and is inherited. In Cryptobia-resistant charr the pathogen is lysed via the Alternative Pathway of Complement Activation. However, some charr can be infected and they have high parasitaemias with no disease (Cryptobia-tolerant). In infected Cryptobia-tolerant charr the metalloprotease is neutralized by a natural antiprotease, α2 macroglobulin. Two vaccines have been developed. A single dose of the attenuated vaccine protects 100% of salmonids (juveniles and adults) for at least 24 months. Complement fixing antibody production and cell-mediated response in vaccinated fish rise significantly after challenge. Fish injected with the DNA vaccine initially have slight anaemias but they recover and have agglutinating antibodies. On challenge, DNA-vaccinated fish have lower parasitaemias, delayed peak parasitaemias and faster recoveries. Isometamidium chloride is therapeutic against the pathogen and its effectiveness is increased after conjugation to antibodies.

1. Introduction

Fish has been and will continue to be one of the major sources of animal protein for humans. It will likely become more important as the population heads towards 8 billion in about 20 years as food production (e.g., growing of crops, breeding of domestic animals) has and will continue to compete with other human activities (e.g., transportation, housing, industry) for the limited usable/inhabitable land. Besides being a more affordable animal protein many species of marine fishes have beneficial health components which include the polyunsaturated fatty acids (e.g., Omega 3). However, the capture-fishery is either stagnant or has been in decline as natural fish stocks in many parts of the world have been reduced significantly because of over and/or indiscriminate fishing and/or the destruction of spawning grounds. Many undesirable discharges (e.g., organophosphates, heavy metals) into the aquatic environments, especially from industries, are known to reduce fish survival and reproduction. In some areas fish are no longer suitable for human consumption because of the high levels of accumulated pollutants, and no new fishing grounds have been discovered. According to the Food and Agriculture Organization, aquaculture continues to be the fastest food producing sector with about a 10% annual increase. It would be higher if not for disease outbreaks [1]. Intensive culture of freshwater and marine fishes in cages is well developed in many countries, especially in those that have large numbers of rivers and lakes and/or long coastlines (e.g., China, Chile, Norway). However, disease outbreaks become more frequent as intensive fish culture tends to facilitate the transmission of parasites between fish in cages and the acquisition of pathogens from feral fishes that are attracted to the uneaten food in cages [2].

The piscine immune system is well developed, and in many ways it is similar to that in mammals (e.g., [3]) which include a comparable set of immunocompetent cells [4]. In general, the adaptive immune response is slower to
develop in fish than in mammals, and this is in part due to its lower body temperature. However, the innate immunity in fish is as well developed and is as responsive as that in mammals. The present discussion is in two parts; the first part (Section 2) is a brief review on Cryptobia and the pathobiology in cryptobiosis—information which are relevant to the discussion on the development of strategies against the pathogen and disease (Section 3).

2. Cryptobia salmositica and Cryptobiosis

2.1. The Parasite. Cryptobia is a parasitic flagellate that has worldwide distribution, and a few species are known to cause disease in marine and freshwater fishes. This extracellular parasite is elongated and is a little larger than a fish red blood cell. Its nucleus is close to the kinetoplast which is located at the anterior end. The parasite has an anterior flagellum and a recurrent flagellum that attaches to the body and exit as a free flagellum at the posterior end [5].

Salmonid cryptobiosis is caused by the haemoflagellate Cryptobia (Trypanoplasma) salmositica (Figure 1). The pathogen has been reported in all species of Pacific salmon, Oncorhynchus spp., along the west coast of North America [5], and outbreaks of cryptobiosis with high fish mortalities have occurred in both freshwater hatcheries and in sea cage cultures [6]. The parasite multiplies by binary fission, and the parasitaemia peaks at about 4-5 weeks after infection (e.g., [7–9]). The severity of the disease (e.g., the anaemia) is directly related to the parasitaemia and clinical signs include exophthalmia (Figure 2), general oedema, abdominal distension with ascites, a microcytic and hypochromic anaemia, positive antiglobulin reaction (or positive Coombs’ test) of red cells (Figure 3), and anorexia [7, 10, 11]. Anorexia is a double-edged sword—it is beneficial to the host in that it reduces the severity of the disease by lowering plasma proteins and subsequently the parasitaemia but it is also detrimental to the fish in that it contributes to the immunodepression [11, 12]. During acute disease the haemolytic activity of complement is significantly lowered [13]. In addition, plasma thyroxine (T3 and T4), protein, and glucose are reduced along with depletion of liver glycogen [14]. The metabolism and swimming performance of infected rainbow trout are also significantly reduced [15], and the bioenergetic cost of the disease in juvenile fish is considerable. These are contributing factors to the retarded growth as there are significant reductions in food consumption, dry weight and energy gained, energy concentration, and gross conversion efficiency. However, the attenuated vaccine strain (Section 3.3.1) has no detectable bioenergetic cost to juvenile fish [16].

2.2. A Cysteine Protease and a Metalloprotease. Two proteases have been identified in the pathogen [17]. The cysteine protease consisting of four polypeptide bands (49, 60, 66, and 97 kDa) is a metabolic enzyme while the metalloprotease (200 kDa) is a histolytic enzyme. The metalloprotease has been isolated and purified (Figure 4). Its proteolytic activity is inhibited by excess of zinc ions [17–19]. The purified enzyme lyses red blood cells [20] by digesting erythrocyte membranes [18]. Consequently, it is an important contributing factor to the anaemia which is a very consistent clinical sign of the disease. Also, the purified metalloprotease readily degrades types I, IV, and V collagens (Figure 5) and laminin [18]. It is secreted by the parasite in fish [21] and in culture [19], and it contributes significantly to the development
Figure 4: Purification of cysteine protease and metalloprotease from Cryptobia salmositica. Lane A: crude cell lysate; lane B: partially purified cysteine protease from a DEAE-agarose column; lane C: partially purified metalloprotease from a DEAE-agarose column; lane D: a single band of purified metalloprotease from a Sephacryl S-300 column; lane M: molecular markers (kDa) (reproduced from Zuo & Woo [18]).

of the disease and histopathological lesions in infected fish [22].

Briefly, metalloprotease activities can be neutralized by either a monoclonal antibody (Section 3.1) or a natural antiprotease (Section 3.2.2) or the antibody against the DNA vaccine (Section 3.3.2); this neutralization essentially “disarms” the pathogen so that the host immune system can more readily control the infection.

3. Strategies against Cryptobia and Cryptobiosis

3.1. Serological. A murine IgG1 monoclonal antibody (mAb-001; Figure 6) has been produced against the 200 kDa glycoprotein (Cs-gp200). The antibody is therapeutic when injected intraperitoneally into infected fish—it significantly lowers the parasitaemias in fish and this is similar to the effects of the inoculation of antiserum from fish that had recovered from cryptobiosis. Also, mAb-001 has prophylactic effects in fish [23]; however, it does not fix complement but agglutinates live parasite. In vitro exposure of the parasite to mAb-001 reduces its survival and infectivity when inoculated back into fish [24]. The monoclonal antibody also inhibits parasite multiplication and its aerobic respiration [25], and it completely neutralizes the activity of the metalloprotease [26]. The Cs-gp200 epitope consists of carbohydrate determinants and conformational polypeptide with internal disulfide bonds. It is hydrophilic and is secreted by the parasite [27]. The epitope has its asparagine-bound N-glycosidically linked hybrid-type carbohydrate chain with the minimum length of a chitobiose core unit. It has a phosphatidylinositol residue which anchors the conformational polypeptide (with disulfide bonds) to the surface of the pathogen. The molecule is extensively posttranslationally modified [28], has high mannose components, and appears as a doublet in the pathogenic strain and as a single band in the attenuated vaccine strain [29].

3.2. Innate (Natural) Immunity. In the present discussion, a distinction is made to distinguish between the two forms of natural immunity—the first is absence of disease in an infected fish (pathogen-tolerant fish) and the second is resistance to infection by a fish (pathogen-resistant fish).

3.2.1. Cryptobia-Resistant Fish. Some laboratory/hatchery raised brook charr, Salvelinus fontinalis, cannot be infected
with *C. salmositica*, and this is innate resistance to infection. Resistance to *Cryptobia* infection is inherited by progeny and it is controlled by a single dominant Mendelian locus. *Cryptobia*-susceptible brook charr are homozygous recessive while the *Cryptobia*-resistant fish are either homozygous or heterozygous dominant for the locus [30]. Consequently, we can now breed resistant fish by initially testing the freshly collected plasma from the brood fish for cryptobiacidal effects. Briefly, under in vitro conditions fresh plasma from *Cryptobia*-resistant charr lyse the parasite via the Alternative Pathway of Complement Activation [31]. There is no detectable difference in the immune responses of both *Cryptobia*-tolerant (Section 3.2.2) and *Cryptobia*-resistant charr to other antigenic stimulations including a commercial vaccine [32]. Not much is known about this type of resistance and its inheritance by progeny; consequently, further studies on innate resistance to infections in other animals would be most rewarding because it can be a good strategy against some pathogens.

3.2.2. *Cryptobia*-Tolerant Fish. Parasitaemias in some infected brook charr are just as high as those in *Oncorhynchus* spp.; however they do not suffer from cryptobiosis (*Cryptobia*-tolerant fish). *Cryptobia*-tolerant brook charr are resistance to disease because the metalloprotease secreted by *C. salmositica* is neutralized by the a2 macroglobulin (a natural antiprotease) in the blood. The amount of a2 macroglobulin is higher in brook charr than in rainbow trout prior to infection and it remains high (about 40%) even at peak parasitaemia while that in trout drops to about 12% [21, 33]. Parasitaemias in both infected rainbow trout and brook charr peak at about 4–6 weeks after infection and as antibodies are produced the parasitaemias decline; however, the parasitaemia fluctuates in rainbow trout while that in infected *Cryptobia*-tolerant charr rapidly declines after peak parasitaemia [32]. Neutralization of the metalloprotease by a2 macroglobulin was demonstrated under both in vivo and in vitro conditions [18, 21, 33]. Since *Cryptobia*-tolerant charr do not suffer from clinical disease, the immune system readily controls the infection and the fish recover much more rapidly than trout from the infection [32].

An obviously option to control cryptobiosis in salmon is to consider producing transgenic *Cryptobia*-tolerant salmonids. It is expected that the transgenic salmon will maintain high levels of a2 macroglobulin in their blood, essentially to neutralize the metalloprotease secreted by the pathogen—the additional a2 macroglobulin will eliminate or at least reduce the severity of the disease. Since the disease is absent or less severe, the fish immune system can more effectively control the infection. This proposal is a novel approach to the management of an infectious disease in animals and it perhaps needs further discussions. An obvious “downside” with this approach is that it may increase the pool of reservoir animals (with infections but no disease) in the population but one very obvious advantage is that no further human interventions (e.g., vaccination, chemotherapy) are required once the transgenic animal is produced.

3.3. Adaptive (Acquired) Immunity. Adaptive immunity has also been exploited to protect the susceptible *Oncorhynchus* spp. from cryptobiosis. Two distinctly different experimental vaccines (a live attenuated vaccine and a metalloprotease-DNA vaccine) have been developed. Fish inoculated with the live attenuated *Cryptobia* vaccine are protected from infection when challenged with the parasite. However, the metalloprotease-DNA vaccine does not prevent an infection in vaccinated fish but antibodies produced in the vaccinated fish neutralize the disease-causing factor secreted by the pathogen. Although the DNA-vaccinated fish is infected, it does not suffer from cryptobiosis, and it essentially turns the pathogenic *Cryptobia* into a nonpathogenic flagellate as in the case of the *Cryptobia*-tolerant brook charr (Section 3.2.2).

3.3.1. Live Vaccine. *C. salmositica* was attenuated by prolonged in vitro culture and the strain has been cloned. The attenuated parasite is maintained in tissue culture medium and it has remained avirulent since 1990. It produces a low infection in rainbow trout, does not cause disease, circulates in the blood for at least 6 months, and is protective when the fish is challenged with the pathogen [34]. The vaccine strain is smaller in size and has lost a few polypeptides. It is adapted to in vitro culture and hence multiplies much more readily than the pathogenic strain in tissue culture medium [35, 36]. A single injection of the strain protects 100% of vaccinated fish and consequently it is used routinely as an experimental vaccine to study the development and mechanism of protective immunity in salmonids and the pathobiology of the disease. The vaccine has no detectable bioenergetic cost to juvenile rainbow trout [16], and it protects various species of juvenile and adult salmonids from the pathogen (e.g., [8, 9, 37–41]).

Rainbow trout vaccinated in fresh water and transferred to sea water are also protected on parasite challenge [42]. A single dose of the vaccine protects rainbow trout for at least 24 months [8]. Vaccinated fish are partially protected if they are challenged at 2 weeks postvaccination (wpv) while all vaccinated fish are protected (e.g., no drop in packed cell volume and virtually no detectable infection after parasite challenge) at 4 wpv. Protection is via the production of complement fixing antibodies and under in vitro conditions activated macrophages from head kidneys of vaccinated fish show antibody-independent and antibody-dependent cytotoxicities. Also, in the presence of antiserum macrophages are very efficient in engulfing living parasites (Figure 7).

The complement fixing antibody titres (e.g., [8]) and cell-mediated response (e.g., [40]) in vaccinated fish rise significantly soon after parasite challenge (classical secondary responses), and the responses in vaccinated and challenged fish are similar to those in naive fish at 6 weeks after infection. Humoral and cell-mediated immunity are important
components of the protection against *C. salmositica* in both vaccinated and recovered fish (e.g., [8, 9, 40, 43]).

### 3.3.2. Metalloprotease-DNA Vaccine

As indicated earlier (Sections 3.1 and 3.2.2) the disease causing metalloprotease (200 kDa glycoprotein) can be neutralized. This is accomplished either by the α2 macroglobulin (a natural antipro- tease) in *Cryptobia*-tolerant brook charr [18, 21, 33] where the parasitaemia does not fluctuate and the fish recovers rapidly [32] or by an antibody (mAb-001) produced against the 200 kDa glycoprotein [26]. The monoclonal antibody (mAb-001) agglutinates the parasite and reduces its survival and infectivity [24]. Neutralization of the metalloprotease by antibodies in vaccinated fish is the basis of our current DNA vaccine.

Briefly, the metalloprotease and cysteine protease genes of *C. salmositica* were sequenced [45, 46] and inserted into plasmid vectors (pEGFP-N) to produce a metalloprotease-plasmid vaccine and a cysteine-plasmid vaccine [47]. Rainbow trout and Atlantic salmon, *Salmo salar*, injected intramuscularly with the metalloprotease-plasmid vaccine consistently had lower packed cell volume (as metalloprotease was secreted into the blood) than controls (fish inoculated either with plasmid alone or with cysteine-plasmid vaccine) at 2–4 wpv. However, the packed cell volume in metalloprotease-vaccinated fish returned to normal by 5 wpv—this was because the metalloprotease was neutralized as antibodies were produced. Agglutinating antibodies against *C. salmositica* were detected 5–7 wpv in the blood (and not before 5 wpv) in metalloprotease-vaccinated fish, but not in fish injected with either the cysteine-plasmid or plasmid alone injected fish. Fish were challenged with the pathogen at 7 wpv and the metalloprotease-vaccinated fish had lower parasitaemia, delayed peak parasitaemia, and faster recovery than control infected fish. In a recent review on the use of DNA vaccines in aquatic organisms Kurath [48] confirms that this is the “... first published demonstration of protective effects of a fish parasite DNA vaccine in fish.”

Many protozoa that are of medical and economic importance (e.g., *Trypanosoma* spp., *Leishmania* spp.) have metalloprotease and cysteine protease [49]. Some of these pathogens also modify their surface coats to evade the host immune response (e.g., antigenic variations as in the *Glossina*-transmitted mammalian trypanosomes in Africa); consequently vaccines based on surface membrane epitopes are not effective. However, an enzyme-based vaccine may be worth serious considerations as enzymes are quite conserve. Also, an enzyme-based vaccine will most likely protect against all isolates of the pathogen including those from different geographical locations which may have different surface membrane antigens. Enzymes are generally quite conserve and this is true even among different pathogens; for example, sequences of PCR-derived fragments of the metalloprotease gene of *C. salmositica* [45] are similar to those in other kinetoplastids, such as *Leishmania chagasi*, *L. donovani* [50], *Trypanosoma cruzi* [51], and *T. brucei* [52]. Major surface protease (MSP) also known as GP63 or leishmanolysin is a highly abundant zinc metalloprotease present on the cell surface of *Leishmania* spp. The NCBI-conserved domain search shows that the alignment for the metalloprotease of *C. salmositica* has 78.3% similarity with peptidase_M8, leishmanolysin domain [45].

### 3.4. Chemotherapy and Immunoochemotherapy

Chemotherapy is essentially differential toxicity; that is, the drug is more toxic to the target organism than it is to the host. Severity of the side effects of chemotherapy is dependent partly on tissue damage and adverse reactions by the host to the drug. However, the drug can be directed more specifically to the pathogen if it is conjugated to an antibody specific for the target organism. Immunoochemotherapy will obviously increase costs and is generally not meant for routine use; it may however be a useful tool under certain circumstances as it reduces the drug dosage and its side effects. For example, it can be used to treat infected brood fish as about 50% of brood fish annually die from cryptobiosis in some hatcheries on the west coast of North America [6]. It is expected the side effects and accumulation of drug residues in host tissues will be reduced in immunoochemotherapy, and this may also lower the risk of the development of drug-resistance by the pathogen. Reduction in drug residue in host tissues is also an important consideration if the fish are for human consumption.

#### 3.4.1. Chemotherapy

In tropical Africa isometamidium chloride (Samorin) is widely used against trypanosomiasis in domestic animals [53], and it is also used as a prophylactic drug against bovine trypanosomiasis [54]. In fish, Samorin (1.0 mg/kg weight) reaches peak level in the blood 2–3 weeks after intramuscular injection [55]. The drug is therapeutic against *C. salmositica* in rainbow trout during pre- and post-clinical phases of the disease. However, it is not effective during acute disease partly as we believe that the drug “modifies” surface epitopes of the parasite so that they are not lysed by complement fixing antibodies [56]. The drug is more effective in infected Atlantic salmon, and at a higher dose (2.5 mg/kg) the infection is eliminated in about 30% of adult fish and significantly reduces the parasitaemias in remaining fish. Also all infected juvenile chinook salmon, *Oncorhynchus tshawytscha*, treated with isometamidium chloride (1.0 mg/kg) survived the disease.
Mean parasitaemia from a recovered fish (reproduced from Ardelli and Woo [59]).

The drug also has prophylactic value, and it reduces infectivity of the parasite to fish and changes the surface glycoprotein antibody-receptor sites of the parasite. This alteration of surface epitopes explains the protection of the parasite while the unconjugated drug accumulates only in the noninfected parasites. In contrast, the mAb-00-conjugated drug does not lyse *C. salmositica* but agglutinates it. After in vitro exposure to PAIC the infectivity of the parasite and subsequent parasitaemias in inoculated fish were significantly decreased after drug exposure with very significant increases in secretion of glycolytic products (lactate and pyruvate) as the parasite switches from aerobic respiration to glycolysis after its mitochondrion is damaged by the drug [44]. Also, in vitro exposure to sublethal levels of the drug reduces infectivity of the parasite to fish and changes the surface glycoprotein antibody-receptor sites of the parasite. This alteration of surface epitopes explains the protection of some parasites from lysis by complement fixing antibodies when rainbow trout with acute infections were treated with the drug [56].

### Table 1: Infectivity of *Cryptobia salmositica* to chinook salmon after in vitro exposure to isometamidium chloride to polyclonal antibodies from a recovered fish (reproduced from Ardelli and Woo [59]).

| Weeks After Infection | Group 1 PAIC | Group 2 Drug | Group 3 PAI | Group 4 Antibody | Group 5 Untreated Controls |
|-----------------------|--------------|--------------|-------------|------------------|---------------------------|
| 1                     | 1/10 ± 0.95  | 7/10         | 0/10        | 0/10             | 8/10                      |
| 2                     | 1/10         | 6/10         | 2/10        | 0/10             | 10/10                     |
| 3                     | 2/10         | 33,250 ± 38,207 | 1,950 ± 4,310 | 0     | 35,625 ± 29,978 |
| 4                     | 2/10         | 302,000 ± 254,142 | 2,500 ± 5,270 | 5,558 ± 16,665 | 5,487,500 ± 5,439,838 |
| 5                     | 2/10         | 9,395,000 ± 16,925,911 | 54,740 ± 112,499 | 556,944 ± 1,500,001 | 3,175,000 ± 3,639,196 |
| 6                     | 1/10         | 3,750 ± 11,858 | 13,475,000 ± 15,298,624 | 2,600,000 ± 5,577,465 | 3,243,750 ± 5,416,196 |
| 7                     | 1/10         | 11,250 ± 35,575 | 18,305,000 ± 52,500,000 | 928,860 ± 1,326,575 | 44,383,334 ± 129,150,260 |

* Number of infected fish/number of fish inoculated.
* Mean parasitaemia ± standard deviation, determined by HCT or haemacytometer.

3.4.2. *Immunochemotherapy*. Ardelli and Woo [59] conjugated isometamidium chloride to polyclonal antibodies (from “recovered” fish) and the monoclonal antibody (mAb-001, Section 3.1). The conjugated drug is on the entire parasite while the unconjugated drug accumulates only in the kinetoplast (Figure 9). Before drug conjugation both antibodies agglutinate living parasites but they react differently after drug conjugation. Polyclonal antibodies-conjugated drug (PAIC) lyses most of the parasite and it no longer agglutinates the parasite. In contrast, the mAb-00-conjugated drug does not lyse *C. salmositica* but agglutinates it. After in vitro exposure to PAIC the infectivity of the parasite and subsequent parasitaemias in inoculated fish were significantly

![Figure 8: Ultrastructural lesions in *Cryptobia salmositica* after in vitro exposure to isometamidium chloride. (a) Parasite kinetoplast (K) not exposed to the drug; (b) condensation of kinetoplast DNA after exposure to the drug; (c) vacuole (V) formation after drug exposure; (d) swelling of mitochondrial cristae (C) after drug exposure; (e) vacuole formation in cytoplasm after drug exposure (reproduced from Ardelli and Woo [44]).](image-url)
lowered. Fish survival (Table 1) was much higher in juvenile chinook salmon infected with parasites exposed to the polyclonal antibodies-conjugated drug (PAIC) than to drug alone (Drug) or to polyclonal antibodies alone (Antibody) or to drug plus polyclonal antibody (PAI). Also, preliminary studies indicate the drug-antibody conjugate to be effective to drug only, note accumulation of drug (in red) in the kinetoplast; exposure to drug conjugated to polyclonal antibodies from a recovered fish, note that the drug (in red) is throughout the organism (reproduced from Ardelli and Woo [59]).

4. Conclusions

Our concerted efforts to better understand the biology of Cryptobia and the mechanism of the disease have allowed us to develop more rational strategies against the pathogen and disease. We have been relatively successful in exploiting the piscine immune system to protect salmonids against C. salmositica and cryptobiosis. This is an ongoing and evolving research program and there is obviously a great deal of work that needs to be conducted. However, I hope our research will be of interest and perhaps be useful to colleagues who are also developing control measures against similar pathogenic organisms. The research has been both challenging and fascinating, and I would like to think the best is yet to come.

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