
Putative antiviral activity in hemolymph from adult Pacific oysters, *Crassostrea gigas*

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Abstract: Innate, non-specific resistance mechanisms are important to pathogens, particularly for delaying virus replication at the onset of infection. Innate immunity constitutes the first line of defense in vertebrates and is the only one in invertebrates. Little is known about possible antiviral substances in invertebrates. The present work concerns a study of antiviral substances in hemolymph from adult *Crassostrea gigas* oysters. Despite the detection of cytotoxicity in fresh filtered hemolymph for both mammalian (CC50: 750 µg/ml) and fish cells (CC50: >2000 µg/ml for EPC cells and 345 µg/ml for RTG-2 cells), an antiviral substance was detected. Fresh filtered hemolymph was capable of inhibiting the replication of herpes simplex virus type 1 in vitro at an EC50 of 425 µg/ml (total proteins) and the replication of infectious pancreatic necrosis virus in EPC and RTG-2 cells at 217 and 156 µg/ml (total proteins), respectively.

Keywords: Oyster; *Crassostrea gigas*; Antiviral defense; HSV-1; IPNV; VHSV; Hemolymph

1. Introduction

Several molecules extracted from marine invertebrates, including bivalves, possess broad spectrum anti-microbial activities, affecting the growth of bacteria, fungi and yeasts (Nakamura et al., 1988; Mitta et al., 2000; Zasloff, 2002). However, there is relatively little information available on the antiviral defense mechanisms of bivalves. Antiviral substances (paolin I and II) have been reported in clam, *Mya arenaria* (Li et al., 1972 ; Prescott et al., 1966), and *in vitro* assays were used to detect a neutralising activity against T3 coliphage in haemolymph from the Pacific oyster, *Crassostrea gigas* (Bachère et al., 1990). A peptide that inhibits HIV-1 protease was also isolated from *C. gigas* hydrolysate (Lee and Maruyama, 1998).

The first bivalve virus was reported in adult eastern oysters, *C. virginica*, and had a particle morphology indicating membership of the family *Herpesviridae* (Farley et al., 1972). Since 1991, high mortalities of *C. gigas* larvae and juveniles have been observed regularly in association with herpesvirus infections in countries around the world (Renault et al., 2001; Arzul and Renault, 2002). Adult oysters appear less sensitive to herpesvirus infections. However, although abnormal mortality has not occurred among *C. gigas* adults in France, the presence of viral DNA was demonstrated in adults of normal appearance (Arzul et al., 2002). Thus, like other herpesviruses, ostreid herpesvirus 1 (OsHV-1) seems to be able to persist in adult *C. gigas*, which are evidently able to maintain effective immunity. Thus, *C. gigas* appears to be an appropriate invertebrate species in which to study antiviral innate immunity. The present study led to the detection of an activity in *C. gigas* acellular haemolymph that inhibits growth of a mammalian virus, herpes simplex virus type 1 (HSV-1; family *Herpesviridae*) *in vitro* at concentrations causing minimum cytotoxicity. Antiviral activity was also evaluated on fish cell lines infected by two fish viruses, viral haemorrhagic septicaemia virus (VHSV, family *Rhabdoviridae*) and infectious pancreatic necrosis virus (IPNV, family *Birnaviridae*).

2. Materials and Methods

2.1. Sources of oysters and haemolymph collection

Three year-old Pacific oysters, *C. gigas*, were obtained from shellfish farms in the Marennes-Oléron Bassin (Charente Maritime, France) on the French Atlantic coast. Oysters were collected during the winter of 2002-3 and the summer of 2003. Haemolymph was collected from the pericardial cavity using a sterile syringe (1 ml, 23G, n°16, Terumo). An acellular fraction was obtained by filtering the crude haemolymph through a 0.22 µm sterile filter. The filtered material was stored in aliquots at -80°C. Total concentrations of proteins in the extracts were determined by the Lowry method (Lowry et al., 1951).

2.2. Cells and viruses

African green monkey kidney cells (Vero, ATCC CCL-81) were grown in Eagle's minimum essential medium (MEM, Eurobio) supplemented with 8% fetal calf serum (FCS, Eurobio) and 1% PCS (10000 IU/ml penicillin, 25000 IU/ml colimycin, 10 mg/ml streptomycin; Sigma). HSV-1 (wild type strain 17, sensitive to acyclovir) was obtained from Pr. Ingrand (Hôpital A. Bécélère, Reims, France).

The fibroblastic RTG-2 cell line, derived from rainbow trout (*Oncorhynchus mykiss*) gonad tissue, and the epithelial EPC (*Cyprini epithelioma papulosum*) cell line derived from carp, *Cyprinus carpio*, were incubated at 14°C in Eagle's Glasgow medium (MEM, BioWhittaker) supplemented with 2% FCS and antibiotics (100 IU/ml penicillin, 0.1 g/ml streptomycin **ARE THESE FINAL CONCENTRATIONS?**). IPNV (VR₂₉₉ strain) and VHSV (07.71 strain) were propagated in RTG-2 and EPC cells, **RESPECTIVELY?** aliquoted and stored at -80°C.

Virus titers were estimated from cytopathogenicity according to the Reed and Muench (1938) dilution method, and expressed as 50% infectious doses per milliliter (ID₅₀/ml). The HSV-1 stock had a titre of 2x10^{5.80} ID₅₀/ml. The IPNV and VHSV stocks had titres of 2x10^{5.26} ID₅₀/ml.

2.3. Cytotoxicity assay by cell viability

Using the Vero cell/HSV-1 model, cytotoxicity was evaluated by incubating cellular suspensions (3.5x10⁵ cells/ml) with various dilutions (total protein concentration from 200 to 1100 µg/ml, 4 wells per concentration) of oyster haemolymph in 96-well plates (48 hours, 37°C, 5% CO₂) in Eagle's MEM containing 8% FCS. The cells were examined daily under a phase-contrast microscope to determine the minimum concentration of haemolymph that induced alterations in cell morphology, including swelling, shrinkage, granularity and detachment. Cytotoxicity by cell viability was tested using the neutral red dye method (McLaren et al., 1983). Optical density (OD) was measured at 540 nm using a spectrophotometer (SpectraCountTM, Packard). The 50% cytotoxic concentration (CC₅₀) was defined as the concentration that reduced the OD of treated cells to 50% of that of untreated cells. CC₅₀ values were expressed as the percentage of destruction (%D): [(OD_c)C – (OD_c)MOCK / (OD_c)C] x 100. (OD_c)C and (OD_c)MOCK were the OD values of the untreated cells and treated cells, respectively.

Using fish models, cytotoxicity was evaluated by incubating RTG-2 cells (4.75x10⁵ cells/ml) or EPC cells (12.5x10⁵ cells/ml) with various dilutions of oyster haemolymph (total protein concentration from 70 to 550 µg/ml, 2 wells per concentration) in 96-well plates (48 hours at 14°C) in Eagle's MEM containing 2% FCS. Cytotoxicity was measured as absorbance at 590 nm after incubation for 45 minutes in crystal violet (Renault et al., 1991). CC₅₀ values were derived as described above.

2.4. Antiviral assays by cell viability

Using the Vero cell/HSV-1 model, 100 µl of cellular suspension (3.5x10⁵ cells/ml) in Eagle's MEM containing 8% FCS was incubated with 50 µl of a dilution of filtered haemolymph (total protein concentration from 200 to 1100 µg/ml) in 96 well-plates (48 hours, 37°C, 5% CO₂). Three replicates were infected using 50 µl of medium or a virus suspension at a MOI of 0.001 ID₅₀/cells. After incubation, antiviral activity was evaluated by the neutral red dye method. The antiherpetic compound acyclovir (9-(2-hydroxyethoxymethyl)guanine) was used as reference inhibitor. The 50% effective antiviral concentration (EC₅₀) was expressed as the concentration that achieved 50% protection of virus-infected cells from virus-induced destruction. The OD was related directly to the percentage of viable cells, which was inversely related to the cytopathic effect (CPE) **THE MEANING OF THE ORIGINAL SENTENCE WAS NOT CLEAR**. The linear regression was determined for each assay on the basis of cell controls (0% CPE) and virus controls (100% CPE). Data were expressed as a percentage of protection (%P): [((OD_t)virus – (OD_c)virus) / ((OD_c)MOCK – (OD_c)virus)] x 100. (OD_t)virus was the OD of the test sample, (OD_c)virus was the OD of the

virus control (no haemolymph), and (ODc)MOCK was the OD of the mock-infected control (Langois et al., 1986).

Using fish RNA viruses, 50 μ l of cellular suspension (RTG-2, 4.75×10^5 cells/ml or EPC, 12.5×10^5 cells/ml) in Eagle's MEM containing 2% FCS was incubated with 25 μ l of various dilutions of oyster haemolymph (70 to 550 μ g/ml) in 96 well-plates (48 hours, 14°C). At each concentration, two replicates were infected by 25 μ l of medium or virus suspension at a MOI of 0.01 ID₅₀/cell. After incubation, antiviral activity was evaluated by the crystal violet method. ODs were determined at 590 nm, and EC₅₀ values were calculated as described above.

2.5. Mechanism of action

Virucidal assay. A virus suspension containing 0.001 ID₅₀/cell of HSV-1 or 0.01 ID₅₀/cell of IPNV was incubated with an equal volume of medium with or without haemolymph dilutions (70 to 550 μ g/ml of total proteins) for 1 h at 37°C for HSV-1 and overnight at 14 °C for IPNV. One hundred μ l of mixed suspension was then added to 100 μ l of cellular suspension (3×10^5 Vero cells/ml or 4.75×10^5 RTG-2 cells/ml) in culture medium (Damonte et al., 1994, 1996; Bergé et al., 1999). After incubating for 48 hours, the virucidal effect was determined using the neutral red dye method for HSV-1 and the crystal violet method for IPNV.

Effect before infection. To determine whether a cellular anti-viral state could be induced by haemolymph, cells were incubated with haemolymph (24 hours, 37°C, 5% CO₂) and were then washed with PBS. Cells were inoculated with virus and incubated for 48 hours (37°C, 5% CO₂) (Damonte et al., 1994, 1996; Bergé et al., 1999). The effect on virus multiplication was determined by the neutral red dye method for HSV-1 and the crystal violet method for IPNV.

Effect of haemolymph addition time. Monolayers of Vero cells were inoculated with HSV-1 at 0.001 ID₅₀/cell, and haemolymph was added simultaneously or after 1, 2, 3 or 5 h following infection. After 48 h of incubation, the effect on HSV-1 replication was determined by the neutral red dye method. RTG-2 cells were inoculated with IPNV at 0.01 ID₅₀/cell. Haemolymph was added simultaneously or after 1, 2, 3 or 5 h following infection (Damonte et al., 1994, 1996; Bergé et al., 1999). After 48 h of incubation, the effect on IPNV replication was determined by the crystal violet method.

Virus adsorption assay. The inhibitory effect of haemolymph on virus adsorption was measured on confluent monolayers of Vero cells infected with HSV-1 at 0.001 ID₅₀/cell, under different treatments (Damonte et al., 1994, 1996; Bergé et al., 1999). In treatment I, cells were exposed to HSV-1 in the presence of various haemolymph dilutions. After virus adsorption (1 h at 4°C), cells were washed with PBS to remove both haemolymph and unadsorbed virus and further incubated with medium. In treatment II, cells were exposed to HSV-1 and after a virus adsorption period (1 h at 4°C), unadsorbed virus was removed and cells were further incubated with the medium containing different concentrations of haemolymph. In treatment III, haemolymph was present both during and after the adsorption period. The effect on HSV-1 adsorption was determined after 2 days by the neutral red dye method.

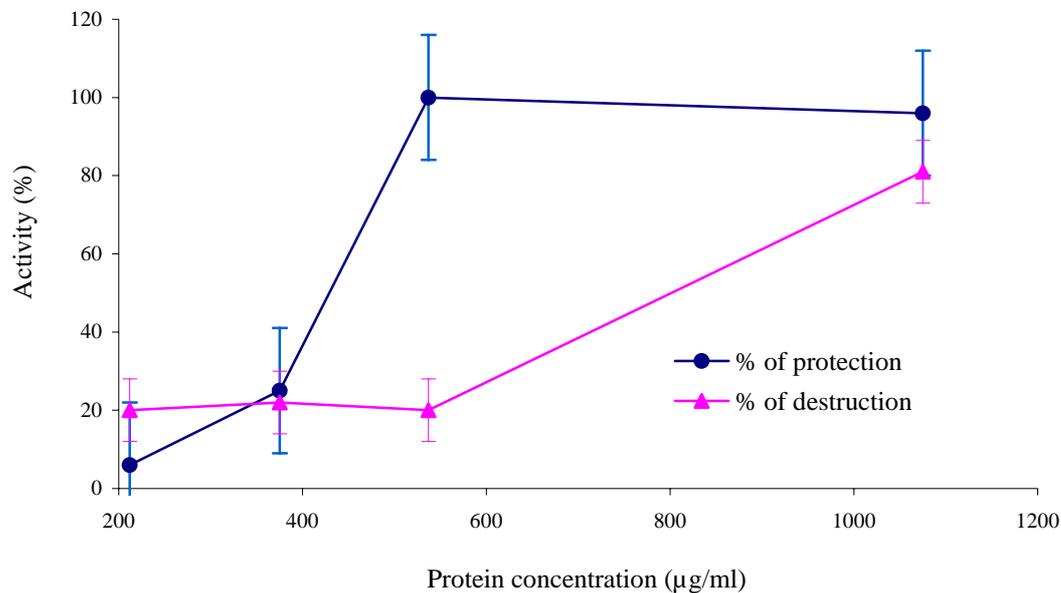
Figure 1: Putative anti-HSV-1 activity of filtered oyster haemolymph.

Figure 1 shows the antiviral and cytotoxic activities of acellular haemolymph observed after incubating for 48 hours a 96-well plate containing Vero cells infected by HSV-1 (MOI 0.001 ID₅₀/cell) with various concentrations of haemolymph. Vero cells are mammalian fibroblastic cells and HSV-1 is herpes simplex virus type 1.

Antiviral activity is expressed as the percentage of viable virus-infected cells (% of protection). Cytotoxic activity is observed as the percentage of viable mock-infected cells (% of destruction). The 50% effective antiviral concentration (EC₅₀) was 425 µg/ml of total protein. The 50% cytotoxic concentration (CC₅₀) was 750 µg/ml of total protein.

Evaluation was carried out three times.

Prescott et al. (1966) reported that molecules (paolins) from clams and oysters were active *in vitro* and *in vivo* against micro-organisms, including HSV-1. Moreover, Lee and Maruyama (1998) isolated a peptide from a hydrolysate of the Pacific oyster, *C. gigas*, which inhibited HIV-1 protease. Previous studies hardly suggested that broadly non-specific antiviral substances are present in tissue extracts from crustaceans, including shrimp, crayfish and crab (Pan et al., 2000) **THE ORIGINAL MEANING IS NOT CLEAR AND I HAVE NOT CHANGED IT**. Although the selective index (1.76) (Table 1) is low, this study highlights the presence of an antiviral activity in adult oyster haemolymph.

Table 1: Haemolymph activity (EC_{50} and CC_{50}) on fish viruses.

	EPC/IPNV	RTG-2/IPNV	EPC/VHSV	RTG-2/VHSV	HSV-1/Vero
EC_{50} ($\mu\text{g/ml}$)	217.3	156.4	>2000	>2000	425
CC_{50} ($\mu\text{g/ml}$)	>2000	346.2	>2000	346.2	750
SI	9.20	2.21	<1.00	<0.17	1.76

Table 1 shows the antiviral and cytotoxic activities of acellular haemolymph observed after incubating for 48 hours a 96-well plate containing fish cells infected by fish viruses in the presence of different concentrations of haemolymph. EPC are carp epithelial cells, RTG-2 are rainbow trout gonad fibroblastic cells, and Vero are mammalian (simian) fibroblastic cells. IPNV is infectious pancreatic necrosis virus, VHSV is viral haemorrhagic septicaemia virus, and HSV-1 is herpes simplex virus type 1. Results are expressed as EC_{50} (the 50% antiviral effective concentration), CC_{50} (the 50% cytotoxic concentration) and SI, the selective index (CC_{50}/EC_{50}).

Evaluation was carried out in triplicate.

A first approach to determining the mode of action of haemolymph was initiated by carrying out different treatments on the HSV-1/Vero model, and the results are shown in Table 2. Results are expressed as percentage of viable virus-infected cells (% of protection: %P) and as the percentage of viable mock-infected cells (% of destruction: %D).

Hm : Haemolymph

Addition time : Hm was added simultaneously, or 1, 2, 3 or 5 hours after virus inoculation

Treatment I : Virus + cells + Hm \rightarrow (1h, 4°C) \rightarrow PBS \rightarrow cells

Treatment II : Virus + cells \rightarrow (1h, 4°C) \rightarrow PBS \rightarrow cells + Hm

Treatment III : Virus + cells + Hm \rightarrow (1h, 4°C) \rightarrow PBS \rightarrow cells + Hm

Effect before infection : cells and Hm were incubated for 24 h at 37°C before virus inoculation

Virucidal assay : virus and Hm were incubated for 1 h at 37°C before addition to cell suspension

Evaluation was carried out in triplicate.

3.2. Antiviral activity against fish viruses

The evaluation of antiviral activity in fish models provided complementary information. The CC_{50} of filtered haemolymph on EPC cells was greater than 2000 $\mu\text{g/ml}$, but values reached 346.2 $\mu\text{g/ml}$ of proteins on RTG-2 cells (Table 1).

No inhibition of VHSV growth was observed on EPC and RGT-2 cells (Table 1). However, haemolymph had an antiviral effect against IPNV (a non-enveloped RNA virus) in fibroblastic and epithelial cells. It completely inhibited (100% of protection) the growth of IPNV in RTG-2 cells at a total protein concentration of 137.5 $\mu\text{g/ml}$ **THIS IS NOT**

CONSISTENT WITH THE DATA IN TABLE 1, AS THIS VALUE IS LESS THAN THE EC₅₀. The effect was greater in fibroblastic cells (EC₅₀ 156.4 µg/ml) than in epithelial cells (EC₅₀ 217.3 µg/ml).

The results using fish models confirm the presence of an antiviral activity in adult oyster haemolymph. The selective index in fish models (2.21 in the RTG-2/IPNV model and 9.20 in the EPC/IPNV model) was greater than that in the mammalian model (1.76). In contrast to the epithelial fish cell line (EPC), haemolymph produced major cytotoxicity on both fibroblastic cell lines (RTG-2 and Vero cells), with CC₅₀ values of 346.2 and 750 µg/ml, respectively. EC₅₀ values in the IPNV/RTG-2 and HSV-1/Vero models were close to the CC₅₀ values, and thus it is difficult to differentiate cytotoxicity from an antiviral effect. Nevertheless, an effect was discerned with models. THE LAST TWO SENTENCES WERE NOT CLEAR, AND I HAVE GUESSED A BIT. Filtered haemolymph did not inhibit VHSV, an enveloped RNA virus, but exerted an antiviral activity against IPNV, a non-enveloped RNA virus. IS THIS OK? Moreover, no cytotoxic effect was detected on EPC cells.

Antiviral substances from invertebrates have been reported previously. Halocyamins A IS THIS NAME RIGHT AND DOES IT HAVE TO BE A PLURAL? (Azumi et al., 1990) isolated from haemocytes of ascidie WHAT IS THIS? induced an inhibition of IPNV growth in RTG-2 cells for 100 µg/ml of pure peptide. Moreover, Pan et al. (2000) demonstrated that crustacean tissue extracts from crab, shrimp and crayfish are broadly antiviral against a variety of viruses, including DNA and enveloped and non-enveloped RNA viruses.

3.3. First approach to the mechanism of action

A first approach to determining the mode of action of haemolymph was initiated by carrying out different treatments in the HSV-1/Vero and IPNV/RTG-2 models. WHERE ARE THE DATA FOR IPNV?

Virucidal assay. Preincubation of the virus with haemolymph did not protect Vero cells against HSV-1 (Table 2). IPNV suspension mixed for one night at 14°C with haemolymph did not protect RTG-2 cells against infection. These results indicate that haemolymph does not irreversibly neutralise HSV-1 or IPNV.

Table 2: A first approach to determining the mechanism of action of filtered oyster haemolymph.

Mechanisms		%P	%D	EC ₅₀ control
Virucidal assay	48 h	0.00	30.00	0.27
Effect before infection	48 h	0.00	35.00	>2.00
Effect of time of haemolymph addition	0 h	89.00 (±21)	20.00 (±4)	0.32
	1 h	98.00 (±4)	38.00 (±24)	0.32
	2 h	100.00 (±0)	33.00 (±0)	0.40
	3 h	0.00 (±4)	33.00 (±1)	0.45
	5 h	0.00 (±0)	41.00 (±3)	0.53
Virus adsorption assay	Treatment I	35.50	16.00	0.90
	Treatment II	74.00	35.00	0.14
	Treatment III	98.50	44.00	0.16

Effect before infection. Vero cells were not protected from HSV-1 infection when haemolymph was present only before virus infection (Table 2). The haemolymph did not induce a durable antiviral state in the target cells.

Effect of haemolymph addition time. Vero cells were optimally protected (89-100%) when haemolymph was added at 0-2 hours after infection (Table 2). After 3 hours, antiviral activity decreased dramatically. The cytotoxicity increased from 20 to 41% between 0-5 hours (Table 2). In comparison, the EC₅₀ of acyclovir increased from 0.32 to 0.53 µg/ml between 0-5 hours (Table 2). Similar results using haemolymph were observed in the fish model (data not shown).

Virus adsorption assay. With treatment I, a low antiviral activity (35% of protection) and 16% of cell destruction were reported. With treatment II, a high percentage of cell protection (74%) was observed. With treatment III, a higher percentage of protection than in treatment II (98%) was reported (Table 2). Vero cells were not protected from HSV-1 infection when haemolymph was present before infection (Table 2).

Haemolymph did not exert a virucidal effect on HSV-1. Moreover, virus inhibition required the simultaneous presence of haemolymph, virus and cells, suggesting the reversible nature of the inhibition. The antiviral activity might be mediated throughout an intracellular mechanism only in infected cells. Li et al. (1972) also reported a virus inhibition by paolins from clams that takes place in cells. Oyster haemolymph inhibits HSV-1 replication at an early stage of the replication cycle, between 0 and 2 hours after initiation of infection. It may thus interfere with the adsorption process, resulting in inhibition of virus attachment to the cell surface. Because of the low selective index observed due to a high degree of cytotoxicity, we could not determine the mode of action of haemolymph. Nevertheless, we suggest that acellular haemolymph contains different components which could induce cytotoxicity as well as antiviral activity.

Knowledge of the immune mechanisms activated after viral infection of vertebrates would suggest an interferon-type mechanism. However, the presence of interferons has not yet been observed in invertebrates, except for the interference mechanism in shrimps and insects (Bulet et al., 1999; Chernysh et al., 2002) **THIS IS NOT CLEAR**. Two antiviral and antitumoral peptides called alloferons have been isolated from the blood of the blow fly *Calliphora vicina* (Chernysh et al., 2002). This study showed that the alloferon molecules induced cytokine-like synthesis and were able to modulate the activity of human natural killer cells **IS THIS OK?**

Despite a high level of cytotoxicity detected in oyster haemolymph, a putative antiviral substance with a potential defence role was detected, and is of potential interest in understanding invertebrate immunity. This study should open the way to more effective veterinary medicine in various cultivated species, both in terms of antiviral therapeutic agents and the identification of new targets involved in controlling infections. Further investigations are in progress to confirm the presence of an antiviral molecule in adult *Crassostrea gigas*.

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