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# 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 ($CC_{50}$: 750 μg/ml) and fish cells ($CC_{50}$: >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 $EC_{50}$ 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 antimicrobial 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 and Traxler, 1972; Prescott et al., 1966), and in vitro assays were used to detect a neutralizing activity against T3 coliphage in hemolymph 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 literature does not clearly mention the existence of antiviral substances in *C. gigas*.

The first bivalve virus was reported in adult eastern oysters, *Crassostrea 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 to study antiviral innate

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immunity. The present study led to the detection of an activity in *C. gigas* acellular hemolymph that inhibits growth of a mammalian virus, *herpes simplex* virus type 1 (HSV-1; family *Herpesviridae*) in vitro at concentrations causing moderate cytotoxicity. Antiviral activity from oyster hemolymph was also evaluated in fish cell lines infected by two fish viruses, viral hemorrhagic septicemia virus (VHSV, family *Rhabdoviridae*) and infectious pancreatic necrosis virus (IPNV, family *Birnaviridae*).

## 2. Materials and methods

### 2.1. Sources of oysters and hemolymph collection

Three year-old Pacific oysters, *C. gigas*, were obtained from shellfish farms in the Marennes-Olèron Bassin (Charente Maritime, France) on the French Atlantic coast. Oysters were collected during the winters of 2002–2003 and the summer of 2003. Hemolymph was collected from the pericardial cavity using a sterile syringe (1 ml, 23 G, no. 16, Terumo). An acellular fraction was obtained by filtering the crude hemolymph 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% of antibiotics PCS (10,000 IU/ml penicillin, 25,000 IU/ml colimycin, 10 mg/ml streptomycin; Sigma). HSV-1 (wild type strain 17, sensitive to acyclovir) was obtained from Pr. Ingrand (Hôpital A. Béclère, Reims, France).

The fibroblastic RTG-2 cell line, derived from rainbow trout (*Oncorhyncus 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 0.1% of a 1000× antibiotics solution (100 IU/ml penicillin, 0.1 g/ml streptomycin). IPNV ($VR_{299}$ strain) and VHSV (07.71 strain) were propagated in both RTG-2 and EPC cells lines, 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 ($ID_{50}$/ml). The HSV-1 stock had a titre of $2 \times 10^{5.80}$ $ID_{50}$/ml. The IPNV and VHSV stocks had titers of $2 \times 10^{5.26}$ $ID_{50}$/ml.

### 2.3. Cytotoxicity assays based upon cell viability

Using the Vero cell/HSV-1 model, cytotoxicity was evaluated by incubating cellular suspensions ($3.5 \times 10^5$ cells/ml) with various dilutions (total protein concentration from 200 to 1100 μg/ml, 4 wells per concentration) of oyster hemolymph in 96-well plates (48 h, 37 °C, 5% $CO_2$) in Eagle's MEM containing 8% FCS. The cells were examined daily under a phase-contrast microscope to determine the minimum concentration of hemolymph 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 (SpectraCount™, Packard). The 50% cytotoxic concentration ($CC_{50}$) was defined as the concentration that reduced the OD of treated cells to 50% of that of untreated cells. $CC_{50}$ values were expressed as the percentage of destruction (%*D*): [(ODc)C − (ODc)MOCK/(ODc)C] × 100. (ODc)C and (ODc)MOCK were the OD values of the untreated cells and treated cells, respectively (Langlois et al., 1986).

Using fish models, cytotoxicity was evaluated by incubating RTG-2 cells ($4.75 \times 10^5$ cells/ml) or EPC cells ($12.5 \times 10^5$ cells/ml) with various dilutions of oyster hemolymph (total protein concentration from 70 to 550 μg/ml, 2 wells per concentration) in 96-well plates (48 h at 14 °C) in Eagle's MEM containing 2% FCS. Cytotoxicity was measured as absorbance at 590 nm after incubation for 45 min in crystal violet (Renault et al., 1991). $CC_{50}$ values were derived as described above.

### 2.4. Antiviral assays based upon cell viability

Using the Vero cell/HSV-1 model, 100 μl of cellular suspension ($3.5 \times 10^5$ cells/ml) in Eagle's MEM containing 8% FCS was incubated with 50 μl of a dilution of filtered hemolymph (total protein concentration from 200 to 1100 μg/ml) in 96 well-plates (48 h, 37 °C, 5% $CO_2$). Three replicates were infected using 50 μl of medium and a virus suspension at a MOI of 0.001 $ID_{50}$/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 ($EC_{50}$) 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 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*): [((ODt)virus − (ODc)virus)/((ODc)MOCK − (ODc)-virus)] × 100. (ODt)virus was the OD of the test sample, (ODc)virus was the OD of the virus control (no hemolymph), and (ODc)MOCK was the OD of the mock-infected control (Langlois et al., 1986).

Using fish RNA viruses, 50 μl of cellular suspension (RTG-2, $4.75 \times 10^5$ cells/ml or EPC, $12.5 \times 10^5$ cells/ml) in Eagle's MEM containing 2% FCS was incubated with 25 μl of various dilutions of oyster hemolymph (70–550 μg/ml) in

96 well-plates (48 h, 14 °C). At each concentration, two replicates were infected by 25 μl of medium or virus suspension at a MOI of 0.01 $ID_{50}$/cell. After incubation, antiviral activity was evaluated by the crystal violet method. ODs were determined at 590 nm, and $EC_{50}$ values were calculated as described above.

### 2.5. Mechanism of action studies

#### 2.5.1. Virucidal assay

A virus suspension containing 0.001 $ID_{50}$/cell of HSV-1 or 0.01 $ID_{50}$/cell of IPNV was incubated with an equal volume of medium with or without hemolymph dilutions (70–550 μg/ml of total proteins) for 1 h at 37 °C for HSV-1 and overnight at 14 °C for IPNV. One hundred microliters of mixed suspension was then added to 100 μl of cellular suspension ($3 \times 10^5$ Vero cells/ml or $4.75 \times 10^5$ RTG-2 cells/ml) in culture medium (Damonte et al., 1994, 1996; Bergé et al., 1999). After incubation for 48 h, the virucidal effect was determined using the neutral red dye method for HSV-1 and the crystal violet method for IPNV.

#### 2.5.2. Effect before infection

To determine whether a cellular antiviral state could be induced by hemolymph, cells were incubated with hemolymph (24 h, 37 °C, 5% $CO_2$) and were then washed with PBS. Cells were inoculated with virus and incubated for 48 h (37 °C, 5% $CO_2$) (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.

#### 2.5.3. Effect of hemolymph addition time

Monolayers of Vero cells were inoculated with HSV-1 at 0.001 $ID_{50}$/cell, and hemolymph 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 $ID_{50}$/cell. Hemolymph 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.

#### 2.5.4. Virus adsorption assay

The inhibitory effect of hemolymph on virus adsorption was measured on confluent monolayers of Vero cells infected with HSV-1 at 0.001 $ID_{50}$/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 hemolymph dilutions. After virus adsorption (1 h at 4 °C), cells were washed with PBS to remove both hemolymph 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 hemolymph. In Treatment III, hemolymph 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.

## 3. Results and discussion

The antiviral evaluation consisted of testing oyster hemolymph on a mammalian fibroblastic cell line (Vero cells) infected by HSV-1. This model is currently used for the screening of antiviral molecules from marine organisms (Bergé et al., 1999; Yasin et al., 2000; Maier et al., 2001). The choice of a heterologous model was imposed by the lack of bivalve cell lines (Prescott et al., 1966; Li and Traxler, 1972; Azumi et al., 1990; Tamamura et al., 1993; Lee and Maruyama, 1998). The antiviral activity of oyster hemolymph was also evaluated in fish cell lines infected by two fish viruses (VHSV and IPNV).

### 3.1. Hemolymph cytotoxicity and anti-HSV-1 activity in a mammalian model

Investigation of antiviral activity in acellular hemolymph was conducted. At 48 h after infection, 100% (±16%) of cellular protection was obtained for a 537 μg/ml protein concentration at a MOI of 0.001 $ID_{50}$/cell ($EC_{50}$ of 425 μg/ml) (Fig. 1). Acyclovir (1 μg/ml) conferred total protection (100%) against HSV-1 with a low percentage of cell destruction (5%). Hemolymph did not inhibit viral production at a MOI of 0.01 $ID_{50}$/cell. After 48 h of treatment, the viability assay showed destruction of the cell monolayer at a $CC_{50}$ of 750 μg/ml. Above 537 μg/ml protein, cytotoxicity increased from 20 to 80%, thus compromising the antiviral activity (Fig. 1).

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. Although the selectivity index (1.76) (Table 1) is low, this study highlights the presence of an antiviral activity in adult oyster hemolymph.

### 3.2. Antiviral activity against fish viruses

The evaluation of antiviral activity in fish models provided complementary information. The $CC_{50}$ of filtered hemolymph on EPC cells was greater than 2000 μg/ml, but values reached 346.2 μg/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, hemolymph 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

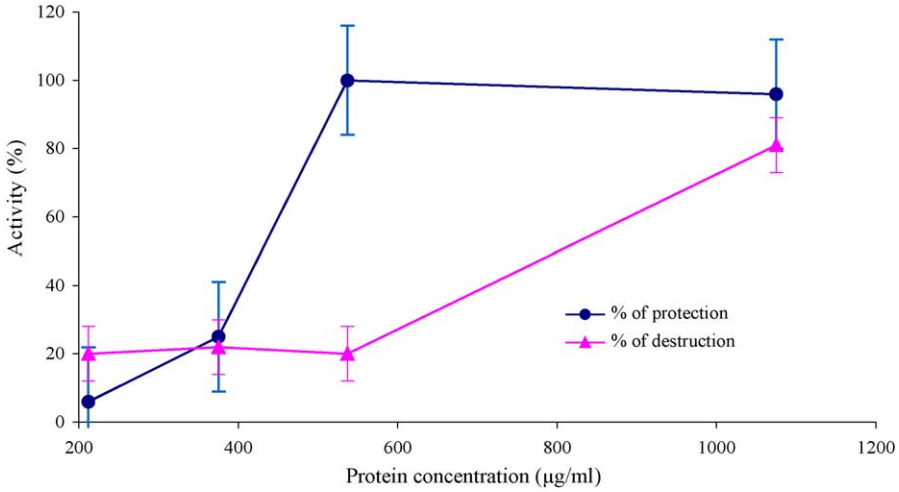


Fig. 1. Putative anti-HSV-1 activity of filtered oyster hemolymph. This figure shows the antiviral and cytotoxic activities of acellular hemolymph observed after incubating for 48 h Vero cells infected by HSV-1 (MOI 0.001 $ID_{50}$/cell) with various concentrations of hemolymph. Vero cells are mammalian 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 ($EC_{50}$) was 425 μg/ml of total protein. The 50% cytotoxic concentration ($CC_{50}$) was 750 μg/ml of total protein. Evaluation was carried out in triplicate.

protein concentration of 156.4 μg/ml. The effect was greater in fibroblastic cells ($EC_{50}$: 90 μg/ml) than in epithelial cells ($EC_{50}$: 156.4 μg/ml).

The results using fish models confirm the presence of an antiviral activity in adult oyster hemolymph. The selectivity index in fish models (3.84 in the RTG-2/IPNV model and 12.78 in the EPC/IPNV model) was greater than that in the mammalian model (1.76). However, hemolymph produced major cytotoxicity in both fibroblastic cell lines (RTG-2 and Vero cells), with $CC_{50}$ values of 346.2 and 750 μg/ml, respectively. $EC_{50}$ values in the HSV-1/Vero models were close to the $CC_{50}$ values, and it is thus difficult to differentiate cytotoxicity from antiviral activity. Filtered hemolymph did not inhibit VHSV, an enveloped RNA virus, but exerted an antiviral activity against IPNV, a non-enveloped RNA virus. Moreover, no cytotoxic effect was detected on EPC cells.

Antiviral substances from invertebrates have been reported previously. Halocyamin (Azumi et al., 1990) isolated from ascidian hemocytes 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 antivirally active against a variety of viruses, including DNA and enveloped and non-enveloped RNA viruses.

### *3.3. First approach to determining the mechanism of action*

A first approach to determine the mode of action of hemolymph was initiated by carrying out different treatments in the HSV-1/Vero and IPNV/RTG-2 models.

#### *3.3.1. Virucidal assay*

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

#### *3.3.2. Effect before infection*

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

Table 1
Hemolymph activity ($EC_{50}$ and $CC_{50}$) against fish viruses

| | EPC/IPNV | RTG-2/IPNV | EPC/VHSV | RTG-2/VHSV | HSV-1/Vero |
|---|---|---|---|---|---|
| $EC_{50}$ (μg/ml) | 156.4 | 90.0 | >2000 | >2000 | 425 |
| $CC_{50}$ (μg/ml) | >2000 | 346.2 | >2000 | 346.2 | 750 |
| SI | 12.78 | 3.84 | <1.00 | <0.17 | 1.76 |

This table shows the antiviral and cytotoxic activities of acellular hemolymph observed after incubating for 48 h a 96-well plate containing fish cells infected by fish viruses in the presence of different concentrations of hemolymph. 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 hemorrhagic septicemia 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.

Table 2
Approach to determine the mechanism of action of filtered oyster hemolymph

| 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 hemolymph 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 |

An approach to determine the mode of action of hemolymph was initiated by carrying out different treatments on the HSV-1/Vero model, and the results are shown in this table. 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: Hemolymph, Addition time: Hm was added simultanously, or 1, 2, 3 or 5 h after virus inoculation. Treatment I: Virus + cells + Hm → (1 h, 4 °C) → PBS → cells; Treatment II: Virus + cells → (1 h, 4 °C) → PBS → cells + Hm; Treatment III: Virus + cells + Hm → (1 h, 4 °C) → PBS → 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.

### 3.3.3. *Effect of hemolymph addition time*

Vero cells were optimally protected (89–100%) when hemolymph was added at 0–2 h after infection (Table 2). After 3 h, antiviral activity decreased dramatically. The cytotoxicity increased from 20 to 41% between 0 and 5 h (Table 2). In comparison, the $EC_{50}$ of acyclovir increased from 0.32 to 0.53 μg/ml between 0 and 5 h (Table 2). Similar results using hemolymph were observed in the fish model (data not shown).

### 3.3.4. *Virus adsorption assay*

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

Hemolymph did not exert a virucidal effect on HSV-1. Moreover, virus inhibition required the simultaneous presence of hemolymph, virus and cells, suggesting the reversible nature of the inhibition. The antiviral activity might be mediated throughout an intracellular mechanism in infected cells. Li and Traxler (1972) also reported virus inhibition by paolins from clams in infected cells. Oyster hemolymph inhibits HSV-1 replication at an early stage of the replication cycle, between 0 and 2 h 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 hemolymph. Nevertheless, we suggest that acellular hemolymph 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. However, antiviral molecules have been isolated in shrimps and insects (Bulet et al., 1999; Chernysh et al., 2002). Two antiviral and antitumoral peptides called alloferons have been isolated from the blood of the blow fly *Calliphorta 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.

Despite a high level of cytotoxicity detected in oyster hemolymph, a putative antiviral substance 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 marine 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 antiviral molecules in adult *C. gigas*.

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