Antiviral activity of hemolymph of *Podalia* against rubella virus

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Abstract Many active principles produced by animals, plants and microorganisms have been employed in the development of new drugs for the treatment of human diseases. Among animals known to produce pharmacologically active molecules that interfere in human cell physiology. Rubella virus (genus *Rubivirus*, family *Togaviridae*) is a single stranded RNA virus of positive genome polarity. Rubella virus infection of susceptible women during the first trimester of pregnancy often results in long-term virus persistence in the fetus causing multiple organ abnormalities. Potent antiviral activity against rubella virus (RV) has been observed in the hemolymph of *Podalia* sp. (Lepidoptera: Megalopygidae). This study

evaluated the effect of hemolymph on RV infected Statens Serum Institute Rabbit Cornea (SIRC) cells. Results of cell viability and cell proliferation assays indicated that hemolymph was not toxic to cultured SIRC cells. Viral binding assay, antiviral assay, PCR, real-time PCR, and transmission electron microscopy were used to demonstrate that hemolymph in post-treatment could inhibit the production of infectious RV particles. Specifically, hemolymph was found to inhibit RV adsorption to the SIRC cells.

Keywords Rubella virus · Congenital Rubella Syndrome · Hemolymph · Antiviral

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Introduction

Rubella virus (RV; genus *Rubivirus*, family *Togaviridae*) is a single-stranded RNA virus of positive genome polarity. RV infection of susceptible women during the first trimester of pregnancy often results in long-term virus persistence in the fetus, causing multiple organ abnormalities (Hobman and Chantler 2007; Atreya et al. 2004). RV can establish persistent infection in the developing fetus, where it continuously replicates and can induce multiple pathological changes. Annually, there are an estimated 110,000 cases of congenital rubella syndrome (CRS) worldwide (Curti et al. 2013). Vaccination with live attenuated rubella vaccine is the most

effective means to prevent CRS. Vaccination efforts have proven successful for preventing and eradicating some viral infections. Alternative means of control include the use of antiviral drugs; however, there are currently not many licensed and efficacious drugs available for prophylactic and therapeutic antiviral treatment. Insects have been highlighted in this scenario since several studies have demonstrated the antiviral effect of substances synthesized by this group. Antiviral activity from insect hemolymph (Chernysh et al. 2002) has also been described; however, the antiviral defense mechanism and any interactions between antiviral and other antimicrobial defenses remain unexplored (Popham et al. 2004). Other reports have demonstrated the presence of pharmacologically active substances in the hemolymph of *Lonomia obliqua* (Mendonça et al. 2008; Raffoul et al. 2005; Souza et al. 2005). The supplementation of cultures with hemolymph proteins has been reported to have a positive effect against viral replication and on recombinant protein production (Raffoul et al. 2005; Souza et al. 2005). Carmo et al. (2012) described a system for protein expression in Sf9/baculovirus cells using recombinant DNA produced from a sequence coding for a protein from the *L. obliqua* caterpillar that displayed potent antiviral action. Antiviral treatments for RV infection are usually limited, since commonly used antiviral drugs against this virus, such as acyclovir or immunoglobulin, are inefficient in eliminating the virions from chronically infected hosts (Gualberto et al. 2013). To the authors best knowledge, this study is the first to investigate and report on the antiviral effects of *Podalia* hemolymph against RV in vitro.

Materials and methods

Hemolymph sampling

Hemolymph (HL) of *Megalopygidae Podalia* sp. larvae was collected from immobilized larvae and clarified by centrifugation (10,000×g, 10 min, 4 °C). The supernatant was removed, filtered through a 0.2 µm, and stored at −20 °C. The total soluble protein content of the extract was quantified using the Bradford method (1976) with bovine serum albumin as a standard.

Cell culture

SIRC cells (Statens Serum Institute Rabbit Cornea ATCC CCL-60, ATCC, Manassas, VA, USA) were grown to confluence in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 20 mM L-glutamine (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere with 5% CO₂.

Observation of cell morphology

The morphology of cells from treated and untreated samples was assessed using an ACCU-Scope 3002 microscope with an attached camera. SIRC cells were plated in 6-well plates and grown for 24 h, and then different concentrations of hemolymph (30, 60, 120, and 180 mg/mL) were added to the wells. After 1 h, the hemolymph was removed by aspiration, and the cells were washed with phosphate-buffered saline (PBS). Fresh medium was added to the wells, and the cells were examined for morphological changes after an additional 24 h of incubation.

Cytotoxicity assay

SIRC cells were seeded at a concentration of 10⁴ cells/well in 96-well plates and grown at 37 °C. After a further 48 h, the cells were treated with different concentrations of hemolymph (0, 8, 16, 32, 68, 88, 136, and 272 mg/mL) or PBS (control negative). After 72 h of treatment, cell viability was determined using MTT (3-[4,5-dimethylthiazol 2-yl]-2,5 diphenyltetrazolium bromide), which reacts with the mitochondrial dehydrogenase of viable cells to give a blue formazan product that can be measured spectrophotometrically. The concentration required to reduce the cell viability by 50% (CC₅₀) was obtained. Dimethyl sulfoxide (DMSO; 1%), which shows no effect on cell viability, was used for the control.

Virus titration

SIRC cells (1 × 10⁴ cells/mL) were cultivated in 96-well plates in DMEM supplemented with 20 mM L-glutamine and 10% FBS (Invitrogen Carlsbad, CA, USA). The stock RV strain RA 27/3 (Meruvax II; Merck, Sharp & Dohme, Sao Paulo,

Brazil) was quantified by infecting cell cultures with a virus multiplicity of infection (MOI) of 0.01. The confluent cell cultures were inoculated with 100 µL of the diluted virus in quadruplicates. After 1 h adsorption at room temperature, each well received 200 µL of DMEM with 2% FBS, and the plates were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Uninfected cultures were also prepared and treated identically as controls. Plate cultures were observed daily for cytopathic effects during 7 days, when the test was concluded. The titers are given as MOI.

Antiviral activity evaluation

The antiviral activity of hemolymph against RV was evaluated using quantitative real-time PCR (qPCR). The SIRC cells were plated in 96-well plates at a concentration of 3.0×10^5 cells/mL in a volume of 100 µL per well and incubated during 48 h at 37 °C and 5% CO₂. The confluent SIRC cells were washed twice with PBS and treated with different concentrations (32, 68, 88, 136 and 180 mg/mL) of hemolymph for 1 h at 37 °C prior to infection (pre treatment) or after infection (post treatment) with RV (MOI = 0.1). DMSO alone was added to cells infected with RV (MOI = 0.1) as a control. After 1 h, the free virions were rinsed off, and the cells were incubated with DMEM supplemented with 2% FBS. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO₂. After 72 h, the hemolymph effects on the infected cells were determined using qPCR. The experiment was carried out in triplicates.

Binding assay

The binding assay was performed at 4 °C, a temperature that allows RV to bind to cell receptors but not to enter the cells. Prior to infection, the RVs (MOI = 0.1) were treated with different concentrations of hemolymph (68, 136 and 200 mg/mL) for 1 h at 37 °C. SIRC cells were plated in 24-well plates and allowed to reach confluency. The cells were treated with infected or untreated RV (MOI = 0.1) dilutions and allowed to adsorb for 1 h at 4 °C. Unabsorbed virions were then aspirated, and the cells were washed twice with PBS. The binding assay was carried out in triplicates. The results were analyzed using PCR and qPCR.

RT-PCR for RV

Total RNA from SIRC cells treated with hemolymph and inoculated with RV or untreated RVs, as described above, was extracted using the MagNA Pure extraction kit (Roche, Basel, Switzerland). RT-PCR was performed using the Access RT-PCR System (Promega, Madison, WI, USA). RV RNA was detected by a RT-PCR method previously described by the US Centers for Disease Control and Prevention (CDC/USA) (Abernathy et al. 2009; Figueiredo et al. 2012). The primers amplify a 185 nucleotide region in the E1 coding region (nucleotides 8807–8991). After PCR amplification, the presence of the product was confirmed by agarose gel electrophoresis and ethidium bromide staining.

Quantitative real-time PCR

Total RNA from the binding assay and antiviral activity (pretreatment, post-treatment, and RV-treated) assay were extracted using the MagNA Pure extraction kit (Roche). To amplify the RV genomic sequence, RT-PCR was performed using the Superscript III Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. For qPCR of the results from the RV assay, a set of primers described by CDC/USA (Abernathy et al. 2009) for amplifying the RV gene E1 was used. The assay was performed in triplicates with 25 µL reaction mixtures containing reaction buffer (Invitrogen, Carlsbad, CA, USA), 0.5 U of a Superscript-Taq enzyme mixture, 0.2 µM of each primer, 0.1 µM of the labeled probe (Sigma-Aldrich, USA), and 5 µL of RNA. The assay was carried out in triplicate for each sample, including a no-template control. The thermal cycling was carried out with an Applied Biosystems 7500 thermal cycle as follows: 50 °C for 10 min; 95 °C for 2 min; and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The presence of intact RNA in the samples was confirmed with primers specific for RNase P RNA. Standard curves were prepared by qPCR using serial dilutions of known copy numbers of the purified amplification product for RV. The copy number of the samples was calculated from the standard curves. The percentage of reduction was defined as follows: $[\text{copy no. of infected cells} - \text{copy no. of treated cells} / \text{copy no. of infected cells} \times 100]$. All experiments were carried out in triplicates. Four positive controls

containing known copy numbers of transcribed RV RNA were run on each plate as quantitation standards. A reaction mixture containing water as the template was run on each plate as the negative control. The data were analyzed with SDS software (version 2.1; Applied Biosystems, Grand Island, NY, USA).

Transmission electron microscopy

SIRC cells were cultivated on Aclar film seeded in 24-well plates and incubated for 48 h at 37 °C in a humidified atmosphere with 5% CO₂. SIRC cells were infected with RVs (MOI = 0.1) and treated with 136 or 200 mg/mL of hemolymph for 1 h at 37 °C after to cell infection. DMSO was used as the negative control. Unadsorbed virions were then aspirated, and the cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂. After 48 h, the cells were fixed with 2.5% glutaraldehyde (Sigma, St. Louis, MO, USA) in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at 4 °C. After rinsing twice with cacodylate buffer, the cultures were post-fixed in a solution containing 1% osmium tetroxide, 0.8% ferrocyanide, and 5 mM calcium chloride and then washed in 0.1 M sodium-cacodylate buffer, dehydrated in graded acetone, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using Exstat software. For analyses of the cell viability data and the antiviral activity by mRNA quantification, the Student's *t* test was used with the *p* value corrected by the Bonferroni–Sidak method.

Results

SIRC cells were exposed to different concentrations (30, 60, 120 and 180 mg/mL) of hemolymph and observed 8 h later by phase contrast microscopy. No significant changes in cell morphology were observed at any of the tested concentrations of hemolymph (data not shown). The cell viability was then determined by MTT assay. The results show that hemolymph was not significantly toxic to SIRC cells (Fig. 1). To evaluate

Fig. 1 Cell viability of SIRC cells treated with different concentrations of hemolymph. The number represent the mean of three replicates

the antiviral activity of hemolymph (32, 68, 88, 136 and 180 mg/mL), cells were treated with hemolymph prior to RV infection (pre-treatment cells) and after infection (post-treatment cells). Figure 2 shows that hemolymph post-treatment reduced the number of copies of RV in the cell lysates. Remarkably, treatment with 180 mg/mL hemolymph resulted in high inhibition from virus replication. Thus, these results confirm our hypothesis that hemolymph has high antiviral activity. In addition, to determine if treatment of RV with hemolymph disrupts virions from binding to the SIRC cellular receptor, a binding assay was performed. This assay is performed at 4 °C,

Fig. 2 Inhibitory effect of hemolymph on rubella viruses RNA synthesis in SIRC cells as analyzed by qRT-PCR. Cell culture lysates were collected after pre-treatment and post-treatment with different concentrations from hemolymph (32, 68, 88, 136 and 180 mg/mL) for 72 h. The inhibitory effect was determined using qPCR. The infectivity from RV decrease after post treatment with hemolymph. The error bars represent the SD calculated from three independent experiments in triplicate. Note the post-treatment the % inhibitory RV infection was more 90%

a temperature that allows viral binding but not cellular penetration. After incubation at 4 °C, the cells were incubated at 37° C for virus replication complete its cycle. Overall, the results indicate that treatment of RV with different concentrations of hemolymph (68, 136, and 200 mg/mL) was sufficient to stop the virus from binding and infecting SIRC cells. Although the cytopathic effect was seen in the cells infected with both untreated and DMSO-treated RVs, no such effect was observed with RVs treated with 68, 136, and 200 mg/mL hemolymph. The results (repeated in triplicates) indicate that hemolymph is able to inhibit the binding of virions to the cells (Figs. 3, 4).

In addition, SIRC cells infected with RV and treated with hemolymph were analyzed by transmission electron microscopy. No RV-like particles could be found in the cytoplasm of cells analyzed (Fig. 5). These results confirm the strong specificity of hemolymph for inhibiting RV and its replication.

Discussion

Rubella virus infection during the early stages of pregnancy can lead to serious birth defects, known as the congenital rubella syndrome. This study evaluated the effect of hemolymph on RV infected SIRC cells. Results of the cell viability and cell proliferation assays indicate that hemolymph is not toxic to cultured

Fig. 3 Binding assay of untreated RV or RV treated with hemolymph (68, 136 and 200 mg/mL). Infectivity was determined by qPCR. The numbers represent the mean triplicate

Fig. 4 Agarose gel of products obtained by conventional RT-PCR of representative RV glycoprotein E1. Products obtained by RT-PCR for the 185-bp amplicon. The samples in each lane are as follows: *Lane 1* DNA ladder, with visible bands identified to the left in base pairs (bp). *Lane 2* SIRC cells inoculated with RV (DNA amplified with primers for RV). The *lanes 3, 4 and 5* cells treated with 68, 136 and 200 mg/mL of hemolymph. *Lane 6* negative sample

SIRC cells. The viral binding assay, antiviral assay, PCR, qPCR and Transmission Electron Microscopy were used to demonstrate that hemolymph could inhibit RV adsorption to the SIRC cells.

Our results demonstrated that hemolymph had a inhibitory effect on the RV. Moreover, the binding assay suggested that hemolymph inhibits RV entry into SIRC cells by interfering with the binding/adsorption of the virions to the cellular receptor. Beside this, the cells treated with hemolymph after RV infection did not only show not only a reduction of viral titers but also a decrease of cytopathic effects and viral protein synthesis. Chernysh et al. (2002) isolated two peptides (alloferon 1 and 2) from the hemolymph of *Calliphora vicina*, which controlled viral infection when added prior to viral addition to the cells. Similarly, phenol oxidase, an enzyme obtained from the tobacco budworm (*Heliothis virescens*) hemolymph, was reported to exhibit antiviral activity against herpes simplex virus 1 (HSV-1), vesicular stomatitis virus, human parainfluenza virus 3, coxsackie virus B3, and Sindbis virus (Ourth and Renis 1993). Olicard et al. (2005) observed that the addition of the hemolymph of *Crassostrea gigas* inhibited HSV-1 infection. Furthermore, Cruz et al. (2005) demonstrated the ability of *Crotalus durissus terrificus* venom to induce production and liberation of nitric oxide and cytokines in murine macrophages as well as interfere with the morphological, functional, and biochemical changes of these immune cells. The experimental approaches used in this study demonstrated that hemolymph has a powerful inhibitory effect on RV.

Fig. 5 SIRC cells were cultivated on Aclar film and after 48 hrs were treated or untreated with hemolymph and infected with RV were processed by TEM. **a–c** SIRC cells inoculated with RV treated with hemolymph. Note the Golgi system (GC), Mitochondria (M). Note that in SIRC cells infected with RV and treated with hemolymph no replication complex or viral

particles were found. **c** SIRC cells morphology (elongated fibroblaste-like cells). **d–f**. SIRC cells inoculated with RV but untreated with hemolymph. Note the replication complex (CPV) and RV like particles. **f** SIRC cells showed cytopathic effect (CPE)

The SIRC cells infected with RV and treated with hemolymph caused a reduction in infectivity in SIRC cells, as measured by qPCR and transmission electron microscopy. In vitro, cells cultivated with RV strains are highly cytopathic. In our study, SIRC cells cultivated with RA 27/3 showed clear and readily detectable cytopathic effects and growth of the virus, results that are in agreement with Figueiredo et al. (2000). Our findings showed inhibition of the cytopathic effect and viral growth in cells infected with hemolymph-treated RV. SIRC cells infected with RV for 48 h showed the virus replication complex, which is a typical replication complex with RV-like particles. RV exhibits the ability to rearrange cellular membranes, presumably to facilitate efficient viral replication. In infected cells, the endoplasmic reticulum, Golgi complex, and mitochondria are often closely arranged around the virus replication complex (Frey 1994; Lee and Bowden 2000; Risco et al. 2003; Fontana et al. 2007). This arrangement of organelles was not detected in cells infected with hemolymph-treated RV. The results obtained by qPCR were

corroborated by those obtained through transmission electron microscopy of SIRC cells treated with RV treated with hemolymph. Beside the absence of a typical replication complex, rubella virions and RV-like particles were not detected in the cytoplasm of analyzed cells. These tests suggested that hemolymph incubated with the virus interfered with the virus structure or viral elaborate necessary for adsorption, invasion, and/or entry into host cells. In addition the binding assay suggest that hemolymph inhibits RV entry into target cells interfering with the binding/absorption of the virions to the cellular receptor. Our group had demonstrated the presence of pharmacologically active substances in the hemolymph of *Lonomia obliqua*. A single antiviral protein of approximately 20 kDa from the *L. obliqua* hemolymph, when added to cultures 1 h before infection, led to a 157-fold reduction in measles virus production and a 61-fold reduction in polio virus production (Greco et al. 2009). Recently, this antiviral protein was cloned and expressed. It was able to inhibit the replication of picornavirus, rubella, and herpes simplex virus (Carmo et al.

2015). In this study, we have demonstrated the antiviral activity of the hemolymph of *Podalia* sp. against RV in vitro.

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