Antiviral Research 84 (2009) 84–90

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Antiviral activity of the hemolymph of *Lonomia obliqua* (Lepidoptera: Saturniidae)

Katia N. Greco[a], Rita Maria Z. Mendonça[b], Roberto H.P. Moraes[a], Dalva. A.P. Mancini[b], Ronaldo Z. Mendonça[a,*]

[a] *Laboratório de Parasitologia e Entomologia, Instituto Butantan, São Paulo, Brazil*
[b] *Laboratório de Virologia, Instituto Butantan, São Paulo, Brazil*

ARTICLE INFO

Article history:
Received 20 May 2009
Received in revised form 27 July 2009
Accepted 29 July 2009

Keywords:
Antiviral
Lonomia obliqua
Purification
Hemolymph
Measles
Polio
Influenza

ABSTRACT

Potent antiviral activity against measles, influenza and polio viruses was observed in the hemolymph of *Lonomia obliqua*. The antiviral protein responsible for this activity was isolated, purified by gel filtration chromatography using a gel filtration column system (Superdex 75) and further fractionated using a Resource-Q ion exchange column system. Experiments with the purified protein led to a 157-fold reduction (from $3.3 \pm 1.25 \times 10^7$ to $2.1 \pm 1.5 \times 10^5$ $TCID_{50}$ mL^{-1}) in measles virus production and a 61-fold reduction (from $2.8 \pm 1.08 \times 10^9$ to $4.58 \pm 1.42 \times 10^7$ mL^{-1}) in polio virus production. Heating and freezing seem to have no influence over its antiviral activity. Also, the protein does not display virucidal activity and does not act on receptors on the cell membrane. The observations suggest an intracellular mechanism of action and that the protein may act as a constitutive agent that affects the innate antiviral immune response.

1. Introduction

Insects are regularly exposed to environmental contaminants. Several studies have detected the presence of peptides, enzymes and metabolites in the hemolymph of insects that curb the proliferation of bacteria and fungi. The immune response of insects to bacterial or fungal infections and to filarial or parasitoid infestations is well documented (Gillespie et al., 1997; Carton and Nappi, 1997; Hultmark, 2003; Leulier et al., 2003). However, unlike vertebrates, insects have none of the well-characterized mechanisms for recognizing viruses or virus-infected cells. Antiviral agents have been isolated from different sources (Petricevich and Mendonça, 2003). Antiviral activity from insect hemolymph (Chernysh et al., 2002) have also been described; however, antiviral defense and any interactions between antiviral and other antimicrobial defenses remain unexplored (Popham et al., 2004). Similarly, the phenol oxidase, an enzyme obtained from the tobacco budworm (*Heliothis virescens*) hemolymph, was reported to exhibit antiviral activity against several vertebrate viruses *in vitro* (herpes simplex viruses types 1 and 2, vesicular stomatitis virus, human parainfluenzavirus 3, coxsackievirus B3 and Sindbis virus) (Ourth and Renis, 1993). Interestingly, Popham et al. (2004) observed that, although *Hz*SNPV, a wild-type of virus of *Helicoverpa zea* single capsid nucleopolyhedrovirus, was substantially deactivated by *H. virescens* plasma, the level of inhibition was much lower than that observed against vertebrate viruses, for which *H. virescens* is not the host. Recently, some studies have demonstrated the presence of pharmacologically active substances in the hemolymph of *Lonomia obliqua* (Maranga et al., 2003; Souza et al., 2005; Raffoul et al., 2005; Mendonça et al., 2008). The supplementation of cultures with hemolymph proteins has been reported to have a positive effect on viral replication (Souza et al., 2005) and on recombinant protein production (Mendonça et al., 2008). Recently, Kanaya and Kobayashi (2000) isolated and characterized a protein from silkworm hemolymph able to increase the activity of a recombinant protein (luciferase) by approximately 6000 times. Nevertheless, few studies have succeeded in isolating and characterizing the factors involved in these effects (Nussbaumer et al., 2000). These factors, once identified and isolated, can be of great importance in the optimization of cell growth, viral replication or recombinant protein production and can lead to more efficient cell cultivations and to final products at lower costs. The objectives of this work were the identification, isolation, characterization of the hemolymph protein from *L. obliqua* and the evaluation of its antiviral activity against human viruses.

2. Materials and methods

2.1. VERO cell cultures

VERO and MDCK cells were grown in plastic T-flasks or on multiwell plates using Leibovitz-15 (L15) medium containing 0.9 g L^{-1}

* Corresponding author at: Laboratório de Parasitologia, Instituto Butantan, Av. Vital Brazil 1500, CEP 05503-900, São Paulo, SP, Brazil. Tel.: +55 011 3726 722/2128; fax: +55 011 3726 1505.
E-mail address: zucatelli@uol.com.br (R.Z. Mendonça).

0166-3542/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2009.07.018

of D-galactose, 0.3 g L^{-1} of L-glutamine and supplemented with 5% fetal bovine serum (FBS). Viable cell counts were performed on Neubauer chambers using Trypan blue (0.05%) exclusion.

2.2. Virus and cell infection

Influenza (H1N1), Polio (Sabin 1) and Measles (Edmonston) viruses were used to determine the antiviral activity of the hemolymph. VERO cells were infected with the measles and polio viruses and MDCK cells with the influenza virus on the 3rd day post-inoculation at an MOI of 0.1. End-point dilution assays were performed on 96-well microtiter plates to measure virus titers.

2.3. Hemolymph collection

The hemolymph of *L. obliqua* was collected from sixth instar larvae after setae had been cut off. The collected hemolymph was clarified by centrifugation at 1000 × *g* for 10 min. Afterwards, the supernatant was heat-treated at 60 °C for 30 min. The heat-treated hemolymph was then filtered through a 0.2 μm membrane and stored at 4 °C.

2.4. Hemolymph fractionation by chromatography

After centrifugation and filtration, 1 mL of hemolymph was loaded on a gel filtration chromatography system equipped with a Superdex 75 column (Amersham Pharmacia Biotech) and eluted with sodium phosphate buffer at 0.5 mL/min. The elution was monitored at 280 nm and 0.5 mL fractions were collected. The protein fractions were then analysed by SDS-PAGE electrophoresis and added to VERO and MDCK cell cultures for antiviral activity determination. The semi-purified fractions exhibiting antiviral activity were further fractionated on a Resource-Q ion exchange column (Amersham Pharmacia Biotech) at a flow rate of 1 mL/min and eluted with a linear gradient (0–100%) of Tris–HCl 20 mM/Tris–HCl 20 mM–NaCl 1 M, pH 8.0. All fractions collected were also added to infected VERO and MDCK cell cultures for antiviral activity determination.

2.5. Effect of hemolymph on infected cells

In order to investigate the antiviral effect of the hemolymph on measles, polio and influenza viruses, whole hemolymph and semi-purified and purified hemolymph fractions (1, 3 and 5% (v/v), respectively) were added to the culture medium at different times (1 h before infection, at the time of inoculation and 1 h after infection). Samples of the cell cultures were taken daily and analysed to determine the percentage of cells with cytopathic effect and the virus titers.

2.6. Analytical procedures

2.6.1. SDS-PAGE analysis

SDS-polyacrylamide gel electrophoresis (15%) was carried out to analyse the whole hemolymph and each of its chromatographic fractions. Protein standards from the LMW-SDS Marker Kit (Amersham Pharmacia Biotech) were used. The electrophoresis was carried out at 50 mA for 90 min. The gel was stained with Gel Code Blue Stain Reagent (Pierce, Rockford, IL).

2.6.2. Flow cytometry

For the determination of the hemolymph effect on the infected cells, flow cytometry analysis was performed. VERO cell cultures at a concentration of 1×10^6 cells/mL were supplemented with hemolymph (whole at 1% and purified fractions at 5%) 1 h prior to infection with the measles virus at an MOI of 0.1. Non-supplemented VERO cell cultures were also carried out under the same conditions. At 72 h post-infection, a sample of each type of culture was collected, washed twice with PBS (pH 7.4) and centrifuged at 800 × *g* for 5 min. The pellet was stained with IgG/FITC anti-measles virus (Light Diagnostics™ – Chemicon International). After 10 min, cells were centrifuged (1000 × g/5 min) and the pellet was resuspended in 1 mL of FACS buffer. Samples were processed on a Becton Dickinson FACSort equipped with an Ar laser (excitation and emission wavelengths were 488 and 620 nm, respectively). Ten thousand events were analysed per sample and the percentage of cells infected showing fluorescence was determined.

2.6.3. Hemagglutination

The antigen activity of the influenza virus was evaluated as hemagglutinating activity (HA) as described by Rosen (1961). Serial twofold dilutions of the antigen were made in PBS to a final volume of 0.05 mL/well. An equal volume of 0.5% guinea pig red blood cells was added to each well and incubated at 37 °C for 60 min. The hemagglutination unit (HAU) is defined as the reciprocal of the highest dilution of the virus that causes complete agglutination.

2.6.4. Determination of the virus infectious dose

In order to determine the amount of virus produced in cultures infected with the polio and measles viruses, in treated or not treated culture, VERO cells culture were seeded at a concentration of 5×10^4 cells mL^{-1} on 96-well plates. After 24 h, these cells were infected with the viruses from the infected cultures, at dilution rates of 10^{-1} to 10^{-10}. The microwell plates containing polio-infected and measles-infected cells were then incubated at 37 °C for 3 days and 5 days, respectively, as described by Griffiths and Thornton (1982). Virus titers were determined by monitoring the *cytopathic effect* (*CPE*) in an *endpoint dilution assay*. They were expressed as $TCID_{50}$ (the highest dilution of virus able to induce CPE in 50% of cells) and calculated by the method of Reed and Muench (1938).

2.6.5. Cytotoxicity activity

The cytotoxic effects of the hemolymph were assessed by using a standard VERO cell assay. Briefly, exponential phase VERO cells (day 3) were exposed to different amounts of hemolymph (0.5, 1, 2, 5 and 10%, v/v) and incubated for different at different times. Daily, the supernatants were removed and the remaining living cells were counted after being fixed and stained with crystal violet (0.2% in 20% methanol). Cytotoxicity percentage values were calculated by the formula: $(A_{control} - A_{sample}/A_{control}) \times 100$.

2.6.6. Statistical analysis

Data are expressed as the mean ± SD. Statistical analysis was performed using the Student's *t*-test and the level of significance was set at $p \leq 0.05$.

3. Results

3.1. Determination of the cytotoxicity of the hemolymph

In this study, the antiviral properties of the hemolymph of *L. obliqua* (Hb) were examined. The well-known lethal and toxic properties of its venom were tested by performing a cytotoxicity assay. As described previously, VERO cells were exposed to different amounts of hemolymph and the cytotoxicity was quantified. Fig. 1 shows the effect of the hemolymph on cell viability. Hemolymph concentrations lower than 2% (v/v) were not toxic to VERO cells, as high percentages of viable cells were observed throughout the three experiments. In the other experiments, cell viability decreased as the amount of Hb increased. On day 3, 25.5% and 49.2% reductions in viable cells took place at 5% and 10% Hb, respectively. After 6 days,

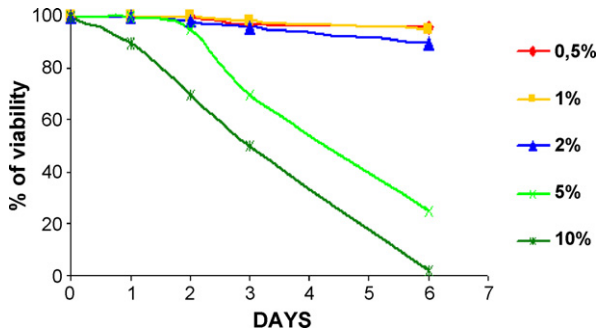

Fig. 1. Effect of the hemolymph of *Lonomia obliqua* on the viability of VERO cells exposed to different amounts of Hb. Daily, the supernatants were removed and the remaining living cells were counted after being fixed and stained with crystal violet (0.2% in 20% methanol). Each point represents the mean value of three experiments.

100% of cells were dead in the culture supplemented with 10% of Hb.

3.2. Virus deactivation by the hemolymph

A proteolytic enzyme could be responsible for the virus deactivating property of the hemolymph (Hb). In order to determine if the Hb was able to directly deactivate the polio, influenza and measles viruses, a standard inoculum of each virus was exposed to 1% (v/v) of Hb and kept at room temperature for 2 h. The virus titers obtained are presented in Table 1. While the fragility of the influenza and measles viruses due to the ease degradation of the protein in their envelopes has been reported, the poliovirus is known to be very resistant to degradation, even under non-ideal environmental conditions. As indicated in Table 1, the hemolymph seemed to have no direct deactivating effect on the polio, measles and influenza viruses.

3.3. Hemolymph effect on the adsorption step

The supernatant of infected cultures was assayed for remaining virus to analyse the effect of Hb on the adsorption step. VERO or MDCK cell cultures were supplemented with whole hemolymph (1%) at different times and infected with the polio, measles or influenza at an MOI of 0.1. Non-supplemented cell cultures were also carried out under the same conditions. Two hours after infection, a sample of supernatant was harvested and titration was carried out on 96-well plates containing VERO or MDCK cells to compare the amount of virus remaining in the supernatant of the hemolymph supplemented cultures to that in the control virus-infected culture. It was concluded that hemolymph does not hinder the adsorption or penetration of the viruses by acting on membrane cell receptors since the amount of virus remaining in the supernatant of supplemented cultures after 2 h was similar to that obtained in the supernatant of the control culture. Considering that no difference in virus titers in the supernatant between supplemented and the control non-supplemented cultures was observed, it can be suggested that the hemolymph of *L. obliqua* does not avoid the adsorption or penetration of the virus into the cells via action on the cell membrane.

3.4. Optimal conditions for cell treatment and infection

For the determination of the best time for hemolymph addition, VERO and MDCK cell cultures were supplemented with 1% (v/v) whole hemolymph at different times (1 h before infection and 1 h after infection) and supplemented with a mixture of hemolymph and virus, which had remained suspended for 2 h. The infection of cells was performed with polio, measles or influenza viruses. The virus titers obtained are exhibited in Table 2. The lowest virus production was observed in cultures supplemented with 1% hemolymph 1 h before infection. Since the hemolymph neither directly deactivates viruses nor affects the adsorption step, the mechanism of antiviral action may be intracellular. While the addition of hemolymph after infection caused reduction in the viral production, the addition before infection led to much lower virus titers. It suggests that the hemolymph may inhibit some important mechanisms for viral replication or may stimulate a non-specific mechanism of defense, such as interferon alpha. When hemolymph was added 1 h prior to infection, the titer of measles virus produced by VERO cells was approximately 65 times lower (from 97×10^6 $TCID_{50}$ mL^{-1} in the control infected culture to 1.5×10^6 $TCID_{50}$ mL^{-1} in the hemolymph supplemented culture). The polio virus titer dropped from 15×10^8 $TCID_{50}$ mL^{-1} (control infected culture) to 0.4×10^8 $TCID_{50}$ mL^{-1} (supplemented culture), which corresponds to a 157-fold reduction. Therefore, the smallest amount of virus produced in the culture supplemented with hemolymph 1 h prior to infection could indicate an intracellular mechanism of antiviral action, which may be initiated before the virus is exposed to the cell. The statistical analysis of the data showed that the difference between the control and the hemolymph supplemented (1 h before infection) cultures was significant ($p = 0.0007$).

Flow cytometric analysis was carried out to assess the hemolymph effect on the percentage of virus-infected cells. Fig. 2 summarizes the data obtained through the analysis of flow cytometry of VERO cells infected with the measles virus and stained with FITC conjugated anti-measles antibody. It was observed that 96.24% of the cells exhibited fluorescence in the infected and non-supplemented culture. As expected, the percentage of fluorescent cells was not significant in the non-infected. In the virus-infected cultures supplemented with hemolymph 1 h before infection, 77.35% of cells displayed fluorescence. While in the infected and non-supplemented culture, 81.95% of cells show intense fluorescence, an approximately 2.5-fold lower percentage (34.63%) was obtained in the hemolymph enriched culture (1 h before infection). On the other hand, fluorescence was exhibited by 88.57% of cells in the cultures supplemented after infection. Of these, 44.38% displayed intense fluorescence. Thus, hemolymph supplementation was able to reduce the number of cells with intense fluorescence by 50%. No morphological changes of VERO cells were observed due to hemolymph addition to the infected cultures. FACs analysis of VERO cells from cultures enriched with hemolymph before and

Table 1
Direct deactivating effect of hemolymph on polio, measles and influenza viruses. The viruses were exposed to 1% whole hemolymph (Hb) and kept at room temperature for 2 h. Virus titers are expressed as $TCID_{50}$ mL^{-1} or hemagglutinating units (HAU).

Virus	Initial virus titers	Virus titers after 2 h (without Hb)	Virus titers after Hb exposure – 2 h before infection
Polio	2.0×10^9 $TCID_{50}$ mL^{-1}	2.0×10^9 $TCID_{50}$ mL^{-1}	2.0×10^9 $TCID_{50}$ mL^{-1}
Measles	6.4×10^7 $TCID_{50}$ mL^{-1}	5.12×10^7 $TCID_{50}$ mL^{-1}	6.4×10^7 $TCID_{50}$ mL^{-1}
Influenza	512 HAU	128 HAU	256 HAU

$TCID_{50}$: tissue culture infectious dose 50%; HAU: hemagglutinating unit.
The values represent the average of three experiments.

Table 2
Production of the polio, measles and influenza viruses in cell cultures infected at an MOI of 0.1 and supplemented with 1% (v/v) whole hemolymph and with a mixture of hemolymph and virus. The virus titers are expressed as $TCID_{50}$/mL or hemagglutinating units (HAU).

	Poliovirus titers 48 h after infection ($TCID_{50}$ mL^{-1})	Measles virus titers 72 h after infection ($TCID_{50}$ mL^{-1})	Influenza virus titers 48 h after infection (HAU)
Control infected culture	$15 \times 10^8 \pm 9 \times 10^8$	$97 \times 10^6 \pm 1.25 \times 10^6$	64
Hb 1 h after infection	$1.6 \times 10^8 \pm 1.02 \times 10^8$	$57 \times 10^6 \pm 1.47 \times 10^6$	16
Hb 1 h before infection	$0.4 \times 10^8 \pm 0.7 \times 10^7$ *	$1.5 \times 10^6 \pm 0.8 \times 10^6$ *	8
Hb and virus (previously in suspension for 2 h)	$1.0 \times 10^8 \pm 2.0 \times 10^8$	$19 \times 10^6 \pm 4.5 \times 10^6$	16

$TCID_{50}$: tissue culture infectious dose 50%; HAU: hemagglutinating units.
The values represent the average of three experiments.
* $p < 0.05$.

after infection showed similar profiles for cell size (FSC-volume-axis) and cell granularity (the axis SSC-complexity/granulosity).

3.5. Purification of whole hemolymph

Whole hemolymph was loaded onto a gel filtration column (Superdex 75) so that the protein responsible for antiviral activity could be identified. The semi-purified fractions obtained were collected, organized in three pools and tested with respect to their cytotoxic effect on VERO cells. Cell viability was monitored daily using optical miscroscopy. The pools of the semi-purified protein proved to be non-cytotoxic within the range of concentrations tested (data not shown). The three pools were also added to VERO cells cultures 1 h before infection to assess the effect of each over viral replication. Viral titrations were performed at the end of the cultures. The results for virus production are presented in Table 3 and discussed below.

3.6. Antiviral activity of the pools on the replication of the polio virus

In order to study the antiviral effect of the semi-purified protein on virus replication, each pool (1–3) was added to virus-infected cell cultures at a concentration of 3% (corresponding to approximately 70 μg of total protein). Whole hemolymph and semi-purified fractions (pools) were added 1 h before infection with the polio virus at an MOI of 0.1. After 48 h, a sample was taken from each culture and titrated on 96-well plates to determine virus titers. As shown in Table 3, the amount of virus produced in cultures supplemented with 1% whole hemolymph was approximately 74 times lower than that obtained in the control infected culture. The final virus titer in the culture enriched with pool 2 (0.32×10^8 $TCID_{50}$ mL^{-1}) was approximately 87 times lower than that in the control culture (28×10^8 $TCID_{50}$ mL^{-1}). Thus, these data suggested that the protein responsible for the antiviral activity of the hemolymph of *L. obliqua* is present in pool 2.

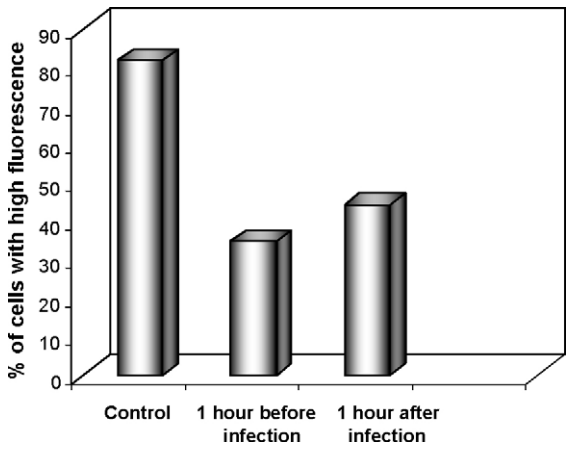

Fig. 2. Flow cytometric analysis of virus-infected VERO cells stained with FITC conjugated antibody using indirect immunofluorescence. VERO cell cultures at a concentration of 1×10^6 cells/mL were supplemented and not supplemented with whole hemolymph (1%) 1 h before and after infection with the measles virus at an MOI of 0.1 ($p = -0.0001$).

3.7. Antiviral activity of the pools on the replication of the measles virus

The same routine was followed as in the experiment with the polio virus. After 72 h post-infection, a sample was taken from each culture and titrated on 96-well plates to determine virus titers. Like the experiments carried out with the polio virus, both measles-infected cell cultures supplemented with whole hemolymph and pool 2 showed a decrease in virus production (1.7×10^6 and 2.24×10^6 $TCID_{50}$ mL^{-1}, respectively) in comparison to the control infected culture (1.63×10^7 $TCID_{50}$ mL^{-1}), indicating, once again, that the protein responsible for the antiviral activity of the hemolymph of *L. obliqua* was present in pool 2.

3.8. Antiviral activity of the pools on the replication of the influenza virus

The same routine was followed as in the experiments discussed above. As indicated in Table 3, virus production was reduced from 64 HAU to 8 HAU in the influenza virus-infected culture enriched with pool 2, which corresponds to an eightfold reduction. A similar result was obtained when whole hemolymph was added, indicating that the hemolymph of *L. obliqua* has a potent antiviral activity against enveloped and non-enveloped viruses.

3.9. Purification of pool 2 and assessment of antiviral activity of purified fractions against polio virus production

An additional chromatographic step was carried out for the identification of the protein from pool 2 responsible for the antiviral effect. A 1 mL sample of the pool was loaded onto a Resource-Q ion exchange column. Afterwards, 5% (about 10 μg) of each of the three purified pools obtained (RQ 3–5, RQ 19–25 and RQ 13–18) was added to cell cultures 1 h before infection with the polio virus at an MOI of 0.1 to determine which one exhibited the antiviral activity observed. After 48 h, a sample was taken from each culture and titrated on 96-well plates for the determination of virus production. The final virus titers for the control culture and the cultures enriched with each of the purified fractions are shown in Table 4. The supplementation of the infected culture with the fraction RQ 3–5 resulted in a 61-fold reduction in the final virus titer (from 28×10^8 $TCID_{50}$ mL^{-1} in the control infected culture to 0.46×10^8 $TCID_{50}$ mL^{-1}). On the other hand, statistically similar virus titers were observed in the RQ 13–18, RQ 19–25 and control experiments (37×10^8, 31×10^8 and 28×10^8 $TCID_{50}$ mL^{-1}), indicating that no inhibition of viral replication took place. Thus, it was concluded that

Table 3
Production of the polio, measles and influenza viruses in cell cultures enriched with whole hemolymph (1%) and each pool of protein (3%) 1 h before infection at an MOI of 0.1. The virus titers are expressed as $TCID_{50}$/mL or hemagglutinating units (HAU).

	Poliovirus titers 48 h after infection ($TCID_{50}$ mL^{-1})	Measles virus titers 72 h after infection ($TCID_{50}$ mL^{-1})	Influenza virus titers 48 h after infection (HAU)
Control infected culture	$28 \times 10^8 \pm 18 \times 10^8$	$1.63 \times 10^7 \pm 1.25 \times 10^6$	64
Hb	$0.37 \times 10^8 \pm 2.1 \times 10^{7*}$	$1.7 \times 10^6 \pm 4.15 \times 10^{5*}$	8
Pool 1	$8.9 \times 10^8 \pm 5.84 \times 10^6$	$1.0 \times 10^7 \pm 8.39 \times 10^6$	64
Pool 2	$0.32 \times 10^8 \pm 2.66 \times 10^{7*}$	$2.24 \times 10^6 \pm 2.67 \times 10^{6*}$	8
Pool 3	$7.81 \times 10^8 \pm 2.19 \times 10^8$	$1.0 \times 10^7 \pm 2.95 \times 10^7$	64

$TCID_{50}$: tissue culture infectious dose 50%; HAU: hemagglutinating units.
The values represent the average of three experiments.
* $p < 0.05$.

Table 4
Production of the polio, measles and influenza viruses in cell cultures enriched with 5% of each Pool 2 fractions 1 h before infection at an MOI of 0.1. The virus titers are expressed as $TCID_{50}$/mL or hemagglutinating units (HAU).

	Poliovirus titers 48 h after infection ($TCID_{50}$ mL^{-1})	Measles virus titers 72 h after infection ($TCID_{50}$ mL^{-1})	Influenza virus titers 48 h after infection (HAU)
Control infected culture	$28 \times 10^8 \pm 10 \times 10^8$	$33 \times 10^6 \pm 12 \times 10^6$	128
RQ 3–5	$0.46 \times 10^8 \pm 1.42 \times 10^{7*}$	$0.2 \times 10^6 \pm 1.5 \times 10^{5*}$	2
RQ 13–18	$37 \times 10^8 \pm 21 \times 10^8$	$1.8 \times 10^7 \pm 4.5 \times 10^6$	64
RQ 19–25	$31 \times 10^8 \pm 1.00 \times 10^2$	$37 \times 10^6 \pm 2.09 \times 10^7$	128

$TCID_{50}$: tissue culture infectious dose 50%; HAU: hemagglutinating units.
The values represent the average of three experiments.
* $p < 0.05$.

the protein responsible for the antiviral activity of the hemolymph of *L. obliqua* was present in the purified fraction RQ 3–5.

3.10. Assessment of antiviral activity of purified fractions against measles and influenza viruses

The antiviral activity of the purified fractions were also tested against the measles and influenza viruses. Similarly, 5% of each purified fraction (3–5 RQ, RQ 19–25 and 13–18), was added to infected VERO or MDCK cell cultures 1 h prior to infection. After 72 h, a sample was taken and analysed as described above. As shown in Table 4, the addition of fraction 3–5 RQ caused a 157-fold decrease in measles virus titers in comparison to those in the control culture.

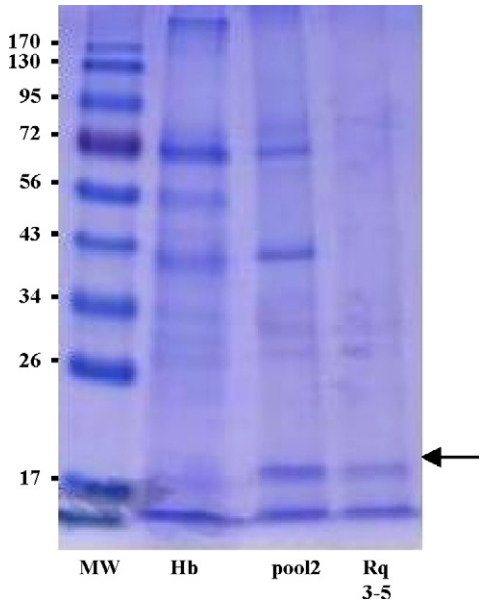

Fig. 3. SDS Polyacrylamide gel electrophoresis (SDS-PAGE) of 15% of the fraction with antiviral activity (Pool 2 and RQ 3–5). The arrow indicates the band of approximately 20 kDa.

Like the experiments carried out with the polio virus, the use of fractions RQ 19–25 and 13–18 did not seem to affect the viral replication of measles, since statistical similarity was observed among the virus titers obtained in these cultures and the control culture (3.7×10^7, 1.8×10^7, 3.3×10^7 $TCID_{50}$ mL^{-1}, respectively). When the purified fraction RQ 3–5 was added to the influenza-infected cell culture, virus production was reduced from 128 HAU to 2 HAU. According to Tables 3 and 4, the purified protein RQ 3–5 proved to be more effective in reducing influenza virus production than whole hemolymph or pool 2 fraction, which contained the antiviral protein.

3.11. Identification of the antiviral protein

The purified RQ fraction 3–5 proved to be the most effective in reducing the replication of the polio (Sabin 1 strain), measles (Edmonston strain), and influenza (H1N1) viruses of all the fractions tested against viral replication. Polyacrylamide gel electrophoresis was performed to assess protein purity. A protein with a molecular mass of approximately 20 kDa was identified by electrophoresis (Fig. 3). The molecular mass of the protein, 20.232, was determined using a MALDI-TOF mass spectrometer (data not shown).

4. Discussion

The cytotoxicity was defined by Nardone (1977) as the set of changes in cellular homeostasis that leads to a series of changes that interfere with the adaptive capacity of the cells and their survival, their proliferation and the metabolic performance of their duties. The intensity of cell damage depends on several factors, such as the concentration of the material tested, the time of exposure, the type of cell, the ability of the compound to penetrate the cell, among others (Hu and Hsiung, 1989). In this study, it was found that the whole hemolymph of *L. obliqua* at concentrations below 2% and its purified fractions within the range of concentrations tested have no deleterious effect on VERO cells.

Petricevich and Mendonça (2003), Karagöz et al. (2003), Ourth (2004) and Olicard et al. (2005) have carried out studies to identify substances with antiviral activity. These authors stress the impor-

tance of the identification of agents that show no toxicity to cells so that their application is feasible.

Whereas the poliovirus is a non-enveloped, positive-stranded RNA virus, the influenza and measles viruses are enveloped, negative-stranded RNA viruses. These differences have led to initial studies, which have aimed to determine and characterize a possible direct effect of hemolymph proteins on viruses and more specifically on the structure of the viral particles. Popham et al. (2004) have reported significant reduction in the titers of the baculovirus HzSNPV due to the action of an antiviral protein present in the hemolymph of *H. virescens* larvae. This protein shows an oxidative activity, catalyzed by a phenoloxidase. Therefore, it seems therefore, it seems possible that the antiviral activity exhibited by the hemolymph of *L. obliqua* in the present study may be also due to a virus-degrading enzyme. Nakayama et al. (1993) and Song et al. (2005) found that tea polyphenols and green tea catechins, respectively, acted directly on the influenza virus envelope, indicating that other compounds in natural extracts could be also involved in the antiviral action.

Olicard et al. (2005) observed that preincubation of the mammal virus HSV-1 and fish virus IPNV (infectious pancreatic necrosis virus), both non-enveloped with a RNA genome, with the hemolymph of *Crassostrea gigas* did not neutralize the viral particles. Exposure of the measles virus to rattlesnake venom also did not result in the inactivation of the virus (Petricevich and Mendonça, 2003), corroborating the data obtained in the present study.

The mechanism of action of the hemolymph of *L. obliqua* against different viruses appears not to be direct, since *in vitro* incubation of viruses with Hb for 2 h (at 20 °C) did not yield significant difference in virus titers. Previous treatment of cells with hemolymph did not affect the subsequent virus penetration, once the amount of virus in the supernatant collected 2 h post-infection was similar to that observed in the control infected culture. This would seem to militate against the mechanisms whereby the uncharacterized factor interferes with the infective process by blocking a required receptor, which could render the virus more resistant to the initial infection. Since the hemolymph neither directly deactivates viruses nor affects the adsorption step, the mechanism of antiviral action may be intracellular. Chernysh et al. (2002) have come to a similar conclusion in a study of the hemolymph of *Calliphora vicina* (a species of fly). These authors isolated two peptides—alloferon 1 and 2 which control viral infection by triggering intracellular when added before infection. The addition of *L. obliqua* hemolymph after infection also reduced viral production, but not as much as the addition prior to infection (Table 2). However, Olicard et al. (2005) observed that the addition of the hemolymph of *C. gigas* to VERO cell cultures prior to infection with the virus HSV-1 did not affect subsequent infection. Thus, the inhibitory action of the hemolymph on virus replication may be through the activation of a non-specific antiviral mechanism such as IFN-α. Recently, Cruz et al. (2005) have demonstrated the ability of *Crotalus durissus terrificus* venom to induce the production and liberation of NO and cytokines as well to interfere with morphological, functional, and biochemical changes in murine macrophages. NO (nitric oxide), a free radical gas and a small molecule mediator of inflammation, is critical to innate immunity. Also, IFN-γ and nitric oxide are recognized as principal mediators of bacterial control in mycobacterial infections (Cooper et al., 2002), are regulators of Trypanosoma cruzy infections (Costa et al., 2006) and have an important role in cell defense against others external agents, such as fungi and viruses. We observed that the hemolymph of *L. obliqua* interferes with NO production (data not shown), which indicates that it can modulate innate immunity. For example, IFN-α and -β are the main cytokines for innate immune responses against viral infections (Ohno et al., 2004) and are an extremely powerful antiviral response that is capable of controlling most, if not all, virus infections in the absence of adaptive immunity (Randall and Goodbourn, 2008). Interferons (IFNs) are secreted from cells infected with viruses. Both IFN-α and -β can induce an antiviral state in cells through the Janus kinase-signal transducers and activators of transcription, the JAK/STAT pathway (Stark et al., 1998). They induce an antiviral state in the cell through complex and indirect mechanisms, which culminate in a direct inhibition of viral replication (Tan and Katze, 2001). The induction of IFNs during viral infection is mediated by a coordinate activation of multiple cellular transcription factors such as interferon regulatory factors (IRFs), NF-κB, and c-Jun/ATF-2 (Wathelet et al., 1998). The mechanism of the hemolymph of *L. obliqua* responsible for the inhibition of viral replication is not known. Once the Hb acts on the production of NO, known to be an agent that interferes with innate immunity, it is possible that the hemolymph protein may also act by activating the IFN-α and -β in the cells. A study is now under way to investigate the mechanism suggested.

Extracts of crustacean tissues have showed broad spectrum antiviral activity against *enveloped and non-enveloped* DNA and *RNA viruses*, probably through multiple inhibitors contained in the extracts (Pan et al., 2000). There are also other proteins and peptides within a broad range of molecular weights that exhibit antiviral activity. For instance, Concanavalin A, a high molecular weight lectin with activity against HSV-1, was extracted from the bean *Canavalia ensiformis* (Garrity et al., 1982). A protein of approximately 24 kDa isolated from the hemolymph of silkworm *Bombyx mori* was responsible for inhibiting nucleopolyhedrovirus (Nakazawa et al., 2004). Hultmark et al. (1980) have reported some antimicrobial properties of a protein of 15 kDa isolated from *Hyalophora cecropia* caterpillars. The alloferon, a 12.65 kDa protein purified from the hemolymph of the fly *C. vicina*, effectively inhibited influenza viruses A and B reproduction by triggering intracellular responses when added before virus infection similarly to the interferons of vertebrates (Chernysh et al., 2002). An antiviral peptide of 916 Da, isolated from *H. virescens* hemolymph, provided protection for cultures of cells when added before virus infection, suggesting that its antiviral activity is due to its entry into cells before infection and, once there, where infection takes place, it inhibits mechanisms of assembly and/or budding and subsequent exit of new viral particles from the cell, probably by internally connecting the regions of the plasma membrane (Ourth, 2004). The approximately 20 kDa protein purified from the hemolymph of *L. obliqua* proved to be able to inhibit the replication of all the viruses tested when added to cultures 1 h before infection, indicating that it may act either on the steps of the cycle of replication of viruses that occur intracellularly, similarly to alloferon, or on the late stages of virus infection, similarly to the peptide extracted from *H. virescens*. It was also observed that the hemolymph did not display any virucidal effect, suggesting that it may act on different stages of virus replication, but it seems to be unable either to inhibit the stages before reaching the target cell or to prevent the process of adsorption.

In this study, we have demonstrated the antiviral activity of the hemolymph of *L. obliqua* against human viruses *in vitro*. The protocols used provided enough evidence to suggest its antiviral activity. We are currently working on the expression of the protein isolated from the hemolymph in a baculovirus system.

Acknowledgements

The authors acknowledge the financial support of FAPESP (2006/02108-0).

References

Carton, Y., Nappi, A.J., 1997. Drosophila cellular immunity against parasitoids. Parasitol. Today 13 (June (6)), 218–227.

Chernysh, S., Kim, S.I., Bekker, G., Pleskach, V.A., Filatova, N.A., Anikin, V.B., Platonov, V.G., Bulet, P., 2002. Antiviral and antitumor peptides from insects. Proc. Natl. Acad. Sci. U.S.A. 99, 12628–12632.

Cooper, A.M., Adams, L.B., Dalton, D.K., Appelberg, R., Ehlers, S., 2002. IFN-γ and NO in mycobacterial disease: new jobs for old hands. Trends Microbiol. 10 (5), 221–226.

Costa, V.M.A., Torres, K.C.L., Mendonça, R.Z., Gresser, I., Gollob, K.J., Abrahamsohn, I.A., 2006. Type 1 interferons stimulate nitric oxide production and resistance to *Trypanosoma cruzi* infection. J. Immunol. 177, 3193–3200.

Cruz, A.H., Mendonça, R.Z., Petricevich, V.L., 2005. *Crotalus durissus terrificus* venom interferes with morphological, functional, and biochemical changes in murine macrophage. Mediat. Inflamm. 6, 349–359.

Garrity, P., Szelc, C., Paquete, C., McEvoy, M., Millette, R., Adler, R., 1982. Inhibition of herpes simplex virus replication by cucinyl concanavalin A. Antimicrob. Agent Chemother. 21 (3), 450–455.

Gillespie, J.P., Kanost, M.R., Trenczek, T., 1997. Biological mediators of insect immunity. Annu. Rev. Entomol. 42, 611–643.

Griffiths, B., Thornton, B., 1982. Use of microcarrier culture for the production of herpes simplex virus (type 2) in MRC-5 cells. J. Chem. Technol. Biotechnol. 32, 324–329.

Rosen, L., 1961. Hemagglutination and hemagglutination-inhibition with measles vírus. Virology 13, 138–174.

Hu, J.M., Hsiung, G.D., 1989. Evaluation of new antiviral agents: in vitro perspectives. Antiviral Res. 11 (5–6), 217–232.

Hultmark, D., Steiner, H., Rasmuson, T., Boman, H.G., 1980. Insect immunity. Purification and properties of three inducible bacterial proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. Eur. J. Biochem. 106, 7–16.

Hultmark, D., 2003. Drosophila immunity: paths and patterns. Curr. Opin. Immunol. 15 (February (1)), 12–19, Review.

Kanaya, T., Kobayashi, J., 2000. Purification and characterization of an insect haemolymph protein promoting in vitro replication of the *Bombyx mori* nucleopolyhedrovirus. J. Gen. Virol. 81 (April (Pt 4)), 1135–1141.

Karagöz, A., Onay, E., Arda, N., Kuru, A., 2003. Antiviral potency of mistletoe (*Viscum album* ssp. album) extracts against human parainfluenza virus type 2 in Vero cells. Phytother. Res. 17 (May (5)), 560–562.

Leulier, F., Marchal, C., Miletich, I., Limbourg-Bouchon, B., Benarous, R., Lemaitre, B., 2003. Directed expression of the HIV-1 accessory protein Vpu in Drosophila fatbody cells inhibits Toll-dependent immune responses. EMBO Rep. 4 (October (10)), 976–981, Epub 2 Sep 12.

Maranga, L., Mendonca, R.Z., Bengala, A., Peixoto, C.C., Moraes, R.H.P., Pereira, C.A., Carrondo, M.J.T., 2003. Enhancement ofSf-9 cell growth and longevity through supplementation of culture medium with hemolymph. Biotechnol. Prog. 19, 58–63.

Mendonça, R.Z., Greco, K.N., Sousa, A.P.B., Moraes, R.H.P., Astrai, R.M., Pereira, C.A., 2008. Enhance effect of a protein obtained from *Lonomia obliqua* hemolymph in the recombinant protein production. Cytotechnology 57, 83–91.

Nakayama, M., Suzuki, K., Toda, M., Okubo, S., Hara, Y., Shimamura, T., 1993. Inhibition of the infectivity of influenza virus by tea polyphenols. Antiviral Res. 21, 289–299.

Nakazawa, H., Tsuneishi, E., Ponnuvel, K.M., Furukawa, S., Asaoka, A., Tanaka, H., Ishibashi, J., Yamakawa, M., 2004. Antiviral activity of a serine protease from the digestive juice of *Bombyx mori* larvae against nucleopolyhedrovirus. Virology 321, 154–162.

Nardone, R.M., 1977. Toxicity testing in vitro. In: Rotbblat, G.H., Cristofalo, V.J. (Eds.), Growth, Nutrition and Metabolism of Cells in Culture. Academic, New York, pp. 471–495.

Nussbaumer, C., Hinton, A.C., Schopf, A., Stradner, A., Hammock, B.D.I.J., 2000. Isolation and characterization of juvenile hormone esterase from hemolymph of Lymantria dispar by affinity- and by anion-exchange chromatography. Insect Biochem. Mol. Biol. 30 (April (4)), 307–314.

Ohno, S., Ono, N., Takeda, M., Takeuchia, K., Yanagi, Y., 2004. Dissection of measles virus V protein in relation to its ability to block alpha/beta interferon signal transduction. J. Gen. Virol. 85, 2991–2999.

Olicard, C., Renault, T., Torhy, C., Benmansour, A., Bourgougnon, N., 2005. Putative antiviral activity in hemolymph from adult Pacific oysters, *Crassostrea gigas*. Antiviral Res. 66, 147–152.

Ourth, D.D., Renis, H.E., 1993. Antiviral melanization reaction of *Heliothis virescens* hemolymph against DNA and RNA viruses in vitro. Comp. Biochem. Physiol. 105B, 719–723.

Ourth, D.D., 2004. Antiviral activity against human immunodeficiency virus-1 in vitro by myristoylated-peptide from *Heliothis virescens*. Biochem. Biophys. Res. Commun. 320, 190–196.

Pan, J., Kurosky, A., Xu, B., Chopra, A.K., Coppenhaver, D.H., Singh, I.P., Baron, S., 2000. Broad antiviral activity in tissues of crustaceans. Antiviral Res. 48, 39–47.

Petricevich, V.L., Mendonça, R.Z., 2003. Effect of Crotalus venom on growth of measles virus. Toxicon 42, 01 a 11.

Popham, H.J.R., Shelby, K.S., Brandt, S., Coudron, L.T.A., 2004. Potent virucidal activity in larval *Heliothis virescens* plasma against *Helicoverpa zea* single capsid nucleopolyhedrovirus. Gen. Virol. 85, 2255–2261.

Randall, R.E., Goodbourn, S., 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. 89, 1–47.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

Raffoul, T., Swiech, K., Arantes, M.K., Souza, A.P.B., Mendonça, R.Z., Pereira, C.A., Suazo, C.A.T., 2005. Performance evaluation of CHO-K1 cell in culture médium supplemented with hemolymph. Braz. Arch. Biol. Technol. 48, 85–95.

Stark, G.R., Kerr, I.M., Williams, B.R.G., Silverman, R.H., Schreiber, R.D., 1998. How cells respond to interferons. Annu. Rev. Biochem. 67, 227–264.

Song, J.M., Lee, K.H., Seong, B.L., 2005. Antiviral effect of catechins in green tea on influenza virus. Antiviral Res. 68, 66–74.

Souza, A.P.B., Peixoto, C.C., Maranga, L., Carvalhal, A.V., Moraes, R.H.P., Mendonça, R.M.Z., Pereira, C.A., Carrondo, M.J.T., Mendonça, R.Z., 2005. Purification and characterization of an anti-apoptotic protein isolated from *Lonomia oblique* hemolymph. Biotechnol. Progr. 21, 99–105.

Tan, S.L., Katze, M.G., 2001. How hepatitis C virus counteracts the interferon response: the jury is still out on NS5A. Virology 284 (1), 1–12.

Wathelet, M.G., Lin, C.H., Parekh, B.S., Ronco, L.V., Howley, P.M., Maniatis, T., 1998. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. Mol. Cell 1, 507–518.