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In vivo neutralization of *Cryptobia salmositica* metallo-protease by α2-macroglobulin in the blood of rainbow trout *Oncorhynchus mykiss* and in brook charr *Salvelinus fontinalis*

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ABSTRACT: *Cryptobia salmositica* infects both rainbow trout *Oncorhynchus mykiss* and brook charr *Salvelinus fontinalis*, but it does not cause disease (e.g. anaemia) in brook charr. The natural anti-protease, α2-macroglobulin (α2-M), was found in the blood of rainbow trout and brook charr, but it had much higher activity in brook charr. Under *in vivo* conditions, α2-M of trout and charr were neutralized by *C. salmositica* extracellular protease. The onset and persistence of anaemia in infected rainbow trout were related to low levels of α2-M. Infected brook charr did not develop anaemia as α2-M remained high during the infection. Secreted metallo-protease of *C. salmositica* was detected in the blood of infected rainbow trout when its α2-M was low and the fish was anaemic; however, metallo-protease was not detected in infected brook charr. We suggest that host α2-M plays an important role in defense against cryptobiosis by inhibiting the activity of metallo-protease secreted by the pathogenic *C. salmositica*.

KEY WORDS: α2-macroglobulin · *Oncorhynchus mykiss* · *Salvelinus fontinalis* · *Cryptobia salmositica* · Protease

INTRODUCTION

Cryptobia salmositica Katz, 1951 (Sarcomastigophora: Kinetoplastida) is a pathogenic haemoflagellate of salmonids on the west coast of North America (Woo 1987, 1991, 1994) and the mechanism of the parasitic disease is not well understood (Woo & Poynton 1995). Infected rainbow trout *Oncorhynchus mykiss* are anorexic (Li & Woo 1991, Thomas & Woo 1992), and develop anaemia, exophthalmia, splenomegaly, abdominal distension with ascites and general oedema (Woo 1979). Although parasitaemias are very high, infected brook charr do not show clinical cryptobiosis (Ardelli et al. 1994, Forward et al. 1995).

Proteases are known to contribute to protozoan diseases in mammals (Coombs 1982, Gadasi & Kobiler 1983, Dluzewski et al. 1986, Pupkis et al. 1986), but their roles in fish disease are not well documented. Our earlier study showed that the pathogenic strain of *C. salmositica* has cysteine and metallo-proteases and the parasite losses the metallo-protease as it becomes less virulent during *in vitro* culture (Zuo & Woo 1997a this issue). Also, the nonpathogenic *C. catostomi* of white suckers *Catostomus commersoni* has only cysteine protease and its proteolytic activity is much lower than that in the pathogenic *C. salmositica* (Zuo & Woo 1997a). We earlier suggested that the metallo-protease in the pathogenic *C. salmositica* is related to its pathogenicity in fish (Zuo & Woo 1997a, b).

We showed that plasma α2-macroglobulin (α2-M) in rainbow trout and brook charr inhibited *Cryptobia salmositica* proteases under *in vitro* conditions and that α2-M was neutralized after the parasites were cultured in the fish blood (Zuo & Woo 1997a).

The main objectives of the present study were to determine the *in vivo* neutralization of the natural

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antiprotease α2-M by *C. salmositica* proteases, and the relationship between antiprotease activity and anaemia in infected fish.

MATERIALS AND METHODS

Fishes. Ten laboratory-raised *Cryptobia*-susceptible brook charr *Salvelinus fontinalis* (3 yr old; 1509 to 2180 g) (Forward et al. 1995) and 10 rainbow trout *Oncorhynchus mykiss* (3 yr old; 1459 to 1902 g) from a disease-free hatchery (Wildcat Trout Farm, Thamesford, Canada) were used in this study. All fishes were kept in fibre glass tanks supplied with running well water and continuous aeration and fed to satiety once daily with a 52% protein diet (3 GR trout pellets, Martin Feed Mills, Elmira, Canada).

Bleeding of fish. Fish were anaesthetized in a solution of Tricaine methane sulfonate (MS-222, Finquel Brand, Argent Chemical Laboratories, WA, USA) before bleeding. A disposable sterile needle (25 G⅝) and a 3 ml disposable plastic syringe with heparinized saline [0.1 g heparin added to 10 ml of cold-blooded vertebrate Ringer's solution (CBVR)] were used to draw blood from the caudal vein of infected fish (Forward et al. 1995). Blood samples were kept on ice until use.

Infection of fish with pathogenic *Cryptobia salmositica*. The parasite was obtained from an infected rainbow trout. Each of the 5 brook charr and 5 rainbow trout was inoculated intraperitoneally (i.p.) with 250 000 parasites (in 0.2 ml of CBVR) (experimental group) and 5 charr and 5 trout were inoculated i.p. with 0.2 ml of CBVR (control group). Blood (1 ml) was taken weekly from fish and the number of parasites and packed cell volume were determined. The remaining blood was centrifuged at 1350 × *g* for 10 min at 4°C, and the supernatant (fish plasma) was used fresh or stored at −100°C for <6 mo. Our preliminary study indicated that storage at −100°C did not affect the effectiveness of fish antiproteases.

Parasite count and determination of packed blood cell volume. Parasitaemias were determined using a haemocytometer (Archer 1965). Low parasitaemia were detected using the haematocrit centrifuge technique (Woo & Wehnert 1983). Packed blood cell volume was determined using a haematocrit centrifuge (13 600 × *g* for 4 min) (Woo & Li 1990).

Detection of α2-macroglobulin (α2-M) in fish plasma. The method was that of Ellis (1990) with some modifications. Briefly, 100 µl trypsin (bovine pancreas type III, Sigma) (100 µg ml^{-1} of 0.1 M Tris-HCl, pH 8.2) was incubated with 50 µl of fish plasma for 10 min at 22°C. Tris buffer (0.1 M, pH 8.2) was added to bring the volume to 2.5 ml, and 2 ml of Na-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA, Sigma) was added and then incubated at 22°C for 25 min. The reaction was terminated by the addition of 0.6 ml 30% acetic acid. Fine precipitates were removed by filtration using a 0.22 µm Millipore filter and the optical density of the filtrate was measured at 410 nm using a spectrophotometer against an appropriate blank. The blank was prepared individually by adding acetic acid to the enzyme/plasma mixture prior to addition of the substrate.

Inhibitory capacity of α2-M was expressed as inhibition on trypsin activity, i.e. a percentage of the difference between the control activity (A1) (without the antiprotease) and the activity remaining (A2) (after incubation with the antiprotease) in the control activity (A1). The formula is as follow:

$$\text{Inhibitory capacity (\%)} = \frac{A1 - A2}{A1} \times 100\%$$

Detection of extracellular proteases of *Cryptobia salmositica* in the blood of infected fish. The proteolytic activity in the fish plasma was determined using azocoll as the substrate (Arnesen et al. 1995) with a slight modification. Briefly, 50 µl of fish plasma was incubated with 300 µl HEPES/$CaCl_2$ buffer (0.1 M HEPES, pH 7.5, supplemented with 4 mM $CaCl_2$) and 300 µl azocoll (10 mg ml^{-1} water, Sigma) at 37°C for 12 h. The reaction was stopped by adding 50 µl of 50% trichloroacetic acid (TCA). The absorbance was measured using a spectrophotometer at 520 nm against the blank. The blank was prepared individually by adding the TCA to the protease sample prior to addition of the substrate. To confirm that the protease is a metalloprotease, proteolytic activity was also assayed after the sample was pre-incubated with 1,10-phenanthroline (Sigma, at final concentration of 1 mM), a metalloprotease inhibitor (North et al. 1983).

Polyacrylamide-substrate gel. Electrophoretic separation of the proteases of *Cryptobia salmositica* was performed on non-reducing SDS-polyacrylamide gels (7.5%) copolymerized with 0.1% gelatin (Sigma) using a Mini-protean II dual slab cell (BioRad, Canada) (Britton et al. 1992). A 20 µl of cell lysate (containing 20 µg protein) or 30 µl of fish plasma was mixed with 10 µl of SDS-sample buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 20% glycerol and 0.02% bromophenol blue) and applied to the gel, without boiling. After electrophoresis the gels were immersed in 1 l of 2.5% (v/v) Triton X-100 for 1 h to remove SDS. The protease bands were developed by immersing the gels in the HEPES/$CaCl_2$ buffer (see above) for 12 h at 37°C. Zones of proteolysis were clear bands against a blue background after Coomassie Blue R-250 staining (0.1%) and molecular weights of individual protease were determined based on their mobility relative to those of molecular weight standards (Britton et al. 1992).

Statistical analysis. The Student *t*-test (Wardlaw 1985) was used to determine the significant difference in parasitaemia, packed cell volume, inhibitory capacity of antiprotease and proteolytic activity. Results were considered significant if $p < 0.05$.

RESULTS

Parasitaemias in infected fishes

All 5 rainbow trout were infected post-inoculation of *Cryptobia salmositica* (p.i.) and the mean parasitaemia was high. The parasitaemia peaked at 5 wk p.i. and it fluctuated during the infection (Fig. 1).

All 5 brook charr were also infected and the highest number was at 4 wk p.i. (Fig. 1). Parasitaemias in infected trout and charr at 1 to 4 wk p.i. were not significantly different ($p > 0.05$). The parasitaemia in infected charr declined at 5 wk p.i. and it remained low with no fluctuation (Fig. 1).

Packed blood cell volumes

Significant differences ($p < 0.05$) in packed blood cell volume were detected between control and infected rainbow trout 4 to 7 wk p.i., i.e. infected trout had significantly lower packed blood cell volume ($p < 0.05$) than control fish (Fig. 2A). However, no significant difference ($p > 0.05$) was found between control and infected brook charr (Fig. 2B), indicating that infected charr were not anaemic.

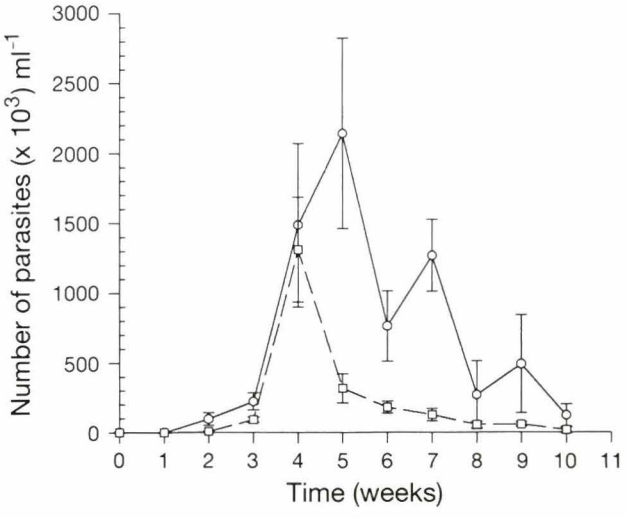

Fig. 1. Parasitaemia of *Cryptobia salmositica* in infected fishes. Each point is the mean ± standard deviation (n = 5). (○) Rainbow trout (*Oncorhynchus mykiss*); (□) brook charr *Salvelinus fontinalis*

Neutralization of plasma α2-M by *Cryptobia salmositica* proteases in infected fishes

The inhibitory capacity of α2-M in rainbow trout were constant for 2 wk prior to infection and for 2 wk p.i. (Fig. 3A); these were not significantly different ($p > 0.05$) from those in uninfected controls. However, from 3 to 8 wk after infection, the inhibitory capacities of α2-M were significantly lower ($p < 0.05$) than those in the control group (Fig. 3A). This indicates that significant amounts of the α2-M were neutralized by *C. salmositica* extracellular proteases. The α2-M activity was lowest in infected trout at 5 wk p.i. (Fig. 3A). This was when fish had the highest parasitaemia (Fig. 1) and the lowest packed blood cell volume (Fig. 2A).

However, there was no significant decrease in the inhibitory capacity of α2-M in infected brook charr compared to uninfected controls except at 4 and 5 wk p.i. (Fig. 3B). At 4 wk p.i. infected charr had the highest parasitaemia and the lowest α2-M; however, the inhibitory capacity of α2-M (Fig. 3B) was still significantly higher ($p < 0.05$) than that in infected trout (Fig. 3A).

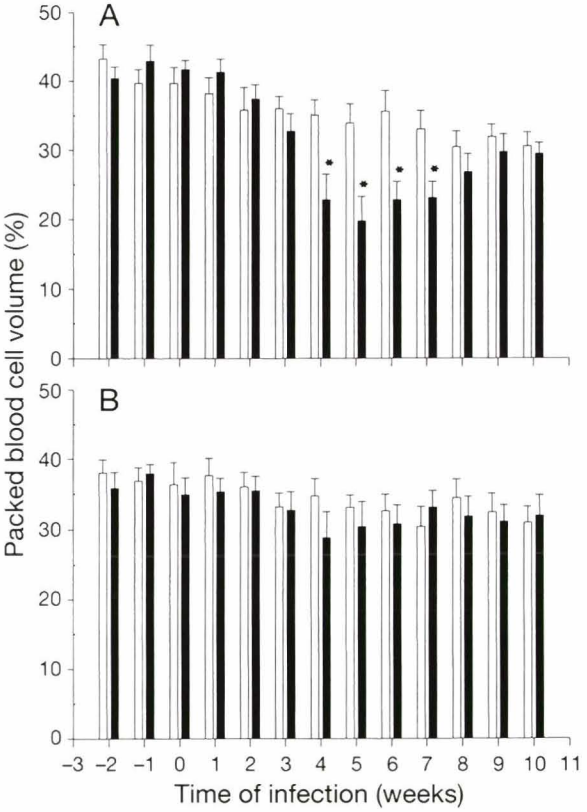

Fig. 2. Packed blood cell volume (%) in fishes. (A) Rainbow trout *Oncorhynchus mykiss*; (B) brook charr *Salvelinus fontinalis*. Each bar represents the mean packed blood cell volume (± standard deviation, n = 5). Open bars: control group (uninfected fish); solid bars: experimental group (infected fish). *Significantly lower ($p < 0.05$) than that in the control group

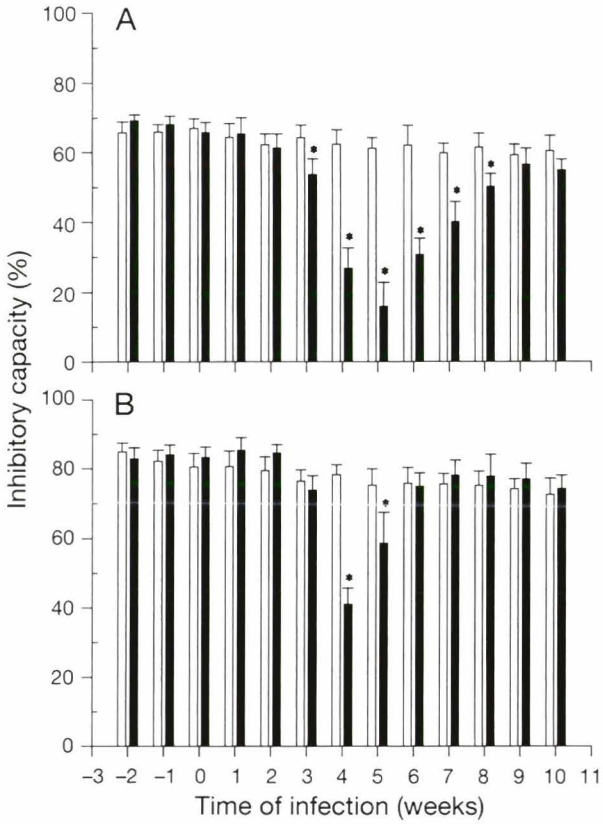

Fig. 3. Inhibitory capacity of α2-M in fishes. (A) Rainbow trout; (B) brook charr. Each bar represents the mean inhibitory capacity (± standard deviation, n = 5). Empty bar: control group (uninfected fish); solid bar: experimental group (infected fish). *Significantly lower ($p < 0.05$) than that in the control group

Secreted metallo-protease of *Cryptobia salmositica* in the blood of infected fishes

No proteolytic activity was detected in plasma of uninfected rainbow trout nor in plasma of uninfected and infected brook charr. However, proteolytic activities were detected in the plasma of infected rainbow trout at 4, 5, and 6 wk p.i. (Table 1). The activities were significantly inhibited by 1,10-phenanthroline, an inhibitor for metallo-proteases (Table 1). This indicates that the secreted protease is a metallo-protease.

Secreted metallo-protease was also detected in plasma of infected trout at 4, 5, and 6 wk p.i. using the gelatin-SDS-PAGE (Fig. 4). This proteolytic activity appeared as a single clear band (about 200 to 205 kDa) after Coomassie blue staining; protease with similar molecular weight but with much higher activity was also found in *C. salmositica* lysate (Fig. 4). No protease band was detected in plasma of uninfected trout (lane 4, Fig. 4), nor in plasma of uninfected and infected brook charr.

Table 1. Protease activity secreted by *Cryptobia salmositica* in the blood of infected rainbow trout *Oncorhynchus mykiss* and the inhibition by 1,10-phenanthroline. The activity is in absorbance assayed using azocoll as substrate (at 520 nm); mean ± standard deviation from 5 individual fish. Control activity is the activity detected without inhibitor; inhibited acitivity is the activity detected with inhibition of 1,10-phenanthroline (1 mM)

Week p.i.	Control activity	Inhibited activity
3	nd	nd
4	0.060 ± 0.014	0.005 ± 0.002* (8.3%)[a]
5	0.092 ± 0.015	0.012 ± 0.004* (13.0%)[a]
6	0.026 ± 0.008	0.004 ± 0.002* (15.4%)[a]
7	nd	nd

*Significantly lower ($p < 0.05$) than the control activity
[a]Activity remaining was given as a percentage in the control activity; nd: no proteolytic activity was detected (the absorbance was ≤ 0)

DISCUSSION

Cryptobia salmositica infects both rainbow trout and brook charr, but it only causes disease (e.g. anaemia) in trout and not in charr although the parasitaemia may be as high or higher in charr (Ardelli et al. 1994, Forward et al. 1995).

The first clinical sign of cryptobiosis in rainbow trout is the anaemia, and this is correlated with increasing parasitaemia (Woo 1979). There are 2 haemolytic components in *Cryptobia salmositica* which contribute to the anaemia: a 'lytic' component which causes lysis of erythrocytes independent of antibody or complement, and an 'immune complex-forming' component which binds to erythrocytes to form immune complex with specific antibody and activates complement which results in haemolysis (Thomas & Woo 1988). These haemolytic components are also secreted by live *C. salmositica* under *in vitro* conditions (Woo & Thomas 1992). However, their biochemical nature is not clear, although they were suggested to be proteinaceous in nature (Thomas & Woo 1989).

We (Zuo & Woo 1997a) showed that the pathogenic *Cryptobia salmositica* has 2 types of proteases (cysteine protease and metallo-protease) and that the parasite has lost its metallo-protease after it becomes avirulent. Also, the nonpathogenic *C. catostomi* of white suckers *Catostomus commersoni* has only cysteine protease. We (Zuo & Woo 1997b) also demonstrated that the purified metallo-protease from the pathogenic *C. salmositica* causes *in vitro* lysis of fish erythrocytes. In the present study, the secreted

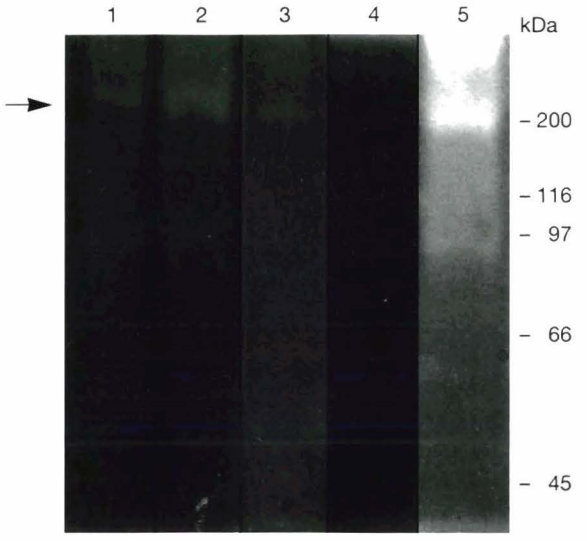

Fig. 4. Protease activity of *Cryptobia salmositica* detected using the gelatin-SDS-PAGE. Lanes 1, 2, 3: secreted protease (indicated by arrow) from plasma of infected rainbow trout at 4, 5, 6 wk p.i., respectively; lane 4: plasma of an uninfected rainbow trout; lane 5: intracellular protease from cell lysate of *C. salmositica*. Standard molecular mass markers (kDa) as indicated

metallo-protease was detected in the blood of infected rainbow trout (Table 1, Fig. 4) at a time when the level of natural antiprotease α2-M was low (Fig. 2). The metallo-protease could be secreted by live parasites or was released from broken parasites lysed by fish complement (Li & Woo 1995). We suggest that the metallo-protease contributes to the anaemia in cryptobiosis and that it is likely the secreted 'lytic component' detected in earlier studies (Thomas & Woo 1988, 1989, Woo & Thomas 1992).

There are good indications that α2-M contributes to defence against diseases caused by pathogens which secrete proteolytic enzymes. It is believed that the extracellular protease and elastase produced by *Pseudomonas aeruginosa*, in burned and compromised patients, are virulence factors and the human α2-M greatly reduces the disease by inhibiting the proteolytic activity of the bacterium (Holder & Haidaris 1979). The protease released by the bovine protozoan *Tritrichomonas foetus* is neutralized by α2-M, but not by α1-PI. This neutralization inhibits the action of the secreted protease (Talbot et al. 1991). The fish pathogen *Aeromonas salmonicida* secretes protease that depletes α2-M in rainbow trout and contributes to pathogenesis (Ellis et al. 1981, Hastings & Ellis 1985), and the fish α2-M plays an important role in defence against furunculosis because it inhibits bacterial protease (Ellis 1987).

In the present study, there was no significant difference in parasitaemias between infected rainbow trout and brook charr at 1 to 4 wk p.i. However, the rainbow trout developed anaemia and the brook charr did not. The α2-M was significantly higher in infected charr than in infected trout, and the α2-M in the trout markedly decreased with the increasing parasitaemia during the infection. Even after infected trout had recovered from the anaemia (at 8 wk p.i.), the α2-M activity was still significantly lower than those in uninfected controls. This indicates that infected trout were slow to produce α2-M. The level of α2-M in infected charr remained high during the infection and there was no anaemia. In another study (Zuo & Woo 1997b) we showed that plasma of rainbow trout and brook charr (containing α2-M) prevents *in vitro* haemolysis caused by the purified metallo-protease from *Cryptobia salmositica*, and that charr has significantly higher α2-M activity than trout in the same amount of plasma. It is likely that a quantitative difference in α2-M between charr and trout is one of the reasons that trout suffers from cryptobiosis while charr does not. We suggest that α2-M, a natural protease inhibitor, is one of defensive factors against cryptobiosis; the high activity of the α2-M in the brook charr before and after infection is the reason that the fish does not suffer from cryptobiosis. Even in the susceptible rainbow trout, its plasma α2-M has a defensive function in that it delays the onset (e.g. anaemia) and also reduces the severity of the disease.

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A systemic parvo-like virus in the freshwater crayfish *Cherax destructor*

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ABSTRACT: Systemic Cowdry Type A inclusions (CAs) were observed in a moribund *Cherax destructor* collected at an aquaculture farm in South Australia. Inclusions were most common in the gills and were associated with multifocal necrosis of the main gill axis and lamellae. The hepatopancreas was necrotic; however, only one CA was observed in the interstitial tissues. CAs were associated with necrosis in the abdominal and gut musculature. CAs were also observed in the spongy connective tissues and the epicardium. Empty capsids (17.5 ± 0.5 nm) and microfilaments were most commonly observed within these inclusions by transmission electron microscopy. Complete icosahedral viral particles (20.8 ± 1.2 nm) were difficult to distinguish within the viroplasm, but were visualised better in aggregates between the viroplasm and the inner nuclear membrane. The nucleolus was closely associated with the developing viroplasm, and was hypertrophied and segregated into its fibrillar and granular components. The virus was named *Cherax destructor* systemic parvo-like virus (CdSPV) on the basis of its histopathology, cytopathology and morphology. CdSPV is the first systemic virus described in a freshwater crayfish.

KEY WORDS: Crayfish · *Cherax destructor* · Systemic parvo-like virus · Histopathology · Cytopathology

INTRODUCTION

Viruses have been described in a number of freshwater crayfish species since the discovery of the initial freshwater crayfish virus, *Cherax quadricarinatus* bacilliform virus, by Anderson & Prior (1992). Most are intranuclear bacilliform viruses which infect the gut (Hedrick et al. 1995, Edgerton 1996, Edgerton et al. 1996). The one exception is *Cherax Giardiavirus*-like virus (CGV), a dsRNA virus which infects hepatopancreatocytes in *C. quadricarinatus* (Edgerton et al. 1994).

Parvoviruses assigned to the *Densovirinae* subfamily infect arthropods (Murphy et al. 1995), including a number of commercially important crustaceans. Hepatopancreatic parvovirus (HPV) infects the hepatopancreatocytes of a range of penaeid species (Lightner & Redman 1985, Lightner 1996), and a morphologically indistinguishable virus also infects the giant freshwater prawn *Macrobrachium rosenbergii* (Anderson et al. 1990). Mari & Bonami (1988) described a systemic parvovirus, which they named PC84, in the crab *Carcinus mediterraneus*. Infectious hypodermal and haematopoietic necrosis virus (IHHNV) is possibly the best known crustacean parvovirus, due to its well documented pathogenicity and its wide host and geographical range (Brock & Lightner 1990). IHHNV has also been described in experimental hybrid penaeids in Australia (Owens et al. 1992). Lymphoidal parvo-like virus was described in 3 penaeid species in Australia (Owens et al. 1991). The viruses PC84, HPV and IHHNV have been characterised physicochemically to confirm their relatedness to the *Parvoviridae* (Mari & Bonami 1988, Bonami et al. 1990, Bonami et al. 1995). The Australian viruses do not react with DNA probes

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for these viruses (Lightner 1996) and are uncharacterised.

A parvo-like virus was associated with Cowdry Type A inclusions in a moribund *Cherax destructor* during an ongoing surveillance of farmed crayfish for disease. This paper describes the histopathology, cytopathology and morphology of the agent.

MATERIALS AND METHODS

One moribund *Cherax destructor* was collected at an aquaculture farm in South Australia. The crayfish was sacrificed immediately by severing the cephalothorax from the abdomen. The cephalothorax was then split longitudinally and placed in Davidson's solution with the abdomen. Tissues were processed and histological sections were prepared conventionally (Culling et al. 1985) on arrival at the laboratory. Sections were stained with H&E, Feulgen and phloxine and tartrazine stains. Histological sections were viewed using a Leitz Orthoplan microscope.

Davidson's fixed gill tissues were also used for electron microscopy. Gill tissue was re-fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C, washed 3 times in 0.1 M cacodylate buffer, post-fixed for 1 h at room temperature in 1% osmium tetroxide in 0.1 M cacodylate buffer, washed 3 times in 0.1 M cacodylate buffer, dehydrated through an acetone series, and embedded in Epon. Ultrathin sections were cut using a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and were viewed using a Jeol 1010 transmission electron microscope.

RESULTS

Gross symptoms

The crayfish was extremely morbid and was easily collected by hand on the pond bank. The ventral surface of the abdomen exhibited patches of opaque musculature.

Light microscopy

Intranuclear, Cowdry Type A inclusions (CAs) were observed systemically in the crayfish and were most common in the gills. Affected nuclei were hypertrophied, had a thin rim of marginated chromatin, and contained a single eosinophilic CA in the centre. The CAs were neutral to very lightly phloxophilic and were Feulgen-negative. The nucleolus, when discernable, was basophilic and was closely associated with the inclusion. Smaller, presumably earlier, inclusions always developed centrally and were lightly eosinophilic.

The gills contained extensive necrotic foci characterised by masses of pyknotic and karyorrhectic nuclei, and major haemocytic infiltration, melanisation and nodulation. Pyknotic nuclei and haemocytic nodules were diffuse throughout non-necrotic tissue. CAs were often observed in gill epithelial cells, and in unidentifiable cells, close to necrotic areas and in areas becoming necrotic (Fig. 1). Gill epithelial cells with hypertrophic nuclei, rarefied chromatin and enlarged nucleoli, but without CAs, were common throughout the gills.

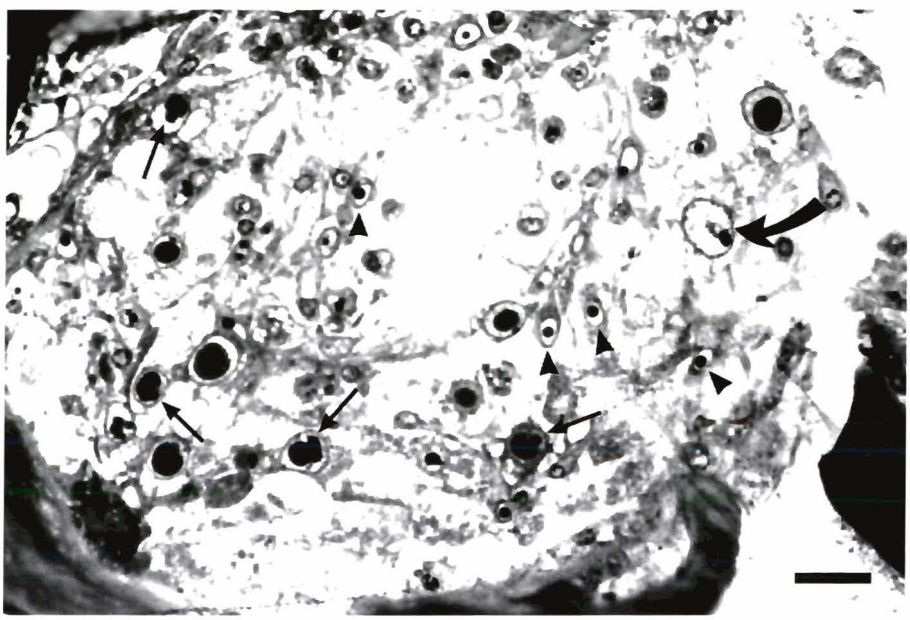

Fig. 1. Light microscopy of *Cherax destructor* systemic parvo-like virus in *Cherax destructor*. Many Cowdry Type A intranuclear inclusions in the gill. Note that the inclusions are rounded and are closely associated with the nucleolus (arrows). Note also the many pyknotic nuclei in the area (arrowheads). This area is near to a completely necrotic and melanised area. A hypertrophic nucleus with rarefied chromatin and hypertrophic nucleolus is also present (curved arrow). Toluidine blue. Scale bar = 27 µm

The hepatopancreas and midgut were extensively necrotic. Hepatopancreatocytes were almost exclusively B-cells. Haemocytic infiltrates and small melanitic foci were common in the interstitial tissues of the hepatopancreas and in the midgut submucosa. Haemolymph vessels were only present peripherally in the hepatopancreas, and the endothelial cells and infiltrating haemocytes were usually voluminous or pyknotic. The nucleus of an unidentified cell associated with a haemolymph vessel in the hepatopancreas contained a CA, as did a hindgut myoepithelial cell. Cellular debris and bacteria were distributed throughout the entire gut.

CAs, pyknotic and karyorrhectic nuclei, and haemocytic infiltrates and nodules were also observed in the epicardium, abdominal muscle and spongy connective tissues. Hypertrophic nuclei with rarefied chromatin and prominent nucleoli, but without inclusions, were also common in these tissues.

Electron microscopy

Gill cells containing CAs were hypertrophied, and had marginated and rarefied chromatin (Fig. 2). A rounded, granular viroplasm was located centrally, closely associated with the hypertrophied nucleolus. The viroplasm contained mostly filamentous structures and rounded 17.5 ± 0.5 nm (n = 7) particles with electron-lucent centres, presumed to be empty capsids (Fig. 3). Electron-dense, 20.8 ± 1.2 nm (n = 6) particles were difficult to distinguish within the inclusion, but were visualised better in aggregates between the viroplasm and the inner nuclear membrane. Some particles were clearly hexagonal or pentagonal, indicating an icosahedral symmetry. In some nuclei, the area between the inclusion and the nuclear membrane was full of empty capsids. Nucleolar components of infected cells had segregated with the filamentous component vacuolated and surrounded by the granular component. The granules of the granular component were more sharply defined and packed densely together, and fully formed virions were adjacent to the body.

In some nuclei, the nucleolus was hypertrophic, had segregated, and the viroplasm was only just forming (Fig. 4). The developing viroplasm was round and consisted of groups of long microfilaments associated with mostly empty capsids.

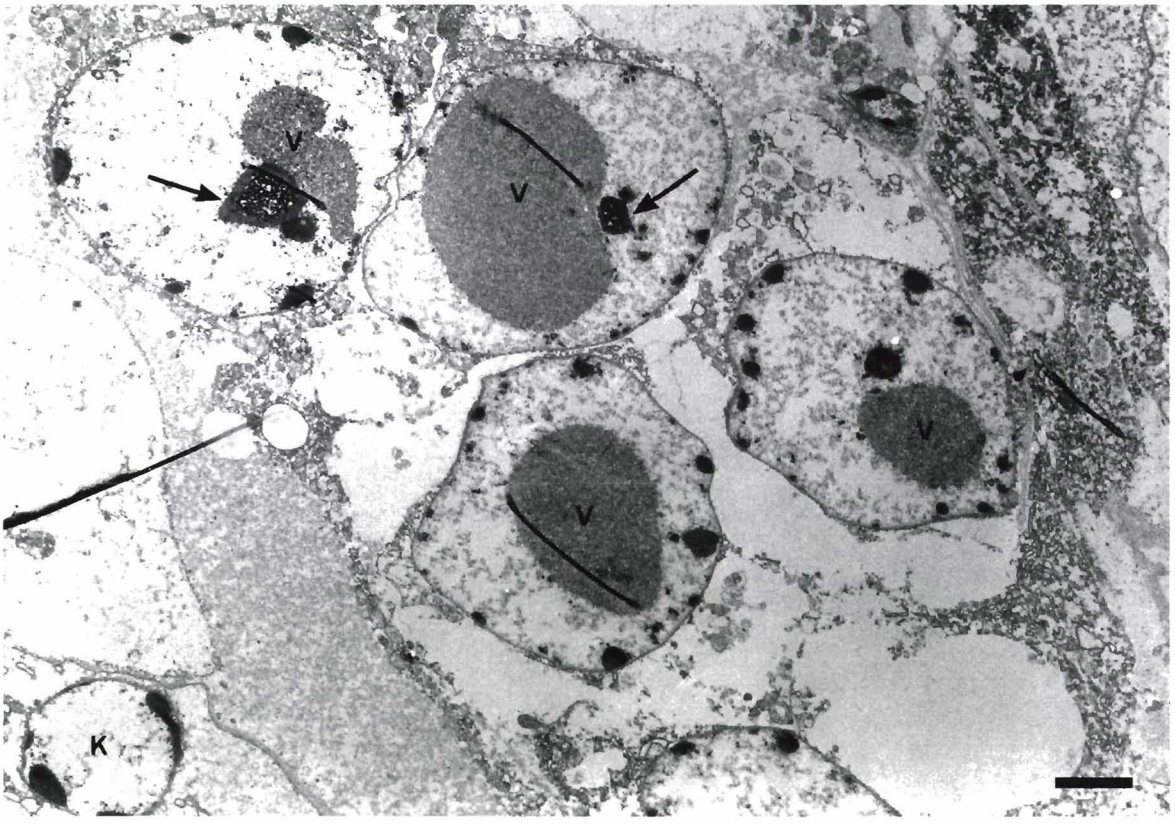

Fig. 2. Electron microscopy of CdSPV in *Cherax destructor* gill. Low magnification of 4 infected nuclei. Note that chromatin is marginated and rarefied, and the rounded viroplasm (V) is closely associated with the segregated nucleolus (arrows). A karyorrhectic nucleus is adjacent (K). Uranyl acetate and lead citrate. Scale bar = 2.4 µm

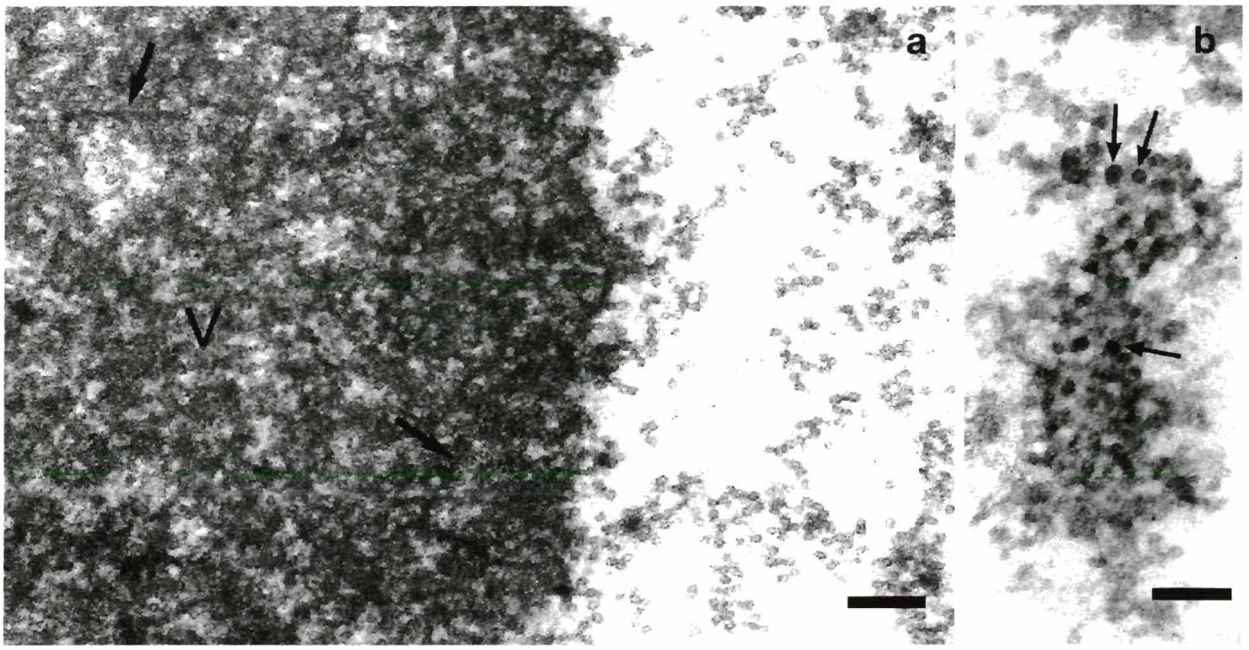

Fig. 3. Electron microscopy of CdSPV in *Cherax destructor* gill. (a) Higher magnification of CdSPV viroplasm (V) and surrounding area. Note that empty capsids and microfilaments (arrows) are most common within the viroplasm. Empty capsids are abundant in the area surrounding the viroplasm. Uranyl acetate and lead citrate. Scale bar = 130 nm. (b) Cluster of complete CdSPV virions. Note that many virions are angular (arrows). Uranyl acetate and lead citrate. Scale bar = 100 nm

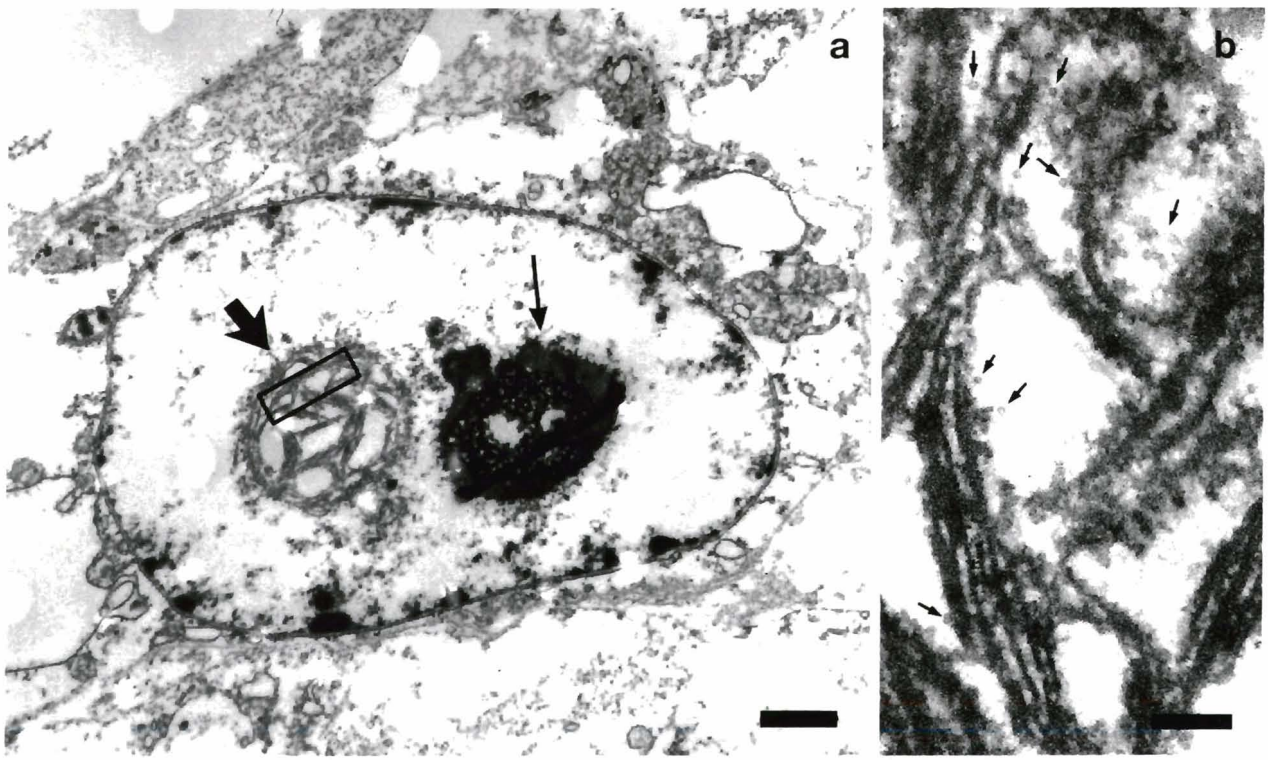

Fig. 4. Electron microscopy of CdSPV in *Cherax destructor* gill. (a) A nucleus in a presumptive early stage of infection. Note that the nucleolus is hypertrophied and segregated (thin arrow); the fibrous component is vacuolated and is completely surrounded by the granular component. The developing viroplasm (thick arrow) is close to the nucleolus and is round. Uranyl acetate and lead citrate. Scale bar = 1.2 µm. (b) Higher magnification of the forming viroplasm within the area indicated on (a). Note that empty capsids (arrows) are associated with long microfilaments. Uranyl acetate and lead citrate. Scale bar = 150 nm

DISCUSSION

This is the first systemic virus found in a freshwater crayfish. This agent is clearly different from bacilliform viruses which infect the gut of freshwater crayfish (Anderson & Prior 1992, Hedrick et al. 1995, Edgerton 1996, Edgerton et al. 1996). It also differs significantly in its tropism, histopathology and cytopathology from CGV which infects hepatopancreatocytes of *Cherax quadricarinatus* (Edgerton et al. 1994). This virus is similar in size and produces cytopathology reminiscent of parvoviruses; it is between 18 and 25 nm in diameter, and it replicates in the nucleus in close association with the nucleolus, which is segregated into its fibrillar and granular components. The agent is therefore named *Cherax destructor* systemic parvo-like virus (CdSPV).

CdSPV was associated with major necroses of vital organs. The CAs were found in close proximity to the large necrotic areas in the gills, and in smaller foci beginning to necrotise. A CA was observed interstitially in the massively necrotic hepatopancreas. CAs were associated with necrotic muscle. Whilst these changes no doubt resulted in the extreme morbidity of this animal, it is impossible to fully understand the involvement of CdSPV with these changes. The pathogenicity of CdSPV is therefore unknown.

The ultrastructural pathology of CdSPV is reminiscent of HPV. Similarly to CdSPV, HPV produces a single rounded virogenic stroma which is closely associated with the nucleolus (Lightner & Redman 1985). Microfibrils or microtubules are also apparent throughout the viroplasm. Intranuclear bodies, which are characteristically embedded in the viroplasm of HPV (Lightner & Redman 1985) and other parvoviruses (Garzon & Kurstak 1976), were not observed in CdSPV. However, the absence of such structures in these preparations may be due to sub-optimal fixation. As the names suggest, HPV has a different tropism to CdSPV. In this respect, CdSPV is similar to PC84 and IHHNV. PC84 also produces microtubules; however, virions were not associated with these, and the authors considered that these structures may characterise abortive morphogenesis (Mari & Bonami 1988). Neither PC84 nor IHHNV produce a rounded virogenic stroma, and nucleoli dissolve following infection by both viruses (Lightner et al. 1983, Mari & Bonami 1988). Nucleolar hypertrophy and segregation is characteristic of densovirus (Garzon & Kurstak 1976) as well as other virus infections; however, this is the first description of such a phenomenon in crustacean parvoviruses. The vacuolation of the fibrous component is reminiscent of canine parvovirus (Paradiso et al. 1982).

CdSPV is the second virus found in *Cherax destructor*. Virtually nothing is known of the prevalence or impact of these viruses on the *C. destructor* aquaculture industry. *C. destructor* is the most economically important freshwater crayfish species in Australia, accounting for over 80% of annual production (various industry sources). Research into the basic biology of these viruses is required to assess the potential threat of these viruses to this developing industry.

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