Antiviral Research, 8 (1987) 1–12
Elsevier

AVR 00248

Antiviral effects of *Melia azedarach* L. leaves extracts on Sindbis virus-infected cells

Mónica B. Wachsman[1], Elsa B. Damonte[1], Celia E. Coto[1] and Ramón A. de Torres[2]

[1]*Laboratorio de Virología, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Buenos Aires, Argentina and* [2]*Departamento de Microbiología e Inmunología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina*

(Received 16 December 1986; accepted 7 April 1987)

Summary

Partially purified extracts from leaves of *Melia azedarach* L. (MA) exert a broad range of antiviral effects on DNA and RNA viruses. The effect of MA on different stages of Sindbis virus replicative cycle in BHK cells was investigated. Under one-step growth conditions MA afforded a greater than 90% inhibition in virus yield if added to the cell cultures 2 h before or after infection, and when added 4 h after infection MA still caused a greater than 80% inhibition.

Analysis of early events following Sindbis virus infection showed that MA did not affect viral adsorption to or penetration in BHK cell. In contrast, viral RNA and protein synthesis was almost totally inhibited in cells pretreated with MA 2 h before infection, while cellular macromolecular synthesis was similar in MA-treated and untreated cell cultures.

Melia azedarach L.; Sindbis virus

Introduction

An intensive search for compounds with antiviral activity in higher plant extracts has led to the discovery of potent inhibitors. In most cases, the chemical nature of the active principle has not been well established but for some plants it has been demonstrated that the reported antiviral activity is associated with proteins [7,14,16], carbohydrates [10] or alkaloids [6,17].

Correspondence to: Mónica B. Wachsman, Laboratorio de Virología, Facultad de Ciencias Exactas y Naturales UBA, Ciudad Universitaria, Pabellón 2, 1428 Buenos Aires, Argentina.

In 1982 we reported that ethanol-water extracts from roots of *Melia azedarach* L. (MA) showed a broad range of in vitro antiviral effects on DNA and RNA viruses, such as herpes simplex, Junin, Tacaribe, Pichinde, polio, Sindbis and vesicular stomatitis virus [18]. The active principle was found also in aqueous extracts obtained from dried and fresh green leaves [1,19].

Virus inhibition occurred when MA was present during the viral replicative cycle; furthermore, MA induced a refractory state of the cells to virus infection [18]. It has also been demonstrated that MA does not exert any direct virucidal action [18]. Partially purified MA extracts have also been found effective in vivo, in protecting neonatal mice against lethal encephalitis caused by Tacaribe virus inoculation [2].

As the antiviral activity of MA obviously results from a virus-cell interaction, the aim of the present work was to investigate more precisely the target point in the viral replicative cycle. Sindbis virus-infected BHK21 cells were selected as the model system, in which the biological and biochemical patterns of viral replication have been well established.

Materials and Methods

Cells

BHK-21 cells were grown as monolayers in minimum essential medium (MEM) supplemented with 6% inactivated calf serum and 50 μg/ml gentamicin. Maintenance medium (MM) consisted of MEM containing 2% inactivated calf serum.

Virus and virus assay

Sindbis virus was plaque-purified three times in BHK cells and virus stocks were prepared by infection of BHK cells at a multiplicity of infection (moi) of 1 pfu (plaque-forming unit) per cell. Virus was harvested 18 – 24 h after infection and titered by plaque formation in BHK cells.

Preparation of the plant extract

Melia azedarach L. leaves were collected from plants in the area of Buenos Aires city. Species taxonomy was confirmed at the Department of Botany, Faculty of Science, Buenos Aires University, where an herbarium specimen has been kept and registered (Argentina, BAFC 1432). The antiviral principle was obtained by blending 300 g of leaves with 300 ml of 10 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.2. The extract was filtered through cheesecloth and then clarified by centrifugation at 10000 × *g* for 1 h. The supernatant was precipitated with 70% saturated ammonium sulphate and the precipitate was dissolved in 30 ml of phosphate buffer, pH 7.2, this solution was passed through a Sephadex G-100 column. All antiviral activity against Sindbis virus eluated in fractions 11, 12 and 13 (consisting of 1 ml each), which were pooled, diluted 1:10 and routinely used as the antiviral prepa-

ration in all the experiments described in this paper. The protein content of this preparation was estimated 0.02 mg/ml using the Bradford reaction. The highest dilution found active against Sindbis virus was 1:40.

Infectious center assay

Monolayers of BHK cells were pretreated for 2 h with MM with or without MA. The supernatant fluids were then removed and all the cultures were washed twice with phosphate buffer solution (PBS) and infected with Sindbis virus at a moi of 1 pfu/cell. After 30 min adsorption at 4°C, inocula were withdrawn and MM was added. After 1 h incubation at 37°C, the cells were washed, dispersed with trypsin, and resuspended in PBS. The cells were gently pelleted, resuspended in MM and counted in an hemocytometer. After adequate dilution, 0.2 ml amounts of the cell suspension containing 4×10^5 cells/ml were plated onto monolayers of BHK cells. The cultures were incubated at 37°C for 2 h and then carefully overlaid with medium containing 0.7% agar. After 48 h incubation at 37°C, the number of plaque-forming cells was counted after staining with 1% crystal violet.

Preparation and titration of anti-Sindbis virus serum

Antiserum to Sindbis virus was obtained in rabbits by four weekly intravenous injections of 1 ml of a virus suspension containing 8×10^8 pfu/ml. The animals were bled 1 week after the last injection. To determine antiserum neutralizing antibodies, 2-fold serum dilutions were incubated for 30 min at 37°C with 100 pfu of Sindbis virus. The serum titer was 16000, expressed as the reciprocal of the highest dilution resulting in a 50% reduction of the number of virus plaques.

Assay of virus penetration

Cultures were preincubated with MA for 2 h at 37°C; the monolayers were then washed and Sindbis virus (moi = 1) was added and allowed to adsorb for 30 min at 4°C. The cultures were again washed with PBS, fed with MM and incubated at 37°C. Anti-Sindbis rabbit antiserum (final dilution 1:10) was added immediately and every 15 min to different groups of cultures, which were incubated further by 30 min. After removing the culture medium, the cells were removed with trypsin and infectious centers were counted. Virus penetration was related to the kinetics of reduction of neutralizable infectious centers.

Uridine and leucine incorporation assays

The method used was an adaptation of that described by Ball et al. [3]. To determine the effect of MA on viral RNA synthesis, 8×10^4 BHK cells grown as monolayers in 5 ml-vials were treated with MA during 2 h and then infected with Sindbis virus at a moi of 10. After 1 h adsorption, virus was removed, the cell sheet was washed with PBS and serum-free medium was added. At 1, 3, 5 and 7 h post-

adsorption, actinomycin D was added to a concentration of 2.5 μg/ml and the cells were incubated at 37°C for a further 60 min period. The medium was then replaced with 0.5 ml of medium containing 2.5 μg/ml actinomycin D and 10 μCi/ml [^{3}H]uridine (New England Nuclear, spec. act. 40.8 Ci/mmol). Two hours later the medium was removed and 4 ml ice-cold PBS was added to each vial. After 30 min at 4°C the PBS was removed and the cells were washed with 3 ml ice-cold 1.5% perchloric acid (PCA) and 3 ml ethanol. Finally, the cells were hydrolyzed with 0.5 ml 5% PCA for 3 h at 80°C, 3 ml of Triton X-100 toluene-based scintillation fluid were added, and radioactivity was determined in a Packard PRIAS 240 CL/D scintillation counter. The rate of RNA synthesis was expressed as cpm/vial of [^{3}H]uridine incorporation and the values represent the means of triplicate vials for each point.

To determine the effect of MA on cellular RNA and protein synthesis, BHK cell cultures grown in 5-ml vials were incubated with MA for 2 or 8 h, then washed, serum-free or leucine-free medium was added, and 1 h later actinomycin D (2.5 μg/ml) was added. After another 1 h incubation, 2 h pulse-labeling with 1 μCi/ml [^{3}H]uridine (New England Nuclear, spec. act. 40.8 Ci/mmol) or 2 μCi/ml [^{3}H]leucine (New England Nuclear, spec. act. 111.1 Ci/mmol), respectively, was carried out. Uridine incorporation was measured as described above. To determine leucine incorporation the vials were processed as follows: 4 ml ice-cold PBS was added to each vial for 15 min and then PBS was removed and 1 ml 1.5% PCA was added. After 15 min incubation, the monolayers were washed twice with PBS and then hydrolyzed with 0.5 ml 0.1 N NaOH for 4 h at 80°C. Radioactivity was measured as described above.

Analysis of protein synthesis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

BHK cells were treated with MA for a period of 2 h. Control cells were treated with MM not containing MA. The MA-treated and untreated cultures were divided in two parts, and one of each was infected with Sindbis virus (moi = 1 pfu/cell) and the other mock-infected. At 1 h post-infection, actinomycin D (2.5 μg/ml) was added to all cultures, and the cells were pulse-labeled with 25 μCi/ml of L-[^{35}S]-methionine (spec. act. 1,030 Ci/mmol: New England Nuclear Corp., U.S.A.) in methionine-free medium (containing 2.5 μg/ml actinomycin D) from 5 – 7 h post-infection. After the pulse-labeling period, the monolayers were washed twice with PBS and lysed in 0.2 ml sample buffer containing 5% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue in 0.0625 M Tris-HCl, pH 6.8. Cell lysates were heated for 2 min in boiling water before loading onto the gels. SDS-PAGE of cell-associated proteins was determined by the method of Laemmli [8] modified by Marsden et al. [9] on 12% acrylamide gels with a 3.6% acrylamide stacking gel. Radiolabeled polypeptides were visualized by fluorography on KODAK X - Omat RP-1 films as described previously [4].

Results

Inhibition of Sindbis virus replication by MA

The effect of MA on Sindbis virus under one-step growth conditions in BHK cells was first explored. For this purpose cells were preincubated with MA for 2 h, washed prior to infection, and after infection MM without MA was added. At various times post-infection supernatant fluid was collected and assayed for infectious virus. MA did not show a toxic effect on BHK cells, since the number of viable cells in cultures incubated with MA for 24 h was similar to that of the control cultures, as determined by the trypan blue dye exclusion test.

In comparison with untreated infected cells, MA-treated infected cultures exhibited a significant reduction in virus production (Fig. 1). Sindbis virus-infected BHK cells showed a 20-fold reduction in the number of productively infected cells following pretreatment with MA. In fact, 1 h after infection the percentage of BHK cells scoring as infective centers diminished from 58% for the untreated cultures to 3% for the MA-pretreated ones.

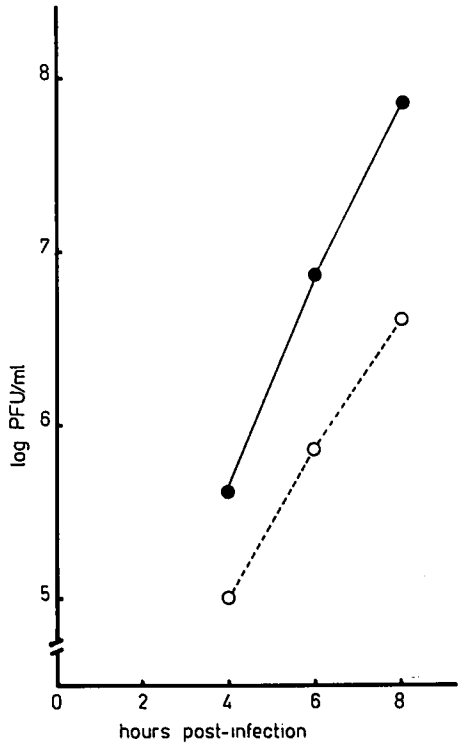

Fig. 1. Growth curve of Sindbis virus in BHK cells pretreated (o--o) or not (•—•) with MA during 2 h before infection (moi=10). Supernatants were harvested at the indicated times and titrated by a plaque assay.

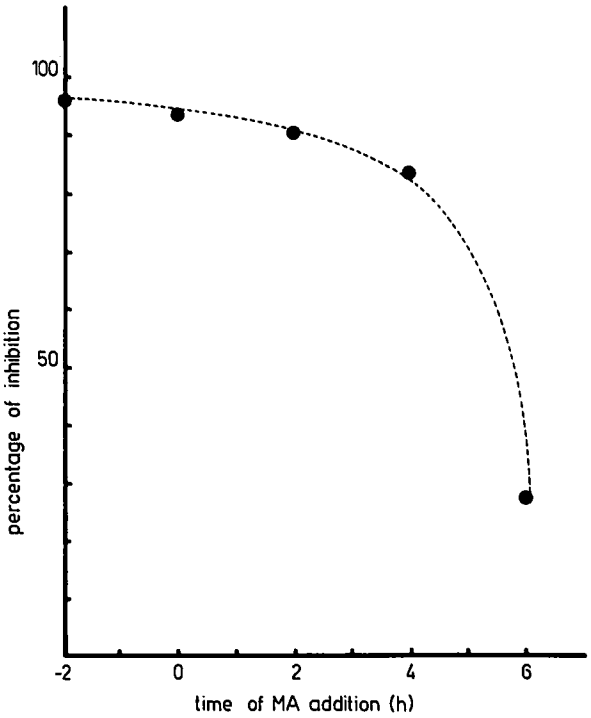

Fig. 2. Inhibition of Sindbis virus multiplication as a function of time of addition of MA. Cells were incubated with MA 2 h before, simultaneously or 2, 4, or 6 h after infection with Sindbis virus (moi=1). At 7 h post-infection the media were harvested and analyzed for infectivity. Results are expressed as percentage of inhibition in MA-treated cultures with respect to control cultures (yield in control cultures: 1.5×10^7 pfu/ml).

To determine whether MA inhibited viral replication during a specific period in the virus cycle, MA was added at various times before and after virus adsorption. In all cases, incubation with MA was continued until 7 h after virus adsorption, at which time the total yield of infectious virus was measured. The results showed

TABLE 1

Effect of MA on Sindbis virus adsorption.

Material	Residual infectivity (pfu/ml)	
	Untreated	MA-treated
Inoculum	1.1×10^6	1.0×10^6
First wash	2.8×10^5	2.5×10^5
Second wash	1.1×10^5	9.0×10^4

BHK cells were pretreated with MA or MM for 2 h, then washed and infected with Sindbis virus (moi=1). After 1 h adsorption at 4°C, inocula were harvested and monolayers were washed twice with PBS. Washes were also harvested and non-adsorbed virus was determined.

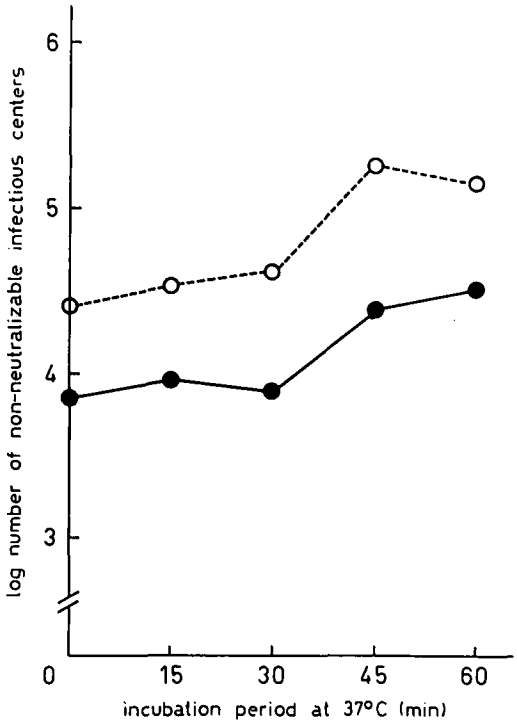

Fig. 3. Effect of MA on virus penetration. Sindbis virus was adsorbed for 30 min at 4°C on BHK cells pretreated during 2 h with MM (o--o) or MA(•—•). Then the cultures were fed with MM and incubated at 37°C. The infectious center number no longer neutralizable by antiserum was determined by addition to the culture medium, at 15 min intervals, of anti-Sindbis serum (final dilution 1:10) and further incubation for 30 min at 37°C. Then cells were trypsinized and assayed for infectious centers.

TABLE 2

Effect of MA on cellular macromolecular synthesis.

Time of incubation with MA (h)	Actinomycin D	[^{3}H]uridine incorporation (ct/min)	[^{3}H]leucine incorporation (ct/min)
Expt. A			
0	+	521	8369
2	+	459	11549
Expt. B			
0	-	8102	2763
8	-	10548	2474

Expt. A: Cell cultures were incubated with MA or MM for 2 h, then washed, serum-free or leucine-free medium was added and 1 h later actinomycin D (2.5 μg/ml) was added. After 60 min, a 2 h-pulse labeling with 1 μCi/ml [^{3}H]uridine or 2 μCi/ml [^{3}H]leucine was performed and then incorporation was measured as described in Materials and Methods.

Expt. B: Cell monolayers were incubated with MA for 8 h, then washed with PBS and serum-free or leucine-free medium containing 1 μCi/ml [^{3}H]uridine or 2μCi/ml [^{3}H]leucine was added for 2 h, then incorporation was measured as described in Materials and Methods.

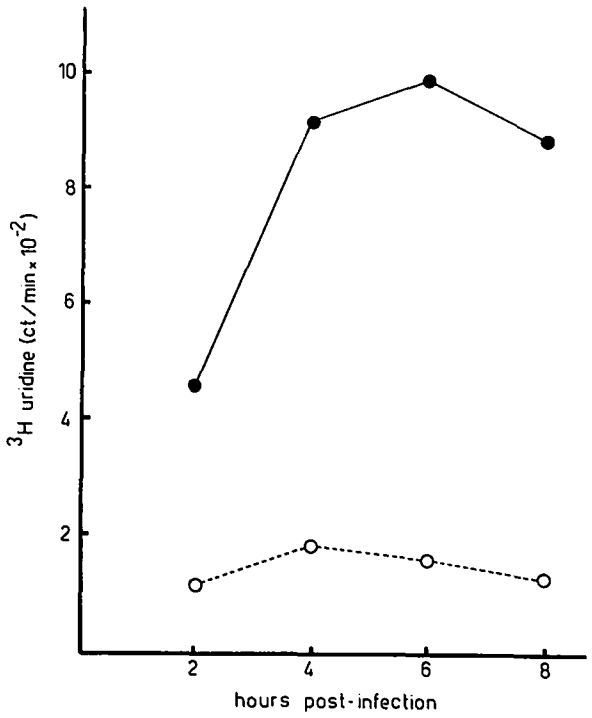

Fig. 4. Effect of MA on viral RNA synthesis. BHK cells grown in 5 ml-vials were treated with MA(o--o) or MM (•—•) 2 h before infection with Sindbis virus. At 2, 4, 6 and 8 h post-infection cultures were pulse-labeled with 10 μCi/ml [^{3}H]uridine for 2 h in the presence of 2.5 μg/ml actinomycin D. Then the incorporation was measured and expressed as cpm/vial.

that pretreatment of cultures with MA 2 h before infection or addition of MA to Sindbis virus-infected BHK cells within 2 h post-infection afforded a maximal inhibition of virus production (Fig. 2). However, MA added as late as 4 h after infection still caused more than 80% inhibition suggesting that MA could act throughout the viral replicative cycle.

Lack of effect of MA on Sindbis virus adsorption and penetration

To investigate whether MA had any effect on virus adsorption, Sindbis virus was adsorbed at 4°C either to MA-pretreated BHK cells or non-pretreated cultures. After 1 h virus adsorption, inocula were harvested and both sets of cultures were thoroughly washed twice. The remaining infectivity in the inocula and washes was almost identical for the treated and non-treated cell cultures (Table 1), indicating that virus adsorption occurred normally in the presence of MA.

The influence of MA on virus penetration was studied by determining the increase with time of the number of infective centers which could no longer be neutralized by anti-Sindbis immune serum. Results are shown in Fig. 3, where each

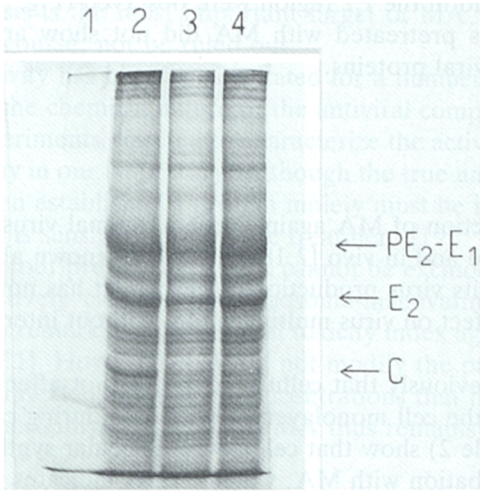

Fig. 5. SDS-PAGE of [^{35}S]methionine-labeled polypeptides of BHK cells treated with MA 2 h before infection with Sindbis virus (lane 3), untreated infected cells (lane 2), and untreated mock-infected cells (lane 4). Lane 1 corresponds to molecular weight markers.

value represents the average of three identical experiments. It can be seen that at all times assayed MA pretreatment reduced the infective center number by 0.6–0.9 log. However, the kinetics of increase of non-neutralizable infective centers was almost identical for cell cultures incubated with or without MA, suggesting that the rate of virus penetration, monitored by the inability of anti-Sindbis serum to neutralize cell-adsorbed virus, was not affected by MA treatment.

Effect of MA on viral RNA and protein synthesis

The lack of toxicity of MA for BHK cells was confirmed by determining its effect on cellular macromolecular synthesis. As can be seen in Table 2, there was no difference in [^{3}H]uridine or [^{3}H]leucine incorporation in MA-pretreated cells in comparison with non-treated cells, irrespective of the presence of actinomycin D. Even after a more extended exposure to MA (8 h), cellular RNA and protein synthesis remained unaffected.

The effect of MA on the rate of incorporation of [^{3}H]uridine into virus RNA was examined according to Stollar et al. [15] as described in Materials and Methods. Untreated infected cells incorporated label into viral RNA, whereas no significant increase in the amounts of label was found during the time course of this experiment for cells treated with MA (fig. 4).

The influence of MA on protein synthesized in infected cells was determined by SDS-PAGE. In Sindbis virus-infected cells, a major band corresponding to capsid protein C, a diffuse broad band corresponding to PE2 (precursor of the enveloped protein E2) and E1 (envelope protein), which comigrated under the conditions

used, and a very weak band in the E2 region were observed (Fig. 5). In contrast, Sindbis virus-infected cells pretreated with MA did not show any band corresponding to either of the viral proteins.

Discussion

Whereas the antiviral action of MA against several animal virus infections has been demonstrated in vitro and in vivo [2,18,19], little is known about the mechanism by which MA inhibits virus production. However, it has now been proven that MA exerts a direct effect on virus multiplication, without interacting with the host cell metabolism.

It has been reported previously that cellular growth is not affected by purified MA either if added after the cell monolayer is formed or during cell seeding [1]. Data presented here (Table 2) show that cell macromolecular synthesis is not impaired by continuous incubation with MA, which clearly indicates that MA is devoid of toxicity for the host cell. From these results and the previously reported observation that MA does not exert any virucidal action [18] it is inferred that a specific step in the viral replicative cycle must be inhibited.

Results presented in Table 1 demonstrate that virus adsorption is not inhibited by MA. Likewise, the kinetics of Sindbis virus penetration in MA-treated cells follow a similar pattern as seen in untreated cells (Fig. 3), indicating that virus penetration is not a main target for the action of MA. However, we cannot yet explain the reduction in the number of non-neutralizable infectious centers observed at the initial stage of infection of the MA-treated cells.

Due to the inability of Sindbis virus to shut off BHK cell RNA and protein synthesis during the first few hours following virus infection [12] the presence of actinomycin D is necessary for examining viral macromolecular synthesis. Usually, cells infected with alphavirus are pretreated with actinomycin D and the drug remains in contact with the cells from the beginning till the end of the infectious cycle [12].

We were not able to work under these conditions because actinomycin D partially prevented the antiviral state induced by MA (unpublished data). Thus, the drug was added once the refractory state was already installed. Nevertheless, a clear and specific inhibition of Sindbis virus RNA synthesis was observed indicating a very early effect of MA on the transcriptional or replicative functions of the Sindbis virus genome. Synthesis of viral specific polypeptides was also inhibited in MA-treated cells as shown by PAGE (Fig. 5), while the pattern of host cell polypeptides remained unchanged.

As early as 2 h after alphavirus infection new viral specific proteins can be detected [13]. Hence, it is possible that MA would exert a selective inhibitory effect on early virus macromolecular synthesis. Whether MA acts as a transcriptional or translational inhibitor cannot be ascertained from the present experiments. At 4 h post-infection almost all viral RNA is synthesized (Fig. 4). Yet, MA added at 4 h post-infection is still able to inhibit virus yield by 85–90% (Fig. 2). Possibly, viral

protein synthesis is the most important target of MA, although a more complex action can, of course, not be ruled out.

Antiviral activity has been demonstrated for a number of plant extracts [5]. Only in a few cases the chemical nature of the antiviral compounds has been fully characterized. Experiments directed to characterize the active principle of MA are currently underway in our laboratory. Although the true nature of the active principle has not yet been established a protein moiety must be involved since the antiviral activity of MA is sensitive to protease treatment [19].

According to our present data, MA cannot be excluded from the group of plant proteins collectively called RIPs (ribosome-inactivating proteins), that have in common a very remarkable differential toxicity index against uninfected and virus-infected cells [11]. However, MA did not modify the pattern of host cell proteins synthesized in infected cultures at concentrations that inhibited Sindbis virus replication. The mechanism of action of MA thus remains subject to further study.

Acknowledgment

This research was supported by a grant from the National Research Council (CONICET-Argentina) PID No. 3-9175-01/85.

References

1 Andrei, G.M., Coto, C.E. and de Torres, R.A. (1985) Ensayos de citotoxicidad y actividad antiviral de extractos crudos y semipurificados de hojas verdes de *Melia azedarach* L. Rev. Argent. Microbiol. 17, 187–194.
2 Andrei, G.M., Lampuri, J.S., Coto, C.E. and de Torres, R.A. (1986) An antiviral factor from *Melia azedarach* L prevents Tacaribe virus encephalitis in mice. Experientia 42, 843–845.
3 Ball, C.R., Van den Berg, H.W. and Poynter, R.W. (1973) The measurement of radioactive precursor incorporation into small monolayer cultures. In: Methods in cell biology, vol. XIII pp. 349–360. Academic Press, New York.
4 Bonner, W.M. and Laskey, R.A. (1974) A film detection method for tritiumlabeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46, 83–88.
5 Chessin, M. (1983) Is there a plant interferon? Bot. Rev. 49, 1–28.
6 Ilven, M., Vlietinck, A.J., Van Den Berghe, D.A. and Tote, J. (1982) Plant antiviral agents. III. Isolation of alkaloids from *Clivia miniata* Regel (Amaryllidaceae). J. Nat. Prod. 45, 564–573.
7 Irvin, J.D. (1983) Pokeweed antiviral protein. Pharm. Ther. 21, 371–387.
8 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680–685.
9 Marsden, H.S., Crombie, I.K. and Subak-Sharpe, J.H. (1976) Control of protein synthesis in herpesvirus-infected cells: analysis of the polypeptides induced by wild type and sixteen ts mutants of HSV strain 17. J. Gen. Virol. 31, 347–372.
10 Moraes, W.B.C., Martins, E.M., Conti, E. and Oliveira, A.R. (1974) The inhibitory activity of extracts of *Abutilon stritum* leaves on plant virus infection. Phytopathol. Z. 81, 145–152.
11 Roberts, W.K. and Selennikoff, C.P. (1986) Plant proteins that inactivate foreign ribosomes. Biosci. Rep. 6, 19–29.
12 Scheefers-Borchel, U., Scheefers, H., Edwards, J. and Brown, D.T. (1981) Sindbis virus maturation in cultured mosquito cells is sensitive to actinomycin D. Virology 110, 292–301.

13 Schlesinger, M.J. (1985) Replication of Togaviruses. In: Virology. (Fields, B.N. ed.). pp. 1021–1032. Raven Press, New York.
14 Stirpe, F., Williams, D.G., Onyon, L.J., Legg, R.F. and Stevens, W.A. (1981) Dianthins, ribosome-damaging proteins with antiviral properties from *Dianthus caryophyllus* L. (carnation). Biochem. J. 195, 399–405.
15 Stollar, V., Peleg, J. and Shenk, T.E. (1973/74) Temperature sensitivity of a Sindbis virus mutant isolated from persistently infected *Aedes aegypti* cell culture. Intervirology 2, 337–344.
16 Teltow, G.M., Irvin, J.D. and Aron, G.M. (1983) Inhibition of herpes simplex virus DNA synthesis by pokeweed antiviral protein. Antimicrob. Agents Chemother. 23, 390–396.
17 Tsuchiya, Y., Shimizu, M., Hiyama, Y., Itoh, K., Hashimoto, Y., Nakayama, M., Horie, T. and Morita, N. (1985) Antiviral activity of natural occuring flavonoids in vitro. Chem. Pharm. Bull. 33, 3881–3886.
18 Wachsman, M.B., Martino, V., Gutkind, G.O., Coussio, J.D., Coto, C.E. and de Torres, R.A. (1982) Antiviral activity of a *Melia azedarach* plant extract. Fitoterapia 53, 167–170.
19 Wachsman, M.B., Andrei, G.M., Daelli, M.G., Coto, C.E. and de Torres, R.A. (1984) Actividad antiviral asociada a una fracción polipeptídica obtenida de extractos de *Melia azedarach* L. Acta Farm. Bonaerense 3, 27–31.